MODULATION OF CENTRAL NEUROENDOCRINE RESPONSE TO PHOTOPERIOD BY SEASONAL FRUITS AND OBESITY.

Cristina Domenech Coca

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Modulation of central neuroendocrine response to photoperiod by seasonal fruits and obesity

PhD DOCTORAL THESIS

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FAIG CONSTAR que aquest treball, titulat "**Modulation of central neuroendocrine response to photoperiod by seasonal fruits and obesity**", que presenta la **Cristina Domenech Coca** 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 poder optar a la Menció Internacional de Doctorat.

HAGO CONSTAR que el presente trabajo, titulado "**Modulation of central neuroendocrine response to photoperiod by seasonal fruits and obesity**", que presenta **Cristina Domenech Coca** para la obtención del título de Doctor, ha sido realizado bajo mi dirección en el **Departamento de Bioquímica y Biotecnología** de esta universidad y que cumple con los requisitos para poder optar a la Mención Internacional de Doctorado.

I STATE that the present study, entitled "**Modulation of central neuroendocrine response to photoperiod by seasonal fruits and obesity**", presented by **Cristina Domenech Coca** for the award of the degree of Doctor, has been carried out under my supervision at the **Department of Biochemistry and Biotechnology** of this university and that this thesis is eligible to apply for the International Doctorate Mention.

Tarragona, 5 de setembre del 2018
Tarragona, 5 de septiembre del 2018
Tarragona, 5th September 2018

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Als de casa

i en especial,

al meu padrí

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"I am among those who think that science has great beauty. A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale"

Marie Curie

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Agraïments

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I en definitiva, MOLTES GRÀCIES A TOTS!

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Content

Abbreviations

ACTH: Adrenocorticotropin hormone

AOAC: Association of Analytical Communities

BAT: Brown adipose tissue

BBB: Blood brain barrier

BDNF: Brain-derived neurotrophic factor

BMAL1: Hydrocarbon receptor nuclear translocator-like 1

CAF: Cafeteria

cAMP: Cyclic adenosine monophosphate

CHGA: Chromogranin A

CLOCK: Circadian locomotor output cycles kaput

Control: Control group

CREB: Cyclic adenosine monophosphate response element binding

CRF: Corticotropin-releasing factor

CRH: Corticotropin releasing hormone

CRY: Cryptochrome

D2R: Dopamine receptor D2

DBP: (Albumin D-box) binding protein

Dio1: Type I iodothyronine deiodinase

Dio2: Type II iodothyronine deiodinase

Dio3: Type III iodothyronine deiodinase

E-box: Enhancer box

EE: Energy expenditure

ELISA: Enzyme-linked immunosorbent assay

ESI: Electrospray ionization

EYA3: Eyes-absent 3

FDA: Food and Drug Administration

FSH: Follicle stimulating hormone

GH: Growth hormone

GnRH: Gonadotropin-releasing hormone

GSPE: Grape seed proanthocyanidin extract

HFD: High-fat diet

HP: Hypothalamic-pituitary

HPA: Hypothalamic-pituitary-adrenal

HPG: Hypothalamic-pituitary-gonadal

HPLC-MS/MS: High-performance liquid chromatography coupled to tandem mass spectrometry

HPT: Hypothalamic-pituitary-thyroid

IL: Interleukin

Kp: Kisspeptin

LD: Long day photoperiod

LH: Luteinizing hormone

LLE: Liquid-liquid extraction

LOD: Limit of detection

LOQ: Limit of quantification

MBH: Mediobasal hypothalamus

ME: Median eminence

MetS: Metabolic Syndrome

MRM: Multiple reaction monitoring

MT1: Melatonin receptor subtype 1

MT2: Melatonin receptor subtype 2

NST: Non-shivering thermogenesis

P: Photoperiod

PAs: Proanthocyanidins

PBMCs: Peripheral blood mononuclear cells

PD: Pars distalis

PER: Period

PPARα: Peroxisome proliferator-activated receptor alpha

PR: Reverse phase

PRL: Prolactin

PT: Pars tuberalis

PxT: Interaction between photoperiod and treatment factors

QC: Quality control

RE: Relative error

REV-ERB: Nuclear receptor subfamily 1, group D, member 1

RF: Rfamides

RFRP: Rfamides-related proteins

RG: Red-grape

RIA: Radioimmunoassay

RORα: Retinoic acid receptor-related orphan receptor alpha

RSDs: Relative standard deviations

SCN: Suprachiasmatic nucleus

SD: Short day photoperiod

SIX1: Sine oculis-binding homeodomain factor 1

SPF: Specific pathogen free

SST: Somatostatin

SST2R: Somatostatin Receptor type 2

SST4R: Somatostatin Receptor type 4

STAT3: Signal transducer and activator of transcription-3

STD: Standard

T: Treatment

T2: Diiodothyronine

T3: Triiodothyronine

T4: Thyroxine

TEF: Thyrotroph embryonic factor

TH: Thyroid hormone

TNF-α: Tumor necrosis factor alpha

TRH: Thyrotropin-releasing hormone

TSH: Thyrotropin

TSHR: Thyrotropin receptor

TSHβ: Beta-subunit of thyrotropin

TX: Thyroidectomy

UCP-1: Uncoupling protein 1

VEGF: Vascular endothelial growth factor

ZT: Zeitgeber

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Summary

Chrononutrition studies the relationship between biological rhythms and metabolism. Nowadays, it has been embraced within nutritional approaches in order to improve dietary advices and their final result. The Xenohormesis theory proposes that animals recognize bioactive compounds from plants, mainly polyphenols, as non-photic cues for checking environmental conditions, allowing them to respond in advance to environmental alterations and to improve their survival.

In this framework, the main aim of the present thesis is to evaluate whether fruit consumption in or out of their natural season produce effects on different key biomarkers of seasonality and neuroendocrine processes in normoweight and cafeteria-fed obese Fischer 344 rats.

We found that in healthy Fischer 344 rats, the oral consumption of red-grape, but not cherry, during its natural season characterized by a short day photoperiod produced behavioral changes, such as decreased locomotor activity and food consumption, and energy expenditure alterations, which could be associated with changes observed on central mechanisms subjected to seasonal control, such as dopaminergic and somatostatinergic systems. Furthermore, considering that these results could be beneficial for the treatment of obesity, CAF fed animals were also studied at the same photoperiodic conditions and supplemented with the red-grape treatment. Results revealed that obesity dampens the effects of fruit consumption in behavior and in central and peripheral controllers of seasonality observed in the normoweight cohort. Finally, photoperiod sensitivity in both phenotypes consuming red-grape was assessed. Results revealed that obesity impairs seasonal control of prolactin release and the response to hypothalamic-pituitary-adrenal, hypothalamic-pituitary-gonadal and hypothalamic-pituitary-thyroidal axis to

changes in photoperiod, therefore producing an insensitivity to photic and non-photic cues.

This thesis work highlights the relevance of seasonality of fruits and its consumption for the modulation of brain function by nutrition, and furthermore, provides new evidences for considering seasonal adaptation as a relevant element for prevention and treatment of diseases.

Resumen

La crononutrición estudia la relación entre los ritmos biológicos y el metabolismo. Actualmente, se ha incorporado en el contexto de la nutrición personalizada, para mejorar los consejos dietéticos y sus resultados. La teoría de la Xenohormesis propone que los animales pueden reconocer compuestos bioactivos de las plantas, principalmente polifenoles, como señales no-fóticas para detectar condiciones ambientales adversas, permitiendo reaccionar por adelantado y mejorando así su nivel de supervivencia.

En este contexto, el objetivo principal de la tesis es evaluar si el consumo de fruta dentro o fuera de su estación produce efectos en los diferentes biomarcadores de estacionalidad y en procesos neuroendocrinos en ratas Fischer 344, utilizando animales normopeso y obesos.

El consumo de uva, pero no de cereza, en animales normopeso durante su época (SD) produce cambios importantes en comportamiento y en gasto energético, cosa que puede estar asociada a cambios en mecanismos centrales controlados por la estacionalidad, como el sistema dopaminérgico y el somatostatinérgico. Además, considerando que estos resultados podrían ser beneficiosos para el tratamiento de la obesidad, animales alimentados con dieta de cafetería fueron estudiados en las mismas condiciones. Los resultados muestran que la obesidad atenúa los efectos del consumo de fruta en el comportamiento y en los marcadores de estacionalidad, tanto a nivel central como periférico. Finalmente, la comparación de la sensibilidad al fotoperiodo entre los dos fenotipos que consumían uva mostró que la obesidad bloquea la respuesta estacional de la prolactina, así como la respuesta de los ejes adrenales, gonadales y tiroideos, mostrando insensibilidad tanto a señales fóticas como no-fóticas.

El trabajo de esta tesis muestra la importancia de la estacionalidad y el consumo de alimentos para la modulación del cerebro a través de la nutrición, y proporciona nuevas evidencias para considerar la adaptación estacional como un elemento preventivo y de tratamiento de enfermedades.

Resum

La crononutrició estudia la relació entre els ritmes biològics i el metabolisme. Actualment, s'ha incorporat en el context de la nutrició personalitzada, per tal de millorar els consells dietètics i els seus resultats. La teoria de la Xenohormesis proposa que els animals poden reconèixer compostos bioactius de les plantes, principalment polifenols, com una senyal no-fòtica per detectar condicions ambientals adverses, permetent que reaccionin per avançat i millorant així la seva supervivència.

En aquest context, l'objectiu principal d'aquesta tesi és avaluar si el consum de fruita dins o fora de la seva estació de maduració produeix efectes en diferents biomarcadors d'estacionalitat i en processos neuroendocrins en rates Fischer 344, utilitzant animals normopès i obesos.

El consum de raïm, però no de cirera, en animals normopès durant la seva època (SD) produeix canvis importants en comportament i en despesa energètica, la qual cosa pot estar associada a canvis en mecanismes centrals controlats per l'estacionalitat, com ara el sistema dopaminèrgic o somatostatinèrgic. A més, considerant que aquests resultats podrien ser beneficiosos pel tractament de l'obesitat, animals alimentats amb dieta de cafeteria van ser estudiats en les mateixes condicions. Els resultats mostren que l'obesitat atenua els efectes del consum de fruita en el comportament i en els controladors d'estacionalitat, tant a nivell central com perifèric. Finalment, la comparació de la sensibilitat al fotoperíode entre els dos fenotips que consumien raïm va mostrar que l'obesitat bloqueja la resposta estacional de la prolactina, així com la resposta dels eixos adrenals, gonadals i tiroidals, mostrant insensibilitat tant a senyals fòtiques com no-fòtiques.

El treball d'aquesta tesi mostra la importància de l'estacionalitat i el consum de determinats aliments per la modulació del cervell a través de la nutrició, i proporciona noves evidències per considerar l'adaptació estacional com un element de prevenció i tractament de malalties.

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Introduction

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1. Circannual Rhythms

The possibility of adapting the physiology of the organism to incoming environmental changes is a key feature for animal survival. In order to adapt to seasonal shifts, animals display a wide range of body alterations, including changes in metabolism, thermogenesis, weight management, hibernation, migration and reproduction[1]. All these processes are synchronized through exogenous factors, which regulate internal pathways that, in turn, modify the intrinsic circannual rhythm of the animal[2,3].

Biological rhythms have been reported to be linked with human health. In this sense, simple rhythmic alterations can induce different kind of diseases, being seasonal affective disorders one of the most popular alterations related with changes in rhythmic patterns[2]. However, other diseases have been associated with seasonal variations. Dopico *et al.* reported that immune system is affected by seasonal switching, showing that, during winter months, European people present a dominant proinflammatory state, according with their increased levels of interleukin (IL) 6 receptor and C-reactive protein[4]. Hanazawa *et al.* had also reported that Japanese population present significant alterations in blood pressure during the year, showing the highest and the lowest levels in January and July, respectively[5]. Accordingly, exogenous factors could be contemplated as a key point in the preservation of animal's health.

Alterations in temperature and food availability are considered to be essential external cues to predict environmental changes for mammals and birds[3]. Moreover,

there are evidences that the most important seasonal environmental stimulus is light length variation between day and night, known as photoperiod. This factor is determined by the circadian system, having an oscillatory pattern of 24 hours approximately[6].

Photoperiod confers a highly specific information for the activation of seasonal adaptative pathways, ensuring that it happens in the correct time during the year[6]. Related with this photoperiodic sensibility, many animals have developed internal long-term systems, the so-called circannual cycle, which allows them to anticipate for the incoming external changes. These intrinsic long-term rhythms are most easily comprehended when it is considered that organisms living in extreme locations of the world, with lack of exogenous time cues, experience profound alterations in their seasonal rhythmic biology[7]. Currently, long-term rhythms are recognized as a critical biological feature in vertebrates.

a) Physiology and mechanisms

Considering the physiological and pathophysiological importance of rhythmic biology and, specifically, seasonal variations, assessment of the anatomy and biology of this adaptation mechanism is currently an active field of research. Despite exact mechanisms are still to be elucidated, a relevant body of literature has been published so far, allowing a comprehensive view of the pathways and effectors that constitute the main elements of seasonal control at the molecular and physiology level.

i) Molecular control of seasonality

The pars tuberalis (PT) of the pituitary has been pointed to be the structure responsible for the control of seasonal changes in metabolism and behaviour due to its function as the first transducer of the photoperiodic signal[8]. This structure is located close to the median eminence (ME), the portal system, the pars distalis (PD)

and also the third ventricle, joining this last one through glial cells known as tanycytes[9] (Figure 1). These elements constitute the main anatomical structures of the seasonal control in animals.

PT is formed by different cell types, which the most important and abundant are the PT-specific cells[9]. This cell type has the higher levels of melatonin receptor MT1 and also have immunoreactivity for the beta-subunit of thyrotropin (TSHβ)[10,11]. TSHβ is also expressed by PD cells, known as thyrotrophs, but its regulation is different between both cell lines. In the case of PD, TSHβ is regulated by the active thyroid hormone (TH) triiodothyronine (T3) or TSH-releasing hormone (TRH) receptors, being included in the regulatory mechanisms of the hypothalamic-pituitary-thyroid (HPT) axis[11]. In contrast, PT-specific cells lack these receptors and TSHβ expression is regulated by photoperiod and melatonin signals[12]. For this reason, PT-specific cells are known as "calendar cells", being associated with the transduction of photoperiodic signals. This information shows PT as the season regulator of the body and could be postulated as the location of the circannual clock.

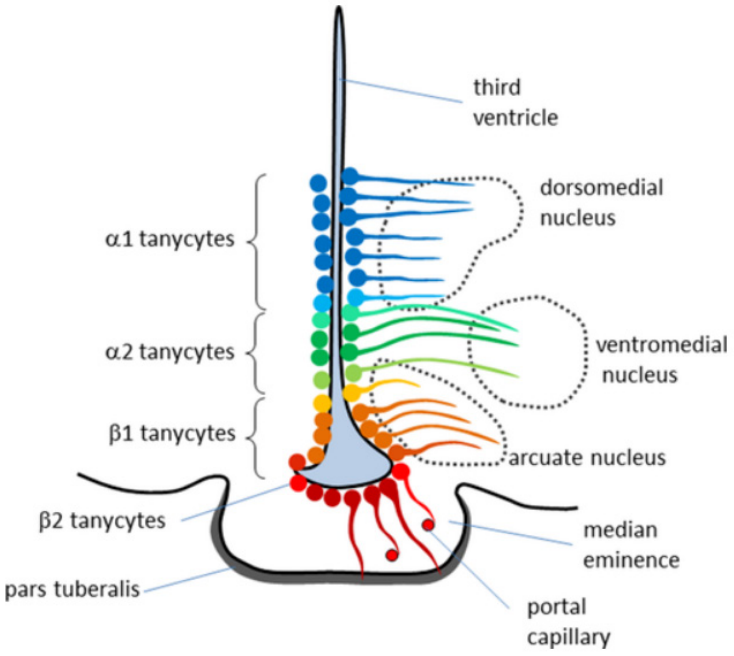

Figure 1. Diagrammatic representation of the structure responsible for the control of seasonal changes in metabolism and behaviour. Figure from Ebling et al., 2017.

Melatonin as a photoperiodic regulator

The PT needs a photoperiodic input to maintain its rhythmicity[2]. In mammals, the photoperiodic information is exclusively acquired by the retina, which, through multisynaptic pathways, is connected to the pineal gland. This organ translates the photic information through the neurohormone melatonin, which has its own rhythmic pattern of secretion. Thus, melatonin is produced only during the night and encode the night length[13]. Hence, its levels are increased at dusk, starting its secretion at early night and stopping at dawn, showing a peak in blood in the middle of dark phase[14,15]. This hormone acts as a transductor of photoperiod change, giving to mammals an intrinsic illustration of external photic alterations[13]. The role of melatonin has been studied during years through studies using pinealectomy and artificial melatonin infusions, which demonstrated the need of a melatonin rhythmic pattern in the development of circannual rhythms[13,16]. Woodfill *et al.* demonstrated that 90 days of daily infusions of melatonin to pinealectomized (PX) and ovariectomized animals is enough to entrain once again the intrinsic annual cycle. The animals were PX and ovariectomized in order to abolishes the modulatory effects of photoperiod in plasmatic melatonin and luteinizing hormone (LH) levels. The delivery of melatonin was calculated to achieve the physiological night time concentrations in serum and the animals were treated with oestradiol, maintaining LH levels invariable during all the experiment. On this basis, periodic swings in LH provides a robust marker of the circannual rhythm of reproductive neuroendocrine activity, with low and high values representing reproductive suppression or induction, respectively. PX-ewes were infused with melatonin from the summer solstice to the autumnal equinox (summer melatonin induction), and they showed not significant differences in LH rhythmic levels compared with the non-PX animals. In the other seasonal inductions, spring and autumn were less effective and winter melatonin induction was totally ineffective[17]. These results show the role of melatonin inducing seasonal rhythms, being the synchronization more effective

when the infusions were done during summer schedule. These works demonstrated that melatonin is a key point in the control of seasonal processes and the importance of time in the rhythmic biology experiments.

PT activity is controlled by melatonin through activation of different kind of receptors. In mammalian PT, it has been identified two subtypes of melatonin receptors, MT1 and MT2, with the highest density corresponding to MT1 subtype in PT-specific cells[10,18]. This receptor is considered an essential factor for the photoperiodic signal transduction[18]. This signal pathway varies among tissues and cell types. However, by using recombinant melatonin receptors in PT, it has been shown that the melatonin receptors are coupled to different G proteins which inhibited cyclic adenosine monophosphate (cAMP) accumulation. Together with this mechanism, intracellular signalling is also mediated by adenylate cyclase, phospholipase C, guanylate cyclase and calcium and potassium channels[19,20].

EYA3 as a critical summer-circannual marker

It is currently considered that eyes-absent 3 (EYA3) protein is a key point in the control of photoperiodic switch, driving seasonal rhythms by a clear response to photoperiod in PT[21]. EYA3 is reported to be involved in the formation of different mammalian structures related with photic signal, such as the eyes, pineal and pituitary gland, as well as with immunology, angiogenesis and cancer metastasis[3,21]. Furthermore, EYA3 protein has a dual role in body physiology, which can act either as phosphatase or a transcriptional factor[22].

Melatonin peaks during the dark phase are considered the major resetting signal in the PT. For this reason, the oscillatory gene expression patterns in this tissue peaks differently regarding to the day length, peaking later in animals housed at long day (LD) compared with those at short day (SD)[23]. In addition, melatonin has been also related with the suppression of a variety of enhancer-box (E-box) controlled genes in PT[24]. In that sense, Dardente *et al.* demonstrated in sheep

housed at LD conditions and exposed to constant light for suppressing the endogenous melatonin secretion, that administration of artificial melatonin had the capacity to supress EYA3 expression[21].

This regulatory model of EYA3 expression by melatonin allows the link between circadian system and the photoperiodic response (Figure 2). EYA3 peaks occurs always after 12 hours of dark phase and melatonin onset, independent of day length, known as the Bünning's external co-incidence timing mechanism. This implies that during SD photoperiod, which has more hours of dark phase and a higher peak of melatonin, EYA3 should peak during the night. But at that time, the high levels of melatonin exert the suppressive effect on EYA3 expression and therefore EYA3 response is mitigated. In contrast, during LD photoperiod, when the light phase is wider and, consequently, levels of melatonin are still low 12 hours after dawn, EYA3 expression peaks during the early morning. In that moment, considering that melatonin levels are minimal and its repressive action cannot take place, EYA3 induction is exacerbated. This mechanism, dependent on external photic information, allows to link a circadian oscillation with a light stimulus and confines the expression of EYA3 to LD photoperiod, related with the summer phenotype[21].

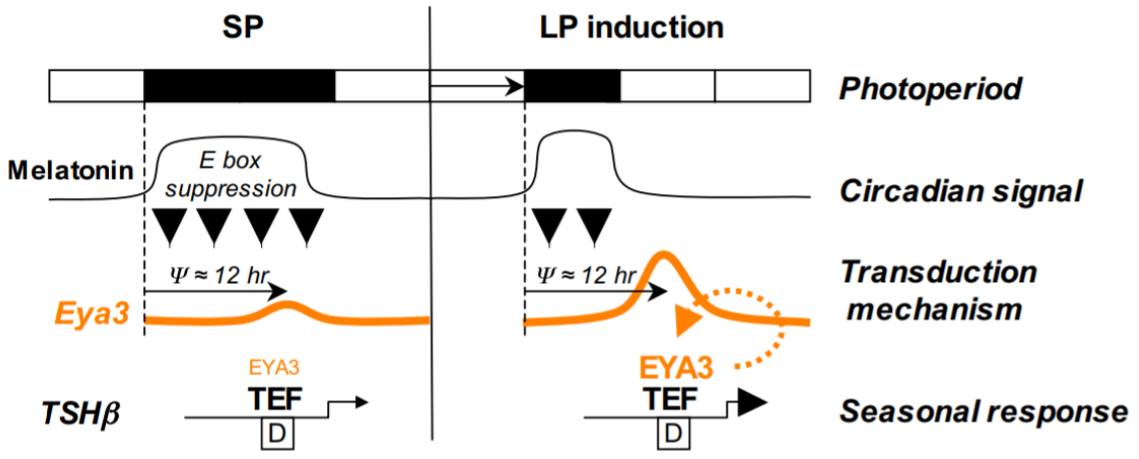

Figure 2. *Model for photoperiodic induction of Eya3 expression in the pars tuberalis. SP, short photoperiod; LP, long photoperiod; E-box, enhancer box; hr, hours; Eya3, eyes-absent 3, TSHβ, beta-subunit of thyrotropin; TEF, thyrotroph embryonic factor. Figure from Dardente et al., 2010.*

CHGA as a robust winter-circannual marker

Nowadays, EYA3 is accepted as a summer marker phenotype. In turn, chromogranin A (CHGA) has been identified as a robust molecular marker for SD photoperiods, related with the winter phenotype. This molecule is expressed specifically in the thyrotrophs of the PT and is crucial for the formation and regulation of hormone secretory granules in PD gonadotrophs and somatotrophs, reflecting a specific physiological SD state[25–27]. Wood *et al.* had demonstrated that both CHGA protein and gene expression are highly increased in response to the SD photoperiod[27].

Thyrotrophs, which contains both EYA3 and CHGA proteins, present a complex and dynamic repertoire of these molecules and its releasing activities, which are specifically modified by changes in photoperiod[27]. Using an immunofluorescence analysis with antibodies for EYA3 and CHGA, it was observed a switch between LD and SD exposition, increasing EYA3 and decreasing CHGA expression after 4 weeks of LD exposure, while the contrary happened at SD (Figure 3). Interestingly, Wood *et al.* only detected 0.01% of cells in PT expressing both EYA3 and CHGA, suggesting that PT may have a binary code within thyrotroph cells, which switch from either LD or SD phenotype, without having both at once. The number of cells in each state determine the general activity of PT and, consequently, the phase of circannual cycle. This fact proposes that PT act as a cell-autonomous timer, presenting a binary phenotype regarding to the circannual cycle[3]. Whether alterations in the levels of EYA3 and CHGA are linked has not been yet established. Despite this fact, the results corroborate that PT presents a binary state exhibiting a LD or SD phenotype, defined by the relative proportion of EYA3 and CHGA molecules which represent a specific phase of the circannual cycle[3,27].

All this information shows that thyrotrophs from PT could be assessed as a circannual timer, presenting a binary switch timing mechanism which regulates the

final rhythmic response. And place EYA3, CHGA and its ratio as an excellent biomarker of seasonality in PT tissue.

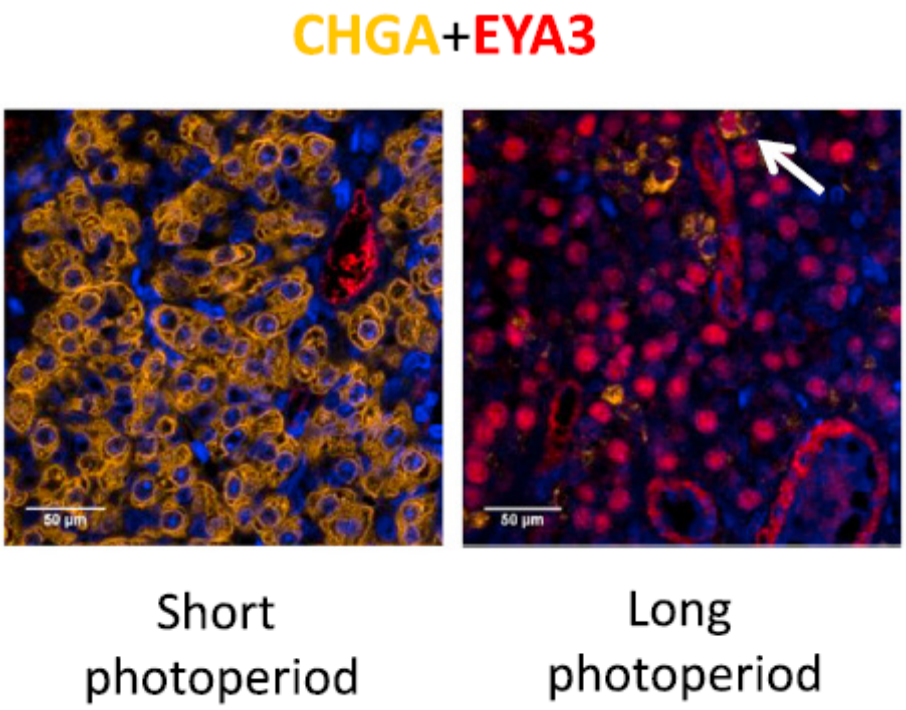

Figure 3. *Immunofluorescence showing expression of EYA3 (red) and CHGA (yellow) in the pars tuberalis on short day and long day photoperiods. CHGA, chromogranin A; Eya3, eyes-absent 3. Figure adapted from Wood et al., 2015.*

Thyroid hormone as seasonal rhythmic generator

Thyroid hormone pathway is also related with photoperiodical changes, being crucial for the expression of seasonal rhythms in different animals[1,7]. Yoshimura *et al.* had reported that hypothalamic T3 levels, the bioactive form of the molecule, is regulated by photoperiodic changes through deiodinase gene expression[28]. Recapitulating into what has been described previously, LD-related melatonin signals activate EYA3 gene expression via MT1. This event triggers a molecular pathway which includes the circadian transcription factor thyrotroph embryonic factor (TEF) and a nuclear complex formed by EYA3 and Six (sine oculis)-binding homeodomain factor 1 (SIX1). The EYA3-SIX1 nuclear complex works synergistically

and activate TSHβ expression, which activity is enhanced by TEF[2,21] (Figure 4). Subsequently, TSHβ binds its specific receptor (TSHR) in the ependymal layer of the infundibular recess[2]. This union results in significant upregulation of type II iodothyronine deiodinase (Dio2), which catalyses the conversion of thyroxine (T4) into the bioactive T3, and the repression of type III iodothyronine deiodinase (Dio3), that converses T3 into the inactive form rT3 or T4 into inactive diiodothyronine (T2). In contrast, short day photoperiod induces the expression of Dio3 and inhibits Dio2 gene[29,30]. These changes in the activity of these two selenoenzymes takes place in the ventral hypothalamic cell layer, also known as tanycytes, which are located between the third ventricle and the mediobasal hypothalamus, with the processes reaching the ME[28]. The final result is that the local T3 concentration in the hypothalamus is regulated in a photoperiod-dependent manner, increasing the levels of bioactive thyroid hormone (TH) during LD exposition, and eventually inducing the summer-like phenotype[1,27] (Figure 4).

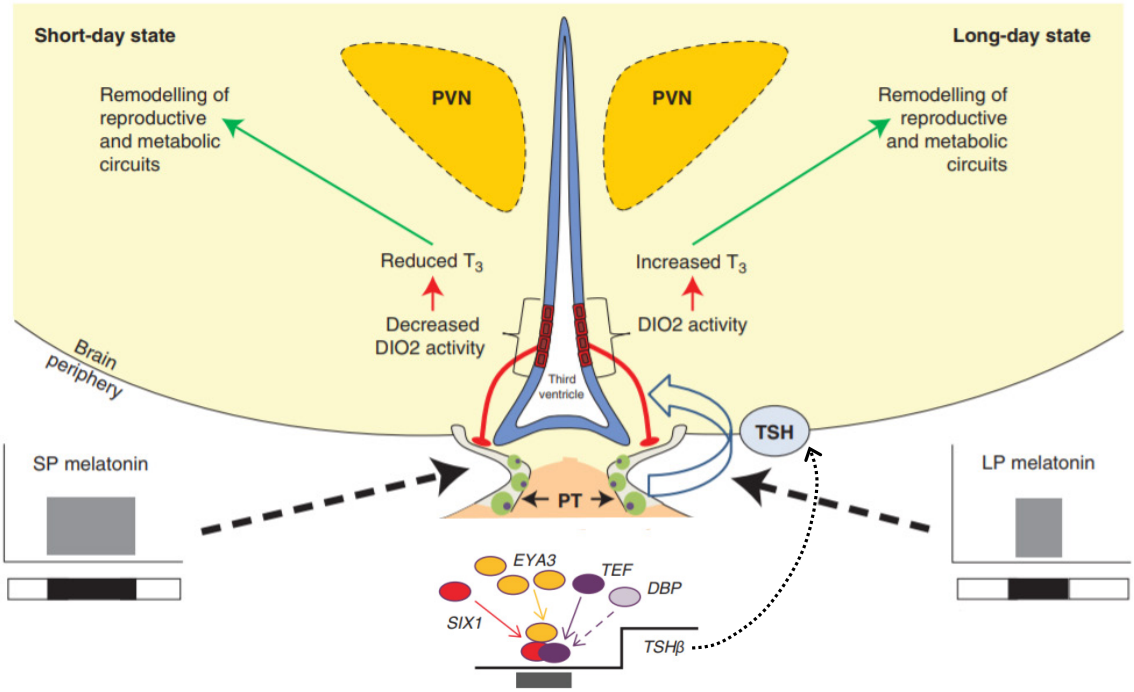

Figure 4. *Modulation of thyroid hormone pathway by photoperiod changes. PVN, paraventricular nucleus; T3, triiodothyronine; Dio2, type II iodothyronine deiodinase; PT, pars tuberalis; TSH, thyrotropin; SP, short photoperiod; LP, long photoperiod; Six1, six (sine oculis)-binding homeodomain factor 1; Eya3, eyes-absent 3; TEF, thyrotroph embryonic factor; DBP, (albumin D-box) binding protein; TSHβ, beta-subunit of thyrotropin. Figure adapted from Wood et al., 2014.*

The nature of this thyroid-activation pathway, which connects the PT with the mediobasal hypothalamus, has been assessed in different organisms[31–34]. Although there seem to be a conserved pathway, there are notable variations between different species in the hypothalamic responses to LD-induction of Dio2 and SD-induction of Dio3. For example, whereas in Siberian hamster (*Phodopus sungorus*) both enzymes are altered when animals are transferred from SD to LD conditions, Syrian hamsters (*Mesocricetus auratus*) only present changes in Dio2, without effects on Dio3 levels[31,32]. In the case of European hamsters (*Cricetus cricetus*) and the photoperiodic-sensitive Fischer 344 rat strain (*Rattus norvegicus*), the switch from SD to LD results in an increase of Dio2 and a decrease of Dio3 expression[33,34].

Therefore, melatonin induces a molecular pathway led by EYA3, which is subsequently translated to neuroendocrine seasonal changes in the hypothalamus by active TH[1]. Besides, Dio2 and Dio3 expression, and mainly their ratio, have been reported as a good biomarker of seasonal control in hypothalamus tissue.

ii) Interaction and coordination with the regulatory components of circadian rhythms

Together with its role as a key structure in circannual control, several studies confirm that the PT is a melatonin-dependent circadian oscillator, exhibiting circadian rhythmicity and expressing clock genes. This tissue contains a molecular clockwork of transcriptional activators, such as the Circadian Locomotor Output Cycles Kaput (CLOCK) and aryl hydrocarbon receptor nuclear translocator-like 1 (BMAL1), and inhibitors, such as Period (PER) and Cryptochrome (CRY)[35]. Clear differences in the final function of each molecule have been reported, depending on the tissue where they are located.

In the suprachiasmatic nucleus (SCN) of the hypothalamus, these proteins regulate circadian gene transcription. Thus, CLOCK forms a heterodimer with BMAL1, which binds to E-box sequences in the promoters of PER and CRY genes,

activating their transcription. In turn, PER and CRY proteins form heterodimers that translocate to the nucleus and inhibit, by direct interaction, CLOCK-BMAL1 activity. Apart from these principal molecules, other factors such as REV-ERB (nuclear receptor subfamily 1, group D, member 1), RORα (retinoic acid receptor-related orphan receptor alpha) and PPARα (peroxisome proliferator-activated receptor alpha) act as regulators of CLOCK-BMAL1 functionality, through the regulation of BMAL1 transcription[36–38].

In the PT, CRY mRNA levels are increased in response to night-melatonin signal, whereas PER expression are down-regulated. Consequently, the relationship between PER and CRY, which track the light onset and offset respectively, indicate the season-specific photoperiodic changes, related with the duration of the melatonin signal. Consequently, the interval phase between PER and CRY during SD is shorter than in response to LD photoperiod[1,3] (Figure 5).

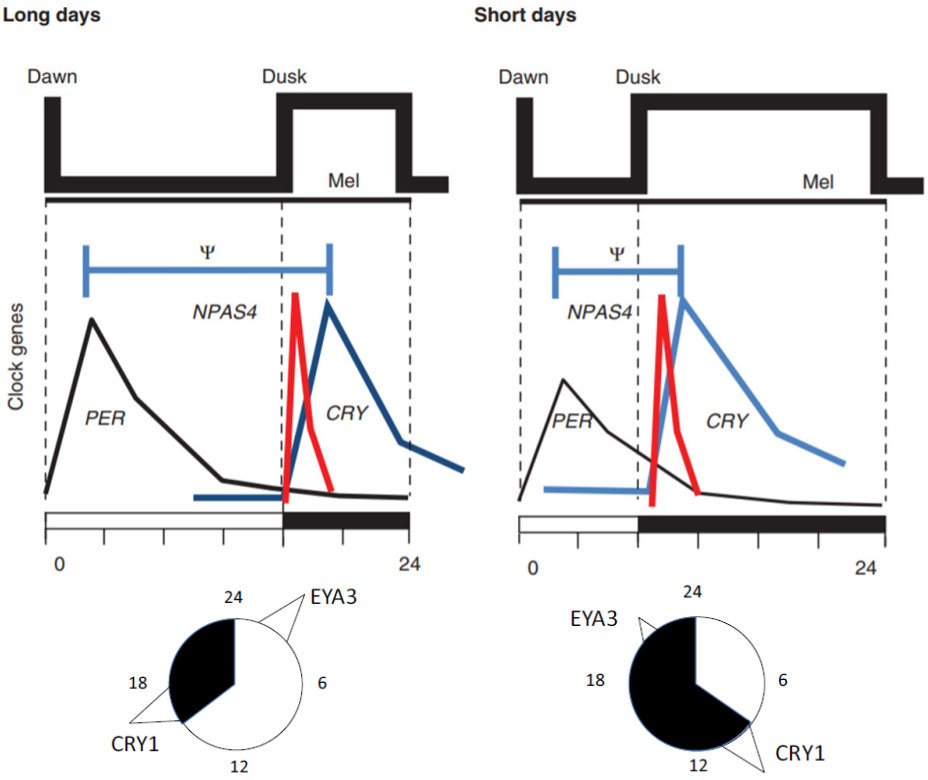

Figure 5. Melatonin release as a photic-cue for seasonal regulation. Mel, melatonin; Per, period; Npas4, neuronal PAS domain-containing protein 4; Cry, cryptochrome; Eya3, eyes-absent 3. Figure adapted from Wood et al., 2014.

EYA3, which has been reported to be the principal seasonality marker, may also be regulated by the circadian system. Dardente *et al.* had identified conserved E-boxes in the EYA3 gene promoter, which might be regulated by the circadian transcription factors CLOCK and BMAL1[21]. Furthermore, CRY proteins, which levels are regulated by PER, are being determined as the principal repressor of CLOCK-BMAL1 transcription. In this sense, melatonin signals produce a peak on CRY transcription during dusk, which repress the dimer CLOCK-BMAL1 until the dawn, when it is produced the activation of EYA3 transcription[3,21,39] (Figure 5). On this basis, it has been reported that the dimer CLOCK-BMAL1 can modulate TSHβ through E-box elements in mice but not in sheep[21,40].

Although all this mechanisms and pathways have been identified, cAMP circadian changes are linked to the seasonal oscillatory pattern of EYA3 expression. This cAMP model proposes that the duration of melatonin signal determines the daily oscillations of cAMP levels. Thus, on SD condition, the continuous secretion of melatonin supress cAMP releasing, and consequently, EYA3 transcription. However, in LD, the peak in EYA3 transcription is in light onset, which may be mediated by the increase of cAMP levels at dawn, which activates cAMP response element binding (CREB) site in the promoter of EYA3[1,3] (Figure 6).

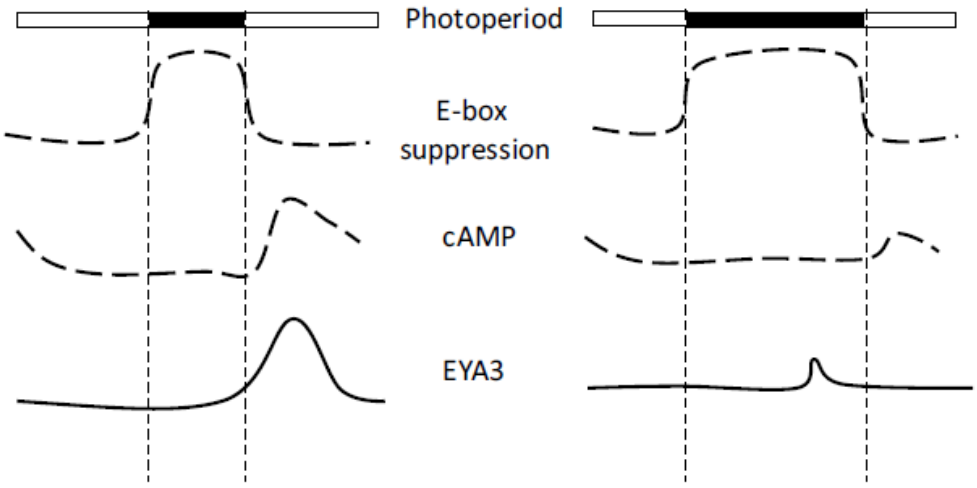

Figure 6. *Modulation of Eya3 levels through changes in cAMP levels. E-box, enhancer box; cAMP, cyclic adenosine monophosphate; Eya3, eyes-absent 3. Figure adapted from Wood et al., 2018.*

In conclusion, all these data show a clear relationship between circadian and circannual rhythms, which are regulated by external photic changes and intrinsic physiological adaptations. Thus, despite more research is still needed in order to underscore the exact relationship between both oscillators, it is accepted that the main elements of circadian rhythms also control circannual rhythms depending on photic signals but also on the timing, phase and amplitude of the changes induced in the signals.

b) Circannual rhythms' relevance for the control of behaviour and physiology

Adaptative changes in behaviour and physiology are the most important aspects for organism's survival in seasonal environments. This concept, which has shaped evolution of species, can be translated into current human societies as the fact that seasonal variations might impact health and disease. For this reason, having a general idea of which are the principal alterations produced by seasonal changes is considered a good starting point for the development of strategies to optimize health status, understood as species' survival in evolutionary terms.

i) Seasonal control of neuroendocrine function

Seasonal rhythmicity has been associated with some structures and mechanisms established as rhythmic generators. Hypothalamus and hypophysis are the main centres of rhythm and recent studies had pointed out the hypophyseal pars tuberalis and the hypothalamic tanycytes as key mechanisms in seasonal timing[28,41]. The PT highly express melatonin receptors and is considered the link between nocturnal secretion of melatonin and photoperiodic changes[10,11]. PT also regulates tanycytes from hypothalamus via paracrine signals. These cells present robust seasonal modulations in their gene expression, related with the transport and metabolism of thyroid hormone[1]. Furthermore, TH is a key element in the control of seasonal responses in mammals, being modulated by melatonin signals from

pituitary[1,41]. These modulatory mechanisms drive the neuroendocrine modulations that are associated with circannual rhythmicity, affecting different axis, such as hypothalamic-pituitary-thyroid (HPT), hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary-adrenal (HPA) axis (Figure7).

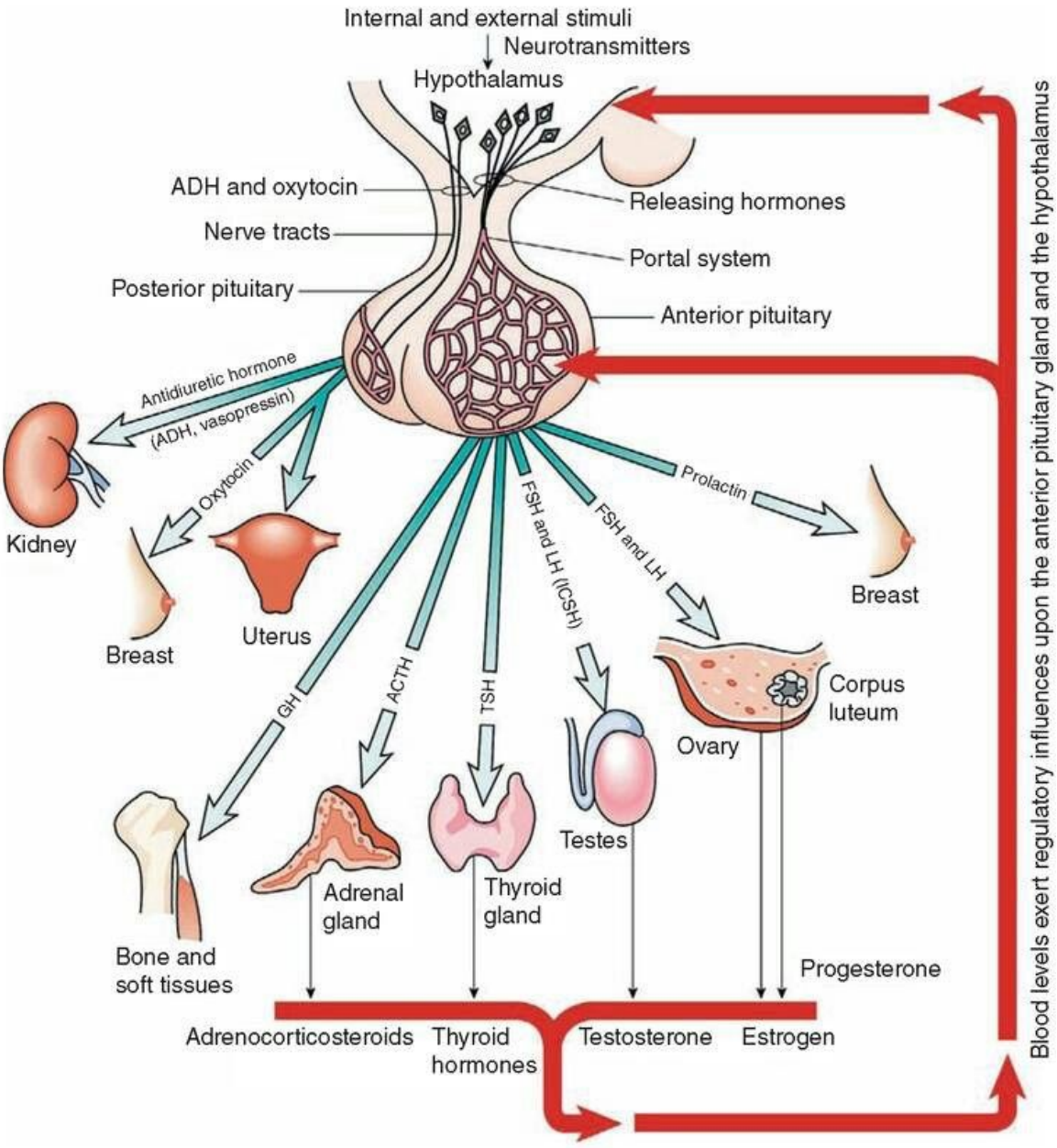

Figure 7. *Hypothalamic-pituitary regulation of different neuroendocrine axis. ADH, antidiuretic hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; ICSH, interstitial cell stimulating hormone; TSH, thyroid-stimulating hormone; ACTH, adrenocorticotropic hormone; GH, growth hormone. Figure from Smeltzer et al., 2000.*

Hypothalamic-pituitary-thyroid axis

HPT axis controls metabolic processes and functionality throughout the organism, in addition of being related with seasonal rhythms. Specifically, it could be related with body heat, metabolic processes that generates energy, tissue growth and the optimum functionality of the nervous, the cardiovascular or the gastrointestinal system[42–44]. The regulatory mechanism of this axis starts with the secretion of TRH from the hypothalamus. This TRH signals to the anterior pituitary, where it increases the secretion of TSH which, in turn, promote the release of T3 and T4 by the thyroid gland. TH (concerning both T3 and T4) act as negative feedback signal on the hypothalamus and hypophysis, blocking the synthesis of extra hormone[42,44] (Figure 8). As has discussed previously for hypothalamus (section 1a), intracellular signalling of TH in peripheral tissues is dependent on the activity of diodinases[29,30] .

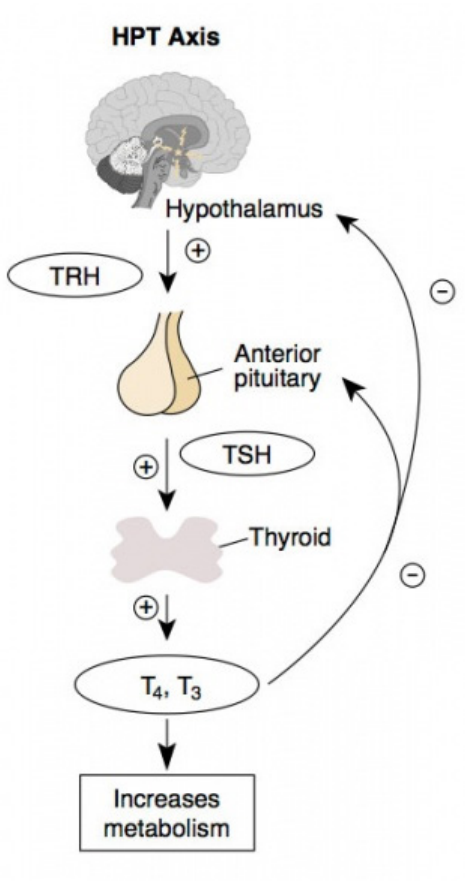

Figure 8. *Schematic representation of the hypothalamic-pituitary thyroid (HPT) axis. TRH, thyroid-releasing hormone; TSH, thyroid-stimulating hormone; T4, thyroxine; T3, triiodothyronine. Figure adapted from Hiller-Sturmhöfel et al., 1998.*

Thyroid hormone pathway had been related with the control of breeding in different species[1,7,39]. Early studies removing the thyroid gland, known as thyroidectomy (TX), which blocks the seasonal response to photoperiod, produces significant changes in the seasonal-related gonadal response in different animals, such as in sheep and quails[45,46]. Currently, it has been reported that a single thyroid hormone administration in PX-animals could restore the seasonal response related

with breeding. In ewes, different studies had reported that T4 replacement after TX produce a direct compensation in the levels of LH, which was used to test the reproductive system alterations[47,48].

Although there is a clear connection between TH metabolism and seasonal breeding, it still remains poorly understood which is the specific regulatory mechanism of that. Rfamides (RF), which includes kisspeptin (Kp), and RF-related peptides (RFRP) are recognized for controlling reproduction in a photoperiod-dependent manner[49]. Kp and RFRP modulates gonadotropin releasing hormone (GnRH) neurons, synchronizing reproduction with season. In this sense, taking into account that its regulation is done by melatonin and not for the seasonal-derived sex hormonal changes, Kp and RFRP could be considered a great candidates for being the key point in the regulation of seasonal breeding with melatonin-related photoperiodic changes[50,51]. Klosen *et al.* have proved in Siberian and Syrian hamsters that TSH increases Dio2 and restores Kp and RFRP expression to LD condition, activating the gonadal axis[52]. Furthermore, T3 administration to Siberian hamsters housed in SD condition activates the gonadal axis and established the RFRP-LD levels[53]. This studies shows that the RFRP neurons are modulated by thyroid hormone pathway, which finally controls GnRH expression, being a link between photoperiod-dependent thyroid hormone modulation and gonadal axis[1].

Exposition to cold temperatures are also related with changes between seasons, which produces the development of adaptative thermogenesis in homeothermic animals. In order to maintain the basal body temperature, two forms of thermoregulatory heat production have been proved. Shivering thermogenesis, which are the based on muscular contractions, and non-shivering thermogenesis (NST), which is focused in the brown adipose tissue of mammals. This NST is controlled by thyroid hormone through increases in Dio2 expression in the brown adipose tissue of small rodents[54].

Hypothalamic-pituitary-gonadal axis

HPG is essential for the reproductive functions of the body, but they also play an important role in the metabolism of carbohydrates and lipids, in the cardiovascular system and are crucial for bone synthesis[42,55]. Hypothalamic gonadotropin-releasing hormone (GnRH) induces the releasing of luteinizing hormone (LH) and follicle stimulating hormone (FSH) by the pars distalis of the anterior pituitary. These two gonadotropins reach sexual organs and activate the synthesis of testosterone in males and oestrogen and progesterone in females. These sexual hormones act as a modulators of the HPG through its inhibitory activity in hypothalamus and pituitary[42,43] (Figure 9).

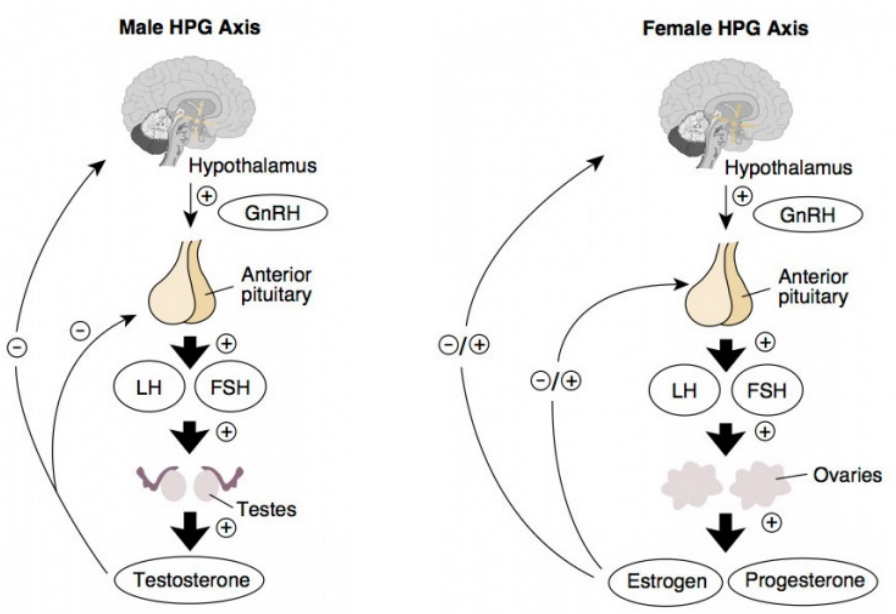

Figure 9. *Schematic representation of the hypothalamic-pituitary gonadal (HPG) axis and its gender-based variations. GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone. Figure adapted from Hiller-Sturmhöfel et al., 1998.*

Mammals can be classified as seasonal or non-seasonal breeders, according to its reproductive mechanisms. Non-seasonal breeders are those animals which live in tropical areas, where the environmental circannual changes are almost unnoticed. However, seasonal breeders develop reproductive physiology and behaviour during a specific time of the year, in order to deliver between spring and early summer,

when the environmental conditions are the most favourable for the survival of the offspring[56,57]. Moreover, seasonal breeding animals could be also classified in LD or SD breeders, based on when the animals are fertile according to day length. For LD breeders reproductive time is from spring to early summer, whereas SD breeders the breeding is during autumn and early winter, depending on their pregnancy length[57].

In vertebrates, gonadal development and steroid hormonal production is regulated by the HPG axis, which shows a photoperiodic response due to its activation during their specific breeding season[57]. In quails, it has been reported that there is a clear seasonal response in the gonadal development and regression. At SD, the changes in the gonads were imperceptibles, whereas in LD, it only takes two weeks to show higher blood levels of gonadotropins and an increase of 100% in the testicular weight[58]. These morphological modulations could be associated with changes in TH, which had been related with seasonal plasticity[1,27]. GnRH neurons reach ME with their terminals rich in TH receptors. Yammamura *et al.* showed, via electron microscopy, that the morphology of GnRH neurons varies according to photoperiod. GnRH end terminals were blocked in SD conditions, while in LD, their terminals contacted with capillaries, allowing GnRH release to the bloodstream[59]. These changes could be related with LD-like phenotype, allowing gonadal modulation by T3 activity in ME[1,27,57].

Hypothalamic-pituitary-adrenal axis

HPA axis is responsible for the control of stress and regulates main body processes, such as digestion, immunity, mood and energy expenditure, in the presence of an external stressful stimulus. Hypothalamic neurons secrete vasopressin and CRF, which increase the adrenocorticotropic hormone (ACTH) release from the pituitary gland. The adrenal cortex is the target organ where ACTH stimulates the secretion of cortisol, which acts back on the hypothalamus and

pituitary to inhibit ACTH release, therefore closing a negative feedback mechanism[60] (Figure 10).

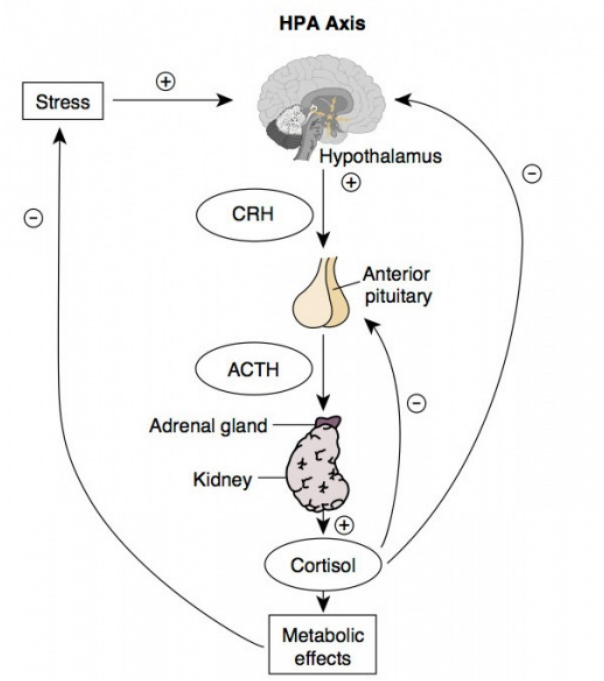

Figure 10. Schematic representation of the hypothalamic-pituitary adrenal (HPA) axis. CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropic hormone. Figure adapted from Hiller-Sturmhöfel et al., 1998.

A bidirectional communication exists between immune system and HPA axis. Immune cells can activate HPA axis through cytokines at hypothalamic, pituitary and adrenal level, stimulating the production of glucocorticoids. In turn, glucocorticoids acts on the receptors of immune cells blocking the induction of proinflammatory response, producing an inhibition of proinflammatory cytokines and increasing the levels of anti-inflammatory molecules. Furthermore, ACTH acts on this pathway producing anti-inflammatory effects through melanocortin system[61].

In mammals, immune system is regulated by the circadian clock, being some related mechanisms modulated by circadian rhythms, such as tissue recruitment of immune cells, antigen presentation or lymphocyte proliferation[62,63]. Furthermore, some diseases which presents an inflammatory profile, such as asthma and

rheumatoid arthritis, vary in severity during the day, showing a circadian oscillatory pattern in their affectation[64,65]. Some studies revealed that alterations in the rhythmic patterns, such as in shift workers or in jet lag, leads to a dysregulation in the immune system[66,67]. Immunes cells present circadian clock components, including macrophages and lymphocytes[68]. Cuesta *et al.* had reported that in humans under constant routine conditions, supplementation with glucocorticoids produce alterations in the circadian rhythmicity of peripheral blood mononuclear cells (PBMCs). This modulation of circadian clock was not related with changes in plasma melatonin or cortisol levels[69]. Thus, this fact link HPA axis regulation with immune cell functionality.

Seasonal regulation of lactotrophic axis

Prolactin (PRL) secretion from PD, specifically from lactotrophs, is known to be independent of the TH regulation and hypothalamic control[39]. Indeed, PRL levels are considered a clear photoperiodic marker because of its invariably response to seasonality, increasing in LD condition in all vertebrates[70,71]. It has been reported that lactotrophs do not express melatonin receptors[3]. In addition, studies with sheep revealed that the control mechanism is also independent from hypothalamus. Surgical disconnection between pituitary gland and hypothalamic region produces a reproductive block due to its lack of GnRH neuronal regulation but the seasonal control in PRL levels were maintained[72]. So, PT controls PRL production and secretion from PD through other photoperiod-sensitive intermediate molecules, which had been designated as "tuberalins"[2].

Various studies have search possible molecules that could be a good candidate for being stablished as tuberalins. Dupre *et al.* showed that PT tachykinins 1 and neurokinin A levels are upregulated in LD-housed sheep. These molecules are related with PRL enhancement in *in vitro* experiments with ovine pituitary cells[70]. Other molecules designed as potential candidates are endocannabinoids, lipid

derivatives with an important role in many biological functions. Experiments with hamsters demonstrated that, 2-arachidonoylglycerol, which is produced in PT, increases prolactin secretion in presence with adenosine or forskolin[73]. But in 2017, Castle-Miller *et al.* had demonstrated that vascular endothelial growth factor (VEGF) influences prolactin cells in PD. This factor is modulated by the melatonin signal length, showing different splice forms with different activity according to the season[74].

Refractoriness

Photoperiodism in seasonal animals is associated to a register system for day length, translating them into a neuroendocrine response. This mechanism is well stablished in vertebrates and invertebrates responding to environmental changes[75]. When maintained for long periods, however, this seasonal response could produce the known refractory state.

Refractoriness is an intrinsic mechanism whereby animals turn out insensitive to an immediate stimulation due to its prolonged exposure to other stimulations. One example could be the seasonal pituitary prolactin rhythmic secretion. In hamsters, activation of prolactin release is done in LD conditions, while SD photoperiods reduce its secretion. This changes in the endocrine system are associated with marked changes in pelage and development of winter-derived coat. Nevertheless, a prolonged exposure to a SD photoperiod produces a return (a refraction) to the levels of prolactin associated to LD (Figure 11).

This kind of responses are prevalent in the vast majority of photoperiodic species, which the first response to photoperiod produce a marked change during weeks or months, but finally, it leads to a reversal of phenotype[1].

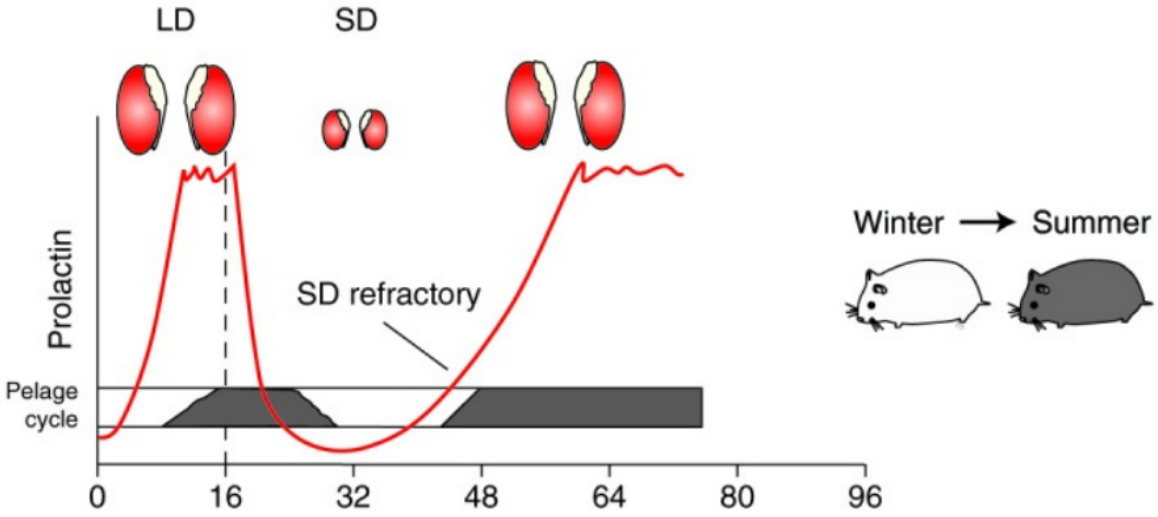

Figure 11. Seasonal prolactin rhythm in male Siberian hamster. LD, long day; SD, short day. Figure adapted from Wood et al., 2014.

ii) Effects of seasonal rhythms on neurotransmitter signalling

It is known that each mammalian neuron can release more than one kind of neurotransmitter, including small-molecules and peptides. These substances can produce a wide range of different effects in their specific target neurons, modifying some molecular mechanisms in adjacent cells. In that sense, the neuronal neurotransmitter fingerprint release is important for comprehending how the system works and its phenotypical response. Thus, the study of the modulation of this fingerprint could be interesting for comprehend neuroplasticity in the adult brain and, consequently, for understanding the final alterations in behaviour and physiology[76].

It has been reported that alterations in photoperiod, which entrains circadian and circannual rhythms, could produce changes in the neurotransmitter profile of mammalian neurons, which, in turn, are related with changes in behaviour. Dulcis *et al.* characterized the mammalian plasticity in response to the dark-light cycle, through changes in two specific neurotransmitters, dopamine and somatostatin (SST)[77]. Dopamine is an important mediator of the brain function, modulating cognition, mood and memory, while SST is implicated in the response to stress[76].

Both of them are also considered critical regulators of functionality in pituitary gland, inhibiting pituitary secretion of different hormones, including TSH, PRL and growth hormone (GH)[76,78]. In that sense, Dulcis *et al.* had reported a clear switch between dopamine and SST expression in rats housed in two different light schedules, LD and SD. The results show that while the number of dopamine-releasing cells were higher at SD and lower at LD, the inverse was seen in the case of SST. These results are consistent with the number of dopamine and SST receptors (D2R and SST2R or SST4R, respectively) in the target neurons, which are increased and decreased to guarantee that the neurotransmitter signal produces a final functional result[77] (Figure 12). Thus, D2R to SST receptors ratio might be used as a photoperiod biomarker to evaluate the response of dopaminergic and somatostatinergic system to seasonality.

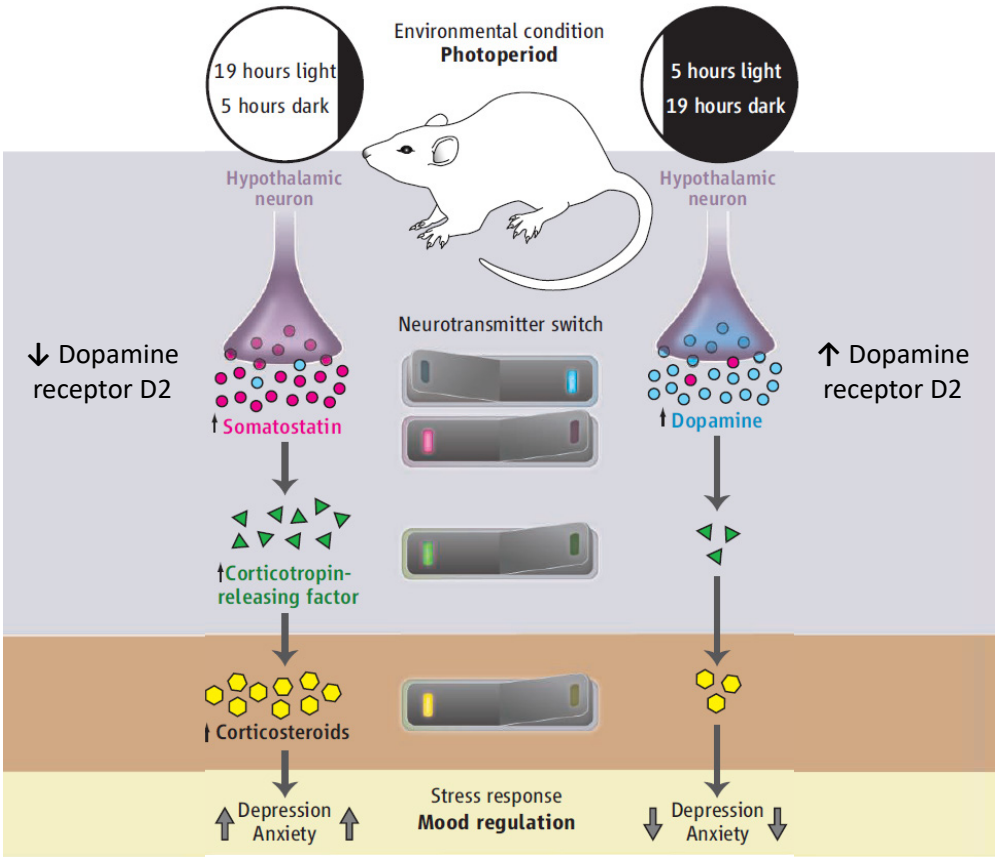

Figure 12. Neurotransmitter switch according to light length and its relation with mood regulation. Figure adapted from Birren et al., 2013.

Considering that photoperiod and dopamine/SST systems had been reported to be linked with mood and behaviour, Dulcis *et al*. studied whereas photoperiodic changes of these neurotransmitters are also linked with changes in behaviour[77,79]. Experiments using different kinds of behavioural tests showed that LD photoperiod is more stressful for rats than SD[77,79,80]. This stress response could be linked with corticotropin-releasing factor (CRF), a protein which secretory pattern is considered photoperiodic-derived. Dulcis *et al*. reported that alterations in SST/dopamine signalling are correlated with changes in CRF levels[77]. CRF, through the HPA axis, increases the levels of circulating corticosteroids, which have been linked with stress and depression mood[80,81]. With this data, Dulcis *et al*. demonstrated the direct relationship between photoperiod, neurotransmitter plasticity and mood regulation.

Therefore, responses to seasonal changes involve different systems in the brain that produce alterations at neuroendocrine, behavioral and physiological level in animals, modulating some pathways and structures considered to govern biological timing, such as hypothalamus and pituitary gland.

2. Biological rhythms and nutrition

Mammals synchronize, through the daily cycle of light and darkness, their behavioural and physiological rhythms with the environment they are living in. They have developed a rhythmic system to adjust their daily activities to these climate conditions, which consists on a central clock, located in the hypothalamic-pituitary zone, and peripheral clocks, found in most organs and tissues[82,83]. Melatonin adjust all clocks to the exactly environmental period. Nevertheless, peripheral clocks have been reported to be synchronized not only by melatonin, but also by hormonal, neural and behavioural signals. Regarding behavioural parameters, one of the most important clock regulators is feeding patterns[83]. The interaction among circadian oscillations and the components of foods, including daily dietary patterns, have gained much attention during the last years due to the relevance that biological rhythms have on human health. This interest has led to the emergence of chrononutrition as a novel discipline[84].

a) Gut-brain axis: Brain modulation by nutrition and obesity

Brain circuitry can be modulated by nutrition through the commonly known as gut-brain axis. This axis also includes, inherently, gut microbiota, which is an important factor in the formation of new signalling molecules from food. This axis is considered bidirectional, wherein the brain regulates the activity of gut and vice versa through different routes and pathways[60]. These circuits integrate all the signals originated in the digestive tract, through immune, neuroendocrine and neural

pathways, which are related with modulation of brain development, function and even behaviour[60,85].

All this information exchange could be divided in two different pathways, the afferent (brain-gut) and efferent (gut-brain) mechanism. Interestingly, the HPA axis represents the principal dual route in the organism. Considering that HPA is the principal stress modulator, its action directly influences the activity of some cell lines included in the enteric nervous system[86,87]. Furthermore, gut microbiota controls the secretion of different immune activators, such as tumor necrosis factor alpha (TNF-α), IL-1β and IL-6, which could reach the brain and finally stimulate the HPA axis[60]. Sudo *et al.* studied the role of microbiota in stress development through experiments with germ-free mice. In these studies, germ-free mice presented altered HPA function, with elevated levels of ACTH and cortisol, as well as reduced brain-derived neurotrophic factor (BDNF), compared with specific pathogen free (SPF) mice. Moreover, when germ-free mice were transplanted with SPF microbiota, they presented a normal HPA axis functionality[88]. This information confirmed the importance of gut microbiota in the normal development of HPA axis and their connection with behaviour.

Immune mediators or food-derived metabolites from microbiota action reach some specific brain regions via median eminence of the neurohypophysis. The median eminence is a brain structure that is located outside the blood-brain barrier (BBB) and is contacted by tanycytes, whose soma is included in the third ventricle in the mediobasal hypothalamus. It has been suggested that these cells possess barrier properties and provide a link between ventricular and blood system, by projecting its processes to the capillaries in the median eminence[41,85,89]. In the last decade, tanycytes have been related not only with being part of the BBB, but also with seasonality, as has been commented previously, and for being nutrient and hormone sensors[89]. This last function is associated with the modulation of its morphology

around the neurosecretory terminals in the median eminence, modulating the brain permeability to different blood components[41]. This characteristic has been reported by Langlet *et al.*, who demonstrated that the decrease in blood glucose under fasting conditions increase the vascular permeability in the brain by changes in the morphology of tanycytes. The modulation of the barrier structure was due to changes in the expression of VEGF, which was reversed when animals were refed[90] (Figure 13). These BBB alterations suggest that the nutritional state of the animal changes the access of blood metabolites to central nervous system, specifically hypothalamic structures.

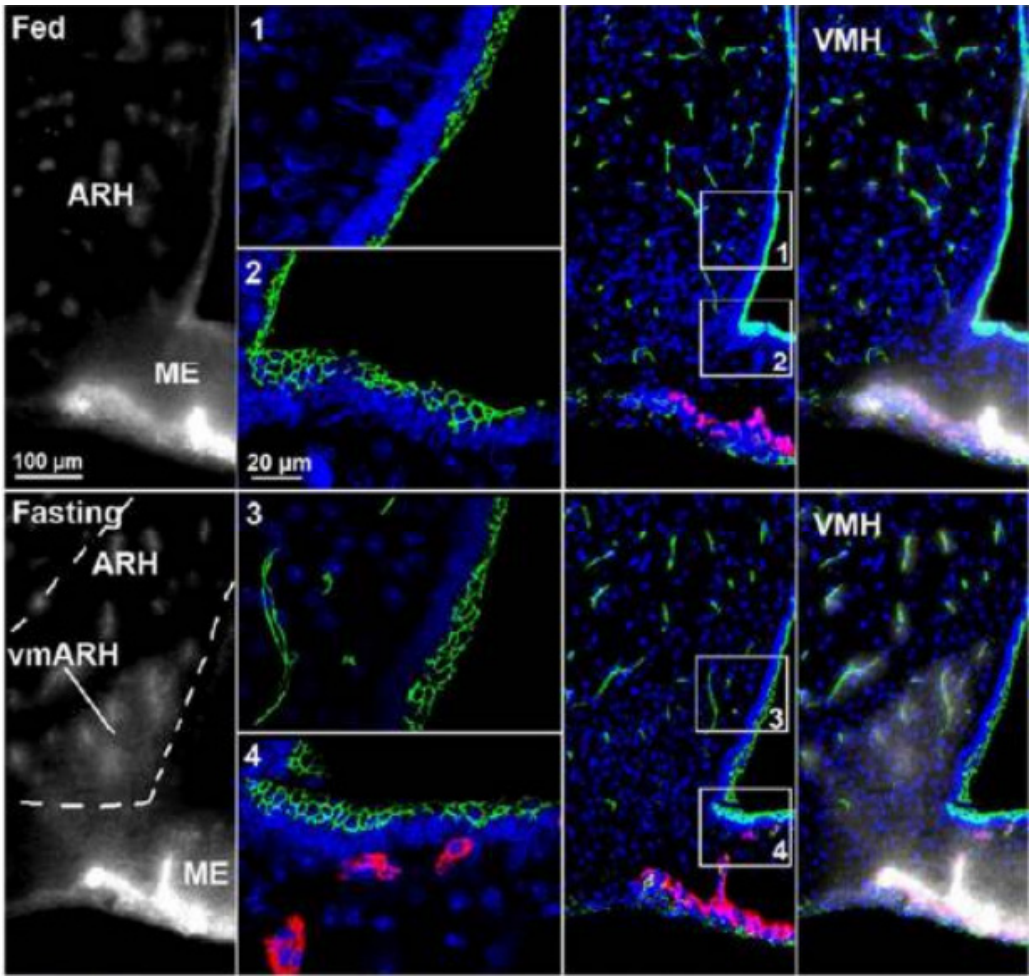

Figure 13. *Fasting-induced structural changes in the hypothalamic tuberal region in fed and fasting mice. ARH, hypothalamic arcuate nucleus; ME, median eminence; VMH, ventromedial nucleus of the hypothalamus. Figure adapted from Langlet et al., 2014.*

But not only nutrition can modulate these brain structures. Also, obesity has been reported to modulate the different components of the gut-brain axis. It has been described that obesity produces changes in gut microbiome, decreasing the gut microbial diversity and changing the proportions of its taxonomic composition[91–93]. Del Bas *et al.* associated these changes with the different physiological effects of diet, suggesting that not only obese state could produce these alterations, but also diet could be responsible of that[93]. Modulation of this proportions could lead to effects on central physiology, modulating the gut-brain axis and, in turn, behaviour and stress response[85,94]. Maternal obesity has been associated with neurological disorders in offspring[95]. In this sense, Buffington *et al.* showed that obese offspring of rats fed with an HFD during their pregnancy present behavioural deficits, which could be associated with alterations in their gut microbiota. Furthermore, the gut microbial diversity of this offspring was reduced and associated with important alterations in the dopamine system[96]. These data show the evident link between obesity, gut health and brain modulation related with social behaviour.

Therefore, the different elements and structures involved in the gut-brain axis might provide a comprehensive view of how food and nutrients may modulate hypothalamic activity and interact with the different components of seasonal, neuroendocrine and neurotransmitter control.

b) Interaction between nutrition and rhythmic regulatory components

Chrononutrition is a field that studies the relationship between nutrition and the biologic rhythmic system. It is known that melatonin signals are considered the principal synchronizer between environmental changes and the rhythmic biology, but currently, feeding had gained importance as a modulatory rhythmic factor. Furthermore, it is important to take into consideration that, beyond the intrinsic

nutrient fingerprint of food, also the meal timing, the feeding/fasting cycles, the nutrient and energy distribution and the consistency of meals can affect the final behavioural and physiological response[84,97]. The rhythmic system is composed by molecular clocks located in all the organism, both in the central system and in periphery. These non-brain areas that contains its own endogenous clock are synchronized by the brain through hormonal and autonomic signals. Therefore, nutritional state of the animal, among other external signals, can also regulate peripheral clocks[98,99].

i) Nutrition and circadian rhythmicity

Food composition has been reported to be related with alterations in circadian rhythms in mammals. Concerning dietary macronutrients, high-fat diets (HFD) have been associated with alterations in expression of clock genes in peripheral tissues, as well as with modulation of behaviour[100–102]. These effects could be a consequence of altering different factors, such as diet composition among others. Thus, it has been described that protein-rich diets modify clock genes in kidneys and liver of mice, compared to a standard diet[103]. Also Oishi *et al.* have reported that locomotor activity and body temperature remained normal in high protein diet fed animals, although they present a slight hypoglycaemia and alterations in plasma insulin and no effects on body weight[103]. Some studies have reported that HFD interfere with circadian rhythmicity, modifying molecular and biochemical circadian rhythmic biomarkers[100,104]. Kohsaka *et al.* assessed how circadian clock system was affected by the consumption of an HFD. Wild-type mice fed with HFD showed significant differences in the free-running period of the clock only two weeks after the beginning of the nutritional intervention, and the alterations persisted during several weeks afterward (Figure 14). Furthermore, HFD animals showed an increase of their daily calorie intake during the light phase. Thus, while standard (STD) fed animals consumed approximately a 20%, those fed the HFD consumed a 30% of the daily intake[100,104]. These studies demonstrated that the consumption of an HFD can

modify biological rhythms at a behavioural and a molecular level, suggesting that food could be considered an external, non-photic cue that influence the rhythmicity of the clock.

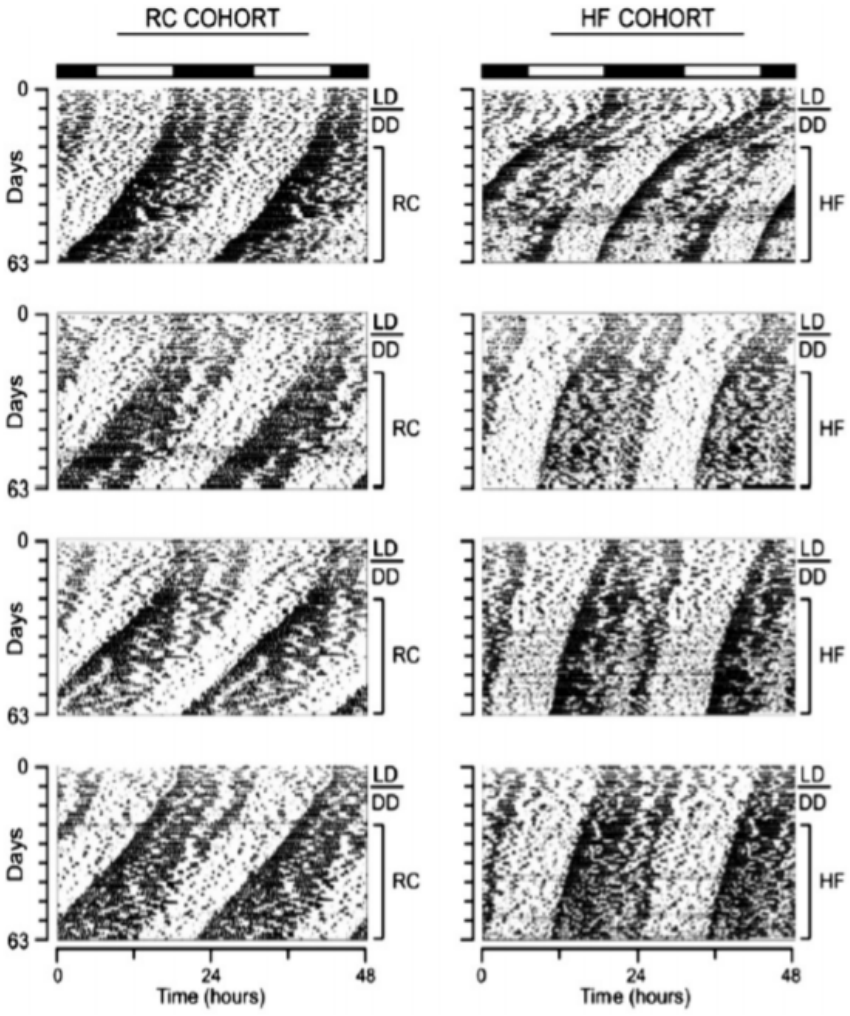

Figure 14. *High-fat diet lengthens the circadian period in mice. RC, regular chow; HF, high fat; LD, light:dark; DD, constant darkness. Figure adapted from Summa et al., 2014.*

HFD have been also related with alterations in neurotransmitter systems, which are related with mood and behaviour and can be modulated by photoperiod[76,77]. First, it is interesting to remark that the modulation of dopaminergic system can be produced by either itself diet or by obesity, factors that may operate independently one of another[105]. It has been reported that the combination of sugar and fats can reduce the dopamine signalling, altering the dopamine control of carbohydrate intake[106]. In agreement, some studies show that cafeteria (CAF) diet decrease

dopamine neurotransmission and produce behavioural changes[107–109]. Moreover, there are also studies which fat intake shows an increase of the release of dopamine[110]. All these data suggest that specific food consumption can modulate photoperiodic-sensitive pathways related with rhythmic biology.

In addition of dietary macronutrients, non-essential compounds are also known to exert an influence on the rhythmic system. Currently, dietary polyphenols, which are found in specific foods and are related with beneficial metabolic effects, are also associated with modulation of circadian rhythmicity. Specifically, resveratrol and proanthocyanidins (PAs) have been reported to induce changes in the circadian clock[111]. Resveratrol, which can be found in red wine and dark chocolate, has been reported to adjust locomotor activity and body temperature rhythms in gray mouse lemurs[112]. Miranda *et al*. described that in rats fed with a HFD, treatment with 30 mg/kg of resveratrol reversed the alterations produced by the diet on the expression of clock genes in the adipose tissue[113]. PAs, with presence in vegetables, cacao, red wine or tea, could modulate peripheral clocks of normoweight and obese rats and the rhythmic pattern expression of clock genes in liver and hypothalamus of healthy rats[114,115]. Moreover, it has been reported that the time of treatment administration modulates the final effect of PAs consumption. Ribas-Latre *et al*. showed that Wistar rats orally gavaged with grape seed proanthocyanidin extract (GSPE) present different response depending on the time of administration. Thus, when treatment was administered at Zeitgeber (ZT) 12 (lights off), i.e. 12 hours after lights are on, GSPE did not modulate the nocturnal expression of clock genes, whereas in rats treated in ZT0 (lights on), i.e. at the moment of lights on, GSPE induced changes on the circadian oscillation of plasma melatonin and different metabolites, and modulated the gene expression level of hypothalamic clock genes *Bmal1* and clock controlled *Nampt*[116]. Furthermore, taking into consideration the HPT axis and its modulatory activity on rhythmic parameters, Da-Silva *et al*. reported that *in vitro* treatment with kaempferol, a polyphenolic bioactive compound present in grapes

among other vegetables, increased the expression of DIO2 in human myocytes. The increase in *Dio2* mRNA levels was associated with increased intracellular T3[117].

Apart from diet, obese condition has also been associated with alterations in circadian rhythmicity, including disruption of clock genes, physiology, eating behaviour and activity[102,118–121]. Mantele *et al.* reported that obese subjects present higher levels of nocturnal plasma melatonin compared with the normoweight group, maintaining the leptin levels invariable among groups[122]. At a molecular level, it has been reported in genetic-obese mice that the rhythmic expression of the major clock genes were attenuated compared with non-obese group[123]. Furthermore, different preclinical works show that diet-induced obesity modulates feeding circadian rhythms and locomotor activity, as well as HPT axis functionality[102,118,124]. Hence, appropriate nutritional approaches with an optimal synchronization between energy intake, energy expenditure and feeding/fasting cycles, are the key point in the maintenance of healthy behavioural and physiological rhythmicity.

ii) Circannual rhythms: modulation of nutritional patterns

Seasonal rhythms have been less studied in the context of nutrition. Moreover, there are few studies which investigate photoperiod effects with a nutritional approach. Fine *et al.* studied if the preference for specific food types could be due to the photoperiod, through modifications in the gustatory processing, in Siberian hamsters[125]. Firstly, it was corroborated that hamsters housed in SD conditions showed marked preferences for carbohydrate- and protein-rich diets, compared with LD animals. A secondary study was performed in order to determine if these differences were caused by the SD exposure or by the reduction in body weight associated with the short winter-like days. Results confirmed that changes in the selection of specific macronutrient-rich diet cannot be associated with a decrease in body weight, but with the change in photoperiod[125]. More recent studies have reaffirmed the preference for high-caloric meals in SD animals, while rats fed with a

HFD increased *ad libitum* consumption of sucrose in SD conditions, compared with a control diet[126]. Behavioural changes could be associated with the symptoms of seasonal affective disorder, which involves depression and a higher preference for carbohydrate-rich meals and increased adiposity depots[126]. Beyond the relationship between photoperiod and food preferences, there is no information about how nutrition modulates some parameters related with alterations in seasonal body rhythms. Further studies focused on this association would be necessary to clarify this issue.

c) Xenohormesis, polyphenols and rhythmic modulation

In recent decades, Howitz and Sinclair developed a theory called Xenohormesis[127]. This concept, which was first named in 2008, is based in two streams of scientific thought. The first concept is the adaptative stress response to physical, chemical and social stressing factors, which are fundamental for organisms' survival[128,129]. Related with this idea, it has been suggested that low levels of stress can enhance organisms' health, stimulating their own compensatory response to stress, known as hormesis[130]. The second concept is based on the link between living organisms on Earth. Success or failure of one species depends on the environmental conditions and the success or failure of other species[131]. Hence, the Xenohormesis concept, whereby *xeno* means stranger or foreigner, is based on the benefits that one organism could take from the chemical stress-derived products of another. Normally, this concept is understood as the capacity of heterotrophs for sensing specific chemical cues synthesized by autotrophs under environmental stressful conditions, thus being able to adapt their physiology, metabolism and behaviour to incoming environmental changes[127] (Figure 15).

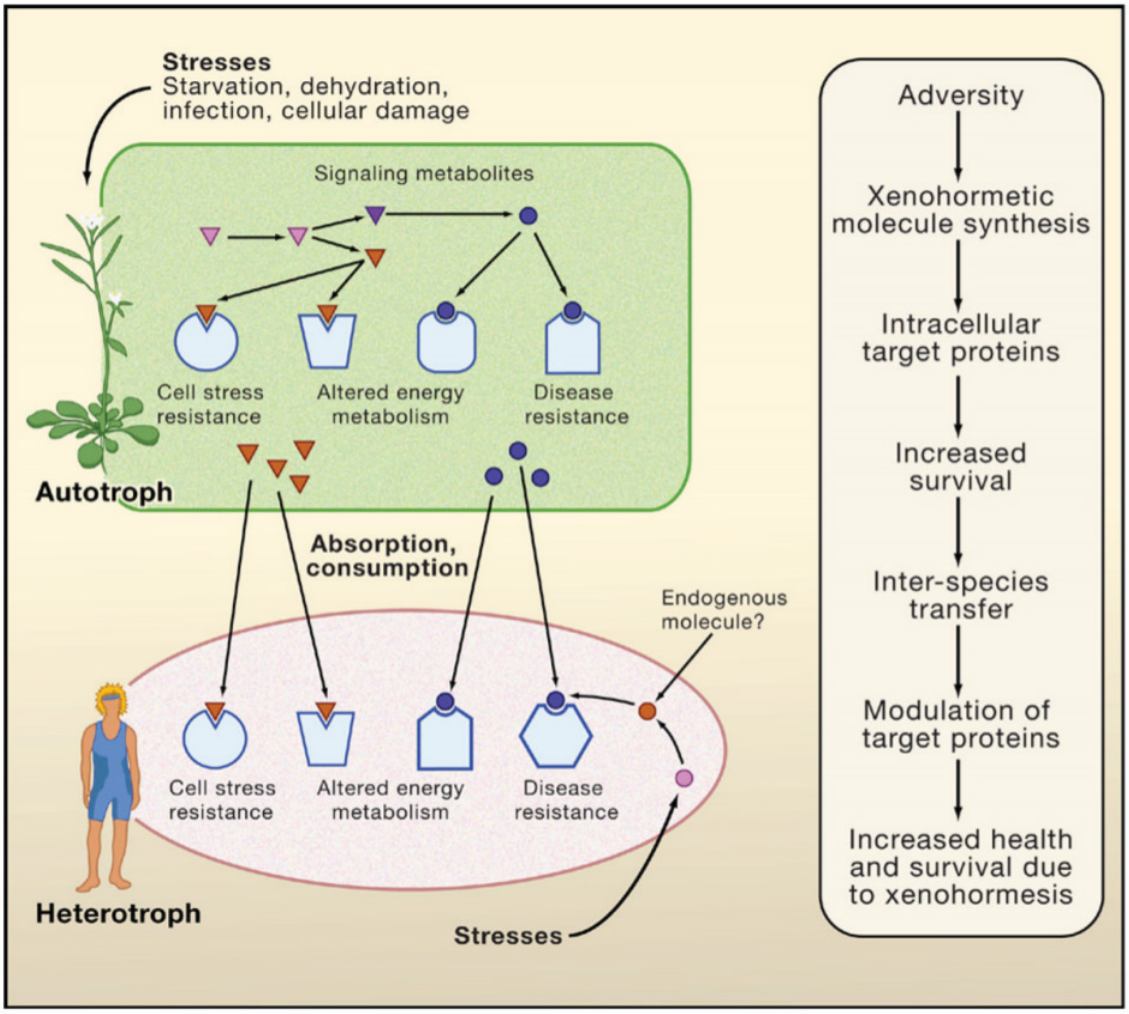

Figure 15. Scheme about the xenohormesis hypothesis. Figure adapted from Howitz et al., 2008.

Polyphenols, a structural class of natural compounds present in a wide range of plants, are secondary metabolites overexpressed under stressful situations, such as water restriction, pathogen infections or ultraviolet radiation[132]. Furthermore, it has been reported that biosynthesis of these compounds is modulated by changes in photoperiod and seasonality[132,133]. Although little is known regarding their biological relevance, it is generally accepted hat this kind of molecules are not essential for the nutrition, growth and development of plants, but might provide defence under awkward situations. In the context of Xenohormesis, polyphenols are proposed as a paradigmatic non-photic cue, indicative of incoming stressful environmental conditions for heterotrophs consuming vegetables as part of their

diet. In the context of human health, such a cue, that induces physiological and behavioural responses increasing the chance of survival for the animal, might be understood as a bioactive compound with beneficial effects for health.

Polyphenols have been shown to present different beneficial properties for health, such as vascular function, inflammatory response, neurodegenerative protection, diabetes and obesity[132,134–136]. Our research group has described that GSPE can modulate central and peripheral circadian clock genes, either in *in vitro* and *in vivo* models[115,116,137]. The rodent model used for the analysis were male Wistar rats, which were orally supplemented with different doses of GSPE. Procyanidins induced changes in the levels of melatonin, the principal photic cue for the regulation of the biological rhythms[116]. Results also showed that proanthocyanidins induce changes at a central level, modulating the expression pattern of different clock genes in the hypothalamus, and also in the periphery, with changes in gut, liver and adipose tissue in a dose-dependent manner[114,116]. Intriguingly, the effects of GSPE on elements of circadian rhythm control were dependent on the moment of administration. Thus, when the treatment was administered at zeitgeber time 0 (ZT0), GSPE blocked the reduction in blood melatonin that takes place during light hours, simulating a signal of dusk. In parallel, circadian modulation of hypothalamic genes *Baml1* and *Nampt* were altered. Moreover, GSPE administration at ZT0 blocked the circadian variation in plasma metabolites, which has been described as a putative indicator of internal body time. In contrast, administration of GSPE at ZT12, coincident with the moment of lights switch off, had no effects in the above mentioned parameters[116]. It was concluded that grape procyanidins were acting as a non-photic signal of dusk, maintaining melatonin, the central clock and the body time in a situation of night time. The different works of our group in the field of chronobiology concluded that polyphenols could be considered as a non-photic signal due to its capacity to modulate biological rhythms depending on its moment of consumption. Those observations were associated with the Xenohormesis and

the mechanisms controlling the response to seasonal changes, driving the conception of new hypothesis that resulted, among other works, in the current thesis.

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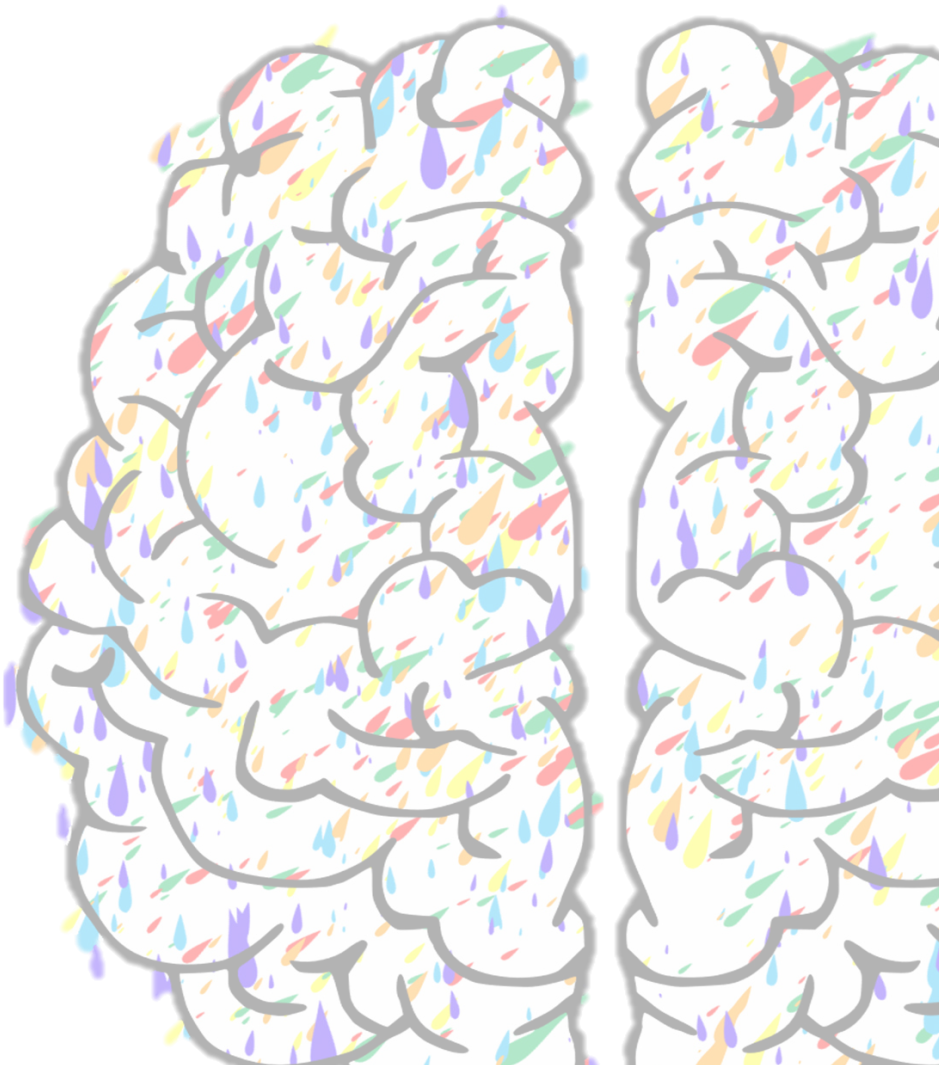
Hypothesis and Objectives



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Hypothesis and Objectives

Chrononutrition is a new discipline built on the close relationship between endogenous rhythmic patterns and metabolism. This discipline considers from intracellular biochemistry to whole-organism physiology. Nowadays, despite the mechanisms involved in such relationship are beginning to be understood, nutritional approaches embrace this new concept, taking into consideration meal timing and circadian variations in key metabolic processes and tissues, including liver, pancreas, white adipose tissue and skeletal muscle, in order to improve dietary advice. The principles of chrononutrition have already been demonstrated to have beneficial effects on human health, as well as to benefit individuals with metabolic diseases. In this scenario, seasonality, or circannual variations, is emerging as a biological rhythm associated to metabolic, neuroendocrine and neurotransmitter modulation with a plausible link with nutrition associated diseases.

Xenohormesis is a recent theory based in two streams of scientific thought. First, the basis of organisms' survival is the adaptability to physical, chemical and social stressing factors. Secondly, the success or failure of one species depends on the environmental conditions and the success or failure of others. Hence, the Xenohormesis concept is a biological principle based on the benefits that one organism could take from the chemical stress-derived products of another. These chemical stress-derived molecules produced by autotrophs, being polyphenols the paradigm, might allow animals to detect future predictable changes when the conditions are still favourable, thus increasing their capacity of survival. Translated into modern human health, these physiologically favourable modulations can be associated with different effects on health and wellness, such as preventing diabetes, cardiovascular diseases and, especially, obesity. Taking into consideration

that the generation of these signals are related with the exposition to different exogenous stimulus, such as photoperiod, seasonal shifts, extreme temperatures or dryness, the molecular fingerprint of plants could be contemplated as a source of biological information, providing information about the seasonal changes that are taking place.

In this context, it is plausible to hypothesize that consumption of seasonal vegetal foods in or out of its natural season might have different consequences in biochemistry, metabolism, physiology or even behaviour. This idea involves that the availability of whichever kind of food in any time of the year, facilitating the consumption of seasonal foods irrespective of its natural season, might result in erroneous signals that can alter or be altered by the control of biological rhythms, promoting the development of metabolic alterations linked to obesity.

Among the different seasonal foods, fruits are a suitable choice to assess such hypothesis, since these products are clearly representative of seasonal foods with an increasing availability all along the year thanks to the global market. Besides, fruits present a considerable number of polyphenolic compounds, standing as an appropriate food to be assessed in the context of the Xenohormesis theory.

Seasonal control is carried out by the interdependence between hypothalamus and pituitary, which directly influence neurotransmitter and neuroendocrine processes. Furthermore, alterations in such systems are a hallmark of obesity, although the consequences of these alterations on the elements controlling the response to seasonal shifts are still unknown. Therefore, the objectives of this thesis are:

1. **To determine the effects of seasonal fruits intake on central controllers of seasonality depending on its consumption in or out of season.**

   To achieve this objective, initially a method for simultaneous measurement of different key biomarkers of seasonality and neuroendocrine processes was developed (manuscript 1). The main objective was addressed by adapting healthy Fischer 344 to LD or SD photoperiod (in order to emulate spring/summer and autumn/winter season, respectively). Animals were orally supplemented with lyophilized cherry or red-grape (determined as spring and autumn fruit, respectively) during 14 weeks. Effects on pituitary and hypothalamic control of seasonality were assessed. This study revealed that red-grape but not cherry induce important changes in feeding behaviour, energy expenditure and locomotor activity in a photoperiod-dependent manner. These alterations could be associated with changes on central mechanisms subjected to seasonal control, such as the somatostatinergic and dopaminergic systems, and suggested beneficial effects in obesity (Manuscript 2).

2. **To determine the impact of cafeteria diet-induced obesity on the photoperiod-dependent modulation of neurotransmitter, neuroendocrine and physiological processes by red-grape.**

   To achieve this objective, Fischer 344 rats fed with a cafeteria diet were adapted to LD or SD photoperiod (in order to emulate spring/summer and autumn/winter season, respectively) and orally supplemented with lyophilized red-grape. Results revealed that cafeteria diet-induced obesity dampens the effects of red-grape in behaviour, in central controllers of seasonality and in peripheral tissues of normoweight animals (Manuscript 2 and 3).

3. **To determine the alterations caused by cafeteria diet-induced obesity in processes controlling the neuroendocrine response to seasonality.**
   To achieve this third objective, the response to photoperiod shifting and red-grape intake were evaluated in both normoweight and cafeteria diet-induced obese Fischer 344 rats. This comparison revealed that cafeteria diet-induced obesity impairs seasonal control of prolactin release and the response of HPA, HPG and HPT axis to changes in photoperiod, blunting the response to photic and non-photic cues (Manuscript 2 and 3).

This PhD doctoral thesis has been developed within the framework of the research project "Illegitimate signalling of fruit consumption and obesity pathogenesis" (AGL2013-49500-EXP), which purpose is to assess whether seasonal fruit consumption in and out of their natural season produce differential effects and might influence the development of metabolic alterations leading to obesity and its related diseases.

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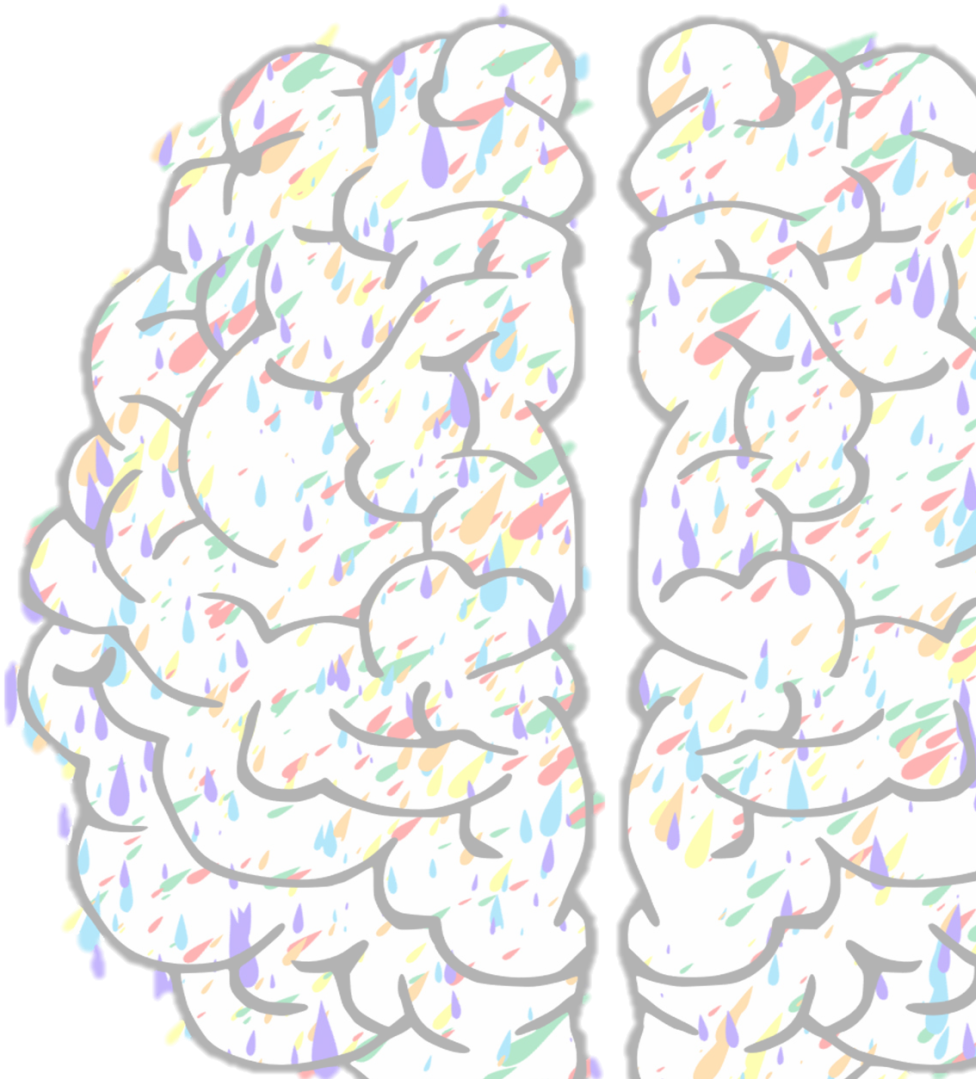
Results and Discussion



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Manuscript 1

Dual liquid-liquid extraction followed by LC-MS/MS method for the simultaneous quantification of melatonin, cortisol, triiodothyronine, thyroxine and testosterone levels in serum: Applications to a photoperiod study in rats.

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Highlights

- A new method for determination of different hormones in rat serum was developed.
- Conditions for the extraction of five endogenous hormones were optimized.
- The method has a good specificity, sensitivity and recovery.
- The method was successfully applied to a photoperiodic rat study.

Conflict of Interest

The authors have no conflicts of interest to declare.

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Abstract

Hypothalamic Pituitary (PH) axes directly affects the functionality of the thyroid gland, the adrenal gland, and the gonads and their alteration has been related to several pathologies. Therefore, the global analysis of a representative hormone from each axis, together with melatonin, would be a very good strategy for therapeutic diagnosis. Hence, an accurate, economic and effective analytical method has been developed and validated for the simultaneous measurement of the melatonin, cortisol, triiodothyronine (T3), thyroxine (T4) and testosterone levels in serum. The protocol consists of two liquid-liquid extractions followed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) analysis with electrospray ionization in positive mode. The isotopically labelled internal standards melatonin-D_4, cortisol-D_4, L-thyroxine-$^{13}C_6$ and testosterone-$^{13}C_3$ were added to serum samples. Multiple reaction monitoring (MRM) mode was performed to target fragment ions for the hormones and internal standards. Excellent linearity ($r^2 \geq 0.993$) of this method was observed within the concentration range of 0.004-0.5 ng/mL for melatonin and 0.4-50 ng/mL for cortisol, T3, T4 and testosterone in rat sera. The mean recovery of all compounds ranged from 62.6% to 97.3%. The relative standard deviations (RSDs) of intra-day and inter-day precision were within the acceptable limits of ±15% at all of the concentrations tested. The method developed here has been successfully applied to study the changes of these hormones induced by the duration of light exposure in rat serum, as a physiological model of HP axes modulation. The results obtained from rat sera showed the suitability of this analytical strategy.

Keywords

Melatonin, cortisol, triiodothyronine, thyroxine, testosterone, LC-MS/MS

1. Introduction

The hypothalamic-pituitary (HP) axis integrates multiple feedback mechanisms to control a large range of physiological systems through the synthesis and secretion of multiple trophic hormones [1]. Specifically, the HP axis directly affects the functions of the thyroid gland, the adrenal gland, and the gonads. In addition, melatonin interacts with HP axis functionality. Thus, because alterations in the levels of these hormones have been related to several pathologies, the global analysis of a representative hormone from each axis together with melatonin would be a very good strategy for medical diagnosis [2]. Specifically, mood disorders and depressive illnesses, such as bipolar depression and seasonal affective disorder, are related with melatonin, cortisol and thyroid hormone alterations [3,4]. This kind of diseases are not easy to identify. In this sense, a non-invasive methodology for their diagnosis could be beneficial for the general population. Other biologic processes in which this group of hormones display a key role are reproductive biology, metabolic pathways and tissue growth, like bone synthesis and adiposity [5–7].

Radioimmunoassay (RIA), a competitive binding immunoassay, is one of the most commonly used method for determining and quantifying hormone levels because of its high sensitivity, specificity and reproducibility [8]. However, this assay presents some limitations, such as cross-reactivity with unknown compounds, lengthy preparation steps, a requirement for large amounts of samples and the use of radioactive isotopes, which requires specialized analysts and equipment and a rigorous manipulation of the samples [9,10]. The enzyme-linked immunosorbent assay (ELISA) is also commonly used as a quantitative analysis of hormone levels because of its simplicity and its high sensitivity. Nevertheless, because each kit uses antibodies with different specificity from diverse sources, the reproducibility of hormone level measurements can be very low among assays [10,11]. The use of mass spectrometry approaches as a powerful tool for the quantification of molecules is gaining importance, as it overcomes all the above mentioned limitations [9,12].

Some authors have developed or improved liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) methods to measure specific hormones in different matrix. As an example, melatonin and cortisol have been quantified in saliva [13,14], thyroid hormones in the liver, heart or the hypothalamus [9] and testosterone levels were analysed in milk [15]. Moreover, in recent years several studies have analysed the circulating levels of these hormones in blood, avoiding the invasive detection analyses in tissues [16–19]. However, only few developed methods have simultaneously analysed the plasma or serum levels of different families of hormones that do not share the same structure or size. Hence, a new economic and effective method for a specific, reproducible and sensitive quantification of different hormones must be developed.

In this study, we present a sensitive and reliable LC-MS/MS method for the simultaneous determination of melatonin, cortisol, triiodothyronine, thyroxine and testosterone levels in serum. In addition, we have validated this method by measuring these hormones in rats placed at different photoperiods as a physiological model of HP axes modulation. Seasonal day length variations affect the secretion of melatonin, gonadotropins, adrenocorticotropins and thyroid hormones [20–23]. Melatonin is the first photoperiodic hormone identified as participating in all HP axes, providing the hormonal signal that transduces day length, whereas T3/T4, cortisol and testosterone are related to the thyroid, adrenal and gonadal axes, respectively [24].

2. Materials and methods

2.1 Chemicals and reagents

The analytes melatonin (≥98%), cortisol (≥98%), 3,3',5-triiodo-L-thyronine (T3) (≥95%), L-thyroxine (T4) (≥98%) and testosterone (≥99%) were obtained from Sigma-Aldrich (St. Louis, MO, USA) (Fig. 1). The internal standards melatonin-D_4 (≥98%), cortisol-D_4 (≥98%) and L-thyroxine-$^{13}C_6$ (≥98%) were purchased from Toronto Research Chemicals (Toronto, Canada); and testosterone-$^{13}C_3$ (≥98%) was obtained from Sigma-Aldrich (St. Louis, MO, USA). All solvents and reagents used in the present study were HPLC grade. HPLC grade water was obtained by ultrafiltration (Millipore Milli Q system, Bedford, MA, USA).

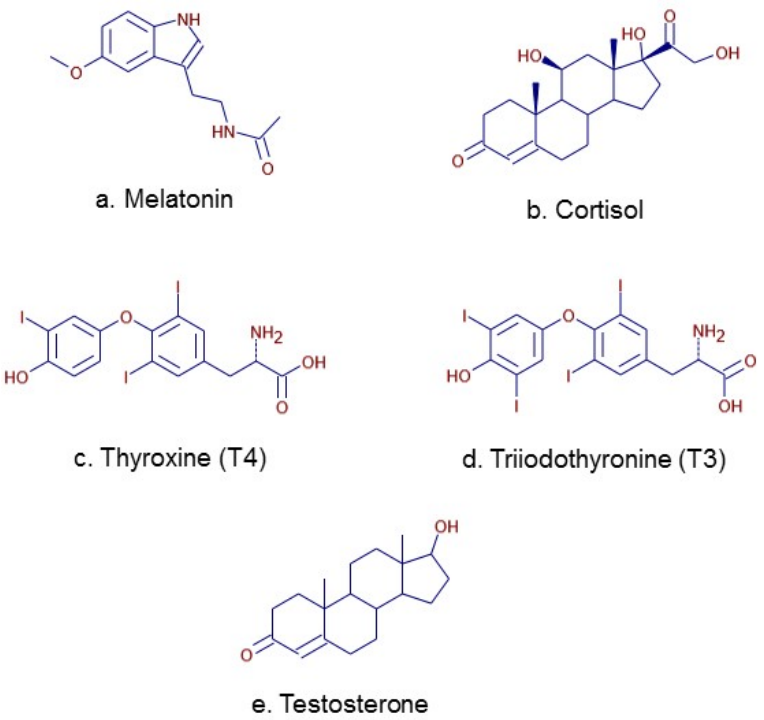

Figure 1. Chemical structures of the analysed hormones: melatonin (a), cortisol (b), 3,3',5-triiodo-L-thyronine (T3) (c), L-thyroxine (T4) (d), and testosterone (e).

2.2 HPLC-MS/MS conditions

Reverse phase (RP) liquid chromatography was performed using a 4.6 mm × 100 mm, 3.5 µm particle Agilent ZORBAX Eclipse Plus C18 (Agilent Technologies, Santa Clara, CA, USA) column. An Agilent 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA, USA) with a binary pump and degasser, a temperature-controlled well plate autosampler and a column compartment were used. The autosampler temperature was 4 °C, the injection volume was 15 µL, and the flow rate was 1 mL/min. The mobile phase consisted of water containing 0.1% (v/v) formic acid (A) and methanol containing 0.1% (v/v) formic acid (B). The following gradient elution program was utilized for chromatographic separation: 0-0.5 min (40% B), 0.5-8 min (40-100% B), 8-10 min (100% B), 10-11 min (100-40% B), and a 3 min post-elution period for re-equilibration.

An Agilent 6490 Triple Quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with an electrospray ionization (ESI) source was operated in the positive ion mode using multiple reaction monitoring (MRM). The flow and temperature of the dry and sheath gases were 18 L/min, 100 °C and 12 L/min, 200 °C, respectively. The nebulizer gas pressure was 25 psi and the capillary voltage was 4.5 kV. Three MRM transitions were monitored for each hormone and a single MRM transition was measured for the internal standards. Table 1 presents the MRM transitions monitored and the collision energy used in the present study. MassHunter Workstation Software (Agilent Technologies, Santa Clara, CA, USA) was used for data acquisition, instrument control and data processing.

Table 1. *Optimized MRM conditions for the identification and quantification of melatonin, cortisol, T3, T4 and testosterone levels*

Compound	MRM transitions (*m/z*)	CE (V)
Melatonin	233.1 → 174 (Q1)	16
	233.1 → 159.1	32
	233.1 → 131.1	40
Melatonin-D_4	237 → 178	12
Cortisol	363.2 → 121 (Q1)	24
	363.2 → 327.2	16
	363.2 → 309.1	16
Cortisol-D_4	367.2 → 121	30
T3	651.8 → 605.7 (Q1)	24
	651.8 → 507.8	24
	651.8 → 478.8	40
T4	777.7 → 731.6 (Q1)	28
	777.7 → 633.7	24
	777.7 → 604.7	40
T4-$^{13}C_6$	783.5 → 737.5	30
Testosterone	289.2 → 97 (Q1)	28
	289.2 → 109	28
	289.2 → 81.1	40
Testosterone-$^{13}C_3$	292.2 → 100	28

CE, collision energy.

2.3 Calibration standards and quality control (QC) samples

The standard stock (1 mg/mL) and internal standard (100 µg/mL) solutions were prepared in methanol. The working and calibration solutions were prepared in a water-methanol solution (1:1, v/v). Calibration curves were constructed in the range of 0.004-0.5 ng/mL for melatonin and 0.4-50 ng/mL for cortisol, T3, T4 and testosterone. Internal standards were added to a final concentration of 0.05 ng/mL for melatonin-D_4 and 10 ng/mL for cortisol-D_4, L-thyroxine-$^{13}C_6$, and testosterone-$^{13}C_3$. For T3, L-thyroxine-$^{13}C_6$ was used as the IS.

QC samples were prepared by spiking six rat serum samples with three different concentrations of each standard (0.125, 0.25, and 0.5 ng/mL of melatonin and 3.125, 12.5, and 50 ng/mL of cortisol, T3, T4, and testosterone). These concentrations were chosen according to the endogenous levels for the hormones in non-spiked QC samples.

2.4 Experimental design and sample collection

The Animal Ethics Committee of the University Rovira i Virgili (Tarragona, Spain) approved all procedures used in the present study.

The animals used in this study were 8-week-old male *Fischer 344* rats (median weight 200 g, n=18) were obtained from Charles River Laboratories (Barcelona, Spain). Animals were divided into three different groups exposed to different light conditions. Rats in the control group (Control, n=6) were housed under a 12 h light/12 h dark cycle, the long day group (LD, n=6) was exposed to an 18 h light/6 h dark cycle and the short day group (SD, n=6) was exposed to a 6 h light/18 h dark cycle, each for 10 weeks. Throughout the experiment, rats were fed *ad libitum* (standard pellet diet) and water. With the exception of room lighting changes, all other environmental conditions were the same between groups (a temperature of 22±2 °C and a relative humidity of 55%).

At the end of the experiment, animals were euthanized by decapitation approximately 1 hour after the lights turned on. Blood and serum were collected and stored at -80 °C until further analysis.

2.5 Sample preparation

Serum was thawed to room temperature before analysis. The extraction was performed by mixing 100 µL of serum and 10 µL of the IS working solution (0.5 ng/mL for melatonin-D_4 and 100 ng/mL for cortisol-D_4, L-thyroxine-$^{13}C_6$, and testosterone-$^{13}C_3$). A liquid-liquid extraction (LLE) process was performed by adding

300 µL of ethyl acetate and vortexing for 1 min. The mixture was shaken on a shaking table at 950 rpm for 15 min at room temperature and centrifuged for 3 min at 4000 rpm. A second LLE step was performed using 300 µL of ethyl acetate containing 10% of formic acid. Then, samples were centrifuged for 3 min at 4000 rpm. Both supernatants were pooled and evaporated to dryness under nitrogen. The resulting pellet was dissolved in 100 µL of a water-methanol solution (60:40, v/v).

2.6 Method validation

The method was validated for selectivity, recovery, matrix effect, linearity, accuracy and precision according to the guidelines for bioanalytical methods established by the United States Food and Drug Administration (FDA) [25]. The selectivity was evaluated by assaying six different lots of samples (blanks) and fortified samples to confirmed that there aren't other substances which interferences with the metabolites and the IS's in the samples. For all the calculations, endogenous peak areas (non-spiked QC samples) for the selected compounds were subtracted. The extraction recovery was evaluated by comparing the peak area obtained from extracted QC samples with pure standard solution at three level concentration. The recovery of IS's were determined at the working concentrations (0.5 ng/mL for melatonin-D_4 and 100 ng/mL for cortisol-D_4, L-thyroxine-$^{13}C_6$, and testosterone-$^{13}C_3$). For the matrix effect, peak areas of serum samples spiked with the standard analytes at three different levels of QC after extraction were compared with original stocks at the same concentration. Calibration curves were prepared with eight concentrations ranged 0.004-0.5 ng/mL for melatonin and 0.4-50 ng/mL for the other hormones. The linear curves were plotted by least-squares regression of relative peak area (analyte/IS) versus the concentration of the calibration standards. Linearity was estimated using the coefficient of determination (r^2). Quantifications of the limit of detection (LOD) and limit of quantification (LOQ) were used to calculate the sensitivity of the method, which was determined at a signal-to-noise ratio of greater than 3 and 10, respectively. Moreover, LOQ was selected as a first

point were the instrumental response was lineal. The intra-day (repeatability) and inter-day (semi-reproducibility) accuracy and precision were confirmed in five replicates measured within a single day and on five different days, respectively. Accuracy and precision were expressed as relative error (%RE) and relative standard deviation (%RSD), respectively, by measuring the ratio between the mean recorded and the spiked sample concentrations.

2.7 Statistical analysis.

Data are reported as the means ± SD. The differences between groups were analysed using one-way ANOVA, followed by Duncan's new multiple range test (as a post hoc test), with the statistical software SPSS Statistics 22 (SPSS Inc., Chicago, IL, USA). Grubbs' test was used to detect outliers, which were discarded from subsequent analyses. The level of significance was set to a bilateral value of 5%.

3. Results and discussion

3.1 Development and optimization of the LC-MS/MS method

This study describes the development and optimization of a new high-performance liquid chromatography method for the simultaneous detection and quantification of melatonin, cortisol, thyroid hormones T3 and T4 and testosterone levels in serum.

3.1.1 Selectivity

Method validation was performed on samples spiked with the different hormones. Positive ESI total ion chromatograms obtained in the analysis of the five endogenous hormones and the four ISs in rat serum are illustrated in *Figure 2*. The hormones were simultaneously separated and detected in rat serum in an 11-min run. The hormones showed well resolved peaks with different retention times (3.75, 6.09, 6.23, 6.85, and 7.51 min for melatonin, cortisol, T3, T4 and testosterone, respectively), and no other matrix substances significantly interfered with their chromatographic peaks. Furthermore, hormones have different parent mass-to-charge ratios and unique MRM transitions; thus, they are spectrally distinguishable. The MS-MS parameters were optimized and the transitions with the highest abundances were selected as monitored quantifier MRM transitions (Q1) (Table 1).

3.1.2 Recovery and matrix effect

The recovery was optimized to ensure the extraction of the highest amounts of hormones from each sample. The recovery of ISs was determined at the working concentrations (0.5 ng/mL for melatonin-D_4 and 100 ng/mL for cortisol-D_4, L-thyroxine-$^{13}C_6$, and testosterone-$^{13}C_3$). The hormone extraction conditions were based on the method reported by Jensen et al. [13]. However, a second LLE step using ethyl acetate containing 10% formic acid as a dissolvent was performed to achieve the highest effective extraction of the thyroid hormones T3 and T4. The pKa of 4'-phenolic hydroxyl group of T4 is 6.7 and 8.5 for T3 (approximately 90% and 10% ionized at pH 7.4, respectively) [26]. Thus, as a highly acidic medium leads to a lower

ionization of the thyroid hormones, their solubility increases in organic solvents. Extraction recoveries ranged between 52.8-74.1% for hormones and the mean recovery of ISs at the working concentrations was 83.0±9.8, values that are considered acceptable based on the criteria of the Association of Analytical Communities (AOAC) (40-120% recovery for an analyte concentration of 1 ppb) (Table 2) [27]. Moreover, the matrix effect on rat serum was negligible at different QC levels (89.7-98.3%) (Table 2).

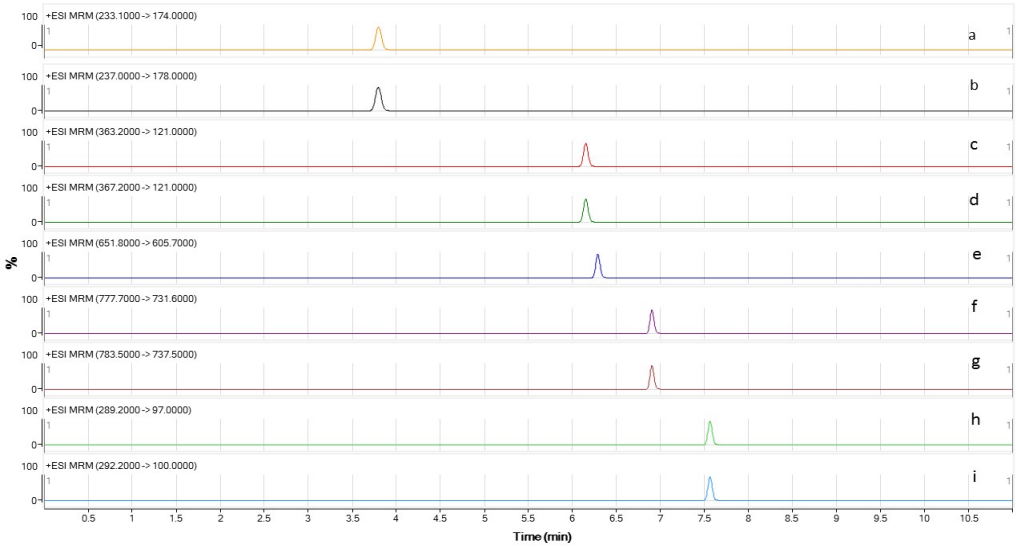

Figure 2. *Typical multiple reaction monitoring (MRM) chromatograms of melatonin (0.0127 ng/mL) (a), melatonin-D4 (0.5 ng/mL) (b), cortisol (0.88 ng/mL) (c), cortisol-D4 (100ng/mL) (d), T3 (1.72 ng/mL) (e), T4 (30.07 ng/mL) (f), L-thyroxine-13C6 (100ng/mL) (g), testosterone (2.26 ng/mL) (h), and testosterone-13C3 (100ng/mL) (i).*

Table 2. *Recovery and matrix effect values (average for 3 QC levels) for the detection and quantification of melatonin, cortisol, T3, T4 and testosterone levels in rat serum using HPLC-MS/MS*

Analyte	Recovery		Matrix effect	
	(%)	RSD (%)	(%)	RSD (%)
Melatonin	70.60	6.27	92.14	5.96
Cortisol	74.10	3.22	95.68	11.36
T3	62.64	0.30	94.48	9.39
T4	52.81	2.53	98.25	4.51
Testosterone	69.32	4.20	89.73	3.53

3.1.3 Linearity, limits of detection and limits of quantification

Calibration curves were prepared with eight concentrations ranging from 0.004 to 0.5 ng/mL for melatonin and from 0.4 to 50 ng/mL for the other hormones. Linear curves were plotted using a least-squares regression of the relative peak area (analyte/IS) versus the concentration of the calibration standards. Good linearity was obtained ($r^2 \geq 0.993$) within the different concentration ranges for each hormone (Table 3).

The LOD and the LOQ results achieved in the present study were less than the concentration ranges used to determine the levels of melatonin, cortisol, testosterone and the thyroid hormones T3 and T4 in rat serum (Table 3). For melatonin, we achieved a LOD similar to a LC-MS/MS method used to quantify melatonin levels in human saliva (0.00095 ng/mL) [13] and more sensitive LOD than an RIA method used to analyse mouse serum (0.0091 ng/mL) and mouse plasma (0.0073 ng/mL) [28]. In human serum, the LOQ using a nanoflow LC-MS/MS (11.65 pg/mL) was higher than the LOQ obtained in this work. The cortisol LOD was 0.002 ng/mL in rat serum, whereas the LC-MS/MS analysis of cortisol reported in the literature showed a 5-fold increase in the concentration in human saliva (LOD = 0.01 ng/mL) [13] and a 350-fold increase in the concentration in human serum (0.7 ng/mL)[29].

Although 99.60% and 99.96% of T3 and T4 hormones, respectively, are bound to circulating proteins in serum, a small fraction of free circulating hormones (fT3 and fT4) are important for diagnostic purposes because of their bioactivity. In this sense, the aim of the described method is determining both fractions, free and bound molecules, in order to know the complete profile of thyroid hormone levels in serum. Consequently, this assay required an additional sensitive step to detect free and bound parts [30–32]. For thyroid hormones, the LODs for total T3 and T4 were 0.003 ng/mL and 0.07 ng/mL, respectively, in rat serum (Table 3), which were lower than

the values obtained by Wang and Stapleton in human serum (0.34 ng/mL for T3 and 0.57 ng/mL for T4) [33]. Finally, the LOD for testosterone using the method developed here was 0.074 ng/mL (Table 3), which was the highest LOD of all the analysed compounds. Tournier et al. [34] obtained an LOD of 0.02 ng/mL using a LC-QTRAP method, but they required an SPE extraction step to analyse testosterone levels in rat serum. Therefore, we obtained LOD and LOQ values within the relevant biological ranges for the simultaneous analysis of five metabolites in rat serum using this method.

Table 3. *HPLC-MS/MS parameters for the detection and quantification of melatonin, cortisol, T3, T4 and testosterone levels in rat serum*

Analyte	Range ng/mL	Regression equation	Correlation coefficient (r^2)	LOD ng/mL	LOQ ng/mL
Melatonin	0.004 - 0.5	y = 20.099x - 0.103	0.998	0.001	0.002
Cortisol	0.4 - 50	y = 0.012x - 0.008	0.994	0.002	0.019
T3	0.4 - 50	y = 0.057x - 0.046	0.996	0.003	0.009
T4	0.4 - 50	y = 0.101x - 0.091	0.993	0.070	0.248
Testosterone	0.4 - 50	y = 0.315x + 0.093	0.995	0.074	0.260

3.1.4 Precision and accuracy

Five replicates at three levels of QC samples (0.125, 0.25, and 0.5 ng/mL of melatonin and 3.125, 12.5, and 50 ng/mL of cortisol, T3, T4 and testosterone) were processed on the same day and on five separate days for the analysis and determination of the intra- and inter-day precision and accuracy of the method, respectively. The %RSD values for intra-day and inter-day precision ranged from 0.57 to 12.72% and the % RE values for accuracy were within 14.82% (Table 4). The assayed values for precision and accuracy showed that our method was reliable and reproducible within the acceptable limits of precision (RSD% ≤ 15%) and accuracy (RE% ≤ ±15%), according to the assayed concentrations [27].

Table 4. *Precision and accuracy parameters, expressed as standard deviation (SD), for the determination of melatonin, cortisol, T3, T4 and testosterone levels in rat serum*

Analyte	QC concentration	Intra-day (n=5)		Inter-day (n=5)	
	(ng/mL)	RSD (%)	SD (%)	RSD (%)	SD (%)
Melatonin	0.125	7.33	29.18	11.81	26.21
	0.25	8.13	22.34	14.56	19.19
	0.5	11.35	14.09	12.10	15.29
Cortisol	3.125	3.50	23.43	6.07	20.53
	12.5	4.49	18.65	12.60	26.43
	50	10.97	12.66	14.29	13.75
T3	3.125	4.10	-5.50	10.62	-1.86
	12.5	2.23	1.57	9.05	1.88
	50	5.79	-0.85	9.50	2.41
T4	3.125	5.07	0.67	11.51	-4.16
	12.5	0.57	0.67	8.17	-0.29
	50	4.52	-1.19	7.62	0.60
Testosterone	3.125	8.03	31.91	10.34	29.16
	12.5	12.72	24.04	6.50	33.14
	50	8.61	8.43	14.91	26.61

QC, quality control sample.

3.2 Application of the validated method

We quantified serum hormone levels in Fischer 344 rats exposed to different light schedules to test the suitability of the validated method. Comparisons of concentrations with previous reports confirmed the robustness and reliability of the method [35–39]. Hormonal levels in rat serum described in the bibliography are normally around 1.5 ng/mL, 1.12 ng/mL, 36.5 ng/mL and 2.62 ng/mL in the case of cortisol, T3, T4 and testosterone, respectively [35–37]. However, in the case of melatonin, its levels present a lot of variation during the day-night cycle. Even so, melatonin levels reported previously are in the same order of magnitude than the ones obtained with our method [38,39].

The quantification of melatonin, cortisol, T3, T4 and testosterone in different groups are summarized in Table 5. Except for testosterone levels, there were no significant differences between groups. The maximum concentrations of melatonin (8.69 pg/mL), cortisol (2.70 ng/mL), T3 (1.89 ng/mL) and T4 (47.87 ng/mL) were observed in the Control group. The lack of changes between different groups may be due to the levels of melatonin, which could be considered a regulatory step and one of the initial points of the following pathways [24]. In contrast, testosterone levels were significantly increased in rats exposed to short day photoperiod compared to Control rats, whereas an increase or decrease in light exposure produced lower serum concentrations of the other hormones. Similar results were observed by Çevik and Aslan, who obtained a decrease in melatonin and cortisol levels in rat serum due to daily exposure to prolonged light periods and shorter dark periods [40].

Table 5. *Serum hormone levels in rats housed under three different light schedules (Control, LD or SD)*

	Control (n=6)	LD (n=6)	SD (n=6)	ANOVA
Melatonin (pg/mL)	8.69 ± 2.52	7.02 ± 1.05	7.81 ± 1.28	p=0.304
Cortisol (ng/mL)	2.70 ± 2.30	2.12 ± 0.79	1.68 ± 1.00	p=0.518
T3 (ng/mL)	1.89 ± 0.64	1.66 ± 0.33	1.72 ± 0.44	p=0.694
T4 (ng/mL)	42.87 ± 27.70	25.69 ± 4.98	25.65 ± 4.45	p=0.214
Testosterone (ng/mL)	1.11 ± 0.70^{a}	1.49 ± 0.48^{a,b}	3.34 ± 2.04^{b}	p=0.039

Data are presented as the means ± SD (n=6). ^{a,b} *Mean values with different letters exhibited significant differences among groups (one-way ANOVA and Duncan's post hoc test, p<0.05). Control, control day photoperiod; LD, long day photoperiod; SD, short day photoperiod.*

Briefly, this method is potentially useful for the simultaneous determination of levels of endogenous melatonin, cortisol, thyroid hormones T3 and T4 and testosterone in rat serum; these hormones represent important endocrine markers.

4. Conclusions

This study describes the development of a new LC-MS/MS method for detecting the melatonin, cortisol, T3, T4 and testosterone levels in serum. The method reported here is highly sensitive, precise and accurate and allows the determination of the serum levels of five different hormones within their relevant biological concentration ranges. Moreover, this method will likely significantly contribute to the clinical laboratory field because the levels of these hormones are used as endocrine markers in some diseases and are relevant diagnostic tools.

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Manuscript 2

Red-grape consumption in natural season modulates dopaminergic system and brown adipose tissue metabolism in Fischer 344 rats

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UNIVERSITAT ROVIRA I VIRGILI
MODULATION OF CENTRAL NEUROENDOCRINE RESPONSE TO PHOTOPERIOD BY SEASONAL FRUITS AND OBESITY.
Cristina Domenech Coca

Red-grape consumption in natural season modulates dopaminergic system and brown adipose tissue metabolism in Fischer 344 rats

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Abstract

The xenohormesis theory proposes that animals recognize chemical cues from plants, mainly polyphenols, to check environmental conditions, allowing them to respond in advance to environmental alterations and to improve their probability of survival. One of these environmental changes is the astronomic season. Thus, because animals have seasonal rhythms, we hypothesized that the specific polyphenol content in seasonal fruits could affect seasonal control in photoperiod-sensitive animals. We studied whether seasonal markers of Fisher 344 male rats, adapted to a short day (SD) or a long day (LD), are modified by red-grape or cherry (autumn and spring fruits, respectively) consumption. We found that red-grape but not cherry significantly modulated the ratio of dopamine receptor 2 to somatostatin receptors 2 and 4 in animals under SD but had minimal effects on other hypothalamic biomarkers such as deiodinase 2 (*Dio2*) to deiodinase 3 (*Dio3*) ratio, pituitary *Eya3* to *Chga* ratio and *Tshβ* expression. In parallel, red-grape decreased food intake and locomotor activity but increased energy expenditure and the ratio *Dio2*/*Dio3* in the brown adipose tissue, suggesting enhanced actions of thyroid hormones and metabolic activity, only in animals living under a SD. In contrast to red-grape, cherry consumption modulated minimally seasonal markers and did not alter energy expenditure or behavioral outcomes. Our results demonstrate that red-grape consumption within its natural season modulates physiology and behavior by interacting with photoperiod-dependent processes. This work highlights the relevance of seasonality of fruits and its consumption for the modulation of brain function by nutrition.

1. Introduction

Xenohormesis is a biological principle based on the observation that environmentally stressed plants produce bioactive compounds which can be considered as interspecific chemical signals for animals. The molecules involved in this responses, mainly polyphenols, might allow animals to detect future predictable changes when the conditions are still favourable, thus increasing their probability of survival[1]. Polyphenols are known to be produced by plants as a response to different factors, such as photoperiod[2] or circannual cycles[3], and stressful situations such as temperature or dryness[4–6]. In the context of the Xenohormesis, these molecules confer different functionalities to the intake of vegetal foods by heterotrophs, which can be associated with the different long-term effects on health and wellness described for polyphenols, such as vascular function, inflammatory response or neurodegenerative protection among others [1,2,7].

Seasonal rhythms are considered dominant features of the biology of mammals and have an important role in the control of several functions, such as reproduction, metabolism, affective disorders and immunity[8]. This adaptive process involves both intrinsic mechanisms and environmental stimuli, whereas the variation of day length between day and night is one of the most important exogenous stimulating signals, known as photoperiod[8,9]. Animal circannual rhythmicity has been reported over the last decades and some structures and mechanisms stablished as rhythmic generators. The primary transducer of photoperiodic signal is the hypophysial pars tuberalis (PT), which controls seasonal behavior and metabolic response of the body[8]. Currently, it is accepted that photoperiodic information arrives into the PT in the form of melatonin, which concentration responds to environmental light. It is known that melatonin restriction on long day photoperiods (LD) is associated with overexpression of the gene eyes absent 3 (*Eya3*) in the pars tuberalis of the pituitary

[10,11]. The resulting protein, *EYA3*, induces the transcription of *Tshβ*, which signals to the hypothalamus, increasing the remodeling of thyroid hormones (TH) by deiodinases *Dio2*, which activates TH into its active form T3, and decreasing *Dio3*, which inactivates TH into the inactive form T4[12]. Through this mechanism animals are confined into a LD state, characterized by increased secretion of prolactin (PRL) into the blood by the pituitary, together with neuroendocrine responses mediated by active TH through increased *Dio2*/*Dio3* ratio in the hypothalamus[13]. In turn, the short day photoperiod (SD) state is characterized by decreased expression of *Eya3* and prevalence of chromogranin A (*Chga*), which in turns results in low expression of *Tshβ*, low levels of blood PRL and hypothalamic *Dio2*/*Dio3* ratios. Therefore, the *Eya3*/*Chga* ratio stands out as a binary switch controlling the response to circannual rhythms, increasing in LD and decreasing in SD[9]. Besides, it has been described that, in rats, photoperiod switching triggers a shift on signaling actions of neurotransmitters dopamine and somatostatin (SST), inducing changes in behavior[14]. Thus, in nocturnal rodents, LD is characterized by increasing signaling of somatostatin and lower dopamine, leading to anxiety and depression behaviors, while in SD increased dopaminergic signaling leads to amelioration of these states. These changes are mediated through photoperiod-driven changes in post-synaptic receptors populations such as dopamine receptor D2 (D2R) and somatostatin receptors 2 and 4 (Sst2R and Sst4R respectively) in the periventricular nucleus of the hypothalamus[14]. Thus, D2R to Sst receptors ratio can be used as a biomarker to assess the response of somatostatinergic and dopaminergic systems to seasonality. Therefore, responses to photoperiod switching involve different signaling systems in the brain that govern neuroendocrine function, behavior and physiology through structures placed in the hypothalamus and the pituitary.

Regulation of brain circuitry by nutrition can be understood by the concept of gut-brain axis, which is defined as the network of physiological links that mediate

signaling from the gut to different structures of the brain. The axis comprises direct communication from the enteric nervous system to the brain by the vagal and spinal cord nerves and indirect communication through the systemic milieu [15]. The last pathway includes signals from the immune system, such as interleukins and other mediators, and direct signaling of food or microbiota metabolites to specific brain regions, such as the hypothalamus through the median eminence of the neurohypophysis. This structure is outside the blood-brain barrier, and tanycytes are in direct contact with the blood through fenestrated vessels[10]. These glial cells might act as nutrient sensors, responding to changes in the concentration of different components of blood, such as glucose or fasting conditions, or might act as barriers themselves, changing their morphology depending on the nutritional state and consequently modulating the permeability of the hypothalamus to components of the systemic circulation[10,16].

We have described that a grape seed procyanidins extract (GSPE) modulates core controllers of the circadian clock, either in *in vitro* models[17], in peripheral tissues[18] or in the hypothalamus of rats fed with these polyphenols[19]. In the last work, administration of GSPE resulted in changes in the rhythmicity of blood melatonin concentration accompanied by altered rhythms of hypothalamic clock genes such as *Bmall1* and *Nampt*. Intriguingly, changes in blood melatonin concentrations were observed only if GSPE was administered during the light period but not during the night. These evidences prompted us to consider polyphenols as a non-photic cue with capacity of modulating biological rhythms depending on the presence of light. According to the xenohormesis theory[1], bioactive compounds in foods, such as polyphenols, may act as a secondary signal, complementary to photoperiod, allowing heterotrophs to adjust different metabolic and behavioral parameters to environmental conditions of a given ecosystem. Consequently, intake of fruits in or out of season might affect differently to the organism and therefore have different

consequences for health. We hypothesized that consumption of fruits of different seasons but with a similar polyphenolic fingerprint would elicit different responses in mammals depending on their environmental photoperiod, and that these changes would be reflected in biomarkers and molecular controllers of seasonality and/or in photoperiod-dependent processes. Therefore, our objective was to assess the effects of consuming spring and autumn fruits, such as cherry and red-grape, respectively, on central controllers of seasonality. To this aim, we adapted Fisher 344 rats to SD and LD and supplemented them with lyophilized cherry or red-grape for 14 weeks. During the experiment, different parameters were monitored, such as food intake, energy expenditure or spontaneous locomotor activity and different biomarkers of seasonality control were assessed in the brain. We show here that red-grape but not cherry elicited important changes in feeding behavior, energy expenditure and locomotor activity in a photoperiod-dependent fashion, which can be associated to changes on central processes subjected to seasonal control, such as the somatostatinergic and dopaminergic systems. Our results highlight the relevance of considering the environmental photoperiod for designing nutritional interventions and interpret its outcomes.

2. Materials and methods

Animals

The Animal Ethics Committee of the University Rovira i Virgili (Tarragona, Spain) approved all the procedures. The animals used were 8-week-old male Fischer 344 rats (Charles River Laboratories, Barcelona, Spain) housed in pairs in cages at 22ºC under two different light schedules in order to emulate different season's day lengths: a long day photoperiod (n=18, LD, 18:6 h light/dark cycle) or a short day photoperiod (n=17, SD, 6:18 h light/dark cycle). After 1 month under these conditions, animals under each photoperiod were orally supplemented with 2 different kinds of lyophilized fruit (100 mg per kg of body weight/day) for 10 weeks: red-grape (V*itis vinifera*, variety garnacha, n=12) was provided as an autumn fruit and cherry (*Prunus avium*, variety royal down, n=11) as a spring fruit. Another group, used as a control, received only the vehicle (control, n=12). Rats were fed a standard diet (2.90 kcal·g^{-1}; A04, Panlab, Barcelona, Spain) *ad libitum*. During the experiment, food intake was recorded weekly. After 14 weeks, the animals were deprived of food for 1 h and killed by decapitation. Blood was collected, and serum was obtained by centrifugation; the samples were stored at -80ºC until analysis. The experiments were conducted between 10:00 and 11:00 to minimize possible circadian variations. The hypothalamus, pituitary gland and brown adipose tissue were rapidly removed after death, weighed, frozen in liquid nitrogen and stored at -80ºC until further analysis.

Physical activity measurements

The OxyletPro^{TM} system (Panlab, Barcelona, Spain) was used to evaluate animal physical activity and respiratory metabolism. The measurements were performed

during weeks 11-12 of the study, under *ad libitum* conditions and for 21 hours (from 12:00 to 09:00). Data collected during the first hour of acclimation were discarded.

The animals were transferred to a standard rodent home cage (LE 1331 OxyletPro™ Home Cage, Panlab) that used an airtight lid (LE 1332 OxyletPro™ Airtight Lid, Panlab) to ensure a contained sample environment. The software program Metabolism 3.0.00 (Panlab, Barcelona, Spain) calculated the energy expenditure (EE, $kcal \cdot day^{-1} \cdot kg^{-0.75}$) as "oxygen consumption x 1.44 x [3.815 + (1.232 x (carbon dioxide production/oxygen consumption))]", according to the Weir formula[20]. The cages were placed on a platform with strain weight transducers to register movements.

Gene expression analysis

The total RNA, containing the micro RNA, was extracted from the hypothalamus, pituitary gland and brown adipose tissue using TRIzol Reagent (Thermo Fisher Scientific, Barcelona, Spain) according to the manufacturer's protocol. To isolate both total and small RNA species, the 10-minute incubation with 100% isopropanol was changed into an overnight incubation at -20ºC. The RNA yield was quantified on a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

To analyze the expression of the samples, cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Barcelona, Spain). A Labnet MultiGene Gradient PCR Thermal Cycler (Sigma-Aldrich, Madrid, Spain) was used for reverse transcription. The reaction was performed according to the instructions of the manufacturer. The cDNA was subjected to a quantitative reverse transcriptase polymerase chain reaction amplification using iTaq Universal SYBR Green Supermix (Bio-Rad, Madrid, Spain) in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Madrid, Spain). The primers used for the different genes are described in Supplementary Table 1 and were obtained from Biomers.net (Ulm, Germany). The fold changes in the mRNA levels were calculated as a percentage of

the LD-Control group using the $-2^{\Delta\Delta Ct}$ method[21] with *PPIA* gene as an endogens control.

Supplementary Table 1. *Nucleotide sequences of primers used for PCR amplification in hypothalamus, pineal gland and interscapular brown adipose tissue.*

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Ref. or Acc. No.
BMAL1	GTAGATCAGAGGGCGACGGCTA	CTTGTCTGTAAAACTTGCCTGTGAC	NM_024362.2
CHGA	AGGATTCTGACAAGGGGCAA	CACTGGGACCTCTCTCACTG	NM_021655.2
CRY1	TGGAAGGTATGCGTGTCCTC	TCCAGGAGAACCTCCTCACG	NM_198750.2
D2R	TGAACAGGCGGAGAATGGAT	CAGGACTGTCAGGGTTGCTA	NM_012547.1
DIO2	TTATGGGGTAGCCTTTGAACG	CCAGCCAACTTCGGACTT	NM_031720.4
DIO3	CTCCAGCAGTTCCGCATATG	CATTCGCACATGAGCTTCGA	NM_017210.4
EYA3	TGGTCCCATGTTGTGCTTTG	CAGTGCTATGGAAACGGTCG	NM_001107910.1
MT1	ACCGGAACTCTCCAGTACGA	GTTTGCTGTCCGGTTTCACC	NM_053676.2
NAMPT	CTCTTCACAAGAGACTGCCG	TTCATGGTCTTTCCCCCACG	NM_177928.3
PER2	CGGACCTGGCTTCAGTTCAT	AGGATCCAAGAACGGCACAG	NM_031678.1
PPIA	CTTCGAGCTGTTTGCAGACAA	AAGTCACCACCCTGGCACATG	NM_017101.1
SST	CTGGAGCCTGAGGATTTGCC	GGATCAGAGGTCTGGCTGAG	NM_012659.2
SST2R	CCCGCTATGTAATCTCGT	TGCAAGAGGGATGCTG	NM_019348.1
SST4R	AGCATGAACACGCCTGCAA	CATCAGCGACGGCCAGGTT	NM_013036.2
TSHb	TCGTTCTCTTTTCCGTGCTTT	CCGTGTCATACAATACCCAGC	NM_013116.2

BMAL1, hydrocarbon receptor nuclear translocator-like 1; CHGA, chromogranin A; CRY1, cryptochrome 1; D2R, Dopamine Receptor D2; DIO2, Type II iodothyronine deiodinase; DIO3, Type III iodothyronine deiodinase; EYA3, eyes absent homolog 3; MT1, melatonin receptor 1; hydrocarbon receptor nuclear translocator-like 1; PER2, period 2; PPIA, Peptidylprolyl Isomerase A; SST, somatostatin; SST2R, somatostatin receptor subtype 2; SST4R, somatostatin receptor subtype 4; TSHβ, Thyroid stimulating hormone subunit beta.

Hormone analysis

The serum melatonin concentration was measured using high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) analysis as previously described by Domenech-Coca *et al.*[22].

The serum prolactin (PRL) concentration was measured by Luminex xMAP technology (BioRad, Madrid, Spain). The assay was run according to the

manufacturer's recommended procedures. Briefly, magnetic beads were coated with specific capture antibodies. Fluorescence detection antibodies were then applied to bind the hormone-capture antibody complex on the bead set. Each hormone was recognized by the differences in fluorogenic emission for each bead set, detected using a Luminex 200 (BioRad, Madrid, Spain), a flow cytometry-based instrument, and then converted into hormone concentrations (pg/mL) using the Bio-Plex Manager software.

Statistical analysis

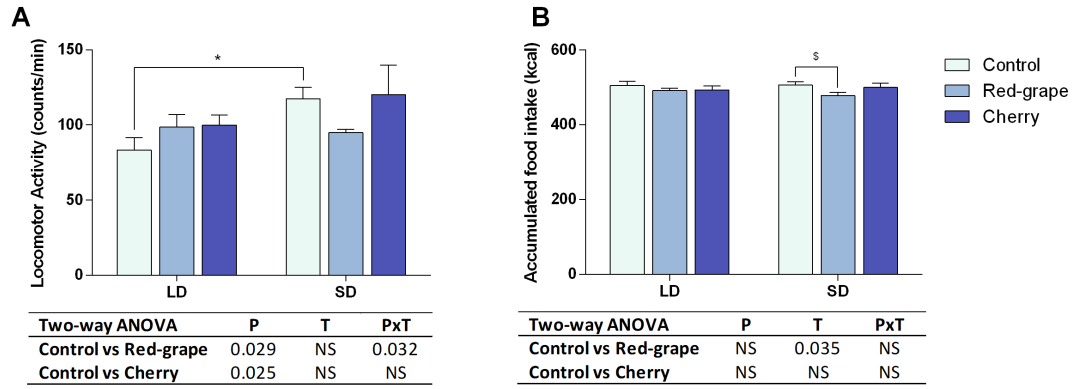
Data are expressed as the means ± S.E.M. Differences between experimental groups were assayed by two-way ANOVA with factorial designs photoperiod (P) and treatment (T) considering control groups and red-grape or cherry groups separately. One-way ANOVA followed by Duncan's new multiple range test (as a post hoc test), or Student's t-test, as indicated in the respective figure legend were applied in order to explore the origin of outcomes of Two-way ANOVA analyses. The linear relationship between key variables was tested using Spearman's correlation coefficient. All the statistical analyses were performed using the statistical software package SPSS Statistics 22 (SPSS Inc., Chicago, IL, USA). Grubbs' test was used to detect outliers, which were discarded for subsequent analyses. The level of significance was set at a bilateral 5%.

3. Results

Red-grape consumption affects energy expenditure and behaviour.

It has been reported that photoperiods can modulate the daily patterns of different metabolic and physiological parameters, such as locomotor activity, energy expenditure and feeding behavior, in mammals[23]. Analysis of spontaneous locomotor activity (figure 1A) by two-way ANOVA revealed that SD increased locomotor activity in all groups. Red-grape intake resulted in a significant interaction between P and T factors (PxT) in 21 hours spontaneous locomotor activity respect control groups. These differences were due to decreased locomotor activity in rats consuming red-grape during the short day (SD) but not during the long day (LD). Accumulated food intake (Figure 1B) was not affected by photoperiod but by red-grape consumption, mainly during the SD ($p=0.045$) and minimally during the LD ($p=0.341$) as assessed by Student's T test comparing vehicle and red-grape fed groups for each photoperiod. Energy expenditure (Figure 1C) was clearly increased by P as a response to SD. Fruit intake significantly affected this parameter when vehicle and cherry groups were assayed by Two-way ANOVA, but not when the same analysis was applied to vehicle and red-grape fed animals. Both fruits resulted in an interaction between photoperiod and treatment factors (PxT), due to increased energy expenditure in the LD but not in the SD, as revealed by one-way ANOVA analyses. Since energy expenditure is directly dependent on locomotor activity, the ratio between both variables was used to assess differences due to abnormal energy expenditure. Results of this approach (Figure 1D) revealed that energy expenditure was similar for all animals when locomotor activity was considered, with the exception of those fed with red-grape. In this case, two-way ANOVA revealed a significant effect of the photoperiod only when animals treated with the vehicle and

red-grape were considered. Closer analysis by one-way ANOVA revealed that red-grape consumption increased the ratio between energy expenditure/locomotor activity only in animals living in the SD, suggesting increased metabolic processes leading to additional dissipation of energy that is not explained by changes in locomotor activity. Therefore, red-grape consumption had relevant effects on behavior and physiology when consumed during the SD but not the LD, in contrast to cherry consumption.



Two-way ANOVA	P	T	PxT
Control vs Red-grape	0.029	NS	0.032
Control vs Cherry	0.025	NS	NS

Two-way ANOVA	P	T	PxT
Control vs Red-grape	NS	0.035	NS
Control vs Cherry	NS	NS	NS

Two-way ANOVA	P	T	PxT
Control vs Red-grape	0.000	NS	0.038
Control vs Cherry	0.000	0.000	0.010

Two-way ANOVA	P	T	PxT
Control vs Red-grape	0.005	NS	NS
Control vs Cherry	NS	NS	NS

*Figure 1. Behavioral parameters of rats adapted to a long day or short day photoperiod and supplemented with red-grape or cherry for 14 weeks. A: Locomotor activity. B: Accumulated food intake. C: Energy expenditure. D: Ratio between energy expenditure and locomotor activity. The data are presented as the mean ± S.E.M. (n=5-6). P, the effect of the photoperiod; T, the effect of each fruit treatment; PxT, the interaction of the photoperiod and the fruit treatment (two-way ANOVA, $p<0.05$). * Mean values were significantly different among groups (one-way ANOVA and Duncan's post hoc test, $p<0.05$). $ Significantly different between groups (Student's t-test, $p<0.05$). LD, long day; SD, short day.*

Red-grape treatment impacts somatostatinergic and dopaminergic systems.

Somatostatinergic and dopaminergic systems drive behavior[24] and are modulated by photoperiod[14]. Interestingly, we found a significant interaction effect between photoperiod and red-grape consumption on somatostatin (*Sst*) expression in the hypothalamus (figure 2A): *Sst* expression increased during the SD. In addition, after 2-way ANOVA analysis, red-grape treated animals also displayed a significant increase in the gene expression levels of *Sst2R* respect vehicle groups (figure 2B). A similar tendency, though not statistically significant, was observed for *Sst4R* (figure 2C) together with a significant effect on the levels of *D2R* mRNA, mainly due to downregulation of the expression in SD animals (figure 2D). Accordingly, red-grape consumption induced a clear treatment effect on *D2R/Sst2R* (Figure 2E) and *D2R/Sst4R* (Figure 2F) ratios, which were more evident in SD animals (80% and 70% of reduction for *D2R/Sst2R* and *D2R/Sst4R*, respectively) than in LD groups (50% and 40%). On the other hand, as for the behavioral parameters, cherry consumption did not show statistically significant effects, when assessed by 2-way ANOVA, in hypothalamic expression of somatostatin or dopaminergic systems.

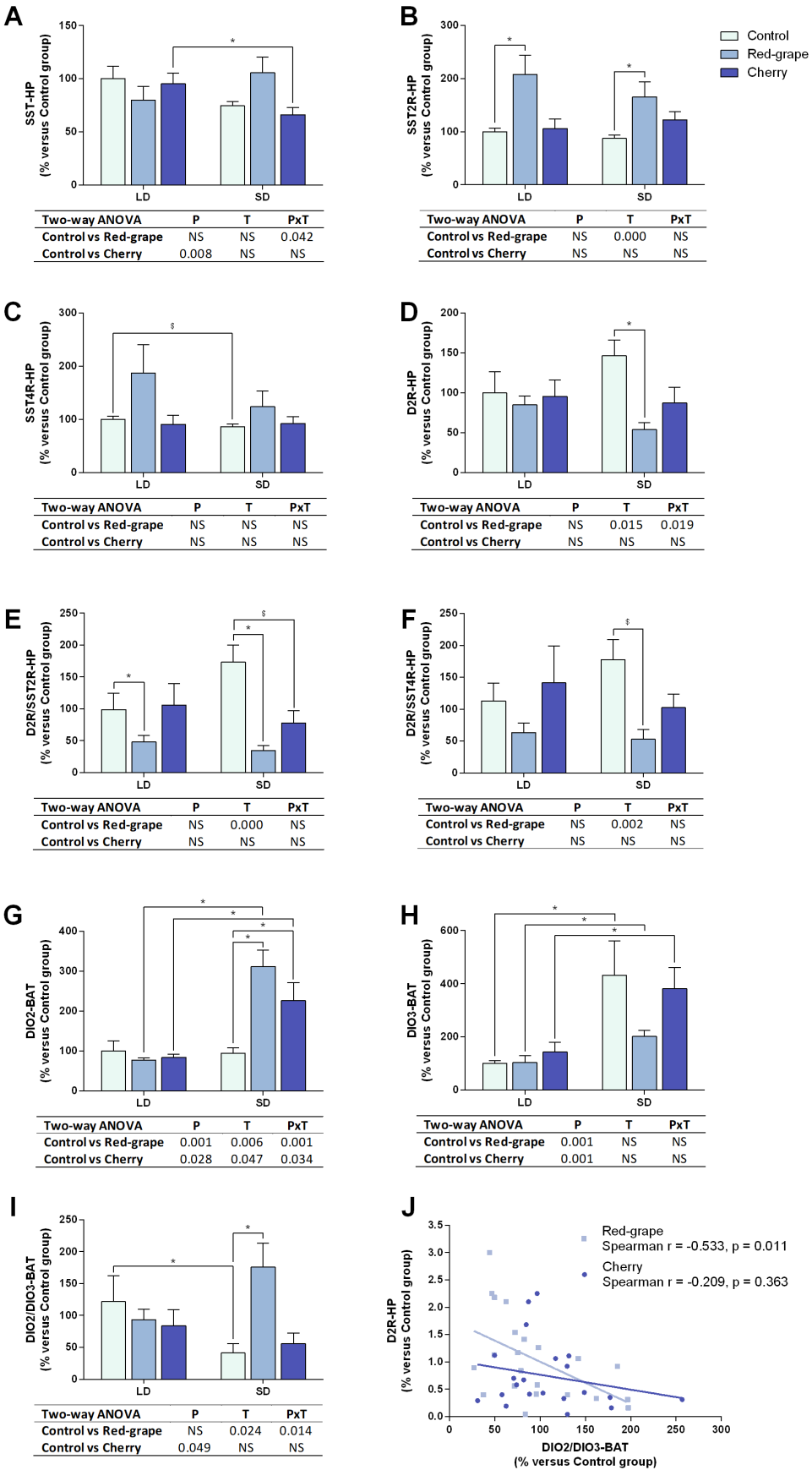
Red-grape intake alters brown adipose tissue gene expression in a photoperiod-dependent manner

The changes observed in energy expenditure prompted us to study the brown adipose tissue. Notably, the *Dio2*/*Dio3* ratio provides relevant information about the metabolic activity of BAT[25]. Results show that *Dio2* (figure 2G) was significantly changed by the intake of both red-grape and cherry. These effects were not due to changes in LD animals, but in those maintained under SD, which showed a 300% and a 200% overexpression as a result of consuming red-grape and cherry, respectively. In contrast, *Dio3* (figure 2H) was not significantly altered by cherry but

presented a clear repression in animals on SD consuming red-grape. As a result, the *Dio2/Dio3* ratio (figure 2I) resulted in T and PxT effects by 2-way ANOVA when red-grape groups were compared with vehicle groups, due to a 300% increase in the comparison between vehicle and red-grape treated animals under SD. When this parameter was analyzed in groups consuming cherry and vehicle, only a P effect was reported by the 2-way ANOVA.

Since the expression of *D2R* affects energy expenditure by modulating the thermogenic activity of the BAT[26] and the hypothalamus and BAT were dissected at the same time, correlational analyses were performed using the expression of hypothalamic *D2R* and the ratio of *Dio2/Dio3* in BAT (figure 2J). Of note, a significant correlation between both parameters was evident for rats consuming red-grape, thus suggesting a link between the repression of *D2R* at the hypothalamic level and the level of BAT metabolic activation. In addition, there was no correlation between the expression of hypothalamic *D2R* and the ratio of *Dio2/Dio3* in the BAT in animals consuming cherry.

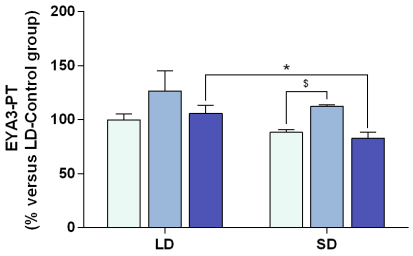
Figure 2. (Next page) Hypothalamic and brown adipose tissue biomarkers related to the somatostatin and dopaminergic systems of rats adapted to a long day or short day photoperiod and supplemented with red-grape or cherry for 14 weeks. *A: Hypothalamic Sst gene expression levels. B: Hypothalamic Sst2R gene expression levels. C: Hypothalamic Sst4R gene expression levels. D: Hypothalamic D2R gene expression levels. E: Ratio between the hypothalamic gene expression levels of D2R and Sst2R. F: Ratio between the hypothalamic gene expression levels of D2R and Sst4R. G: Brown adipose tissue Dio2 gene expression levels. H: Brown adipose tissue Dio3 gene expression levels. I: Ratio between Dio2 and Dio3 gene expression levels in brown adipose tissue. J: Correlation measures between the gene expression levels of hypothalamic D2R and the ratio of Dio2 and Dio3 in brown adipose tissue. The data are presented as the mean ± s.e.m. (n=5-6). P, the effect of the photoperiod; T, the effect of each fruit treatment; PxT, the interaction of the photoperiod and the fruit treatment (two-way ANOVA, $p<0.05$). * Mean values were significantly different among groups (one-way ANOVA and Duncan's post hoc test, $p<0.05$). $ Significantly different between groups (Student's t-test, $p<0.05$). LD, long day; SD, short day; SST, somatostatin; HP, hypothalamus; SST2R, somatostatin receptor subtype 2; SST4R, somatostatin receptor subtype 4; D2R, Dopamine Receptor D2; DIO2, Type II iodothyronine deiodinase; BAT, brown adipose tissue; DIO3, Type III iodothyronine deiodinase.*


A
SST-HP (% versus Control group)
LD
SD
Two-way ANOVA | P | T | PxT
Control vs Red-grape | NS | NS | 0.042
Control vs Cherry | 0.008 | NS | NS
B
SST2R-HP (% versus Control group)
Control
Red-grape
Cherry
Two-way ANOVA | P | T | PxT
Control vs Red-grape | NS | 0.000 | NS
Control vs Cherry | NS | NS | NS
C
SST4R-HP (% versus Control group)
Two-way ANOVA | P | T | PxT
Control vs Red-grape | NS | NS | NS
Control vs Cherry | NS | NS | NS
D
D2R-HP (% versus Control group)
Two-way ANOVA | P | T | PxT
Control vs Red-grape | NS | 0.015 | 0.019
Control vs Cherry | NS | NS | NS
E
D2R/SST2R-HP (% versus Control group)
Two-way ANOVA | P | T | PxT
Control vs Red-grape | NS | 0.000 | NS
Control vs Cherry | NS | NS | NS
F
D2R/SST4R-HP (% versus Control group)
Two-way ANOVA | P | T | PxT
Control vs Red-grape | NS | 0.002 | NS
Control vs Cherry | NS | NS | NS
G
DIO2-BAT (% versus Control group)
Two-way ANOVA | P | T | PxT
Control vs Red-grape | 0.001 | 0.006 | 0.001
Control vs Cherry | 0.028 | 0.047 | 0.034
H
DIO3-BAT (% versus Control group)
Two-way ANOVA | P | T | PxT
Control vs Red-grape | 0.001 | NS | NS
Control vs Cherry | 0.001 | NS | NS
I
DIO2/DIO3-BAT (% versus Control group)
Two-way ANOVA | P | T | PxT
Control vs Red-grape | NS | 0.024 | 0.014
Control vs Cherry | 0.049 | NS | NS
J
D2R-HP (% versus Control group)
Red-grape
Spearman r = -0.533, p = 0.011
Cherry
Spearman r = -0.209, p = 0.363
DIO2/DIO3-BAT (% versus Control group)

Red-grape intake modulates biomarkers of central regulation of seasonal control

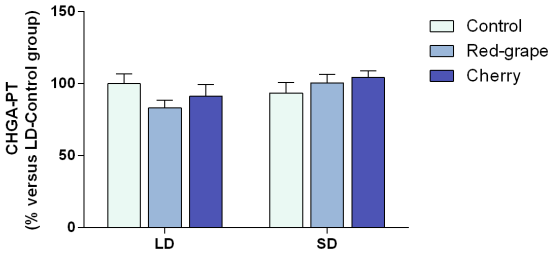
Photoperiod affected *Eya3* expression, as expected, only when vehicle and cherry groups were assessed by 2-way NOVA (Figure 3A). Red-grape consumption slightly, but significantly, caused the overexpression of *Eya3* (27% increase during both in the LD and the SD), masking the effect of the photoperiod reported above. None of the treatments had significant effects on *Chga* expression (Figure 3B). Consequently, the *Eya3*/*Chga* ratio (figure 3C) presented a significant effect of the photoperiod when vehicle and cherry groups were considered and assessed by 2-way ANOVA. Again, red-grape intake resulted in a significant increase of the ratio regardless of the photoperiod (an increase of 46% and 21% during the LD and the SD, respectively). *Dio2*/*Dio3* ratio (figure 3D) showed a similar pattern to that of *Eya3*/*Chga* ratio, being affected by RG treatment mainly in LD animals. These effects were not paralleled by plasma melatonin (figure 3E) or melatonin receptor (*MT1*) expression (figure 3F), which was affected by photoperiod only when red-grape and vehicle treated animals were considered. On the other hand, cherry consumption did not modify any of these seasonal markers. Quantification of plasma PRL (figure 3G) revealed that photoperiod modulated its concentration as expected, but neither red-grape nor cherry affected the levels of the hormone, suggesting that, despite red-grape induced the overexpression of *Eya3*, this was not translated into changes in blood PRL. Similarly, quantification of *Tshβ* mRNA levels (figure 3H) revealed a significant effect of the photoperiod, resulting of increased levels during LD.

A



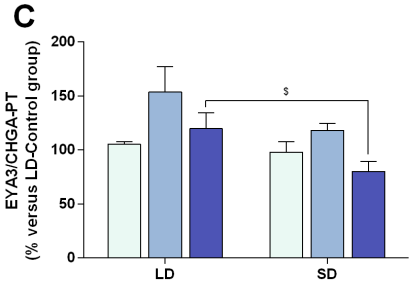
Two-way ANOVA	P	T	PxT
Control vs Red-grape	NS	0.029	NS
Control vs Cherry	0.005	NS	NS

B



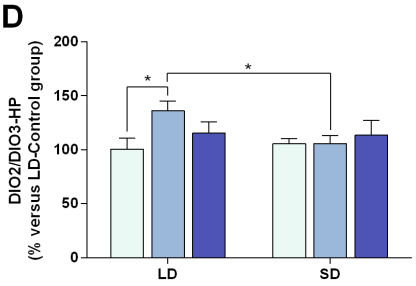
Two-way ANOVA	P	T	PxT
Control vs Red-grape	NS	NS	NS
Control vs Cherry	NS	NS	NS

C



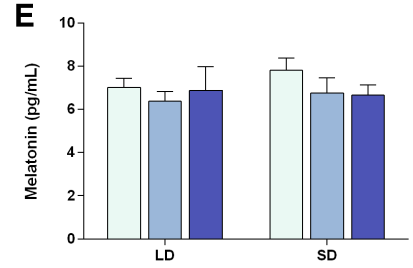
Two-way ANOVA	P	T	PxT
Control vs Red-grape	NS	0.024	NS
Control vs Cherry	0.030	NS	NS

D



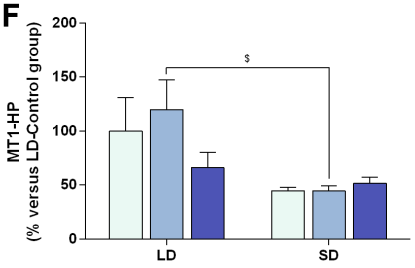
Two-way ANOVA	P	T	PxT
Control vs Red-grape	NS	0.042	0.043
Control vs Cherry	NS	NS	NS

E



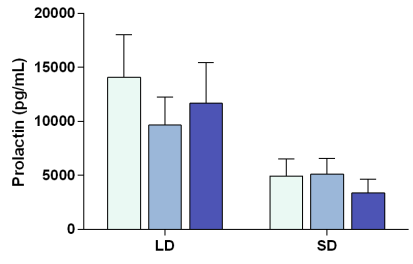
Two-way ANOVA	P	T	PxT
Control vs Red-grape	NS	NS	NS
Control vs Cherry	NS	NS	NS

F



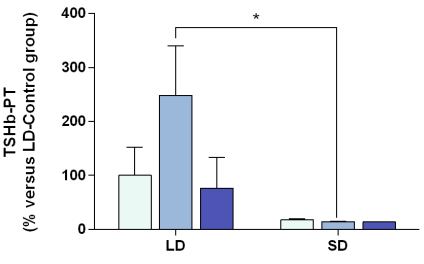
Two-way ANOVA	P	T	PxT
Control vs Red-grape	0.017	NS	NS
Control vs Cherry	NS	NS	NS

G



Two-way ANOVA	P	T	PxT
Control vs Red-grape	0.028	NS	NS
Control vs Cherry	0.009	NS	NS

H



Two-way ANOVA	P	T	PxT
Control vs Red-grape	0.002	NS	NS
Control vs Cherry	0.023	NS	NS

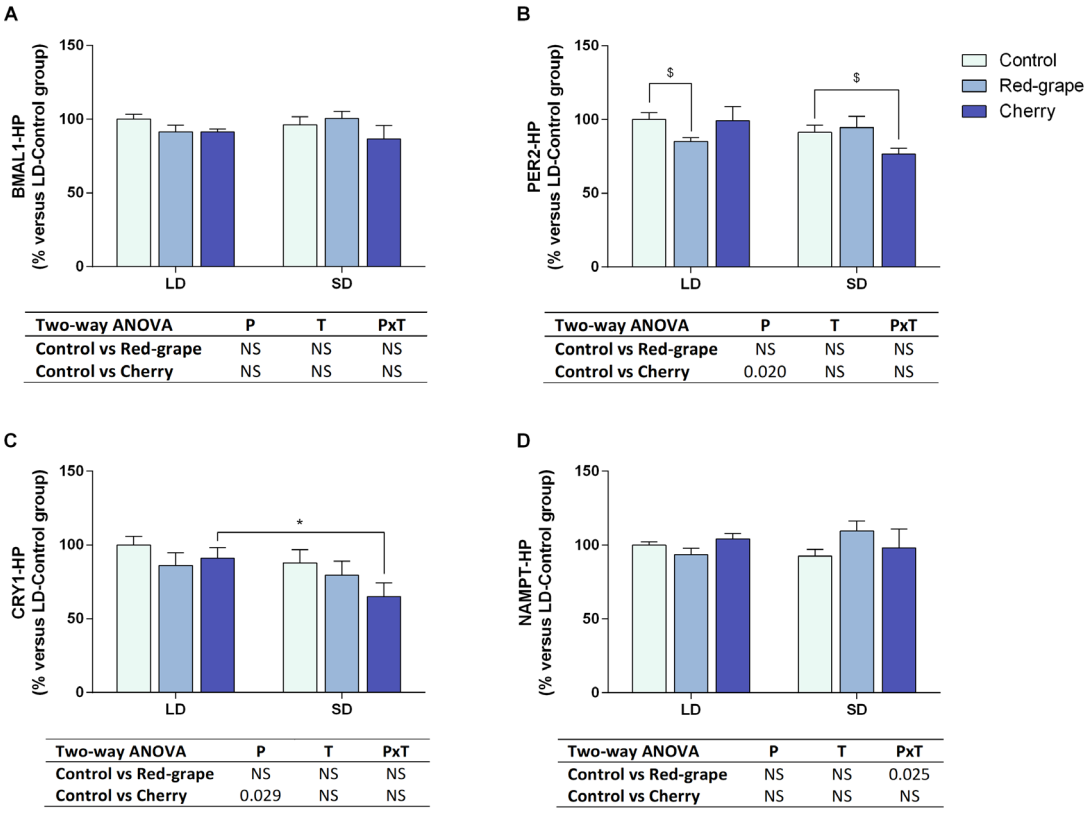
Figure 3. Seasonal controllers of rats adapted to a long day or short day photoperiod and supplemented with red-grape or cherry for 14 weeks. *A: Hypophyseal Eya3 gene expression levels. B: Hypophyseal Chga gene expression levels. C: Ratio between the hypophyseal gene expression levels of Eya3 and Chga. D: Ratio between Dio2 and Dio3 gene expression levels in hypothalamus. E: Melatonin plasma levels. F: Hypothalamic MT1 gene expression levels. G: Prolactin plasma levels. H: Hypophyseal Tshβ gene expression levels. The data are presented as the mean ± s.e.m. (n=5-6). P, the effect of the photoperiod; T, the effect of each fruit treatment; PxT, the interaction of the photoperiod and the fruit treatment (two-way ANOVA, p<0.05). * Mean values were significantly different among groups (one-way ANOVA and Duncan's post hoc test, p<0.05). $ Significantly different between groups (Student's t-test, p<0.05). LD, long day; SD, short day; EYA3, eyes absent homolog 3; PT, pineal gland; CHGA, chromogranin A; DIO2, Type II iodothyronine deiodinase; DIO3, Type III iodothyronine deiodinase; HP, hypothalamus; MT1, melatonin receptor 1A; TSHβ, Thyroid stimulating hormone subunit beta.*

4. Discussion

We have shown that fruit consumption induces different effects on physiology, behavior and central dopaminergic and somatostatinergic signaling in a photoperiod dependent manner. Comparison of the effects of cherry and red-grape, spring and autumn fruits respectively, has revealed profound differences among fruits that might be attributed to their slight different polyphenolic fingerprint.

A limitation of this work is that gene expression studies were done on a single time point, and therefore the circadian rhythm of the different genes could not be assessed. It is known that expression of *Eya3* depends on melatonin, which peaks at dusk in response to lack of light[13]. Melatonin inhibits *Eya3* expression, which levels rise only if light is co-incident with a phase under 12 h after dark onset, a condition that can be achieved only under LD[13]. Consequently, it has been shown that, in the sheep, overexpression of *Eya3* is photo-dependent, confined to the photophase, and peaks at ZT4 (4 hours after lights on), while before and after the peak its expression is maintained at basal levels[27,28]. We decided to sacrifice animals at ZT1, 1 hour after lights on, regardless of the photoperiod in order to avoid interferences due to melatonin signaling and circadian rhythmicity. It is therefore expected that, at ZT1, the levels of *Eya3* expression remained unchanged respect the basal levels. This is the reason that explains the apparent lack of effects of the photoperiod on the expression of *Eya3* and *Eya3*/*Chga* ratios when comparing LD and SD groups in our experiment. Nonetheless, the clear higher expression of *Tshβ* in LD, which transcriptional response to photoperiod is regulated by *Eya3*, is consistent with the literature and supports the expected response to the photoperiod, since it has been shown that *Tshβ* expression remains constantly upregulated in LD during the 24 hours[13]. Similarly, *Dio2* to *Dio3* ratio, which can be considered as the hypothalamic equivalent to PT *Eya3* to *Chga* ratio, was not significantly changed by photoperiod

in our experiment. Again, it has been shown that *Dio2* is subjected to circadian control by Bmal1[29], and therefore dependence to photic cues can also be considered in order to explain the apparent lack of response to the photoperiod in our conditions. This possibility is reinforced by the lack of changes in central clock genes (supplementary figure 1) and in the concentration of melatonin, indicating that all animals were sacrificed at the same central clock timing. The response to photoperiod is evident in other parameters, such as *Tshβ* expression and serum PRL. It is well known that signals from the PT induce the release of PRL from the pars distalis of the pituitary responding invariably to seasonality and increasing during LD periods in all vertebrates[28,30]. Therefore, results are consistent with the idea that animals were not in a refractory state.



Two-way ANOVA	P	T	PxT
Control vs Red-grape	NS	NS	NS
Control vs Cherry	NS	NS	NS

Two-way ANOVA	P	T	PxT
Control vs Red-grape	NS	NS	NS
Control vs Cherry	0.020	NS	NS

Two-way ANOVA	P	T	PxT
Control vs Red-grape	NS	NS	NS
Control vs Cherry	0.029	NS	NS

Two-way ANOVA	P	T	PxT
Control vs Red-grape	NS	NS	0.025
Control vs Cherry	NS	NS	NS

Supplementary Figure 1. Hypothalamic clock genes of rats adapted to a long day or short day photoperiod and supplemented with red-grape or cherry for 14 weeks. *A: Hypothalamic Bmal1 gene expression levels. B: Hypothalamic Per2 gene expression levels. C: Hypothalamic Cry1 gene expression levels. D: Hypothalamic Nampt gene expression levels. The data are presented as the mean ± s.e.m. (n=5-6). P, the effect of the photoperiod; T, the effect of each fruit treatment; PxT, the interaction of the photoperiod and the fruit treatment (two-way ANOVA, p<0.05). * Mean values were significantly different among groups (one-way ANOVA and Duncan's post hoc test, p<0.05). $ Significantly different between groups (Student's t-test, p<0.05). LD, long day; SD, short day; BMAL-1, hydrocarbon receptor nuclear translocator-like 1; PER2, period 2; CRY1, cryptochrome 1; NAMPT, nicotinamide phosphoribosyl transferase.*

Despite the biomarkers of seasonality mentioned above did not show a remarkable response to photoperiod, we could observe effects of red-grape intake increasing the expression of *Eya3* and the *Eya3/Chga* ratio in both photoperiods. These effects were also found in the *Dio2/Dio3* ratio. Those results could be indicative of modulatory properties of red-grape consumption on the mechanisms controlling seasonality. Nevertheless, the lack of changes in the levels of plasma PRL, which is considered the main biomarker of seasonality because it depends exclusively on PT activity[28,31] and can be modulated by non-photic dependent processes as in the case of refractoriness[13], suggests that the effects of red-grape consumption on the central control of seasonality are not translated into effective alterations at the systemic level. The meaning of the slight but significant increase of *Dio2/Dio3* and *Eya3/Chga* ratio remains therefore unexplained.

In contrast with the results exposed above, we identified several changes in the somatostatinergic and dopaminergic systems in rats treated with either red-grape or cherry that can be associated with physiological and behavioral alterations. According to Dulcis *et al.*, adult rats display an increase of dopamine releasing neurons when exposed to SD and a decrease when maintained under LD, whereas the contrary occurs in the case of Sst, and these changes are reflected by the populations of receptors, i.e. *D2R* and *Sst2R/4R* at the expression level[14]. Our results are consistent with this work. Moreover, according to Dulcis *et al.* this

neurotransmitter switching induced by the photoperiod results in changes in mood and behavior, consistent with different works supporting that the LD photoperiod is more stressful for rats[14,32,33]. Our results show that in rats under SD, red-grape intake resulted in decreased locomotor activity and accumulated food intake compared to vehicle treated rats, what could be interpreted as anxiety-like behavioral changes. Concomitantly, those rats showed increased expression of hypothalamic *Sst*, *Sst* receptors and lower expression of *D2R*, resulting in important decreases in *D2R/SSTR* ratios. These results are consistent with the observed behavioral changes associated to neurotransmitter switching[14]. Since it has been shown that perturbations in photoperiod are under different mood and behavior alterations [34–37], our results show that intake of certain foods, i.e. red-grape, might be as relevant as photoperiod shifting for altering mood and behavior at different levels.

Together with changes in mood and behavior, the dopaminergic system is related to the control of energy expenditure. Thus, dopamine activity, through central D2R, has been shown to reduce food intake and increase energy expenditure in experiments using wildtype and D2R-/- mice[38]; and stimulation of dopaminergic system by D2R and D3R mixed agonists has been shown to reduce cold-induced thermogenesis in rats[26]. In both cases, changes in energy expenditure were promoted by modulation of BAT metabolic activity. We have shown that rats maintained under SD conditions and treated with red-grape presented decreased expression of hypothalamic *D2R* and increased energy expenditure respect animals of the same photoperiod treated with the vehicle when this parameter was corrected by locomotor activity. These results suggest that the extra energy expenditure in red-grape treated rats was not due to physical activity, but to increased metabolic processes such as non-shivering thermogenesis in the BAT[39,40]. The activity of the BAT respond, among other stimuli, to the levels of intracellular active thyroid hormone, which depends on the expression levels of the *Dio2* and *Dio3* enzymes[40,41]

that, in turn, respond to adrenergic signaling[42]. Therefore, the ratio *Dio2*/*Dio3* might be considered as a surrogate measurement of the metabolic activity of the BAT. Since we dissected both tissues, hypothalamus and BAT, at the same time, we assessed the correlation between the expression of hypothalamic *D2R* and BAT *Dio2*/*Dio3* ratio, finding a significant negative correlation, which reinforces the idea that the effects of red-grape consumption on hypothalamic *D2R* drive an increase in thermogenesis that underlies the increase of energy expenditure. This hypothesis is reinforced by increased expression of uncoupling protein 1 (*Ucp-1*) observed in the BAT of SD animals treated with red-grape (data not shown).

Despite diet induced obesity has been associated to deficits in dopamine signaling[43–46], Kim *et al* reported decreased food intake as a result of D2R ablation, likely due to enhancement of the leptin signaling system in the hypothalamus[38]. This agrees with our results in rats under SD consuming red-grape compared to vehicle. Consistently, intake of grape seed proanthocyanidins has been shown to enhance leptin signaling, reducing hyperphagia and peripheral leptin resistance associated to a high fat diet[47], and to decreased food intake and increased energy expenditure in aged rats[48]. Therefore, our results are consistent with the effects ascribed to purified polyphenols from red-grape, and highlight the relevance of this fruit in the context of obesity[49,50]. Considering this scenario, the decrease in locomotor activity observed in SD rats treated with red-grape could be understood as a mechanism to save energy in order to compensate decreased food intake and increased thermogenesis. Overall, these results highlight that consumption of red-grape might be beneficial for weight maintenance, though its effects might depend on seasonality and the state of the dopaminergic system.

Previously, we have described photoperiod-dependent effects of cherry intake in the metabolism of the rats studied in this work[51]. Nevertheless, cherry intake did not exert any effect on the parameters studied in the present work. In contrast, we have

found several effects of red-grape consumption. Regardless of the fact that both fruits contain similar classes of polyphenols, they differ in the qualitative and quantitative composition of individual polyphenols[52]. Thus, each fruit can generate different circulating metabolites or microbiota-derived metabolites with different signaling properties; this difference could explain the differential effect on seasonal markers and physiological parameters from red-grape and cherry consumption. The mechanisms by which these metabolites can interact with brain structures through the gut-brain axis remains elusive.

Remarkably, the dopaminergic system is sensitive to signals transported by the blood due to the anatomy of the median eminence of the neurohypophysis[53]. This structure is outside the blood-brain barrier, and tanycytes are in direct contact with the blood through fenestrated vessels[10,16] and their processes reach dopaminergic neurons[54,55]. Therefore, a possible sensitivity of tanycytes to polyphenol metabolites or other molecules derived from fruit consumption might fall under the observed modulatory properties of fruits on the hypothalamus. Moreover, morphological changes of tanycytes[53] might facilitate the diffusion and direct contact of polyphenol metabolites with hypothalamic components of the hypothalamus-pituitary axes and, therefore, modulate their function. Alternatively, our results could also be explained by direct actions of fruit consumption on the enteric nervous system and its connections, via the vagus nerve, with the central nervous system[15].

The effects of red-grape consumption on physiological and behavioral parameters are photoperiod-dependent. These observations can be explained by the different modifications, such as physiological, molecular and even anatomical, that the photoperiod program induces in the brain[9,31]. This photoperiod-dependent response to fruits might be postulated for other nutritional challenges, therefore expanding the factors that should be considered when studying the gut-brain axis and the modulation of brain function by nutrition. Interestingly, the effects of red-

grape intake have been found when the fruit was consumed in its natural season. This evidence strengthens the idea that heterotrophs have adapted their metabolism and behavior to specific cues provided by food through the evolutionary process.

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Author contributions

CB, LA, AC, and JdB designed the studies. RM-C, CD-C, AC, and JdB performed the experiments and analyzed the data. JdB, CD-C, and CB wrote the manuscript. All authors read, discussed, and approved the final version of the manuscript.

Author information

The authors have no conflicts of interest to declare.

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MODULATION OF CENTRAL NEUROENDOCRINE RESPONSE TO PHOTOPERIOD BY SEASONAL FRUITS AND OBESITY.
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Manuscript 3

Cafeteria diet-induced obesity impairs normal neuroendocrine response to photoperiod and red grape consumption and abolishes prolactin seasonal variation

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Abstract

Animals are sensitive to environmental light, and adapt their metabolism and behavior to seasonal photoperiod. The xenohormesis theory proposes that animals also recognize bioactive compounds in vegetal foods, mainly polyphenols, as non-photic cues. We have previously shown that, in Fischer F344 rats, red-grape intake in its natural season, corresponding to short days (SD), decreased locomotor activity and food intake and increased energy expenditure. In SD, red-grape repressed the expression of hypothalamic dopamine receptor and increased the expression of thermogenic genes in the brown adipose tissue (BAT). Therefore, we hypothesized that red-grape might be beneficial to obesity. In this work, eight groups of rats were placed in SD (6h light:18h darkness) or long day (LD) conditions (18hlight:6h darkness), fed with a cafeteria diet (CAF) or standard chow diet, and treated with red-grape or vehicle. CAF abolished the physiological and behavioral effects of red-grape and exacerbated the response of hypothalamic-pituitary-thyroid and hypothalamic-pituitary-adrenal axis to photoperiod. In contrast, CAF abolished the normal seasonal variation of plasma prolactin found in the normoweight cohort. Moreover, red-grape affected the thyroid axis in both phenotypes, although in CAF animals only thyroid hormones were affected, whilst in normoweight animals red-grape increased more than 3-fold the ratio between deiodinases 2 and 3 in peripheral tissues such as liver and BAT, suggesting increased metabolic activity. Our results suggest that CAF induced obesity disrupts neuroendocrine homeostasis, impairing the response to photic and non-photic cues. Although more research is still needed, this work provides new evidences for considering seasonal adaptation as a relevant element for prevention and treatment of diseases.

1. Introduction

Xenohormesis is a biological principle based on the observation that environmentally stressed plants produce bioactive compounds which can be considered as interspecific chemical signals for animals. The molecules involved in this responses, mainly polyphenols, might allow animals to detect future predictable changes when the conditions are still favourable, thus increasing their probability of survival[1]. Polyphenols are known to be produced by plants as a response to different factors, such as photoperiod[2] or circannual cycles[3], and stressful situations such as temperature or dryness[4–6]. In the context of the Xenohormesis, these molecules confer different functionalities to the intake of vegetal foods by heterotrophs, which can be associated with the different long-term effects on health and wellness described for polyphenols, such as vascular function, inflammatory response or neurodegenerative protection among others [1,2,7].

Seasonal rhythms are considered dominant features of the biology of mammals and have an important role in the control of several functions, such as reproduction, metabolism, affective disorders and immunity[8]. This adaptive process involves both intrinsic mechanisms and environmental stimuli, whereas the variation of day length between day and night is one of the most important exogenous stimulating signals, known as photoperiod[8,9]. Animal circannual rhythmicity has been reported over the last decades and some structures and mechanisms stablished as rhythmic generators. Recent findings have revealed a key role of thyroid hormone (TH) in the control of seasonal responses in mammals[10,11]. Thus, sustained signaling of melatonin to the pituitary on long day photoperiods (LD) induces the activity of thyroid hormones in the hypothalamus, which drive the neuroendocrine and neurotransmitter changes associated to circannual rhythms[11]. It has been described that, in rats, photoperiod switching triggers a shift on signaling actions of

neurotransmitters dopamine and somatostatin (SST), inducing changes in behavior[12]. LD is characterized by increasing signaling of SST and lower dopamine, leading to anxiety and depression behaviors, while in short day (SD) increased dopaminergic signaling leads to amelioration of these states. These changes are mediated through photoperiod-driven changes in post-synaptic receptors populations such as dopamine receptor D2 (D2R) and somatostatin receptors 2 and 4 (SST2R and SST4R respectively) in the periventricular nucleus of the hypothalamus[12]. Therefore, D2R to SST receptors ratio can be used as a biomarker to assess the response of somatostatinergic and dopaminergic systems to seasonality.

Hypothalamic seasonality control also affects different neuroendocrine axis, such as the hypothalamic-pituitary-thyroid (HPT) axis, in which thyrotropin-releasing hormone (TRH) induces the release of thyrotropin (TSH) from the pars distalis of the pituitary into the systemic circulation through the vessels of the median eminence[13]. Circulating TSH acts on thyrocytes through the TSH receptor to stimulate synthesis and release of THs, mainly thyroxine (T4). In turn, circulating TH inhibits *Tsh* by a negative-feedback loop that represses hypothalamic *Trh* expression and pituitary TSH release in order to fine tune the thyroid function[14]. Intracellular signaling of TH in peripheral tissues is dependent on deiodinase enzymes. Type 1 and 2 deiodinases (DIO1 and DIO2 respectively) convert the TH precursor T4 into its active form triiodothyronine (T3). In turn, type 3 deiodinase (DIO3) converts T4 into an inactive form (rT3) or T3 into inactive diiodothyronine (T2). Therefore, the ratio DIO2/DIO3 determines the amount of intracellular bioactive T3 that signals to cell nucleus[15]. Together with the HPT axis, the hypothalamic-pituitary axis controls reproduction through the hypothalamic-pituitary-gonadal (HPG) axis, based on the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) by the pars distalis of the anterior pituitary after stimulation by hypothalamic gonadotropin releasing

hormone (GnRH)[16]. Gonadotropins signal to testes in males, inducing the release of testosterone that, among different actions, inhibits gonadotropins release closing a negative feedback control. The third relevant axis is the hypothalamic-pituitary-adrenal, which governs response to stress[17]. This axis starts with the expression of hypothalamic corticotropin releasing hormone (*Crh*), which induces the secretion of adrenocorticotropic hormone (ACTH) by the pars distalis into the systemic circulation. In turn, ACTH induces the release of glucocorticoids such as cortisol or corticosterone, and catecholamines such as epinephrine, norepinephrine and aldosterone from the adrenal cortex. Despite the known photoperiodic control, other factors such as stress, mood and feeding disorders, hormone imbalance or reproductive rhythmicity might modulate the function of these axis[18]. In contrast, prolactin (PRL) release from the pituitary is not subjected to hypothalamic control, and responds increasing during LD periods in all vertebrates[19,20]. Together with PRL, other pituitary biomarkers of seasonality are the ratio between eyes absent 3 (EYA3) and chromogranin A (CHGA), which is exacerbated in LD[9], and expression of *Tshβ*, which drives seasonal signaling to the hypothalamus[11].

According to the xenohormesis theory, bioactive compounds in foods may act as signals secondary to photoperiodic cues, allowing heterotrophs to adjust different metabolic and behavioral parameters to the environmental conditions of a given ecosystem. Based on this idea, we have assessed the effects of seasonal fruit consumption in different photoperiods simulating either autumn-winter conditions by maintaining animals under a SD photoperiod consisting on 6 hours of light and 18 hours of darkness, or spring-summer conditions with a LD photoperiod of 18 hours of light and 6 hours of darkness. As a model, we chose the photosensitive Fischer 344 rat. In a previous work (Manuscript 2), we have shown that administration of red-grape (RG), an autumn fruit, in a dose that resembled human consumption, decreased global locomotor activity, food intake and increased energy expenditure

when consumed during SD, its natural seasonal photoperiod, but not during LD. These effects were paralleled by an exacerbated increase of *Dio2* to *Dio3* ratio in the interscapular brown adipose tissue (BAT) that negatively correlated with decreased expression of *D2R* in the hypothalamus. Therefore, our results suggested that RG intake could be proposed as a strategy for correcting obesity and its associated alterations by modulating energy expenditure and food intake acting through central control, but on a photoperiod-dependent fashion.

Therefore, our objective was to assess the effects of RG intake on the above mentioned parameters in Fisher F344 rats fed with cafeteria diet, a model that mimics metabolic syndrome in humans, inducing obesity, dyslipidemia, insulin resistance among other abnormalities[21–23]. Moreover, due to our previous results and the relevance of the gut-brain axis in the modulation of neuroendocrine function, we assessed different biomarkers representative of the HPT, HPG and HPA axis in order to better characterize the effects of RG in a model of obesity living in different photoperiods. Results show that cafeteria diet induced obesity completely blunted the effects of RG on energy expenditure, food intake and locomotor activity, impaired seasonal control of prolactin release and the response of HPA, HPG and HPT axis to photoperiod. Moreover, RG modulated HPT axis on a phenotype-dependent mode. Our results suggest that diet induced obesity or obesogenic diet itself alters the normal neuroendocrine response to photoperiod and to nutritional interventions.

2. Materials and methods

Animals

The Animal Ethics Committee of the University Rovira i Virgili (Tarragona, Spain) approved all the procedures.

Experiment 1: The methods for this experiment have been described previously (Manuscript 2). Twenty-four 8-weeks-old male Fischer 344 rats (Charles River Laboratories, Barcelona, Spain) were housed in pairs in cages at 22ºC under two different light schedules in order to emulate season's day length: long day photoperiod (n=12, LD, 18:6h light/dark cycle) or short day photoperiod (n=12, SD, 6:18h light/dark cycle). After 1 month under these conditions, animals under each photoperiod were orally supplemented with 100 mg per kg of body weight/day of red-grape (RG, V*itis vinifera*, variety garnacha, n=12) for 10 weeks. Another group, used as a control, received only the vehicle (control, n=12). Rats were fed *ad libitum* with a standard diet (2.90 kcal·g-1; Teklad Global 14% Protein Rodent Diet 2014, ENVIGO, Sant Feliu de Codines, Barcelona, Spain). During the experiment, food intake was weekly recorded. After 14 weeks, the animals were deprived of food for 1h and killed by decapitation.

Experiment 2: Forty 8-weeks-old male Fischer 344 rats (Charles River Laboratories, Barcelona, Spain) were housed and fed in the same conditions described in the first experiment. After 1 month of adaptation under these conditions, animals in each photoperiod were switched to a cafeteria diet (CAF) for 7 weeks, which is a period of time that allows the appearance of obesity and Metabolic Syndrome (MetS)-related alterations in CAF-fed rats[24]. During these 7 weeks, animals were also orally supplemented with 100 mg per kg of body weight/day of red-grape (RG, V*itis vinifera*, variety garnacha, n=20). Another group used as control received the vehicle

(Control, n=20). The cafeteria diet contained bacon, biscuit with pâté, biscuit with cheese, carrots, muffins and milk with sugar (220 g/L), and its caloric distribution was 58.1% carbohydrate, 31.9% lipid and 10.0% protein. During the experiment, food intake was weekly recorded. After 11 weeks, the animals were deprived of food for 1h and killed by decapitation.

In both experiments, the blood was collected and the serum was obtained by centrifugation and stored at -80ºC until analysis. The two experiments were conducted between 10:00 and 11:00 to minimize possible circadian variations. Hypothalamus, pituitary gland, brown adipose tissue and liver were rapidly removed after death, weighed, frozen in liquid nitrogen and stored at -80ºC until further analysis.

Physical activity and respiratory metabolism measurements

The OxyletProTM system (Panlab, Barcelona, Spain) was used to evaluate animal respiratory metabolism and physical activity. The measurements were performed at 7 weeks of study in the animals of experiment 2 in *ad libitum* conditions and for 21 hours (from 12:00 to 09:00). Monitored data during the first hour of acclimation were discarded.

The animals were transferred to a standard rodent home cage (LE 1331 OxyletProTM Home Cage, Panlab), which uses an airtight lid (LE 1332 OxyletProTM Airtight Lid, Panlab) to ensure the sample environment. The software program Metabolism 3.0.00. (Panlab, Barcelona, Spain) calculated the Energy Expenditure (EE, kcal·day-1·Kg-0.75) as "oxygen consumption x 1.44 x [3.815 + (1.232 x (carbon dioxide production/ oxygen consumption))]", according to the Weir formula[25]. Cages were placed on a platform with extensiometric weight transducers to register the movements.

Gene expression analysis

The total RNA containing micro RNA was extracted from hypothalamus, pituitary gland, brown adipose tissue and liver using TRIzol Reagent (Thermo Fisher Scientific, Barcelona, Spain) according to the manufacturer's protocol. To isolate both total and small RNA species, 10 minutes of incubation with 100% isopropanol were changed into an over-night incubation at -20ºC. The RNA yield was quantified on a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

To analyse the expression of the samples, cDNA was synthetized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Barcelona, Spain). A Labnet MultiGene Gradient PCR Thermal Cycler (Sigma-Aldrich, Madrid, Spain) was used for reverse transcription. The reaction was performed according to the instructions of the manufacturer. The cDNA was subjected to a quantitative reverse transcriptase polymerase chain reaction amplification using the iTaq Universal SYBR Green Supermix (Bio-Rad, Madrid, Spain) in the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Madrid, Spain). The primers used for the different genes are described in Supplementary Table 1 and were obtained from Biomers.net (Ulm, Germany). The fold change in each mRNA levels was calculated as a percentage of the LD-Control group, using the -2ΔΔCt method[26] with PPIA gene as an endogenous control.

Supplementary Table 1. *Nucleotide sequences of primers used for PCR amplification in central and peripheral tissues.*

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	Access Number
CHGA	AGGATTCTGACAAGGGGCAA	CACTGGGACCTCTCTCACTG	NM_021655.2
D2R	TGAACAGGCGGAGAATGGAT	CAGGACTGTCAGGGTTGCTA	NM_012547.1
DIO2	TTATGGGGTAGCCTTTGAACG	CCAGCCAACTTCGGACTT	NM_031720.4
DIO3	CTCCAGCAGTTCCGCATATG	CATTCGCACATGAGCTTCGA	NM_017210.4
EYA3	TGGTCCCATGTTGTGCTTTG	CAGTGCTATGGAAACGGTCG	NM_001107910.1
PPIA	CTTCGAGCTGTTTGCAGACAA	AAGTCACCACCCTGGCACATG	NM_017101.1
SST	CTGGAGCCTGAGGATTTGCC	GGATCAGAGGTCTGGCTGAG	NM_012659.2
SST2R	CCCGCTATGTAATCTCGT	TGCAAGAGGGATGCTG	NM_019348.1
SST4R	AGCATGAACACGCCTGCAA	CATCAGCGACGGCCAGGTT	NM_013036.2
THRalpha	ATCCACGTTGCTACAGAGGC	TGACTGGCCAATGTCATCCG	NM_001017960.1
THRbeta	GATCATGTCCCTCCGAGCTG	AGATCGCATCTGACACCACC	NM_001270854.1
TSHb	TCGTTCTCTTTTCCGTGCTTT	CCGTGTCATACAATACCCAGC	NM_013116.2

CHGA, chromogranin A; D2R, Dopamine Receptor D2; DIO2, Type II iodothyronine deiodinase; DIO3, Type III iodothyronine deiodinase; EYA3, eyes absent homolog 3; PPIA, Peptidylprolyl Isomerase A; SST, somatostatin; SST2R, somatostatin receptor subtype 2; SST4R, somatostatin receptor subtype 4; THRα, thyroid hormone receptor subunit alpha; THRβ, thyroid hormone receptor subunit beta; TSHβ, Thyroid stimulating hormone subunit beta.

Hormone analysis

Serum adrenocorticotropin (ACTH from Cusabio, CSB-E06875r, Abyntek, Vizcaya, Spain), luteinizing hormone (LH from Cusabio, CSB-E12654r, Abyntek, Vizcaya, Spain), thyroid-stimulating hormone (TSH from DRG, EIA-5296, Ricardo Esteller, Valencia, Spain) and prolactin (PRL from MyBiosource, MBS2512489, Abyntek, Vizcaya, Spain) concentrations were measured using different rat ELISA kits

Serum cortisol, testosterone, triiodothyronine (T3) and thyroxine (T4) concentrations were measured using liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) analysis as previous described by Domenech-Coca et al.[27].

Statistical analysis

Data are expressed as means ± S.E.M. The differences between groups were performed by two-way ANOVA, with factorial designs photoperiod (P) and treatment (T), one-way ANOVA, followed by Duncan's new multiple range test (as a post-hoc test), or Student's t-test, as indicated in the figure legends. All the statistical analyses were done with the statistical software SPSS Statistics 22 (SPSS Inc., Chicago, IL, USA). Grubbs' test was used to detect outliers, which were discarded for subsequent analyses. The level of significance was set at bilateral 5%.

3. Results

Photoperiod and red-grape consumption effects on behavior and central dopaminergic biomarkers in obese rats

Monitoring of behavioral parameters during 21 hours resulted in no differences among groups on free locomotor activity (figure 1A), energy expenditure (figure 1B) or energy expenditure normalized by locomotor activity (figure 1C). Nevertheless, accumulated food intake (figure 1D) during all the experiment was significantly changed by photoperiod, decreasing in animals under SD respect those living under a LD condition, as reported by the 2-way ANOVA analysis. Overall, no effects of RG treatment were observed. In our previous work, we associated changes in behavior to profound effects of RG consumption on the somatostatinergic and dopaminergic systems.

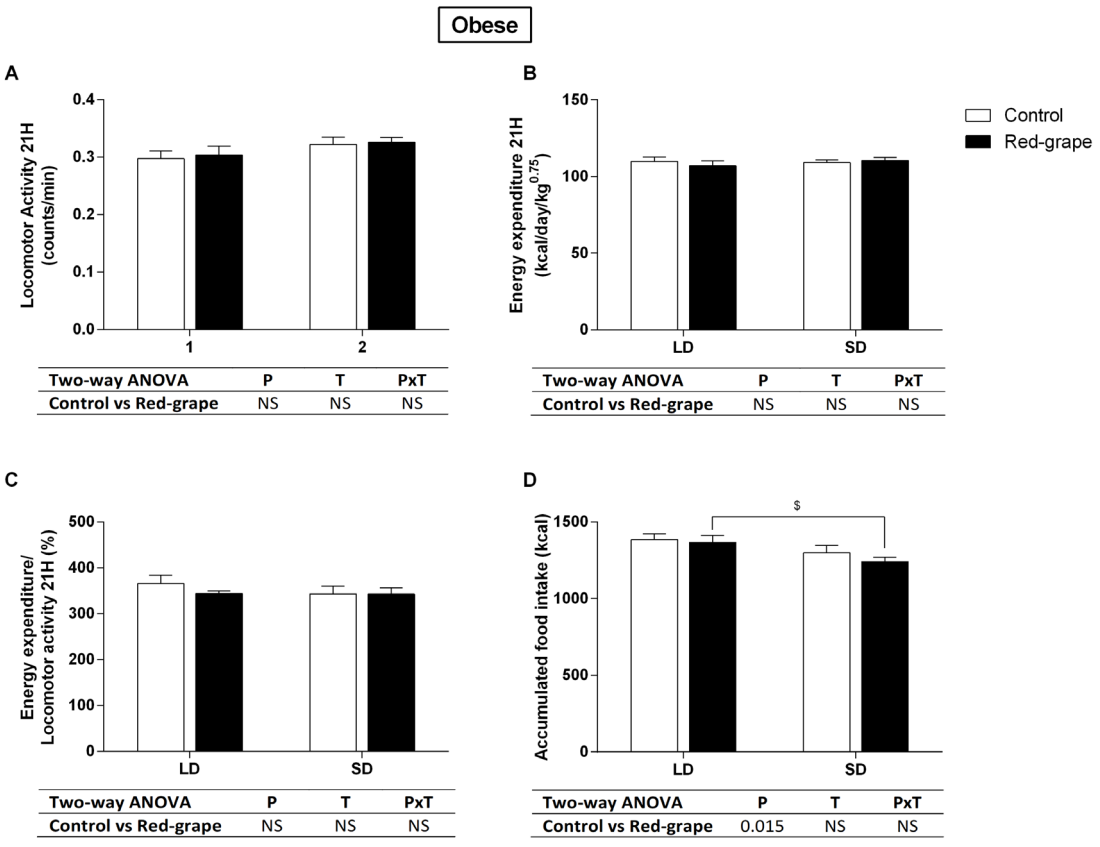


Figure 1. Behavioral parameters of cafeteria-fed rats adapted to a long day or short day photoperiod and supplemented with red-grape for 11 weeks. *A: Locomotor activity. B: Energy expenditure. C: Ratio between energy expenditure and locomotor activity. D: Accumulated food intake. The data are presented as the mean ± S.E.M. (n=10). P, the effect of the photoperiod; T, the effect of each fruit treatment; PxT, the interaction of the photoperiod and the fruit treatment (two-way ANOVA, p<0.05). $ Significantly different between groups (Student's t-test, p<0.05). LD, long day; SD, short day.*

Evaluation of the same biomarkers revealed no differences on somatostatin expression (figure 2A). No significant differences were observed for mRNA levels of dopamine receptor D2R (figure 2B) after 2-way ANOVA analysis, although its expression was decreased a 44% by RG treatment in LD, reaching statistical significance by Student's T test (p=0.039). Somatostatin receptors SST2R (figure 2C) and SST4R (figure 2D) were not changed by either photoperiod or RG treatment. Nevertheless, the D2R to somatostatin receptors ratios showed a tendency to decrease in animals treated with RG during the LD. While the tendency did not reach significant differences for D2R/SST2R (figure 2E), an effect of the treatment was reveled for the D2R/SST4R ratio when assessed by 2-way ANOVA (figure 2F).

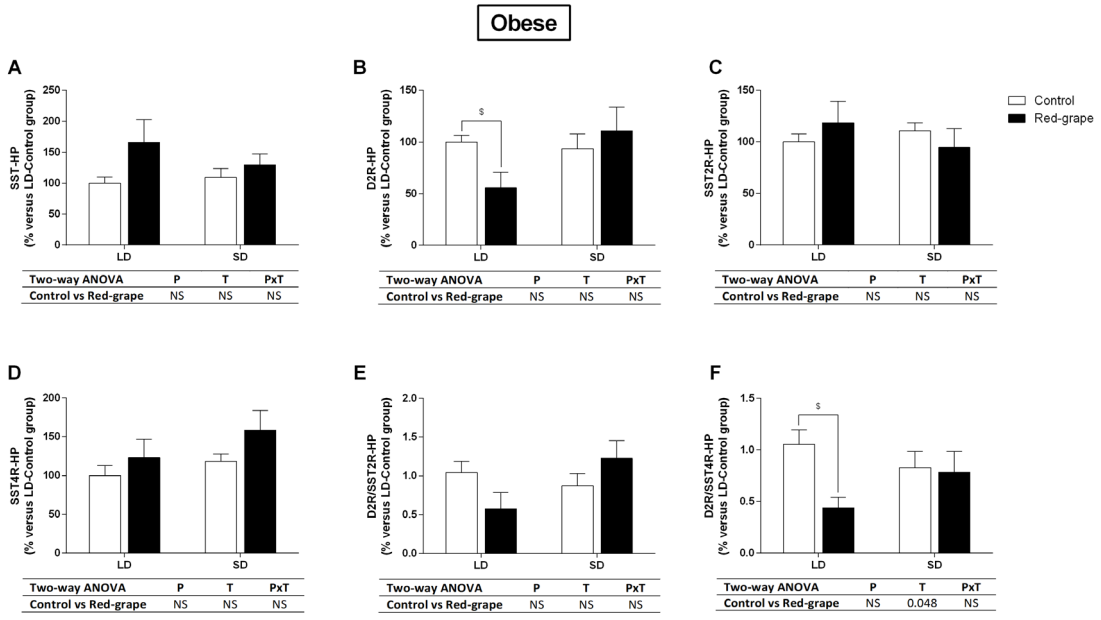


Figure 2. Hypothalamic biomarkers related to the somatostatin and dopaminergic systems of cafeteria-fed rats adapted to a long day or short day photoperiod and supplemented with red-grape for 11 weeks. *A: Hypothalamic Sst gene expression levels. B: Hypothalamic D2R gene expression levels. C: Hypothalamic Sst2R gene expression levels. D: Hypothalamic Sst4R gene expression levels. E: Ratio between the hypothalamic gene expression levels of D2R and Sst2R. F: Ratio between the hypothalamic gene expression levels of D2R and Sst4R. The data are presented as the mean ± s.e.m. (n=5). P, the effect of the photoperiod; T, the effect of each fruit treatment; PxT, the interaction of the photoperiod and the fruit treatment (two-way ANOVA, p<0.05). $ Significantly different between groups (Student's t-test, p<0.05). LD, long day; SD, short day; SST, somatostatin; HP, hypothalamus; D2R, Dopamine Receptor D2; SST2R, somatostatin receptor subtype 2; SST4R, somatostatin receptor subtype 4.*

Cafeteria diet affects photoperiod-dependent neuroendocrine control and sensitivity to red grape intake.

Since both factors, photoperiod and RG treatment, differentially modulated behavior and hypothalamic dopaminergic and somatostatinergic systems depending on the phenotypical traits, e.g. normoweight and cafeteria diet induced obesity, we wondered whether hypothalamic-controlled neuroendocrine function could also be dependent on these phenotypical differences. We analyzed key elements of the axis HPA, HPG and HPT in both normoweight and animals with obesity induced by cafeteria diet living in LD and SD and treated with RG or vehicle (table 1). Results show no differences between normoweight and obese animals associated with photoperiod or RG intake on the levels of ACTH. Nevertheless, while photoperiod did not affect plasma cortisol in normoweight groups, obese animals resulted more sensitive to changes in photoperiod, with statistically significant lower levels of cortisol in animals under SD. The HPG axis was similarly affected by photoperiod in normoweight and obese animals, being LH and testosterone higher during SD in both phenotypes and not affected by RG treatment as revealed the 2-way ANOVA analysis. In contrast, we found that the HPT axis responded differently to both photoperiod and RG treatment in normoweight and obese animals. Thus,

TSH was significantly affected by the RG treatment in normoweight groups, increasing mainly during LD and slightly during SD. When obese groups were analyzed, no differences were found between vehicle and RG treatments. Levels of T3 were not changed by either photoperiod or RG treatment in normoweight animals. Instead, obesity resulted in a clear increase of plasma bioactive T3 in animals living in SD. Moreover, T4 was not affected by photoperiod or RG treatment in normoweight animals. Again, SD photoperiod significantly increased this hormone in obese rats. Besides, RG treatment also increased plasma T4 levels only in the SD obese phenotype. Consequently, in obese animals, both photoperiod and RG treatment significantly affected the T4 to T3 ratio, indicative of the proportion of inactive to active TH, and the T3 to T3+T4 ratio, an index indicating the proportion of active form relative to the total amount of TH. Thus, the T4/T3 ratio was increased by RG treatment, while the significant photoperiod effect was due to lower concentration in animals under SD. In turn, the T3/T3+T4 index was decreased by RG treatment and increased by SD photoperiod. These results suggest that active TH is higher during SD than during LD in obese rats, while intake of RG decreases the relative amount of the active hormone. None of these effects were observed on the normoweight cohort, suggesting that phenotype and/or diet conditioned the response of the HPT axis to both photoperiod and RG intake.

Table 1. Hormonal plasma levels related with hypothalamic-pituitary-adrenal, hypothalamic-pituitary-gonadal and hypothalamic-pituitary-thyroidal axis.

			HPA		HPG		HPT				
			ACTH	**Cortisol**	**LH**	**Testosterone**	**TSH**	**T3**	**T4**	**T4/T3**	**T3/T3+T4**
			pg/mL	ng/mL	ng-mIU/mL	ng/mL	ng/mL	ng/mL	ng/mL	%	ng/mL
Normoweight	LD	Control	8.55 ± 0.77	2.12 ± 0.32	0.81 ± 0.13^{a}	1.49 ± 0.20	0.83 ± 0.08^{a}	1,66 ± 0,14	25,69 ± 2,03	15,69 ± 1,24	0,06 ± 0,00
		Red-grape	9.00 ± 1.19	2.56 ± 0.56	0.59 ± 0.19^{a}	1.25 ± 0.50	1.90 ± 0.23^{b}	1,68 ± 0,17	46,49 ± 11,23	27,71 ± 6,23	0,04 ± 0,01
	SD	Control	10.43 ± 2.67	1.68 ± 0.41	1.74 ± 0.23^{b}	3.34 ± 0.83	1.76 ± 0.36^{b}	1,72 ± 0,18	25,65 ± 1,99	16,81 ± 1,83	0,06 ± 0,01
		Red-grape	8.90 ± 1.28	3.79 ± 1.08	2.04 ± 0.34^{b}	2.29 ± 0.55	1.80 ± 0.16^{b}	1,87 ± 0,22	24,70 ± 2,29	14,46 ± 1,60	0,07 ± 0,01
		Two-way ANOVA	NS	NS	*P* (p=0,000)	*P* (p=0,032)	*T* (p=0,008)	NS	NS	NS	NS
Obese	LD	Control	0,85 ± 0,06	2,12 ± 0,31^{a}	3,71 ± 0,47^{a}	1,77 ± 0,22^{a}	1,36 ± 0,06	0,39 ± 0,07^{a}	16,07 ± 0,55^{a}	45,15 ± 6,10^{a}	0,02 ± 0,00^{ab}
		Red-grape	0,90 ± 0,06	1,39 ± 0,17^{b}	4,03 ± 0,48^{a}	1,56 ± 0,17^{a}	1,36 ± 0,10	0,23 ± 0,05^{a}	16,89 ± 0,45^{a}	107,68 ± 26,05^{b}	0,01 ± 0,00^{a}
	SD	Control	0,92 ± 0,05	0,83 ± 0,17^{b}	5,33 ± 0,72^{a}	2,87 ± 0,67^{ab}	1,46 ± 0,09	0,91 ± 0,14^{b}	19,52 ± 0,92^{b}	25,25 ± 3,29^{c}	0,04 ± 0,01^{c}
		Red-grape	1,01 ± 0,05	0,87 ± 0,24^{b}	7,00 ± 0,80^{b}	4,45 ± 0,88^{b}	1,42 ± 0,05	0,75 ± 0,11^{b}	21,56 ± 0,83^{c}	32,75 ± 3,83^{ac}	0,03 ± 0,00^{bc}
		Two-way ANOVA	NS	*P* (p=0,001)	P (p=0,001)	P (p=0,001)	NS	*P* (p=0,000)	P (p=0,000) *T* (P=0,049)	P (p=0,000) *T* (P=0,006)	P (p=0,000) *T* (P=0,037)

LD, long day; SD, short day; HPA, hypothalamic-pituitary-adrenal; HPG, hypothalamic-pituitary-gonadal; HPT, hypothalamic-pituitary-thyroidal; ACTH, adrenocorticotropic hormone; LH, luteinizing hormone; TSH, thyrotropin; T3, triiodothyronine; T4, thyroxine.

Cafeteria diet blunted the response of deiodinases to red grape consumption in peripheral tissues

The results found on plasma elements of the HPT axis prompted us to assess the expression of deiodinases in peripheral tissues relevant for metabolism such as BAT and liver. In BAT (figure 3), neither photoperiod nor RG treatment affected the expression of *Dio2* (figure 3A) or *Dio3* (figure 3B) when assessed by 2-way ANOVA, although in the latter, significant differences were found by Duncan post-hoc test analysis when comparing vehicle treated animals between LD and SD, indicating mild effect of the photoperiod in control conditions. Analysis of the *Dio2/Dio3* ratio (figure 3C) revealed a significant interaction between photoperiod and treatment, resulting in decreased ratio in RG treated animals in LD but increased ratio in RG treated rats in SD. The same analysis has been reported previously for the normoweight cohort (Manuscript 2). Analysis of mRNA levels of *TH receptors alpha* in this tissue revealed no effects of either photoperiod or RG treatment in obese animals (figure 3D) but a marked increase on the expression of this gene in SD groups of the normoweight cohort (figure 3E). In the case of *TH receptor beta*, exposure to SD slightly but significantly decreased its expression in both obese (figure 3F) and normoweight (figure 3G) animals. These results point out slight effects of RG treatment on the expression of deiodinases genes on the BAT of obese rats, contrasting with the results obtained in normoweight rats and published previously (Manuscript 2).

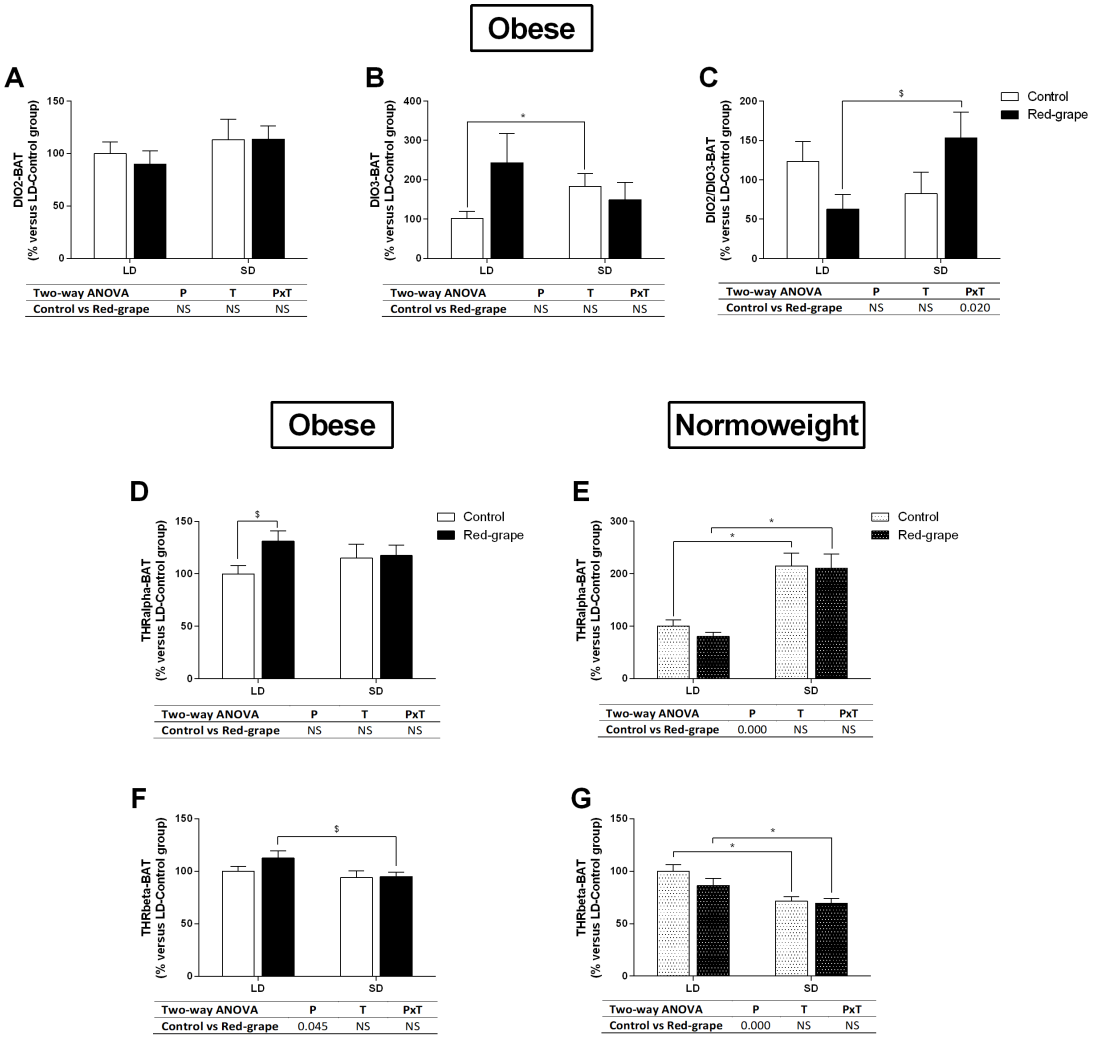
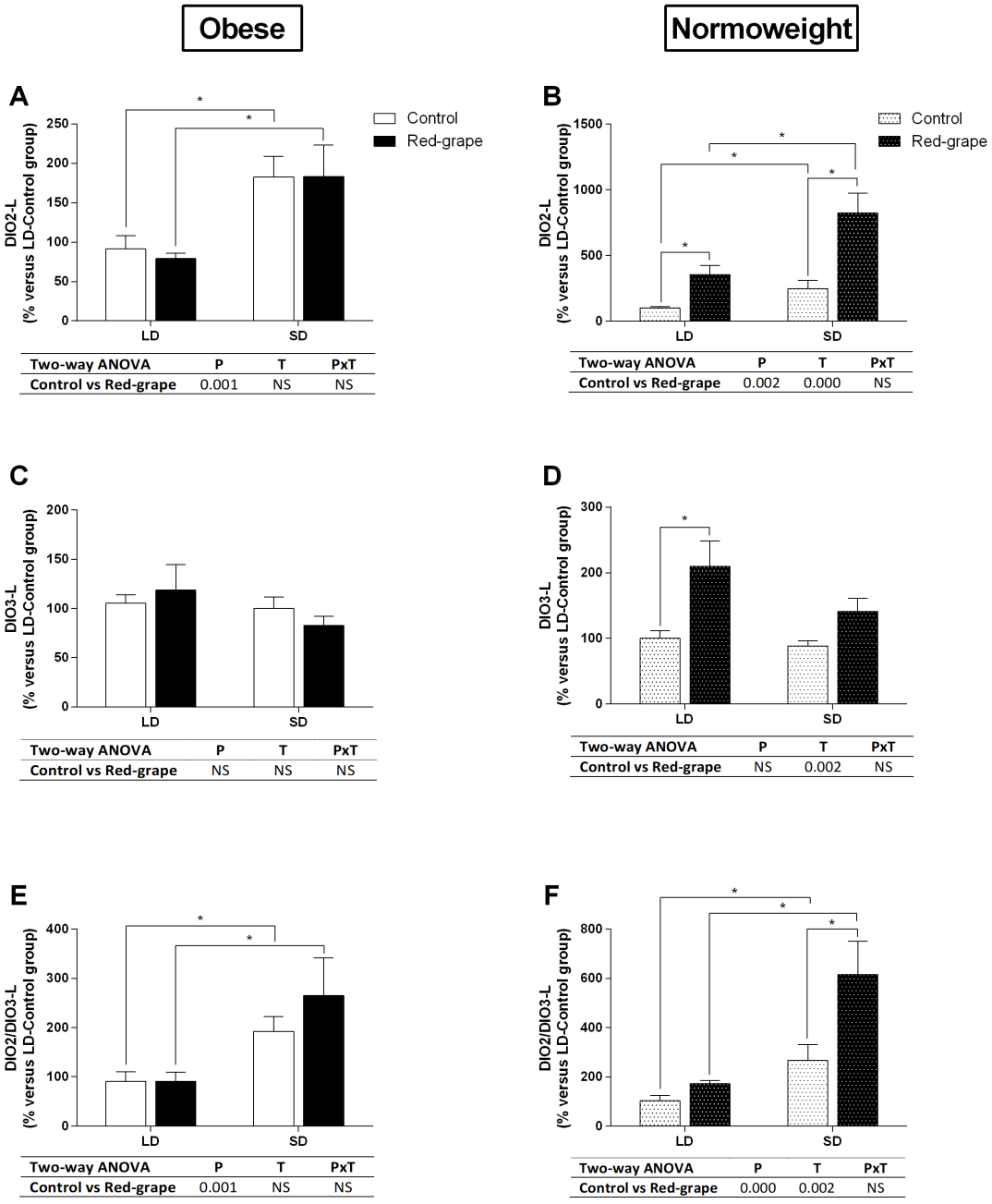


Figure 3. Brown adipose tissue biomarkers related to the thyroid system of cafeteria- or standard-fed rats adapted to a long day or short day photoperiod and supplemented with red-grape for 11 weeks. *A: Brown adipose tissue Dio2 gene expression levels of obese animals. B: Brown adipose tissue Dio3 gene expression levels of obese animals. C: Ratio between Dio2 and Dio3 gene expression levels in brown adipose tissue of obese animals. D: Brown adipose tissue Thrα gene expression levels of obese animals. E: Brown adipose tissue Thrα gene expression levels of normoweight animals. F: Brown adipose tissue Thrβ gene expression levels of obese animals. G: Brown adipose tissue Thrβ gene expression levels of normoweight animals. The data are presented as the mean ± s.e.m. (n=10). P, the effect of the photoperiod; T, the effect of each fruit treatment; PxT, the interaction of the photoperiod and the fruit treatment (two-way ANOVA, p<0.05). * Mean values were significantly different among groups (one-way ANOVA and Duncan's post hoc test, p<0.05). $ Significantly different between groups (Student's t-test, p<0.05). LD, long day; SD, short day; DIO2, Type II iodothyronine deiodinase; BAT, brown adipose tissue; DIO3, Type III iodothyronine deiodinase, THRα, thyroid hormone receptor subunit alpha; THRβ, thyroid hormone receptor subunit beta.*

Differences in deiodinases expression between phenotypes were magnified in the liver (figure 4). Thus, *Dio2* was increased in obese animals living in SD respect LD (83% and 100% for animals treated with vehicle and RG, respectively), but no significant differences were found for the treatment (figure 4A). In contrast, in normoweight animals (figure 4B), SD induced a statistically significant 2-fold increase respect the LD and the RG treatment a significant 4-fold overexpression respect vehicle treated animals regardless of the photoperiod. *Dio3* was not affected neither by photoperiod nor by RG treatment in the obese cohort (figure 4C). Again, analysis in normoweight rats revealed a clear effect of the RG treatment, increasing 2-fold the expression of *Dio3* (figure 4D) in LD and a 53% in SD. As a result of these changes, the *Dio2/Dio3* ratio was affected only by the photoperiod in obese animals (figure 4E), while in normoweight rats, both photoperiod and treatment significantly affected this parameter (figure 4F), with a marked 2.5-fold and a 2-fold increase in SD and LD respectively in response to RG treatment.

Figure 4. (Next page) Liver biomarkers related to the thyroid system of cafeteria- or standard-fed rats adapted to a long day or short day photoperiod and supplemented with red-grape for 11 weeks. *A: Liver Dio2 gene expression levels of obese animals. B: Liver Dio2 gene expression levels of normoweight animals. C: Liver Dio3 gene expression levels of obese animals. D: Liver Dio3 gene expression levels of normoweight animals. E: Ratio between Dio2 and Dio3 gene expression levels in liver of obese animals. F: Ratio between Dio2 and Dio3 gene expression levels in liver of normoweight animals. The data are presented as the mean ± s.e.m. (n=10). P, the effect of the photoperiod; T, the effect of each fruit treatment; PxT, the interaction of the photoperiod and the fruit treatment (two-way ANOVA, $p<0.05$). * Mean values were significantly different among groups (one-way ANOVA and Duncan's post hoc test, $p<0.05$). LD, long day; SD, short day; DIO2, Type II iodothyronine deiodinase; L, liver; DIO3, Type III iodothyronine deiodinase.*



Exposure to a cafeteria diet blunts the photoperiod response of prolactin and TSHβ.

The differential response to photoperiod and RG treatment observed between normoweight and obese rats prompted us to assess *Eya3*, *Chga* and *Tshβ* expression in the pituitary together with the levels of plasma prolactin. Expression of *Eya3* (figure 5A), *Chga* (figure 5B) and the *Eya3/Chga* ratio (figure 5C) followed a similar

expression pattern to that observed in normoweight rats (Manuscript 2). Nevertheless, plasma prolactin (figure 5D) and *Tshβ* expression (figure 5E) did not respond to photoperiod shifting. This situation contrasts with the clear results reported for normoweight rats, and suggests that cafeteria diet completely blunted the seasonal response of prolactin and TSHβ in the pars tuberalis in Fischer 344 rats.

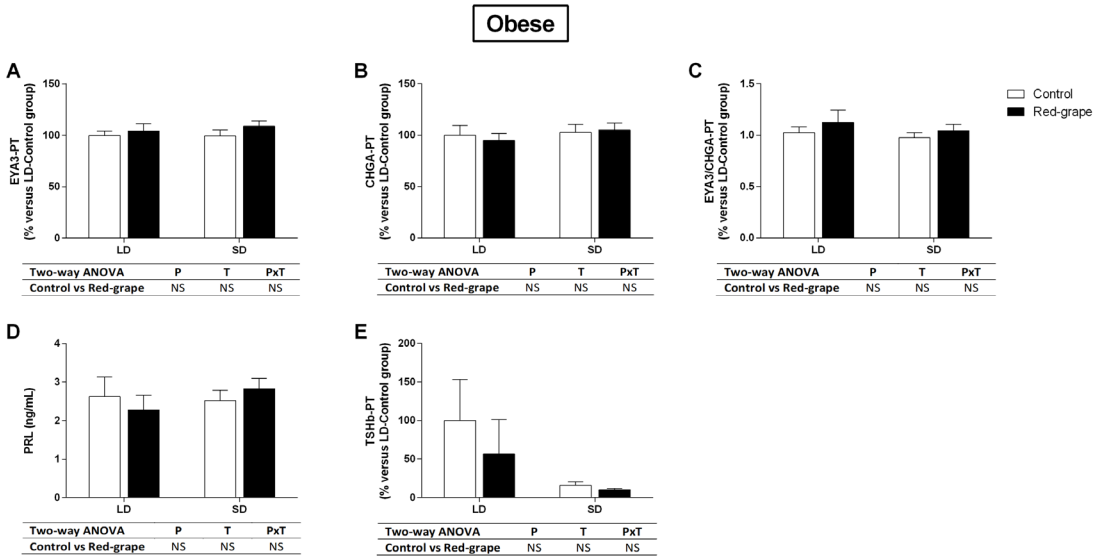


Figure 5. Seasonal controllers of cafeteria-fed rats adapted to a long day or short day photoperiod and supplemented with red-grape 11 weeks. *A: Hypophyseal Eya3 gene expression levels. B: Hypophyseal Chga gene expression levels. C: Ratio between the hypophyseal gene expression levels of Eya3 and Chga. D: Prolactin plasma levels. E: Hypophyseal Tshβ gene expression levels. The data are presented as the mean ± s.e.m. (n=5-10). P, the effect of the photoperiod; T, the effect of each fruit treatment; PxT, the interaction of the photoperiod and the fruit treatment (two-way ANOVA, p<0.05). LD, long day; SD, short day; EYA3, eyes absent homolog 3; PT, pineal gland; CHGA, chromogranin A; PRL, prolactin; TSHβ, Thyroid stimulating hormone subunit beta.*

4. Discussion

In a previous work we described that RG consumption has different behavioral and physiological effects when consumed in season. Among others, it decreases food intake, locomotor activity and increases energy expenditure without affecting body weight in normoweight Fischer F344 rats. The mechanisms proposed to explain those results, at least in part, were increased BAT thermogenic activity associated and correlated with important changes in hypothalamic dopaminergic system. These promising results were found in rats under a SD photoperiod. Therefore, photoperiod and seasonal sensitivity stands out as a key feature for enhancing the health promoting effects of RG intake. In view of these results, we decided to apply the same approach to rats with obesity induced by a cafeteria diet in order to evaluate whether RG might be helpful against obesity and its associated alterations. Surprisingly, our results suggest that cafeteria diet blunted the effects of RG treatment on energy expenditure, food intake and locomotor activity. The lack of effects was evident as well on the different elements of the hypothalamic somatostatinergic and dopaminergic systems, such as the ratios between dopamine and somatostatin receptors, which were clearly increased by RG in normoweight rats. In these animals, treatment with RG induced a clear 4-fold increase in *Dio2/Dio3* ratio in the BAT, suggesting enhancement of the metabolic activity of the tissue. Thus, hypothalamic *D2R* expression and BAT *Dio2/Dio3* ratio were correlated in normoweight animals, suggesting that the known central dopaminergic control of thermogenesis could explain, at least in part, our results since dopamine activity, through central D2R, has been shown to reduce food intake and increase energy expenditure in experiments using wildtype and *D2R*-/- mice[28]; and stimulation of dopaminergic system by D2R and D3R mixed agonists has been shown to reduce cold-induced thermogenesis in rats[29]. In the present work, the effects on the same parameters were abolished in rats fed with a cafeteria diet. The explanation can be

found in the effects of obesogenic diets on reward systems. Thus, diet composition can alter dopamine control of carbohydrate intake, being the combination of sugar and fats a determinant factor reducing dopamine signaling [30]. Moreover, Davis JF *et al.* showed that diet itself can modulate the dopaminergic system independently of obesity. Thus, consuming a high fat diet decreased dopamine turnover in the mesolimbic system, attenuating psychostimulant reward[31]. In agreement, Rada P *et al*. described that fat intake can stimulate the release of dopamine[32]. Moreover, cafeteria diet has been shown to cause deficits in dopamine neurotransmission[33] as well as relevant behavioral changes [24,34]. Remarkably, Fordahl SC *et al*. described that, in C57BL6 mice, prolonged consumption of high fat diets results in attenuation of dopaminergic function, mitigating the effect of pharmacological challenges on locomotor activity independently of obesity[35]. Moreover, different works have demonstrated that diet induced obesity disrupts feeding circadian rhythms and general locomotor activity, suggesting alterations in neurotransmitter processes subjected to circadian rhythms such as dopamine signaling[36]. Therefore, considering that the effects on dopaminergic system and behavior observed in normoweight rats treated with RG were dependent on circannual rhythm and probably on circadian rhythms, it is likely that disruption of rhythmical patterns of neurotransmitter signaling by cafeteria diet is the cause of impaired physiological and behavioral effects of RG in obese rats.

In contrast with these differences between phenotypes, we have found that RG treatment affects the HPT axis in both normoweight and obese animals. Thus, in normoweight, RG affected plasma TSH concentration and expression of deiodinases in peripheral tissues. Nonetheless, concentration of T3 and T4 hormones, or the different ratios among these hormones, were not altered. On the other hand, RG intake resulted in slight changes in T3 and a clear increase of T4 concentration in cafeteria diet-induced obese rats, but did not affect TSH. These results suggest that

RG consumption might modulate the HPT axis in both conditions, but the mechanisms are phenotype-dependent.

In normoweight rats, the increase on *Dio2/Dio3* induced by RG in peripheral tissues suggests a higher TH metabolism and intracellular signaling, which might result in the observed slight increase of TSH release by the pituitary gland in order to respond to this demand. Da-Silva *et al.* described that the polyphenolic molecule kaempferol induced a 3-fold upregulation of *Dio2* expression in isolated human myocytes, resulting in 2.6-fold increase in the production of intracellular active T3, suggesting that this polyphenol might regulate TH metabolism and signaling in peripheral tissues[37]. Interestingly, kaempferol is abundant in RG[38]. Our results agree with these observations, suggesting that RG treatment impacts the peripheral component of the HPT axis, consequently modulating the whole axis. Enhancement of *Dio2* expression and TH signaling in peripheral tissues is expected to have multiple consequences at the metabolic level[13,39]. We and others have demonstrated that grape polyphenols modulate different elements of the metabolism[40–46]. Therefore, more research is needed in order to clarify whether the effects of these bioactive compounds on intracellular activity of TH could be underlying their metabolic actions. In contrast with normoweight rats, peripheral tissues of obese animals remained insensitive to the effects of RG on the expression of deiodinases. Moreover, increased levels of circulating TH were not accompanied by lower *Tsh*, a result that would imply a disruption of the negative feedback that controls the homeostasis of these hormones, a situation that has been described for obesity[47]. Therefore, discrepancies between the effects of RG in both phenotypes could be related to the alterations that obesity exerts on the HPT axis[47,48]. Despite this is a considerably unknown field, it is accepted that the effects of obesity on thyroid function are caused by disruption of the hypothalamic-pituitary axis due to its link with adipose tissue metabolism and mechanisms of energy control depending on

hormones such as leptin, orexigenic peptides (e.g. neuropeptide Y, agouti-related peptide) and anorexigenic peptides (e.g. alpha-melanocyte-stimulating hormone, and cocaine and amphetamine-related transcript)[49–52]. In this scenario, it could be hypothesized that, in cafeteria fed animals, RG affects plasma TH concentrations and peripheral response because the central control has been impaired by obesity. Alternatively, the discrepancies found between phenotypes could be related to the effects of cafeteria diet and obesity on the gut microbiome[22,53,54]. Thus, differences in gut bacterial populations might result in absorption of different polyphenol metabolites with different activities[55–58]. Indeed, different polyphenols exert different actions, either positive or negative, in thyroid function[37,59–61]. Therefore, alterations induced by cafeteria diet, or obesity itself, in metabolism, microbiota or both could be underlying the phenotype-dependent differences observed for RG activity on the HPT axis.

The effects of RG on normoweight rats were dependent on the photoperiod, taking place under SD conditions. In obese rats, the lack of effects of RG is paralleled by a lack of response to photoperiod shifting in concentration of plasma prolactin. Plasma prolactin is invariably increased in LD in all seasonal animals[11], and that was the case in the normoweight cohort, as expected. Surprisingly, obese rats presented no differences between LD and SD conditions in plasma prolactin levels. These results could be interpreted as the obese rats entering into a refractory state, a phenomenon that takes place in seasonal animals exposed to SD for long periods that eventually restore and retain the LD phenotype, mainly those traits associated with reproduction, despite the lack of photic cues[11]. Nevertheless, in the current work, the experiment took 11 weeks whilst the experiment with the normoweight cohort, which showed no signs of refractoriness, lasted for 14 weeks. This means that if refractoriness took place in the present study, it appeared considerably faster in obese than in normoweight animals. On the other hand, the reproductive seasonal

variation, driven by the HPG axis, is maintained in the obese cohort. Since the reproductive rhythm is not necessarily phased to that of prolactin[11], this might be indicative of no refractoriness, but of specific blunting of the prolactin response caused by diet induced obesity. Regardless of the mechanism, the effects of obesogenic diets on neuroendocrine control could explain the surprising results concerning prolactin. It has been described that intake of high fat diets interfere with the circadian rhythm, lengthening the free-running period of the clock (i.e., the duration of 1 full circadian cycle in constant conditions without any external timing cues) and blunting the rhythmicity of molecular and biochemical biomarkers of circadian cycle [62,63]. Also obesity, either diet induced or genetic has been associated to disruption of circadian rhythms of core clock genes[64], physiology, eating behavior and activity[36,65]. Therefore, it is likely that the blunting of circadian response by diet and obesity could result in similar effects on seasonal rhythms of prolactin. Prolactin is secreted from the pars distalis of the pituitary by lactotrophs, and its photoperiodic response is independent of hypothalamic seasonal controllers, depending exclusively on the secretagogue from the pars tuberalis to the pars distalis of the pituitary[66]. Therefore, our results suggest that cafeteria diet induced obesity directly impacts pituitary function. To our knowledge, it is the first time that such effects of diet are described. Although more research is still needed in order to reveal the pathophysiological consequences of these findings, the implications for health might be considerable, since impairment of prolactin has been associated to different alterations such as dysregulation of immune function, bone metabolism, insulin resistance and cancer[67–70]. Other markers of seasonality, such as *Eya3* and *Eya3/Chga* ratio were not altered, though a possible explanation has been discussed previously (Manuscript 2). Nevertheless, *Tshβ*, which responded to photoperiod as expected in normoweight animals (Manuscript 2), remained unaltered in the obese cohort. Since *Tshβ* is expressed and released from thyrotrophs in the pars tuberalis

of the pituitary, together with the results obtained for prolactin, these results suggest altered pituitary function in obese animals.

Contrasting with the lack of response of prolactin and *Tshβ* to different photoperiods, we have found that obese animals present increased response of HPT and HPA axis than normoweight animals. These differences suggest that mechanisms controlling the homeostasis of neuroendocrine processes are altered by cafeteria diet induced obesity. Indeed, obesity has been described as a situation associated to disruption of endocrine control[48,71]. In view of our results, it is plausible to propose that this lack of control might affect hormonal homeostasis, partially blunting the buffering capacity of the different axis, i.e. HPT and HPA, for responding to challenges such as photoperiod shifting. Interestingly, HPT, HPA and HPG axis, are controlled by the hypothalamus, while photoperiod response of prolactin and *Tshβ* relies exclusively on the pituitary. It could be hypothesized that cafeteria diet induced obesity impairs homeostatic control of hypothalamic-dependent processes and a complete insensitivity to photoperiod in pituitary function. The alteration of neuroendocrine seasonal control by obesity and/or obesogenic diets might have considerable implications for human health since the different axis assessed herein are key elements regulating homeostatic elements of the organism.

In conclusion, together with our previous work, we have described that RG administration at doses that resemble habitual human intake can modulate neuroendocrine control in a photoperiod- and phenotype-dependent fashion. And that cafeteria diet-induced obesity disrupts the control of neuroendocrine axis, seasonal response of prolactin and blunts the photoperiod-dependent response of the organism to the intake of RG. Therefore, it seems that cafeteria diet induced obesity impairs the response to both type of cues, photic and non-photic. Despite more research is needed to underscore the mechanisms responsible for these observations, our results suggest that both diet and photoperiod are key factors to

be considered for designing nutritional interventions addressed to prevent the development of diseases and to correct the alterations associated to obesity.

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Author contributions

CB, LA, AC, and JdB designed the studies. RM-C, CD-C, AC, and JdB performed the experiments and analyzed the data. JdB, CD-C, and CB wrote the manuscript. All authors read, discussed, and approved the final version of the manuscript.

Author information

The authors have no conflicts of interest to declare.

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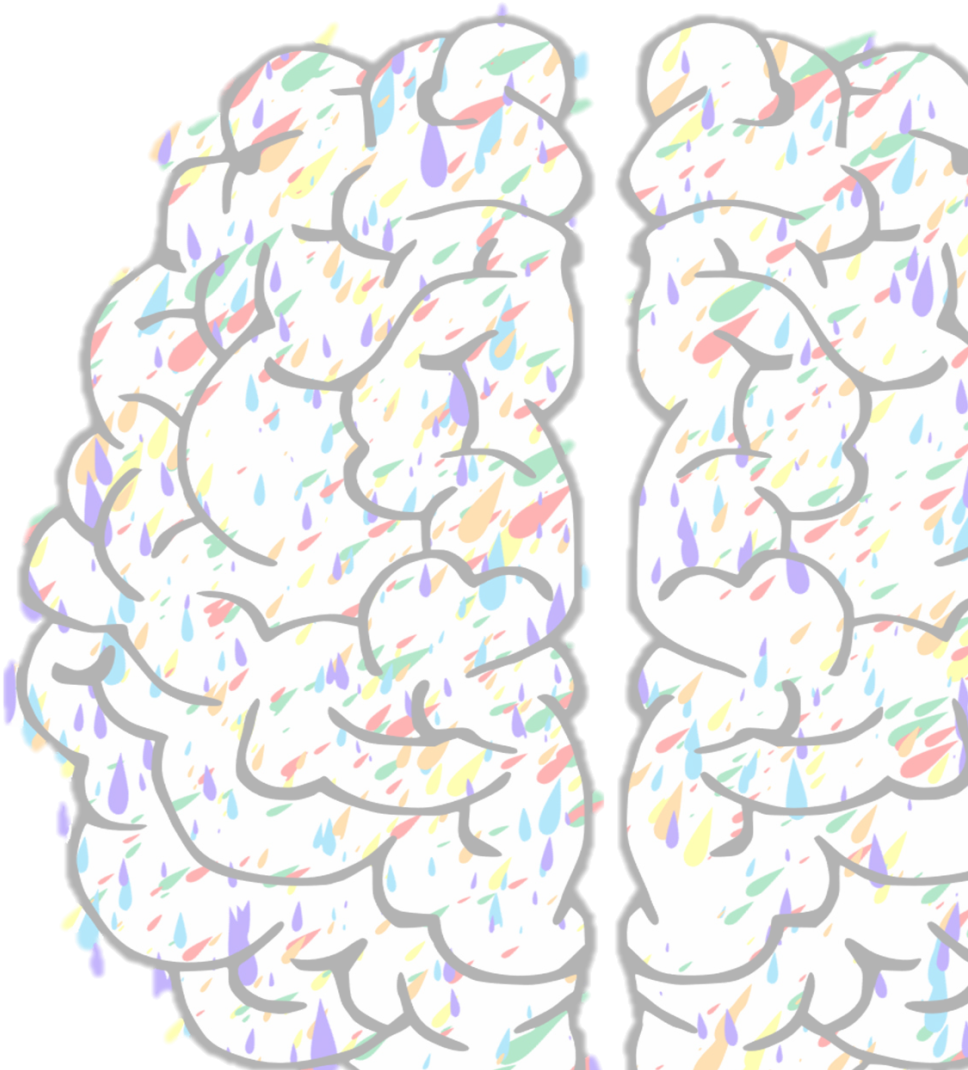
General Discussion



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General Discussion

The Xenohormesis theory proposes that animals can sense chemical cues in foods in order to adapt their metabolism to incoming environmental conditions. Therefore, this kind of signals may be integrated with other cues. This is the case of the present work, in which the primary signal was photoperiod. In our research we have demonstrated that RG intake presents important effects on different processes that govern the animal response to photoperiod shifting. Thus, we propose that the effects of RG consumption on dopaminergic and somatostatinergic systems are related, at least in part, with increased activity of BAT, resulting in increased energy expenditure that cannot be explained by changes in locomotor activity, but by non-shivering thermogenesis. In agreement with this hypothesis, Ootsuka Y et al demonstrated that stimulation of central nervous system D2R inhibits cold induced thermogenesis in BAT[1]. Moreover, leptin signaling in the hypothalamus of D2R-/- mice was enhanced, promoting a decrease of energy intake and activity and increased energy expenditure by the BAT[2]. Therefore, our hypothesis is compatible with increased central leptin signaling in rats treated with RG. This mechanism is in agreement with the effects described for grape procyanidins enhancing leptin signaling in the hypothalamus, activating signal transducer and activator of transcription-3 (STAT3)[3]. Nevertheless, more research is still needed in order to completely underscore the mechanisms of RG treatment in our conditions, since circulating dopamine has been described to enhance BAT thermogenesis by direct stimulation, although the mechanism described is based on the type 1 receptor[4]. Regardless of the mechanism, the effects of RG on locomotor activity, food consumption and energy expenditure are intriguing. These effects have been found in rats under the SD photoperiod, which represents the natural season of red grape maturation and consumption. In contrast, the same treatment in rats under a LD photoperiod resulted in no physiological or behavioral effects. Therefore, according

to the xenohormesis theory, the effects of RG should be interpreted as the result of a chemical cue provided within the correct season. Assuming that the chemical cue is of a polyphenolic nature, it is expected a response of the subject directed to adapting its physiology and behavior to environmental conditions that promote polyphenols accumulation in berries of plants such as *vitis vinifera*, which usually are stressful conditions, such as low temperature or hydric stress[5–8]. Therefore, in our experiments, RG consumption involves increased polyphenols intake synthesized by an autumn fruit, and therefore might be understood as the exposure to chemical cues that might be interpreted by the organism as an incoming challenging environmental scenario. The response of animals fed RG in SD is decreased locomotor activity and decreased food intake, which could be understood as an adaptation for saving energy and decrease the need of food, respectively, in an environment that, according to non-photic cues provided by RG, might result in though conditions and food scarcity. But in this situation, increased energy expenditure by non-shivering thermogenesis contrasts with this interpretation, at first glance. Nevertheless, it has been described that hibernating mammals present increased BAT metabolic activity during cold months[9–11]. Thus, despite mitochondrial metabolism is generally suppressed during torpor compared with active seasons[11–13], BAT mitochondria have enhanced function during hibernation compared with the active seasons in order to assist the animal in the topor arousal that takes place on a rhythmic basis[11,14]. Our results strongly suggested increased metabolic activity of the BAT in animals consuming RG during SD, which is consistent with the mechanisms of hibernation. Therefore, considering that the F344 rat is not a hibernating mammal, it could be speculated that consumption of RG during SD photoperiods induces a hibernating-like state, consisting in reduced activity and food intake and enhanced BAT metabolism that take place only if animals are in SD photoperiods. Such a response would allow animal survival in conditions of winter time, characterized by food deprivation and

cold. Despite this explanation is coherent with the Xenohormesis theory, it falls in the field of speculation. Nevertheless, our results open new possibilities for validating the Xenohormesis theory through experimental approaches based, initially, in the experimental designs that we have proposed all along this work. More research is still needed in order to assess whether RG intake enhances survival possibilities of animals under though environmental conditions.

According to our hypothesis, effects of RG treatment in animals living in a LD photoperiod could be considered as erroneous signaling, since the chemical cues of RG are provided to the animal out of season. In LD, RG intake caused mild increases in biomarkers of pituitary and hypothalamic seasonal control such as the EYA3/CHGA ratio and DIO2/DIO3 ratio, respectively. Although these results could be interpreted as altered response to photoperiod shifting, the levels of key biomarkers of seasonality, such as plasma prolactin and pituitary TSHβ remained unaltered, suggesting that these changes do not result in relevant consequences. Besides, RG induced a 2-fold increase in plasma TSH with no effects on the levels of TH. This situation, compatible with subclinical hyperthyroidism, has been described as well in association with obesity[15–18], and explained as a response to increased adiposity. Nevertheless, we have shown that RG affects different elements of TH function in both overweight and normoweight animals regardless of the photoperiod. Therefore, despite the response of the thyroid axis is different in both phenotypes, its photoperiod-independent alteration by RG suggests that modulation of the HPT axis by RG is not confined to a specific season.

Or results suggested that RG intake during SD photoperiods could be beneficial in a situation of obesity, but feeding a cafeteria diet resulted in abolishment of RG actions. As discussed previously in detail, these effects could be attributed to the effects that obesity or obesogenic diets exert on the mechanisms of reward, altering neurotransmitter actions, including dopamine but also other orexigenic and

anorexigenic peptides and hormones such as leptin[19–24]. Nevertheless, in the context of weight management strategies, dietary habits must be changed together with increased physical activity[25]. Therefore, inclusion of RG in weight management diets could be suggested as an effective element for enhancing energy expenditure and modulate food intake. To our knowledge, no studies with whole grape have been conducted to date, being a new possibility to explore. Nevertheless, we have associated the effects of RG consumption mainly with its polyphenolic content. Polyphenols from grape have been extensively studied in preclinical models and, to a lower extent, in clinical trials. Grape proanthocyanidins have been shown to enhance leptin signaling, reducing hyperphagia and peripheral leptin resistance associated to a high fat diet[3], and to decreased food intake and increased energy expenditure in aged rats[26]. In hamsters, doses of grape polyphenols in doses equivalent to daily human consumption resulted in decreased adiposity due to enhanced metabolism of white adipose fatty acid metabolism[27]. Similar effects have been described for higher doses of grape procyanidins[26]. Moreover, grape procyanidins have shown different effects against obesity associated alterations, such as dyslipidemia[28–31], inflammation[32,33] or insulin resistance[32,34] in different animal models. Despite all these evidences in preclinical studies, trials in human volunteers consuming polyphenols have provided contrasting results [35–44]. It is tempting to propose that the variability of results obtained in clinical interventions with polyphenols targeting obesity could be due, among other factors, to the lack of control on seasonality. Currently, the interaction among circadian oscillations and the components of foods, including daily dietary patterns, have focused great attention, emerging as a new discipline known as chrononutrition[25,45–47]. Our results suggest that circannual cycle should be also considered when analyzing the interactions between nutrition and biological rhythms, providing a new tool for the design of personalized interventions.

The interaction between obesity or obesogenic diets and biological rhythms has gained attention during the last years. Different works have shown that obesity itself, or consumption of obesogenic diets alter response to photoperiod, either behavioral or metabolic. It has been described that intake of high fat diets interfere with the circadian rhythm, lengthening the free-running period of the clock (i.e., the duration of 1 full circadian cycle in constant conditions without any external timing cues) and blunting the rhythmicity of molecular and biochemical biomarkers of circadian cycle[48,49]. Also obesity, either diet induced or genetic has been associated to disruption of circadian rhythms of core clock genes[50], physiology, eating behavior and activity[20,51]. Therefore, it is likely that the blunting of circadian response by diet and obesity could result in similar effects on seasonal rhythms. Indeed, one of the most striking results of our works was the total suppression of plasma prolactin response to photoperiod by cafeteria diet induced obesity, despite this hormone is thought to be invariably increased in LD in all seasonal animals[52]. Prolactin is secreted from the pars distalis of the pituitary by lactotrophs, and its photoperiodic response is independent of hypothalamic seasonal controllers, depending exclusively on the secretagogue from the pars tuberalis to the pars distalis of the pituitary, although the actual signals involved in this regulatory pathway have not been identified yet[53]. Therefore, our results suggest that cafeteria diet induced obesity directly impacts pituitary function. To our knowledge, this is the first time that such effects of diet are described. Although more research is still needed in order to reveal the pathophysiological consequences of these findings and their translation to human physiology, the implications for human health might be considerable, since impairment of prolactin has been associated to different alterations such as dysregulation of immune function, bone metabolism, insulin resistance and cancer[54–57].

Together with prolactin, we have found that cafeteria diet induced obesity also alters the response to photoperiod of HPT and HPA axis when compared with standard chow fed rats. Overall, our results highlight the profound changes that dietary habits and obesity might have on neuroendocrine control, impairing hypothalamic, pituitary function or both. Indeed, obesity has been described as a situation associated to disruption of endocrine control[15,58], although, to our knowledge, this is the first time that these effects are described in the context of photoperiod shifting. Our results suggest that obesity, obesogenic diets or both factors, impair the normal neuroendocrine response to both photic and non-photic cues.

Overall, our work provides new evidences supporting the idea that seasonal foods are a source of chemical cues that can modulate behavior and metabolism on a photoperiod-dependent fashion. As a proof of concept, our results can be interpreted under the scope of the Xenohormesis theory. In the field of nutrition and health research, the current results are relevant for understanding how biological rhythms, as a universal environmental factor, can influence and be influenced by nutrition in health and disease.

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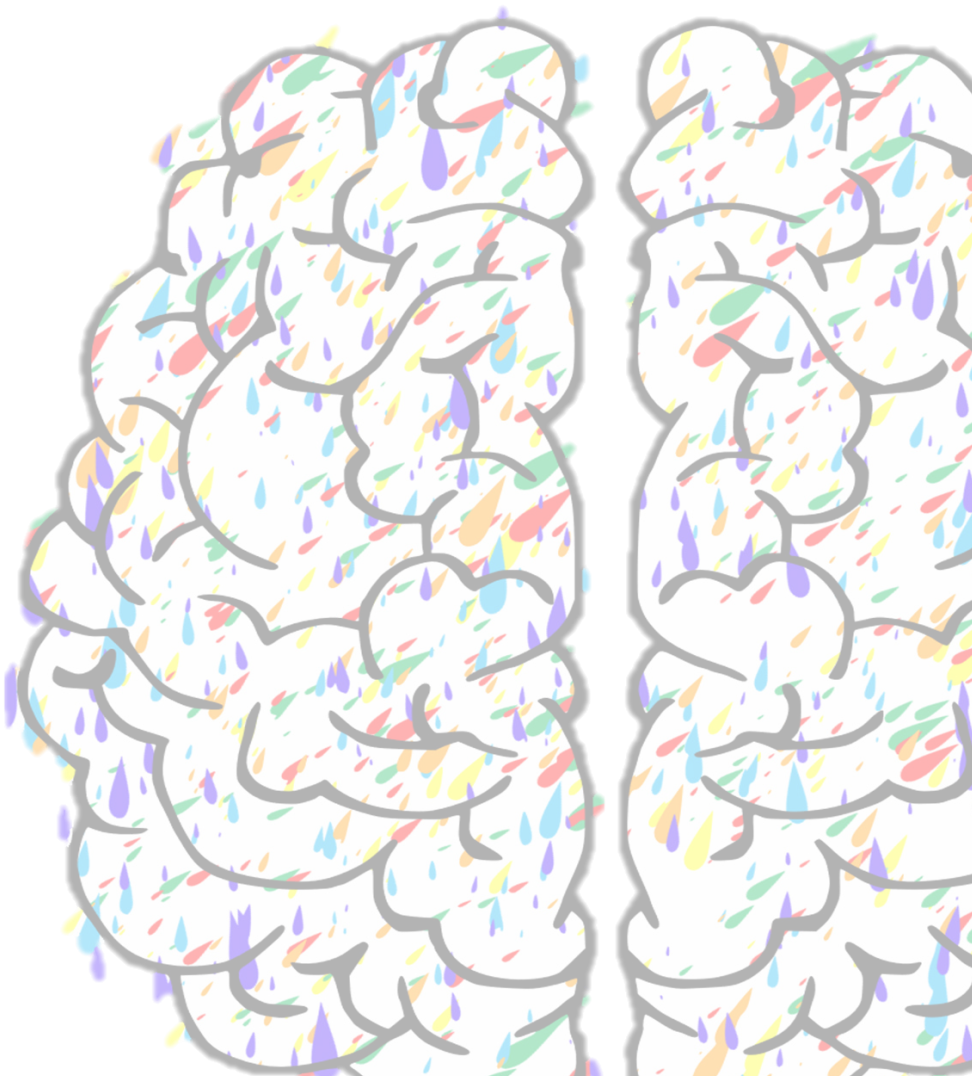
Conclusions



UNIVERSITAT ROVIRA I VIRGILI
MODULATION OF CENTRAL NEUROENDOCRINE RESPONSE TO PHOTOPERIOD BY SEASONAL FRUITS AND OBESITY.
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Conclusions

1. **Modulation of central neurotransmitter and neuroendocrine control by seasonal fruits consumption in or out of season is fruit-specific in Fischer 344 rats.**

   Red-grape but not cherry elicited important changes in different markers of seasonal control. Specifically, it has been observed a clear modulation on somatostatinergic and dopaminergic systems when red-grape was consumed during SD conditions. Moreover, when the consumption is done at LD, it produces significant changes in the mRNA expression levels of *Eya3* and *Dio2* genes, reported in the literature as seasonal marker genes.

2. **Red-grape intake by Fischer 344 rats modulates behaviour and energy expenditure when consumed in its natural season.**

   Administration of red-grape to normoweight Fischer 344 rats reduces food intake and locomotor activity and increases energy expenditure when it is consumed during its natural seasonal photoperiod, SD, but not during LD. The intrinsic mechanisms behind these changes are related with a higher activity of thyroid hormone in peripheral tissues, such as liver or BAT. Therefore, our results suggest that red-grape intake during its natural season could be proposed as a beneficial strategy for correcting obesity and its associated alterations, besides its own properties as a fruit.

3. **Red-grape consumption modulates hypothalamic-pituitary-thyroid axis of Fischer 344 rats.**

   Red-grape intake modulates the HPT axis irrespective of the organism phenotype, although the final outcome is dependent of this parameter. In

normoweight animals, red-grape intake produced changes in plasma TSH concentrations and in the expression of deiodinases in peripheral tissues, while there were not significant changes in T3 and T4 plasmatic levels. On the other hand, obese rats showed slight changes in T3 and clear variations in T4 levels, but without modulation of plasmatic TSH. This mechanism may explain the metabolic effects associated with the consumption of red-grape polyphenols.

4. **Cafeteria diet-induced obesity completely abolishes the effects in energy expenditure and behaviour derived of red-grape consumption.**

   Cafeteria diet fed animals did not show the effects produced by red-grape consumption on energy expenditure, food intake and locomotor activity in normoweight animals. These lack of effects in obese animals was also evident in the elements of dopaminergic and somatostatinergic systems, which might be postulated as the cause of impaired response to red-grape.

5. **Cafeteria diet-induced obesity impairs the natural response of plasma prolactin to photoperiod shifting.**

   Obese animals did not show any differences between LD and SD condition in plasma prolactin levels. This lack of response contrasts with the situation observed in normoweight animals, whose prolactin plasma levels were increased during LD, as expected for all seasonal mammals. This finding is relevant for its implications in human health, since the modulation of prolactin levels have been associated with the origin and development of different diseases, such as immune function, bone metabolism, insulin resistance and cancer.

6. Cafeteria diet-induced obesity modulates the seasonal response of hypothalamic-pituitary-thyroid and hypothalamic-pituitary-adrenal axis.

Obese animals present increased response in HPT and HPA axis compared with normoweight animals. Taking into consideration that both axes are controlled by the central pacemaker, these alterations would be related with the impairment of hypothalamic control mechanisms and the fewer sensibility of pituitary gland to changes in photoperiod.

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