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Nathane Rosa Rodrigues

PRIVAÇÃO DE SONO EM *Drosophila melanogaster* RESULTA EM ALTERAÇÕES NA HOMEOSTASE REDOX, MITOCONDRIAL E NA EXPRESSÃO GÊNICA DE REGULADORES DA FUNÇÃO CIRCADIANA E METABÓLICA.

Santa Maria, RS
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Prof. Dr. Jeferson Luis Franco (UNIPAMPA)
(Presidente/Orientador)

Prof. Dr. Eduardo Rico (UNESC)

Prof. Dr. Elgion Lucio Loreto (UFSM)

Prof. Dra. Marina Prigol (UNIPAMPA)

Prof. Dr. Denis Brook Rosemberg (UFSM)

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RESUMO

PRIVAÇÃO DE SONO EM *Drosophila melanogaster* RESULTA EM ALTERAÇÕES NA HOMEOSTASE REDOX, MITOCONDRIAL E NA EXPRESSÃO GÊNICA DE REGULADORES DA FUNÇÃO CIRCADIANA E METABÓLICA.

AUTOR: NATHANE ROSA RODRIGUES
ORIENTADOR: JEFERSON LUIS FRANCO

A privação do sono e as doenças relacionadas à essa condição, como os distúrbios metabólicos, são consequências do estilo de vida agitado da sociedade moderna. O sono é um estado de descanso crucial para a sobrevivência e regulado pelos ritmos circadianos. As alterações nesses ritmos afetam os sistemas de defesa antioxidantes, causam danos oxidativos e deficiências metabólicas, porém os mecanismos envolvidos nessas alterações ainda não estão bem esclarecidos. Neste estudo foi avaliado os efeitos causados pela privação do sono em moscas *Drosophila melanogaster* mantidas em condição de luz constante. A exposição de moscas à condição de luz contínua foi capaz de causar alterações nos padrões de sono, caracterizando a regulação homeostática do sono. A privação do sono de moscas pela exposição à luz durante 24 horas causou diminuição da atividade locomotora, aumento na atividade das enzimas GST, SOD e TRxR, bem como a diminuição da atividade de CAT. Também foi observado o aumento dos níveis de espécies reativas de oxigênio e peroxidação lipídica, disfunção mitocondrial, diminuição dos níveis de glicose, triglicerídeos e glicogênio e aumento da atividade das caspases 3/7. Além disso, moscas privadas do sono apresentaram alterações na expressão de genes envolvidos nas vias do estresse oxidativo, controle circadiano e regulação metabólica. Sendo observado o aumento na expressão dos genes *Nrf2, p38β, Pp2a, pale, Akt1, Clk, cyc, per, tim, cry, pdf*, e nos genes *dilp2, 3* e *8*. Por outro lado, os genes *dbt* e *dilp4, 5, 6* e *7* apresentaram uma diminuição significativa na sua expressão. Em conjunto nossos dados sugerem uma estreita relação entre privação do sono, controle circadiano, estresse oxidativo e regulação metabólica, indicando que a privação do sono, mesmo por um curto período de tempo, é capaz de causar efeitos deletérios ao organismo, e ainda reforça o uso de *Drosophila melanogaster* como um organismo modelo para estudos relacionados ao sono e metabolismo.

Palavras-chave: Privação do sono, danos mitocondriais, estresse oxidativo, relógio circadiano, distúrbios metabólicos, Peptídeos semelhantes à insulina.

ABSTRACT

SLEEP DEPRIVATION IN *Drosophila melanogaster* RESULTS IN CHANGES IN REDOX, MITOCHONDRIAL HOMEOSTASIS AND GENE EXPRESSION OF CIRCADIAN AND METABOLIC FUNCTION REGULATORS

AUTHOR: NATHANE ROSA RODRIGUES
ADIVISOR: JEFERSON LUIS FRANCO

Sleep deprivation and related diseases, such as metabolic disorders, are consequences of the busy lifestyle of modern society. Sleep is a crucial resting state for survival and regulated by circadian rhythms. Changes in these rhythms affect antioxidant defense systems, cause oxidative damage and metabolic deficiencies, but the mechanisms involved in these changes are still unclear. In this study we evaluated the effects of sleep deprivation on *Drosophila melanogaster* flies maintained in constant light. Exposure of flies to the continuous light condition was able to cause changes in sleep patterns, characterizing homeostatic sleep regulation. Sleep deprivation of flies by 24-hour light exposure caused decreased locomotor activity, increased GST, SOD, and TRxR enzymes, as well as decreased CAT activity. Increased levels of reactive oxygen species and lipid peroxidation, mitochondrial dysfunction, decreased glucose, triglycerides and glycogen levels and increased caspase 3/7 activity were also observed. In addition, sleep deprived flies showed alterations in the expression of genes involved in oxidative stress pathways, circadian control and metabolic regulation. The increase in gene expression of *Nrf2, p38β, Pp2a, pale, Akt1, Clk, cyc, per, tim, cry, pdf* and in the *dilp2, 3* and *8* genes. On the other hand, the *dbt* and *dilp4, 5, 6* and *7* genes showed a significant decrease in their expression. Taken together, our data suggest a close relationship between sleep deprivation, circadian control, oxidative stress, and metabolic regulation, indicating that sleep deprivation, even for a short period of time, is capable of causing deleterious effects on the body, and further enhances use of *Drosophila melanogaster* as a model organism for studies related to sleep and metabolism.

Key words: Sleep deprivation, mitochondrial damage, oxidative stress, circadian clock, metabolic disorders, Insulin-like peptides.

APRESENTAÇÃO

No item **INTRODUÇÃO**, consta uma breve revisão da literatura sobre os temas trabalhados nesta tese. A metodologia realizada e os resultados obtidos que fazem parte desta tese estão apresentados sob a forma de um artigo e um manuscrito, que se encontram no item **RESULTADOS**. No mesmo constam as seções Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas referente a cada trabalho. O item **DISCUSSÃO** apresenta uma breve discussão dos resultados de maneira geral. O item **CONCLUSÕES**, encontrado no final desta tese, apresenta interpretações e comentários gerais sobre os resultados do manuscrito presentes neste trabalho. As **REFERÊNCIAS** referem-se somente às citações que aparecem nos itens **INTRODUÇÃO, DISCUSSÃO** e **CONCLUSÕES** desta tese.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

AKT	Proteína quinase B
ATP	Trifosfato de adenosina
CAT	Catalase
CLK	Do inglês *Clock*
CRY	do inglês *Cryptochrome*
CTE	Cadeia Transportadora de Eletons
CYC	do inglês *Cycle*
DA	Dopamina
DBT	do inglês *Doubletime*
dFOXO	do inglês *forkhead Box O*
DILP	Peptídeos semelhantes à insulina/ do inglês *Insulin-like peptides*
DNA	Ácido desoxirribonucléico
DNs	Neurônios dorsais
ERA / ARE	Elemento Responsivo Antioxidante/ do inglês *Antioxidant response element*
ERO	Espécies Reativas de Oxigênio
GABA	Ácido γ-aminobutírico
GPDH	Glicerol 3 fosfato desidrogenase
GPx	Glutationa Peroxidase
GSH	Glutationa reduzida
GST	Glutationa S-transferase
HSP83	Proteína de choque térmico 83/ do inglês *Heat-shock protein 83*
IPC	Células produtoras de insulina/ do inglês *Insulin producing cells*
lLNVs	Neurônio lateral ventral grande/ do inglês *Largue lateral ventral neuron*
LNds	Neurônios laterais dorsais/ do inglês *Dorsal lateral neurons*
MB	Corpos Pedunculados/ do inglês *Mushroom Bodies*
mTOR	Alvo da Rapamicina/ do inglês *Target of Rapamycin*
NREM	Movimento rápido dos olhos/ do inglês *non-rapid eye movement*

NRF2	Fator nuclear eritróide 2/ do inglês *Nuclear factor (erythroid-derived 2)-like 2*
OXPHOS	Fosforilação Oxidativa/ do inglês *Oxidative phosphorylation*
P38 MAPK	Proteínas quinases P38 ativadas por mitógenos/ do inglês *P38 mitogen-activated protein kinases*
PDF	do inglês *Pigment dispersing Factot*
PER	do inglês *Period*
PI	do inglês P*ars intercerebralis*
PP2A	Proteína fosfatase 2A/ do inglês *Protein phosphatase 2A*
PRx	Peroxiredoxina
RNA	Ácido ribonucléico
SLNVs	Neurônio lateral ventral pequeno/ do inglês *Small lateral ventral neuron*
SOD	Superóxido dismutase
SWA	Atividade de ondas lentas/ do inglês *Slow Wave Activity*
TH	Tirosina hidrolase
TIM	do inglês *Timeless*
ZT	Zeitgeber- do alemão *zeit* "tempo" e *geber* "doador" (termo científico que se refere a elementos ambientais chave capazes de regular um ciclo de relógio biológico).

SUMÁRIO

1. INTRODUÇÃO

1.1 SONO: CARACTERÍSTICAS GERAIS

O sono é um estado espacial de descanso caracterizado pela imobilidade, o que torna os indivíduos incapazes de interagir com o ambiente, buscar por alimentos e mais susceptíveis a predação (DONELSON; SANYAL, 2014; HARBISON; MACKAY; ANHOLT, 2009; SHAW, 2003). No entanto o sono é crucial para a sobrevivência, uma vez que experiências em ratos (RECHTSCHAFFEN et al., 1989) e em moscas (SHAW et al., 2002; SHAW; FRANKEN, 2003) demonstraram que a privação do sono à longo prazo resulta em morte. A perda do sono prejudica amplamente as funções cognitivas, evidências sugerem que o sono, não apenas o repouso, é requerido para a recuperação desses efeitos prejudiciais (GOEL; BASNER; DINGES, 2015). Assim, perturbações na qualidade, duração ou temporização do sono intensificam a sua homeostase, permitindo a recuperação do sono perdido (DONELSON; SANYAL, 2014; HARBISON; MACKAY; ANHOLT, 2009; HUBER et al., 2004).

Um grande número de evidências sugerem o papel fisiológico do sono no processo metabólico, resposta ao estresse e a inflamação, recuperação de reservas de energia, entre outros (KNUTSON et al., 2007; TRIVEDI et al., 2017). A importância do sono em processos fundamentais ao organismo é indicada pelo rigoroso processo de regulação que ele apresenta (ALLADA; CIRELLI; SEHGAL, 2017). A regulação do sono ocorre devido a dois processos que podem ocorrer independentemente, sendo, um relógio circadiano que regula principalmente o tempo de sono e, um mecanismo homeostático que detecta e responde à necessidade de sono (SEIDNER et al., 2015; SHAW et al., 2002).

O sono é uma atividade conservada entre as espécies (CAMPBELL; TOBLER, 1984; JOINER, 2016), os mecanismos principais de regulação do sono são similares entre vertebrados e invertebrados (DONELSON; SANYAL, 2014). Aqui o foco será dado para os mecanismos de regulação do sono em *Drosophila melanogaster*, organismo modelo que tem sido muito utilizado para investigar os mecanismos envolvidos nas alterações do sono (FRANKEN; DIJK, 2009; MA; MA; XU, 2015; PARISKY et al., 2016; SEHGAL; MIGNOT, 2011), por apresentar características conservadas com o sono de mamíferos (HARBISON; MACKAY; ANHOLT, 2009).

1.2 *Drosophila melanogaster*

Grande parte do entendimento dos mecanismos que levam ao desenvolvimento das doenças tem sido obtido graças aos modelos experimentais realizados com animais, sejam eles vertebrados ou invertebrados. O modelo experimental se faz valer pela capacidade deste em representar com fidelidade o fenômeno natural. Esse modelo animal, obrigatoriamente, deve permitir a avaliação de fenômenos biológicos naturais ou comportamentais induzidos, que possam ser comparados aos fenômenos em questão que ocorrem naturalmente. (FERREIRA, HOCHMAN, BARBOSA, 2005).

O grande número de pesquisas realizadas a partir da utilização de insetos contribui para a compreensão dos mecanismos biológicos existentes em quase todos os seres vivos (MORALES, 2008). Dentre os organismos modelos, a mosca *Drosophila melanogaster* **(Fig. 1)** é um invertebrado de grande utilização em estudos genéticos, bioquímicos, toxicológicos e moleculares (STRANGE, 2016) por ser de fácil manipulação devido às vantagens advindas de seu ciclo biológico, como o rápido desenvolvimento. Outra vantagem é a ausência de mitose celular nas moscas em fase adulta, desse modo, a mosca na fase adulta tem envelhecimento sincronizado das suas células, exceto as células nas gônadas e algumas no intestino (JIMENEZ-DEL-RIO, MARTINEZ, PARDO, 2009).

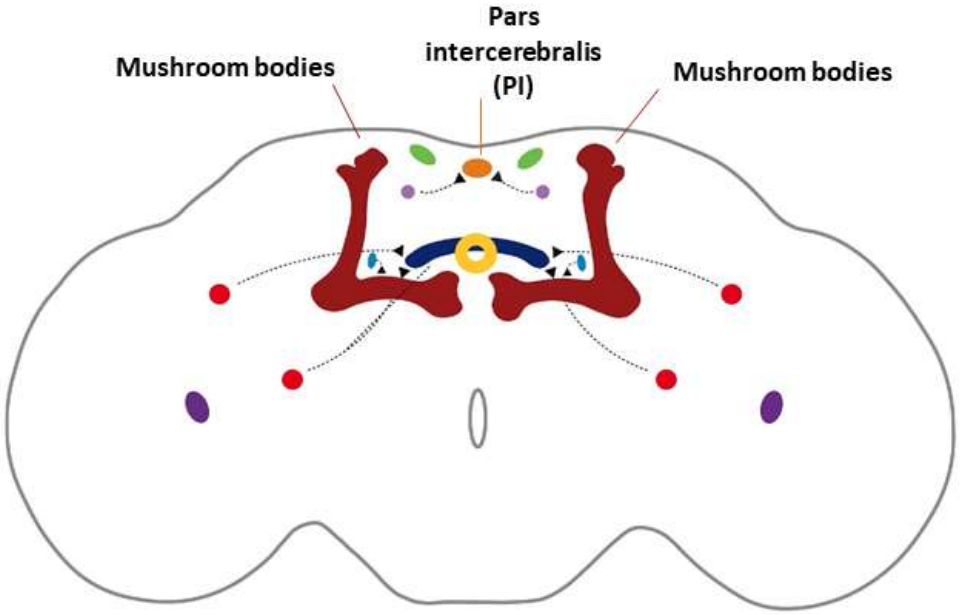
Além disso, a *Drosophila* tem sido utilizado para investigar os mecanismos envolvidos em alterações do sono (FRANKEN; DIJK, 2009; MA; MA; XU, 2015; PARISKY et al., 2016; SEHGAL; MIGNOT, 2011), uma vez que exibe as mesmas características comportamentais do sono de mamíferos e permite o controle tanto genético como do meio ambiente com precisão (HARBISON; MACKAY; ANHOLT, 2009). Como nos mamíferos, o sono é abundante em moscas jovens e reduzido em moscas mais velhas e também é modulado por estimulantes como a cafeína e por hipnóticos, como os anti-histamínicos (PAUL J. SHAW et al., 2000). Semelhante aos humanos, as duas principais características do sono são conservadas em *Drosophila*; a interação entre os ritmos circadianos internos e pistas externas confiáveis e previsíveis sobre a hora do dia (zeitgeber) e; uma necessidade de sono natural regulada pela homeostase (DONELSON; SANYAL, 2014).

Figura 1. *Drosophila melanogaster*. Organismo modelo.

Fonte: **https://encrypted-tbn0.gstatic.com/im...**

O sistema nervoso de *Drosophila* possui vários centros de controle do sono / vigília. Os corpos pedunculados (*mushroom bodies*- MB) são considerados o centro de regulação do sono (ARTIUSHIN; SEHGAL, 2017). Localizado no *protocerebrum* de *Drosophila*, o MB é uma estrutura que foi primeiramente estabelecida como um centro associativo essencial para aprendizagem olfativa e memória, entretanto estudos baseados na excisão ou inibição dos MB identificaram seu papel na regulação do sono (PITMAN et al., 2006; YI et al., 2013). Uma outra região de controle do sono/vigília é identificada como *pars intercerebralis* (PI), uma população de neurônios neuropeptidérgicos semelhante ao hipotálamo de mamíferos (FOLTENYI; GREENSPAN; NEWPORT, 2007). Em PI as células produtoras de insulina (IPCs) são promotoras do despertar mediado pela octopamina (CROCKER; SEHGAL, 2010) **(Fig. 2)**. No entanto, a complexidade do controle do sono se estende a outros circuitos e mecanismos regulatórios.

Figura 2. Imagem representativa da localização dos centros de controle/vigília no cérebro de *Drosophila*. A imagem mostra a localização dos corpos pedunculados- *mushroom bodies* (em marrom), e da região *pars intercerebralis*- PI (em laranja).



Fonte: Adaptado de LY; PACK; NAIDOO, (2018).

Em 2017, pesquisas com *Drosophila* que colaboraram para decifrar a regulação do ritmo circadiano renderam o Prêmio Nobel de Fisiologia ou Medicina aos pesquisadores Jeffrey C. Hall, Michael Rosbash e Michael W. Young. Todas essas características, juntamente com o desenvolvimento de ferramentas para observação e análise do sono, fazem da *Drosophila* um organismo modelo atraente e válido para estudos do sono.

1.3 REGULAÇÃO CIRCADIANA

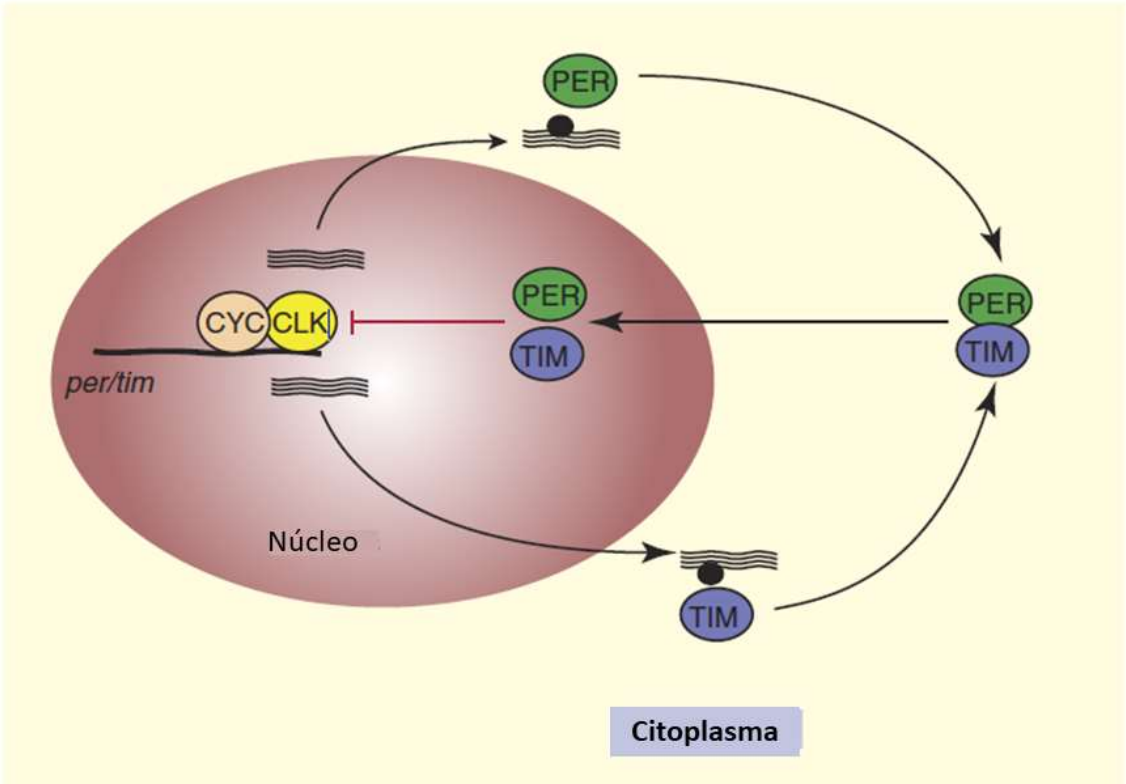
Os ritmos circadianos são eventos controlados por um relógio biológico que ocorre em animais, plantas, fungos e alguns procariotos num período de tempo de 24h, o que permite aos organismos a predição de mudanças rítmicas em seu ambiente (HARDIN, 2005; TATAROGLU; EMERY, 2014). O relógio circadiano influencia aspectos fisiológicos e comportamentais, como ritmos hormonais e metabólicos e também são responsáveis pela regulação do sono (COLIN-GONZALEZ et al., 2015; TATAROGLU; EMERY, 2014).

As alterações diárias nos períodos de repouso e atividade são as oscilações circadianas mais observáveis tanto em vertebrados quanto em invertebrados (SHAW, 2003), podendo ser influenciadas por fatores ambientais como mudança de temperatura, ciclos de luz e disponibilidade de alimento, o que leva ao arraste do relógio circadiano (TATAROGLU; EMERY, 2014). Estudos demonstram que alterações nos ritmos circadianos e nos padrões de exposição à luz são capazes de provocar assincronia em neurônios circadianos (KAYSER; YUE; SEHGAL, 2014; XIANG et al., 2010; YUAN et al., 2011) e danos oxidativos (ESCRIBANO et al., 2014; HIRAYAMA; CHO; SASSONE-CORSI, 2007; KRISHNAN; DAVIS; GIEBULTOWICZ, 2008).

Em *Drosophila*, a regulação do relógio circadiano é baseada em ciclos de retroalimentação (HARDIN, 2009). O ciclo de retroalimentação básico que forma o mecanismo de relógio envolve os genes *Clock* (*Clk*) e *Cycle* (*cyc*) que codificam proteínas que ativam a transcrição dos genes *period* (*per*) e *timeless* (*tim*) **(Fig. 3)**. As proteínas PER e TIM acumulam-se nos núcleos celulares, onde o PER reprime os ativadores CLK / CYC, levando à supressão da transcrição *per* e *tim* (HARDIN, 2005). O ritmo circadiano é controlado por um oscilador circadiano, que mantém a sincronização com os ciclos ambientais para promover a regulação comportamental, fisiológica e metabólica em momentos adequados do dia (HARDIN, 2009). Este oscilador pode ser arrastado por ciclos ambientais como a temperatura, comida e luz. A luz arrasta o oscilador ativando um fotorreceptor, que é então capaz de alterar um dos componentes do oscilador (COLLINS et al., 2006; HARDIN, 2005). O fotorreceptor *cryptochrome* (CRY) medeia a entrada de luz para os osciladores circadianos no cérebro e no tecido periférico e é responsável pela degradação TIM induzida pela luz (CERMAKIAN et al., 2002; COLLINS et al., 2006). O funcionamento normal deste mecanismo, com a ativação e degradação de cada proteína

envolvida, no período correto do dia é primordial para o funcionamento de diversos processos celulares que estão sob controle regulação circadiano (CIRELLI, 2006; MCDONALD; ROSBASH, 2001).

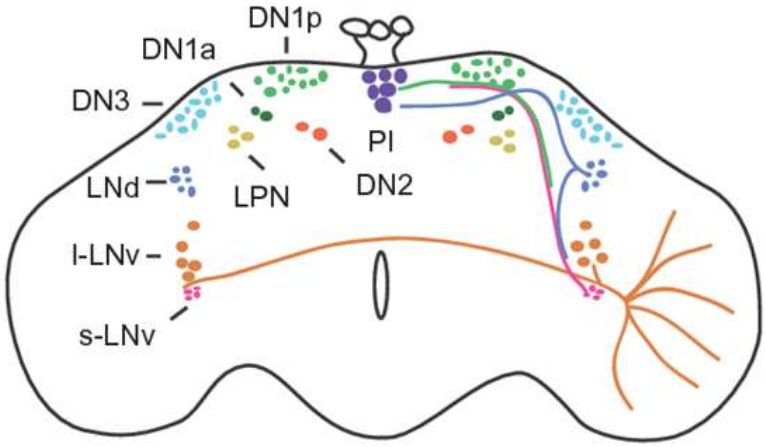
Figura 3. Ciclo de retroalimentação básico do relógio circadiano. A expressão dos genes *per* e *tim* é promovida pelos fatores de transcrição heterodiméricos CLK-CYC e atinge um pico no final do dia. A tradução dos RNAs de *per* e *tim* leva à acumulação e dimerização graduais das proteínas PER e TIM no citoplasma. Os níveis de proteína atingem o pico durante a noite, período durante o qual eles entram separadamente no núcleo para inibir a atividade transcricional de CLK-CYC.



Fonte: Adaptado de NITABACH; TAGHERT, (2008)

Além disso, o cérebro da mosca possuí aproximadamente 150 neurônios marcapassos que formam o relógio central com função de regular os ritmos do sono / vigília (HE et al., 2017). Os neurônios do relógio, que possuem os componentes moleculares do relógio circadiano, constituem uma rede difusa, composta pelos neurônios laterais pequenos e grandes (sLNVs e lLNVs), os neurônios dorsais (DN1s, DN2s e DN3s) e os neurônios laterais dorsais (LNds) **(Fig. 4)**. O fator de dispersão do pigmento (PDF- do inglês *Pigment dispersing factor*) é um neurotransmissor expresso tanto nos sLNVs quanto nos lLNVs (HARDIN, 2009; NITABACH; TAGHERT, 2008). Os LNVs estão envolvidos na regulação do sono durante o dia, promovida, em parte, pela atividade de PDF (CONG et al., 2015b; PARISKY et al., 2008).

Figura 4. Rede de neurônios do marcapasso circadiano no cérebro de *Drosophila* adulta. Os neurônios laterais dorsais LNds estão destacados em azul escuro. Os l-LNvs encontram-se destacados em laranja e os s-LNvs em rosa. Os neurônios dorsais DN1p estão marcados em verde, os DN1a em verde escuro, DN2 em laranja escuro e DN3 em azul claro. O subconjunto de neurônios *pars intercerebralis* (PI) estão marcados em roxo e, em amarelo, os neurônios laterais posteriores LPNs.



Fonte: Adaptado de FLOURAKIS; ALLADA, (2015)

Contudo, a regulação circadiana é muito mais complexa e envolve uma série de mecanismos regulatórios adjacentes (para uma revisão detalhada HARDIN, 2009, 2011). Essa complexidade enfatiza a importância dos ciclos circadianos na regulação do sono e de muitas outras respostas comportamentais e moleculares.

1.4 REGULAÇÃO HOMEOSTÁTICA

A regulação homeostática ocorre em resposta a necessidade de recuperar o tempo de sono perdido, ou seja, a pressão para ir dormir aumenta de acordo com o tempo desperto, e é refletida na duração e intensidade do sono recuperado (CIRELLI; BUSHEY, 2008; HUBER et al., 2004). Embora os mecanismos moleculares envolvidos na regulação circadiana estejam melhor caracterizados, os mecanismos moleculares que regulam a homeostase do sono são menos definidos. Acredita-se que esses mecanismos de regulação sejam neuronais, entretanto, a privação do sono ou outras condições de estresse podem induzir diferentes vias de homeostase, sugerindo que existem vias homeostáticas dependentes do contexto (ALLADA; CIRELLI; SEHGAL, 2017; HILL et al., 2018).

Os processos homeostáticos dependem do tempo anterior de vigília, assim a avaliação da regulação homeostática pode ser realizada após um longo tempo de vigília espontânea ou após a privação do sono induzida (ALLADA; CIRELLI; SEHGAL, 2017; HUBER et al., 2004). As alterações do ciclo sono/vigília podem ser avaliadas em todo o cérebro, partes específicas do cérebro ou em neurônios específicos. As mudanças nos grupos neuronais específicos conhecidos por desempenhar um papel no início e / ou manutenção do sono ou da vigília, como acetilcolina, dopamina, glutamato, ácido γ-aminobutírico (GABA), são de especial interesse para compreender a homeostase do sono (ALLADA; CIRELLI; SEHGAL, 2017).

Em mamíferos a avaliação da homeostase do sono incluí um marcador fisiológico bem estabelecido, a atividade de ondas lentas (SWA- *Slow Wave Activity*), que é utilizado para avaliar a intensidade e necessidade do sono durante o sono NREM (do inglês *non-rapid eye movement*- movimento rápido dos olhos) (DEBOER, 2013). Já em *Drosophila*, em substituição ao SWA (ALLADA; CIRELLI; SEHGAL, 2017), a homeostase é a avaliada por parâmetros comportamentais pós privação do sono, que é seguida por um sono mais longo e

mais consolidado, caracterizado por um número reduzido de breves despertares, episódios de sono mais longos e elevado limiar de excitação (CIRELLI; BUSHEY, 2008; HENDRICKS, 2003; PAUL J. SHAW et al., 2000). Em *Drosophila* o sono é definido como qualquer período de imobilidade comportamental ininterrupta com duração maior que 5 minutos (Shaw et al., 2000; Huber et al., 2004). Equipamentos como o *Drosophila Activity monitor* (DAM) da TriKinetics® permitem a avaliação da qualidade e duração do sono através do monitoramento da atividade diária das moscas. Diversos *softwares* como pySolo (GILESTRO; CIRELLI, 2009), Rethomics (GEISSMANN et al., 2019), ShinyR-DAM (CICHEWICZ; HIRSH, 2018), entre outros, convertem os dados obtidos do DAM para dados gráficos de atividade, ciclos de sono, quantidade do sono e fragmentação, permitindo a avaliação da homeostase do sono em *Drosophila*.

1.5 PRIVAÇÃO DO SONO

A privação do sono é decorrente do sono irregular, o que ocasiona um desempenho reduzido, alerta inadequado e deterioração da saúde. O estilo de vida agitado da sociedade moderna causa várias consequências prejudiciais à saúde, incluindo a privação do sono e as doenças relacionadas, sendo estimado que 20% da população adulta seja afetada por essa condição (ABRAMS, 2015). Uma gama de processos essenciais, como cognição e memória, estado de alerta, atividade metabólica e função imunológica são prejudicados pela privação do sono (COUSINS; FERNÁNDEZ, 2019; DURMER; DINGES, 2005; FOSTER; WULFF, 2005).

A quantidade de sono necessária é variável entre os indivíduos, no entanto, são necessárias em média de 7 a 8 horas de sono noturno e, mesmo que o tempo de sono seja superior a 8 horas por noite, a privação de sono ainda pode ocorrer se a qualidade desse sono for ruim (ABRAMS, 2015). Os problemas do sono e assincronia circadiana são frequentemente observadas em pessoas com trabalhos por turnos e muitos desses trabalhadores apresentam distúrbios metabólicos (BASS, 2013; POGGIOGALLE; JAMSHED; PETERSON, 2018). Especificamente, a duração da privação de sono por 1 a 2 semanas provoca intolerância à glicose, enquanto apenas uma noite de privação do sono é capaz de causar resistência à insulina (BUXTON et al., 2010; DONGA et al., 2010). Além disso, é possível que a privação do sono possa influenciar as atividades metabólicas através de

mudanças nos processos oxidativos, uma vez que a elevação sistêmica dos níveis de espécies reativas de oxigênio (ERO) foi relatada em animais após a privação do sono (VILLAFUERTE et al., 2015). Assim, a privação do sono causa muitos efeitos deletérios, incluindo o maior risco de acidente vascular cerebral, obesidade, risco elevado de diabetes, déficits cognitivos permanentes, depressão ou ansiedade, osteoporose, risco de desenvolver doença cardiovascular entre outros (ABRAMS, 2015).

1.6 MITOCÔNDRIAS

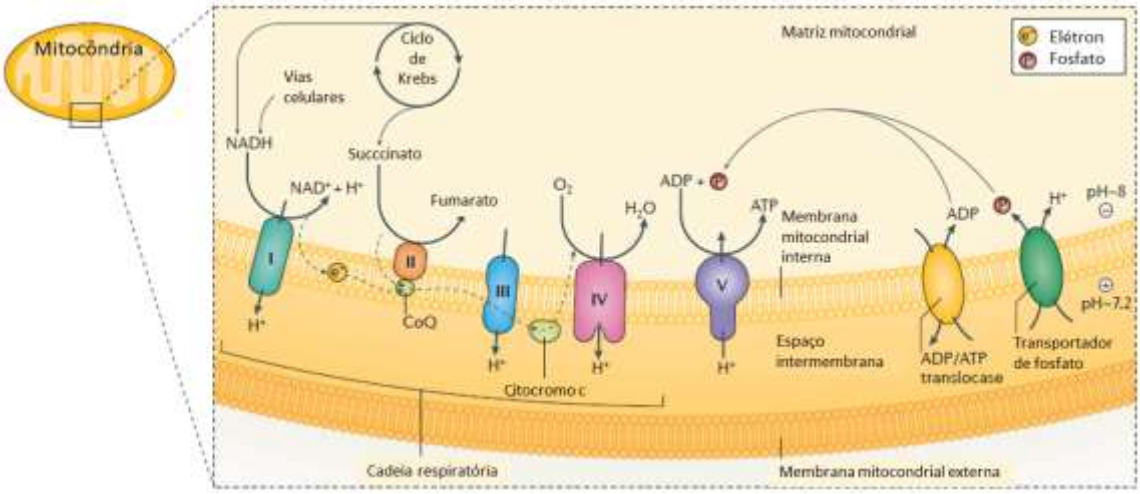
As mitocôndrias são organelas intracelulares presentes em eucariotos e estão envolvidas em várias vias metabólicas celulares, incluindo a fosforilação oxidativa (OXPHOS) **(Fig. 5)**, ciclo do ácido cítrico, oxidação de ácidos graxos, ciclo da ureia, gliconeogênese e cetogênese (GORMAN et al., 2016). As mitocôndrias desempenham um papel significativo nas células, metabolizando nutrientes e produzindo o trifosfato de adenosina (ATP), responsável por vários processos, como metabolismo energético, geração de radicais livres, homeostase do cálcio, sobrevivência e morte celular (CRAVEN et al., 2017). A maior parte da energia celular do corpo (>90%) é produzida pelas mitocôndrias na forma de ATP através do ciclo do ácido cítrico e da cadeia de transporte de elétrons (BHATTI; BHATTI; REDDY, 2017).

Além da geração de ATP, as mitocôndrias medeiam a produção de espécies reativas de oxigênio para a sinalização redox (BAILEY; UDOH; YOUNG, 2014). Atualmente, se acredita que alterações fisiopatológicas nas mitocôndrias no envelhecimento e em muitos outros distúrbios metabólicos estejam ligados a disfunção mitocondrial, como diminuição da capacidade oxidativa e defesa antioxidante pela maior geração de espécies reativas de oxigênio, redução da fosforilação oxidativa e diminuição da produção de ATP (BHATTI; BHATTI; REDDY, 2017).

Os danos e disfunções mitocondriais estão ligados a inúmeras doenças. Devido à estreita conexão entre os ritmos circadianos e o metabolismo, tem sido proposto que várias funções mitocondriais podem ser reguladas pelo relógio circadiano e, possivelmente, podem servir como um coordenador central entre o relógio e o metabolismo energético celular (LANGMESSER; ALBRECHT, 2006). Além de seu papel fundamental no metabolismo energético celular, as mitocôndrias parecem contribuir para a patogênese de muitas doenças degenerativas, envelhecimento, câncer e doenças metabólicas como a diabetes (FUJIMAKI;

KUWABARA, 2017; MOLNAR; KOVACS, 2018). Assim as mitocôndrias desempenham um papel importante para manutenção da homeostase celular.

Figura 5. Representação esquemática da fosforilação oxidativa. A fosforilação oxidativa é uma via metabólica que as células usam para oxidar nutrientes, liberando energia na forma de ATP. A via respiratória inclui os complexos I a IV da cadeia respiratória e o complexo V, uma ATP sintase. O complexo I oxida NADH com a redução da coenzima Q10 (também conhecida como CoQ) de sua forma de ubiquinona (CoQ; Q) para ubiquinol (QH2), gerando um gradiente eletroquímico através da membrana mitocondrial interna. O complexo II liga intrinsecamente o ciclo de Krebs à cadeia respiratória. O complexo II oxida o succinato com a redução da CoQ da sua forma de ubiquinona para o ubiquinol. O complexo III catalisa a redução do citocromo c pela oxidação do ubiquinol com a geração de um gradiente eletroquímico. O complexo IV é responsável pela reação enzimática terminal da cadeia respiratória que transfere elétrons (e–) para o oxigênio molecular e gera um gradiente eletroquímico. O complexo V converte a energia do gradiente de prótons eletroquímicos transmembrana (H +) em energia mecânica, que catalisa a energia de ligação química entre ADP e fosfato (P) para formar ATP.



Fonte: Adaptado de GORMAN et al., (2016)

1.7 ESTRESSE OXIDATIVO E SISTEMA DE DEFESA ANTIOXIDANTE

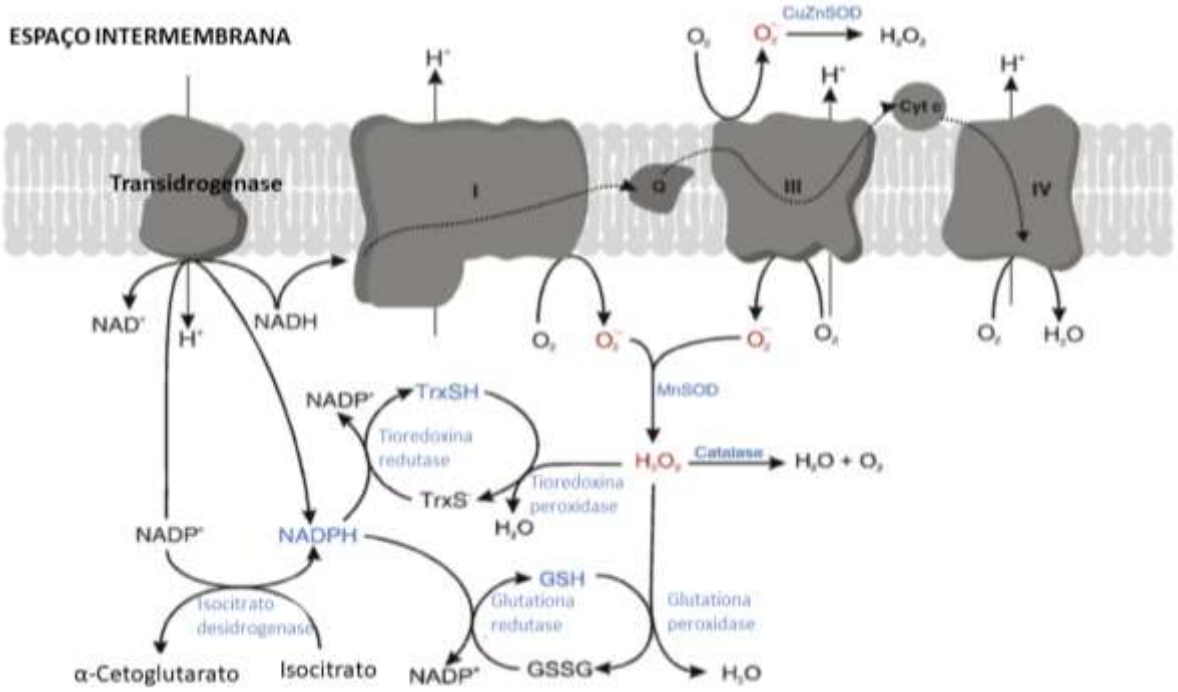
O dano oxidativo celular decorre da produção celular excessiva de espécies reativas, principalmente as espécies reativas de oxigênio (ERO). As ERO são subprodutos do metabolismo normal das mitocôndrias, formadas, em parte, durante o processo de transferência de elétrons que ocorre ao longo da cadeia transportadora de elétrons (CTE), com o objetivo de produzir energia na forma de ATP em conjunto com a fosforilação oxidativa (KOWALTOWSKI et al., 2009) **(Fig. 6)**. Em condições normais, as ERO participam na regulação da resposta imunológica contra infecções e atuam como fatores de transcrição na sinalização intracelular, induzindo a apoptose (BIESALSKI, 2002; HALLIWELL, 1994). Porém, quando produzidas em excesso podem ocasionar a peroxidação lipídica, oxidação proteica e alterações na atividade das enzimas antioxidantes (SIES; BERNDT; JONES, 2005), além de danificar proteínas mitocondriais, membranas e DNA, levando à interrupção na geração de ATP e disfunção mitocondrial (BHATTI; BHATTI; REDDY, 2017).

Fisiologicamente, é muito baixa a concentração de ERO dentro das células, devido a presença dos sistemas antioxidantes celulares que catalisam a remoção ou impedem a formação de ERO (OGA, CAMARGO, BATISTUZZO, 2008). No decorrer da evolução, os seres vivos desenvolveram mecanismos adaptativos capazes de lhes permitirem coexistir com a exposição a ERO. Além dos antioxidantes naturais oriundos da dieta, as células possuem a capacidade de detoxificação de ERO específicas, ajudando a manter os níveis fisiológicos destas espécies reativas. O desequilíbrio entre a produção e eliminação das ERO leva a uma desregulação fisiológica, caracterizando o estresse oxidativo (SIES; BERNDT; JONES, 2005).

Para controlar o balanço na produção de ERO e evitar a ocorrência do estresse oxidativo, a célula possui um sistema de neutralização que inclui a produção de enzimas detoxificantes como catalase (CAT), superóxido dismutase (SOD), glutationa peroxidase (GPx), peroxirredoxina (PRx), glutationa S-transferase (GST) e antioxidantes gerados fisiologicamente como as vitaminas A e E, glutationa (GSH) e ácido úrico (GLOU et al., 2012). A enzima CAT atua na remoção de H_2O_2, possuindo o mais alto poder de catálise conhecido dentre as enzimas, dismutando o H_2O_2 em oxigênio e água diminuindo assim sua permanência nas células (ROJKIND et al., 2002; KLICHKO, RADYUK, ORR, 2004). A enzima SOD possui por função catalisar a dismutação do radical superóxido; a GPx, catalisar

a redução de H_2O_2 através da glutationa reduzida (GSH) para formação de água e glutationa oxidada (GSSH) (OGA, CAMARGO, BATISTUZZO, 2008). As GSTs promovem a conjugação de GSH com produtos endógenos causadores de danos oxidativos, como radicais hidroxila citotóxicos, peróxidos de lipídios de membrana e produtos de degradação oxidativa do DNA, visando sua detoxificação (YAMUNA, BHAVAN, GERALDINE, 2012). Em insetos muitas isorformas de GSTs desempenham o papel de peroxidase e a glutationa age como cofator para a atividade GST (MA; CHANG, 2007).

Figura 6. Metabolismo Mitocondrial de ERO. Os ânions radicais superóxido ($O_2^{\cdot -}$) são formados pela redução de O_2, principalmente nos Complexos I e III da cadeia respiratória. $O_2^{\cdot -}$ é dismutado em H_2O_2 por Cu, Zn-SOD no espaço intermembranoso e Mn-SOD na matriz. O H_2O_2 pode ser removido pela catalase ou tiol peroxidases mitocondriais tais como glutationa e tioredoxina peroxidase, usando glutationa reduzida (GSH) e tiorredoxina (TrxSH) como substrato, respectivamente. A glutationa oxidada (GSSG) e a tiorredoxina (TrxS−) são reduzidas por suas respectivas redutases, usando o NADPH como fonte de elétrons. O NADP pode ser mantido reduzido pela atividade da transidrogenase NAD / NADP, com transporte de prótons para a matriz, fornecendo uma ligação entre o potencial de membrana interna e a capacidade redox mitocondrial. Alternativamente, o $NADP^+$ é reduzido pela isocitrato desidrogenase.

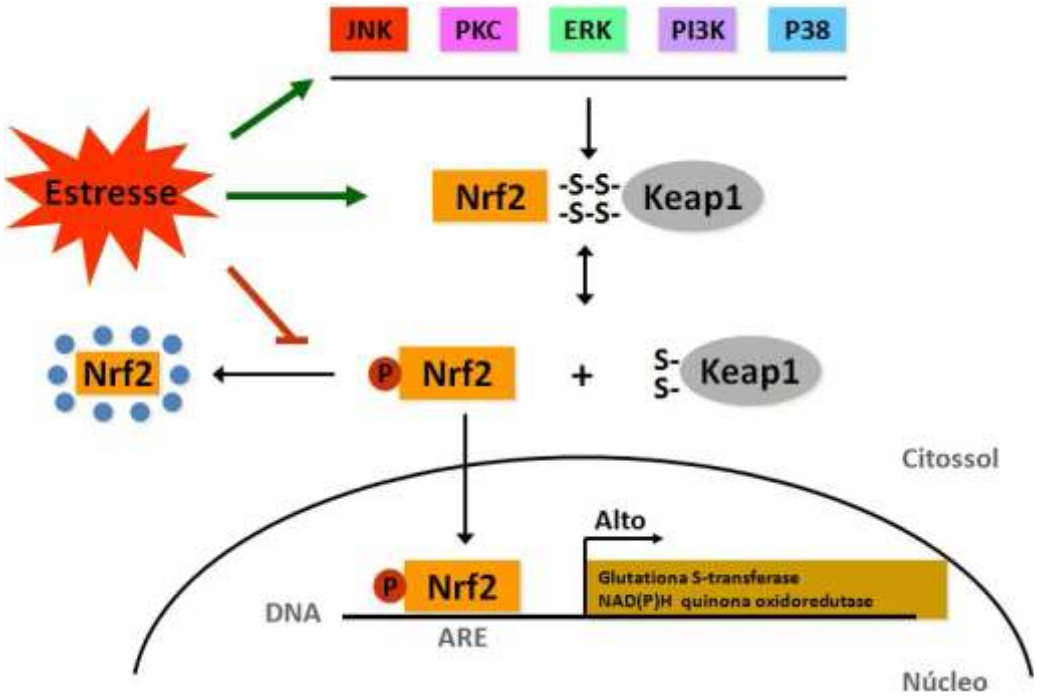


Fonte**:** Adaptado de KOWALTOWSKI et al., (2009).

A regulação de sistemas antioxidantes e de resposta celular ao estresse oxidativo envolve diversas moléculas sinalizadoras, entre elas o NRF2 (fator nuclear eritróide 2) **(Fig.**

7), fator de transcrição que regula a expressão de uma variedade de enzimas mediante ligação ao ERA (elemento de resposta antioxidante) (CHEN et al., 2015). A partir da interação do NRF2 com o ERA são expressas enzimas antioxidantes e de detoxificação de fase II (GST, GPX, SOD, TRX, entre outras) (OSBRURN & KENSLER, 2008). Proteínas quinases ativadas por mitógenos (MAPKs) compreendem um grande número de quinases serina/treonina envolvidas na regulação da proliferação, diferenciação, adaptação ao estresse e apoptose. Em *Drosophila melanogaster*, a via da MAPK é conhecida por estar envolvida em numerosos processos durante o desenvolvimento normal e na regulação da resposta imunitária (STRONACH & PERRIMON, 1999). MAPKs são efetoras *dowstream* nas respostas antioxidantes e suas atividades são manifestadas na ativação de vários fatores de transcrição incluindo NRF2 (LIMÓN-PACHECO et al, 2002). ERK e $P38^{MAPK}$ também regulam positivamente a atividade de NRF2 para iniciar a transcrição de genes antioxidantes (ZIPPER & MULCAHY, 2000). Sendo assim, a via de sinalização do NRF2 é considerada uma das mais importantes na defesa celular contra o estresse oxidativo (ZANG, 2006; KOBAYASHI & YAMAMOTO, 2006; COPPLE et al, 2008).

Figura 7. Modelo de ativação de NRF2 mediada por estresse oxidativo. O estresse oxidativo pode agir diretamente no complexo Nrf2-Keap-1, ou alternativamente ativar as quinases PI3K, p38, ERK, PKC e JNK, causando a liberação de NRF-2 do seu estado inibitório. O fator de transcrição NRF-2 ativado, transloca-se para o núcleo, onde este se liga ao elemento responsivo ao estresse (ERA) na região promotora de genes alvo, tais como genes antioxidantes ou de detoxificação de fase II.



Fonte: Adaptado de SON; CAMANDOLA; MATTSON, (2008).

Muitos antioxidantes e enzimas que protegem a célula do estresse oxidativo exibem ciclos diários em sua expressão ou níveis de atividade, sugerindo que os ritmos circadianos têm um impacto significativo no sistema de defesa antioxidante. Em *Drosophila* e mamíferos, três categorias de genes foram demonstrados como sendo consistentemente positivamente regulados durante a vigília e privação do sono, incluindo genes envolvidos no metabolismo energético, na potencialização sináptica e na resposta ao estresse celular (CIRELLI, 2006). A expressão rítmica de genes envolvidos em vias metabólicas e de resistência ao estresse foi sugerida a partir de estudos de *microarrays* em genoma completo de *Drosophila* (MCDONALD; ROSBASH, 2001). E também várias enzimas antioxidantes, como SOD,

CAT, GSH, GPx, Prx, e GST, apresentam oscilação na sua expressão ou no nível de atividade de acordo com o ritmo circadiano, da mesma forma, os níveis de subprodutos do estresse oxidativo, como os que indicam danos ao DNA, dano proteico ou peroxidação lipídica, também oscilam em diferentes momentos do dia (WILKING et al., 2013), sugerindo que essa ritmicidade nas defesas antioxidantes pode proteger o organismo de níveis excessivos de ERO e dos danos resultantes em macromoléculas biológicas (KRISHNAN; DAVIS; GIEBULTOWICZ, 2008)

O estresse oxidativo e o ritmo circadiano estão relacionados com o desenvolvimento de várias doenças, incluindo as relacionadas ao envelhecimento, doenças cardíacas, câncer, diabetes e distúrbios neurodegenerativos (WILKING et al., 2013). Além disso, o estresse oxidativo parece desempenhar um papel indutor do sono, na tentativa de recuperar a função antioxidante (VILLAFUERTE et al., 2015), uma vez que o sono pode desempenhar uma função antioxidante eliminando os radiais livre e ERO produzidas durante a vigília (TRIVEDI et al., 2017).

1.8 PEPTÍDEOS SEMELHANTES À INSULINA- DILPS

A insulina é um dos hormônios peptídicos mais extensivamente investigados devido ao seu papel crítico no metabolismo dos carboidratos e, portanto, na diabetes e na obesidade. A insulina e os peptídeos semelhantes à insulina foram identificados em um grande número de animais invertebrados, como nematóides, moluscos, insetos e cordados (NÄSSEL; BROECK, 2016). *Drosophila* e mamíferos (incluindo humanos) possuem reguladores moleculares comuns das principais vias metabólicas, incluindo hormônios, fatores transcricionais e moléculas de sinalização que governam o metabolismo celular. A insulina desempenha um papel especial entre esses reguladores, controlando muitos aspectos do metabolismo de carboidratos e lipídios (SEMANIUK et al., 2018).

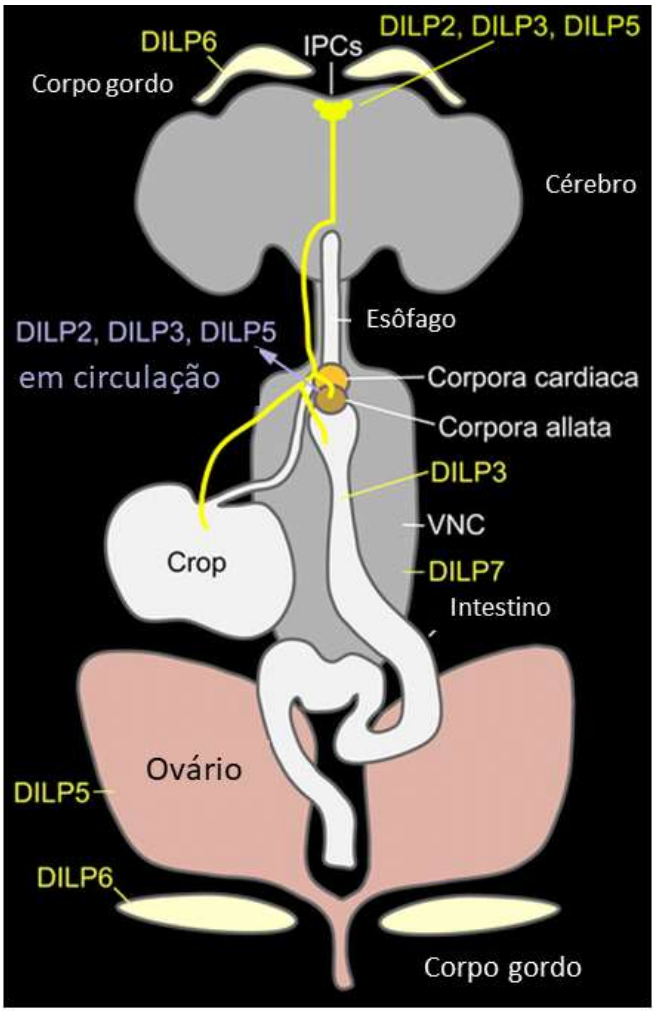
A *Drosophila* possuí oito peptídeos semelhantes à insulina (DILPs) que estão envolvidos na regulação das concentrações de carboidratos na hemolinfa, bem como no acúmulo de metabólitos de armazenamento (SEMANIUK et al., 2018). DILPs 1-7 interagem com um único receptor de insulina do tipo tirosina quinase (DInR) e têm funções redundantes no crescimento, metabolismo, resistência ao estresse, reprodução e longevidade, enquanto

DILP8, recentemente descoberto, interage com um único receptor acoplado à proteína G (GPCR), Lgr3 e mostrou coordenar o estado de crescimento dos tecidos de acordo com o tempo de desenvolvimento (LIN et al., 2017; NÄSSEL; BROECK, 2016).

Os diferentes DILPs são produzidos em um padrão espaço-temporal diferente, sugerindo que cada peptídeo exerce uma função distinta, entretanto funções redundantes já foram descritas entre os DILPs. Uma característica conservada entre os insetos é que certas ILPs são produzidas em um conjunto de células neurossecretoras medianas na *pars intercerebralis* do cérebro **(Fig. 8)** (NÄSSEL et al., 2013). O local principal para a síntese de DILPs são as células neurossecretoras IPCs onde os DILPs 1, 2, 3 e 5 são expressos (BROGIOLO et al., 2001; BROUGHTON et al., 2008; COLOMBANI; ANDERSEN; LÉOPOL, 2012). A perda dos IPCs durante os estágios larvais iniciais causa profundas consequências ao crescimento larval e à homeostase metabólica, e os níveis totais de lipídios, trealose e glicogênio são elevados nesses animais, o que sugere que os DILPs provenientes dos IPCs são reguladores críticos do metabolismo da glicose, e que a função fisiológica dos IPCs se assemelha à das células β pancreáticas de mamíferos (MATTILA; HIETAKANGAS, 2017) .

A DILP6 é estruturalmente e funcionalmente semelhante ao IGF (do inglês *insulin-like growth factors*- Fator de crescimento Insulínico) e são produzidos nas células adiposas do corpo gorduroso (BAI; KANG; TATAR, 2012; RAUSCHENBACH et al., 2017). Na mosca adulta, a expressão do DILP3 ocorre no intestino, DILP5 nos ovários e túbulos de Malpighi e DILP7 em neurônios dos gânglios abdominais (GRÖNKE et al., 2010; MIGUEL-ALIAGA; THOR; GOULD, 2008). Já o DILP8 foi encontrado principalmente em discos imaginários da larva (COLOMBANI; ANDERSEN; LÉOPOL, 2012; GARELLI et al., 2012; GONTIJO; GARELLI, 2018). Sobre os DILP1 e DILP4 a informação é escassa e a expressão parece ser temporariamente limitada durante o desenvolvimento (BROGIOLO et al., 2001).

Figura 8. Visão geral dos locais de produção e liberação de DILPs no SNC e outros órgãos da *Drosophila* adulta. DILP2, 3 e 5 são produzidos nas células produtoras de insulina (IPCs) do cérebro. DILP3 é adicionalmente produzido pelo músculo intestinal e DILP5 nos ovários e nos túbulos de Malpighi (não mostrados). O DILP6 é produzido principalmente pelos adipócitos do corpo gordo na cabeça e no corpo da mosca. Finalmente, a DILP7 é produzida por cerca de 20 neurônios nos neurômeros abdominais do cordão nervoso ventral (VNC) e pode ser liberada no intestino posterior e no oviduto, bem como dentro do SNC.



Fonte**:** Adaptado de NÄSSEL; BROECK, (2016).

A relação entre regulação do sono e peptídeos semelhantes à insulina foi demonstrada em mutantes de *Drosophila* com expressão reduzida para os sete genes DILPs e o receptor DInR. Moscas com ausência de todos os DILPs e do receptor de insulina mostraram uma redução no tempo total de sono, exceto no mutante DILP4. Além disso, este estudo

identificou a expressão de DILP em neurônios LNd e LNv, sugerindo um papel dos DILPs no sistema de regulação do sono (CONG et al., 2015), reforçando a conexão entre os ciclos circadianos e os ciclos metabólicos.

1.9 JUSTIFICATIVA E HIPÓTESE

O estilo de vida agitado da sociedade moderna causa consequências prejudiciais à saúde. Diariamente o ser humano é exposto a fatores capazes de interferir na regulação circadiana e do sono, e condições de arritmia do ciclo circadiano estão envolvidas no aparecimento de diversos distúrbios biológicos. As alterações nos padrões de sono também são observadas em condições patológicas, como distúrbios metabólicos, geralmente observadas em pessoas com trabalhos por turnos. No entanto os mecanismos relacionados aos danos causadas pela privação do sono ainda não estão bem esclarecidos. Assim, pretendeu-se avaliar como a privação do sono a curta prazo age sob o metabolismo redox e energético através da utilização do organismo modelo *Drosophila melanogaster*.

As seguintes hipóteses foram testadas:

- A exposição de *Drosophila* à luz constante seria capaz de causar a privação do sono?

- A privação do sono a curto prazo seria capaz de causar danos oxidativos?

- A privação do sono a curto prazo seria capaz de alterar a regulação circadiana?

- A privação sono a curto prazo seria capaz de causar alterações metabólicas?

2 OBJETIVOS

2.5 OBJETIVO GERAL

Avaliar os efeitos da privação de sono sobre a homeostase redox, atividade mitocondrial, regulação circadiana e controle metabólico em *Drosophila melanogaster*.

2.6 OBJETIVOS ESPECÍFICOS

Avaliar o efeito da privação de sono induzida pela exposição de *Drosophila melanogaster* à luz noturna sobre:

1. Os padrões de sono e comportamento motor;
2. O sistema de transferência de elétrons e fosforilação oxidativa mitocondrial*;*
3. A expressão de marcadores de estresse oxidativo, apoptose e resposta celular antioxidante;
4. A expressão gênica de marcadores chave na regulação circadiana;
5. A expressão de genes relacionados ao controle metabólico.

3. RESULTADOS

Os resultados que fazem parte desta tese estão apresentados sob a forma de um artigo e um manuscrito onde estão os itens Materiais e Métodos, Resultados, Discussão e Referências Bibliográficas. O primeiro está disposto na forma como foi publicado e o segundo na forma em que foi submetido para publicação.

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Original article

Short-term sleep deprivation with exposure to nocturnal light alters mitochondrial bioenergetics in Drosophila

Nathane Rosa Rodrigues[a,b], Giulianna Echeverria Macedo[a], Illana Kemmerich Martins[a], Karen Kich Gomes[a], Nélson Rodrigues de Carvalho[a], Thaís Posser[a], Jeferson Luis Franco[a,b,*]

[a] Oxidative Stress and Cell Signaling Research Group, Centro Interdisciplinar de Pesquisas em Biotecnologia – CIPBIOTEC, Universidade Federal do Pampa, Campus São Gabriel, RS, Brazil

[b] Departamento de Bioquímica e Biologia Molecular, CCNE, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil

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ABSTRACT

Many studies have shown the effects of sleep deprivation in several aspects of health and disease. However, little is known about how mitochondrial bioenergetics function is affected under this condition. To clarify this, we developed a simple model of short-term sleep deprivation, in which fruit-flies were submitted to a nocturnal light condition and then mitochondrial parameters were assessed by high resolution respirometry (HRR). Exposure of flies to constant light was able to alter sleep patterns, causing locomotor deficits, increasing ROS production and lipid peroxidation, affecting mitochondrial activity, antioxidant defense enzymes and caspase activity. HRR analysis showed that sleep deprivation affected mitochondrial bioenergetics capacity, decreasing respiration at oxidative phosphorylation (OXPHOS) and electron transport system (ETS). In addition, the expression of genes involved in the response to oxidative stress and apoptosis were increased. Thus, our results suggest a connection between sleep deprivation and oxidative stress, pointing to mitochondria as a possible target of this relationship.

1. Introduction

The hectic lifestyle of modern society causes several harmful consequences to health. Sleep deprivation and related-diseases are among them being 20% of the adult population affected by this condition [1]. Two processes are responsible for regulating sleep: (i) a circadian clock that regulates sleep time and (ii) a homeostatic mechanism that perceives and responds to lack of sleep [2,3]. The daily variations during rest and activity periods are among the most observable circadian changes [4] and can be influenced by environmental factors, such as temperature changes, light cycles and food availability, which leads to the trapping of the circadian cycle [5].

Sleep is a biological event observable in most species, however, its functions are not clear [6]. However, it is known that sleep plays a critical role in learning and consolidating newly acquired memories [7], as well as in survival since studies in rats and flies have shown that long-term sleep deprivation results in death [3,8,9]. In addition, increasing peaces of evidence suggests the physiological role of sleep in the metabolic process, response to stress and inflammation, energy recovery among others [10,11].

Sleep deprivation has been related to elevated levels of reactive oxygen species (ROS) [12], however this relationship is not well understood. ROS are by-products of the normal metabolism of mitochondria, formed in part during the electron transfer process that occurs along the electron transport chain (ETC), in order to produce energy in the form of adenosine triphosphate (ATP) during oxidative phosphorylation (OXPHOS) [13]. In addition to the production of ATP, mitochondria exert essential functions for the cell, such as energy metabolism, production and elimination of ROS, calcium homeostasis, survival and cell death. Thus, mitochondrial dysfunctions such as decreased oxidative capacity and antioxidant defense, reduced OXPHOS and decreased ATP production are related to metabolic disorders [14]. A study in sleep-deprived mice demonstrated a decrease in mitochondrial complexes activity and provides evidence that mitochondrial dysfunction is involved in the regulation of sleep recovery [15].

In Drosophila and mammals, three gene categories have been shown to be consistently up-regulated during wakefulness and sleep deprivation, including genes involved in energy metabolism, synaptic potentiation, and cellular stress response [16]. The rhythmic expression of genes involved in metabolic pathways and resistance to stress was suggested from studies of microarrays in complete Drosophila genome [17]. Many antioxidants and enzymes that protect the cell from

* Corresponding author at: Oxidative Stress and Cell Signaling Research Group, Centro Interdisciplinar de Pesquisas em Biotecnologia – CIPBIOTEC, Universidade Federal do Pampa, Campus São Gabriel, São Gabriel, RS 97300-000, Brazil.
E-mail address: jefersonfranco@unipampa.edu.br (J.L. Franco).

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oxidative stress such as superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), glutathione peroxidase (GPx) and peroxiredoxin (Prx) show oscillation in their expression or levels of activity according to the circadian rhythm, in the same way, levels of oxidative stress by products, such as those indicating DNA damage, protein damage or lipid peroxidation, also oscillate at different times of the day [18], suggesting that this rhythmicity in antioxidant defenses can protect the body from excessive levels of ROS and its resulting damage to biological macromolecules [19]. However, the phase, amplitude and levels of different antioxidants and/or pro-oxidants may vary depending on specific tissues and organisms [20]. For instance, in humans, some antioxidant enzymes have a morning peak, while melatonin and lipid peroxidation show evening peaks [18].

In the last decade, *Drosophila melanogaster* emerged as a powerful model to investigate sleep and its characteristics [21–24]. Drosophila has many similarities to mammalian sleep, such as prolonged periods of immobility associated with increased excitation thresholds, varying amounts of sleep required by age (young flies sleep longer), responses to chemical stimulants, gene modulation for sleep and wakefulness [25,26]. Furthermore, the two driving forces for sleep are both conserved in Drosophila: the interaction between intrinsic circadian rhythms and external environmental cues and a need for natural sleep regulated by sleep homeostasis [27]. Recently, fruit fly research that collaborated to decipher the regulation of circadian rhythm was awarded the Nobel Prize in Physiology or Medicine in 2017 to researchers Jeffrey C. Hall, Michael Rosbash and Michael W. Young. All these characteristics, together with the development of tools for observation and sleep analysis, make Drosophila an attractive and valid model for sleep studies.

Thus, this study aimed to investigate the relationship between changes in sleep patterns, mitochondrial functions and expression of oxidative pathways genes in the *Drosophila melanogaster* model, using a simple sleep deprivation protocol via exposure to nocturnal light as an environmental cue.

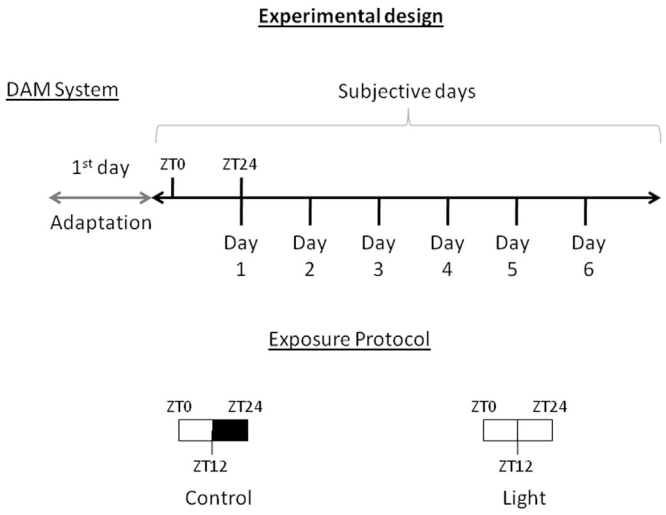
2. Methods

2.1. Fly stock and treatments

D. melanogaster (Oregon strain) was obtained from the National Species Stock Center, Bowling Green, OH, USA. The flies were maintained in incubators at 25 ± 1 °C, 12 h dark-light photoperiod and 60–70% relative humidity with free access to food as previously described [28]. For the experiments, female flies (1–4 days-old) were placed in tubes containing agar meal during 24 h under controlled conditions of light as follows control group (12 h light/12 h dark cycle), and Light group (12 h light/12 h light cycle), both at 25 ± 1 °C (Scheme 1). After treatments period, always finished at 12:00 p.m., the flies were submitted to behavioral tests, biochemical and molecular analysis.

2.2. Sleep analysis

For the sleep analysis and circadian rhythm measurements we used the Drosophila Activity Monitor (DAM) (DAMSystem 308-TriKinetics Inc, Waltham, MA) in which a single fly at a time is allocated in glass tubes (5 × 65 mm) containing agar medium (1.5%) at one end and arranged horizontally on specific monitors supporting up to 32 tubes. During the experiments, the monitors were maintained in a DigiTherm® CircKinetics™ incubator (Tritech Research Inc, Los Angeles, CA) set at 25 °C. As the fly passes through the monitor's infrared beams, its activity is registered. Thus, female flies with 1–4 days-old were maintained under treatments conditions for six days, the first day was not considered in the sleep analysis since at this period flies were allowed to adapt to the system's conditions. As stated, control group was exposed to 12:12 h light/dark cycle, and the Light group was exposed to constant light 12:12 h light/light (Scheme 1). Aiming to evaluate the sleep deprivation without circadian clock interferences, a separated experiment was performed in which flies were allocated into DAM's glass tubes containing agar medium and arranged horizontally on specific monitors. The monitors were then placed over the platform of an orbital shaker (Labnet Enduro™ Mini Mix™ 3D S0600) set at 20 rpm and the experiment was conducted at 12:12 h light/dark cycle (Shake group). For the sleep, circadian rhythm and activity analysis the first 24 h after adaptation period were considered. Sleep patterns were analyzed on pySolo program which converts the results from DAM to graphical activity data, sleep cycles, sleep quantity, and sleep fragmentation among others [29]. For sleep experiments, a total of sixty-four individual flies were used per group (n = 64). All experiments were performed in duplicates.



Scheme 1. Experimental design. *D. melanogaster* with 1–4 days old were maintained in the DAMSystem (Drosophila Activity Monitor) for 7 days. The first day was disregarded (phase of adaptation to the system). Under standard conditions (Control) the Zeitgeber (ZT) started at 7:00 a.m. with the lights ascending (ZT0) and hung up at 7:00 p.m. with the lights off (ZT12), each day of experiment was considered to complete ZT cycle (ZT0–24 − 7:00–6:59 h). For the Light group, the light remained constantly on throughout the experimental period (7 days). For the other experiments performed outside the DAM system, the control group flies were kept in a light / dark cycle of 12 h and the flies of the Light group were kept under constant light for a total period of 24 h, both were kept under temperature of 24 °C.

2.3. Locomotor activity

Locomotor activity was determined as negative geotaxis behavior (climbing ability) in individual flies [30] with some modifications. In brief, after the treatment is finished, a total number of 20 flies *per* group were anesthetized on ice and individually placed in vertical glass tubes (length 25 cm, diameter 1.5 cm) closed with cotton wool. After 30 min of recovery the flies were gently tapped to the bottom of the tube and the time taken by each fly to climb 6 cm in the glass column was recorded. The test was repeated 3 times with 20 s intervals for each fly. A total of twenty individual flies were used per group (n = 20). All experiments were performed in duplicates. The results were expressed as percentage of control.

2.4. Enzyme assays

For measurements of enzymes activity, twenty flies per group were homogenized in 20 mM HEPES buffer (pH 7.0). The homogenate was centrifuged at 20,000*g* for 30 min (4 °C) (Eppendorf 5427R, rotor FA-45-30-11). The supernatant was isolated and used for measuring the activity of antioxidant enzymes (superoxide dismutase, catalase,

glutathione S-transferase and thioredoxin reductase) based on protocols described below. The protein concentration at all samples was determined by the method of Bradford (1976) [31].

Glutathione S-transferase (GST) activity was assayed following the procedure of [32] using 1-chloro 2,4-dinitrobenzene (CDNB) as substrate. The assay is based on the formation of the conjugated complex of CDNB and GSH at 340 nm. The reaction was conducted in a mix consisting of 0.1 M phosphate buffer pH 7.0, 1 mM EDTA, 1 mM GSH, and 2.5 mM CDNB. Catalase activity was assayed following the clearance of H_2O_2 at 240 nm in a reaction media containing 0.05 M phosphate buffer pH 7.0, 0.5 mM EDTA, 10 mM H_2O_2, 0.012% TRITON X100 according to the procedure of [33]. Superoxide dismutase (SOD) activity consists in the inhibition of superoxide driven oxidation of quercetin by SOD at 406 nm. The complete reaction system consisted of 25 mM phosphate buffer, pH 10, 0.25 mM EDTA, 0.8 mM TEMED and 0.05 μM quercetin [34]. Thioredoxin reductase (TrxR) activity was measured according to [35], which consists in measuring the rate of reduction of DTNB by NADPH. The assay medium consisted of 0.1 M phosphate buffer (0.0025 M EDTA, 0.25 M K_2HPO_4, 0.25 M KH_2PO_4, pH 7.0), 10 mM EDTA, 5 mM DTNB, 0.2 mg mL-1 BSA and 0.2 mM NADPH. The rate of reduction of DTNB in the absence of NADPH was used as a blank. One unit of enzyme activity was considered the amount of enzyme that catalyzes the formation of 1.0 μmol of TNB per minute at 25 °C, pH 7.0. All spectrophotometric assays were performed in an Agilent Cary 60 UV/VIS spectrophotometer with an 18-cell holder accessory coupled to a Peltier Water System temperature controller. The values were normalized by protein concentration and expressed as percentage of control. Approximately 200 flies were used, divided into 3–9 biological replicates per group (n = 3–9), performed in triplicates.

2.5. Mitochondrial activity, lipid peroxidation and ROS production

After treatments were finished, twenty flies per group were homogenized in 1000 μL of mitochondrial isolation buffer (220 mM mannitol, sucrose 68 mM, KCl 10 mM, 10 mM HEPES, 1% BSA) following centrifugation at 1000*g* for 10 min (4 °C). The mitochondrial-enriched supernatant was used for determination of mitochondrial activity (Resazurin assay), ROS formation (DCF-DA assay) and lipid peroxidation (TBARS assay).

The activity of mitochondrial enriched-fractions obtained from treated flies was used as an index of toxicity. Mitochondrial activity was measured by resazurin assay (fluorescence). Resazurin assay is based on the ability of viable mitochondria to convert resazurin into a fluorescent end product (resorufin). Nonviable samples rapidly lose metabolic capacity and thus do not generate a fluorescent signal [36]. The fluorescence was monitored at regular intervals of 1 h using a fluorescence plate reader (Perkin Elmer Enspire 2300) at 544 nm_{ex}/590 nm_{em}.

Lipid peroxidation end products were quantified as thiobarbituric acid reactive substances (TBARS) following the method of [37] with minor modifications. The mitochondrial-enriched supernatant was incubated in 0.45 M acetic acid/HCl buffer pH 3.4, 0.28% thiobarbituric acid, 1.2% SDS, at 95 °C for 60 min and absorbance then measured at 532 nm. The TBARS values were normalized by protein concentration. The results were expressed as % of control.

We also quantified 2',7'-dichlorofluorescein diacetate (DCFDA) oxidation as a general index of ROS production [38]. The DCF fluorescence resulting from DCF-DA oxidation was monitored at regular intervals at 485 nm_{ex}/530 nm_{em}. The values were normalized by protein concentration and expressed as percentage of control. Approximately 200 flies were used, divided into 9 biological replicates per group (n = 9), in triplicates.

2.6. High-resolution respirometry (HRR)

Changes in mitochondrial bioenergetics patterns were evaluated by high-resolution respirometry using Oxygraph-2k (O2k, Oroboros Instruments, Innsbruck, Austria). Drosophila mitochondria preparations were obtained according to previously published protocols [39]. For each mitochondrial preparation, 50 flies from each experimental group were anesthetized on ice and homogenized in 2 mL ice-cold mitochondrial isolation buffer containing 250 mM sucrose, 0.1% bovine serum albumin, 2 mM EGTA and 5 mM Tris-HCl (pH 7.4). The resulting homogenate was filtered to remove the tissue particles with a nylon filter membrane (10 μm pore size) and centrifuged at 200*g* for 3 min. The supernatant was collected and a further centrifugation at 9000*g* for 10 min was performed. Next, the mitochondrial pellet was carefully resuspended in 2 mL of ice-cold albumin free isolation medium, followed by centrifugation at 9000*g* for 10 min. This final pellet was resuspended in 100 μL of albumin free isolation medium; this resuspension contained the isolated mitochondria and was used in the respiration assay. All the above procedures were performed at 4 °C. Protein concentration was determined using a Bradford assay using bovine serum albumin Standards [31].

Isolated mitochondria from *D. melanogaster* (0.05 mg mL^{-1}) were transferred to 2 mL of MiR05 solution (110 mM sucrose, 60 mM K-lactobionate, containing 0.5 mM EGTA, 3 mM MgCl2, 20 mM taurine, 10 mM KH2PO4, 20 mM HEPES and 0.1% essentially fatty acid-free BSA, pH 7.1) [40]. All experiments were performed at 24 °C using DatLab 4.0 software (Oroboros Inc., Austria), with continuous stirring at 750 rpm. Through titration protocols, the abilities of a series of substrates and inhibitors to influence mitochondrial function, as a reflex in the difference in respiratory states evaluated. L-Proline + pyruvate + malate + succinate + ascorbate and tetramethyl-p-phenylenediamine (TMPD) will be used as oxidizable substrates in all experiments [39]. Respiratory rates, changes in mitochondrial respiratory chain complexes, mitochondrial membrane integrity and production of oxidative oxygen species were determined.

After signal stabilization, the basal respiration supported by endogenous substrates, the complex I (CI)-mediated leak (LEAK) respiration was determined using 5 mM pyruvate, 5 mM L-proline and 1 mM malate. CI-mediated OXPHOS (OXPHOS) was determined using ADP (2.5 mM). The functional integrity of the outer mitochondrial membrane (CIc_{OXPHOS}) was determined by addition of exogenous cytochrome *c* (cyt *c*) from equine heart (10 μM). Outer mitochondrial membrane disruption is associated with cyt *c* release; so, the respiration stimulated after the cyt *c* addition is proportional to membrane damage [41]. Respiratory control ratios (RCR = CI_{OXPHOS}/CI_{LEAK}) and the increase of oxygen flux after injection of cyt *c* (CIc_{OXPHOS}/CI_{OXPHOS}) were used as quality control for isolated mitochondria. The convergent electron flow during the maximal OXPHOS respiration (CIc + CII_{OXPHOS}) was determined with substrates of CIc and CII (10 mM succinate). The ETS respiration represents the noncoupled respiration using FCCP (optimum concentration reached between 0.5 and 1.5 μM); CIc + CII-mediated ETS respiration (CIc + CII_{ETS}) was determined using FCCP (optimum concentration reached between 0.5 and 1.5 μM). CII-mediated ETS respiration (CII_{ETS}) was determined with 0.5 μM rotenone. The addition of 2.5 μM antimycin A inhibited complex III, resulting in non-mitochondrial respiration, the residual oxygen consumption (Rox) with small contributions from electron leak in the uncoupled state. The CIV respiration was determined adding ascorbate 0.5 mM and TMPD 1 mM. For HRR, 6 independent mitochondrial preparations from each experimental group were used (n = 6). All HRR experiments were performed in duplicates.

2.7. Quantitative real-time qRT-PCR and gene expression analysis

Approximately 1 μg of total RNA from 20 flies was extracted using the Trizol Reagent (Invitrogen™) according to the manufacturer's suggested protocol. After quantification, total RNA was treated with DNase I (DNAse I Amplification Grade – Invitrogen™, NY) and cDNA was synthesized with iScript cDNA Synthesis Kit and random primers again according to the manufacturer's suggested protocol (BIORAD). The

primers were picked by NCBI Primer-Blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) and acquired from Invitrogen™, NY. Quantitative real-time polymerase chain reaction was performed in 10 µL reaction volumes containing water treated with diethyl pyrocarbonate (DEPC), 200 ng of each primer and 0,2 × SYBR Green I (molecular probes) using a 7500 real time PCR system (Applied Biosystems, NY). The qPCR protocol was the following: activation of the reaction at 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles of 15 s at 95 °C, 60 s at 60 °C, and 30 s at 72 °C. All samples were analyzed as technical and biological triplicates with a negative control. Threshold and baselines were automatically determined, SYBR fluorescence was analyzed by 7500 software version 2.0.6 (Applied Biosystems, NY), and the CT (cycle threshold) value for each sample was calculated and reported using the $2^{-\Delta\Delta CT}$ method [42]. The GPDH gene was used as endogenous reference genes presenting no alteration in response to the treatment. For each well, analyzed in quadruplicates, a ΔCT value was obtained by subtracting the GPDH CT value from the CT value of the interest gene (Table 2.). The ΔCT mean value obtained from the control group of each gene was used to calculate the ΔΔCT of the respective gene ($2^{-\Delta\Delta CT}$).

2.8. Caspase assay

Caspase 3/7 (DEVDase) activity was measured with the aid of a commercial kit Promega Apo ONE homogeneous caspase 3/7 assay (Promega®, WI, USA) according to manufacturer's instructions. For each group, twenty flies were homogenized in 500 µL of 20 mM HEPES buffer pH 7.0, and centrifuged at 20,000*g* for 1 min at 4 °C. The supernatant was then incubated for 30 min with substrate (Z-DEVD-R110) (Z-ASP-GLU-VAL-ASP)2-R110 in a 1:1 ratio and fluorescence was captured in a plate reader (Perkin Elmer Enspire 2300) (499 nm/ 521 nm, excitation/emission, respectively). The results were calculated as a percentage in relation to the control group and values were normalized by protein concentration. Approximately 150 flies were used, divided into 6 biological replicates per group (n = 6), in triplicates.

2.9. Statistical analysis

Statistical analysis was performed using two-way ANOVA and Bonferroni post hoc test, or unpaired *t*-test when necessary. Differences were considered statistically significant between groups when $p \leq 0.05$.

3. Results

3.1. Exposure to nocturnal light alters the behavior and sleep cycles in Drosophila

The flies sleep/wake cycle was evaluated during six days in DAMSystem. The data obtained were analyzed in the pySolo software and showed the change in sleep patterns in flies kept under constant light during the six days of exposure when compared to control (Fig. S1a). The total sleep analysis demonstrate that flies kept under constant light showed a tendency to increase sleep in relation to the control group, with total sleep time being significantly increased on days one, three and four ($p \leq 0.05$) (Fig. S1b). Similarly, flies maintained in constant light were less awake in the first three days of exposure (Fig. S1c). Therefore, the first 24 h of exposure, after adapting to DAM system conditions, were chosen for the subsequent analyzes.

The waking activity obtained from pySolo analysis after 24 h exposure indicated a significant decrease ($p \leq 0.05$) in flies maintained in constant light compared to the control (Fig. 1a). The analysis of sleep bout demonstrated a significant increase ($p \leq 0.001$) in the time (min) of sleep during subjective day (ZT 0–12) in flies maintained to constant light. However, flies at continuous light exhibited a decrease on the same proportion ($p \leq 0.001$) in the sleep bout during subjective night (ZT 12–24) (Fig. 1b). These results were equally observed in the duration of sleep bout on flies exposed to constant light (Fig. 1c). On the other hand, the sleep fragmentation has significantly decreased ($p \leq 0.001$) in flies maintained under constant light only at daytime (Fig. 1d).

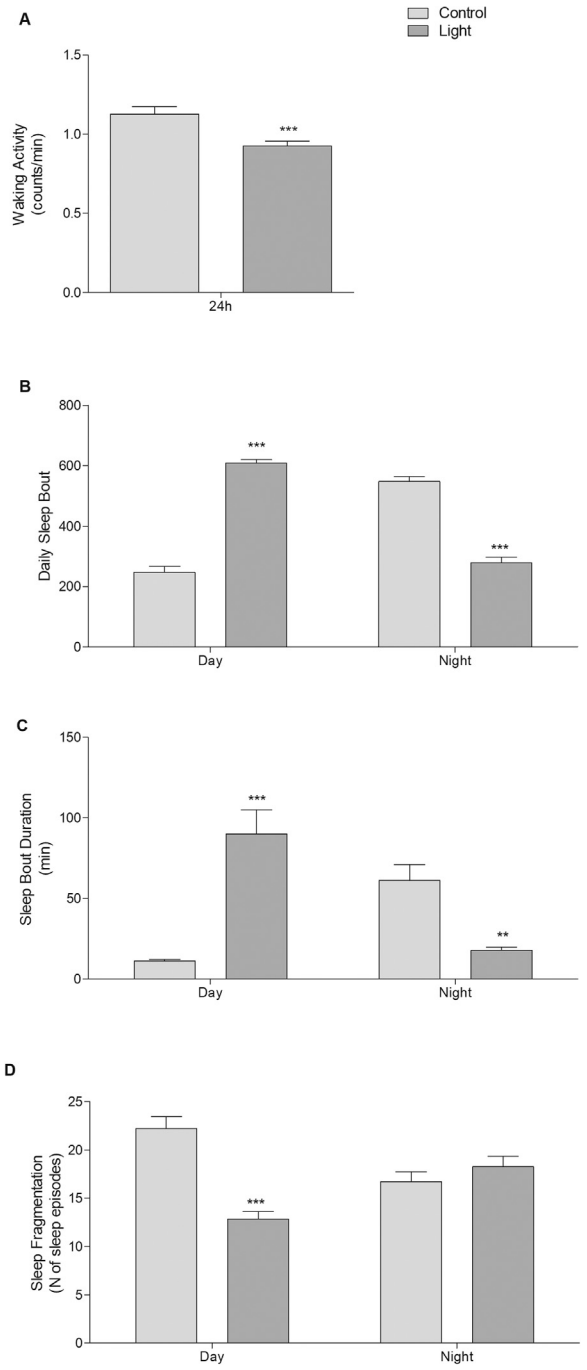


Fig. 1. Effect of constant light on the sleep patterns of flies. (A) Waking Activity, (B) Daily Sleep Bout, (C) Sleep Bout Duration, (D) Sleep Fragmentation. Results are expressed as mean ± SEM. A total of sixty-four individual flies per group (n = 64) was used; experiments were performed in duplicates; **p ≤ 0.01; ***p ≤ 0.001 compared to control group.

For evaluation of the locomotor activity, flies were submitted to the geotaxis negative test. Under normal conditions, flies exhibit a trend to climb a glass column. This natural behavior is called negative geotaxis. In this assay, flies are challenged to climb up to a 6 cm mark in a glass tube and time to climb is computed, control flies are expected to quickly

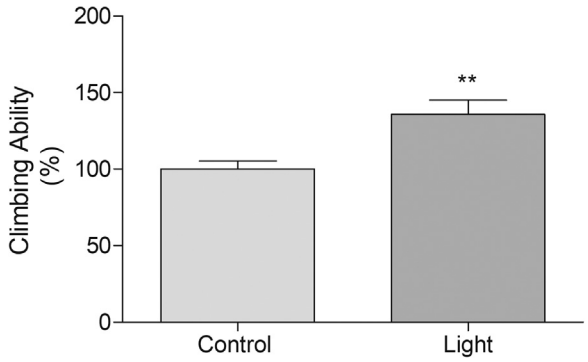


Fig. 2. Effects of sleep deprivation induced by constant light exposure on the locomotor performance of flies. Locomotor activity was assessed by negative geotaxis assay in flies exposed to constant light during 24 h. Values represent the time taken by each fly to climb a marked glass tube. Data are mean ± SEM; a total of twenty individual flies were used per group (n = 20), in duplicates; **p ≤ 0.01 compared to control group.

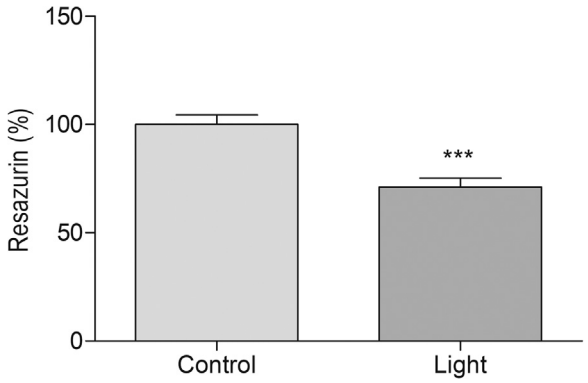


Fig. 3. Effects of sleep deprivation induced by constant light exposure on flies' mitochondrial activity. Levels of resazurin reduction were determined in a mitochondrial enriched fraction prepared after flies' exposure to the treatment conditions. Results are expressed as percentage of control (mean ± SEM); approximately 200 flies, divided into 9 biological replicates *per* group (n = 9) were used. Measurements were performed in triplicates; ***p ≤ 0.001 compared to control.

escalate to the top of the tube. In a condition in which a locomotor deficit is present, flies are expected to take longer times to reach the top. Flies exposed to constant light showed a significant increase (p ≤ 0.05) in the time taken cross a 6 cm mark in a glass column (climbing time) compared to the control (Fig. 2a).

3.1.1. Sleep cycles are altered by mechanical stimulation

Aiming to evaluate sleep deprivation without circadian clock influences, we set a separated experiment in which flies were exposed to a protocol of mechanically induced sleep deprivation, as described in material and methods section. The results revealed that, similarly to flies exposed to continuous light, the mechanically sleep-deprived flies demonstrated a significant increase (p ≤ 0.05) in the time (min) of sleep during subjective day (ZT 0–12) and a decreased (p ≤ 0.001) during subjective night (ZT 12–24) together with a proportionally decreased duration of sleep bout (Fig. S2 a-b). The sleep fragmentation was increased (p ≤ 0.001) at nighttime when compared to the control group (Fig. S2 c).

3.2. Mitochondrial activity and function is affected by sleep deprivation in flies

The mitochondrial activity was determined by resazurin assay, which is a measurement of general mitochondrial dehydrogenase activity. Flies exposure to continuous light during 24 h presented a significant decrease (p ≤ 0.001) in mitochondrial activity (Fig. 3).

To investigate the effects of light exposure on the mitochondrial physiology, the oxygen flux through mitochondrial complexes was measured to determine the mitochondrial bioenergetics function, using high-resolution respirometry (HRR) (Fig. 4a). The basal and CI_{leak} respiration showed no significant differences between control and light exposed group. On the other hand, the addition of saturating ADP to induce OXPHOS indicated that flies exposure to continuous light was able to decrease (p ≤ 0.01) CI_{OXPHOS} and CIc + CII_{OXPHOS} respiration when compared to the control. Similarly, the addition of FCCP to uncoupling respiration indicated a significant decrease on ETS at the CIc + CII_{ETS} (p ≤ 0.01) and CII_{ETS} (P ≤ 0.05) respiration. The addition of antimycin A, used to evaluate the residual oxygen consumption (ROX), decreased the O_2 flux to the basal levels without any significant differences between treatment groups. The addition of TMPD plus ascorbate to available the CIV respiration did not result in significant differences between the control and the light-exposed groups.

The mitochondrial bioenergetics capacity was determined by subtracting the ADP-induced CI_{OXPHOS} values from the CI_{LEAK} values. The exposure of flies to continuous light was able to significantly reduce (p ≤ 0.01) the mitochondrial bioenergetics efficiency, as compared to control (Fig. 4b).

To evaluate mitochondrial functionality, the RCRs were determined as an indicator of the state of mitochondrial coupling, no significant differences were observed between control and light exposed group. Likewise, the integrity of mitochondrial outer membrane was tested by adding cytochrome *c*, and the rates with and without cyt *c* were compared. A satisfactory ratio of membrane integrity was observable (considering values lower than 1.1), as described in the literature [43]. As expected, respiration rates before and after cyt *c* additions were similar in all preparations, attesting the integrity of mitochondrial preparations used here (Table 1).

3.2.1. Mechanical sleep deprivation causes disruption of mitochondrial respiration

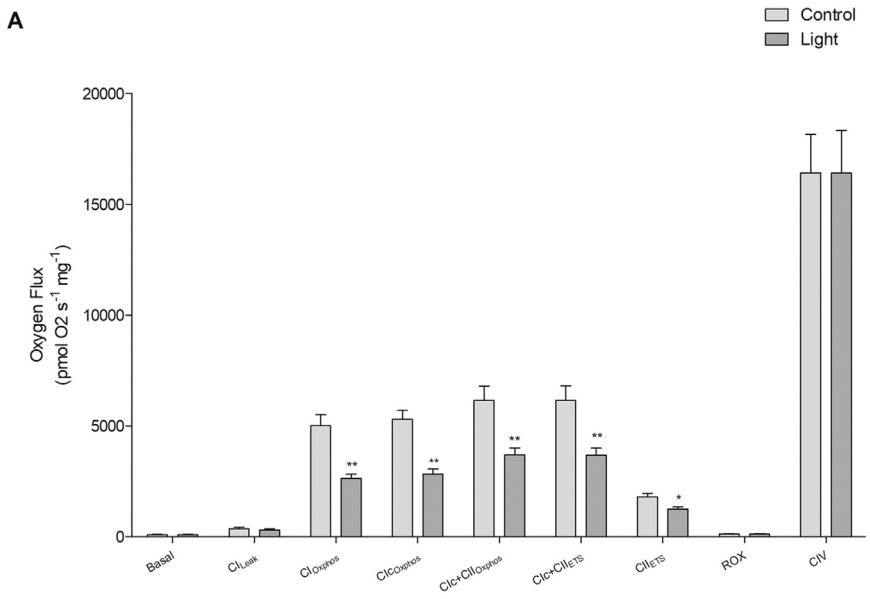
To compare if results observed in mitochondrial metabolism were due to sleep deprivation, *per se*, or due to circadian clock alterations, a group flies was submitted to a mechanical sleep deprivation protocol, as described elsewhere, then the high-resolution respirometry was conducted. The respiratory rates observed were similar to the Light group (continuous light sleep deprivation protocol); however more emphasized. The OXPHOS respiration was decreased (p ≤ 0.01) at all respiratory states, similarly, the uncoupled respiration in ETS also decreased (p ≤ 0.01), compared to control (Fig. S3 a). The mitochondrial bioenergetics efficiency was also significantly decreased (p ≤ 0.01) in the Shake group (Fig. S3 b).

3.3. Sleep deprivation promotes ROS production and lipid peroxidation

The exposure of flies to constant light conditions during 24 h was able to cause increased reactive oxygen species production. Flies maintained under light conditions had a significant increase in the ROS production (p ≤ 0.001) when compared to the control group (Fig. 5a). The levels of lipid peroxidation were increased (p ≤ 0.05) in flies exposed to 24 h of constant light (Fig. 5b).

3.4. The enzymatic antioxidant system is disturbed by sleep deprivation

The activity of antioxidant enzymes known to be modulated under oxidative stress conditions was measured. Catalase is an enzyme involved in H_2O_2 detoxification, flies exposed to light conditions showed a significant decrease in CAT activity (p ≤ 0.001) (Fig. 6a). The GST activity also was measured, and flies had a significant increase after constant light exposure (p ≤ 0.01) (Fig. 6b). The enzyme SOD had its activity increased (p ≤ 0.05) in flies maintained to light conditions



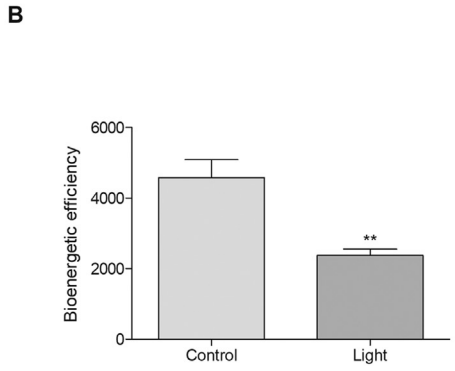


Fig. 4. Effects of sleep deprivation induced by constant light exposure on flies' mitochondrial function (HRR). (A) O2 flux measured in the mitochondria of flies exposed to constant light for 24 h. Mitochondrial function is presented with the abbreviation(s) of the complex(es) involved followed by the state of respiration measured in the presence of L-proline + pyruvate + malate (CI_{LEAK}), + ADP (CI_{OXPHOS}), + cytochrome *c* (CIc_{OXPHOS}), + succinate (CIc + CII_{OXPHOS}), + FCCP (CIc + CII_{ETS}), + rotenone (CII_{ETS}), + antimycin A (ROX) + TMPD/ascorbate (CIV). (B) Analysis of bioenergetics capacity. Results are presented as means ± S.E.M; a total of 6 independent mitochondrial preparations (50 flies each) were used per group (n = 6); *p ≤ 0.05, **p ≤ 0.01 compared to control.

Table 1
Effects of light exposure on the values of respiratory control ratio, uncoupling control ratio and cytochrome *C* effect in the mitochondria of *D. melanogaster*.

	RCR for complex I	Cyt *C* effect
Control	12,59 ± 4,11	1,06 ± 0045
Light	11,23 ± 2,45	1,07 ± 0036

Respiratory Control Ratio (RCR) for complex I (RCR = CIOXPHOS/CILEAK) and cytochrome *C* effect (CIcOXPHOS/CIOXPHOS). Results are means ± SD, n = 7 for control and n = 5 for light group.

(Fig. 6c). TRxR also had its activity altered, in flies exposed to constant light conditions was observed a significant increase (p ≤ 0.05) in the activity of this enzyme when compared to the control (Fig. 6d).

3.5. Gene expression is modulated in flies under sleep deprivation

The exposure of flies to continuous light was able to alter the expression of genes involved to cellular homeostasis, oxidative stress, apoptosis and sleep regulation (Table 2). qRT-PCR analysis revealed a significantly increased (p ≤ 0.05) Nrf2, Pp2a, Akt1 and Hsp83 gene expression. Likewise, the p38β and Pale genes also showed a significant increase (p ≤ 0.01) in their expression (Fig. 7a- e).

3.6. Light-induced sleep deprivation increases caspase activity

As a pro-apoptotic index, caspase 3/7 activity was evaluated. These proteins play a key role in the execution-phase of cell apoptosis. Flies exposure to 24 h of constant light presented a significantly increased (p ≤ 0.001) caspase 3/7 activity (Fig. 8), suggesting an induction of apoptotic cell death.

4. Discussion

The sleep regulation occurs due to two processes that can occur independently: (i) a circadian clock that mainly regulates the sleep time and an (ii) homeostatic mechanism that detects and responds to the need of sleep [2,3]. *Drosophila melanogaster* has been used as a model to investigate the mechanisms involved in sleep disorders [21–24] since it exhibits similar behavioral characteristics of mammalian sleep and allows for both genetic and environmental studies on a high trustworthiness [26]. Here we demonstrated that exposure of flies to 24 h constant light is able to cause changes in sleep patterns similar to sleep deprivation protocols, including sleep homeostatic response. In parallel, locomotor activity deficits and changes in antioxidant defense system together with increased ROS production, lipoperoxidation, apoptosis, mitochondrial dysfunction and changes in gene expression were also evident.

Our results showed that Drosophila exposure to constant light condition was able to cause changes in circadian rhythm and modulate

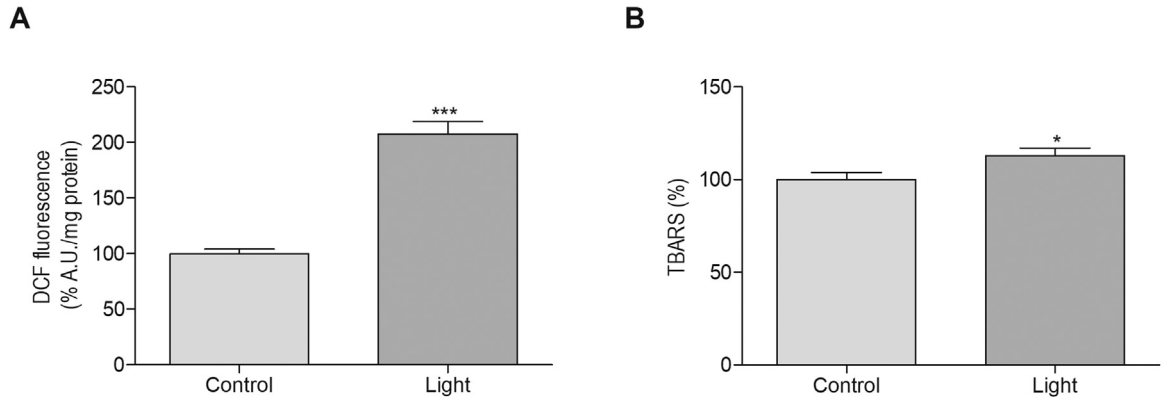


Fig. 5. Effects of sleep deprivation induced by constant light exposure on flies' ROS production and lipid peroxidation. (A) DCF-DA fluorescence as an index of ROS production. (B) Lipid peroxidation assessed by TBARS assay. Results are expressed as percentage of control (mean ± SEM); a total of 200 flies, divided into 9 biological replicates per group (n = 9) in triplicates were used; ***p < 0.001 compared to control.

sleep patterns when compared to flies maintained under normal circadian conditions (12 h light / dark cycle). Flies maintained in constant light condition showed a greater consolidation of sleep during the day, indicating an arrhythmia in the circadian cycle. And the locomotor activity of flies kept in this condition was also affected. Taken together, our data suggest a homeostatic sleep regulation of Drosophila maintained under constant light conditions. Our data corroborate previous studies in which *Drosophila melanogaster* submitted to a sleep deprivation protocol, presented alterations in sleep patterns and decreased locomotor performance, affecting the duration and intensity of sleep after a 24-h deprivation. Sleep recovery was less fragmented and with longer sleep episodes. The same study also showed that the homeostatic sleep response is a well-conserved phenotype among more than 100 wild-type Drosophila strains [25].

Sleep deprivation caused by exposure to constant light conditions was able to impair mitochondrial activity, cause lipid peroxidation and increase the production of reactive oxygen species, as well as the activity of antioxidant enzymes in Drosophila. Like our results, a study realized in splenocytes from mice sleep deprived for 72 h showed an increase in the enzymatic activity of CuZnSOD and MnSOD after sleep deprivation, and the catalase activity was significantly lower after sleep deprivation [44]. Finally, the study suggested that sleep deprivation acts by triggering the antioxidant system, influencing splenocytes homeostasis and interfering with physiological responses [44]. In another study conducted to determine the effect of photoperiod on markers of oxidative damage of rats submitted to different light/dark

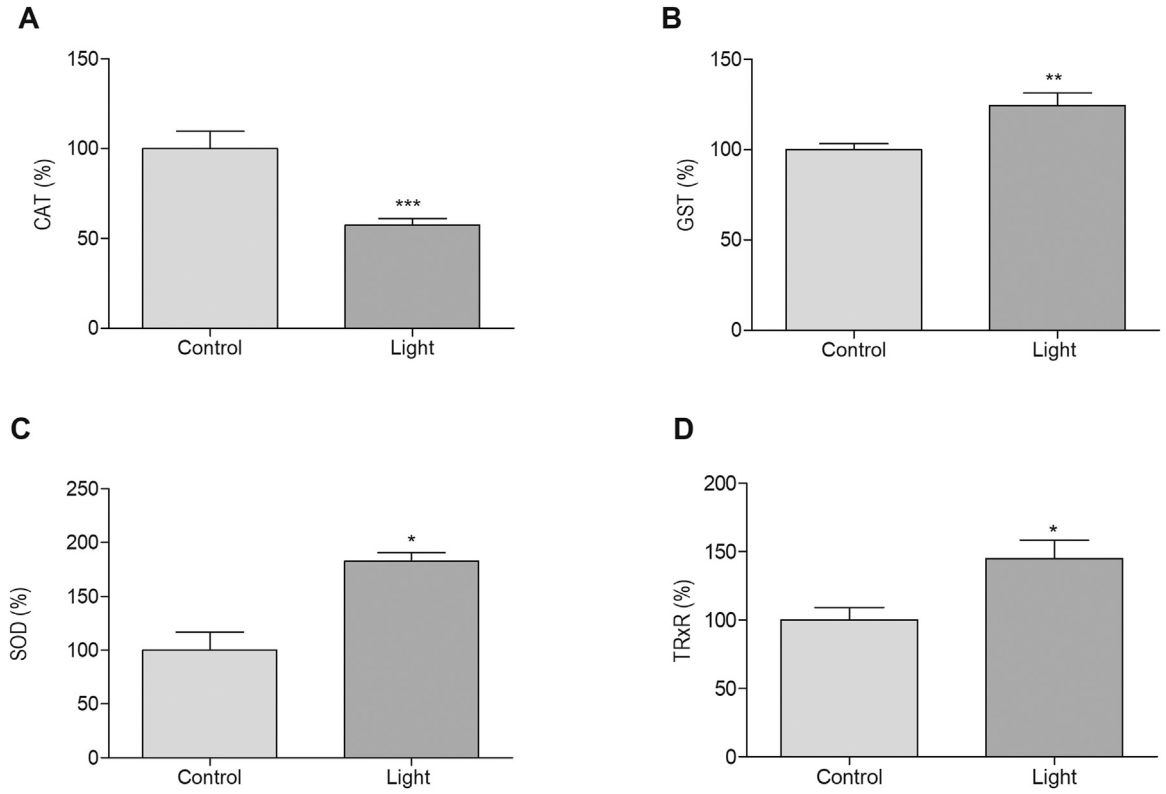


Fig. 6. Effects of sleep deprivation induced by constant light exposure on flies' antioxidant enzyme activity. (A) Catalase (CAT), (n = 9); (B) Glutathione S-transferase (GST), (n = 6); (C) Superoxide dismutase (SOD), (n = 3); (D) Thioredoxin reductase (TrxR) (n = 6). Results are expressed as percentage of control (mean ± SEM); approximately 200 flies, divided into 3–9 biological replicates per group (*n* represents the number of biological replicates used for both control and Light groups), performed in triplicates; **p < 0.01, ***p < 0.001 compared to control.

Table 2
Genes tested by quantitative real-time RT-PCR analysis and used forward and reverse primers.

Gene	Primer Sequences	
Gpdh	Forward	5'ATGGAGATGATTCGCTTCGT
	Reverse	5'GCTCCTCAATGGTTTTTCCA
Nrf2	Forward	5'CGTGTTGTTACCCTCGGACT
	Reverse	5'AGCGCATCTCGAACAAGTTT
P38 β	Forward	5'CGCCGATCTGAACAACATAA
	Reverse	5'GTCCTCGTTTACCGCAATGT
Pale	Forward	5'CATGCCAATCTGGAGAACCT
	Reverse	5'TCCTCGGTGAGACCGTAATC
Akt1	Forward	5'ATAGCAGCGGCGTTAAGAAA
	Reverse	5'CCACCTCGTCCTTTTGGATA
Pp2a	Forward	5'TTGATCTGCAAAAGGCTGTG
	Reverse	5'ACCTGGTTGACCTTGTCCAG
Hsp83	Forward	5'CAAATCCCTGACCAACGACT
	Reverse	5'CGCACGTACAGCTTGATGTT

patterns, and using adriamycin as an inducer of oxidative stress, it was observed that similar to the group treated with adriamycin, the control group exposed to permanent light patterns exhibited lipoperoxidation and impaired antioxidant enzyme activity, demonstrating that exposure to permanent light is capable of causing oxidative damage, these results were related to the increase of leptin and decrease of melatonin [45]. In *Drosophila melanogaster* circadian regulation was shown to be involved in the response to oxidative stress. Null mutant flies to the *period* (*per*) clock gene showed an increase in the susceptibility to hydrogen peroxide compared to wild flies, coinciding with the increase in generation of mitochondrial H_2O_2 and decrease in catalase activity due to oxidative damages [19].

To evaluate the effects of the alteration in the sleep patterns of *D. melanogaster* at the mitochondrial level, we evaluated the mitochondrial bioenergetics function through high-resolution respirometry. The decrease in respiratory rate observed in our results may be due to a compromise of the mitochondrial OXPHOS system and/or to an inhibition of the mitochondrial complexes, along with the decrease in the electron transport (ETS). Changes in the flow of electrons can induce the generation of ROS together with the pro-oxidant effects, in this case, caused by constant exposure to light, and oxidative damage changes the fluidity of the mitochondrial membrane, resulting in the leakage of radicals [13]. In parallel with these data, it was possible to observe a decrease in mitochondrial bioenergetics efficiency, which may cause a compromise in mitochondrial ability to re-phosphorylate ADP at OXPHOS level [39]. In another study aimed at characterizing the function of the mitochondrial electron transport chain in the brain using the paradoxical sleep deprivation model (PSP) in mice, and the ability to recover sleep in reversing the alterations found after the PSP indicated that sleep deprivation was able to impair the transport of electrons from complex I to complex III in the hypothalamus and that sleep recovery after 24 h led to a decrease in the ability of complexes I and II to transfer electrons to complex III via coenzyme Q10 and cytochrome c, respectively, leading to excessive production of reactive oxygen species and causing oxidative damage. The authors suggest that sleep dysfunction leads to a decrease in the activity of the electron transport chain, increasing ROS production and causing oxidative damage, which in turn activates the apoptosis regulatory pathways [15].

Several studies report the relationship between sleep deprivation and oxidative stress, as well as the influence of circadian rhythms in the regulation of oxidative metabolism [3,19]. Although these mechanisms act independently in the sleep regulation, both are simultaneously active in the organism [46]. Furthermore, sleep in Drosophila can be modulated by environmental factors such as light, temperature, feeding, among others, and some of these environmental factors can act on both circadian and homeostatic circuitry simultaneously [46].

Therefore, to prove the importance of sleep in the regulation of oxidative metabolism, flies were submitted to one protocol of mechanically induced sleep deprivation. The changes observed in the sleep of flies under shake conditions were similar to those found in flies exposed to constant light, confirming the effectiveness of both protocols. Interestingly, the respiratory rates observed in flies submitted to mechanically induced sleep deprivation were similar to those presented by flies exposed to constant light; however in a sharper manner. Likewise, the mitochondrial bioenergetics efficiency was further impaired in flies under shake conditions. These data suggest the importance of sleep on the oxidative metabolism and point sleep deprivation as one main contributor to the establishment of oxidative stress.

Reinforcing this hypothesis, a study in sleep-deprived mice using a Sleep Interruption Apparatus (SIA) demonstrated that the chronic sleep deprivation was able to cause alterations in the antioxidant defense biomarkers, like glutathione levels, catalase and superoxide dismutase activities and the levels of lipid peroxidation [47]. The same study also showed that the 20(S)-protopanaxadiol (PPD), a ginsenoside metabolite, with antioxidant activity, effectively restored the levels/activities of antioxidant defense biomarkers in the cortex and hippocampus of chronic sleep-deprived mice [47]. In another study, the overnight total sleep deprivation negatively influenced systemic (plasma-derived) redox metabolism of young adult humans, revealing that glutathione, ATP, cysteine, and homocysteine levels were significantly reduced following one night of sleep deprivation, supporting the idea that sleep deprivation has a crucial role in inducing oxidative stress and ATP depletion [11].

To identify possible pathways involved in the response to the oxidative damage induced by the alteration in sleep patterns of *D. melanogaster* flies, the expression of genes involved in the cellular response to stress and circadian regulation was evaluated. The organisms have systems responsible for protection against damage caused by reactive species, the signaling pathway of the Nrf2 transcription factor (erythroid nuclear factor 2) is considered to be one of the best defense systems against oxidative stress [48]. Nrf2 is a transcription factor that controls the basal and inducible expression of a variety of antioxidant and detoxification enzymes, including GST, SOD, CAT, TRxR, GPx and others [49]. Our results show an increase in Nrf2 expression that may be related to changes in the activities of antioxidant enzymes in response to possible oxidative damage. Nrf2 activation is conditioned to its phosphorylation and nuclear translocation and, in vivo and in vitro models, demonstrate that ERK and p38 MAPK positively regulate the activity of Nrf2 to initiate the transcription of antioxidant genes [49,50]. In addition, one study reported increased expression of Nrf2 mRNA in mitochondrial isolates of the cortex of mice subjected to sleep deprivation [51]. Taken together these facts corroborate the idea that increased expression of the Nrf2 gene may be related to oxidative damage induced by changes in sleep patterns in response to light.

In addition to regulating Nrf2, p38 MAPK has been reported in circadian control. In hamster suprachiasmatic nuclei, phosphorylation of p38 MAPK increases during the day, and exposure to light during the dark phase induces its phosphorylation [52]. In Drosophila, it has been shown that both activation and inhibition of p38β in clock neurons result in circadian arrhythmia and that p38β interacts with the period gene to regulate the length of the period and the strength of rhythmicity, in the end the authors suggest that p38β can affect circadian locomotor rhythms by regulating multiple post-transcriptional pathways [53]. Thus, the increase in p38β expression observed in our results may be related to the increase in Nrf2 expression, as well as changes in circadian rhythm induced by light exposure.

The activation of P38 MAPK was also related to the increase in tyrosine hydroxylase (TH) expression of *D. melanogaster* [54] and in the regulation of human TH [55]. Tyrosine hydroxylase (also known as Pale in Drosophila) is the rate-limiting enzyme of dopamine biosynthesis (DA). A study in *Xenopus laevis* larvae indicated that in the presence of bright light the number of TH+ neurons (used as marker of dopaminergic neurons) was increased, allowing fast adaptation to light [56]. In

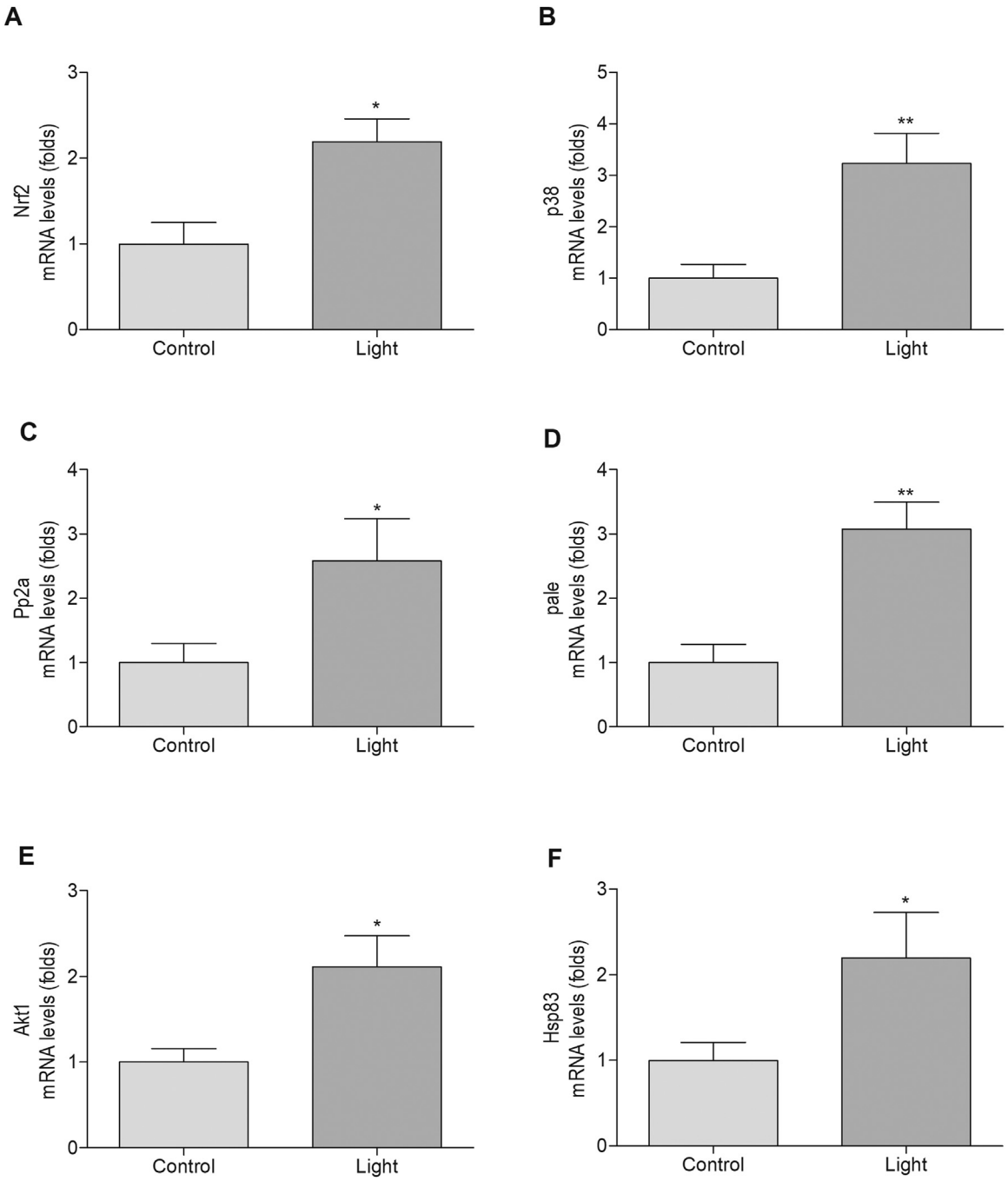


Fig. 7. Effects of sleep deprivation induced by continuous light exposure on flies' gene expression. Gene expression was evaluated by qRT-PCR. (A) Nrf2; (B) p38β; (C) Pp2a; (D) Pale (TH); (E) Akt1; (F) Hsp83. Results are shown as fold increases/decreases as compared to control (considered as 1) (mean ± SEM); a total of 6 biological replicates (n = 6), in triplicates were used; *p ≤ 0.05, **p ≤ 0.01 compared to control.

another report, Zebrafish retinal tissues showed an increase in TH expression in light adaptation, followed by increased concentration of 3,4-dihydroxyphenylacetic acid (DOPAC) and dopamine, suggesting that changes in dopamine concentration are due to adaptive changes in the synthesis, release and metabolism of dopamine in response to light [57]. In Drosophila, studies report that dopamine is strongly linked to sleep and wakefulness regulation, suggesting a simple relationship between DA and the amount of sleep, where more DA leads to less sleep, and less DA leads to more sleep [58,59]. Thus, the observed increase in pale expression may suggest that dopamine levels in flies exposed to light for 24 h are also increased as a likely effect of altered sleep patterns.

Akt or PKB is a serine/threonine kinase that play critical role in regulating diverse cellular functions like, growth, proliferation, metabolism, survival, transcription and protein synthesis. Recent studies have indicated the role of Akt in the regulation of Nrf2 activity [60,61]. A study realized in cultured human retinal pigment epithelium (RPE) cells demonstrated that Akt activation is sufficient to increase the Nrf2 activity, suggesting that one of the signaling mechanisms by which PI3K/Akt promotes cell survival is to facilitating induction of the Nrf2-dependent antioxidant defensive system [62]. Another study also performed in RPE cells, aimed to evaluate the potential activity of SC79, an activator to Akt, against oxidative damage and apoptosis caused by Ultra-violet (UV) radiation, indicated that Akt-Nrf2 cascade activation

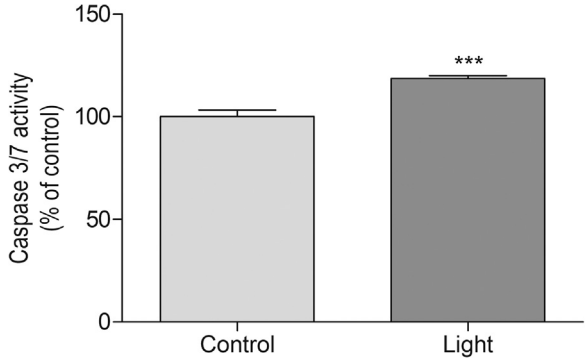
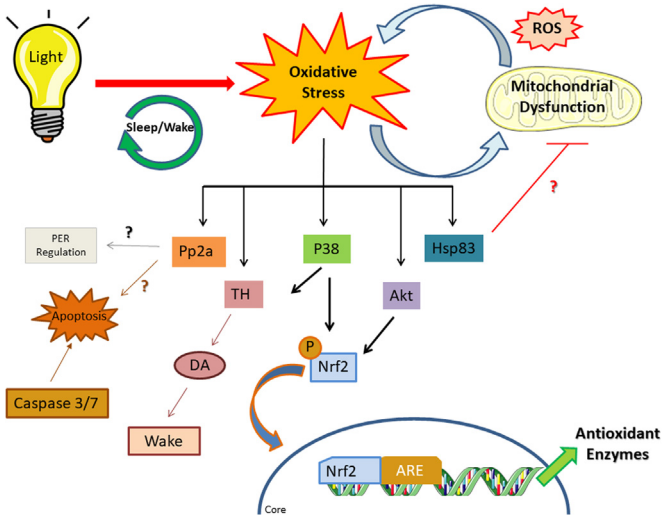


Fig. 8. Effects of sleep deprivation by continuous light exposure on flies' caspase activity. Caspases 3/7 activity assay was performed by using commercial kits according to manufacturer instructions. Results are expressed as percent control (mean ± SEM); a total of 6 biological replicates per group (n = 6), in triplicates were used; *** $p < 0.001$ compared to the control.



Scheme 2. Authors suggestions on potential mechanisms involved in nocturnal light exposure of *D. melanogaster*. The exposure of flies to constant light for 24 h was able to alter sleep patterns and sleep/wake cycle. These changes lead to the occurrence of oxidative damage, promoted mitochondrial dysfunction and generation of reactive oxygen species, accentuating the state of oxidative stress. As a response to oxidative stress, expression of stress-related genes Hsp83, Pp2a, pale (TH), Akt1, p38β were up-regulated, and consequently, Nrf2. Akt and p38β can act on the phosphorylation of Nrf2 [50,60]. When phosphorylated, the Nrf2 is activated and translocated to the nucleus, where it binds to ARE (the antioxidant responsive element), initiating the transcription of antioxidant enzymes [49]. p38β may also influence tyrosine hydroxylase [55]. The increased expression of TH may result in increases in dopamine (DA) levels [57], and consequently increasing the waking state of the flies. We have observed a stimulation of caspase 3/7 activity. The expression of Pp2a may be involved with the activation of apoptosis pathways [67]. On the other hand, this phosphatase may be related to the regulation of the rhythmic protein PER [69], but this interaction needs further clarification.

is required for SC79-mediated cytoprotection against UV in primary RPE cells, suggesting that SC79 activates Akt to possibly phosphorylate Nrf2 at Ser-40, causing Nrf2 accumulation and activation, attenuating UV-induced oxidative stresses and RPE cell apoptosis [63]. Our results demonstrate the increased in the Akt1 mRNA levels, probably in response to the oxidative stress induced by sleep deprivation in exposed flies to constant light, causing the observed increase in the expression of the Nrf2 gene. A link between oxidative stress and circadian rhythms was demonstrated in Drosophila, where flies *foxo* mutant showed lost behavioral rhythms driven by the central clock, and the overexpression of signaling molecules that affect FOXO activity, such as the insulin receptor or Akt, in the fat body of flies also increased susceptibility of the central clock to oxidative stress [64].

In a review of Cirelli [16], three gene groups have been reported to be involved in the waking and short-term sleep deprivation in several species. Among them are included the chaperones heat shock proteins and genes involved in the response to cellular stress. In our study, we demonstrated a significant increase in Hsp83 gene expression in sleep-deprived flies by exposure to light for 24 h, this increase may reflect a defense response against cell stress caused by sleep deprivation. In rodents, chaperone protein levels were also demonstrated to be increased after sleep deprivation [65]. One study demonstrated that Drosophila carrying a mutation for the heat-shock protein Hsp83 showed an exaggerated homeostatic response and died after being submitted to sleep deprivation. The same study also showed that flies cyc^{01} gene mutant had a reduction in the expression of heat shock genes after sleep loss, and the activation of heat shock chaperones before to sleep deprivation was able to prevent this mortality. However, the preheating did not prevent the mortality in Hsp83 mutant flies, suggesting that chaperone proteins protect against the lethal effects of sleep deprivation [3].

Protein phosphatase 2A (PP2A) is a cytoplasmic serine/threonine phosphatase, known to control various cellular processes, including metabolism, kinase signaling cascade, cell growth and apoptosis [66]. In mice osteoblasts, PP2A was shown to mediate apoptosis induced by oxidative stress, suggesting that lipid peroxidation products activate PP2A, in addition, inhibition of PP2A partially prevented osteoblastic apoptosis under oxidative conditions [67]. PP2A protein is also related to circadian regulation, acting on the regulation of PER through dephosphorylation [68]. In Drosophila S2 cells, PP2A played a role in the cyclic expression of PER protein, where the reduction of PP2A activity reduced PER expression in central clock neurons, resulting in long periods and arrhythmia. The authors showed that PP2A also affects the phosphorylation of PER *in vitro* and *in vivo*, suggesting that post-translator mechanisms that drive PER cycling require the rhythmic expression of PP2A [69]. Thus, the increase in Pp2a gene expression observed in our results may suggest the activation of an apoptotic pathway induced by changes in the circadian rhythm of Drosophila exposed to constant light conditions.

A previous study has also reported the establishment of neuroinflammation, microglial activation and neuronal apoptosis in the hippocampus of sleep-deprived mice even after 3 weeks of recovery [70]. Our results demonstrated a significant stimulation of caspases 3/7 activity in sleep-deprived flies. Caspases are a family of cysteine proteases commonly known as effectors of apoptosis [71]. Therefore, our results in flies corroborate the pro-apoptotic effects of sleep deprivation in other models.

Therefore, we suggest (Scheme 2.) that Drosophila exposure to constant light condition for 24 h promotes changes in sleep/wake cycle. These changes lead to the occurrence of oxidative damage, promoted mitochondrial dysfunction and generation of reactive oxygen species, accentuating the state of oxidative stress. As a response to oxidative stress, expression of stress-related genes Hsp83, Pp2a, pale (TH), Akt1, p38β were up-regulated, and consequently, Nrf2. Akt and p38β can act on the phosphorylation of Nrf2 [50,60]. When phosphorylated, the Nrf2 is activated and translocated to the nucleus, where it binds to ARE (the antioxidant responsive element), initiating the transcription of antioxidant enzymes [49]. p38β may also influence tyrosine hydroxylase [55]. The increased expression of TH may result in increases in dopamine (DA) levels [57], and consequently increasing the waking state of the flies. We have observed a stimulation of caspase 3/7 activity. The expression of Pp2a may be involved with the activation of apoptosis pathways [67]. On the other hand, this phosphatase may be related to the regulation of the rhythmic protein PER [69], but this interaction needs further clarification.

Taken together our results emphasize the relationship between sleep deprivation, mitochondrial dysfunction and oxidative stress,

highlighting the importance of sleep in health, demonstrating that even short-term sleep deprivation is capable of causing deleterious effects to organisms. However, further investigation on the relationship between constant light exposure and activation of apoptotic pathways, as well as the relationship with Drosophila circadian oscillator genes is required for a better understanding of changes in sleep patterns induced by light exposure conditions.

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Conflict of interest

Authors declare no conflict of interest

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2018.04.549.

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Disturbed sleep induces disruption of circadian clock and metabolism regulation genes in drosophila

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Corresponding Author: Professor Jeferson Luis Franco, Ph.D.

Corresponding Author's Institution: Universidade Federal do Pampa

First Author: Nathane Rodrigues, MsC

Order of Authors: Nathane Rodrigues, MsC; Giulianna Macedo, MsC; Illana Martins, MsC; Patrícia Vieira, PhD; Karen Gomes; Thais Posser, PhD; Jeferson Luis Franco, Ph.D.

Abstract: Circadian rhythms are biological events that influence physiology and behavior, being present in all living organisms. Sleep is a crucial resting state for survival and is regulated by circadian rhythms. Several studies have shown the effect of sleep on various pathological conditions, including metabolic diseases; however the mechanisms involving sleep, circadian clock and metabolism regulation are not well understood. In this study we evaluated the expression of genes of the circadian clock and Drosophila insulin-like peptides (DILPs) together with metabolic markers (glucose, triglycerides and glycogen) in fruit-flies submitted to a short-term sleep deprivation protocol with exposure to nocturnal light. Exposure of flies to sleep deprivation caused increased expression of the main circadian clock genes, modulated DILP genes and decreased the glucose, triglycerides and glycogen levels. Moreover, we demonstrated concomitant changes in DILPs, AKT phosphorylation and dopamine levels in sleep deprived flies. Thus, our results suggest a connection between sleep deprivation, circadian rhythms and metabolism disruption, demonstrating the importance of sleep in health maintenance

Suggested Reviewers: Marcelo Farina
Universidade Federal de Santa Catarina
marcelo.farina@ufsc.br
Professor Farina is an outstanding researcher in the field of biological sciences and his comments would certainly improve our manuscript

Thomas Merritt
Laurentian University
tmerritt@laurentian.ca
Professor Merritt is an outstanding researcher, expert in drosophila metabolism and function and his comments would certainly improve our manuscript

CM Peterson
University of Alabama at Birmingham
cpeterso@uab.edu
Professor Peterson is an outstanding researcher, expert in metabolism and circadian rhythms, his work has been cited within this manuscript and his comments would certainly improve our manuscript

O Lushchak
Vasyl Stefanyk Precarpathian National University, Ivano-Frankivsk, Ukraine
olehl@pu.if.ua
Professor Lushchak is an outstanding researcher and expert in drosophila insulin-like peptides. His comments would certainly improve our manuscript

C Wegener
University of Würzburg
christian.wegener@biozentrum.uni-wuerzburg.de
Professor Wegener is an outstanfing researcher and expert in Neuropeptide signalling in Drosophila and other insects, Hormonal and circadian regulation of behaviour and physiology, Circadian regulation of neuroendocrine
systems, Neurochemistry, Peptidomics and architecture of peptidergic systems in
insects. His comments would certainly improve our manuscript

J Tennessen
Indiana University
jtenness@indiana.edu
Professor Tennessen is an outstanding researcher and The Tennessen lab uses the fruit
fly, Drosophila, to study the coordinate regulation of growth and metabolism. His comments would certainly improve our manuscript

Opposed Reviewers:

UNIVERSIDADE FEDERAL DO PAMPA
CAMPUS SÃO GABRIEL
Av. Antonio Trilha, 1847, Centro. São Gabriel, RS, Brazil 97300-000

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Dear Editor

We are submitting a manuscript entitled " **Disturbed sleep induces disruption of circadian clock and metabolism regulation genes in drosophila"** from authorship of Rodrigues et al.

This is a report on the effects of exposure of flies to a sleep deprivation protocol which caused increased expression of the main circadian clock genes, modulated DILP genes and decreased the glucose, triglycerides and glycogen levels. Moreover, we demonstrated concomitant changes in DILPs, AKT phosphorylation and dopamine levels in sleep deprived flies. Thus, our results suggest a connection between sleep deprivation, circadian rhythms and metabolism disruption, demonstrating the importance of sleep in health maintenance, which might be of interest to the readership.

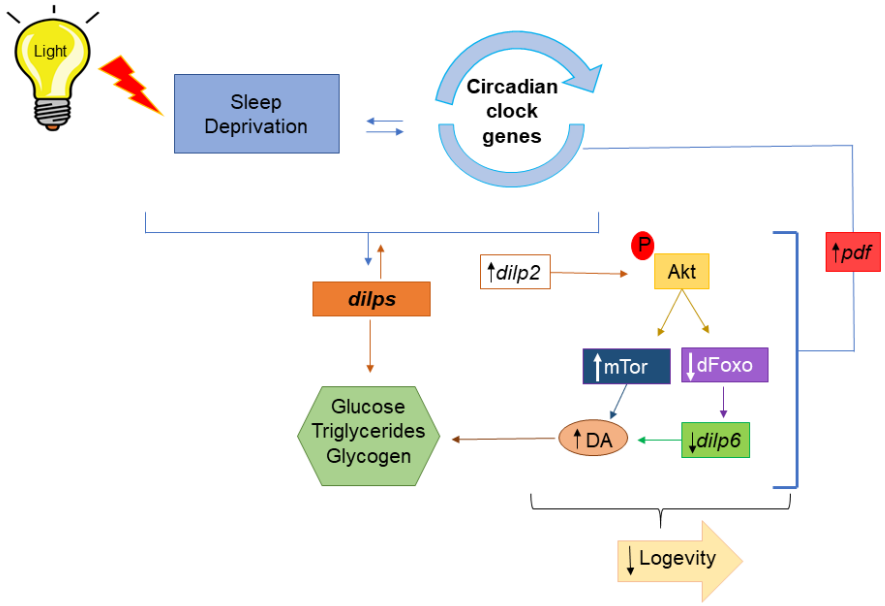
In the hope that our manuscript meets the standards of the Journal of Molecular Biology, we are looking forward your appreciation.

Sincerely,

Jeferson Luis Franco, Ph.D

Universidade Federal do Pampa
Campus São Gabriel
São Gabriel – RS (97.300-162)
jefersonfranco@unipampa.edu.br
jefersonfranco@gmail.com

***Graphical Abstract (for review)**



***Research Highlights**

Highlights

- Sleep deprivation modulates metabolic markers in drosophila;
- Sleep deprivation increase dopamine levels in drosophila;
- Sleep deprivation active AKT phosphorylation in drosophila;
- Sleep deprivation alters clock genes in drosophila;
- Sleep deprivation alters metabolism response genes in drosophila.

Disturbed sleep induces disruption of circadian clock and metabolism regulation genes in drosophila

Nathane Rosa Rodrigues[a,b], Giulianna Echeverria Macedo[a], Illana Kemmerich Martins[a], Patrícia de Brum Vieira[a], Karen Kich Gomes[a], Thaís Posser[a], Jeferson Luis Franco[a,b].

[a] Oxidative Stress and Cell Signaling Research Group, Centro Interdisciplinar de Pesquisas em Biotecnologia – CIPBIOTEC, Universidade Federal do Pampa, Campus São Gabriel, RS, Brazil.

[b] Departamento de Bioquímica e Biologia Molecular, CCNE, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil

To whom correspondence should be addressed at Oxidative Stress and Cell Signaling Research Group, Centro Interdisciplinar de Pesquisas em Biotecnologia – CIPBIOTEC, Universidade Federal do Pampa, Campus São Gabriel, São Gabriel, RS 97300-000, Brazil. E-mail: jefersonfranco@unipampa.edu.br

Abstract

Circadian rhythms are biological events that influence physiology and behavior, being present in all living organisms. Sleep is a crucial resting state for survival and is regulated by circadian rhythms. Several studies have shown the effect of sleep on various pathological conditions, including metabolic diseases; however the mechanisms involving sleep, circadian clock and metabolism regulation are not well understood. In this study we evaluated the expression of genes of the circadian clock and Drosophila insulin-like peptides (DILPs) together with metabolic markers (glucose, triglycerides and glycogen) in fruit-flies submitted to a short-term sleep deprivation protocol with exposure to nocturnal light. Exposure of flies to sleep deprivation caused increased expression of the main circadian clock genes, modulated DILP genes and decreased the glucose, triglycerides and glycogen levels. Moreover, we demonstrated concomitant changes in DILPs, AKT phosphorylation and dopamine levels in sleep deprived flies. Thus, our results suggest a connection between sleep deprivation, circadian rhythms and metabolism disruption, demonstrating the importance of sleep in health maintenance.

Key words- sleep deprivation, clock genes, DILP, dopamine, mTOR, dFOXO.

Introduction

Circadian rhythm comprehend events controlled by the biological clock that occur in animals, plants, fungi and prokaryotes over a 24h period, allowing organisms to predict rhythmic changes in their environment and optimize most of their bodily functions with the day / night cycle including the sleep / wake cycle [1,2]. Sleep is an observable biological event in most species, but its actual functions are unclear [3]. Increasing evidence suggests the physiological role of sleep in the metabolic process, stress and inflammation response, energy recovery [4,5], and survival, as studies in rats and flies have shown that long-term sleep deprivation results in death [6,7].

The hectic lifestyle of modern society causes several detrimental health consequences. Sleep deprivation and related diseases are among these consequences, with 20% of the adult population affected by this condition [8]. Sleep problems and circadian disruption are often observed in shift workers and many of these workers have metabolic disorders. [9,10].

A circadian oscillator is responsible for maintaining circadian time, being dragged by environmental signals such as light and activating rhythmic outputs at the appropriate day time [1]. In addition, several autonomously acting genes are involved in circadian timing. In *Drosophila melanogaster*, the basic feedback mechanism that forms the clockwork involves the Clock (*clk*) and Cycle (*cyc*) genes that encode proteins that activate the transcription of period (*per*) and timeless (*tim*) genes. PER and TIM proteins accumulate in cell core, then PER protein repress the CLK / CYC activators, causing the suppression of *per* and *tim* transcription [11]. Several genes involved in circadian regulation have been shown to affect sleep patterns in drosophila. [7,12,13].

Drosophila and mammals (including humans) have common molecular regulators of major metabolic pathways, including hormones, transcriptional factors, and signaling molecules that govern cellular metabolism and insulin plays a special role among these regulators, controlling many aspects of carbohydrate and lipid metabolism [14]. The *D. melanogaster* genome contains eight genes encoding insulin-like peptides (DILPs) that participate in the regulation of carbohydrate concentrations and accumulation of reserve metabolites [14]. DILPs 1-7 interact with a single tyrosine kinase insulin receptor (DInR) and have redundant functions in growth, metabolism, stress resistance, reproduction and longevity, while newly discovered DILP8 interacts with a single G protein coupled receptor (GPCR), Lgr3 and showed to coordinate the state of tissue growth according to developmental time [15,16]. A study using fragile X mutant fly, which displays defects in the circadian output pathway and memory revealed elevated levels of Dilp2 in insulin-producing cells and elevated insulin signaling in fly brain, moreover, the reduction of the insulin pathway rescued circadian and memory defects in the fragile X mutant fly [17]. In addition, a study demonstrated an interaction between sleep regulation and the insulin-like peptide system in *D. melanogaster* [18], which supports the notion that circadian cycles are interconnected with metabolic cycles. However, the mechanisms relating the circadian cycle and the metabolic response still need to be clarified.

Drosophila is considered a valuable model for investigating sleep and its characteristics, as well as energy metabolism, since many of these characteristics are conserved between drosophila and mammals [19–22]. Thus, the aim of this study is to evaluate the relationship between sleep deprivation and changes in gene expression of circadian cycle and metabolic regulation in *Drosophila melanogaster* exposed to a sleep deprivation protocol through exposure to nocturnal light as an environmental stimulus.

Results

Sleep-deprived flies undergo changes in circadian cycle gene expression

The genes of circadian cycle regulation were assessed, and as expected, flies sleep deprived at night by light showed alterations in genes expression. The genes *pdf*, *cry*, C*lk*, *cyc* and *tim* had their expression significantly increased ($p \leq 0.05$). Similarly, the expression of *per* gene showed a significant increase ($p \leq 0.01$) in flies under nocturnal light. Otherwise, *dbt* gene was significantly decreased ($p \leq 0.05$) (Fig. 1), as compared to control group.

Sleep deprivation causes metabolism disruption.

The exposure of flies to constant light conditions during 24h was able to cause alterations in metabolic markers levels. Flies maintained under nocturnal light had a significant decreased in the glucose ($p \leq 0.001$), triglycerides ($p \leq 0.01$) and glycogen ($p \leq 0.001$) levels when compared to the control group (Fig. 2).

Sleep deprivation enhanced dopamine levels in Drosophila

The dopamine levels were assessed by HPLC. Flies maintained under light conditions had a significant increase in the dopamine levels ($p \leq 0.01$) when compared to the control group (Fig. 3).

Drosophila insulin-like peptides genes are modulated in sleep-deprived flies.

The exposure of flies to sleep deprivation by light was able to alter the expression of genes involved in insulin metabolism. qRT-PCR analysis revealed a significant increase in Dilp 2 ($p \leq 0.01$), 3 and 8 ($p \leq 0.05$) gene expression. Unlike, the Dilp 4, 6, and 7 genes showed a significant decrease ($p \leq 0.05$) in their expression, likewise, Dilp 5 also showed a decreased expression ($p \leq 0.01$) compared to the control group (Fig. 4).

Sleep deprivation operates in mTOR and dFOXO signaling induced by AKT phosphorylation in Drosophila

The *mTor* gene expression was increased ($p \leq 0.05$) in sleep deprived flies compared to the control group, on the other hand, *dFoxo* expression was decreased in flies sleep deprived (Fig. 5). According to this data, sleep deprived flies by nocturnal light showed an increased AKT phosphorylation (Fig. 6).

Discussion

Sleep is a complex biological process, and although its function is not yet well understood, it is involved in most of the body's regulatory processes, and many issues surrounding sleep and its mechanisms remain unclear. In a previous work we showed that exposure to flies at constant light for 24 hours was able to cause changes in sleep patterns, mitochondrial dysfunction, apoptosis and induced oxidative stress. Concomitant with these results, flies have been shown to undergo homeostatic sleep regulation in response to sleep deprivation. [23]. In the present study we evaluated the effect of sleep deprivation on the circadian cycle and metabolism regulation.

Sleep is regulated by two processes, a homeostatic mechanism that perceives and responds to lack of sleep, and a circadian clock that regulates sleep time [7,24]. Circadian clock regulation is based on two transcription–translation feedback loops, the *per / tim* loop and the *Clk* loop. The *per*/*tim* loop is required for the function of both loops. *Per* and *tim* genes transcription is started by regulatory complex CLK-CYC, before, PER and TIM proteins form heterodimers and accumulate in the cell nuclei repressing CLK-CYC transcriptional activity and thus suppressing their own transcription. Each loop interacts with diverse regulation factors that facilitate or delay the feedback [1].

Among these regulators, Doubletime (DBT) also known as Discs overgrown or DCO, acts in PER phosphorylation signaling to further degradation, preventing PER accumulation [25–27]. CLK is also destabilized, via DBT phosphorylation, and degraded, and CLK non-phosphorylated or hypophosphorylated accumulation leads to heterodimerization with CYC and another cycle of *per* and *tim* transcription [1]. Our results showed a decrease in *dbt* gene expression and *per*, *tim*, *Clk* and *cyc* overexpression in sleep deprived flies (Fig. 1. A-E). In our previous work, we

demonstrated increased Protein phosphatase 2A (*Pp2a*) gene expression [23]. PP2A is a cytoplasmic serine/threonine phosphatase that in circadian regulation acts on the regulation of PER through de-phosphorylation [1]. A study using Drosophila S2 cells showed that the reduction of PP2A activity reduced PER expression in central clock neurons, resulting in long periods and arrhythmia, moreover, the authors also showed that PP2A also affects the phosphorylation of PER *in vitro* and *in vivo*, suggesting that post-translational mechanisms driving PER cycling requires the rhythmic expression of PP2A [28]. Thereby, our results suggest that the *per* overexpression in sleep deprived flies is a reflection of stabilization by PP2A (suggested by the increase in mRNA levels) and of the *dbt* decreased expression, as an attempt to regulate the feedback loop by interrupting the unregulated transcription of *per* and *tim*.

The circadian oscillator maintains synchronization with environmental cycles to promote behavioral, physiological and metabolic regulation at appropriate times of day, however, this oscillator can be dragged by environmental cycles such as food, temperature and light. The light drags the oscillator by activating a photoreceptor, which is then able to alter a component of the circadian oscillator [1,29] .

In drosophila the cryptochrome photoreceptor (CRY) mediates light input to circadian oscillators in the brain and peripheral tissue and is responsible for light-induced TIM degradation[30,31]. In this work we observed that flies sleep deprived at night by light exposure had increased *cry* and *tim* mRNA expression (Fig 1. D and F), however, the levels of their respective proteins were not evaluated. Together to increase in *cry* expression, we also showed that flies sleep deprived had an increase in dopamine levels (Fig. 3). A study using Drosophila circadian clock mutant to exhibit nocturnal behavior reported that increased nighttime activity is mediated by high CRY levels,

moreover, the authors found that *cry* expression is also required for nocturnal activity in mutants that have high dopamine signaling [32].

Clock genes are the determinants of the temporary organization of homeostasis [33]. Here, we evaluated the clock genes expression and our data showed that exposure of drosophila to sleep deprivation caused an overexpression in *Clk*, *cyc*, *per*, *tim* and *cry* genes (Fig 1. A-D and F). A study that investigated the effects of protein rich and low-protein diets on lifespan of fruit flies overexpressing core clock genes, revealed that the overexpression of *Clk*, *per*, *tim* and *cry* genes decreased lifespan in females flies under protein rich diet [34]. Similar, transgenic drosophila overexpressing clock genes in the nervous system showed increase in stress-resistance when *cry*, *per* e *tim* were overexpressed, however, overexpression of *cry*, *clk*, *per* e *cyc* decreased thermoresistance. Moreover, the *cyc* overexpression decreased the lifespan in female flies [35].

The presence of oxidative stress in sleep deprived flies was shown in our previous work, together with apoptosis activation [23], thus, the overexpression in the clock genes observed here may be related with the previous results.

The fly brain consists of approximately 150 pacemaker neurons that form the central clock that regulate sleep / wake rhythms and respond to environmental cues such as the presence of light [36,37]. Pigment dispersing factor (PDF) is a neurotransmitter expressed in small ventral lateral neurons (sLNVs) and large ventral lateral neurons (lLNVs) [36,37]. The LNVs are involved in daytime sleep regulation by wake promoting, in part by PDF activity [18,38]. Our results showed a *pdf* overexpression mRNA level (Fig. 1. G) in flies sleep deprived, strengthening the role of PDF in wake promotion.

Several studies have shown a close relationship between circadian regulation, sleep time and metabolism [18,22,39–42], however many questions still need to be clarified, mainly about relationship between circadian cycle and metabolic regulation changes in sleep deprivation models. Here we show that exposure of flies to nocturnal light-induced sleep deprivation, in addition to altering the expression of genes involved in circadian control, also caused changes in glucose, triglyceride and glycogen levels. Flies sleep deprived had glucose and triglycerides levels decreased after 24h of light exposure (Fig. 2 A-B). Similarly, a study in rats submitted to the paradoxical sleep deprivation (PSD) protocol was able to induce basal hypoglycemia over all time periods evaluated, and was most pronounced at 96 and 192 hours of PSD. The same study also reported a decrease in the plasma triglycerides concentrations in all periods of PSD, which confirms the influence of sleep deprivation on metabolism [40].

Glycogen levels were also decreased after sleep deprivation in flies (Fig. 2. C). A study using drosophila eye absent (eya2) submitted of rest deprivation (RD) showed decreased brain glycogen levels after 3h of RD, and after 3 and 6h the glycogen levels were depleted in flies body [22]. The glycogen depletion in response to sleep deprivation has also been reported in brains of rats deprived of sleep for 12 or 24 hours, the levels were decreased to approximately 40%, and the sleep recovery during 15h after 12 hr of sleep deprivation was able to reverse the decreases in glycogen [43]. Benington and Heller, in 1995, postulated the sleep energy hypothesis, where they suggest that glycogen levels are reduced in response to wakefulness, which leads to a decrease in the ATP / AMP ratio, promoting adenosine accumulation, resulting in sleep promotion, which in turn restores glycogen levels, thus decreasing glycogen levels would be an impulse for sleep [42–44]. In this paper we show that sleep deprivation at night in light-exposed flies led to a significant decrease in glycogen levels, together

with these data, in a previous study we showed that sleep deprivation caused mitochondrial dysfunction leading to decreased oxidative phosphorylation (OXPHOS) ability and consequent decrease in ATP levels; this same study also showed that flies underwent a homeostatic regulation of sleep, restoring sleep levels over time [23]. Thus, altogether these data corroborate with the hypothesis of Benington and Heller.

Dopamine (DA) levels has been reported to be promoter of waking in Drosophila, where more DA leads to less sleep, and less DA leads to more sleep [45,46]. Moreover, studies have linked the DA levels with the stimulation of insulin secretion by glucose. An *in vitro* pancreatic islets study demonstrated that higher concentrations of dopamine (10^{-7}– 10^{-4} M) inhibited glucose-induced insulin secretion in the presence of both 4 mM and 20 mM glucose, suggesting that a low concentration of dopamine is necessary for the stimulation of insulin secretion by glucose [47]. Another study in Drosophila submitted to heat stress indicated that o increased to dopamine lead to decreased of glucose and trehalose levels but brings them to values close to normal following the stress exposure, suggesting the dopamine participation the regulation of carbohydrates metabolism, tending to normalize it after stress [48]. Our data show a significant increase of dopamine levels in sleep deprived flies (Fig 3), together with decreased at glucose levels, proposing that sleep deprivation lead to increased DA and consequently glucose levels reduction.

Insulin is the hormone responsible for regulating carbohydrate, fat and protein metabolism [14,49,50]. Insulin-like peptides are conserved proteins responsible for regulating metabolism, growth, reproduction, stress and longevity [15,51]. Drosophila have eight insulin-like peptides (DILPs) and two known receptors (dInR and Lgr3) [16] and although they are differently expressed they seem to perform redundant functions in the organism [15,16,52].

Dilp2,3 and *5* are produced by neurosecretory cells, the insulin producing cells (IPC) [16], in adult Drosophila, glucose regulates DILPs secretion through interaction with IPCs [50]. *Dilp 2* modulates longevity and carbohydrate metabolism, *dilp 5* mediates protein metabolism, while *dilp 3* is thought to regulate lipid metabolism. [53,54]. However, studies have shown compensatory regulation between *Dilps2*, *3* and *5* [55,56], as DILP2 knockdown leads to increased DILP3 and 5 levels [56]. DILP2 and 5 appear to play a role in glycogen regulation, flies lacking *dilp2* and *5* showed a reduction in glycogen levels, and loss of *dilp5* function also resulted in decreased glucose and triglyceride levels. [14,54]. Our results indicate *dilp2* and *3* overexpression, in contrast to a decrease in *dilp5* expression in flies sleep deprived at night (Fig 4 A, B and D), which may be related to the observed decrease in glucose, triglycerides and glycogen levels, and *dilp2* overexpression may be explained by the decrease in *dilp5*, since loss of *dilp5* increases the expression of *dilp2* [54].

Dilp2 and *5* are also involved in AKT phosphorylation [15,54], AKT phosphorylation negatively regulates forkhead Box O (FOXO) expression [15,51,54,57]. *dFoxo* is expressed in fat bodies and controls the expression of *dilp6* (like Insulin Growth Factor- IGF), which is involved in carbohydrate and lipid storage, oxidative stress resistance and longevity [51]. AKT also acts in Target of Rapamycin (TOR) [58,59]. The TOR inhibition by rapamycin in flies increased night sleep duration, but not affected daytime sleep, moreover, this study reported that day activity and night sleep were regulated through distinct mechanisms, day activity by the dFOXO, adipokinetic hormone, and octopaminergic signaling, whereas night sleep was mediated through TOR and dopaminergic signaling [60]. In our study, sleep deprived flies by nocturnal light showed *mTor* overexpression (Fig 5). Thus we suggest that the decrease in nocturnal sleep may be related to AKT phosphorylation, which in turn

promotes mTOR activation, acting on dopaminergic regulation, promoting increased night waking, however, dFOXO downregulation promotes daytime sleep, suggesting a role in homeostatic sleep regulation, according to the results observed in our previous study [23].

Dilp2 and *dilp6* play a reverse role in longevity. Flies lacking *dilp2* show increased longevity, while overexpression of *dilp6* provides similar effect [51,52,61]. However, increasing dilp2 leads to the decline of *dilp6*, as *dilp2* acts by phosphorylating AKT, which leads to a decrease in dFOXO signaling, inhibiting the expression of *dilp6*. Flies expressing *dilp6* show a reduction in both DILP2 transcripts and peptides [51]. Here we show that sleep-deprived flies overexpressed the *dilp2* gene (Fig. 4 A) together with increased AKT phosphorylation (Fig. 6 B). In addition, *dilp6* had reduced expression (Fig. 4 E), which could be interfering with longevity of sleep deprived flies. In our previous work, we showed that sleep deprivation was able to activate the apoptotic pathway of caspase 9 [23]. Together these results suggest the DILPs involvement in survival pathways as a result of depletion of energy reserves.

Furthermore, DILP6 modulates DA metabolism, a study with *dilp6* null mutant flies showed increased in alkaline phosphatase (ALP) and tyrosine hydroxylase (TH) activity [62]. The increase in DILP6 expression was also related to the increase in triglyceride, glycogen and trehalose levels [51]. In this study, *dilp6* mRNA levels were downregulated in flies exposed to light (Fig 4 E), possibly linked to increased dopamine levels and decrease in metabolic markers.

We also evaluated the *dilp4* and *7* (Fig. 4 C and F) expression and saw a decrease in both mRNA levels in sleep deprived flies. *Dilp4* expression was observed in embryonic mesoderm and larvae midgut [63], however adult expression of *dilp4* was not identified [52]. *Dilp7* was located in abdominal neuromeres in both embryonic and

adult drosophila stages, it is also expressed in populations of ventral cord neurons and various neurons in the brain [63,64]. Despite that, the role of these DILPs are not yet well established.

Dilp8 was identified more recently, and interacts with a single G protein coupled receptor (GPCR), Lgr3 relaxin-like. *Dilp8* expression was primarily observed in larvae imaginal discs, being involved with the timing control of the metamorphosis onset and bilateral development [65,66]. Our results showed an increased in *dilp8* mRNA levels in flies sleep deprived at night (Fig 4 G). In the larvae, *dilp8* overexpression led to downregulation of insulin signaling in imaginal discs and a reduction in growth rate [65,66]. However, there is no literature information about DILP8 in adult flies.

The relationship between sleep regulation and insulin-like peptides was demonstrated in drosophila mutants to the seven DILPs genes and the DInR receptor. Flies with lack to all DILPs and the insulin receptor showed a reduction in total sleep time, except in DILP4 mutant. Moreover, this study identified the DILP expression in LNd and LNv clock neurons, demonstrating that upregulated *dilp2* in *pdf* neurons significantly increased the total sleep only during the daytime, suggesting a role of DILPs in sleep regulation system [18].

Here, we demonstrated that sleep deprivation by exposing drosophila to nocturnal light was able to increase expression of main genes of circadian clock control. Moreover, the expression of genes involved in metabolism were affected in parallel to lower levels of glucose, triglycerides and glycogen, reinforcing the importance of sleep in metabolic regulation. Therefore, we suggest (Scheme 1) that sleep deprivation flies by nocturnal light exposure was able to change circadian clock genes expression and modulates the DILPs genes expression. These changes leading to decreased of glucose, triglycerides and glycogen levels, since DILP2, 5 and 3 were related to modulate this

response [14,53,54]. In special, the *dilp2* overexpression may be involved with the AKT phospholylation, that in turn can be acting on mTOR and dFOXO signaling [15,51,54,58]. DILP6 expression is under dFOXO control and has been related to dopaminergic regulation [62]. So, the decrease in *dilp6* expression possibly related to the increase in dopamine levels, consequently due to the *mTor* overexpression. In turn, the increase in dopamine levels may be favors the decrease in glucose, triglyceride and glycogen levels, since this response was showed to dopamine [47,48]. The mTOR and dFOXO signaling pathways are related to sleep/wake cycle according to period of day [60], thus, the modulation of *mTor* and *dFoxo* expression can be suggest a relationship with the *pdf* overexpression, since this is thought to promote wakefulness [36,37,67].

Taken together our results emphasize the relationship between sleep deprivation, circadian rhythms and metabolism disruption, highlighting the importance of sleep in health, demonstrating that even short-term sleep deprivation is capable of causing harmful effects to organisms. In addition, this study reinforces the use of *Drosophila melanogaster* as a model organism for sleep and metabolic regulation investigations.

Methods

Fly stock and Treatments

D. melanogaster (Oregon strain) was obtained from the National Species Stock Center, Bowling Green, OH, USA. The flies were maintained in incubators at 25± 1°C, 12 h dark-light photoperiod and 60–70% relative humidity with free access to food as previously described [68]. For the experiments, female flies (1-4 days-old) were placed in tubes containing agar meal during 24h under controlled conditions of light as follows, control group (12h light/12h dark cycle), and light group (12h light/12h light cycle), both at 25± 1°C. After treatments period, always finished at 12:00 pm, the flies were submitted to biochemical and molecular analysis.

Quantitative real-time qRT-PCR and gene expression analysis

Approximately 1 µg of total RNA from 20 flies was extracted using the Trizol Reagent (Invitrogen™) according to the manufacturer's suggested protocol. After quantification, total RNA was treated with DNase I (DNAse I Amplification Grade – Invitrogen™, NY) and cDNA was synthesized with iScript cDNA Synthesis Kit and random primers again according to the manufacturer's suggested protocol (BIORAD). The primers were picked by NCBI Primer-Blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) and acquired from Invitrogen™, NY. Quantitative real-time polymerase chain reaction was performed in 10 µL reaction volumes containing water treated with diethyl pyrocarbonate (DEPC), 200 ng of each primer and 0,2 X SYBR Green I (molecular probes) using a 7500 real time PCR system (Applied Biosystems, NY). The qPCR protocol was the following: activation of the reaction at 50 °C for 2 min, 95 °C for 2

min, followed by 40 cycles of 15 s at 95 °C, 60 s at 60 °C, and 30 s at 72 °C. All samples were analyzed as technical and biological triplicates with a negative control. Threshold and baselines were automatically determined, SYBR fluorescence was analyzed by 7500 software version 2.0.6 (Applied Biosystems, NY), and the CT (cycle threshold) value for each sample was calculated and reported using the $2^{-\Delta\Delta CT}$ method [69]. The GPDH gene was used as endogenous reference genes presenting no alteration in response to the treatment. For each well, analyzed in quadruplicates, a ΔCT value was obtained by subtracting the GPDH CT value from the CT value of the interest gene (Table 1.). The ΔCT mean value obtained from the control group of each gene was used to calculate the ΔΔCT of the respective gene ($2^{-\Delta\Delta CT}$).

Metabolic Markers

Energetic metabolism of Drosophila was determined by colorimetric kits. Twenty flies were weighted and homogenized in 1000 µL of 20 mM HEPES buffer, pH 7.0, and centrifuged at 14,000 rpm for 30 min at 4 °C. The supernatant was used for analysis of glucose, glycogen and triglycerides levels accordingly to the manufacturer's suggested protocol (Labtest®, MG) with minor modifications. The results were calculated as a percentage in relation to the control group and values were normalized by weight of each treatment group.

Dopamine quantification

Dopamine levels were determined using high performance liquid chromatography (HPLC), as described previously with some modifications [70]. The HPLC apparatus consisted of a Shimadzu Prominence UFLC high performance chromatographic equipped with a LC-6AD bomb and SPD-20AV UV detector. The software LC Solution was applied for analyzing retention time, chromatograms, and evaluated peak area. A Kromasil® C18 reversed-phase column (250mm× 4.6mm, d.i.; 5 µm) was used at ambient temperature. Twenty female flies were homogenized in a PowerLyser® with 100 µL of 0.1M $HClO_4$ and centrifuged at 1000 rpm for 5 min at 4°C. The supernatant was transferred for a new tube and 100 µL of sodium phosphate buffer (0.1 M, pH 7.4) containing 1.0 mM ethylenediamine tetraacetic acid (EDTA), followed by centrifugation at 3000 rpm for 5 min at 4 °C. Then, samples were filtered, transferred for a new tube, and an additional centrifugation was performed at 10000 rpm for 10 min at 4 °C. The supernatant was injected into the HPLC. The mobile phase consisted of a mixture of 0.2% TFA and methanol with a ratio of 80:20 (v/v). The flow rate was 1.0 mL/min and the retention time was 6.580 min. Results were expressed as µM (mean ± SEM) and the dopamine levels were assayed by comparison with standard curve.

Western Blotting

Western blotting was performed according to Martins et, al. (2018) with minor modifications [71]. Briefly, the flies were homogenized at 4°C in Tris NaF buffer pH 7.0 (50 mM Tris, 1 mM EDTA, 0.1 mM phenyl methyl sulfonyl fluoride, 20 mM Na_3VO_4, 100 mM sodium fluoride and protease inhibitor cocktail). The homogenate was then centrifuged (1.000 g) for 10 min at 4°C, the supernatants was collected and 10

μL of sample was taken out for protein analysis. The remaining sample was added to 4% SDS stop solution (50 mM Tris, 100 mM EDTA, pH 6.8), 25% glycerol sample (40% glycerol, 25 mM Tris and bromophenol blue, pH 6.8) and then β-mercaptoethanol was added to samples to a final concentration of 8%. Subsequently, samples were frozen at −80°C for further analysis. The proteins (30 μg per well) were separated by SDS-PAGE using 15% gels and electrotransferred to nitrocellulose membranes for approximately 3 hours using a GE Health Care TE22 Mini Tank Transfer System at 4°C. The membranes were blocked with 5% skimmed milk for 1 hour. Then, membranes were washed three times in Tris-buffered saline with Tween (TBS-T) containing 100 mM Tris–HCl, 0.9% NaCl, and 0.1% Tween-20, pH 7.5 and incubated overnight (4°C) with primary antibodie anti-rabbit phospho AKT (1:1000) and anti β-Actin HRP conjugated (1:10.000). Finally, membranes were incubated with specific secondary antibodies anti-rabbit IgG alkaline phosphatase (1:6.000) during 1 h at room temperature (except for β-Actin HPR conjugated). The immunoblots were visualized on a Bruker IS4000MM Pro imaging system using ECL Western Blotting substrate Kit (Promega) and BCIP/NBT phosphatase substrate. The densitometric analysis of immunoreactive bands was performed using Scion Image® software. The density of the bands was measured and expressed as a rate (%) of increase in relation to control (slices treated only with media).

Statistical Analysis

Statistical analysis was performed using unpaired *t*-test. Differences were considered statistically significant between groups when $p \leq 0.05$.

Conflict of interest

Authors declare no conflict of interest.

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Legends

Figure 1. Effects of sleep deprivation on flies' circadian clock gene expression. Gene expression was evaluated by qRT-PCR. **(A)** *Clk*; **(B)** *cyc*; **(C)** *per*; **(D)** *tim*; **(E)** *dbt*; **(F)** *cry*, **(G)** *pdf*. Results are shown as fold increases/decreases as compared to control (considered as 1) (mean ± SEM); a total of 6 biological replicates per group (n= 6), were used; *$p \leq 0.05$, **$p \leq 0.01$ compared to control.

Figure 2. Effects of sleep deprivation on flies' metabolic markers. **(A)** Glucose, **(B)** Triglycerides, **(C)** Glycogen. Results are expressed as percentage of control (mean ± SEM); a total of 6 biological replicates per group (n= 6), were used; **$p < 0.01$, ***$p < 0.001$ compared to control.

Figure 3. Effects of sleep deprivation on flies' dopamine levels. Dopamine assay was performed by HPLC. Results are expressed in µM of dopamine (mean ± SEM); a total of 5 biological replicates per group (n= 5); were used *** $p < 0.001$ compared to the control.

Figure 4. Effects of sleep deprivation on flies' insulin-like peptides gene expression. Gene expression was evaluated by qRT-PCR. **(A)** *dilp2*; **(B)** *dilp3*, **(C)** *dilp4*, **(D)** *dilp5*, **(E)** *dilp6*, **(F)** *dilp7*, **(G)** *dilp8*. Results are shown as fold increases/decreases as compared to control (considered as 1) (mean ± SEM); among 3-6 biological replicates per group (n=3 to 6) were used; *$p \leq 0.05$, **$p \leq 0.01$ compared to control.

Figure 5. Effects of sleep deprivation on flies' target of rapamycin (*mTor*) and forkhead Box O (*dFoxo*) gene expression. **(A)** *mTor*, **(B)** *dFoxo*. Gene expression was evaluated by qRT-PCR. Results are shown as fold increases/decreases as compared to control (considered as 1) (mean ± SEM); a total of 6 biological replicates per group (n= 6), were used; *$p \leq 0.05$, **$p \leq 0.01$ compared to control.

Figure 6. Effects of sleep deprivation on flies' AKT phosphorylation. AKT phosphorylation was performed by Western Blotting analysis. **(A)** AKT phosphorylation levels were normalized by β-actin and results compared with control group. **(B)** Densitometric quantification of immunoreactive bands. Results are expressed as percentage of control (mean ± SEM); a total of 4 biological replicates per group (n= 4) were used; * $p < 0.005$ compared to the control.

Scheme 1. Authors suggestions on potential mechanisms involved in *D. melanogaster* sleep deprivation by nocturnal light exposure. The exposure of flies to nocturnal light was able to change circadian clock genes expression and modulate the DILPs gene expression. These changes lead to decreased glucose, triglycerides and glycogen levels. Particularly, the *dilp2* overexpression might be related to the observed AKT phospholylation that acts on mTor and dFoxo signaling. *Dilp6* expression is under *dFoxo* regulation, and its decrease might be related to the increased dopamine levels, possibly due to the increase in *mTor*. In turn, the increase in dopamine levels favors the decrease in glucose, triglyceride and glycogen levels. The mTOR and dFOXO signaling pathways are related to sleep/wake cycle according to the period of the day, which

suggest a relationship with the *pdf* overexpression, since this is thought to promote wakefulness.

Table 1. Genes tested by quantitative real-time RT-PCR analysis and used forward and reverse primers

Gene	Primer Sequences	
Gpdh	Forward	5'ATGGAGATGATTCGCTTCGT
	Reverse	5'GCTCCTCAATGGTTTTTCCA
Clk	Forward	5'GTGGGCTACTTTCGTAACG
	Reverse	5'CGCAGGTTGTCCATTTGAG
cyc	Forward	5' GAGCAGCTATCCTCACTGG
	Reverse	5' AACATGGTCTTCGCATCGA
per	Forward	5' ACAACAAGAAATACACGGACAG
	Reverse	5' ATGAAGGACGAGTAGAAGGAG
tim	Forward	5' ATGAAGATGAGGACGAAGTG
	Reverse	5' GGTTGTTGTGTTAAGTTTAGGG
dbt	Forward	5' ACAAAGCCATAAGCGACTG
	Reverse	5' GATTTGCGTTGCCTTTCTG
cry	Forward	5'CTTCTGTTGGATGAGCGTCTTA
	Reverse	5' TTGGCGAGTCGTGGATATTG
pdf	Forward	5' CCACTCTCTGTCGCTATCCG
	Reverse	5' ATGAAGGACGAGTAGAAGGAG
dilp2	Forward	5'ATCCCGTGATTCCACACAAG
	Reverse	5'GCGGTTCCGATATCGAGTTA
dilp3	Forward	5'CCGAAACTCTCTCCAAGCTC
	Reverse	5'GCCATCGATCTGATTGAAGTT
dilp4	Forward	5'GGCACTGGATGTGATTTGTG
	Reverse	5'CGTTTCCTGTTCAATGTCCTC
dilp5	Forward	5'GCCTTGATGGACATGCTGA
	Reverse	5'CATAATCGAATAGGCCCAAGG
dilp6	Forward	5'CCCTTGGCGATGTATTTCC
	Reverse	5'CACAAATCGGTTACGTTCTGC
dilp7	Forward	5'AAGAAGACAACCACGGAACC
	Reverse	5'CGCAGAAACATGTTGGCATA
dilp8	Forward	5'AACGCCACTAAAATGAGTTCAAA
	Reverse	5'GCAACAGACTCCGATGACC
mTor	Forward	5'GGTGAACACATTGCTGTTGG
	Reverse	5'CCTTCTTCTTGTCGCGGTAG

Figure 1

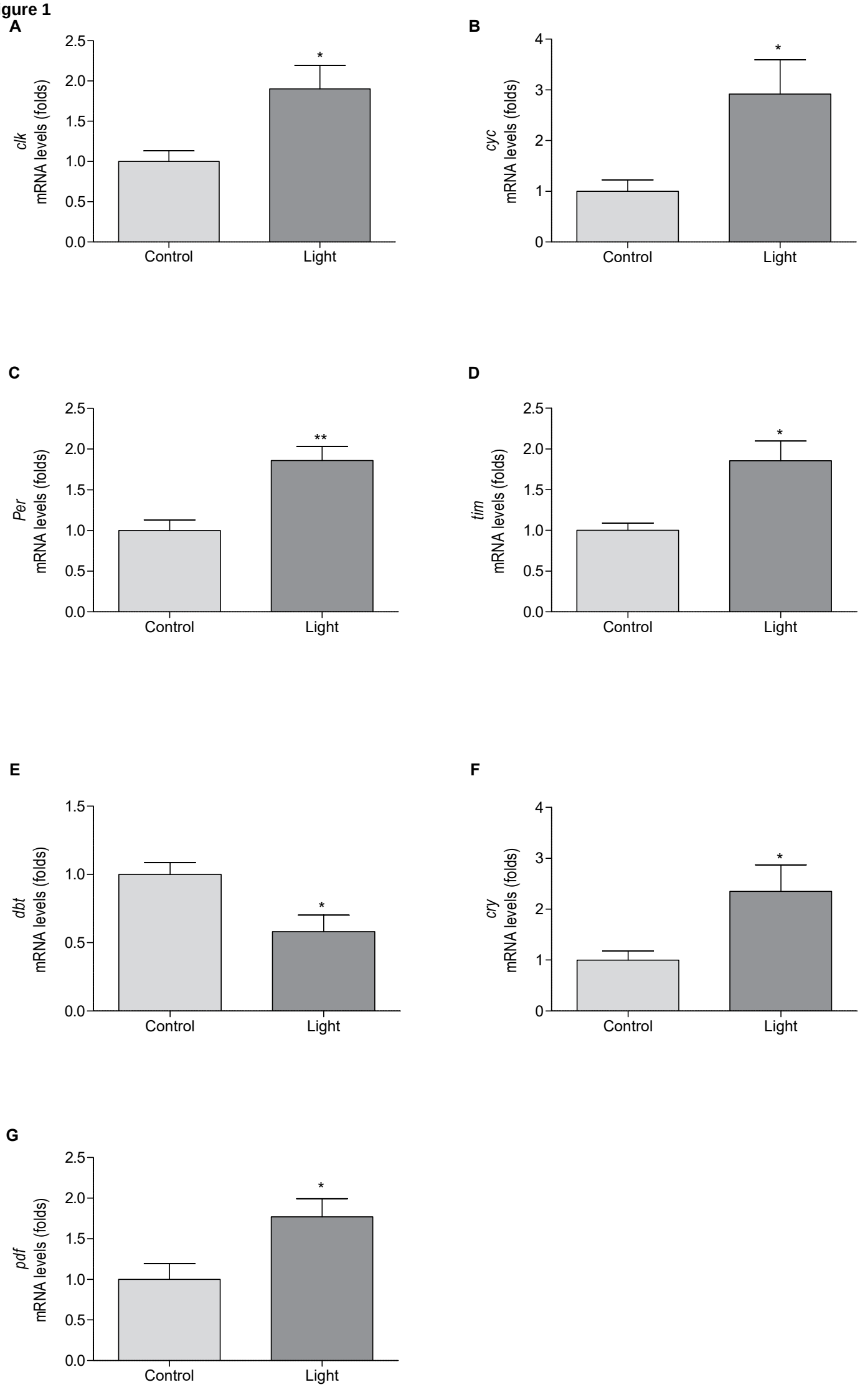

A
clk mRNA levels (folds)
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
Light
B
cyc mRNA levels (folds)
4
3
2
1
0
*
Control
Light
C
Per mRNA levels (folds)
2.5
2.0
1.5
1.0
0.5
0.0
**
Control
Light
D
tim mRNA levels (folds)
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
Light
E
dbt mRNA levels (folds)
1.5
1.0
0.5
0.0
*
Control
Light
F
cry mRNA levels (folds)
4
3
2
1
0
*
Control
Light
G
pdf mRNA levels (folds)
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
Light

Figure 2

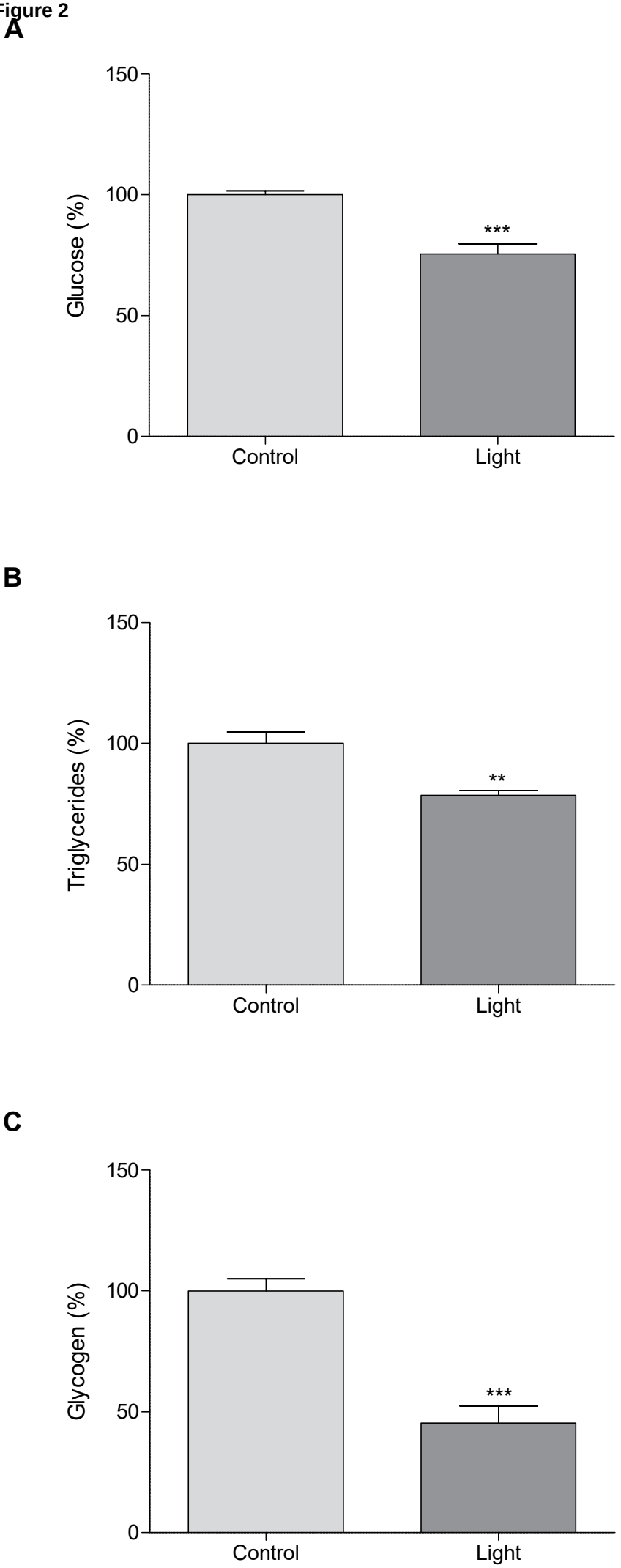

A
150
100
50
0
Glucose (%)
***
Control
Light
B
150
100
50
0
Triglycerides (%)
**
Control
Light
C
150
100
50
0
Glycogen (%)
***
Control
Light

Figure 3

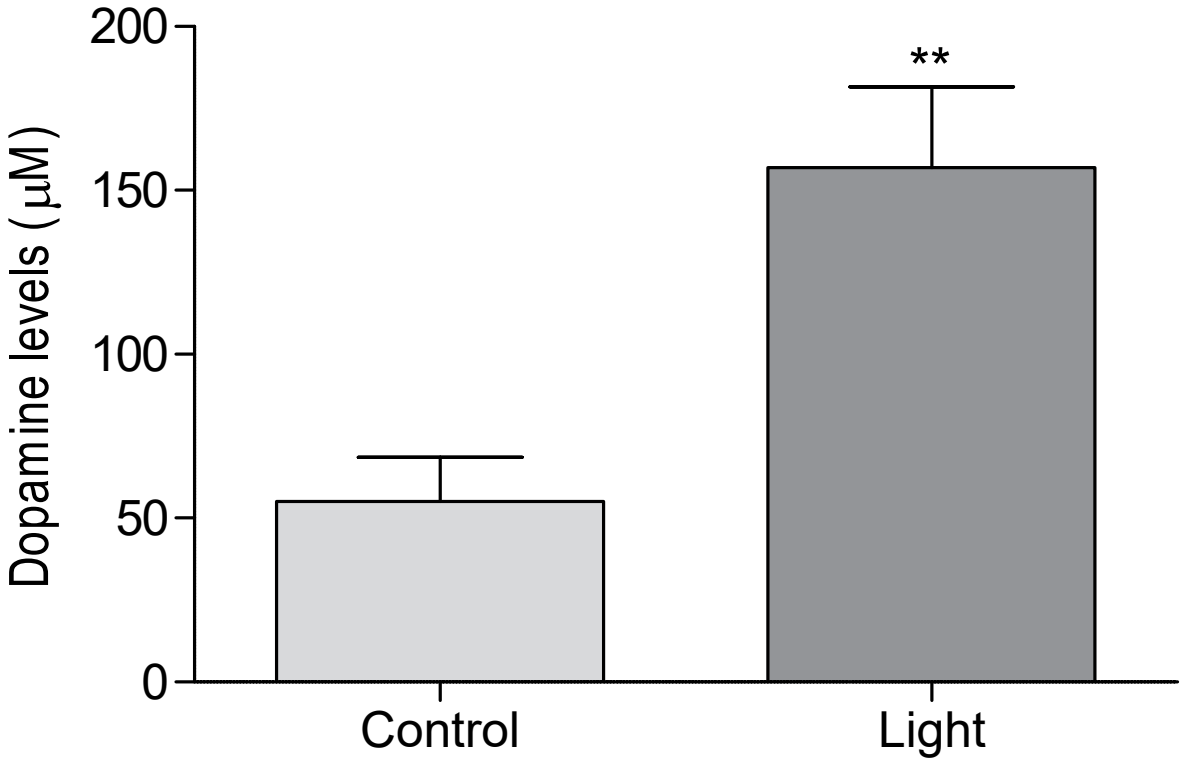


Figure 4

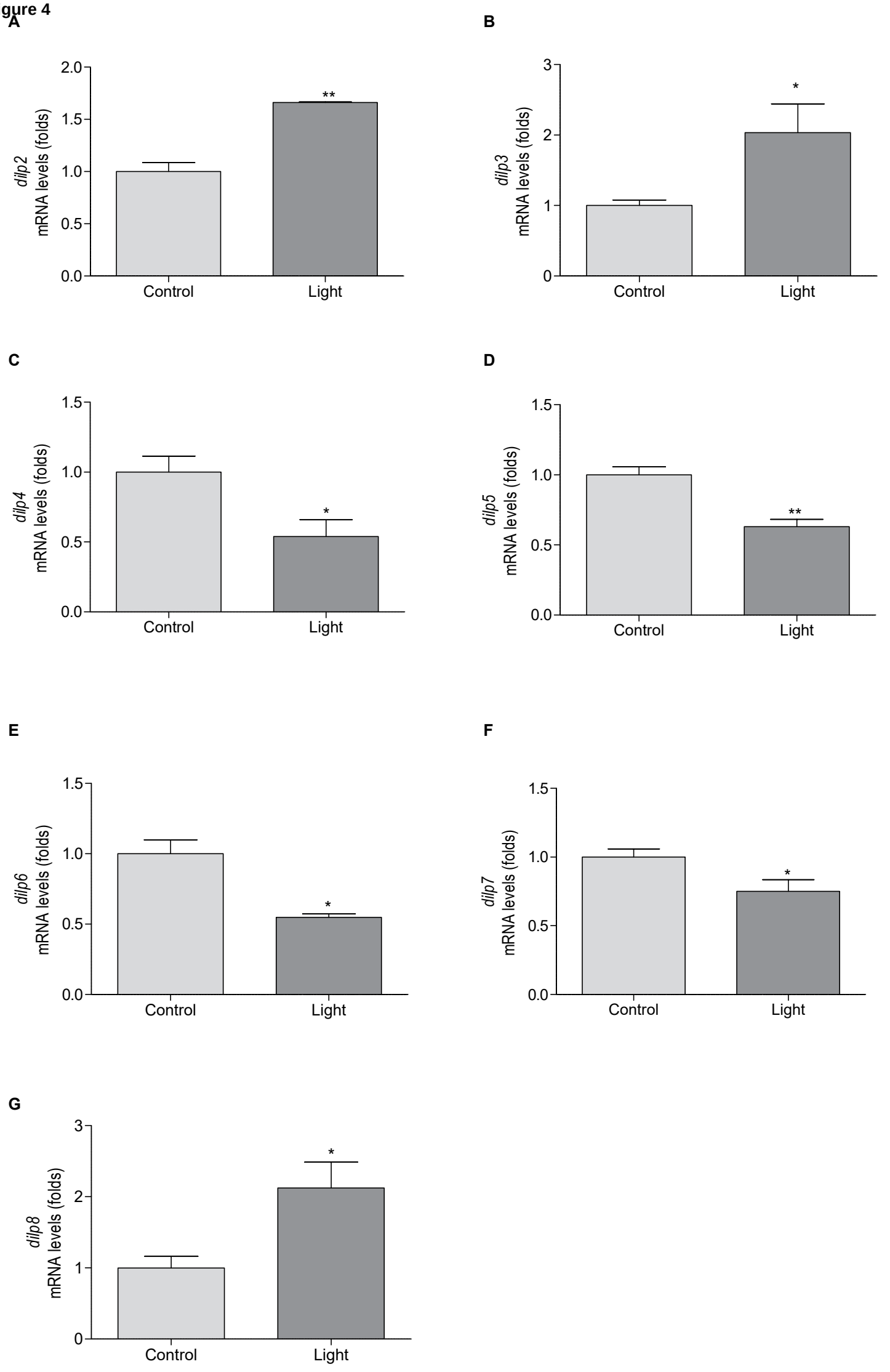

A
dilp2 mRNA levels (folds)
2.0
1.5
1.0
0.5
0.0
**
Control
Light
B
dilp3 mRNA levels (folds)
3
2
1
0
*
Control
Light
C
dilp4 mRNA levels (folds)
1.5
1.0
0.5
0.0
*
Control
Light
D
dilp5 mRNA levels (folds)
1.5
1.0
0.5
0.0
**
Control
Light
E
dilp6 mRNA levels (folds)
1.5
1.0
0.5
0.0
*
Control
Light
F
dilp7 mRNA levels (folds)
1.5
1.0
0.5
0.0
*
Control
Light
G
dilp8 mRNA levels (folds)
3
2
1
0
*
Control
Light

Figure 5

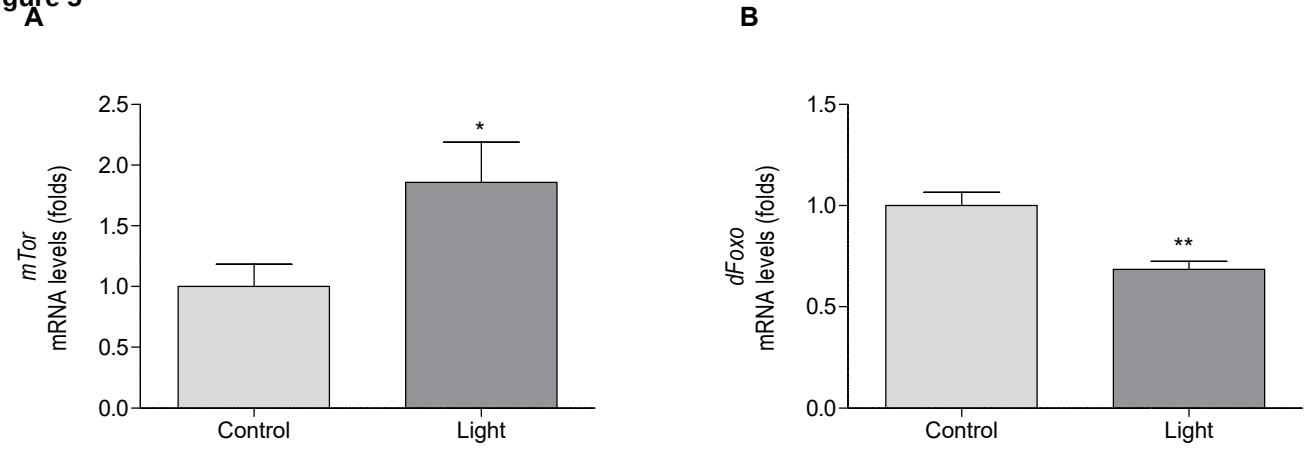
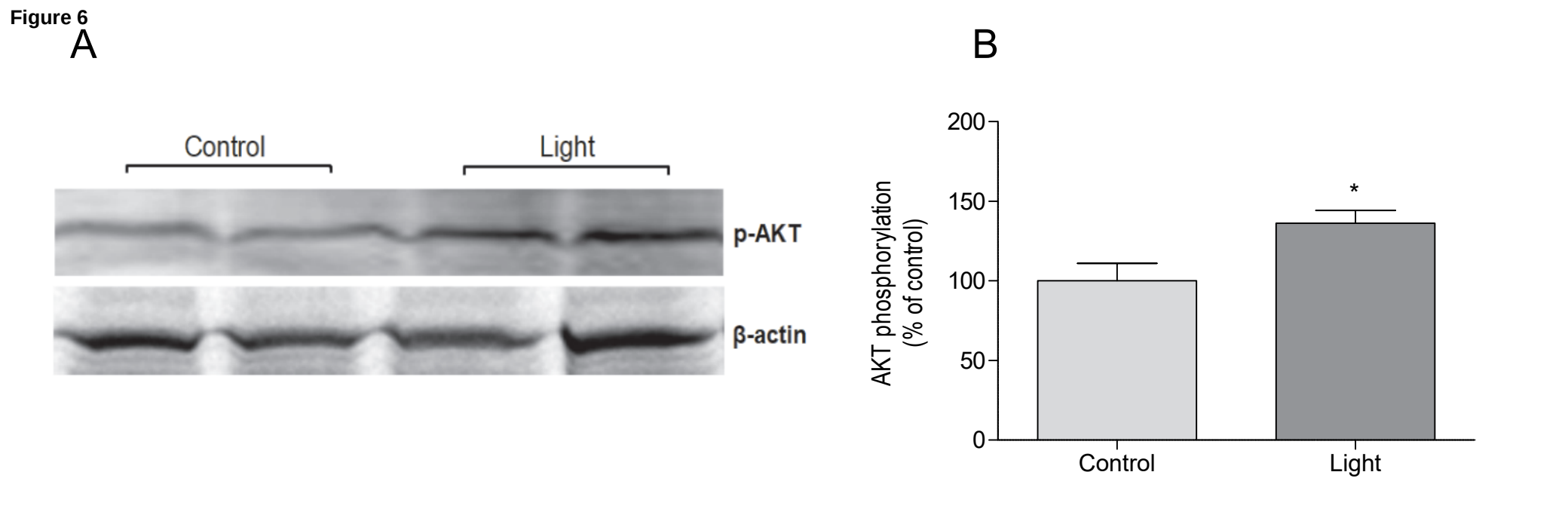
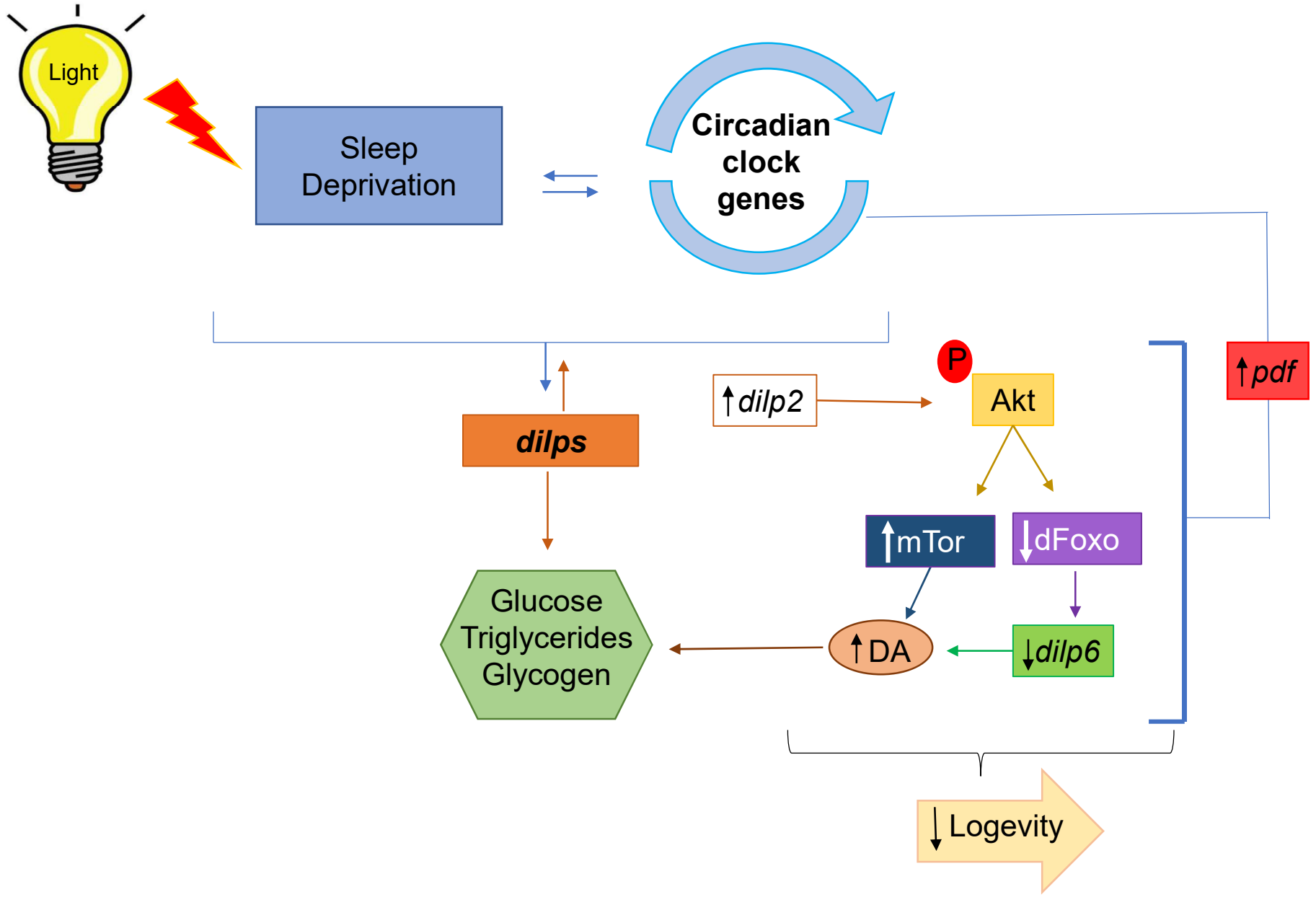

A
mTor
mRNA levels (folds)
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
Light
B
dFoxo
mRNA levels (folds)
1.5
1.0
0.5
0.0
**
Control
Light

Figure 6


A
Control
Light
p-AKT
β-actin
B
AKT phosphorylation
(% of control)
200
150
100
50
0
*
Control
Light

Scheme 1


Light
Sleep Deprivation
Circadian clock genes
dilps
↑dilp2
P
Akt
↑pdf
↑mTor
↓dFoxo
Glucose
Triglycerides
Glycogen
↑DA
↓dilp6
↓Logevity

3. DISCUSSÃO

Os resultados apresentados neste trabalho demonstram que a privação do sono pela exposição das moscas à luz constante foi capaz de alterar o ciclo sono/vigília e afetar a atividade locomotora, caracterizando uma regulação homeostática. Semelhante aos nossos resultados, *Drosophila melanogaster* submetidas a um protocolo de privação do sono, apresentaram alteração nos padrões de sono e diminuição no desempenho, afetando a duração e intensidade do sono após uma privação de 24h. A recuperação do sono foi menos fragmentada e com episódios de sono mais longos. E este estudo também mostrou que a resposta homeostática do sono é um fenótipo bem conservado entre mais de 100 cepas de *Drosophila* do tipo selvagem (HUBER et al., 2004).

As moscas privadas do sono pela exposição à luz apresentaram diminuição da atividade mitocondrial, aumento da peroxidação lipídica e na produção de espécies reativas de oxigênio, bem como a modulação de enzimas antioxidantes, caracterizando o dano oxidativo. Em um estudo realizado em ratos demonstrou que a exposição a padrões de luz permanentes foi capaz de causar lipoperoxidação e afetar a atividade de enzimas antioxidantes, sugerindo que a exposição à luz permanente é capaz de causar danos oxidativos (ESCRIBANO et al., 2014). Em *Drosophila melanogaster* a regulação circadiana foi demonstrada para estar envolvida na resposta ao estresse oxidativo, moscas mutantes nulo para o gene do relógio *period (per)* apresentaram um aumento na susceptibilidade ao peróxido de hidrogênio em comparação com moscas selvagens, coincidindo com o aumento da geração de H_2O_2 mitocondrial e diminuição na atividade da catalase devido a danos oxidativos (KRISHNAN; DAVIS; GIEBULTOWICZ, 2008).

Estudos em camundongos demonstram que a privação do sono foi capaz de prejudicar o funcionamento da cadeia de transporte de elétrons, levando a uma produção excessiva de espécies reativas de oxigênio, provocando danos oxidativos e ativando as vias reguladoras da apoptose (ANDREAZZA et al., 2010). De maneira similar, a diminuição nos níveis de glicose, triglicerídeos e glicogênio foram observados em ratos privados do sono, acarretando na diminuição da produção de ATP (BRIANZA-PADILLA et al., 2015; ZIMMERMAN et al., 2004, KONG et al., 2018). Neste trabalho, a privação do sono de *Drosophila* pela

exposição a luz constante provocou diminuição nos níveis de glicose, triglicerídeos e glicogênio, a atividade mitocondrial também foi afetada, consequentemente causando a diminuição nos níveis de ATP. A disfunção mitocondrial observada também pode estar relacionada com o dano oxidativo e ativação da via apoptótica, observada através do aumento da atividade das caspases 3/7.

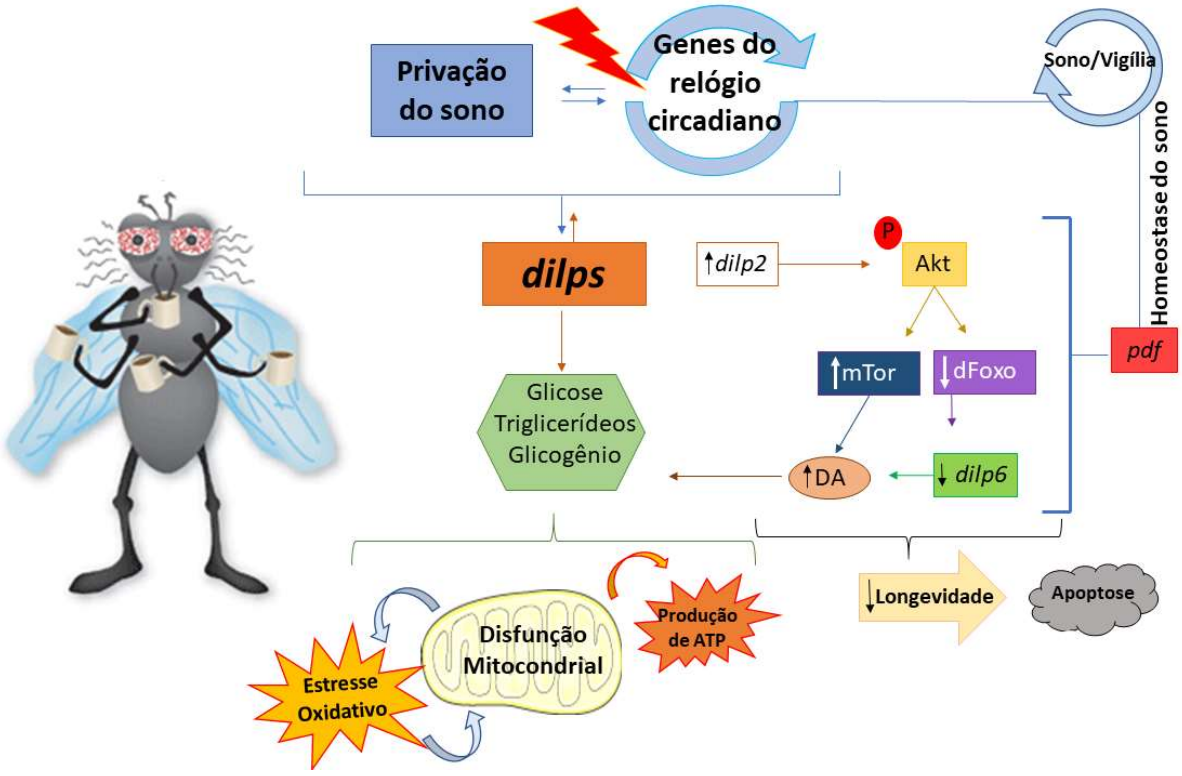
A privação do sono nas moscas foi capaz de modular a expressão dos genes *Nrf2*, *p38*β, *Akt, pale*, *hsp83* e *Pp2a,* envolvidos na via do estresse oxidativo, bem como dos genes envolvidos na regulação circadiana, *Clk*, *cyc*, *per*, *cry* e *pdf.* Vários estudos relatam a relação entre privação do sono e estresse oxidativo, bem como a influência dos ritmos circadianos na regulação do metabolismo oxidativo (KRISHNAN; DAVIS; GIEBULTOWICZ, 2008; SHAW et al., 2002). Três grupos de genes foram relatados como envolvidos na vigília e privação de sono a curto prazo em várias espécies, entre eles estão incluídos os genes que codificam as proteínas chaperonas de choque térmico e os genes envolvidos na resposta ao estresse celular (CIRELLI, 2006).

Vários estudos mostraram uma estreita relação entre regulação circadiana, tempo de sono e metabolismo (BRIANZA-PADILLA et al., 2015; CONG et al., 2015; SPIEGEL et al., 2011; ZIMMERMAN et al., 2004), porém muitas questões ainda precisam ser esclarecidas, principalmente sobre a relação entre o ciclo circadiano e a regulação metabólica nos modelos de privação de sono. As alterações metabólicas observadas nas moscas privadas do sono neste estudo podem estar relacionadas com a modulação dos genes dilps, que por sua vez interagem com os genes mtor e dfoxo, envolvidos na regulação do ciclo sono/vigília. Concomitantemente, a dopamina (DA), relatada por ser um promotor da vigília em Drosophila (ANDRETIC; VAN SWINDEREN; GREENSPAN, 2005; VAN SWINDEREN; ANDRETIC, 2011) e modulada pelo DILP6 (RAUSCHENBACH et al., 2017), apresentou níveis elevados nas moscas privadas do sono por exposição constante à luz. Além disso, estudos associaram os níveis de dopamina à estimulação da secreção de insulina pela glicose (SHANKAR; SANTHOSH; PAULOSE, 2006).

De uma maneira geral, a privação do sono causa alterações nos genes do ciclo circadiano e genes *dilp*. DILP2 e DILP6 estão relacionados na diminuição da longevidade, sugerida neste trabalho pela ativação da via apoptótica. A sinalização mTOR- dFOXO está relacionada com a regulação do ciclo sono/vigília através do controle da atividade de vigília por DA e PDF. A DA também está relacionada com a diminuição nos níveis de glicose,

triglicerídeos e glicogênio. Que por sua vez podem estar envolvidos na disfunção mitocondrial, através da diminuição nos níveis de ATP, acarretando no aumento de EROS e promoção do estresse oxidativo **(Fig. 9)**.

Figura 9. Resumo gráfico. Esquema representativo dos resultados.



Fonte: Autor

4. CONCLUSÕES

A exposição de *Drosophila melanogaster* à luz noturna causou:

- ✓ Déficit locomotor e alteração nos padrões de sono caracterizando uma regulação homeostática;
- ✓ Os níveis de marcadores metabólicos foram diminuídos;
- ✓ A viabilidade mitocondrial foi afetada em conjunto com a disfunção mitocondrial;
- ✓ Aumento na produção de ERO e peroxidação lipídica, em conjunto com a alteração da atividade de enzimas antioxidantes, caracterizando o estresse oxidativo;
- ✓ Aumento nos níveis de dopamina;
- ✓ Modulação de genes envolvidos na via do estresse oxidativo, controle circadiano e metabólico;
- ✓ Ativação da via apoptótica.

Tomados em conjunto, nossos resultados mostraram claramente que a privação de sono, mesmo por um curto período de tempo, é capaz de causar efeitos deletérios ao organismo, indicando uma estreita relação entre privação do sono, controle circadiano, estresse oxidativo e regulação metabólica. Além de reforçar o uso de *Drosophila melanogaster* como um organismo modelo para investigações de regulação do sono e metabolismo.

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ANEXO A- MATERIAL SUPLEMENTAR- ARTIGO

Short-term sleep deprivation with exposure to nocturnal light alters mitochondrial bioenergetics in Drosophila

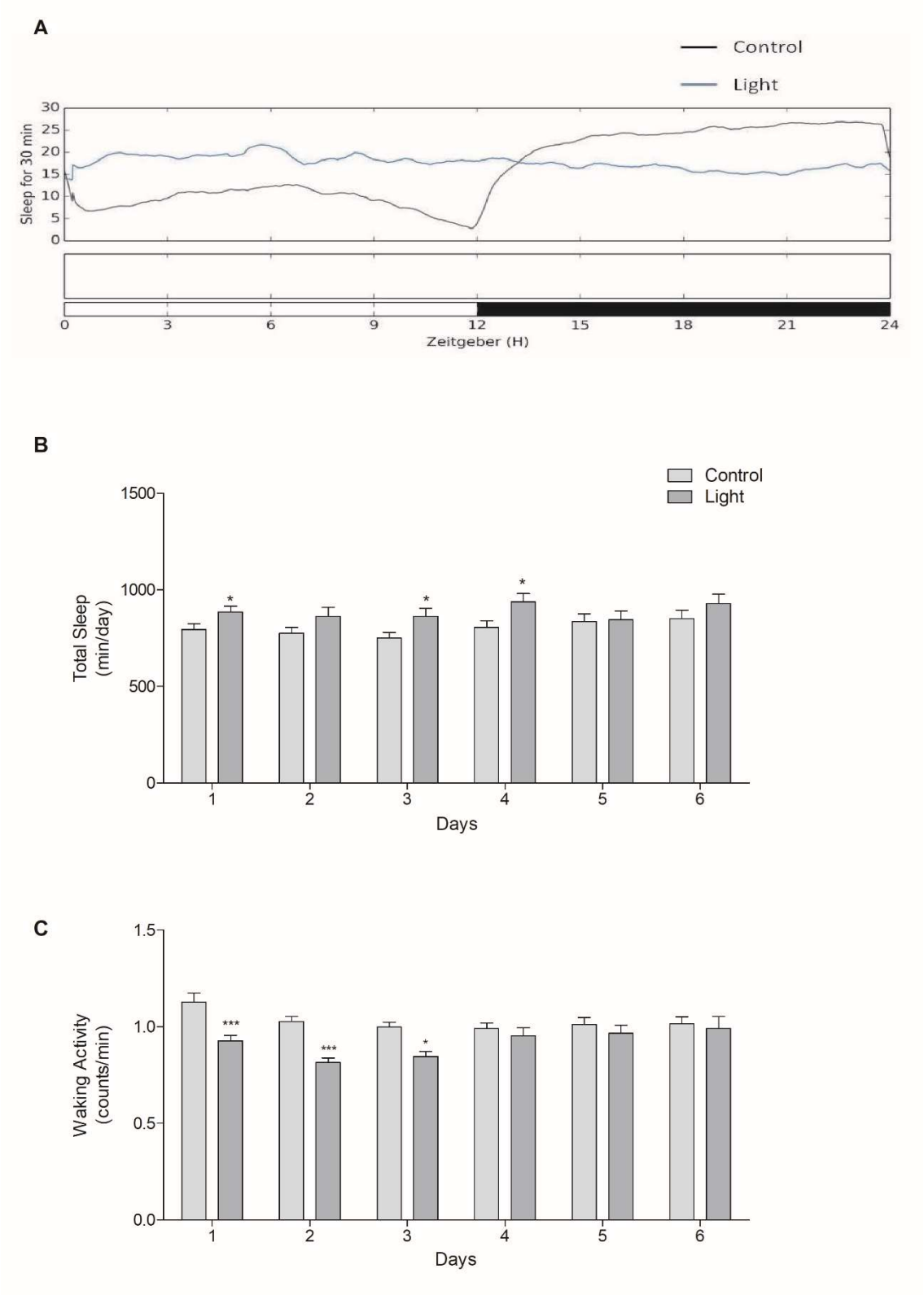
Figure legends

Figure S1. Effect of exposure to constant light during six days on the sleep patterns of flies. **(A)** pySolo analysis of daily average sleep for 30 min, dark line= control group and blue line= Light group. **(B)** Total sleep for six 917 days. **(C)** Waking activity for six days. Results are presented as means ± S.E.M; a total of sixty-four individual flies per group (n=64), in duplicates were used; *$p \leq 0.05$, ***$p \leq 0.001$ compared to control.

Figure S2. Sleep patterns of flies exposed to a mechanically sleep deprivation protocol. **(A)** pySolo analysis of daily average sleep for 30 min, dark line= control group and blue line= Shake group **(B)** Daily Sleep Bout, **(C)** Sleep Bout Duration, **(D)** Sleep Fragmentation. Results are expressed with mean ± SEM, a total of sixty-four individual flies per group (n=64), in duplicates were used; *$p \leq 0.05$; ***$p \leq 0.001$ compared to control group.

Figure S3. Effects of sleep deprivation on the flies respiratory mitochondrial function determined by HRR. **(A)** O2 flux measured in the mitochondria of *D. melanogaster* exposed to different sleep deprivation protocols for 24h. Mitochondrial function is presented with the abbreviation(s) of the complex(es) involved followed by the state of respiration measured in the presence of L-proline + pyruvate + malate (CILEAK), + ADP (CIOXPHOS), + cytochrome c (CIcOXPHOS), + succinate (CIc + CIIOXPHOS), + FCCP (CIc + CIIETS), + rotenone (CIIETS), + antimycin A (ROX) + TMPD/ascorbate (CIV). **(B)** Analysis of bioenergetics capacity. Results are presented as means ± S.E.M, a total of 6 independent mitochondrial preparations (50 flies each) were used per group (n=6); *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ compared to control.

Fig. S1.


A
Control
Light
Sleep for 30 min
30
25
20
15
10
5
0
0
3
6
9
12
15
18
21
24
Zeitgeber (H)
B
Control
Light
Total Sleep (min/day)
1500
1000
500
0
*
*
*
1
2
3
4
5
6
Days
C
Waking Activity (counts/min)
1.5
1.0
0.5
0.0
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*
1
2
3
4
5
6
Days

Fig. S2.

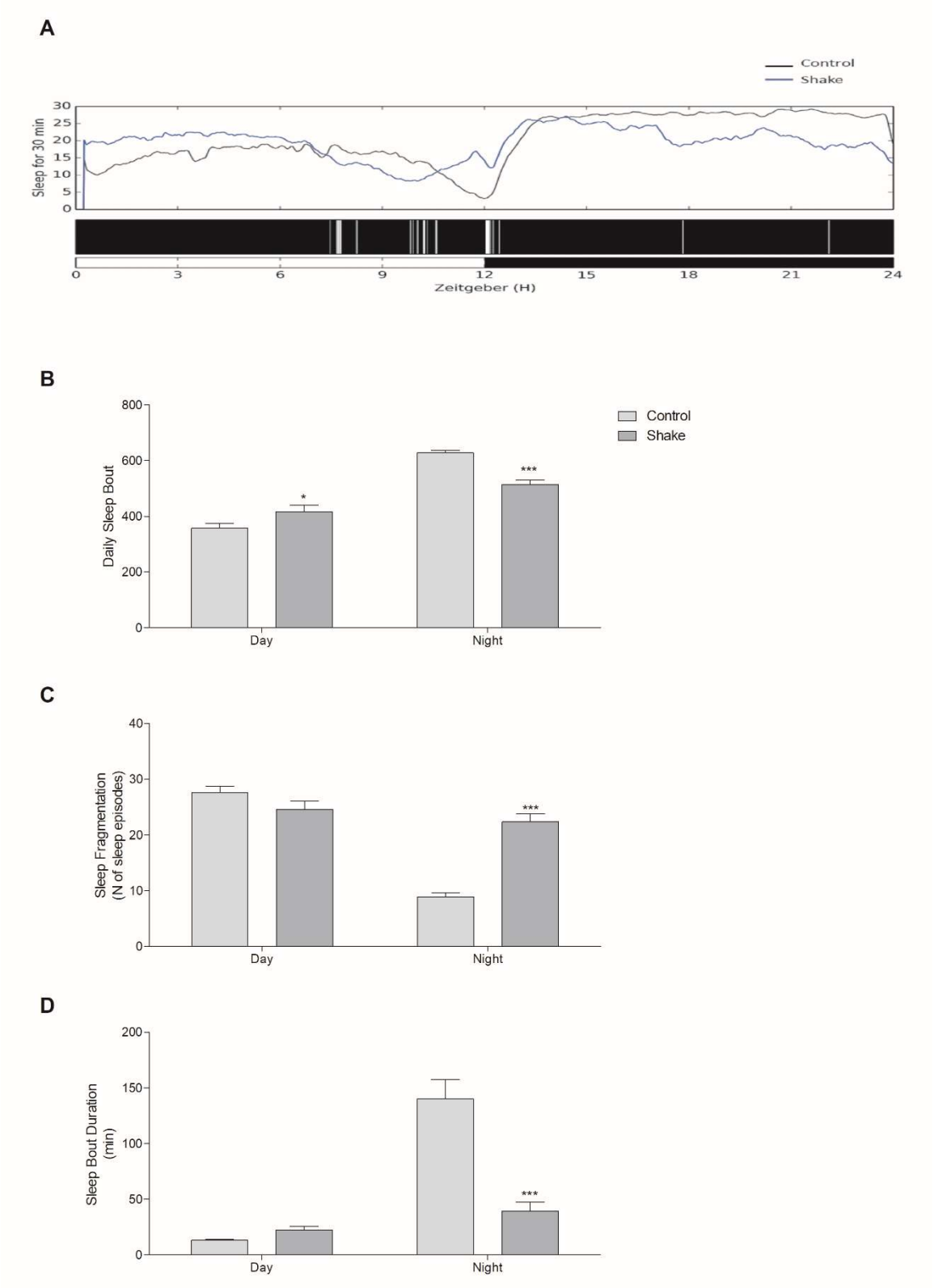
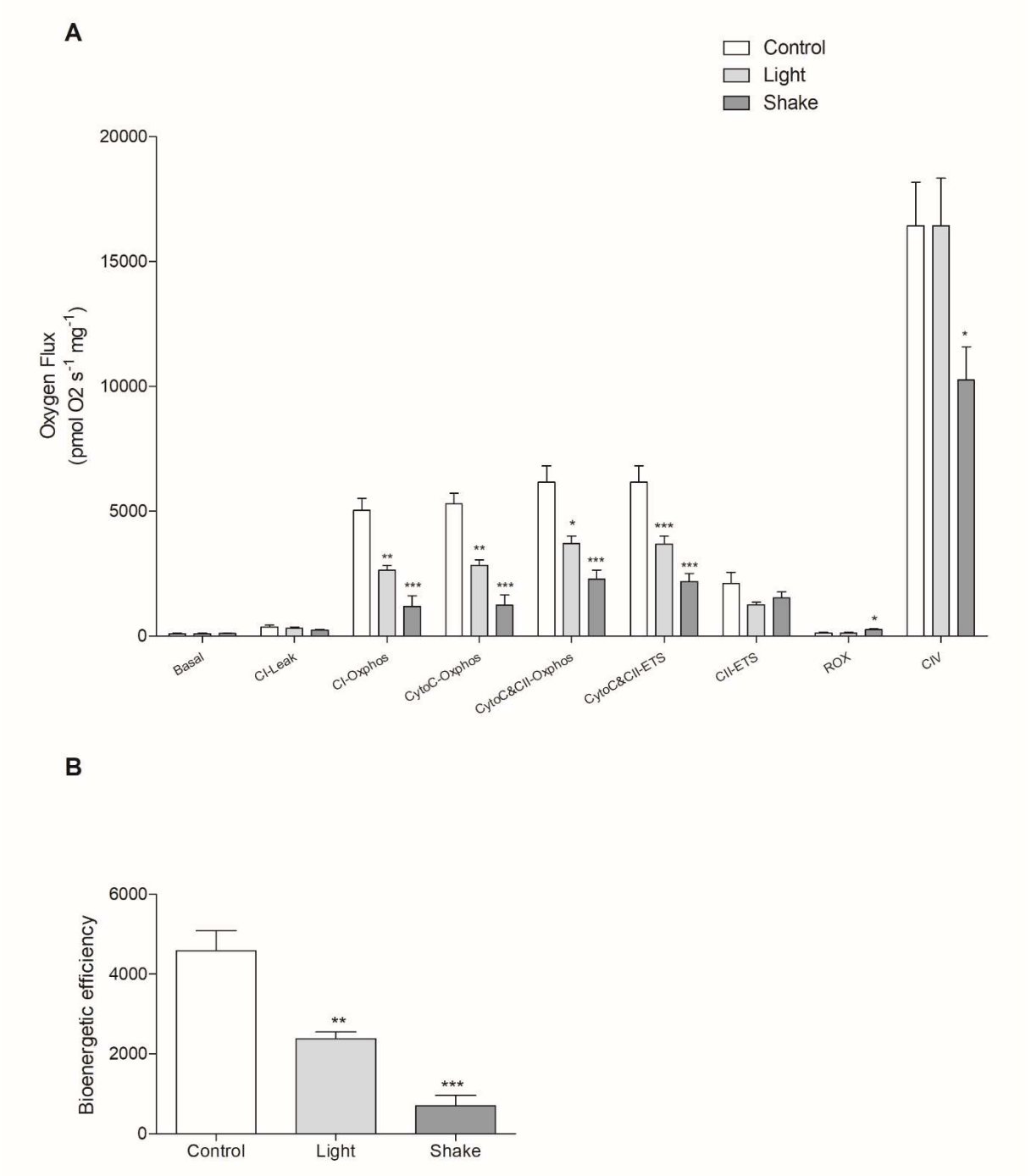

A
Control
Shake
Sleep for 30 min
30
25
20
15
10
5
0
0
3
6
9
12
15
18
21
24
Zeitgeber (H)
B
Control
Shake
Daily Sleep Bout
800
600
400
200
0
*
***
Day
Night
C
Sleep Fragmentation
(N of sleep episodes)
40
30
20
10
0
***
Day
Night
D
Sleep Bout Duration
(min)
200
150
100
50
0
***
Day
Night

Fig. S3.


A
Control
Light
Shake
Oxygen Flux
(pmol O2 s-1 mg-1)
20000
15000
10000
5000
0
Basal
CI-Leak
CI-Oxphos
CytoC-Oxphos
CytoC&CII-Oxphos
CytoC&CII-ETS
CII-ETS
ROX
CIV
B
Bioenergetic efficiency
6000
4000
2000
0
Control
Light
Shake