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MARIANE TRINDADE DE PAULA

ALTERAÇÕES METABÓLICAS INDUZIDAS POR UMA DIETA RICA EM GORDURA EM *Drosophila melanogaster* E OS EFEITOS DA HESPERIDINA

Uruguaiana
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ALTERAÇÕES METABÓLICAS INDUZIDAS POR UMA DIETA RICA EM GORDURA EM *Drosophila melanogaster* E OS EFEITOS DA HESPERIDINA

Tese apresentada ao programa de Pós-graduação Stricto sensu em Bioquímica da Universidade Federal do Pampa, como requisito parcial para obtenção do Título de Doutor em Bioquímica.

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“Talvez não tenha conseguido fazer o melhor, mas lutei para que o melhor fosse feito. Não sou o que deveria ser, mas Graças a Deus, não sou o que era antes”.

Marthin Luther King

RESUMO

Os efeitos deletérios de dietas como, por exemplo, as dietas ricas em gorduras (DRG) estão, cada vez mais, sendo estudados uma vez que eleva os níveis de triglicerídeos (TGC), causando um acúmulo de gordura, diminuindo a tolerância ao estresse e, consequentemente, propiciando o desenvolvimento de doenças e complicações que levam a diminuição da expectativa de vida e/ou morte precoce dos indivíduos. A hesperidina (Hesp), um flavonóide cítrico, já demonstrou ter propriedades antioxidantes representando um potencial agente protetor contra danos ligados à obesidade. Assim, o objetivo deste estudo foi investigar as alterações comportamentais, bioquímicas e genéticas induzidas por uma DRG em exemplares adultos e descendentes da mosca da fruta *Drosophila melanogaster*, bem como os efeitos terapêuticos da Hesp frente a estas alterações. Através deste estudo avaliou-se os danos oxidativos, a atividade e expressão de enzimas antioxidantes, como a superóxido dismutase (SOD) e catalase (CAT), e expressão de mRNA de enzimas envolvidas no metabolismo dos ácidos graxos como acetil-CoA sintetase (ACeCS 1) e acil-CoA sintetase (ACSL 1) e modulação da via de sinalização de estresse. Como resultados, nosso estudo demonstrou pela primeira vez que as moscas progenitoras adultas alimentadas por sete dias com DRG têm um desempenho locomotor reduzido, um aumento da ACeCS 1 e ACSL 1, aumento na produção de espécies reativas e substâncias reativas ao ácido tiobarbitúrico, aumento da expressão da proteína de choque térmico 83 (HSP83) e proteína quinase ativada por mitógenos 2 (MPK2), encurtando a vida útil destas moscas. Além disso, DRG quando adicionada durante todo o período de desenvolvimento embrionário, causa nas moscas descendentes um aumento nos níveis de TGC e glicose, juntamente com um aumento no nível de expressão de mRNA de DILP6, uma diminuição na enzima ACeCS1 presente no metabolismo de ácidos graxos, SOD e CAT em níveis de expressão de mRNA e diminuição da atividade de CAT. Os resultados observados a partir do co-tratamento com a Hesp e a DRG, este composto mostrou ser eficaz na redução de alguns parametros de alterações metabólicas, melhorou os níveis de TGC, glicose, parametros de estresse oxidativo, manteve a viabilidade celular e mitocondrial, melhorou o desempenho locomotor e atividade da enzima acetilcolinesterase (AChE), aumentando a expectativa de vida das moscas progenitoras. Em relação às moscas descendentes, a Hesp melhorou os níveis de TGC, diminuiu os níveis de glicose protegendo contra um possível desenvolvimento de sintomas pré-diabéticos, melhorando a taxa de eclosão dos ovos e expectativa de vida. Portanto, através deste estudo revelamos um eficiente papel protetor do flavonóide Hesp no tratamento de moscas progenitoras ou descendentes que receberam DRG demonstrando assim que este composto é capaz de atenuar alterações metabólicas, estresse oxidativo e sinalização de proteínas causadas pela obesidade.

Palavras-chave: Estresse oxidativo, flavonóide, Acetil-CoA Sintetase, Acil-CoA Sintetase, tempo de vida, sobrevivência.

ABSTRACT

Deleterious effects of diets such as high fat diets (DRG) are increasingly being studied as it raises triglyceride levels (TGC), causing fat accumulation, decreasing stress tolerance, and , Consequently, favoring the development of diseases and complications that lead to the decrease of the life expectancy and / or early death of the individuals. Hesperidin (Hesp), a citrus flavonoid, has already been shown to have antioxidant properties as a potential protective agent against obesity-related damage. Thus, the objective of this study was to investigate the behavioral, biochemical and genetic alterations induced by a DRG in adults and descendants of the fruit fly Drosophila melanogaster, as well as the therapeutic effects of Hesp in face of these alterations. This study evaluated oxidative damage, activity and expression of antioxidant enzymes, such as superoxide dismutase (SOD) and catalase (CAT), and mRNA expression of enzymes involved in the metabolism of fatty acids such as acetyl-CoA synthetase (ACeCS 1) and acyl-CoA synthetase (ACSL 1) and modulation of the stress signaling pathway. As a result, our study demonstrated for the first time that adult progenitor flies fed seven days with DRG have reduced locomotor performance, increased ACeCS 1 and ACSL 1, increased production of reactive species and thiobarbituric acid reactive substances, increased Expression of heat shock protein 83 (HSP83) and mitogen-activated protein kinase 2 (MPK2), shortening the lifespan of these flies. In addition, DRG when added during the entire embryo development period causes in the downward flies an increase in TGC and glucose levels, together with an increase in the level of DILP6 mRNA expression, a decrease in the ACeCS1 enzyme present in acid metabolism Fatty acids, SOD and CAT at mRNA expression levels and decreased CAT activity. The results observed from the co-treatment with Hesp and DRG, this compound showed to be effective in reducing some parameters of metabolic alterations, improved TGC levels, glucose, oxidative stress parameters, maintained cellular and mitochondrial viability, Improved the locomotor performance and activity of the enzyme acetylcholinesterase (AChE), increasing the life expectancy of the progenitor flies. For descending flies, Hesp improved TGC levels, decreased glucose levels protecting against a possible development of pre-diabetic symptoms, improving egg hatching rate and life expectancy. Therefore, through this study we have revealed an efficient protective role of flavonoid Hesp in the treatment of progenitor or descendant flies that received DRG demonstrating that this compound is able to attenuate metabolic alterations, oxidative stress and protein signaling caused by obesity.

Keywords: Oxidative stress, flavonoid, Acetyl-CoA Synthetase, Acyl-CoA Synthetase, lifespan, survival.

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LISTA DE ABREVIATURAS

ACeCS 1 – Acetyl-Coenzyme A Synthetase

Ache – Acetilcolinesterase

ACP – proteína carreadora de acila

ACSL 1 – Acyl-Coenzyme Synthetase

ATP – Adenosine triphosphate

AVC - Acidente Vascular Cerebral

CAT – Catalase

cDNA – Complementary Deoxyribonucleic acid

CIP/BIOTEC – Centro interdisciplinar em Biotecnologia

cm – centímetro (s)

DCF-DA - 2´, 7’ diclorodihidrofluoresceina diacetato

CAPES – Coordenação de Aperfeiçoamento de Pessoal de Nível Superior

CNPq – Conselho Nacional de Desenvolvimento Científico e Tecnológico

DILP – Drosophila insulin-like peptide

dL – decilitro (s)

DMSO – Dimethyl sulfoxide

DNA - Deoxyribonucleic acid

DRG – Dieta Rica em Gordura

DTNB – 5,5’-dithiobis(2-nitrobenzoic acid)

DTT – Ditiotreitol

ECL – Enhanced chemiluminescence

EDTA – ethylenediaminetetraacetic acid

ENG – England

EO - Estresse Oxidativo

ER - Espécies reativas

FABP – Proteínas transportadoras de ácidos graxos

FAPERGS – Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase

g – grama (s)

GSH – Glutationa

GPx – Glutationa Peroxidase

h – Hora (s)

HCl – Ácido clorídrico

HDL - proteínas de alta densidade

HEPES – sodium orthovanadate, 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid

Hesp - Hesperidina

HFD – High-fat diet

HSP83 - Proteína de choque térmico ou chaperona

IBGE – Instituto Brasileiro de Geografia e Estatística

IC – Insuficiência cardíaca

IKK – Inibitor-kappa B quinase

IMC – Índice de Massa Corporal

ITU – Infecção do Trato Urinário

JNK – c-jun N-terminal kinase

Kcal – Quilocaloria (s)

LDL – Lipoproteína de baixa densidade

M – molar

MA – Massachusetts

MAPK – Mitogen activated protein kinase

MCP – proteínas quimiotáticas de monócitos

mg – Miligrama (s)

MG – Minas Gerais

mL - mililitro (s)

mM – milliMolar

MMLV – Moloney Murine Leukemia Virus

MPK2 – mitogen-activated protein kinase homolog 2

mRNA – Ribonucleic acid messenger

MTT – 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide

mU – Mili unidades

NAD^+ -Nicotinamide adenine dinucleotide

ng – Nanograma (s)

nm – nanômetro(s)

NT – Neurotensina

NY – New York

OCV – Óleo de Coco Virgem

OMS – Organização Mundial da Saúde

pH - potencial de hidrogênio iônico

PKC – Proteína C quinase

qRT-PCR – Quantitative reverse transcription polymerase chain reaction

RD – Regular diet

Resazurin – 7-hydroxy-3H-phenoxazin-3-one 10-oxide

RNA - Ácido ribonucleico

RL – Radicais Livres

ROS – Espécies Reativas de Oxigênio

RS – Reactive Species Levels

RT-PCR – Reverse transcription polymerase chain reaction

s – segundo (s)

SBEM – Sociedade Brasileira de Endocrinologia e Metabologia

SDS - Dodecil sulfato de sódio

SDS - PAGE - Eletroforese unidimensional em gel de poliacrilamida e SDS

SE – Standard error

SM – Síndrome Metabólica

SOD - Superóxido dismutase

SP – São Paulo

TBA – thiobarbituric acid

TBARS - Substâncias reativas ao ácido tiobarbitúrico

TEMED - N, N, N', N'-tetramethylethylenediamine

TGC – Triglicerídeo (s)

TNF-α – Fator de necrose tumoral

Tris - Hodroximetil aminometano

TrxR1 – Tiorredoxina Redutase

USA – United States of America

Xg – Força gravitacional

µL – microlitro (s)

APRESENTAÇÃO

Nos itens **INTRODUCÃO** e **DESENVOLVIMENTO** constam uma 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 artigo científico e manuscritos, que se encontra no item **ARTIGO CIENTÍFICO** e **MANUSCRITOS 1** e **2.** No mesmo constam as seções: Materiais e Métodos, Resultados, Discussão e Referencias Bibliográficas.

Os itens **DISCUSSÃO** e **CONCLUSÕES**, encontradas no final desta tese, apresentam descrições, interpretações e comentários gerais sobre os resultados dos artigos presentes neste trabalho.

As **REFERÊNCIAS** dizem respeito às citações que aparecem nos itens **INTRODUCAO**, **DESENVOLVIMENTO**, **DISCUSSÃO** e **CONCLUSÕES** desta tese.

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1 INTRODUÇÃO

Os estudos sobre a ingestão de alimentos vêm ganhando maior atenção pois, além de ser a característica fundamental para a manutenção do equilíbrio de energia e sobrevivência dos seres vivos, também pode ser um componente da incidência de alterações metabólicas, tais como obesidade, dislipidemia, síndrome metabólica, diabetes, dentre outras (Garlapow et al., 2015). Segundo a Organização Mundial da Saúde (OMS) o consumo de dietas inadequadas e as complicações subsequentes a isso estão entre os dez principais fatores de mortalidade. Por conseguinte, o papel da dieta e consumo de alimentos vem sendo amplamente avaliado em estudos clínicos, bioquímicos e epidemiológicos e, cada vez mais, se faz necessária a implementação urgente de políticas públicas ligadas à prevenção e ao tratamento destas alterações metabólicas em resposta às mudanças na dieta, e o desenvolvimento de estratégias farmacológicas e não farmacológicas inovadoras (Baker e Thummel, 2007; Lottenberg, 2009; Smith et al., 2014). Este impacto crescente sobre os danos a saúde humana levou ao aumento no interesse em compreender estes mecanismos, muitas vezes dependendo de modelos de ratos, camundongos ou outros vertebrados na busca da compreensão da sua biologia e fisiologia (Burggren e Warburton, 2007; Lieschke e Currie, 2007; Hunziker et al., 2009; Chianese et al., 2011).

O metabolismo compreende diversos processos celulares, dentre eles os que regulam o armazenamento de lipídios e a utilização do substrato para assegurar que a quantidade de energia suficiente esteja disponível para as funções vitais diante das contínuas mudanças nas demandas energéticas e na disponibilidade de alimentos (Figlewicz, 2003; Lam et al. 2009; Morrison ET AL., 2010). Um elevado consumo de dieta hiperlipídica ou também chamada dieta rica em gordura (DRG), é caracterizado por causar danos em níveis celular e molecular, desencadear estresse oxidativo ativando assim diferentes respostas como as vias de sinalização para proteção e modificando proteínas e conferindo resistência à insulina (Toye et al., 2007; Newgard et al., 2009; Sitnick et al., 2009; Satapati et al., 2012; Heinrichsen et al., 2014). Por isso, o consumo adequado de nutrientes é fundamental para a manutenção do equilíbrio metabólico uma vez que está diretamente ligado ao metabolismo e à imunidade. A quantidade e o tipo de gordura alimentar podem exercer influência direta sobre o desenvolvimento de múltiplas patologias como diabetes, insuficiência cardíaca (IC), acidente vascular cerebral (AVC), doença arterial periférica, insuficiência renal, retinopatia, apoplexia, apneia do sono dentre outros (Marshall, 2006; Ogden et al. , 2007; Lottenberg, 2009; Heinrichsen et al., 2014).

Além disso, as DRG são bem descritas por modificarem o perfil dos ácidos graxos essenciais e o perfil lipídico bem como alterar o desenvolvimento e crescimento de proles (Al et al., 2000; Lee et al., 2014; Gunasekaran et al., 2017). Por isso, o óleo de coco virgem (OCV) tem servido como objeto para modulação de uma DRG uma vez que é fonte, principalmente, de gorduras saturadas e já se demonstrou que o consumo em excesso causa diversas alterações metabólicas em adultos. Em proles, o OCV acarretou diversos prejuízos como a alteração no crescimento fetal, resistência à insulina, esclerose múltipla, deficiência endotelial de óxido nítrico sintase, déficits espaciais, altos níveis circulantes de adipocinas, as quais são citocinas derivadas do tecido adiposo que contribuem para o desenvolvimento de SM, respostas inflamatórias e obesidade (Boney et al., 2005; Carlsen et al., 2014; Albert et al., 2016; Ferrari et al., 2016; Hong et al., 2016; Hsieh et al., 2016; Saben et al., 2016). Além disso, o consumo exacerbado de DRG leva a uma maior probabilidade de mortalidade, principalmente, por doenças coronarianas e cardiovasculares, diminuindo a expectativa de vida da população (Lima et al., 2000; Cornier et al., 2008).

Por isso, estudos que envolvam a regulação metabólica e os sistemas de *feedback* fisiológico de indivíduos são áreas centrais para todos os aspectos da vida pós-embrionária (Baker e Thummel, 2007). Porém, a complexidade multicelular de organismos superiores impõe limitações em determinadas pesquisas, tais como vias regulatórias de sinalização, interações entre genes, metabolismo e mudanças na expressão de proteínas a nível transcricional (Oldham e Hafen, 2003; Baker e Thummel, 2007; Leopold e Perrimon, 2007; Perez e Van Gilst, 2008; Diop e Bodmer, 2012).

Na última década, o modelo da mosca da fruta *Drosophila melanogaster* (*D. melanogaster*) tem sido um dos principais sistemas para o estudo de alterações no metabolismo e/ou mudanças na dieta combinado com o estresse oxidativo (Arque e Neckameyer, 2014). A gordura corporal em insetos, como a *D. melanogaster*, desempenha um papel fundamental durante todo o seu desenvolvimento, buscando atender a novas necessidades fisiológicas e energéticas (Arrese e Soulages, 2010). Diversas alterações fenotípicas que se assemelham àquelas demonstradas por humanos têm sido bem descritas neste modelo. Moscas expostas à dieta rica em ácidos graxos demonstraram acúmulo de gordura, disfunção cardíaca, aumento dos níveis de triglicerídeos (TGC), diminuição da tolerância ao estresse e tempo de vida reforçando a ideia da utilização da *D. melanogaster* como um excelente modelo de estudo (Ogden et al., 2007; Birse et al., 2010; Heinrichsen e Haddad, 2012; Boden e Salehi, 2013).

A dificuldade no tratamento destas alterações metabólicas e uma melhor compreensão de seu desenvolvimento e suas causas em modelos alternativos de estudo realçam a utilização de compostos bioativos de produtos naturais como potenciais tratamentos (Pandey e Rizvi, 2009; Visioli et al, 2011; Del Rio et al, 2013). Os polifenóis são antioxidantes conhecidos por ajudar na estimulação das vias de sinalização celular relacionadas com o estresse desencadeando a biogênese mitocondrial e influenciando o sistema vascular (Somerville et al., 2017). A principal classe dos polifenóis são os flavonóides, conhecidos compostos fenólicos encontrados abundantemente em plantas, cascas de frutas, sementes e flores com amplas propriedades antioxidantes, anticancerígenas, anti-inflamatórias, dentre outras (Manthey, et al., 2001; Yu, et al., 2005). Naturalmente, os flavonóides são encontrados na dieta humana em frutas, legumes e bebidas derivadas de plantas tais como sucos de frutas, chá, café e vinho tinto (Scalbert e Williamson, 2000).

Existe um número extenso de artigos científicos sobre os benefícios de produtos naturais como os flavonóides, como cita Kuntic et al. (2014), atualmente as ferramentas de pesquisas no meio eletrônico podem ser encontrados mais de 58.000 referências sobre estes compostos. Ainda para Kuntic et al. (2014), apesar deste assunto ser amplamente estudado, é necessário dar uma maior atenção para a hesperidina (Hesp). Dessa maneira, a Hesp é um bioflavonóide encontrado, principalmente, em frutos cítricos e, pode ser uma alternativa eficaz uma vez que ela já demonstrou efeitos benéficos tanto *in vitro* como *in vivo* (Pari et al., 2015). Ademais, a Hesp desempenha diversos efeitos, dentre eles: capacidades antioxidante, antimicrobiana, anti-inflamatória, anticancerígena e antialérgica (Garg et al., El-Sayed et al., 2007; 2001; Roohbakhsh et al., 2014, Parhiz et al, 2015).

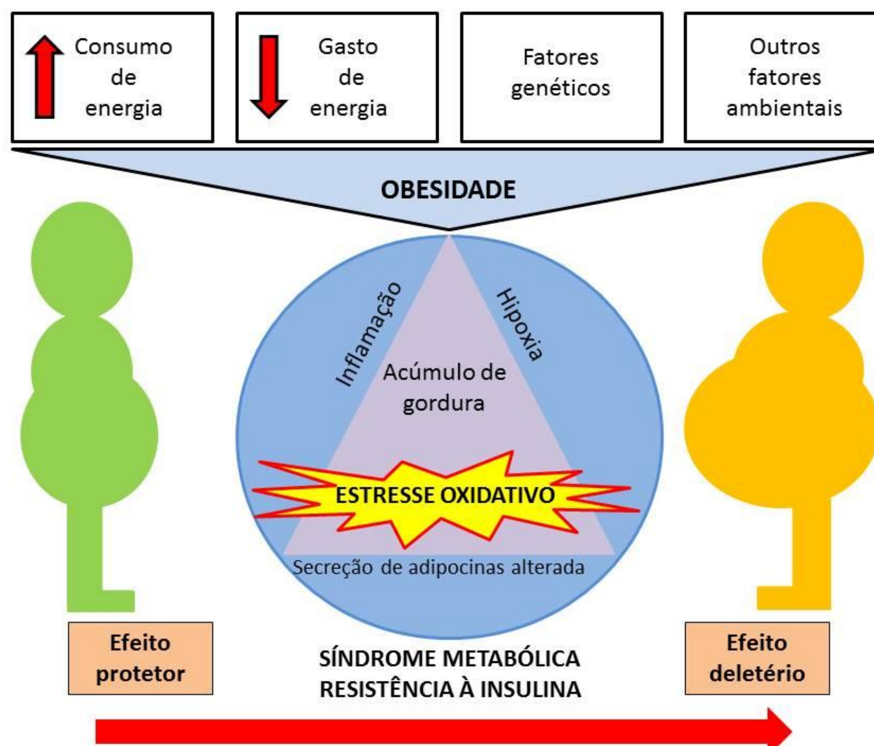
Tendo em vista isso, e a busca por tratamentos naturais e não medicamentosos, o presente estudo objetivou investigar o efeito de uma DRG sobre as alterações metabólicas, comportamentais e genéticas, bem como estresse oxidativo e dano celular em exemplares da mosca da fruta *D. melanogaster*, adultos e descendentes, bem como o potencial terapêutico da Hesp. frente à essas alterações.

2 DESENVOLVIMENTO

2.1 Obesidade e disfunções metabólicas associadas

A obesidade é um problema de caráter pandêmico multifatorial (Figura 1) e multiétnico que ocorre em países de alta, média e baixa renda acometendo tanto homens, quanto mulheres em todas as faixas etárias (Wannmacher, 2016). Segundo a Sociedade Brasileira de Endocrinologia e Metabologia (SBEM), a obesidade é caracterizada pelo acúmulo excessivo de gordura corporal em um determinado indivíduo. A avaliação nutricional de um adulto requer o conhecimento das reservas energéticas e da massa metabolicamente ativa dos indivíduos pelo qual é utilizado o parâmetro de avaliação índice de massa corporal (IMC) que é calculado dividindo-se o peso do indivíduo pela sua altura elevada ao quadrado (Anjos 1992). Todavia, o IMC não permite aferir diferenciadamente o peso de músculos e gordura (Wannmacher, 2016). Para um diagnóstico mais preciso, a Organização Mundial da Saúde (OMS) recomenda aliar ao IMC a aferir circunferência da cintura e relação cintura-quadril e, uma vez esta relação alterada, estes dados servem como um dos critérios para caracterizar a síndrome metabólica (SM) (Wannmacher, 2016).

Figura 1 – Diagrama esquemático do caráter multifatorial da obesidade.



Fonte: arquivo próprio (2017) adaptado de Rupérez et al. (2014).

Dados globais apontam que em 2014 mais 1,9 bilhão de adultos estavam com sobrepeso, sendo 600 milhões desses obesos (OMS, 2014). O Brasil, segundo o levantamento do Ministério da Saúde revelou que 51% da população brasileira está acima do peso, sendo que esta estatística atinge a maioria os homens (54%) seguidos das mulheres (48%). Além disso, 17% da população é considerada obesa e, índices como estes são cada vez mais preocupantes, principalmente porque o sobrepeso e a obesidade podem trazer diversas consequências para a saúde da população, entre elas doenças cardiovasculares, diabetes, alguns tipos de câncer, apneia e hipertensão arterial sistêmica (Marshall, 2006; Ogden et al. , 2007; Heinrichsen et al., 2014). A causa fundamental da obesidade e sobrepeso é o desequilíbrio entre o consumo de calorias e o gasto calórico, onde a ingestão de dietas ricas em carboidratos e gorduras é abundante associada ao grande índice de sedentarismo da população urbana (Wannmacher, 2016). Todavia, não se pode excluir que a obesidade é uma doença multifatorial e a interação de fatores é fundamental para a sua etiologia, bem como a interação de fatores genéticos, disfunções endócrinas e condições do ambiente.

A obesidade e o sobrepeso em adultos são fatores de predisposição para o desenvolvimento de doenças cardiovasculares, diabetes, osteoartrite, alguns tipos de câncer, insuficiências respiratórias, infertilidade masculina, transtornos psicossociais e hipertensão arterial sistêmica (Kahn et al., 2006; Labounty et al., 2013; Bastien et al., 2014; Chandra et al., 2014; Ko et al., 2014; Previs et al., 2014; James et al., 2015; Xiang e Na, 2015). Já em crianças, a obesidade associa-se a maior chance de morte prematura, incapacidade funcional na fase adulta, dificuldades respiratórias, maior probabilidade de fraturas, efeitos psicológicos e precoces indicadores de doença cardiovascular e resistência à insulina (OMS, 2012).

Devido a estes dados alarmantes e crescentes nos índices de indivíduos obesos e com sobrepeso, crescem o número de estudos que investigam a saciedade, peso corporal e auxiliam no desenvolvimento de estratégias farmacológicas e não farmacológicas inovadoras para combater a obesidade (Smith et al., 2014). Para Wannmacher (2016), a obesidade e o sobrepeso devem ser preferencialmente manejados com medidas não medicamentosas, com ênfase em dietas (dietas mais adequadas que limitem a ingestão total de gorduras, açúcares e sal), atividade física regular (60 minutos/dia para crianças e 150 minutos/semana para adultos) e consumo de alimentos naturais (consumo de frutas, vegetais e grãos) (SMITH et al., 2014; OMS, 2015).

2.1.1 Obesidade gestacional e síndrome metabólica na infância

A prevalência de sobrepeso e obesidade tem aumentado substancialmente nas últimas décadas onde, especialmente nas mulheres em idade reprodutiva (20 a 49 anos), este dado é ainda mais alarmante (Seligman et al., 2006). Muitas são as complicações causadas pela obesidade durante a gravidez tanto nas mães quanto nos filhos, aumentando assim riscos de diabetes, hipertensão, angústia fetal, hemorragias, endometrite, fenômenos tromboembólicos, anemia, infecções do trato urinário (ITU), prematuridade e morte fetal (Vahratian et al., 2005; Seligman et al., 2006).

Para Crespo et al. (2007), a relação entre obesidade, SM e diabetes tipo 2 já está bem estabelecida em adultos mas, esta associação, também pode ser sugerida em crianças e, por isso, esta síndrome durante a infância precisa ser urgentemente e claramente estabelecida. Os recém-nascidos de mães obesas têm demonstrado, em muitas vezes, baixo ou alto peso ao nascer, crescimento acelerado, resistência à insulina periférica dentre outras complicações (Higgins et al., 2001; Sina et al., 2002; Cruz e Goran, 2004). Contudo, a redução de peso da mãe é totalmente necessária no período gestacional a fim de proporcionar ao feto um período de desenvolvimento mais saudável e, além disso, aliar na rotina diária a prática de exercícios físicos, redução de ácidos graxos saturados, gorduras trans e carboidratos mantendo uma dieta equilibrada (Crespo et al., 2007).

2.2 Dieta rica em gordura (DRG)

Os hábitos alimentares exercem grande influência sobre o crescimento, desenvolvimento e saúde geral dos indivíduos (Neutzling et al., 2007). O perfil alimentar da população está fortemente associado a aspectos psicológicos, culturais, socioeconômicos e demográficos, tornando-se necessário uma melhor compreensão desses aspectos no entendimento do comportamento alimentar (Birch e Fisher, 1998). O metabolismo possui a propriedade de converter a energia química dos alimentos em substâncias próprias do organismo, como fosfato, creatina e, principalmente, adenosina-trifosfato (ATP). A energia do ATP pode ser utilizada para o trabalho mecânico (músculos), para a síntese de numerosas substâncias como proteínas estruturais, enzimas, dentre outros (Silbernagl e Despopoulos, 2009).

O consumo de dietas inadequadas ou a alteração nos componentes primordiais da dieta causam diversas mudanças no metabolismo dos seres vivos podendo levar ao desenvolvimento de doenças ou alterações moleculares (Cooper et al., 2016). Dietas hiperlipídicas ou também conhecidas como dietas ricas em gordura (DRG) são bem descritas por serem os principais fatores externos para o desenvolvimento de diversas patologias como: casos de pré-diabetes clínicos, diabetes mellitus tipo II, hiperglicemia, apneia do sono, apoplexia, retinopatia, insuficiência renal, doença arterial periférica, insuficiência cardíaca (IC), acidente vascular cerebral (AVC), obesidade, doenças crônicas, patologias da vesícula, osteoartrite, hipertensão dentre outros (Bertolami e Bertolami, 1986; Watts et al., 1996; Metz et al., 1997; Oliver, 1997; Cesaretti, e Kohlmann Junior, 2006; Marshall, 2006; Ogden et al. , 2007; Gigante et al., 2009; Clemmensen et al., 2012; Speakman, 2013; Heinrichsen et al., 2014). Além disso, já está comprovado que em populações cujas dietas têm excessivo teor de gordura também se encontram os maiores índices de mortalidade, principalmente, por doenças coronarianas, cardiovasculares, câncer, doenças renais e hepáticas, dentre outras, o que reduz drasticamente a expectativa de vida quando comparada à população que consome dieta normal e equilibrada (Lima et al., 2000; Cornier et al., 2008). Ainda assim, estudos já comprovaram que a obesidade está cada vez mais em alta devido às más condições alimentares e, que isso, pode em breve emergir como uma das principais causas de morte precoce em todo o mundo (Kitahara et al., 2014).

Em diversos países, cada vez mais a população vêm fazendo uso de dietas compostas por até 30% a 40% de calorias na forma de gorduras, consumindo assim maior quantidade de ácidos graxos saturados e gorduras do tipo trans (Garbarino e Sturley, 2009). Segundo estudos, no Brasil estes dados não são diferentes e, aproximadamente, um terço (32,7%) dos adultos de ambos os sexos consomem frequentemente alimentos ricos em gordura (alimentos ricos em gordura saturada) e, é isso que gera o aumento do risco para o desenvolvimento das doenças crônicas não transmissíveis (Lopes et al., 2005; Monteiro et al., 2008; Vitigel, 2010; Rombaldi et al., 2014).

O princípio destas DRG é que se aumentando a ingestão de gorduras e, consequentemente diminuindo-se a quantidade de carboidratos, desenvolve-se um quadro de oxidação lipídica promovendo um efeito de saciedade e, com isso, decompondo as gorduras em glicerol e ácidos graxos livres que formam pares de dois compostos de carbono, denominados corpos cetônicos, resultando em um novo ácido graxo que poderá ser utilizado como combustível energético (Bonnie et al., 2014). Não sendo diferente em animais, as DRG são conhecidas pela significativa redução na ingestão de alimentos e, o conteúdo de energia,

proteína e micronutrientes em uma DRG precisa ser proporcionalmente mais elevado do que os animais que recebem uma dieta padrão (DP) já que ingerem menos a dieta por alcançarem a saciedade mais rapidamente (Blundel et al., 1996; Duca et al., 2013). O hipotálamo é quem coordena todos os processos vegetativos e a maioria dos endócrinos e assim, é conhecido por ser o centro regulador da massa corporal com seu núcleo arqueado e os conectados "centros" da "saciedade" (núcleo paraventricular) e da "fome" (hipotálamo lateral) (Silbernagl e Despopoulos, 2009). Junto a isso, a leptina, um hormônio peptídico de 16 kDa, é produzido pelas células adiposas e cuja concentração plasmática aumenta com a gordura, informa o hipotálamo sobre o tamanho dos depósitos de gordura; sobre a disponibilidade de glicose, o hipotálamo é informado por meio da concentração de insulina. Quando a concentração de leptina aumenta é relatado que diminui a ingestão de alimentos e aumentam o consumo de energia. Porém, quando a concentração de leptina baixa é desencadeado um processo inverso (Silbernagl e Despopoulos, 2009).

De modo geral, dados publicados na literatura relatam que uma dieta considerada hiperlipídica, ela precisa necessariamente fornecer de 10-40% de lipídeos (Jobgen et al., 2009) com isso, em modelos de DRG em ratos, os mesmos autores utilizaram 23,6% de gordura para a DRG e apenas 4,3% de gordura para a dieta regular e, a partir daí, observaram que essas alterações alimentares induzem estresse oxidativo, disfunção mitocondrial e comprometem a síntese e a ação da insulina. Estes resultados observados corroboram com dados da literatura que relatam os efeitos de uma DRG causando danos em níveis celulares e moleculares desencadeando o processo de estresse oxidativo que possibilita o organismo gerar diferentes respostas como a ativação de vias de sinalização implicadas na proteção de células contra danos oxidativos, tais como proteínas de choque térmico (HSP) e proteínas quinases ativadas por mitógenos (MAPK), peroxidação de lipídios, modificação de proteínas e resistência à insulina (Toye, et al., 2007; Newgard et al., 2009; Sitnick et al., 2009; Satapati et al., 2012; Heinrichsen et al., 2014;).

Como uma rede interligada do metabolismo e suas respostas, o consumo adequado de nutrientes ou uma boa proporção na ingestão de macronutrientes presentes nos alimentos para fornecimento de energia é fundamental para a manutenção do equilíbrio metabólico e preservação da função imunológica (Lottenberg, 2009). Tanto macrófagos (células do tecido conjuntivo que fagocitam elementos estranhos ao corpo) quanto adipócitos (células de armazenamento de gordura) possuem origem embrionária em comum e, por sua vez, produzem os mesmos componentes (Wellen e Hotamisligil, 2005). Quando em condições normais, os adipócitos apresentam função de regular a homeostase metabólica e armazenar

lipídeos enquanto que os macrófagos estão relacionados com as respostas inflamatórias. Todavia, quando a ingestão de lipídeos é alta gerando alterações no metabolismo, estas vias metabólicas e inflamatórias se sobrepõem e a expressão de genes torna-se similar em ambas as células. Além do mais, os macrófagos passam a expressar proteínas normalmente produzidas pelos adipócitos como as proteínas transportadoras de ácidos graxos (FABP) que modulam o acúmulo de lipídeos no adipócito e de colesterol no macrófago, simultaneamente a citocinas inflamatórias como TNF-α, interleucina-6 e proteínas quimiotáticas de monócitos (MCP) (Hotamisligil, 2005).

Indivíduos que ingerem DRG são caracterizados por desencadear processos inflamatórios a partir de alterações como o aumento de moléculas de adesão vascular e intercelular, interleucina-6, TNF-α e selectina podendo induzir ainda processos apoptóticos das células endoteliais (Nappo et al., 2002; Coenen et al., 2007). Ainda assim, os lipídeos podem ativar processos de sinalização celular como, por meio das FABP, ativam quinases intracelulares ("inhibitor-kappa B quinase" (IKK), "c-jun N-terminal kinase" (JNK) e proteína quinase C (PKC) que também podem ser ativadas através do estresse do retículo endoplasmático) causando a não sinalização do receptor de insulina corroborando com o processo inflamatório (Hotamisligil, 2005). Portanto, quando há presença de processos inflamatórios, devido ao elevado consumo de gorduras, várias citocinas e proteínas de fase aguda se encontram aumentadas por alterações causadas nos adipócitos o que acarreta em diversas doenças (Vellosa et al., 2013).

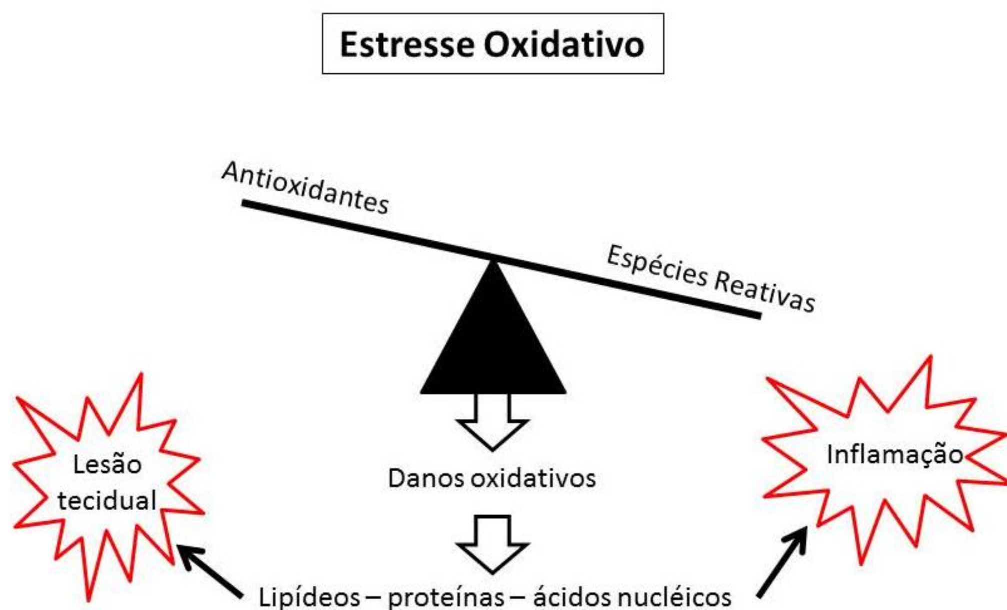
Diretamente ligado ao aumento da ingestão de gorduras pela dieta pelo qual ativa a cascata inflamatória pelo excesso da disponibilidade de gordura, podemos também interligar um aumento excessivo na produção de ER causando um desequilíbrio no balanço energético das células (Menon et al., 2003; Axelsson et al., 2004; Beddhu, 2004; Axelsson et al., 2005; Lubrano et al., 2013). É importante lembrar que as ER possuem diversas funções fisiológicas quando em concentrações normais e apresentam papéis fundamentais na manutenção de estados celulares normais (Valko et al., 2007).

Com o consumo de DRG e maior disponibilidade de gorduras, o organismo se adapta para aumentar a oxidação de ácidos graxos nas mitocôndrias e assim geração de energia (Hall, 2011). Porém, uma disfunção mitocondrial pode ser observada nestas situações de aumento de disponibilidade de gorduras uma vez que podem acumular danos oxidativos, aumentar a geração de ER de modo que as defesas antioxidantes não sejam suficientes para proteger contra os danos oxidativos e assim gerar um processo de estresse oxidativo (EO) (Valko et al., 2007; Rupérez et al., 2014). O estresse oxidativo é caracterizado por um desequilíbrio redox

entre a geração de RL e ER e os mecanismos de defesas celulares antioxidantes como, por exemplo, as enzimas superóxido dismutase (SOD), catalase (CAT) glutationa peroxidase (GPx), tiorredoxina redutase (TrxR1) e não enzimáticas como a glutationa reduzida (GSH), tocoferóis (vitamina E), ácido ascórbico (vitamina C), albumina, polifenóis, entre outros (Figura 1) (Comporti, 1989; Marks et al., 1996; Finkel e Holbrook, 2000; Shulman et al., 2011). Altas concentrações de ácidos graxos livres foram caracterizados por gerar altos níveis de peróxido de hidrogênio (H_2O_2) em mitocôndrias de ratos e seres humanos (Furukawa et al., 2004; Anderson et al., 2009). Assim, segundo Rupérez et al. (2014), a ligação entre DRG e estresse oxidativo aumentado pode ser devido às mudanças que ocorrem no metabolismo de um indivíduo obeso como: hiperglicemia, defesas antioxidantes diminuídas e inflamação crônica.

Desse modo, uma DRG causa diferentes danos desencadeando um quadro de estresse oxidativo e, assim, fazendo que com cada indivíduo gere respostas como, por exemplo, ativação de vias de sinalização celular, peroxidação lipídica, ativação ou inibição de enzimas e defesas antioxidantes endógenas (Sitnick et al., 2009; Satapati et al., 2012; Heinrichsen et al., 2014). Além disso, todas estas alterações que acometem o metabolismo normal do corpo causado pela mudança na ingestão de alimentos, contribuem, então, ao aparecimento de problemas de saúde (Figura 2) (Rupérez et al., 2014).

Figura 2 – Esquema de estresse oxidativo quando há um excesso de radicais livres sobre as defesas antioxidantes.



Fonte: arquivo próprio (2017) adaptado de Kelly, 2003.

Estas alterações na saúde, bem como também alterações ambientais e maus hábitos, são fatores primários associados à longevidade em estudos populacionais (Allen et al., 2016). Dada a grande problemática do consumo inadequado de dietas não saudáveis e, consequentemente, aumento de doenças e sobrepeso é determinante para a diminuição da expectativa de vida da população bem como aumento do risco de morte prematura devido às diversas complicações que levam à diagnósticos desfavoráveis às condições de saúde (Seidell e Halberstadt, 2016). Ademais, a indução de uma alteração metabólica causada pela DRG é caracterizada por desencadear um quadro de estresse oxidativo e inflamação e, ligado à isso, pode-se observar a indução de distúrbios cognitivos e impactos negativos nas funções cerebrais, pontos considerados chaves na progressão da idade e envelhecimento (Palomera-Ávalos et al., 2017).

2.3 Óleo de coco virgem (OCV)

O coqueiro é uma fonte de diversos produtos pelos quais podem sem explorados e, por isso, é considerado uma planta de múltiplas funcionalidades sendo reconhecido mundialmente como um recurso vegetal (EMBRAPA, 2014). Além de serem fontes de alimentos, muitas populações de regiões tropicais e subtropicais acreditam na capacidade dos produtos derivados do coco na medicina popular (Chan et al., 2006). Relatos indicam as propriedades dos derivados do coco vão desde a eficácia em casos de bronquite, febre e gengivite até os benefícios em relação à perda de cabelo, queimaduras e controle de doenças cardíacas (Debmandal et al., 2011).

Contudo, a parte mais utilizada comercialmente é a polpa seca, também conhecida como copra, principal fonte da extração e produção do óleo de coco virgem (OCV) sob condições de temperatura moderada e controlada, a fim de evitar a perda de extração de componentes menores como provitamina A, provitamina E e polifenóis devido a radiação UV da luz do sol durante a extração de outros tipos de óleos (Nevin e Rajamohan, 2008; Dauber, 2015). As indústrias têm oferecido diversas opções de produtos como recursos no tratamento de doenças e, o OCV vem se destacando no mercado com a promessa de possuir diversas ações benéficas ao organismo como, por exemplo, ação antibacteriana, antiviral e antifúngica, ação termogênica atuando como coadjuvante na perda de gordura corporal, perda de peso e significativa redução de gordura abdominal, atuar como um fortificante imunológico tanto de

recém nascidos pela ingestão do leite materno humano como de mães e antinflamatório (Fife, 2002; Fife, 2006; Natue, 2015). Ainda para explicar estes efeitos, a indústria relata aos consumidores que este efeito benéfico se dá pela presença do ácido láurico, um ácido graxo saturado com 12 carbonos, que ao ser ingerido chega ao intestino e é quebrado pelas enzimas lipases se transformando em monolaurina que é absorvida e alcança a corrente sanguínea podendo destruir a membrana de vírus e bactérias, estimular a glândula tireóide, reduzem a oxidação de LDL e tendem a diminuir a compulsão de carboidratos (Kabara e Vrable, 1977; Dauber, 2015). Além da comercialização do OCV para estes fins, a indústria também faz o uso para a produção de cosméticos, biocombustível e componente para enriquecimento de rações animais como aves, bovinos, equinos, caprinos e suínos (Lovett et al., 2003; Nascif et al., 2004; Mourad, 2006; Kumar et al., 2010; Kumar, 2011; Vysakh et al., 2014; Wang et al., 2015).

O óleo de coco é um óleo altamente saturado (cerca de 90%) onde, aproximadamente, 60% de sua composição é composta de ácidos graxos de cadeia média (6-12 átomos de carbono) e tem sido muito utilizado como principal fonte de gorduras saturadas (Tabela 1) para modelar uma DRG (Bhatnagar et al., 2009). Além do ácido graxo láurico, o OCV é composto, sucessivamente, pelos ácidos graxos saturados: mirístico, palmítico, caprílico, cáprico, esteárico e capróico.

Tabela 1 – Composição em ácidos graxos de uma marca comercial de óleo de coco virgem.

Ácidos graxos		**Quantidade (%/100g)**
C 6:0	Ácido Capróico	0,38
C 8:0	Ácido Caprílico	5,56
C 10:0	Ácido Caprico	4,99
C 12:0	Ácido Laurico	45,78
C 14:0	Ácido Mirístico	18,56
C 16:0	Ácido Palmítico	8,85
C 18:0	Ácido Esteárico	3,39
C 18:1	Òmega 9 oléico	5,65
C 18:2	Ômega 8 linoléico	0,94

Fonte: Pró-Ervas® (2016).

O consumo de óleo de coco contribui para aumentar os níveis de energia na dieta, porém o principal fator de risco para o seu consumo é o uso indiscriminado deste óleo pela população (fisiculturistas, obesos, sedentários) sem o acompanhamento de um profissional habilitado que forneça informações quanto às quantidades benéficas a serem consumidas, prescrição de uma alimentação adequada e incorporação de hábitos saudáveis (Noguchu et al., 2002; Clegg, 2010; Natue, 2015; Gunasekaran et al., 2017). O OCV quando consumido em excesso e sem prescrição, já demonstrou efeitos prejudiciais ao metabolismo normal do organismo como alteração no perfil de ácidos graxos essenciais e lipídicos em camundongos, alterou o desenvolvimento e crescimento de proles, dentre outros (Al et al., 2000; Lee et al., 2014; Gunasekaran et al., 2017).

2.4 Metabolismo de ácidos graxos

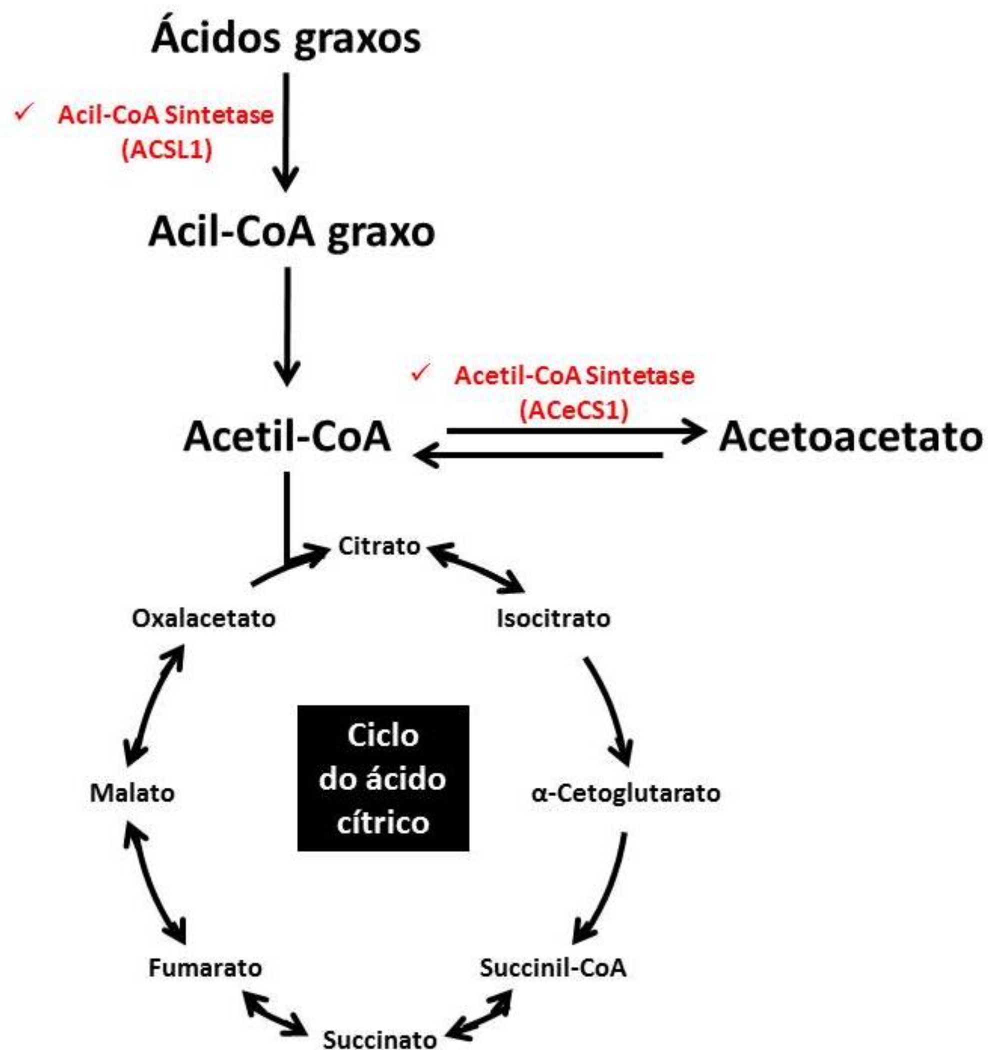
O metabolismo converte a energia química dos alimentos em substâncias próprias do organismo, como fosfato e creatina e, sobretudo, para a produção de energia na forma de ATP (Silbernagl e Despopoulos, 2009). Em especial, o metabolismo de lipídeos compreende, em parte, a síntese e degradação de ácidos graxos, ou seja, a mobilização de ácidos graxos para tecidos de reserva como o tecido adiposo, oxidação de ácidos graxos para geração de energia ou ainda o excesso de proteínas ou carboidratos da dieta podem ser convertidos em ácidos graxos que são armazenados na forma de triglicerídeos nos adipócitos.

2.4.1 Degradação de ácidos graxos

A degradação de ácidos graxosque compreende a oxidação de ácidos graxos a acetil-CoA é uma via central de produção de energia em diversos organismos e tecidos, sendo denominada de β-oxidação. Os lipídeos são completamente metabolizados na presença de O_2 em CO_2 e H_2O no organismo pelas mitocôndrias, com um valor calórico fisiológico de 38,9 kJ/g, valor este mais alto do que o de carboidratos e proteínas (Hall, 2011). Logo que ingerida as gorduras são quebradas a ácidos graxos que por sua vez é transportado para as mitocôndrias, processo este mediado através de um transportador que usa a carnitina como substancia carreadora (Hall, 2011). No interior das mitocôndrias, a β-oxidação tem início onde em seu primeiro estágio quatro reações (desidrogenação de carbonos, hidratação de ligação dupla, desidrogenação e clivagem dependente de CoA) removem cada unidade de Acetil-CoA da extremidade carboxil de um acil graxo-CoA saturado (Nelson, 2011). Ao final

destas quatro reações são formados acetil-CoA e acil graxo-CoA encurtada de dois carbonos que entra novamente na sequência. A enzima acil-CoA Sintetase (ACSL1) é uma enzima necessária para síntese lipídica onde esta enzima ativa os ácidos graxos em duas reações: 1) formação de um intermediário acil-AMP graxo com a liberação de pirofosfato e 2) formação de Acil-CoA graxo com a liberação de AMP (Mashek et al., 2007). Esta formação de Acil-CoA graxo é fundamental para a formação do Acetil-CoA e consequente utilização para a produção de ATP no ciclo do ácido cítrico (ciclo de Krebs) (Figura 3).

Figura 3 – Esquema da degradação de ácidos graxos até a entrada no ciclo do ácido cítrico e formação de acetoacetato.



Fonte: arquivo próprio (2017).

O segundo estágio da β-oxidação compreende a entrada da molécula de acetil-CoA no Ciclo de Krebs, associando-se, em primeiro lugar, ao ácido oxalacético para formar ácido cítrico que então é degradado em CO_2 e átomos de hidrogênio. Os hidrogênios são encaminhados à cadeia transportadora de elétrons e oxidado pelo sistema quimiosmótico oxidativo liberando grande quantidade de ATP (Silbernagl e Despopoulos, 2009; Hall, 2011). Desta forma, assim como no metabolismo da glicose as moléculas de acetil-CoA formadas se ligam ao ácido pirúvico no ciclo de Krebs, os ácidos graxos também são degradados de maneira semelhante. Todavia, quando ácidos graxos insaturados são oxidados, este processo requer a presença de duas enzimas adicionais (enoil-CoA-redutase e 2,4-dienoil-CoA-redutase) não encontradas na oxidação de ácidos graxos saturados.

Este processo de β-oxidação pode ser quando a ingestão ou disponibilidade de carboidratos não é suficiente, reduzindo-se acentuadamente os níveis de secreção pancreática de insulina e diminuindo o armazenamento de gorduras uma vez que quase toda a energia produzida pelo corpo então deverá derivar da oxidação dos ácidos graxos (Hall, 2011).

O destino do acetil-CoA formado na β-oxidação de ácidos graxos pode ser a entrada no ciclo de Krebs como relatado anteriormente ou também sofrer a conversão a corpos cetônicos quando a quantidade de ácido oxalacético não é suficiente para a quantidade de demanda de acetil-CoA formado. Por isso, são biotransformados a corpos cetônicos como a acetona, acetoacetato e D-β-hidroxibutirato para encaminhamento destes a outros tecidos pela saída do interior mitocondrial para a corrente sanguínea. A enzima acetil-CoA sintetase (ACeCS1)(Figura 3) catalisa a ligação de acetato e CoA para assim produzir acetil-CoA, e sua atividade é regulada por alterações no estado nutricional e hormonal do animal, ou seja, sua atividade cai sob estado de inanição e é reativada após realimentação (Woodnutt e Parker, 1986). Logo, Ikeda et al. (2001) reafirmam que quando a produção de acetato é elevada podem-se observar níveis elevados de ACeCS1 como forma do organismo suportar estes altos níveis de acetato, de maneira que se possa metabolizá-lo de forma eficiente.

O destino da acetona, produzida em quantidades menores em relação aos demais corpos cetônicos pelo fígado, é ser eliminada pela urina (cetonúria) e expelida pela boca o que confere o odor característico “hálito cetônico” em pacientes com diabetes mellitus ou em estado de jejum prolongado. Já o acetoacetato e o D-β-hidroxibutirato são transportados para tecidos extra-hepáticos onde são convertidos em Acetil-CoA que, por sua vez, é oxidado no ciclo de Krebs fornecendo energia necessária para tecidos cerebrais, musculares, cardíacos e o córtex renal (Nelson, 2011). Os níveis sanguíneos aumentados de acetoacetato e D-β-

hidroxibutirato alteram o pH do sangue deixando-o mais ácido que o normal desencadeando acidez sanguínea conhecida como cetose (Araguaia, 2017).

2.4.2 Síntese de ácidos graxos

Além dos lipídios obtidos na dieta, os mesmos também podem ser sintetizados no organismo a partir de outras fontes, principalmente carboidratos e também proteínas. O excesso de carboidratos ou proteínas da dieta são convertidos em ácidos graxos no fígado ou tecido adiposo, o que torna o principal fator no controle da taxa de lipogênese o estado nutricional do indivíduo. Os ácidos graxos sintetizados deverão combinar-se por esterificação com o glicerol que será ativado até glicerofosfato por transferência de um grupo fosfato do ATP a fim de produzir os triacilgliceróis. Uma vez formados, os triacilgliceróis são armazenados nos adipócitos como principal fonte de reserva energética (Nelson e Cox, 2011).

Diferentemente da oxidação dos ácidos graxos, a sua biossíntese ocorre por diferentes vias e são catalisadas por diferentes grupos de enzimas localizando-se em diferentes compartimentos das células (Nelson e Cox, 2011). A síntese de ácidos graxos ocorre no citosol onde a partir de seis atividades enzimáticas e uma proteína transportadora de acila (ACP) na ácido graxo sintase. A primeira reação ocorre a partir do acetil-CoA (transportado para fora da mitocôndria) condensa-se com CO_2 e dá-se a formação de malonil-CoA pela enzima transcarboxilase (acetil-CoA carboxilase). O grupo malonil é transferido à proteína carreadora de acila (ACP) carregando um grupamento (coenzima) 4-fosfopanteína; Como um processo cíclico a acila é adicionada há uma unidade de dois carbonos oriunda de malonila, o grupamento ceto é reduzido a álcool, ocorre uma retirada de água para a formação de uma ligação insaturada que é reduzida, a acila formada é transferida de ACP para outro grupamento. A síntese de ácidos graxos é regulada na etapa de formação de malonil-CoA.

2.5 Modelo de estudo *Drosophila melanogaster*

Modelos animais vertebrados (roedores, sapos, peixes) têm contribuído, cada vez mais, para a compreensão da biologia e fisiologia humana (Burggren e Warburton, 2007; Lieschke e Currie, 2007; Hunziker et al., 2009; Chianese et al., 2011). No entanto, há uma grande problemática na investigação científica nestes modelos uma vez que estes são geneticamente e metabolicamente complexos, limitando a capacidade de fornecimento detalhado de processos fundamentais como, por exemplo, vias de sinalização celular (Diop e

Bodmer, 2012). Dada a complexidade da resposta do organismo a mudanças na dieta, modelos de organismos invertebrados podem servir como ferramentas úteis na descoberta de interação entre genes, vias de sinalização e metabolismo (Oldham e Hafen, 2003; Baker e Thummel, 2007; Leopold e Perrimon, 2007; Perez e Van Gilst, 2008).

Os invertebrados da classe dos insetos compreende um dos maiores grupos classificados na escala zoológica. Possuem o corpo segmentado e dividido basicamente em três regiões: cabeça (percepção sensorial), tórax (principal função locomotora onde asas e pernas estão inseridas) e abdômen (localizam-se as vísceras incluindo componentes do sistema digestivo, excretor e reprodutor) (Apidianakis e Rahme, 2011). Em especial, a conservação de estruturas moleculares, celulares e teciduais entre moscas e mamíferos podem ser utilizadas para investigar vários aspectos da biologia que são relevantes às doenças humanas (Apidianakis e Rahme, 2011). Estas similaridades metabólicas e sinalizadoras compartilhadas por invertebrados e mamíferos dão credibilidade à pesquisas com modelos alternativos, por exemplo, a neurotensina (NT) é um peptídeo predominantemente situado em células especializadas do intestino delgado e liberado para o lúmen quando há ingestão de gordura facilitando assim a translocação de ácidos graxos em ratos (Li et al., 2016). Todavia, consistentes com os achados em ratos, a expressão de NT no invertebrado *D. melanogaster* também foi positivamente descoberta e através deste modelo foi possível concluir que a expressão deste peptídeo pode fornecer um marcador de prognóstico da obesidade no futuro e um alvo potencial para prevenção e tratamento de seus efeitos deletérios (Li et al., 2016).

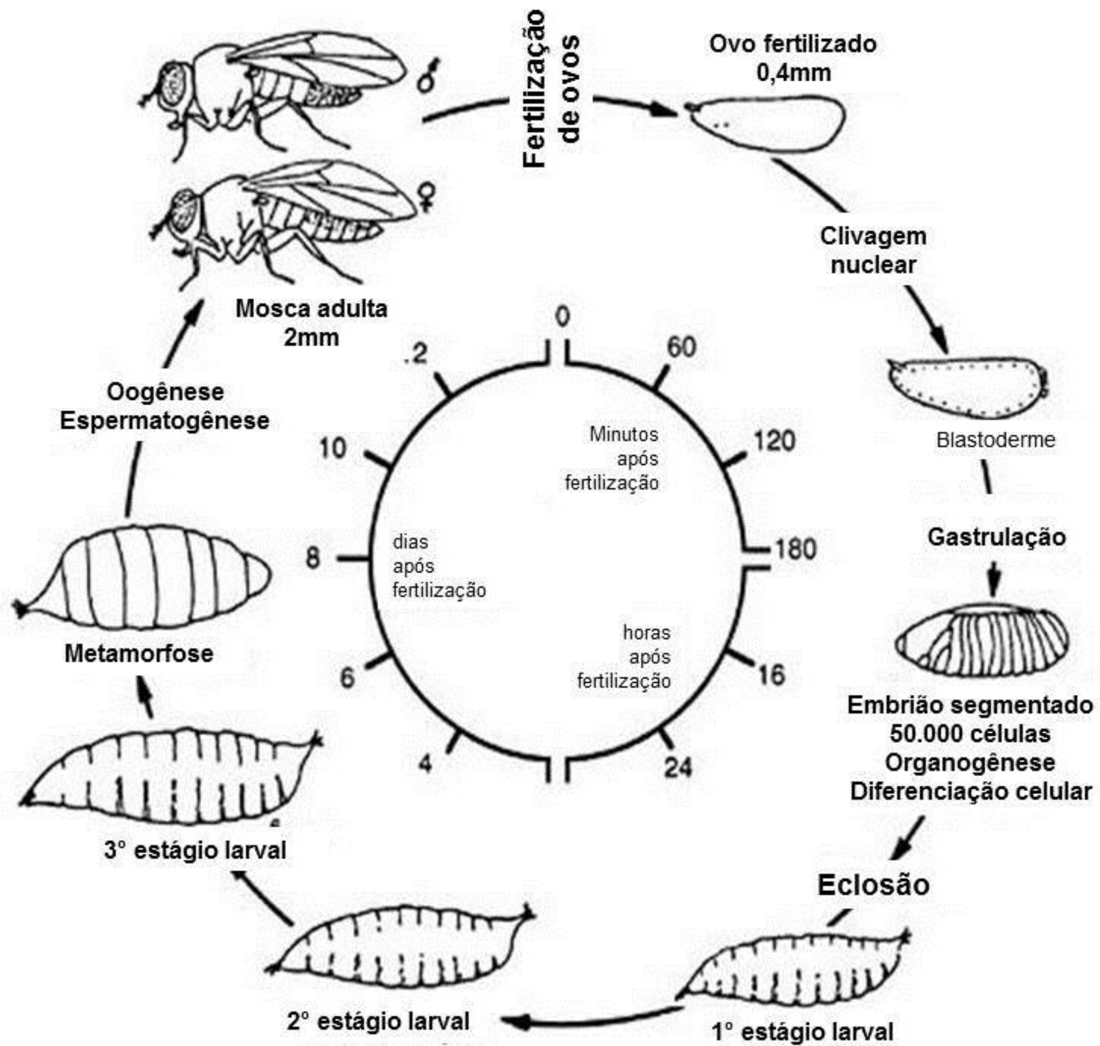
Atualmente, cresce a demanda por modelos animais alternativos que representem uma ferramenta satisfatória para pesquisa bioquímica (Hosamani, 2009; Jeibmann e Paulus, 2009). O inseto *D. melanogaster* (Figura 4), é conhecido popularmente como a mosca da fruta e é geralmente encontrada no dia-a-dia em volta de frutas em estado de putrefação. Esta espécie está classificada na ordem Díptera e família Drosophilidae e, têm se tornado cada vez mais um excelente modelo para diversos estudos sendo eles: análises genéticas, estudos de toxicidade já que é considerado um bioindicador na detecção de poluentes, modelo de doenças neurológicas como Parkinson e Mal de Alzheimer, muito eficaz em testes de ação biológica de substâncias naturais, dentre outros (Nichols, 2006; Benton, 2008; Bharucha, 2009; Hong e Park, 2010; Bagatini et al., 2011).

Figure 4 – Exemplar adulto de *Drosophila melanogaster*

Fonte: Arquivo próprio (2017).

Além disso, *D. melanogaster* compartilha inúmeros genes análogos a humanos, conservam vias metabólicas e sinalizadoras, evidências de conservação em nível comportamental e de seus mecanismos moleculares, incluindo ritmos circadianos, aprendizagem, memória e sono (Nichols, 2006; Benton, 2008; Bagatini et al., 2011; Paula et al., 2012). Ademais, este modelo de estudo torna-se uma boa ferramenta para pesquisas pelas quais envolvam metodologias testadas durante o desenvolvimento embrionário e ciclo de vida já que além do modelo ser livre de impedimentos éticos, o ciclo de vida destes animais é relativamente rápido (Figura 5) e ocorrem no meio externo permitindo a realização satisfatória de estudos relacionados à exposição a compostos ou metais durante este ciclo (Bonilla-Ramirez et al, 2011; Ternes et al., 2014).

Figura 5 – Ciclo de vida da *Drosophila melanogaster*



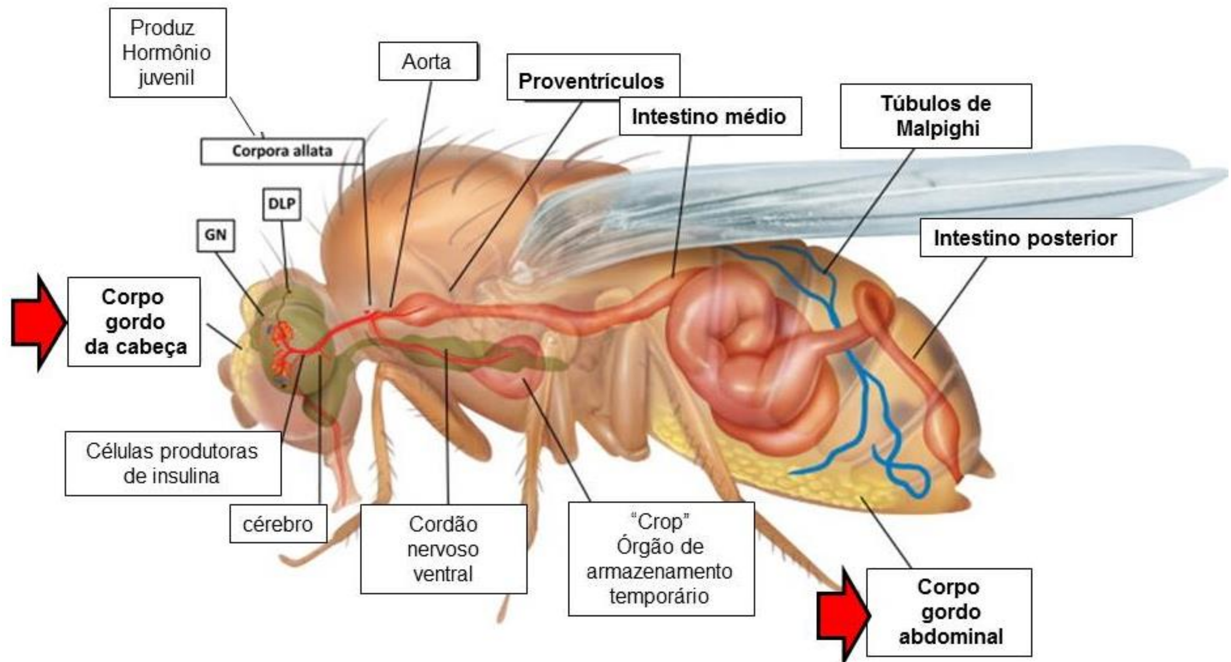
Fonte: Arquivo próprio adaptado de National Research Council (2000).

2.5.1 Metabolismo de gorduras no modelo de *D. melanogaster*

Considerando a grande analogia dos insetos com vertebrados, grande parte de tecidos podem ser estudados de maneira satisfatória em ambos (Law e Wells, 1989). O corpo de gordura de um inseto, como em *D. melanogaster*, é relativamente considerado um grande órgão distribuído por todo o corpo do animal, preferencialmente localizado abaixo do tegumento e em torno do intestino e órgão reprodutivo (Figura 6), sendo considerado um tecido solto conectado diretamente à hemolinfa (Dean et al., 1985). Este corpo gordo desempenha um papel fundamental no metabolismo do animal bem como no armazenamento de gorduras, funcionando, principalmente, como um depósito de nutrientes e energia, ou seja,

é considerado um órgão que apresenta intensa atividade metabólica (Arrese e Soulages, 2010).

Figura 6 – Modelo de *Drosophila melanogaster* representando a localização do corpo de gordura no animal



Fonte: Arquivo próprio adaptado de Tatar et al. (2014)

Considerando que as moscas são insetos holometábolos, animal que durante o seu desenvolvimento apresenta metamorfose completa, este corpo gordo torna-se essencial para a manutenção da vida destas espécies uma vez que em fases larvais devem acumular significativa quantidade de nutrientes para a sobrevivência em fases de inanição e metamorfose (Keeley, 1985). Ao mesmo tempo em que o corpo de gordura dos insetos atua respondendo às suas próprias exigências metabólicas, ele ainda possibilita mobilizar energia para outros tecidos conforme for sua necessidade, atuando também como um órgão endócrino, produzindo vários peptídeos antimicrobianos e participando ativamente em processos de desintoxicação (Hoshizaki, 2005; Jiang et al., 2005; Cheng et al., 2006; Ferrandon et al., 2007).

Por isso, os insetos armazenam reservas de energia na célula básica do corpo de gordura, os adipócitos, caracterizados pela presença de numerosas gotículas lipídicas (Arrese e Soulages, 2010). Estas gotículas lipídicas são compostas, exclusivamente, sob a forma de TGC seguidas de glicogênio e proteínas (Dean et al., 1985). Além disso, é importante ressaltar que em fase adulta, as moscas utilizam o suprimento dietético no preparo para a

reprodução e, assim, para o desenvolvimento do ovo que exige uma mobilização substancial das reservas do corpo gordo para os ovários (Arrese e Soulages, 2010). Porém, quando estes indivíduos adultos não são alimentados dependem exclusivamente das reservas energéticas para a manutenção do equilíbrio metabólico. Por isso, durante períodos de intensa demanda de energia o organismo acessa as reservas de lipídeos através da atuação de enzimas denominadas lipases para então dar início ao processo de lipólise (Brasaemle, 2007; Bickel et al., 2009). Por isso, o corpo gordo de uma mosca adulta está sujeito a uma sobrecarpa lipídica induzida por DRG e, por isso, o modelo de *D. melanogaster* torna-se um bom análogo a estudos em humanos uma vez que este corpo gordo representa um órgão com função análoga ao fígado e tecido adiposo (Canavoso, 2001; Heinrichsen e Haddad, 2012).

Estudos recentes têm investigado os efeitos prejudiciais causados pela ingestão de DRG em modelo de *D. melanogaster* tendo demonstrado um fenótipo claramente deletério aumentando os níveis de triglicerídeos e glicose, diminuindo a tolerância ao estresse, encurtando o tempo de vida podendo induzir acúmulo de gordura e disfunção cardíaca (Birse et al., 2010; Heinrichsen e Haddad, 2012). Portanto, o modelo invertebrado *D. melanogaster* têm se mostrado eficaz no estudo de obesidade já que demonstra alterações fenotípicas, apresentam vias de sinalização do metabolismo de gorduras e insulina conservadas, possuem sistemas de órgãos análogos e que se assemelham aos encontradas em seres humanos (Canavoso et al., 2001; Fontaine et al., 2003; Van Gaal et al., 2006; Odgen et al., 2007; Wang et al., 2007; Wolf e Rockman, 2008; Diangelo e Birnbaum, 2009; Boden e Salehi, 2013).

As vias sinalizadoras de insulina também são peças-chave para a interpretação e controle do metabolismo (Saltiel e Kahn 2001; Tártara et al., 2003; Vellai et al., 2003; Arking et al., 2005; Wang et ai 2005). A espécie *D. melanogaster* possui oito peptídeos semelhantes à insulina (dilp1-8) que são proteínas evolutivamente conservadas e regulam o crescimento, metabolismo, reprodução e longevidade destes invertebrados (Brogiolo et ai, 2001;. Gronke et al., 2010). Estudos já demonstraram que a expressão de dilp6 atua diretamente sobre o crescimento, sobrevivência, armazenamento de carboidratos e gorduras e resistência ao estresse oxidativo (Gronke et al., 2010). Além disso, a expressão de dilp6 no corpo de gordura de moscas pode estar diretamente relacionada à da sinalização de insulina (Bai et al., 2012).

Portanto, *D. melanogaster* e mamíferos compartilham interações recíprocas entre a gordura corporal e a sinalização sistêmica da insulina e identificar os mecanimos responsáveis por estas interações, bem como papéis de reguladores e sinalizadores celulares, pode fornecer uma boa fonte para entender os mecanismos centrais do envelhecimento e sua interação metabólica (Bai et al., 2012). No entanto, a maioria dos estudos que utilizam este modelo de

D. melanogaster se detém a análises genéticas, mas, é imprescindível ampliar os horizontes de pesquisas para este modelo uma vez que moscas e outros insetos também possuem defesas antioxidantes como, por exemplo, as enzimas SOD e CAT (Missirlis et al., 2001).

2.6 Terapias antioxidantes naturais

Nas últimas décadas, a busca por compostos naturais e/ou sintéticos com ação antioxidante tem aumentado notavelmente. Os compostos bioativos, oriundos de produtos naturais como, por exemplo, as plantas e seus compostos polifenólicos têm demonstrado diferentes efeitos benéficos para a saúde humana (Scalbert et al, 2005; Pandey e Rizvi, 2009; Visioli et al, 2011; Del Rio et al, 2013).

Os polifenóis compreendem uma ampla classe de substâncias biologicamente ativas estando distribuídos nas plantas como metabólitos secundários, proporcionando à planta cor e sabor diferente, desempenhando diversas funções como proteção contra radiações e toxinas mostrando, também, um papel fundamental na resistência contra agentes patogênicos (Quideau et al., 2011; Daglia, 2012; Iranshahi et al., 2015). Dentre os polifenóis mais conhecidos e estudados têm-se os flavonóides, conhecidos como compostos bioativos, exibem efeitos medicinais interessantes e benéficos para a saúde humana exibindo propriedades biológicas como: antioxidante, anti-inflamatória, anticancerígena, antibacteriana, imunoestimulantes e atividades antivirais (Harborne e Williams, 2000; Chahar et al, 2011; Spatafora e Tringali, 2012; Nabavi et al, 2013a).

Embora os compostos contendo polifenóis, na sua grande maioria, apresentem baixa toxicidade, é importante ressaltar que a presença de açúcar em sua estrutura pode afetar e alterar a microbiota natural do intestino de alguns indivíduos (Iranshahi et al., 2015). Uma vez que altere esta microbiota, estas substâncias podem perder seu papel protetor e tornar-se um problema na terapia fazendo-se sempre necessário a realização de estudos prévios que determinem as doses adequadas e estruturas para a utilização por seres humanos (Iranshahi et al., 2015).

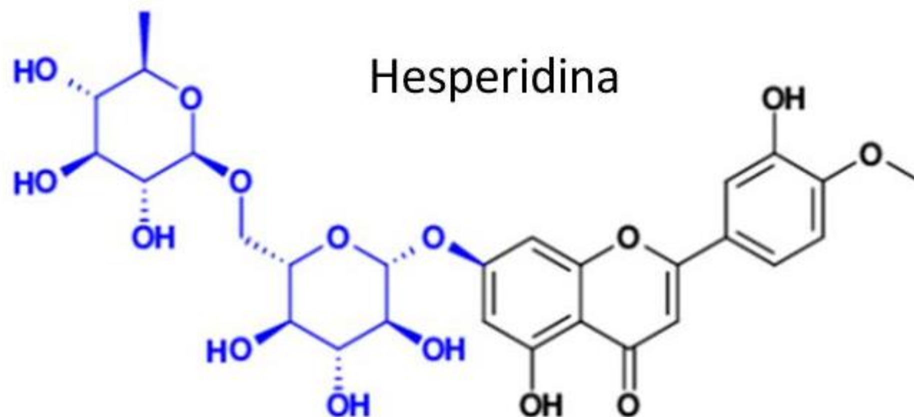
2.6.1 Hesperidina

A hesperidina (Hesp) é um dos constituintes bioativos mais comuns, um flavonóide, encontrado e isolado a partir da casca de uma espécie de Citrus por Lebreton em 1827. A

Hesp é amplamente encontrada no epicarpo, mesocarpo, endocarpo e sucos de frutas cítricas como, por exemplo, o limão e a laranja (Garg et al., 2001; Kanaze et al., 2003).

A Hesp (Figura 7) é um glicosídeo de flavanona, composta por uma unidade aglicona, denominada hesperitina, e um dissacarídeo denominado rutinose (Preston et al., 1953; Evans, 1996).

Figura 7 – Estrutura química da Hesperidina



Fonte: Arquivo próprio adaptado de Cohen (2016).

A Hesp é convertida em hesperitina pela microflora do cólon e, que em seguida, é absorvida (Roohbakhsh et al., 2014). Após sua absorção, alguns autores relatam que a Hesp atua no organismo prevenindo lesões oxidantes e a morte celular por diversos mecanismos, tais como a eliminação de RL e ER, protegendo contra a peroxidação lipídica e atuando como substância quelante de íons metálicos (Fraga et al., 1987, Korkina e Afanas'ev, 1997, Miller e Rice Evans , 1997; Jung et al., 2003; El-Sayed et al., 2007).

Especialmente, as propriedades de eliminação de RL da Hesp se dão, segundo estudos, na propriedade direta de doação de hidrogênio para extinguir ER, transferindo os elétrons e prótons reduzindo superóxidos e o hidroperóxidos (Fraga et al., 1987; Ratty e Das, 1988; Yuting et al, 1990; Wang e Zheng, 1992; Jovanovic et al., 1994; Deng et al., 1997; Miller e Rice-Evans, 1997; Miyake et al., 1997; Suarez et al., 1998; Malterud e Rydland, 2000). Além disso, quanto à sua atividade antioxidante, a Hesp demonstrou uma interação direta com o sistema de defesa antioxidante endógeno, uma vez que aumentou as defesas celulares antioxidantes como, por exemplo, vias de sinalização de ERK e Nrf2 (Chen et al., 2010; Elavarasan et al., 2012).

Dentre as propriedades medicinais da Hesp estão relacionadas diversas atuações deste flavonoide como: diminuir a permeabilidade e a fragilidade nas paredes dos capilares pela inibição da enzima hialuronidase e, podendo prevenir doenças como diabetes, insuficiência venosa crônica, hemorroidas, escorbuto, úlceras e hematomas; reduzir significativamente os níveis de colesterol, lipoproteínas de baixa densidade (LDL), lipídeos totais e TGC aumentando os níveis de proteínas de alta densidade (HDL); efeitos anti-hipertensivos devido a sua atividade em sistemas enzimáticos que influenciam no fluxo sanguíneo e efeitos diuréticos devido ao seu potencial inibidor da enzima fosfodiesterase que hidrolisa o AMPc (adenosina monofosfato cíclico) transformando-o em AMP inativando proteínas quinases; efeito anti-inflamatório pela diminuição da liberação de histamina ou pela eliminação de RL; atividades antimicrobianas, bactericidas e antifúngicas demonstradas em estudos *in vitro*; efeito radioprotetor; dentre outras inúmeras outras atividades e propriedades benéficas para a saúde (Morii, 1939; Scarborough, 1940; Higby, 1941; Beiler e Martin, 1947; Choi et al., 1991; Son et al., 1991; Emim et al., 1994; Galati et al., 1994; Jean e Bodinier, 1994; Struckmann e Nicolaides, 1994; Allegra et al., 1995; Monforte et al., 1995; Galati et al., 1996; Islam e Ahsan, 1997; Amiel e Barbe, 1998; Korthuis e Gute, 1999; Kim et al., 2000; Garg et al., 2001; Fardid et al., 2016; Jadeja et al. 2016; Haghmorad et al., 2017; Jawien et al., 2017; Sarkar et al., 2017; Shehata et al., 2017).

Portanto, a Hesp possui efeitos benéficos tanto *in vitro* como *in vivo*, desempenhando papel antioxidante, antimicrobiano, anti-inflamatório, anti-hiperlipidêmico, anticancerígeno e antialérgicos dentre outros (Garg et al., 2001; Roohbakhsh et al., 2014; Parhiz et al., 2015). No entanto, os efeitos benéficos protetores ou estudos de toxicidade da hesperidina em modelo da mosca da fruta *D. melanogaster* ainda precisam ser amplamente investigados.

3 OBJETIVOS

3.1 Objetivo geral

O presente trabalho teve como principal objetivo investigar as alterações comportamentais, bioquímicas e genéticas induzidas por uma dieta rica em gordura em exemplares adultos e descendentes da mosca da fruta *D. melanogaster*, bem como os efeitos terapêuticos da Hesperidina frente a estas alterações.

3.2 Objetivos específicos

- ✓ Analisar a sobrevivência e valores de peso corporal de *D. melanogaster* expostas a diferentes concentrações de uma dieta rica em gordura e ao composto hesperidina.
- ✓ Avaliar o desempenho locomotor de *D. melanogaster* após tratamento com diferentes concentrações de uma dieta rica em gordura e hesperidina.
- ✓ Avaliar a atividade da enzima acetilcolinesterase em resposta à dieta rica em gordura e ao composto hesperidina em *D. melanogaster*.
- ✓ Avaliar a atividade das enzimas antioxidantes em resposta à dieta rica em gordura e ao composto hesperidina em *D. melanogaster*.
- ✓ Avaliar a modulação da fosforilação e expressão de proteínas de enzimas reguladoras da oxidação de ácidos graxos.
- ✓ Realizar uma avaliação de atividade metabólica mitocondrial nas moscas após o tratamento com uma dieta rica em gordura e com hesperidina.
- ✓ Realizar a dosagem dos níveis de glicose e triglicerídeos nas *D. melanogaster* submetidas ao protocolo de exposição.
- ✓ Avaliar os efeitos causados desde o período de desenvolvimento embrionário de *D. melanogaster* expostas à dieta rica em gordura e/ou aliada ao composto hesperidina.

4 ARTIGOS CIENTÍFICOS

A metodologia realizada e os resultados obtidos que fazem parte desta tese estão apresentados sob a forma de artigo científico e manuscritos, que se encontram neste item. Além disso, aqui constam as seções: Materiais e Métodos, Resultados, Discussão e Referencias Bibliográficas.

4.1 Artigo 1: Efeitos da dieta rica em gordura (DRG) em exemplares adultos de *Drosophila melanogaster*

4.1.1 Dieta rica em gordura induz estresse oxidativo e expressão gênica de MPK2 e HSP83 em *Drosophila melanogaster.*

4.2 Manuscrito 1: Efeitos da dieta rica em gordura (DRG) em modelo da mosca da fruta *Drosophila melanogaster* quando expostas ao óleo de coco durante o período de desenvolvimento embrionário.

4.2.1 *Drosophila melanogaster* as model to study maternal obesity effects on expression of antioxidant genes and developmental changes on descendents.

4.3 Manuscrito 2: Efeito da Hesperidina frente às alterações comportamentais, bioquímicas e genéticas induzidas por uma dieta rica em gordura em moscas adultas e descendentes da espécie *Drosophila melanogaster*.

4.3.1 Hesperidin ameliorates metabolic alterations, oxidative stress and protein signaling in the *Drosophila melanogaster* model of high-fat diet

ARTIGO 1

High-Fat Diet Induces Oxidative Stress and MPK2 and HSP83 Gene Expression in *Drosophila melanogaster*

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Research Article

High-Fat Diet Induces Oxidative Stress and MPK2 and HSP83 Gene Expression in *Drosophila melanogaster*

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The consumption of a high-fat diet (HFD) causes alteration in normal metabolism affecting lifespan of flies; however molecular mechanism associated with this damage in flies is not well known. This study evaluates the effects of ingestion of a diet supplemented with 10% and 20% of coconut oil, which is rich in saturated fatty acids, on oxidative stress and cells stress signaling pathways. After exposure to the diet for seven days, cellular and mitochondrial viability, lipid peroxidation and antioxidant enzymes SOD and CAT activity, and mRNA expression of antioxidant enzymes HSP83 and MPK2 were analyzed. To confirm the damage effect of diet on flies, survival and lifespan were investigated. The results revealed that the HFD augmented the rate of lipid peroxidation and SOD and CAT activity and induced a higher expression of HSP83 and MPK2 mRNA. In parallel, levels of enzymes involved in lipid metabolism (ACSL1 and ACeCS1) were increased. Our data demonstrate that association among metabolic changes, oxidative stress, and protein signalization might be involved in shortening the lifespan of flies fed with a HFD.

1. Introduction

Obesity is a chronic multifactorial disease, result of positive energy balance, where food intake is greater than energy expenditure. This overweight predisposes the organism to a series of diseases such as cardiovascular problems, diabetes, and sleep apnea [1, 2]. Given that, excessive food intake is often directly linked to the consumption of foods rich in fat and the increase in the amount of fatty acids in the diet causes an imbalance in the metabolism [3].

A high-fat diet (HFD) causes damage at the cellular and molecular levels, and it triggers an oxidative stress process. Studies have demonstrated that this oxidative stress process generates different responses such as the activation of signaling pathways implicated in protecting cells against oxidative damage, such as heat shock proteins (HSP) and mitogen-activated protein kinase (MAPK) [3], peroxidation of lipids and modification of proteins [4, 5], and insulin resistance [6, 7]. Moreover, a HFD promotes an increased supply of triglycerides and fatty acids and consequently it results in an increase of fatty acids oxidation in order to produce energy.

Therefore, the study of biochemical mechanisms involved in the cellular responses to changes in the diet requires close attention to several metabolic pathways, given the complexity of the organism, since in cellular signaling pathways the interaction between genes and protein expression changes

at the transcriptional level [8–11]. Acyl-CoA Synthetase and Acetyl-CoA Synthetase are enzymes present in the fatty acid metabolism, preserved in *D. melanogaster*, and they can be used as parameters to quantify the production of acetate, one of the main metabolites that play an important role in lifespan regulation [12].

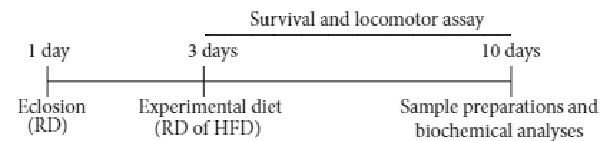
In the last decade, drosophila has been a major model system for the study of obesity and metabolic syndrome combined with oxidative stress [13, 14]. Body fat in insects such as fruit fly *Drosophila melanogaster* plays a key role throughout its development, to meet the new physiological and energy needs [15]. Several phenotypic changes related to obesity in humans can be observed in the *Drosophila melanogaster* model, since flies exposed to a diet rich in fatty acids showed fat accumulation, cardiac dysfunction, increased levels of triglycerides, decreased levels of stress tolerance, and shortening of lifespan, reinforcing the idea of using drosophila as an excellent model [1, 16–18].

Studies focused on cellular stress facing the high consumption of lipids in drosophila are scarce. Thus, this work aimed to investigate the oxidative damage, antioxidant enzymes, and modulation of cells stress signaling pathway in response to a high lipid diet contributing to the knowledge about the cellular response to alteration of diet content in flies.

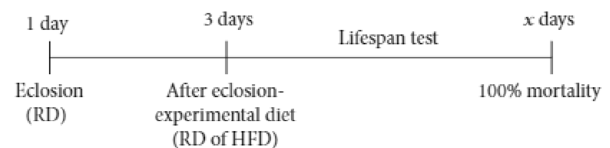
2. Materials and Methods

2.1. Materials and Fly Culture Condition. In order to perform the treatments, the following items were used: virgin coconut oil produced by Pró-Ervas®. Chemicals, including thiobarbituric acid (TBA), 2′,7′-dichlorofluorescein diacetate (DCF-DA), cocktail protease inhibitor, sodium orthovanadate, 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide, ethylenediaminetetraacetic acid (EDTA), quercetin, N,N,N′,N′-tetramethylethylenediamine (TEMED), mannitol, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 7-hydroxy-3H-phenoxazin-3-one 10-oxide (Resazurin), and β-mercaptoethanol, were procured from Sigma-Aldrich Co., LLC, St. Louis, MO, USA. Fatty acid and Lipid Metabolism Antibody Sampler Kit and β-actin antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). Triglycerides liquiform and glucose liquiform were obtained from Labtest (Lagoa Santa, MG, Brazil). The sodium dodecyl sulfate was procured from GE Healthcare Life Sciences (Little Chalfont, Bucks, ENG). Trizol Reagent and DNase I were obtained from Invitrogen (Grand Island, NY). iScript cDNA Synthesis Kit was from Bio-Rad (Laboratories, Montreal, Quebec). Tris(hydroxymethyl)aminomethane, hydrogen peroxide, TRITON X-100, and dimethyl sulfoxide were purchased from Synth (Diadema, SP, Brazil).

Drosophila melanogaster wild type (strain Harwich) was obtained from the National Species Stock Center, Bowling Green, Ohio, USA. The newly hatched flies were maintained for about 3 days in an incubator with controlled temperature of 25°C and 30–50% humidity under a light/dark cycle of 12 h fed on standard medium (1% yeast w/v, 2% w/v sucrose, 1% w/v milk powder, 1% agar w/v, and 0.08% v/w nipagin).



Scheme 1



Scheme 2

2.2. Experimental Diets: Regular Diet (RD) and High-Fat Diet (HFD). All the flies were fed on a regular diet containing corn flour (76.59%), wheat germ (8.51%), sugar (7.23%), milk powder (7.23%), and salt (0.43%). *D. melanogaster* (both genders) aged from 1 to 3 days were divided in three groups of 30 flies each: (1) 0% coconut oil (regular diet, RD); (2) 10% coconut oil; (3) 20% coconut oil. The macronutrient compositions of the regular diet or coconut-supplemented diets are given in Table 1. The concentration of coconut oil used in this protocol is in accordance with Heinrichsen et al. [3].

2.3. Lifespan, Survival, and Body Weight. The flies were exposed to the treatments for 7 days; at the end of the treatments the flies were used for the different assays. The survival rate was evaluated by a daily count of the number of living flies until the end of the experimental period (Scheme 1). In addition, body weight was registered at the beginning and at the end of the treatment.

In order to test the lifespan, three days after the eclosion, the flies were maintained under different experimental diets which were changed every two days, until there were no more flies alive (Scheme 2).

2.4. Locomotor Assay: Negative Geotaxis. The locomotor performance of the flies fed with a RD and a HFD was investigated using the negative geotaxis test, according to Coulom and Birman [19], with minor modifications. Ten flies fed with a HFD and a RD were immobilized under light anesthesia with ice and they were placed separately in a vertical glass column (15 cm long and 1.5 cm in diameter). After 30 minutes of recovery from the anesthesia, the flies were gently tapped to the bottom of the column and the time they took to reach the height of 8 cm was recorded. The tests were repeated five times for each fly at on minute intervals. Results are presented as mean time ± SE (s) of three independent experiments.

2.5. Biochemical Assays

2.5.1. Determination of DCF-DA Oxidation: Reactive Species Levels (RS). For the quantification of reactive species generation (RS) a total of 20 flies were anesthetized on ice and homogenized in 1 mL 10 mM Tris-buffer, pH 7.4, after the end

Table 1: Composition of regular diet (RD) and high-fat diets (HFD).

	RD	HFD 10%	HFD 20%
Energy (kcal/g)	4.039	4.49	4.96
Carbohydrate (weight, %)	89.26	80.13	72.55
Protein (weight, %)	8.66	7.77	7.04
Total fat (weight, %)	2.07	12.08	20.40
Total saturated fat (weight, %)	1.08	10.00	17.71
6:0	0	0.03	0.07
8:0	0	0.05	0.10
10:0	0	0.49	0.94
12:0	0.03	0.61	1.13
14:0	0.13	2.02	3.6
16:0	0.61	1.71	2.47
18:0	0.20	0.61	0.91
20:0	0.01	0.01	0.01
24:0	0.001	0.002	0.002
Total monounsaturated fatty acids (weight, %)	0.60	1.35	1.81
14:1	0.007	0.01	0.01
16:1	0.02	0.04	0.06
18:1	0.55	1.30	1.78
20:1	0.002	0.003	0.003
Total polyunsaturated fatty acids (weight, %)	0.38	0.72	0.88
18:2 n-6	0.40	0.63	0.69
18:3 n-6	0.01	0.02	0.03
18:1t	0.04	0.06	0.08
18:2t	0.005	0.007	0.009

HFD 10% and HFD 20%: high-fat diets were performed with a coconut oil addiction.

of the treatments. The homogenate was centrifuged at 1000 ×g for 5 minutes at 4°C and the supernatant was removed for assay quantification of 2′,7′-dichlorofluorescein diacetate (DCF-DA) oxidation, as a general index of oxidative stress according to the protocol of Pérez-Severiano et al. [20]. The fluorescence emission of DCF resulting from DCF-DA oxidation was monitored after one hour at an excitation wavelength of 485 nm and an emission wavelength of 530 nm in the spectrophotometer. The rate of DCF formation was calculated as a percentage of the fluorescence of the treatments in relation to the RD group.

2.5.2. Determination of Lipid Peroxidation. In order to analyze lipid peroxidation, 20 flies were homogenized in 1 mL of HEPES buffer 20 mM, pH 7.0, and centrifuged at 1000 ×g for 10 minutes (4°C), after the end of the treatments. The supernatant was removed for assay, following the method of Ohkawa et al. [21], with minor modifications. Briefly, the 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 minutes, and the absorbance was then measured at 532 nm. Results represent the mean of three independent experiments (performed in duplicate). The TBARS values were normalized by protein concentration and are expressed as a percentage of TBARS production in relation to the RD group.

2.5.3. Triglyceride, Glucose, and Protein Measurements. After seven days of exposing *D. melanogaster* to the diets, 20 whole flies were anesthetized, homogenized, and prepared, as described by Grönke et al. [22] for the analysis of total triglycerides and as described by Birse et al. [16] for the analysis of glucose. Both were measured by using the specific Labtest® kit. Protein concentrations of the homogenate were determined by the method of Bradford [23], using bovine serum albumin as the standard.

2.5.4. Enzyme Assays. For the analysis of enzymes activity, 20 flies were homogenized in 1 mL of 10 mM Tris-buffer, pH 7.4, and then centrifuged at 1000 ×g for 10 minutes (4°C). An aliquot was taken for the analysis of acetylcholinesterase activity (AChE; EC 3.1.1.7) in a reaction mixture containing phosphate buffer (0.25 M, pH 8.0), 5,5-dithiobis 2-nitrobenzoic acid (5 mM), 50 μL sample, and 25 μL acetylthiocholine iodide (7.25 mM). The reaction was monitored for two minutes at 412 nm. Enzyme activity was expressed as nanomoles of substrate hydrolyzed per minute per milligram protein [24].

The remaining supernatant was centrifuged at 20000 ×g for 30 minutes (4°C) and it was used for the analysis of antioxidant enzymes. Catalase activity (CAT; EC 1.11.1.6) was determined according to Aebi [25] with minor modifications

by Paula et al. [26]. The reaction mixture contained phosphate buffer (0.25 M/EDTA 2.5 mM, pH 7.0), H_2O_2 (10 mM), 0.012% Triton X 100, and 30 μL sample. The decay in H_2O_2 was monitored during one minute at 240 nm and expressed as micromole of H_2O_2 decomposed/min/mg protein.

Superoxide dismutase activity (SOD, EC 1.15.1.1) was measured according to Kostyuk and Potapovich [27], with minor modifications by Franco et al. [28]. It was performed by monitoring the inhibition of quercetin autooxidation. The reaction mixture contained sodium phosphate buffer (0.025 M/EDTA 0.1 mM, pH 10), N,N,N,N-tetramethylethylenediamine (TEMED), and 10 μL sample and it was started by adding 0.15% quercetin dissolved in dimethyl formamide. The reaction was monitored for two minutes at 406 nm, and it is expressed as the amount of protein required to inhibit 50% of quercetin autooxidation.

2.5.5. Preparation of Mitochondrial Enriched Fractions and Metabolic Activity. A mitochondrial enriched fraction was prepared from the whole body of flies using the method of differential centrifugation [29]. Briefly, flies were homogenized in ice-cold Tris-sucrose buffer (0.25 M, pH 7.4) (60 mg fly tissue homogenized in 1000 μL buffer) and centrifuged at 1000 ×g for five minutes (4°C). A fraction enriched with mitochondria was obtained by centrifuging the postnuclear supernatant at 10000 ×g for 10 minutes (4°C). The pellet was washed in mannitol-sucrose-HEPES buffer and resuspended in 200 μL of suspension buffer.

A portion of 200 microliters of the mitochondrial enriched fraction (200 microliters) was incubated with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide MTT (x% solution) for 30 minutes at 37°C. After that, the samples were centrifuged at 10000 ×g for five minutes. The pellet was dissolved in dimethyl sulfoxide (DMSO), incubated for 30 minutes at 37°C, and the absorbance was measured at 540 nm. Results were expressed as percentage of the control.

2.5.6. Dehydrogenases Activity of Drosophila melanogaster. An investigation of the dehydrogenases activity was carried out for the whole flies' homogenate and it was performed by using the CellTiter-Blue® cell viability assay kit [30]. The method is based on the ability of viable cells to reduce resazurin to resorufin, a fluorescent molecule. Whole flies were homogenized in 10 mM Tris-buffer, pH 7.4, and 100 μL pipettedin, a 96-well plate, and an aliquot of CellTiter-Blue was added according to the instructions of the manufacturer. After 1 h, the fluorescence was recorded at $_{ex}$579 nm and $_{em}$584 nm.

2.6. Western Blotting. After the treatments, groups of 40 whole flies were mechanically homogenized at 4°C in 200 μL of buffer (pH 7.0) containing 50 mM Tris, 1 mM EDTA, 20 mM Na_3VO_4, 100 mM sodium fluoride, and protease inhibitor cocktail. Then, the homogenate was centrifuged for 10 min at 1000 ×g at 4°C and the supernatant was collected. After protein determination according to Bradford [23], 4% SDS solution, ß-mercaptoethanol, and glycerol were added to samples to a final concentration of 100, 8, and 25%, respectively, and the samples were frozen for further analysis. The proteins were separated by SDS-PAGE using 10% gels and then electrotransferred to nitrocellulose membranes as previously described by Paula et al. [26]. Membranes were washed in Tris-buffered saline with Tween 100 mmol/L Tris-HCl, 0.9% NaCl, and 0.1% Tween-20, pH 7.5, and incubated overnight (4°C) with specific primary antibodies anti-Acetyl-Coenzyme A Synthetase (ACeCS 1) and anti-Acyl-Coenzyme Synthetase (ACSL 1) and anti-β actin. Subsequently, the membranes were washed in Tris-buffered saline with Tween and incubated for 1 h at 25°C with anti-rabbit Ig-secondary antibodies. Antibody binding was visualized using the ECL Western Blotting Substrate Kit (Promega). Band staining density was quantified using the Scion Image software (Scion Image for Windows). Results are expressed as optical density of ACSL1 or ACeCS1/optical density of respective ß-actin.

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

Genes	Primers sequences
Tubulin	LEFT 5′-ACCAATGCAAGAAAGCCTTG 3′
	RIGHT 5′-ATCCCCAACAACGTGAAGAC 3′
Catalase	LEFT 5′-ACCAGGGCATCAAGAATCTG 3′
	RIGHT 5′-AACTTCTTGGCCTGCTCGTA 3′
Superoxide dismutase	LEFT 5′-GGAGTCGGTGATGTTGACCT 3′
	RIGHT 5′-GTTCGGTGACAACACCAATG 3′
HSP83	LEFT 5′-CAAATCCCTGACCAACGACT 3′
	RIGHT 5′-CGCACGTACAGCTTGATGTT 3′
MPK2	LEFT 5′-GGCCACATAGCCTGTCATCT 3′
	RIGHT 5′-ACCAGATACTCCGTGGCTTG 3′

2.7. Quantitative Real-Time RT-PCR. A Trizol Reagent (Invitrogen, NY) based on RNA extraction was employed using 20 flies, according to the manufacturer's instruction. After quantification, the total RNA was treated with DNase I (Invitrogen, NY) and the cDNA was synthesized with MMLV RNAse H reverse transcriptase contained in the iScript cDNA Synthesis Kit (Bio-Rad), using both oligodT and random primers, following the manufacturer's protocol (Table 2). Quantitative real-time polymerase chain reaction was performed in 15 μL reaction volumes containing 14 μL of SYBR Select Master Mix 1x PCR Buffer plus 1 μL of the sample containing 60 ng/μL of cDNA in a 7500 Fast & 7500 Real-Time PCR System (Applied Biosystems, NY). The qPCR cycling protocol was the following: 50°C for two minutes and 95°C for two minutes, followed by 40 cycles of 15 seconds at 95°C, one minute at 60°C, and 30 seconds at 72°C. All samples were analyzed as technical and biological triplicates with a negative control. Threshold and baselines were manually determined using the StepOne Software v2.0 (Applied Biosystems, NY). RNA input normalization was performed with two genes (Tubulin and GAPDH), and the stability was evaluated and confirmed by geNorm [31]. The $2^{-\Delta\Delta ct}$ method [32] was used to calculate the relative expression.

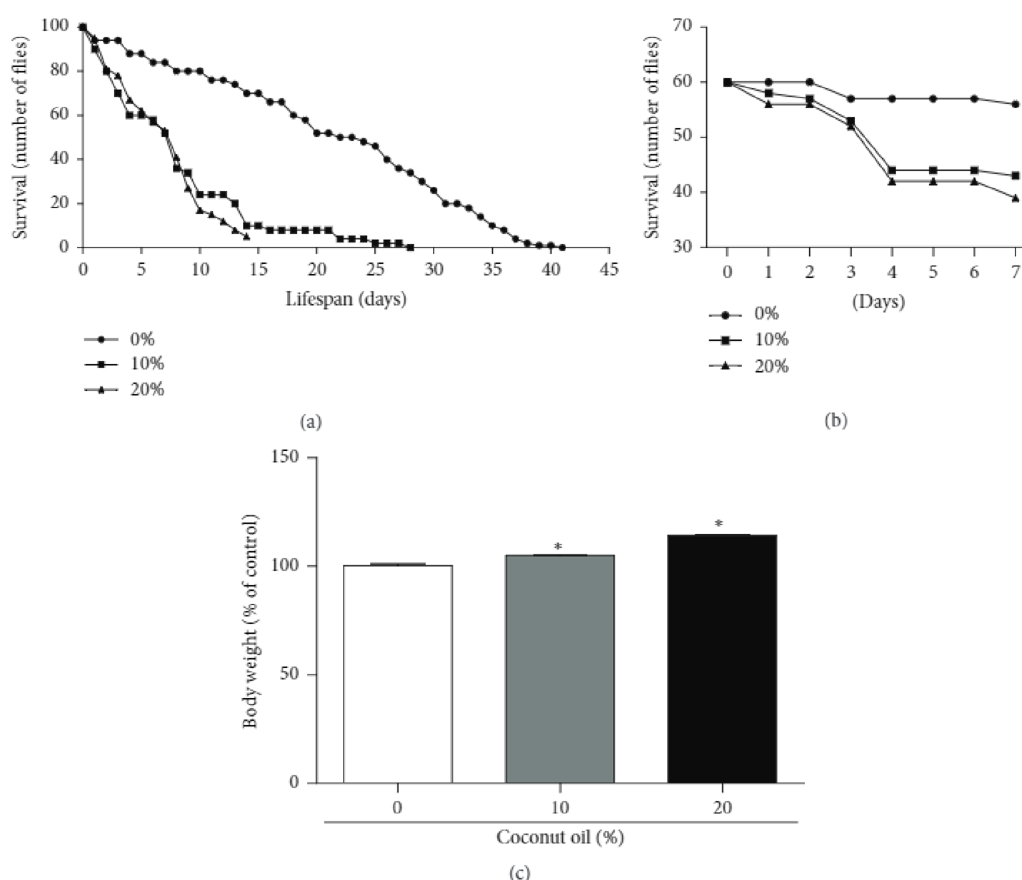


FIGURE 1: Lifespan and survival curves and body weight due to a high-fat diet. Wild type *Drosophila melanogaster* (strain Harwich: both sexes) were placed on regular diet (RD) and high-fat diet (HFD) under controlled conditions. (a) Lifespan: the flies were counted daily until there were no more flies alive. Coconut oil (10% and 20%) in the diet caused a significant decrease in lifespan. Four sets of twenty-five flies were used for each diet condition. (b) Survival curve in seven days: the number of live and dead flies was counted every 24 h during seven days of the treatment and exposure of diets. Coconut oil (10% and 20%) in the diet caused a significant decrease in survival rate. Three sets of twenty flies were used for each diet condition. (c) Body weight was evaluated at first and seventh days after the exposure to the different diets (data expressed in percentage of the 0% coconut oil group, RD). Three sets of twenty flies were used for each diet condition (mean ± standard deviation). $^*p < 0.05$ in relation to RD group.

2.8. Statistical Analysis. Lifespan measurement was determined by comparing the survival curves with a log-rank (Mantel-Cox) test. Other statistical analyses were performed using one-way ANOVA followed by Newman-Keuls *post hoc* test. Differences were considered statistically significant when $p < 0.05$. The Graph Pad Prism 5 Software was used for artwork creation.

3. Results

3.1. High-Fat Diet Reduces Lifespan and Survival Rate Causing Alteration in Body Weight, Triglycerides, and Glucose Levels. According to Figure 1(a), the lifespan of flies receiving a RD was up to 41 days. However, when adding coconut oil at both concentrations tested (HFD: 10% to 20%), the maximum life span drops to 28 and 15 days, respectively. Furthermore, seven days of exposure of flies to a HFD (10% and 20% coconut oil) caused significant reduction on survival (28% and 35%, resp.) when compared with the RD group ($p < 0.05$) (Figure 1(b)). In addition, seven days of exposure to coconut oil in the diet (10% and 20%) caused a significant increase in the body weight of flies (5% and 14%, resp.) (Figure 1(c)).

The levels of triglycerides and glucose were measured in *D. melanogaster* exposed to a HFD. At the concentrations of 10% and 20% of coconut oil, the levels of triglycerides in flies increased 66% and 105%, respectively, when compared to the RD group (0% coconut oil) (Figure 2(a)). However, the HFD (10% and 20% coconut oil) caused a decrease in the glucose levels in 49% and 60%, respectively, when compared to the RD group (0% coconut oil) (Figure 2(b)).

3.2. Locomotor Performance and Acetylcholinesterase Activity. The exposure of flies to a HFD also had a significant deleterious impact on locomotor behavior. Flies fed for seven days with 10% and 20% coconut oil took two to five times longer to reach to the 8 cm measurement on the containers

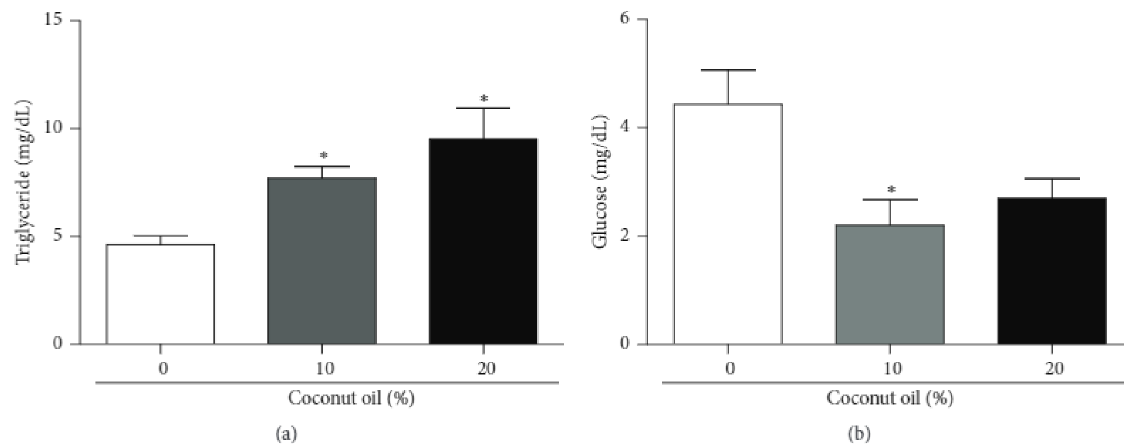


Figure 2: Diet enriched with coconut oil increased augmented triglyceride levels and decreased glucose levels in *D. melanogaster*. After seven days on the diets, three sets of twenty flies of each diet condition were used for triglyceride and glucose measurements. (a) The data expresses triglycerides levels in whole flies homogenate expressed in mg/dL. (b) Glucose levels in flies homogenate expressed in mg/dL. $^*p < 0.05$ in relation to the RD group.

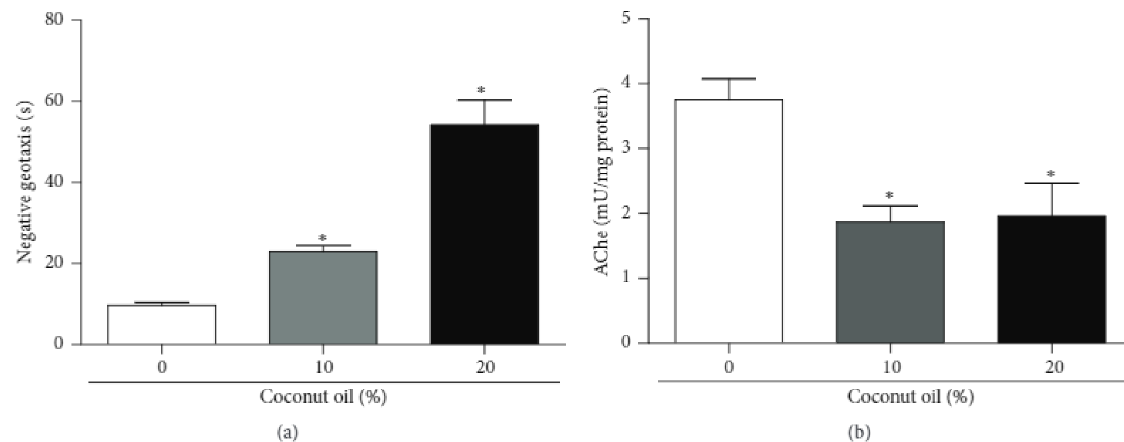


Figure 3: Effects of the exposure to a high-fat diet on locomotor performance and acetylcholinesterase (AChe) activity in *D. melanogaster*. (a) Locomotor ability of flies was analyzed by negative geotaxis after seven days of exposure to coconut oil enriched diet (0%, 10%, and 20%). Three sets of ten flies were used for each diet condition. Results are expressed as mean of time spent to reach 8 cm in a glass tube ± SE of three independent experiments. (b) The data shows the AChe activity in flies' homogenate expressed as mean (mU/mg protein) ± standard deviation. Three sets of twenty flies were analyzed for each diet condition. $*$ indicates a significant difference in the RD and the HFD ($p < 0.05$).

(Figure 3(a)). Thus, it is considered that the higher the concentration of coconut oil in the diet of drosophila, the lower their mobility capabilities in the negative geotaxis test.

Additionally, the activity of acetylcholinesterase (Ache) in *D. melanogaster* exposed to a HFD and coconut oil was also determined, which resulted in a decrease of AChe activity (Figure 3(b)).

3.3. Oxidative Stress Markers and Antioxidant Defenses. Oxidative stress is directly linked to the accumulation of fat in mice and humans; however, the mechanism implicated in flies is not fully understood. In this study we quantify DCF-DA oxidation as a general indicator of oxidative stress and TBARS as an indicator of lipid peroxidation (Figures 4(a) and 4(b)). Flies fed with 20% coconut oil in the diet had a significant increase of 47% in the production of reactive species measured by the oxidation of DCF-DA in the RD group (Figure 4(a)). Regarding the levels of lipid peroxidation, both concentrations tested (10% and 20%) reported a significant increase of 11% and 19%, respectively, compared to the RD group (0% coconut oil) (Figure 4(b)).

The levels of the activity of the antioxidant enzymes CAT and SOD were determined (Figures 4(c) and 4(d)). After

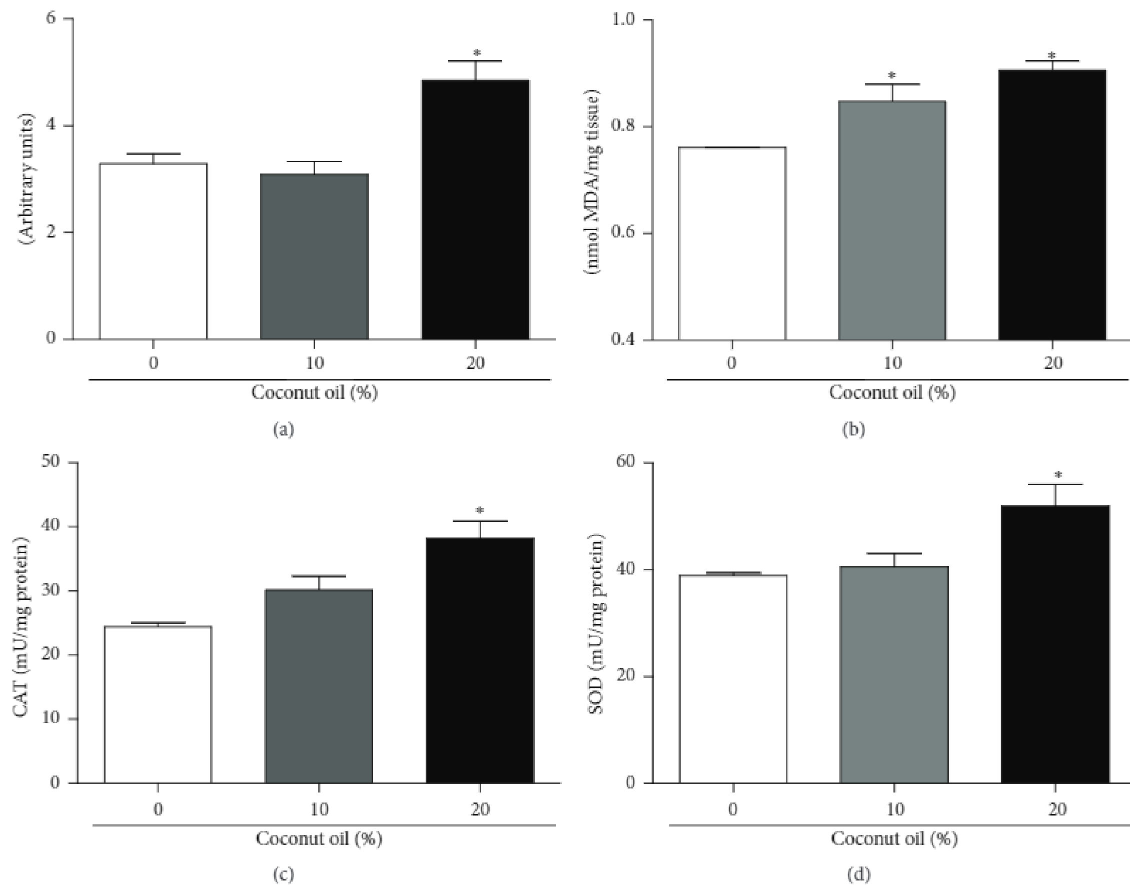


FIGURE 4: Effects on oxidative stress parameters in flies treated for seven days with a regular diet (RD) and a high-fat diet (HFD). After seven days on the diets, flies were homogenized and the supernatant was used for various analyses of stress markers and the activity of antioxidant enzymes. (a) Showing the DCF-DA intensity of fluorescence in total flies homogenate. Three sets of twenty flies were used for each diet condition. (b) End products of lipid peroxidation determined by TBARS assay in total flies homogenate, expressed in MDA nmoles/mg of protein. Three sets of twenty flies were analyzed for each diet condition. (c) Catalase (CAT) activity and (d) superoxide dismutase (SOD) activity in total flies homogenate. Three sets of twenty flies were analyzed for each diet condition. Data are expressed as a mean ± standard deviation in mU/mg protein. * indicates a significant difference in relation to RD ($p < 0.05$).

seven days of treatment, the flies that received 20% coconut oil concentration in the diet demonstrated a significant increase in the activity of both catalase (CAT) and superoxide dismutase (SOD) enzymes.

3.4. Metabolic Activity of Mitochondrial Enriched Fraction and Dehydrogenases Activity in Response to a HFD. Dehydrogenases activity was assessed by the resazurin reduction test and the metabolic activity was assessed by MTT assay. Both tests showed a significant drop in dehydrogenases and metabolic activity for all coconut oil concentrations tested (10% and 20%) (Figures 5(a) and 5(b)).

3.5. Coconut Oil Exposure in Diet by Seven Days Increases the Levels of ACeCS1 and ACSL1. Acetyl-Coenzyme A Synthetase (ACeCS 1) and Acyl-Coenzyme Synthetase (ACSL 1) were investigated in flies exposed to coconut oil (10% and 20%) in the diet during seven days. There was a significant increase in ACeCS1 content in the groups that have consumed 10% and 20% of coconut oil in diet, respectively, when comparing to control group (Figure 6(b)). ACSL1 content was increased in both concentrations tested comparing to control (Figure 6(b)). Data expresses a ratio of optical density of the bands in relation to β-actin.

3.6. Quantitative Real-Time PCR (QRT-PCR) Analysis of HSP83, MPK2, SOD, and CAT mRNA in Flies. Flies were exposed to 0%, 10%, and 20% coconut oil in their food for seven days. We used qRT-PCR to quantify levels of mRNA, relative to the respective RD groups, after exposure. The data were normalized against TUBULIN transcript levels. The concentration of 20% coconut oil causes significant increase in HSP83 and MPK2 expression of mRNA levels (Figures 7(a) and 7(b), resp.). Although the statistical tests did not reveal significant difference, SOD and CAT mRNA levels seem to

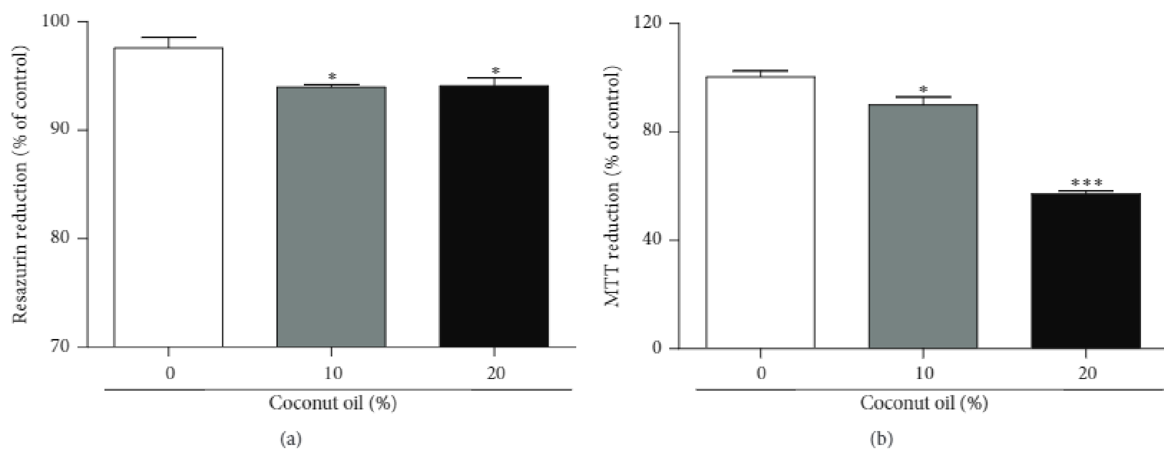


Figure 5: Effect of coconut oil on the metabolic activity and dehydrogenases activity in total homogenate of flies treated during seven days with a RD or a HFD. After seven days on the diets, flies were homogenized and centrifuged according to protocols and the samples were utilized for dehydrogenases activity (three sets of twenty flies were analyzed for each diet condition) by the resazurin reduction test in (a) and the metabolic activity (approximately 60 mg fly tissues were used for each diet condition) by MTT assay in (b). Both graphs express the results as a percentage (%) in relation to the RD group (mean ± standard deviation). $^{*}p < 0.05$ in relation to RD $^{***}p < 0.0001$ in relation RD.

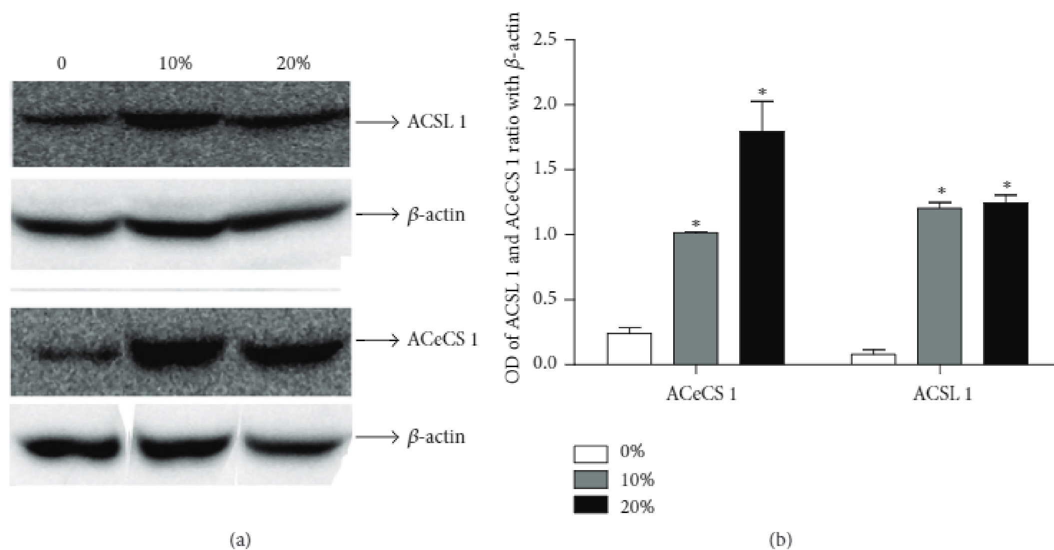


Figure 6: Expression levels of enzymes ACSL1 and ACeCS1 in response to the treatment of *D. melanogaster* with a regular diet (RD) and a high-fat diet (HFD) for seven days. After feeding the flies for seven days with different diets, they were homogenized and the proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. We quantified total content of proteins using specific antibodies. (a) The upper panel is a Western Blot showing expression levels of Acyl-CoA Synthetase (ACSL1) and Acetyl-CoA Synthetase (ACeCS1) with respective contents of β-actin. (b) The graphs are showing the ratio of OD from quantification of immunoreactive bands/β-actin and represent an average ± standard deviation. Three sets of forty flies were used for each diet condition. $*$ indicates a significant difference between the RD and the HFD ($p < 0.05$).

be increased in relation to control group, mainly in higher concentration of oil (Figures 7(c) and 7(d), resp.).

4. Discussion

The results of this study indicate that the coconut oil, when added to the diet of the *Drosophila melanogaster*, causes several changes in its metabolism as observed in studies based in mammal models [32]. Coconut oil in both concentrations tested (10% and 20%) is one of the causes of a significant increase in body weight, triglyceride levels, and a decrease in glucose levels. Additionally, an increase in the enzymes present in the metabolism of fatty acids, such as Acetyl-CoA Synthetase (ACeCS1) and Acyl-CoA Synthetase (ACSL1),

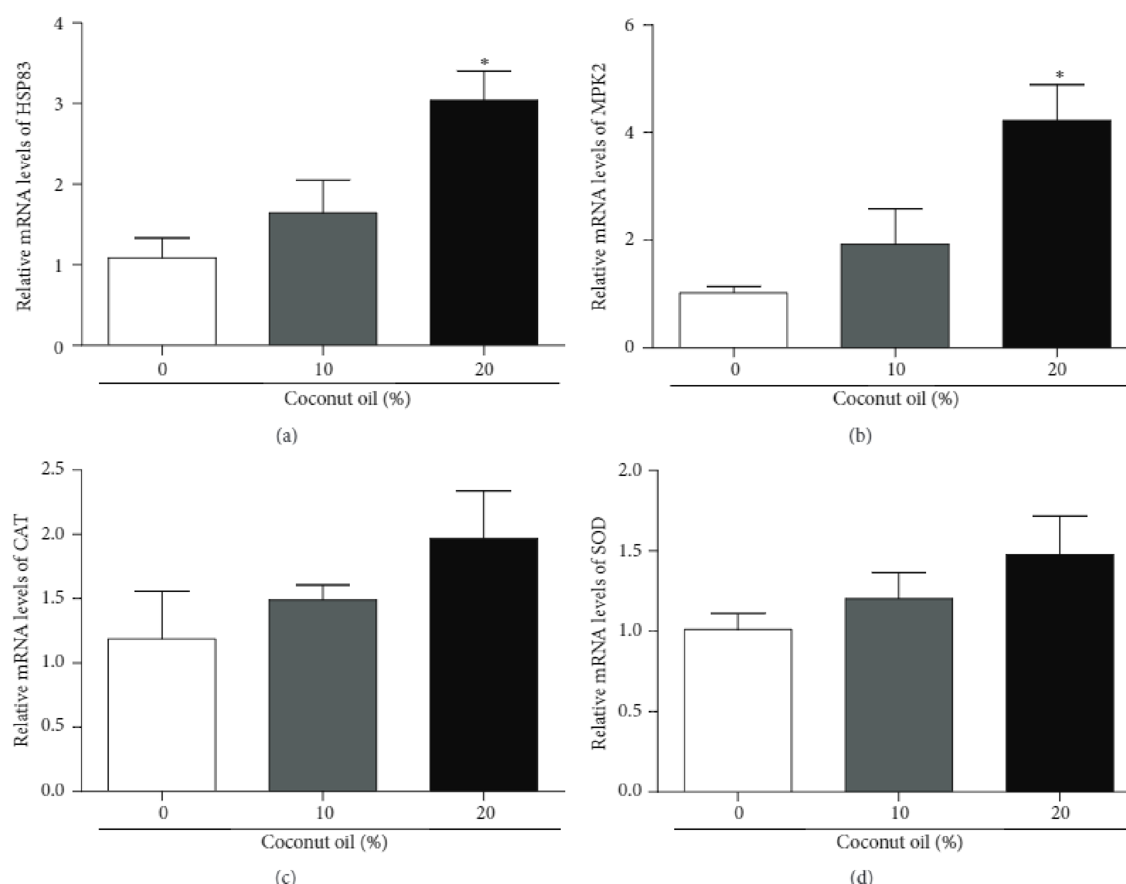


FIGURE 7: Quantitative real-time PCR (qRT-PCR) analysis of Hsp83, MPK-2, CAT, and SOD mRNA in flies exposed to high-fat diet. Flies were exposed for seven days to 0%, 10%, and 20% of coconut oil in their food. qRT-PCR was used to quantify levels of mRNA of each diet after exposure. The data were normalized against TUBULIN transcript levels and each bar represents the mean ± SEM. Three sets of twenty flies were used for each diet condition. * indicates a significant effect of a high-fat diet in comparison with a RD ($p < 0.05$).

and an induction of oxidative stress were observed. Possible serving as an adaptive response to this, there was an increase in the activity of antioxidant enzymes SOD and CAT and augmented expression levels of mRNA of Hsp83 and MPK2. Furthermore, the locomotor performance was impaired and the activity of acetylcholine esterase was reduced. Besides, cellular and mitochondrial viability was decreased with a significant reduction in the lifespan of treated flies.

The addition of coconut oil concentrations in the diet (10% and 20%) increases the body weight and triglycerides levels. Considering the diet being tested, which is high in saturated fat, it offers to flies larger concentrations of fat and, consequently, the increase of body weight is attributed to the increase of fat amount. It has been demonstrated, corroborating with our study, that a high-fat diet induces several consequences on lifespan, stress tolerance, and others in the *Drosophila melanogaster* model [3, 17]. In our study, we observed the decrease in glucose levels in flies fed with 10% and 20% coconut oil concentrations in their diet; however, our result is different from those observed by others [3, 17, 33]. According to Heinrichsen et al. [3] flies that received a high-fat diet (combination of yeast, corn starch, molasses, and 20% coconut oil concentration) demonstrated an increase in glucose levels. We suppose that the increase in the availability of triglycerides and fatty acids alters the use of glucose and its substrates on the oxidative mitochondrial metabolism. In fact, high-fat diets are known to influence the glucose metabolism including the increase in lactate levels in obese human subjects and the increase of lactate and pyruvate in mice that received a HFD [6, 7].

Many of the metabolic regulatory pathways are deranged in models using high-fat diets. A HFD promotes an increased supply of triglycerides and fatty acids and consequently an increase of fatty acids oxidation as a way to get energy. Our work demonstrates for the first time an association between a HFD and the increase on Acyl-CoA Synthetase and Acetyl-CoA Synthetase phosphorylation, enzymes that are present in the fatty acid metabolism, in *Drosophila melanogaster*.

The significant increase in Acyl-CoA Synthetase in the flies is directly connected to Acetyl-CoA by the oxidation

of fatty acids for adenosine triphosphate (ATP) production in the Krebs cycle. Therefore, we suggest that the HFD is being used to produce energy since the glucose levels are not enough. However, we know that the excess of Acetyl-CoA by oxidation of fatty acids can generate ketone bodies and acetate. Some studies demonstrate that acetate levels rise when fatty acid oxidation rises [34]. Acetate freely diffuses to most organs where it is utilized by Acetyl-CoA Synthetase to generate Acetyl-CoA. Then, Acetyl-CoA can be oxidized by peripheral tissues as a source of energy [34]. The increase in Acyl-CoA Synthetase and Acetyl-CoA Synthetase in our study is related to the increase in this metabolic pathway caused by a HFD in the mitochondria.

Given that a HFD accelerates the lipid metabolism route and increases the Acetyl-CoA Synthetase enzyme, we can suppose an increase in the amount of acetate available in the organism of the flies fed with coconut oil and, in part, this is one of the causes of the reduction in the lifespan, as presented in this study, since many authors report than acetate metabolism plays a critical and important role in aging, because it is regulated by NAD^+ dependent protein deacetylases (sirtuins) that have central roles in energy homeostasis and aging.

Mitochondria are cytoplasmic organelles whose main function is the production of most of the phosphate compounds needed for energy balance of the cell [35]. Besides, the mitochondrial dysfunction can accumulate oxidative damage, increase the RS generation, and consequently decrease ATP production and cell viability. The mitochondrial activity in excess by an increase of enzymes ACSL1 and ACeCS1, observed in our study, can be directly related to the increase in the RS production and, consequently, oxidative stress caused by a HFD in our model. In fact, in our study we observed an increase in RS production in the flies fed with 20% coconut oil concentration in the diet. Excessive consumption of fats, as in the cases of obesity, increases mitochondrial oxidative work load, which causes an increase in mitochondrial RS production by the electron transport chain and, consequently, the oxidative stress situation [36]. We believe that the increased oxidative stress is directly related to the change in the diet, providing flies with increased intake of saturated fats present in the commercial coconut oil.

Moreover, the oxidative stress by a HFD also caused an increase in lipid peroxidation. Furthermore, studies described that this high-fat diet model based on coconut oil concentrations is characterized as a genetic model to study obesity in *D. melanogaster* [9, 16, 37]. Increased oxidative stress in accumulated fat is an important pathogenic mechanism of metabolic syndromes associated with obesity, because oxidative damage may favor cell damage processes and inflammatory processes [38]. As a compensatory response to the damage, there is an increase in the activity of the antioxidant enzymes CAT and SOD and, in addition to this, there is a tendency to increase the expression of these enzymes. We believe that this increase in the activity of antioxidant enzymes and expression may represent a compensatory response to oxidative insults, since chronic exposure to coconut oil remained for seven days.

Obesity caused by lipid accumulation in adipose tissue is responsible for triggering cellular stress, a process of chronic inflammation characterized by abnormal production of cytokines by adipose tissue [39]. Once in a state of stress, the major organelles that suffer the consequences of this stress are the mitochondria and the endoplasmic reticulum (ER) [40]. In this study, a decrease in the metabolic activity has been demonstrated confirming the presence of a stress state which may result from apoptotic processes that produce a decrease in dehydrogenases activity caused by coconut oil in the diet of *D. melanogaster*. Besides, in increased nutrition conditions, the adipocyte breaks its ER which begins to generate malformed proteins. In response to this damage, the adipocyte ER begins to generate responses as the formation and increased expression of heat shock proteins and mitogen-activated protein kinases (MAPKs) [41].

In invertebrates, MAPKs and molecular chaperones such as the heat shock family of stress proteins (HSPs) participate in a range of cellular processes, including the development of normal cells, regulating the immune response and cytoprotection [42, 43]. However, there are no studies regarding the effects caused by a HFD with coconut oil in flies on gene expression of MAPK mRNA levels and HSP83 mRNA levels, a heat shock protein. Flies on a HFD with 10% and 20% coconut oil concentrations also led to the cellular response to damage such as increased expression of heat shock protein HSP83 and increased expression of MPK2, which is the *D. melanogaster* protein homologous to the P38 (MAPKs) in mammals.

In our experimental protocol, the exposure of *D. melanogaster* to coconut oil concentrations (10% and 20%) revealed a decrease in locomotor capacity by changes in geotaxis negative, a commonly used behavior addressed to assess neurolocomotor function in *Drosophila melanogaster* [44]. Moreover, a decrease in the activity of the enzyme acetylcholinesterase was also observed. Fournier et al. [45] reported that changes in the enzyme AChe, present in the central nervous system of drosophila, can affect the sensitivity of these insects and their locomotor ability as demonstrated in compounds with insecticidal effects. Furthermore, a reduction in the growth of skeletal muscles which can be directly related to the decrease in protein levels and locomotor capacity has been reported in studies of mice that received a high-fat diet in comparison to the study model reported in this work [3]. In addition to this, we presuppose that because the flies treated with coconut oil showed a significant decrease in glucose levels, the degradation of fatty acids by lipolysis is a compensatory role.

In summary, our study revealed for the first time that flies fed for seven days with a high-fat diet by coconut oil addition (10% and 20%) have a reduced locomotor performance, an increase in Acyl-CoA Synthetase and Acetyl-CoA Synthetase, and, consequently, an increase in the production of reactive species and thiobarbituric acid reactive substances, an increase of HSP83 and MPK2 expression, which generates an oxidative stress situation. Furthermore, the association among metabolic changes, oxidative stress, and protein signalization might be involved in shortening the lifespan of flies fed with a HFD.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

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MANUSCRITO 1

Drosophila melanogaster as experimental model to study maternal obesity effects on antioxidant genes expression and developmental changes on descendents

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Manuscrito

2017

Drosophila melanogaster as experimental model to study maternal obesity effects on antioxidant genes expression and developmental changes on descendents

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ABSTRACT

Maternal obesity and metabolic disease are two of the most important potential exposures dangerous to offspring, given that impairs offspring may cause deficiencies that impair the adult life and health. However, in *Drosophila melanogaster* model, studies with metabolic disorders caused by high fat diet (HFD) during the development are still scarce. This study evaluated the oxidative damage, enzimatic antioxidant defenses and enzymes of fatty acid metabolism as Acyl-CoA Synthetase and Acetyl-CoA Synthetase (levels and expression of mRNA) as well as modulation of cell stress signaling pathway as dilp6 in flies that received a high-fat diet (10% and 20% of coconut oil) throughout their development period. After seven days, the progenitors flies were removed and, daily the eggs were monitored until the eclosion. The descendants were removed for regular diet (RD) to the experiments. To confirm the damage effect of diet on descendants flies, the cumulative eclosion proportion, the number of hatched eggs and life span were investigated. The results revealed that the HFD cause a decrease in: eclosion proportion, lifespan, MTT reduction in mitochondrial enriched fractions, AceCS1 levels, SOD and CAT expression of mRNA levels and in catalase activity in most high concentration of coconut oil tested. In parallel, was demonstrated an increase in the glucose and triglyceride levels, as well upregulation of HSP83 mRNA levels when added 10% of coconut oil and in larger concentration of coconut oil tested (20%) increased considerably the dilp6 mRNA levels. In conclusion, flies that have progenitors fed with HFD can develop metabolic dysfunctions by insulin resistance, causing oxidative insults involved in shortening the lifespan.

Keywords: Acetyl-CoA synthetase; Acyl-CoA synthetase; Coconut oil; Lifespan; Lipid Metabolism; insulin-like peptides; insulin resistance.

1. INTRODUCTION

Maternal obesity outcomes for offspring have been reported in human and animal models, and involves increased risk for being born large for gestational age (Carlsen et al., 2014), endothelial nitric oxide synthase deficiency (Ferrari et al., 2016) high newborn mortality (Albert et al., 2016), spatial deficits and obesity (Hsieh et al., 2016), high circulating levels of adipokines, adipose tissue–derived cytokines that contribute to the development of metabolic dysfunctions, inflammatory responses and insulin resistance (Hong et al., 2016). The conditions by which embryo and fetus are exposed has been related with the origin of chronic adult disease, given that they compromise tissues development and gene expression (Saben et al., 2016).

Considering the importance of this subject, there is the necessity of experimental models that reproduce with reliability the effects observed in mammals on offspring with less practical and ethical obstacles that limit these studies. In this aspect, *D. melanogaster* can be considered a useful model since it shares metabolic and genetic mechanisms and are responsive to the effects from hyperlipidic diet similar to observed in mammals. In fact, in our previous studies we first demonstrated that flies fed seven days with a high fat diet by adding coconut oil (10% and 20%) have reduced locomotor performance, metabolic alterations and oxidative stress situation that might be involved in shortening the lifespan of flies (Paula et a., 2016). Substantially, it is possible to study various metabolic and cellular marker changes that are evolutionarily conserved in simpler organisms subject to systematic genetic analysis, such as the invertebrate *Drosophila melanogaster* (Géminard et al., 2009; Paula et al., 2016).

When flies are exposed to a HFD, metabolic dysfunction is reported and it invludes abnormal glucose and lipid levels as well insulin resistance (Hong et al., 2016). Insulin-like peptides are evolutionary conserved proteins that regulate growth, metabolism, reproduction, and longevity in *D. melanogaster* (Pierce et al., 2001; Brogiolo et al., 2001; Gronke et al., 2010; Bai et al., 2012; Kannan and Fridell, 2013). Eight insulin-like peptides (DILPs) and one insulin receptor (DInR) were reported in this organism (Gronke and Partridge, 2010). The altered expression of genes encoding DILPs2, 3, 5, and 6 affects metabolism and lifespan (Broughton et al., 2005, 2008; Gronke et al., 2010; Bai et al., 2012).

Fatty acid metabolism is a well conserved mechanism. Acyl-CoA Synthetase and Acetyl-CoA Synthetase are enzymes acting in the triacylglycerol synthesis (Oba et al., 2005; Phillips et al., 2010; Paula et al., 2016). Changes in these pathways may result in metabolic

syndrome by development of dyslipidemia and insulin resistance (Phillips et al., 2010). Oxidative stress is implied or is a consequence of exposure to a hyperlipidic environment and it causes differents alterations in metabolism and decrease in lifespan of animals. In flies and other insects the antioxidant defenses consist of a system of enzymes including superoxide dismutase (SOD) and catalase (CAT) which play a key role in maintaining the intracellular redox homeostasis (Missirlis et al., 2001). When the expression and activity of these enzymes is compromised, changes eggs eclosion rate and oxidative stress was reported in Drosophila (Missirlis et al., 2001).

Drosophila melanogaster has served as an experimental model for the studying of various human diseases, including obesity and other metabolic disorders (Cruz et al., 2014; Reis, 2016). Considering maternal condition as a critical event for embryo development and life quality of adult individual, herein we evaluated for the first time the consequences of maternal obesity on embryo of *Drosophila melanogaster,* taking into account parameter such lipid metabolism, antioxidant enzymes, stress responsive gene expression and insulin-like peptide DILP6. Particularly sensitive in alterations of diet, this work aimed to investigate the oxidative damage, antioxidant enzymes and enzymes present in the fatty acid metabolism as Acyl-CoA Synthetase and Acetyl-CoA Synthetase (levels and expression of mRNA), as well modulation of cell stress signaling pathway as dilp6 in flies that received a high-fat diet throughout their development period contributing to the knowledge about the cellular response in flies.

2. MATERIALS AND METHODS

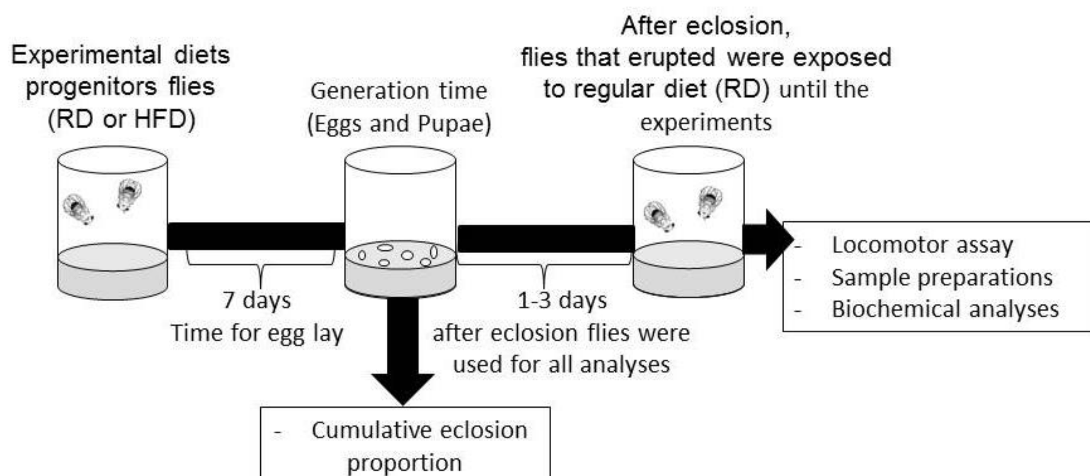
2.1 Chemicals

Fatty Acid and Lipid Metabolism Antibody Sampler Kitand β-actin antibody were purchased from Cell Signaling Technology. 2 ', 7'-dichloroflurescein diacetate (DCF-DA), 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB), 7-Hydroxy-3H-phenoxazin-3-one 10-oxide (Resazurin), Acetylthiocholine iodide, β-Mercaptoethanol, Ethylenediaminetetraacetic acid (EDTA), Mannitol, cocktail protease inhibitor, N,N,N′,N′-Tetramethylethylenediamine (TEMED), Quercetin, Sodium

orthovanadate, thiobarbaturic acid (TBA) were obtained from Sigma-Aldrich. Triglycerides Liquiform and Glucose Liquiform were obtained from Labtest (Brazil). The sodium dodecyl sulfate was procured from GE Healthcare Life Sciences. Trizol Reagent and DNase I was obtained from Invitrogen. iScript cDNA Synthesis Kit was from Bio-Rad. Tris(hydroxymethyl)aminomethane, hydrogen peroxide, TRITON X-100 and Dimethyl sulfoxide were purchased from Synth. Coconut oil were obtained from Pró-Ervas.

2.2 Animal treatment

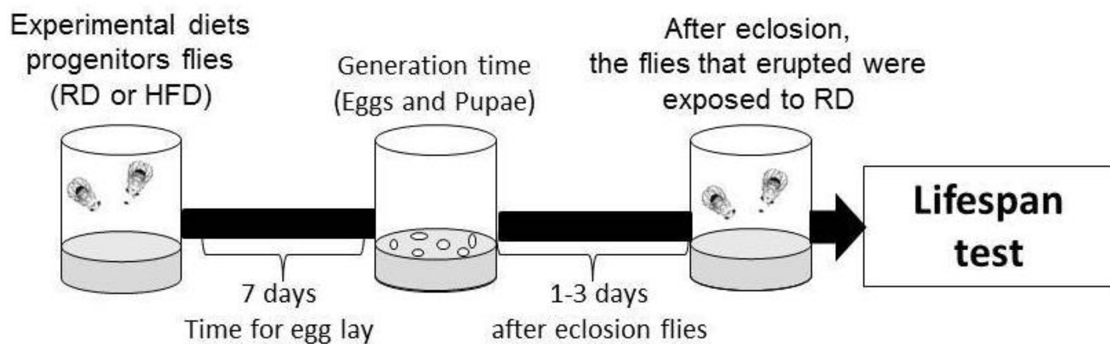
Drosophila melanogaster (Harwich strain) was obtained from the National Species Stock Center, Bowling Green, OH, USA. The progenitors flies were maintained, during seven days, at 25°C on 12 h light/dark cycle in glass bottles containing 5 mL of regular diet (RD) consisting of standard medium (76.59% corn flour, 8.51% wheat germ, 7.23% sugar, 7.23% milk powder and 0.43 % salt) supplemented with 10% or 20% of coconut oil (by Pró-Ervas®)(high fat diet –HFD). Newly ecloded flies grown in RD or HFD with 1-3 days old were used for all analyses (Scheme 1). The coconut oil concentrations were chosen based on previous studies of our group (Paula et al., 2016).



Scheme 1

2.3 Lifespan and cumulative eclosion

The cumulative eclosion proportion was done by counting total number of flies hatched in each diet, until there were no more viable eggs. In order to test the lifespan, three days after the eclosion, the flies were maintained under standard medium which were changed every two days, until there were no more flies alive (Scheme 2).



Scheme 2

2.4 Locomotor assay

Locomotor activity was determined using the negative geotaxis assay as described by Bland et al. (2009) with minor modifications. Briefly, for each assay, a total of five flies in each group, individually, were immobilized on ice and placed separately in a glass tube; this method of immobilization does not affect fly neurology (Deepa et al., 2009). After 15 minutes the flies were gently tapped to the bottom of the tube and the time required to climb up 8 cm of the tube wall was recorded. Each fly was tested 4 times at 1 minute intervals. For each experiment, the climbing mean was calculated.

2.5 Biochemical assays

2.5.1 Triglyceride, glucose and protein measurements

After eclosion, 20 whole flies per group or treatment were anesthetized, homogenized and prepared, as described by Gronke et al. (2003) for the analysis of total triglycerides and as described by Birse et al. (2010) for the analysis of glucose by using the specific Labtest® kit. Protein concentrations of the homogenate were determined by the method of Bradford (1976), using bovine serum albumin as the standard.

2.5.2 Sample Preparation and Enzyme Assays

For enzyme activity, groups of 20 whole flies from each treatment were homogenized in 1 mL of 10 mM tris buffer, pH 7.0, and centrifuged at 1000 x g for 10 min at 4°C. The supernatant was isolated and a 200 µL aliquot separated for determination of acetylcholinesterase activity (AChE; EC 3.1.1.7) in a reaction mixture containing phosphate buffer (0.25 M, pH 8,0), 5,5-dithiobis 2-nitrobenzoic acid (5 mM), 50 µL sample and 25 µL acetylthiocholine iodide (7.25 mM). The reaction was monitored for two minutes at 412 nm. Enzyme activity was expressed as nanomoles of substrate hydrolyzed per minute per milligram protein based on protocols previously described (Ellman et al., 1961).

The remaining supernatant was then centrifuged at 20.000 x g for 30 min (4°C). The resulted supernatant was used for determination of antioxidant enzymes CAT and SOD. CAT (EC 1.11.1.6) was determined according to Aebi (1984) with minor modifications by Paula et al., (2012). The reaction mixture contained phosphate buffer (0.25 M/EDTA 2.5 mM, pH 7.0), H_2O_2 (10 mM), 0.012% Triton X 100 and 30 µl sample. The decay in H_2O_2 was monitored during one minute at 240 nm and expressed as micromole of H_2O_2 decomposed/min/mg protein.

SOD (EC 1.15.1.1) was measured according to Kostyuk and Potapovich (1989), with minor modifications by Franco et al. (2010). It was performed by monitoring the inhibition of quercetin auto oxidation. The reaction mixture contained sodium phosphate buffer (0.025 M/EDTA 0,1 mM, pH 10), N,N,N,N-tetramethylethylenediamine (TEMED), 10 µl sample and it was started by adding 0.15% quercetin dissolved in dimethyl formamide. The reaction was monitored for two minutes at 406 nm, and it is expressed as the amount of protein required to inhibit 50% of quercetin auto oxidation.

2.5.3 Determination of Lipid Peroxidation and DCF-DA Oxidation

Lipid peroxidation products were quantified as thiobarbituric acid reactive substance (TBARS) following the method of Ohkawa et al. (1979) with minor modifications. Briefly, groups of 20 whole flies from each treatment were homogenized in 1 mL 0.1 M phosphate buffer pH 7.0 and centrifuged at 1000 x g for 5 min (4°C). After centrifugation, the supernatant was incubated in 0.45 M acetic acid/HCl buffer pH 3.4, 0.28% thiobarbituric acid, 1.2% SDS, at 958C for 60 min and absorbance then measured at 532 nm. Results represent the mean of three independent experiments. In each experiment, each treatment was done in duplicate. The results were expressed as nmol TBARS/mg tissue.

For the quantification of reactive species generation (RS) a total of 20 flies were anesthetized on ice and homogenized in 1 ml 10 mM Tris buffer, pH 7.4, after the end of the treatments The homogenate was centrifuged at 1000 x g for 5 minutes at 4 ° C and the supernatant was removed for assay quantification of 2 ', 7'-dichloroflurescein diacetate (DCF-DA) oxidation, as a general index of oxidative stress according to the protocol of Pérez-Severiano et al. (2004). The fluorescence emission of DCF resulting from DCF-DA oxidation was monitored after one hour at an excitation wavelength of 485 nm and an emission wavelength of 530 nm in the spectrophotometer. The rate of DCF formation was demonstrated by arbitrary units (AU).

2.5.4 Resazurin reduction test

An investigation to resazurin reduction test of *Drosophila melanogaster* was carried out for the 20 whole flies from each treatment were anesthetized on ice and homogenized and it was perfomed by using the CellTiter-Blue® cell viability assay kit by Franco et al. (2009). The method is based on the ability of viable cells to reduce resazurin to resorufin, a fluorescent molecule. Whole flies were homogeneized in 10 mM tris buffer, pH 7.4 and 100 µL pipetted in a 96-well plate and an aliquot of CellTiter-Blue was added according to the instructions of the manufacturer. After 1 h, the fluorescence was recorded using at ex579nm and em584nm.

2.5.5 Preparation of mitochondrial enriched fractions and MTT reduction test

A mitochondrial enriched fraction was prepared from the whole body of flies using the method of differential centrifugation by Hosamani (2013). Briefly, 0,3 g of flies from each

treatment were homogenized in ice-cold Tris-sucrose buffer (0.25 M, pH 7.4) (60 mg fly tissue homogenized in 1000 µl buffer) and centrifuged at 1000 x g for five minutes (4° C). A fraction enriched with mitochondria was obtained by centrifuging the post nuclear supernatant at 10000 × g for 10 minutes (4° C). The pellet was washed in mannitol–sucrose–HEPES buffer and resuspended in 200 µl of suspension buffer.

A portion of 200 microliters of the mitochondrial enriched fraction was incubated with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) MTT (x% solution) for 30 minutes at 37° C. After that, the samples were centrifuged at 10000 x g for five minutes. The pellet was dissolved in dimethyl sulfoxide (DMSO), incubated for 30 minutes at 37° C and the absorbance was measured at 540 nm. Results were expressed as percentage of the control.

2.6 Western blotting analysis

After the eclosion, groups of 40 whole flies from each treatment were mechanically homogenized at 4°C in 200 µL of buffer (pH 7.0) containing 50 mM Tris,1 mM EDTA, 20 mM Na_3VO_4, 100 mM sodium fluoride and protease inhibitor cocktail according with Paula et al. (2016). Then, the homogenate was centrifuged for 10 min at 1000 x g at 4ºC and the supernatant was collected. After protein determination according to Bradford (1976), 4 % SDS solution, ß-mercaptoethanol and glycerol were added to samples to a final concentration of 100, 8 and 25 %, respectively, and the samples were frozen for further analysis. The proteins were separated by SDS-PAGE using 10% gels and then electrotransferred to nitrocellulose membranes as previously described by Paula et al. (2012). Membranes were washed in Tris-buffered saline with Tween 100 mmol/L Tris-HCl, 0.9% NaCl, 0.1% Tween-20, pH 7.5 and incubated overnight (4° C) with specific primary antibodies [anti-Acetyl-Coenzyme A synthetase (ACeCS 1) and anti-Acyl-Coenzyme synthetase (ACSL 1) and anti-β actin. Subsequently, the membranes were washed in tris buffered saline with tween and incubated for 1 h at 25ºC with anti rabbit Ig-secondary antibodies. Antibody binding was visualized using the ECL Western Blotting substrate Kit (Promega). Band staining density was quantified using the Scion Image software (Scion Image for Windows). Results are expressed as optical density of ACSL1 or ACeCS1/optical density of respective ß-actin.

2.7 Quantitative Real-Time RT-PCR and Gene Expression Analysis

Approximately 1 μg of total RNA from 20 flies was extracted using the Trizol Reagent (Invitrogen) accordingly 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 accordingly to the manufacturer's suggested protocol (BIORAD). Quantitative real-time polymerase chain reaction was performed in 11 μL reaction volumes containing water treated with diethyl pyrocarbonate (DEPC), 200 ng of each primer (described in Table 1), 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 (Livak and Schmittgen, 2001).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 (sequences of tested genes are represented in Table 1). The ΔCT mean value obtained from the control group of each gene was used to calculate the ΔΔCT of the respective gene ($2^{-\Delta\Delta CT}$).

2.8 Statistical Analysis

Lifespan measurement was determined by comparing the survival curves with a log-rank (Mantel-Cox) test. Other statistical analyses were performed using one-way ANOVA followed by Newman-Keuls *post hoc* test. Differences were considered statistically significant when $p < 0.05$.The Graph Pad Prism 5 Software was used for artwork creation.

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

Gene	Primer Sequences	
GPDH	LEFT	5’- ATGGAGATGATTCGCTTCGT
	RIGHT	5’- GCTCCTCAATGGTTTTTCCA
SOD	LEFT	5’- GGAGTCGGTGATGTTGACCT
	RIGHT	5’- GTTCGGTGACAACACCAATG
CAT	LEFT	5’- ACCAGGGCATCAAGAATCTG
	RIGHT	5’- AACTTCTTGGCCTGCTCGTA
HSP83	LEFT	5’- CAAATCCCTGACCAACGACT
	RIGHT	5’- CGCACGTACAGCTTGATGTT
P38 MPK2a	LEFT	5’- GGCCACATAGCCTGTCATCT
	RIGHT	5’- ACCAGATACTCCGTGGCTTG
DILP6	LEFT	5’- CCCTTGGCGATGTATTTCC
	RIGHT	5’- CACAAATCGGTTACGTTCTGC

3. RESULTS

3.1 Diet enriched with coconut oil causes a decrease in eclosion rate, number of hatched eggs and lifespan

According to Figure 1A, the HFD, in both concentrations, demonstrated a significant decrease in cumulative eclosion proportion of flies in comparison to the RD. The same significant decrease caused by HFD (10% and 20%) was also observed in number of eclosion eggs (Figure 1B). Lifespan of descendant flies receiving a RD was up to 50 days. However, when adding coconut oil at both concentrations tested (HFD 10% and HFD 20%), the maximum life span drops to 29 and 17 days, respectively (Figure 1C).

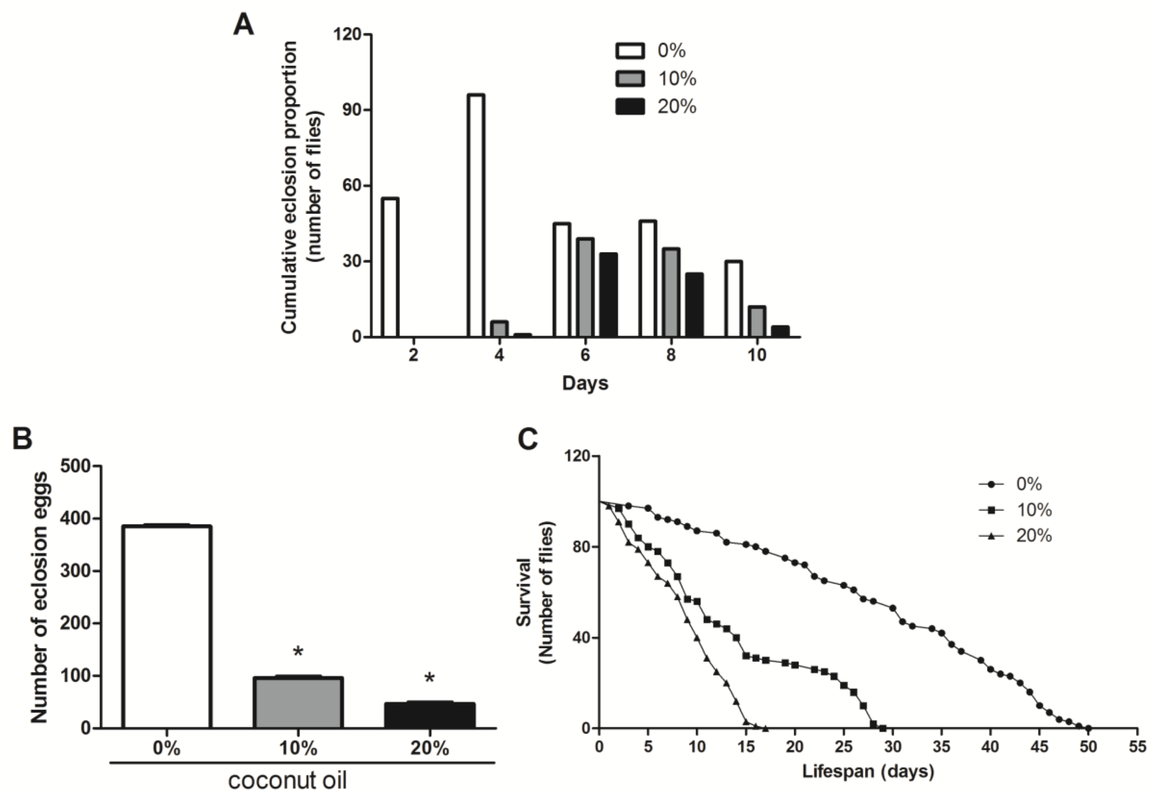


Figure 1. Diet enriched with coconut oil causes a decrease in eclosion rate, number of hatched eggs and life span in *D. melanogaster*. Three sets of ten flies (seven females and three males) in each treatment (exposed to RD and HFD for reproduction). After seven days, the progenitors flies were removed and, daily the eggs were monitored until the eclosion. (A) The data expresses the cumulative eclosion proportion every two days. (B) Number total of hatched eggs. (C) Lifespan: the flies were counted daily until there were no more flies alive.* p< 0.05 in relation with the RD group.

3.2 Descendant flies of progenitors fed by diet enriched with coconut oil develop an increased in triglyceride and glucose levels.

The levels of triglycerides and glucose were measured in *D. melanogaster* descendants exposed to a RD and HFD during embryonic development period. At the concentration of 20% of coconut oil, the levels of triglycerides in flies increased three times more than the RD group (0% coconut oil) (Figure 2A). Moreover, 20% of coconut oil caused an increase two times more elevated than the RD group in the glucose levels (Figure 2B).

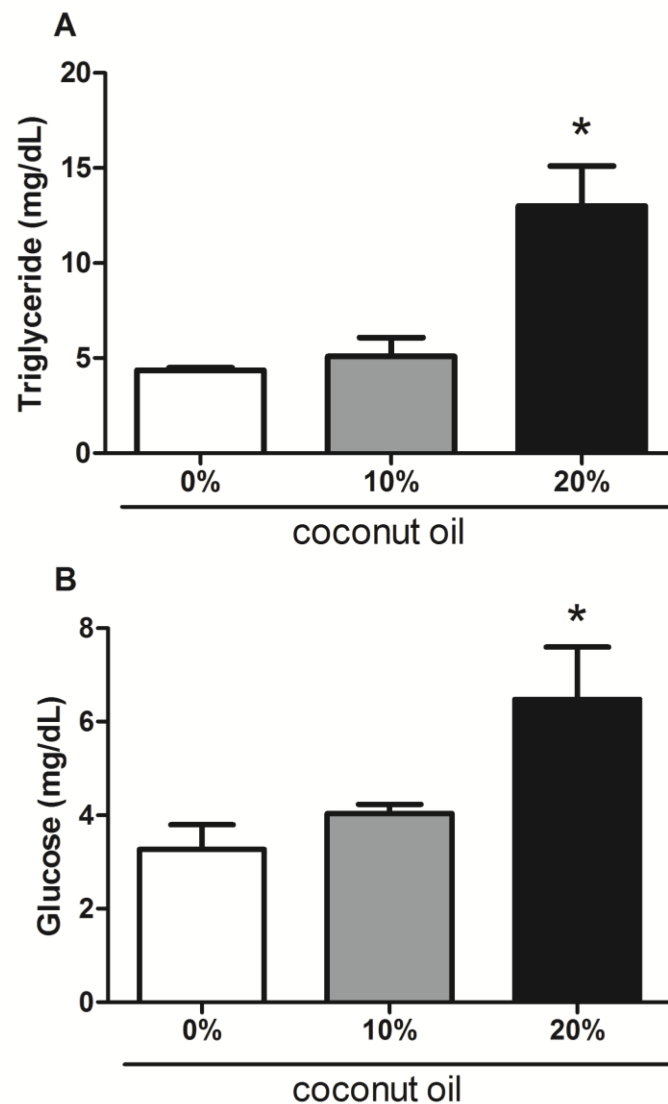


Figure 2. Diet enriched with coconut oil increased augmented triglyceride levels and glucose levels in *D. melanogaster*. Three sets of twenty flies each treatment (exposed to RD and HFD during embryonic development period) were homogeneized for each diet condition and supernatant analyzed to triglycerides levels and glucose levels. (A) The data expresses triglycerides levels in whole flies homogenate expressed in mg/dL. (B) glucose levels in flies homogenate expressed in mg/dL.* $p < 0.05$ in relation with the RD group.

3.3 Locomotor performance and Acetylcholinesterase activity

The exposure of flies to HFD during embryonic development period did not cause a significant loss in the locomotor performance, only a tendency to increase the climbing time

(Figure 3A). Additionally, the activity of Ache in the descendants flies remained unaltered by HFD (Figure 3B).

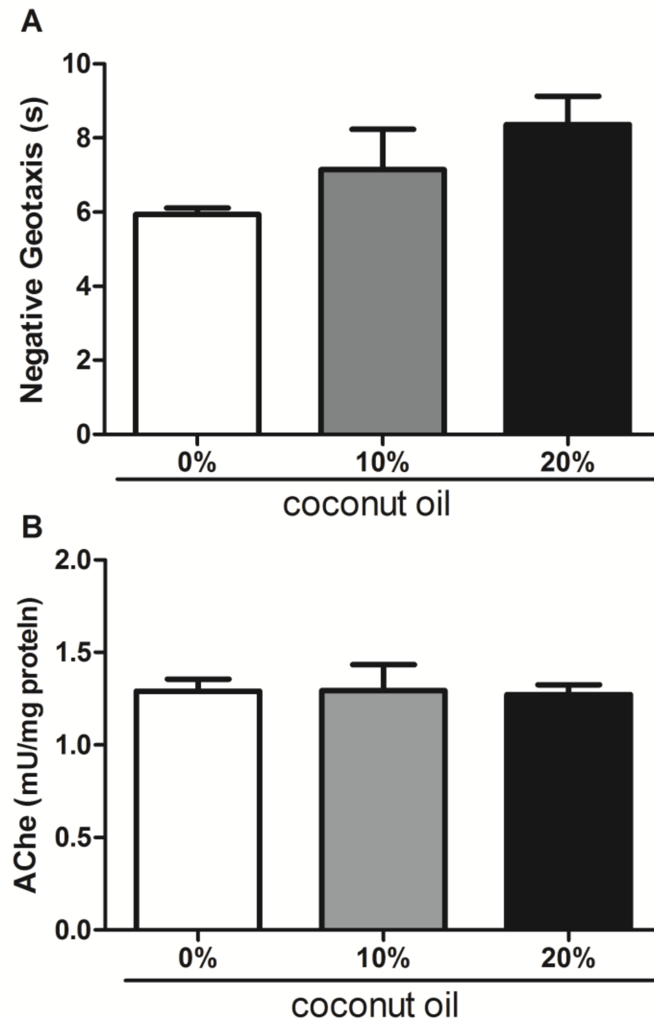


Figure 3. Effects of the exposure to a high-fat diet in *D. melanogaster* exposed during embryonic development period on locomotor performance and acetylcholinesterase (AChe) activity. (A) Flies exposed to RD or HFD during embryonic development period were analyzed for locomotor ability by negative geotaxis. Three sets of ten flies were analyzed for each diet condition. Results are expressed as mean of time spent to reach 8 cm in a glass tube of three independent experiments. (B) The data shows the AChe activity in flies' homogenate expressed as mean (mU/mg protein) ± standard deviation. Three sets of twenty flies were analyzed for each diet condition * indicates a significant difference in the RD and the HFD ($p < 0.05$).

3.4 Oxidative stress markers and antioxidant defenses in *D. melanogaster* descendants exposed to a RD and HFD during embryonic development period.

In this study, we evaluated some of oxidative stress parameters in flies descendants exposed to RD and HFD during embryonic development period. However, the results did not show a significant difference in DCFDA oxidation as a general indicator of oxidative stress (Figure 4A), TBARS as an indicator of lipid peroxidation (Figure 4B) and activity of the antioxidant enzyme superoxide dismutase (SOD) (Figure 4D). But, in flies that received 20% coconut oil concentration during embryonic development period, demonstrated a significant decrease in the activity of the antioxidant enzyme catalase (CAT) (Figure 4C).

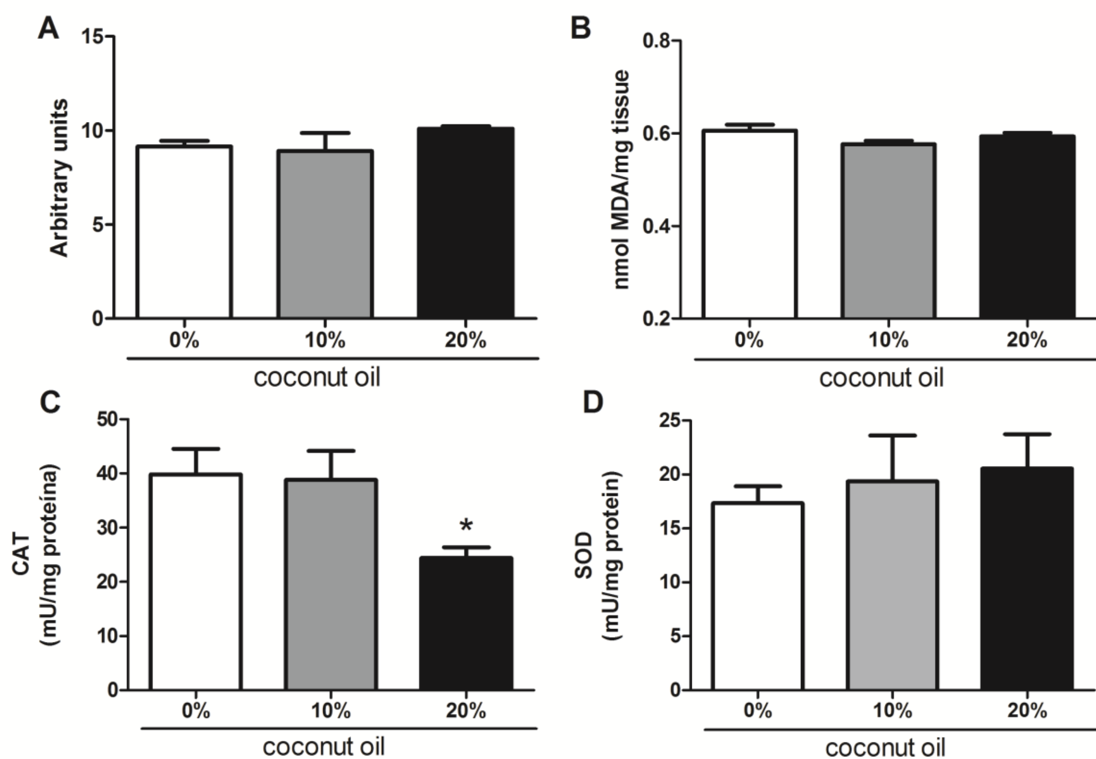


Figure 4. Effects on oxidative stress parameters in flies descendants exposed to a RD and HFD during embryonic development period. Flies exposed to RD or HFD during embryonic development period were homogenized and the supernatant was used for various analyses of stress markers and the activity of antioxidant enzymes. (A) Shows the DCF-DA intensity of fluorescence in total flies homogenate. Three sets of twenty flies were analyzed for each diet condition. (B) End products of lipid peroxidation determined by TBARS assay in total flies homogenate, expressed in MDA nmoles/mg of protein Three sets of twenty flies were analyzed for each diet condition. (C) Catalase (CAT) activity and (D) Superoxide dismutase (SOD) activity in total flies homogenate. Three sets of twenty flies were analyzed for each diet condition. Data are expressed as a mean ± standard deviation in mU/mg protein. * indicates a significant difference in relation with RD ($p < 0.05$).

3.5 Coconut oil exposure during embryonic development caused decreased in MTT reduction in mitochondrial enriched fraction without changing resazurin reduction in viable cells.

The resazurin reduction test did not show significant difference in any of the tested concentrations (Figure 5A). Also, there was a significant decrease in reduction of MTT in mitochondrial enriched fraction for all coconut oil concentrations tested (10% and 20%) (Figure 5B).

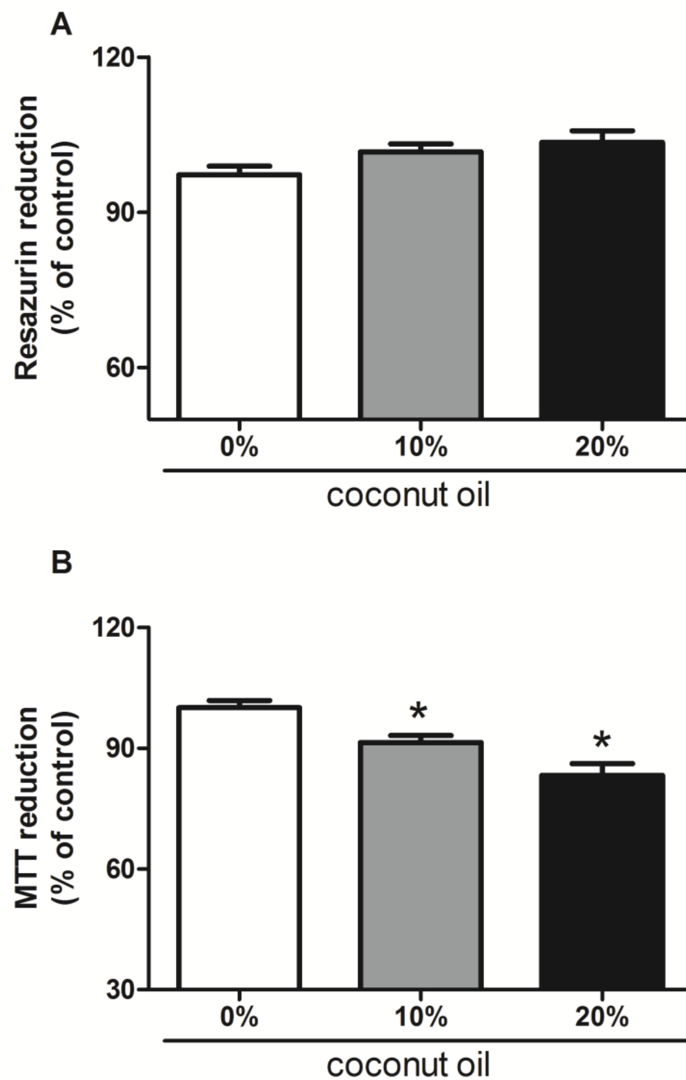


Figure 5. Effect of coconut oil on the metabolic activity and dehydrogenases activity in total homogenate of flies treated during embryonic development period with a RD or a HFD. Flies exposed to RD or HFD

during embryonic development period were homogenized and centrifugated according to protocols and the samples were utilized to dehydrogenases activity (three sets of twenty flies were analyzed for each diet condition) by the resazurin reduction test in (A) and the metabolic activity (approximately 60 mg fly tissue were analyzed for each diet condition) by MTT assay in (B). Both graphs expresses the results as a percentage (%) in relation with the RD group (mean ± standard deviation). * ($p < 0.05$) in relation to RD.

3.6 Coconut oil exposure in diet during embryonic development period of the flies caused alteration in response in the levels of ACeCS1 without changing ACSL1 expression

Acyl-coenzyme synthetase (ACSL 1) and acetyl-coenzyme A synthetase (ACeCS 1) were investigated in flies exposed to coconut oil (10% and 20%) during embrionary development period. There was a significant decrease in ACeCS1 content in both the groups tested, when comparing to control group (Figure 6B). However, ACSL1 content did not show alterations in any of coconut oil concentrations tested (Figure 6B). Data expresses a ratio of optical density of the bands in relation to β-actin.

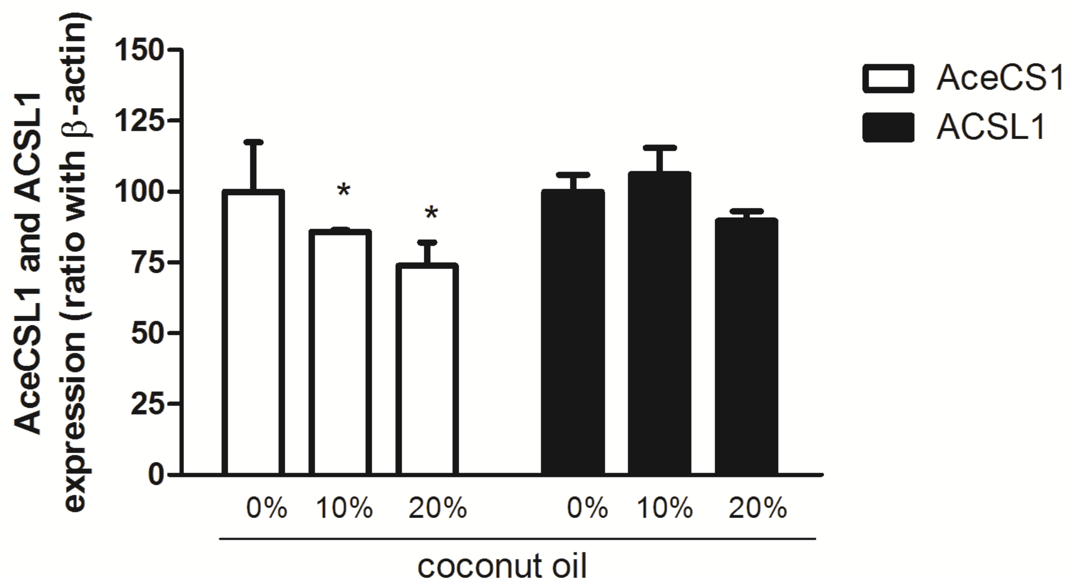


Figure 6. Expression levels of enzymes ACSL1 and AceCS1 in *D. melanogaster* descendants exposed to a RD and HFD during embryonic development period. Flies exposed to RD or HFD during embryonic development period were homogeneized and the proteins were separated by SDS–PAGE, and transferred to nitrocellulose membrane. We quantified total content and phosphorylation of proteins using specific antibodies. (A) The graph shows the ratio of OD from quantification of immunoreactive bands/β-actin and represents an average ± standart deviation of the Acetyl-CoA Synthetase (AceCS1) expression and in (B) the ratio of OD from quantification of immunoreactive bands/β-actin and represents an average ± standart deviation of the Acyl-CoA Synthetase (ACSL1) expression. Three sets of fourty flies were analyzed for each diet condition. * indicates a significant difference between the RD and the HFD ($p < 0.05$).

3.7 Quantitative Real-Time PCR (QRT-PCR): Analysis of SOD, CAT, HSP83 and dilp6 mRNA in *Drospophila melanogaster*

Flies were exposed to HFD during embrionic development and were used qRT-PCR to quantify levels of mRNA, relative to the respective RD groups, after eclosion. The data were normalized against GPDH transcript levels. Both coconut oil concentrations tested (10% and 20%) causes significant decrease in SOD and CAT expression of mRNA levels (Figures 7A and 7B, resp.). Besides, a significant increase in the HSP83 mRNA levels was observed when added 10% of coconut oil in embrionic development of flies (Figure 7C). Additionally, the major concentration of coconut oil tested (20%) increased considerably the dilp6 mRNA levels (Figure 7D).

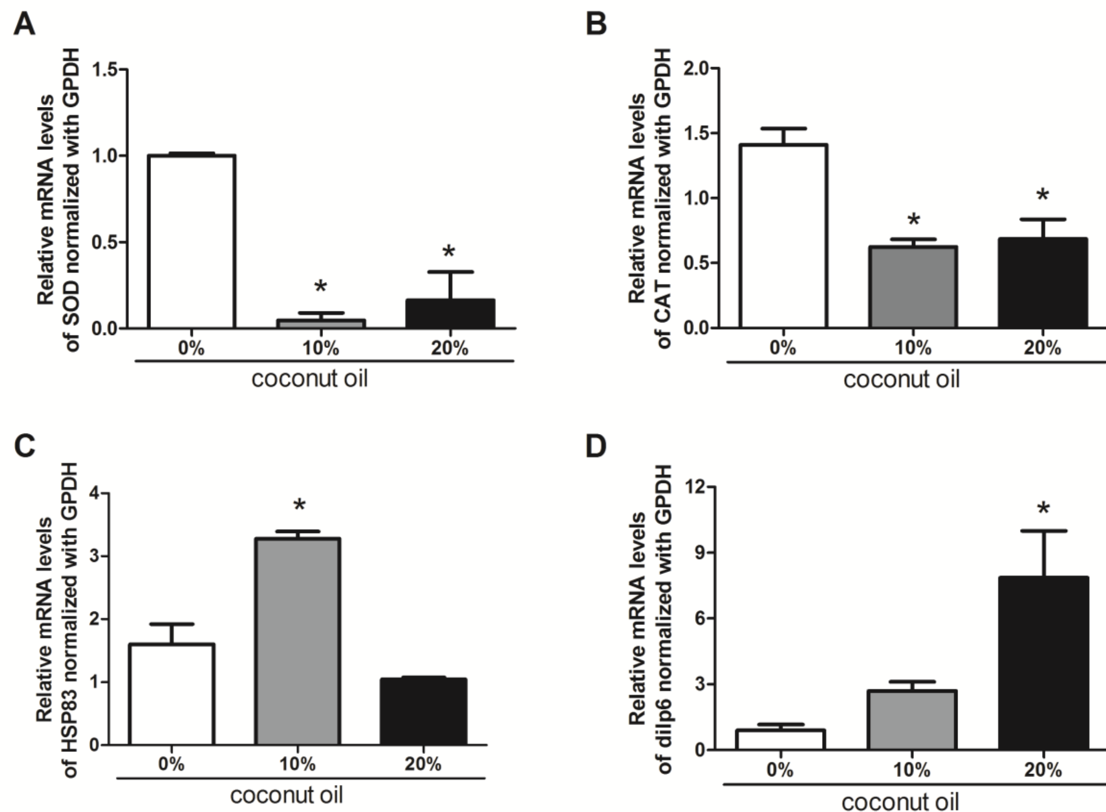


Figure 7. Quantitative real time PCR (qRT-PCR) analysis of dilp6, Hsp83, SOD and CAT mRNA in flies exposed to high-fat diet during embryonic development period. Flies exposed to RD or HFD during embryonic development period were used qRT-PCR to quantify levels of mRNA of each diet after exposure. The

data were normalized against TUBULIN transcript levels and each bar represents the mean ± SEM. Three sets of twenty flies were analyzed for each diet condition. *indicates a significant effect of a high fat diet in comparison with a RD ($p < 0.05$).

4. DISCUSSION

It is well described that obesity leads to widespread systemic disease, reduces quality of life, and shortens life expectancy becoming an epidemic problem in the world (Whitlock et al., 2009; Ng et al., 2014). However, studies have revealed what childhood obesity is closely linked with obesity in either parent once children tend to become obese adults or by genetic influences or shared environmental factors, such as diet and lifestyle (Whitaker et al., 1997; Ng et al., 2014). The studies in *Drosophila melanogaster* model allow us to understand better the delay in the external developmental cycle into adults, embryos and larvae more satisfactory than in more complex models (Vernós et al., 1989; Ternes et al., 2014). In this study, we determined the genetic expression and biochemical alterations in *D. melanogaster* in response to HFD exposure during embryonic development. Besides, coconut oil added to the diet flies during development period caused increase in triglyceride and glucose levels, along with an increase in expression level of mRNA of DILP6, a decrease in enzyme present in the metabolism of fatty acids (ACeCS1), SOD and mRNA CAT levels al well CAT activity.

HFD drastically alters the metabolism in *D. melanogaster* (Heinrichsen et al., 2014). The study of Terashima et al. (2005) revealed that *D. melanogaster* model, when altered the normal diet, observed a decrease in egg laying, which may be caused by deregulation of normal oogenesis, once is regulated by supplies nutrition. Furthermore, Baker and Russel (2009) demonstrated that oogenesis in *D. melanogaster* is a dynamic, highly regulated process, that depends on the correct developmental, environmental and nutritional cues for normal progression and synchronization of processes in the ovary and fat body for successful egg production. In mammal models, coconut oil when added to the diet causes several changes in its metabolism both in adults and in descendeants (Livak and Schmittgen, 2001). In our study, we showed that the addition of coconut oil concentrations in the diet (10% and 20%) of the flies, during embryonic development period, caused changes in the eclosion rate, decreasing the cumulative eclosion proportion eggs and increasing the time for this development. We believe that this change occured because the female progenitors flies had a

reduction in mature oocytes due to alterations in the diet, decreasing the number of viable eggs eclosion. Confirming our findings, Britton and Edgar (1998) suggested that progenitor flies when received a high-fat diet demonstrated a decrease in eclosion rate by decreased in oocyte maturation. Moreover, Britton and Edgar (1998) reported that is during the embrionary development, more specifically during the larval stages, larvae increase their mass about 200 fold during the 3 days which they spend feeding prior to pupariation, thus developing specific tissue to be differentiated in the intended adult of this insect, for example, gut, fat body, salivary glands, malpighian tubules, trachea, muscle and epidermis. Besides that, we understand the larvae need a balanced diet for their mitotic cellular division and normal development that have been altered, causing a loss in maturation flies and, consequently, increase in the time during this development observed in our study.

Considering the diet utilized (group received 20% coconut oil), caracterized by high saturated fat concentration, the flies descendants who managed to hatch showed a significant rise in glucose levels and triglyceride levels as observed in adult flies who also received similar diet in study by Heinrichsen et al. (2014). In fact, we believe that in models of flies descendants of the obese parents may present excess adiposity resulting in abnormal production of cytokines, growth factors, and hormones which in turn causes secondary diseases like insulin resistance (Guilherme et al., 2008; de Ferranti and Mozaffarian, 2008). In this way, we resolved investigate the insulin/insulin-like signaling (IIS) events what in Drosphila are remarkably conserved.

Eight insulin-like peptides (DILPs) are found to regulate physiological processes in *D. melanogaster* such as: growth (Brogiolo et al., 2001; Rulifson et al., 2002; Slaidina et al., 2009; Colombani et al., 2012; Garelli et al., 2012), metabolism (Broughton et al., 2008; Gronke and Partridge, 2010; Bai et al., 2012), lifespan (Broughton et al., 2005; Gronke and Partridge, 2010; Bai et al., 2012). In this study, we evaluated the DILP6 expression in homogenate of the flies descendantes that received coconut oil in the diet. In the group that received 20% of coconut oil, the flies that hatched, showed a significantly increase in the DILP6 expression. Other studies demonstrated that levels of DILP6 during larval development show an increased due ecdysone regulates during larval-pupal transition through unknown effectors (Kannan and Fridell, 2013). However, in the adult flies this increase it is related the regulation of the lifespan (Kannan and Fridell, 2013). We believe that this increase in DILP6 was due to increase the glucose levels generating a modified insulin/IGF signaling in order to extend lifespan.

Long-chain acyl CoA synthetase 1 (ACSL1) and Acetyl-CoA Synthetase (ACeCS1) plays an important role in fatty acid metabolism (Phillips et al., 2010). We observed in previous studies that adult flies when fed a HFD developed a significant decrease in glucose levels and, concomitantly, an increase in triglyceride levels (Paula et al., 2016). Therefore, we suggest that the HFD was being used for the production of energy, given that these enzymes responsible for the metabolism of fatty acids showed a significant increase (Paula et al., 2016). However, when evaluating the expression of ACeCS1 and ACSL1 in flies during development period until eclosion received the HFD (10% and 20% coconut oil) we can observe a different result than that found in adult flies. Here, ACeCS1 levels are significantly reduced and there is a tendency to decrease ACSL1 levels at the highest concentration tested. We consider those difference results in adults and descendants due to modification in to lipid metabolism, ACeCS1 levels is decrease while the ACSL1 levels it is not change. We think that ACSL1 levels it is regular due the flies are not performing extensive mobilization of fatty acids to mitochondrial matrix, a process known as β -oxidation. The product of β-oxidation is a ketone bodies formation and, consequently, acetate. Then, as there is no excess mobilization by β -oxidation, we can consider that acetate levels here in our study are relatively low and, whence, we observed a decreased in ACeCS1 levels. Besides, Phillips et al. (2010) reported that individuals genetically predisposed to metabolic syndrome, caused by exposition a dietary fat during embrionary development, showed develop an adaptation in your metabolism once it is this development period that need a lot of energy to the formation of the tissues that form the individual adult.

In a previous study, we demonstrated that exposure of adults *D. melanogaster* to coconut oil concentrations (10% and 20%) decreased locomotor capacity by changes in geotaxis negative and AChE activity (Paula et al., 2016). However, here evaluating the descendants flies, these changes in behavior and AChE activity not were observed, as well as the parameters of oxidative stress not were altered. We suppose that due to flies have passed by changes its development, has acquired a genetic adaptation to oxidative insults and, connected to this, we can say that in early life there are not many oxidative insults when compared with the course of development and adulthood. However, we saw in our study that the SOD mRNA expression and CAT and CAT activity were changed, presenting a way to reduce these values when compared to the DR group. In view of this, we think that this significant change left the cells of these animals more susceptible to oxidative insults during its development from adulthood to its aging process. Moreover, we should ask ourselves: the

reduction in antioxidant defenses affect the longevity and cause damage to shortening the useful life of these flies?

Based on the above question, we check that the longevity of flies that received coconut oil during the of development embrionary period were decreased confirming our suspicions that a decrease in antioxidant defenses in the descendants it can lead to a detrimental effect later these markers and shortening the useful life of these flies. An older study, it was show that activities of SOD, CAT, and peroxidases (PER) during *D. melanogaster* development are consistently low in larval stages, when compared with adults flies (Nickla et al., 1983). Besides, according to Carvalho et al. (2012), fatty acids available from the diet directly influence the composition of fatty acid moieties in cell membranes. This can be the primary cause of adult development with changes in their cell membranes and hence more susceptible to insult. However, a decrease in antioxidant system defenses demonstrated in the flies descendants (1-3 days after eclosion) of obese progenitors, as well as the significantly decreased in the MTT reduction test, caused the shortening the lifespan these flies. Thus, it seems plausible that strong adherence to high-fat diets by progenitors, detrimental to overall health and longevity, a conclusion which has an empirical support based in study of Lagiou et al. (2007).

In summary, our study revealed for the first time that flies when exposed at high-fat diet by coconut oil addition (10% and 20%), during the embryonic development period of *Drosophila melanogaster* have a reduced eclosion rate, decreasing the number of descendants flies and increasing the time for this development. Consequently, the survivors flies demonstrated a significantly increase in glucose and triglyceride levels they may have acquired a homologous factor to insulin resistance of the mammals, corroborating with data described in the literature that demonstrated increased levels of dilp6 in these cases. However, in this study protocol in descendants not was possible observe an oxidative stress situation momentary, oxidative parameters were not changed, but we believe that in the future these animals may display frames from oxidative insult as the expression of antioxidant enzymes have been shown harmed. In conclusion, flies that have progenitors fed with HFD can develop metabolic dysfunctions by insulin resistance, causing slow oxidative insults involved in shortening the lifespan.

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MANUSCRITO 2

Hesperidin ameliorates metabolic alterations, oxidative stress and protein signaling in the *Drosophila melanogaster* model of high-fat diet

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Manuscrito

2017

Hesperidin ameliorates metabolic alterations, oxidative stress and protein signaling in the *Drosophila melanogaster* model of high-fat diet

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ABSTRACT

The high consumption of saturated fats plays a fundamental role in the development of obesity and its complications. High fat diet (HFD) alter the normal metabolism of adult *Drosophila melanogaster* resulting in shortening of shelf life, oxidative stress and changes in protein signaling. This study evaluated the effects of bioflavonoid hesperidin (Hesp) on changes caused by HFD in adults and descendants flies. For the treatments, the experiments into two parts where treatments were carried out with the progenitor flies and the descending flies, divided in six groups each: regular diet (RD), HFD 10, HFD 20, RD + Hesp, HFD 10 + Hesp, HFD 20 + Hesp. After exposure to the diet, lifespan, survival, locomotor performance, oxidative damage and antioxidant enzymes were evaluated. Moreover, enzymes present in the fatty acid metabolism as acyl-CoA synthetase and acetyl-CoA synthetase (levels and expression of mRNA) and modulation of cells stress signaling pathway as dilp6 in flies that received a different diets. Considering that the reduction of antioxidant defenses and the development of oxidative stress caused by HFD causes a shortening in the life time of the progenitor flies. Hesp treatment was effective in increasing the shelf life and also the fertility of these flies given that the eggs of flies that were co-treated with Hesp during the seven days of HFD were developed in greater number of those who did not receive doses of flavonoid. Therefore, Hesp showed no harmful effects on the descendant flies, even if they were developed in a HFD, in addition to keeping the eclosion rate relatively normal, the offspring that had hatched maintained a lifetime comparable to the control flies. In summary, the study has revealed an efficient role of Hesp in the treatment of obese flies that have received HFD or developed in a HFD demonstrating that this flavonoid acts as a protective agent through analyzes of metabolic alterations, oxidative stress and protein signaling.

Keywords: Hesperidin; Acyl-CoA; high-fat diet; Lifespan; Lipid Metabolism; insulin-like peptides; insulin resistance.

1. INTRODUCTION

Food intake is a key feature for maintaining energy balance and survival (GARLAPOW et al., 2015). In humans, the consumption of excess calories is directly associated with the increased incidence of diseases and disorders, among them obesity (GARLAPOW et al., 2015). Obesity is a chronic metabolic disease that has become a global problem and, in Brazil, according to the World Health Organization (WHO), is the cause of death of 2.8 million people a year, a number that has increased dramatically in the past decades and continues to grow (Ministry of Health, 2013). Although the etiology of obesity is not entirely clear, the lifestyle, including poor diet, lack of physical activity habits and genetic factors are the main aspects contributing to this overweight problem.

The high consumption of saturated fats in the diet play a key role in the development of obesity in humans and other animals (Clemmensen et al, 2012;. Speakman, 2013). In models of vertebrate animals (rodents, frogs, fishes), research with modified diets have increased the understanding of biology and human physiology (Burggren & Warburton, 2007; Lieschke & Currie, 2007; Hunziker et al, 2009; Chianese et al, 2011; Morris et al, 2014).

However, the scientific research is difficult in these models they are genetically and metabolically complex, limiting the detailed supply capacity of the key processes such as cell signaling pathways (Diop & Bodmer 2012). Given the complexity of the organism to changes in diet response, invertebrate models can serve as useful tools in the discovery of interaction between genes, signaling pathways, and metabolism (Oldham & Hafen, 2003; Baker & Thummel, 2007; Leopold & Perrimon, 2007; Perez & Van Gilst, 2008).

Drosophila melanogaster (*D. melanogaster*) models have been extensively studied as to effects caused by eating a high-fat diet (HFD), as shown clearly deleterious phenotype by increasing the levels of triglycerides and glucose, decreasing stress tolerance, shortening the lifetime can induce accumulation of fat causing cardiac dysfunction (Birse et al, 2010;. & Heinrichsen Haddad, 2012). Moreover, in previous studies we have shown that progenitors flies fed for seven days with a HFD, rich in saturated fatty acids, demonstrated alterations in the normal metabolism, survival, lifespan, lipid peroxidation, antioxidant enzymes, expression of HSP83 and MPK2 mRNA and enzymes involved in lipid metabolism, concluding that there is an association among metabolic changes, oxidative stress and protein signalization, involved in shortening the lifespan of flies fed with a HFD (Paula et al., 2016).

Consequently, there are growing studies investigating the molecular mechanisms that control food intake and body weight in order to develop innovative pharmacological and non-

pharmacological strategies to combat obesity (Smith et al., 2014). In recent decades, the search for natural compounds and synthetic antioxidants with therapeutic actions have increased notably. The bioactive compounds from natural products, such as plants and their different polyphenolic compounds, have demonstrated beneficial effects to human health (Pandey & Rizvi, 2009; Visioli et al, 2011; Del Rio et al, 2013). Hesperidin (Hesp) is one of the most abundant natural flavonoids found, and isolated from the bark of a species of citrus with beneficial effects both in vitro and in vivo and plays an antioxidant, antimicrobial, anti-inflammatory, anticarcinogenic and antiallergic activities (Garg et al., 2001; Roohbakhsh et al., 2014; Parhiz et al, 2015). In addition, it is believed that one of the most important roles of Hesp is its antioxidant property since it prevents oxidant injury and cell death caused by free radicals that generate oxidative stress (El-Sayed et al., 2007).

However, the wide range of pharmacological activities and widespread applications of Hesp, as well as their antioxidant, lipid-lowering and insulin-sensitizing properties need to be further investigated in fruit fly models *D. melanogaster*, in view of the difficulty in finding studies that associate a benefit of Hesp in treatment for the effects caused by HFD in flies.

2. MATERIALS AND METHODS

2.1 Reagents

The virgin coconut oil produced by Pró-Ervas®. Chemicals, including thiobarbituric acid (TBA), 2',7'-dichlorofluorescein diacetate (DCFDA), cocktail protease inhibitor, sodium orthovanadate, 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide, ethylenediaminetetraacetic acid (EDTA), quercetin, N,N,N',N'-tetramethylethylenediamine (TEMED), mannitol, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 7-hydroxy-3H-phenoxazin-3-one 10-oxide (Resazurin), and β-mercaptoethanol, were procured from Sigma-Aldrich Co., LLC, St. Louis, MO, USA. Fatty acid and Lipid Metabolism Antibody Sampler Kit and β-actin antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). Triglycerides liquiform and glucose liquiform were obtained from Labtest (Lagoa Santa, MG, Brazil). The sodium dodecyl sulfate was procured from GE Healthcare Life

Sciences (LittleChalfont, Bucks, ENG). Trizol Reagent and DNase I were obtained from Invitrogen (Grand Island, NY). iScript cDNA Synthesis Kit was from Bio-Rad (Laboratories, Montreal, Quebec). Tris(hydroxymethyl)aminomethane, hydrogen peroxide, TRITON X-100, and dimethyl sulfoxide were purchased from Synth (Diadema, SP, Brazil). The virgin coconut oil purchased commercially and produced by Pro-Ervas® and the compound hesperidin (H) was obtained from Sigma-Aldrich (Co. LLC, St. Louis, MO, USA).

2.2 Animals

D. melanogaster (Harwich strain) was obtained from the National Species Stock Center, Bowling Green, OH, USA. The flies, both male and female, were maintained at 25 °C on 12 h light/dark cycle in glass bottles containing 10 mL of regular diet (RD) contaning: corn flour (76.59%), wheat germ (8.51%), sugar (7.23%), milk powder (7.23%), salt (0.43%) and a pinch of methyl paraben. All experiments were performed with the same strain, and both genders were used at random.

2.3 Experimental protocol

2.3.1 High-fat diet (HFD) exposure and Hesp treatments

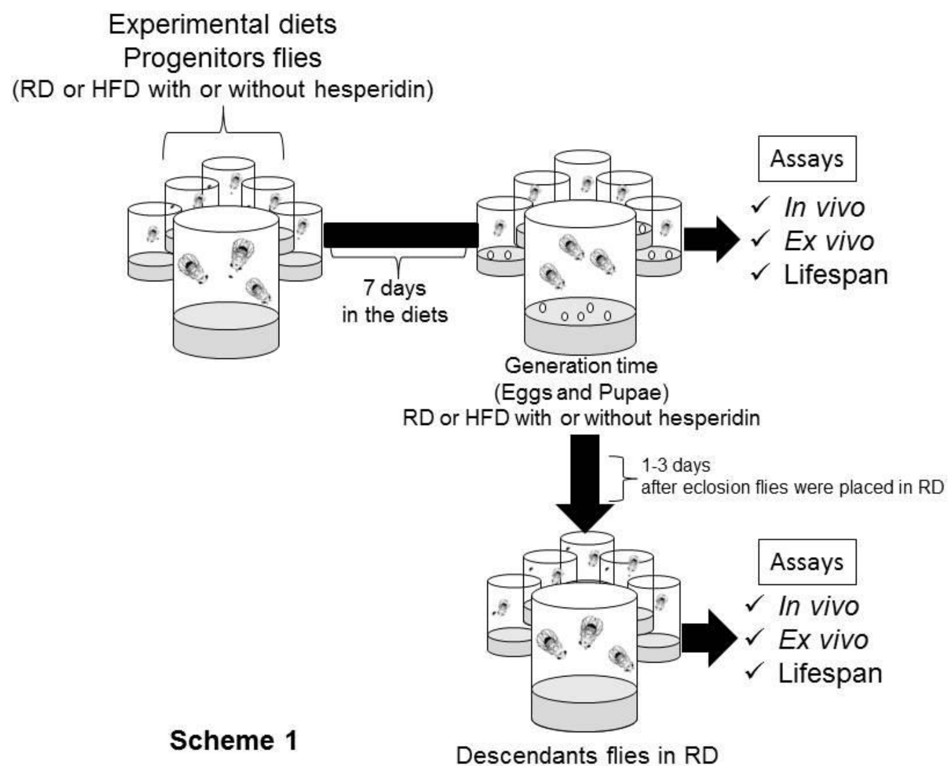
For the treatments, the experiments into two parts where treatments were carried out with the progenitor flies and the descending flies. For the treatment of progenitor flies, were divided in six different groups: RD (regular diet), HFD 10 (high fat diet with 10% coconut oil), HFD 20 (high fat diet with 20% coconut oil), RD + Hesp (regular diet plus hesperidin 10μM), HFD 10 + Hesp (high fat diet with 10% coconut oil plus hesperidin 10μM) and HFD 20 + Hesp (a high fat diet with 20% coconut oil plus hesperidin 10μM). For the treatment of desdendants flies, were also divided into six different groups: RD (regular diet), HFD 10 (high fat diet with 10% coconut oil), HFD 20 (high fat diet with 20% coconut oil), RD + Hesp (regular diet plus hesperidin), HFD 10 + Hesp (high fat diet with 10% coconut oil plus

hesperidin) and HFD 20 + Hesp (a high fat diet with 20% coconut oil plus hesperidin). The methods are presented according to the protocols below.

2.3.1.1 Treatment of progenitors flies

For treatment and diet of progenitors, adult flies (3-4 days) were exposed at coconut oil concentrations (10% and 20%). These concentrations were chosen based on previous studies by Paula et al. (2016). Hesp was added or not at diet at 10μM during seven days. Concentration of Hesp was chosen by dose curve study. After this period of exposure to diet to coconut oil and or Hesp, flies were anesthetized and homogenized for different assays. The media containing the diets were maintained so that the eggs could develop and generate the downward flies (Scheme 1).

2.3.1.2 Treatment of descendants flies



Scheme 1

After seven days laying eggs the progenitors flies were removed. When eggs were newly ecloded the descendants flies of all groups were transferred to a RD maintened in a RD

until the experiments. Experiments were carried out with 1-3 days old flies. The coconut oil concentrations (10% and 20%) were chosen based on previous studies (Paula et al., 2016) and the concentration of Hesp was chosen by dose curve study (10µM) (Scheme 1).

2.4 *In vivo* assays

2.4.1 Survival rate and lifespan of progenitors

The survival rate was evaluated by counting daily the number of living flies until the end of the experimental period (7 days). Around 60 flies *per group* were included in the survival data and the total number of flies represents the sum of three independent experiments (20 flies/each treatment repetition). The lifespan, flies were maintained under different experimental diets for seven days (with or without coconut oil or hesperidin). After this, flies were placed in RD, which were changed every two days, daily counted until there were no more flies alive.

2.4.2 Cumulative eclosion and lifespan of descendants

It was used the cumulative eclosion of eggs proportion obtained of progenitor (see scheme 1) to count the total number of flies which have hatched in each diet, until there were no more viable eggs. In order to test the lifespan, three days after the eclosion, the flies were maintained under standard medium, which were changed every two days, until there were no more flies alive.

The lifespan was performed when, after the eclosion of flies that developed in the different diets (with or without coconut oil or hesperidin), were maintained in RD, which were changed every two days, and until there were no more flies alive.

2.4.3 Analysis of locomotor ability

The locomotor ability of the flies was assessed by climbing test also known as Negative Geotaxis test as described by Jimenez-DelRio et al. (2010) with some modifications. On the last day of treatment of progenitors and the second day after hatching the flies'

descendants ten flies of each treatment group were anesthetized on ice and placed individually in Falcon tubes of 10 mL, stoppered with sponges for airflow. After this and after an average time of 10 minutes of recovery, we recorded the average time each fly took to reach a height of 8 cm, measured from the bottom of the tube. The test was repeated five times for each fly, and ten were separated and evaluated flies in each group and the data analyzed according averaging time.

2.5 *Ex vivo* assays

2.5.1 Analysis of triglycerides, glucose and total protein levels

For sample preparation, a total of 20 flies (without sex distinction) were decapitated, weighed and homogenized in 1ml of 20mM Hepes, pH 7.0. The homogenate was centrifuged at 14,000 rpm for 30min. at 4 ° C. The levels of total triglycerides were assessed by using the Labtest's own kit following protocol Gronke et al. (2003). For the analysis of glucose levels was also used Labtest's own kit following the Birse et al protocol. (2010). Results are expressed in milligrams per deciliter (mg / dL). The sample protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as standard.

2.5.2 Enzymatic assay for the evaluation of acetylcholinesterase activity (Ache)

For the analysis of acetylcholinesterase activity, 20 flies were homogenized in 1 ml of 10 mM Tris buffer, pH 7.4 then centrifuged at 1000 xg for 10 minutes (4 ° C). The analysis of the activity of acetylcholinesterase (AChE, EC 3.1.1.7) occurred following the protocol described by Ellman et al. (1961). Briefly, a reaction mixture containing phosphate buffer is performed (0.25M, pH 8.0), 5,5-dithiobis-2-nitrobenzoic acid (5mM), 25µl sample and 50mL of acetylthiocholine iodide (7,25mM). The reaction was monitored for two minutes at 412 nm. The enzymatic activity was expressed as nanomole of substrate hydrolyzed per minute per milligram protein.

2.5.3 Determination of reactive species (RS) and lipid peroxidation levels

To quantify the generation of reactive species (RS) a total of 10 flies were anesthetized on ice and homogenized in 500µL of 10mM Tris buffer, pH 7.4. The homogenate was centrifuged at 1000 xg for 5 minutes at 4 ° C and the supernatant removed for assay quantification of the oxidation of 2 ', 7'-dichlorofluorescein diacetate (DCF-DA) as a parameter and general index of oxidative stress following protocol Pérez-Severiano et al. (2004). The resulting DCF fluorescence emission of DCF-DA oxidation was monitored after one hour, a 485nm excitation wavelength and a wavelength of 530nm emission in the fluorometer. DCF formation results are shown in arbitrary units and represent the mean of three independent experiments performed in duplicate.

Lipid peroxidation products were quantified as thiobarbituric acid reactive substance (TBARS) following the method of Ohkawa et al. (1979) with minor modifications. Briefly, ten whole flies in each group were homogenized in 1 mL 0.1 M phosphate buffer pH 7.0 and centrifuged at 1000 x g for 5 min (4°C). After centrifugation, the supernatant was removed and added to thiobarbituric acid (0.8% pH 3.2), acetic acid (20%, pH 3.5), sodium lauryl sulfate (SDS) (8%). The mixture was incubated for two hours at 95°C for 60 min and absorbance then measured at 532 nm. Results represent the mean of three independent experiments. In each experiment, each treatment was done in duplicate. The results were expressed as nmol TBARS/mg tissue.

2.5.4 Antioxidant enzymes activity

2.5.4.1 Determination of catalase activity

The catalase activity (CAT; EC 1.11.1.6) was measured following the method of Aebi (1984). Twenty flies in each group were anesthetized on ice and homogenized in 1ml of 10mM Tris buffer, pH 7.4 then centrifuged at 20,000 x g for 30 minutes (4°C). The supernatant was removed and the activity of catalase (CAT, EC 1.11.1.6) was measure in the mixture containing phosphate buffer (0.25M / 2.5mM EDTA, pH 7.0), H_2O_2 (10mM), 0.012% Triton X 100. The reaction was monitored for one minute at 240 nm and expressed as H_2O_2 decomposed micromoles / min / mg protein.

2.5.4.2 Determination of superoxide dismutase activity

Superoxide dismutase activity (SOD, EC 1.15.1.1) was measured according to Kostyuk and Potapovich (1989), with minor modifications by Franco et al. (2010). It was performed by monitoring the inhibition of quercetin auto oxidation. The reaction mixture contained sodium phosphate buffer (0.025 M/EDTA 0,1 mM, pH 10), N,N,N,N-tetramethylethylenediamine (TEMED), 10 μl sample and it was started by adding 0.15% quercetin dissolved in dimethyl formamide. The reaction was monitored for two minutes at 406 nm, and it is expressed as the amount of protein required to inhibit 50% of quercetin auto oxidation.

2.5.5 Resazurin reduction test

Resazurin reduction test of *Drosophila melanogaster* was carried where 20 whole flies from each treatment were anesthetized on ice and homogenized and it was performed by using the CellTiter-Blue® cell viability assay kit by Franco et al. (2009). The method is based on the ability of viable cells to reduce resazurin to resorufin, a fluorescent molecule. Whole flies were homogeneized in 10 mM tris buffer, pH 7.4 and 100 μL pipetted in a 96-well plate and an aliquot of CellTiter-Blue was added according to the instructions of the manufacturer. After 1 h, the fluorescence was recorded using at ex579nm and em584nm.

2.5.6 Preparation of mitochondrial enriched fractions and MTT reduction test

A mitochondrial enriched fraction was prepared from the whole body of flies using the method of differential centrifugation by Hosamani (2013). Briefly, 0,3 g of flies from each treatment were homogenized in ice-cold Tris-sucrose buffer (0.25 M, pH 7.4) (60 mg fly tissue homogenized in 1000 μl buffer) and centrifuged at 1000 *x g* for five minutes (4° C). A fraction enriched with mitochondria was obtained by centrifuging the post nuclear supernatant at 10000 × g for 10 minutes (4° C). The pellet was washed in mannitol–sucrose–HEPES buffer and resuspended in 200 μl of suspension buffer.

A portion of 200 microliters of the mitochondrial enriched fraction was incubated with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) MTT (x% solution) for 30 minutes at 37° C. After that, the samples were centrifuged at 10000 x g for five minutes. The pellet was dissolved in Dimethyl sulfoxide (DMSO), incubated for 30 minutes at 37° C and the absorbance was measured at 540 nm. Results were expressed as percentage of the control.

2.5.7 Protein determination: Acetyl-Coenzyme A Synthetase (ACeCS 1) and Acyl-Coenzyme Synthetase (ACSL 1)

Groups of 40 whole flies were homogenized at 4°C in 200μL of buffer (pH 7.0) containing 50mM Tris, 1mM EDTA, 20mM Na_3VO_4, 100mM sodium fluoride, and protease inhibitor cocktail according with Paula et al. (2016). Then, the homogenate was centrifuged for 10min at 1000 ×g at 4°C and the supernatant was collected. After protein determination following Bradford (1976), 4 % SDS solution, ß-mercaptoethanol and glycerol was added to samples to a final concentration of 100, 8 and 25 %, respectively and the samples frozen for further analysis. Proteins were separated using SDS-PAGE with 10 % gels, and then electrotransferred to nitrocellulose membranes as described by Paula et al. (2012). Membranes were washed in tris-buffered saline with Tween (100 mM tris-HCl, 0.9 % NaCl and 0.1 % Tween-20, pH 7.5) and incubated overnight at 4°C with specific primary antibodies: anti-Acetyl-Coenzyme A Synthetase (ACeCS 1) and anti-Acyl-Coenzyme Synthetase (ACSL 1) and anti-β actin. Following incubation, the membranes were washed in Tris-buffered saline with Tween and incubated for 1 h at 25°C with anti-rabbit Ig-secondary antibodies. Antibody binding was visualized using the ECL Western Blotting Substrate Kit (Promega). Band staining density was quantified using the Scion Image software (Scion Image for Windows). Results are expressed as optical density of ACSL1 or ACeCS1/optical density of respective ß-actin.

2.5.8 Determination of gene expression of SOD, CAT, HSP83 and *dilp*6

Approximately 1 µg of total RNA from 20 flies was extracted using the Trizol Reagent (Invitrogen) accordingly 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 accordingly to the manufacturer's suggested protocol (BIORAD). Quantitative real-time polymerase chain reaction was performed in 11 µL reaction volumes containing water treated with diethyl pyrocarbonate (DEPC), 200 ng of each primer (described in Table 1), 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 (Livak and Schmittgen, 2001).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 (sequences of tested genes are represented in Table 1). The ΔCT mean value obtained from the control group of each gene was used to calculate the ΔΔCT of the respective gene ($2^{-\Delta\Delta CT}$).

2.6 Statistical analysis

Lifespan measurement was determined by comparing the survival curves with a log-rank (Mantel–Cox) test. Other statistical analysis was performed using two-way ANOVA followed by Newman–Keuls post hoc test where appropriate. Differences were considered significant between groups at $p < 0.05$ using the GraphPad Prism5 program.

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

Gene	Primer Sequences	
GPDH	LEFT	5’- ATGGAGATGATTCGCTTCGT
	RIGHT	5’- GCTCCTCAATGGTTTTTCCA
SOD	LEFT	5’- GGAGTCGGTGATGTTGACCT
	RIGHT	5’- GTTCGGTGACAACACCAATG
CAT	LEFT	5’- ACCAGGGCATCAAGAATCTG
	RIGHT	5’- AACTTCTTGGCCTGCTCGTA
HSP83	LEFT	5’- CAAATCCCTGACCAACGACT
	RIGHT	5’- CGCACGTACAGCTTGATGTT
DILP6	LEFT	5’- CCCTTGGCGATGTATTTCC
	RIGHT	5’- CACAAATCGGTTACGTTCTGC

3. RESULTS

3.1 Hesperidin causes an increase in the lifespan and survival of adult flies treated with HFD

According to Figure 1A, the lifespan of flies receiving a RD was up to 41 days. However, when adding coconut oil at both concentrations tested (HFD10 and HFD20), the maximum life span drops to 28 and 15 days, respectively. Yet, when hesperidin was added in both diets, the compound has the property to significant increase this life time to 49 days for the hesperidin group, 34 days for the HFD10 plus hesperidin and 32 days for the HFD20 plus hesperidin.

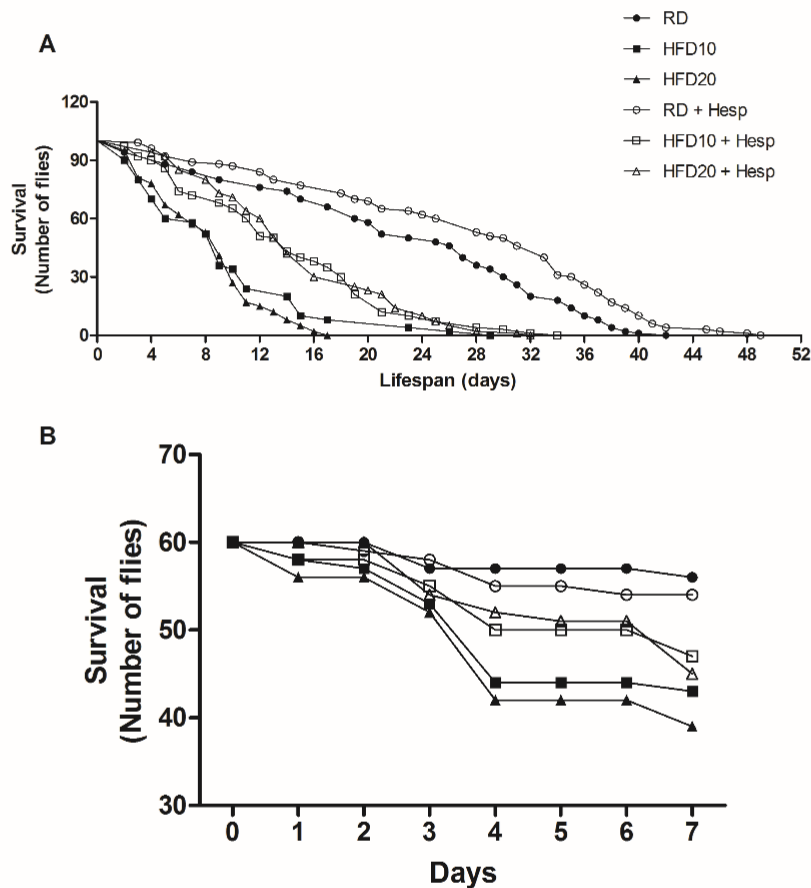


Figure 1. Lifespan and survival curves due to a high-fat diet supplemented with or without hesperidin. Wild type *Drosophila melanogaster* (strain Harwich: both sexes) were placed on regular diet (RD) and high-fat diet (HFD) supplemented with or without hesperidin, under controlled conditions. (A) Lifespan: the flies were counted daily until there were no more flies alive. Hesperidin in the diet caused a significant increase in lifespan of flies treated with HFD. In addition, hesperidin alone is able to increase the life expectancy of flies. Four sets of twenty-five flies were used for each diet condition. (B) Survival curve in seven days: the number of live and dead flies was counted every 24 h during seven days of the treatment and exposure of diets. Coconut oil (10% and 20%) in the diet caused a significant decrease in survival rate. However, when added hesperidin this is

reversed increasing the resistance to death of these flies. Three sets of twenty flies were used for each diet condition. $p < 0.05$ in relation to RD group.

Furthermore, seven days of exposure of progenitors flies to a HFD10 and HFD20 caused significant reduction on survival (28% and 35%, resp.) when compared with the RD group ($p < 0.05$). When hesperidin was added in both diets, the compound has the property to significant increase in the survival flies during seven days (7% in the HFD10 plus hesperidin and 10% in the HFD20 plus hesperidin) (Figure 1B).

3.2 Hesperidin improves the survival rate and restores cumulative eclosion kept flies eggs of *D. melanogaster* treated with HFD

According to Figure 2A, hesperidin demonstrated a significant increase in cumulative eclosion proportion of flies that received RD in comparison to the control group. Furthermore, when adding coconut oil at both concentrations tested (HFD10 and HFD20), this cumulative eclosion proportion is decreased. Yet, when hesperidin was added in both HFD, the accumulative eclosion proportion was increased.

The same significant increase caused by hesperidin was also observed in number of eclosion eggs (Figure 2B). Hesperidin alone does not cause alterations in the number of eclosion and, moreover, increased the number of eclosion in HFD10 (Figure 2B).

Lifespan of descendant flies receiving a RD was up to 50 days. However, when adding coconut oil at both concentrations tested (HFD10 and HFD20), the maximum life span drops to 29 and 17 days, respectively. Yet, when hesperidin was added in both diets, the compound has the property to significantly increase this life time to 55 days for the hesperidin group, 41 days for the HFD10 plus hesperidin and 39 days for the HFD20 plus hesperidin.

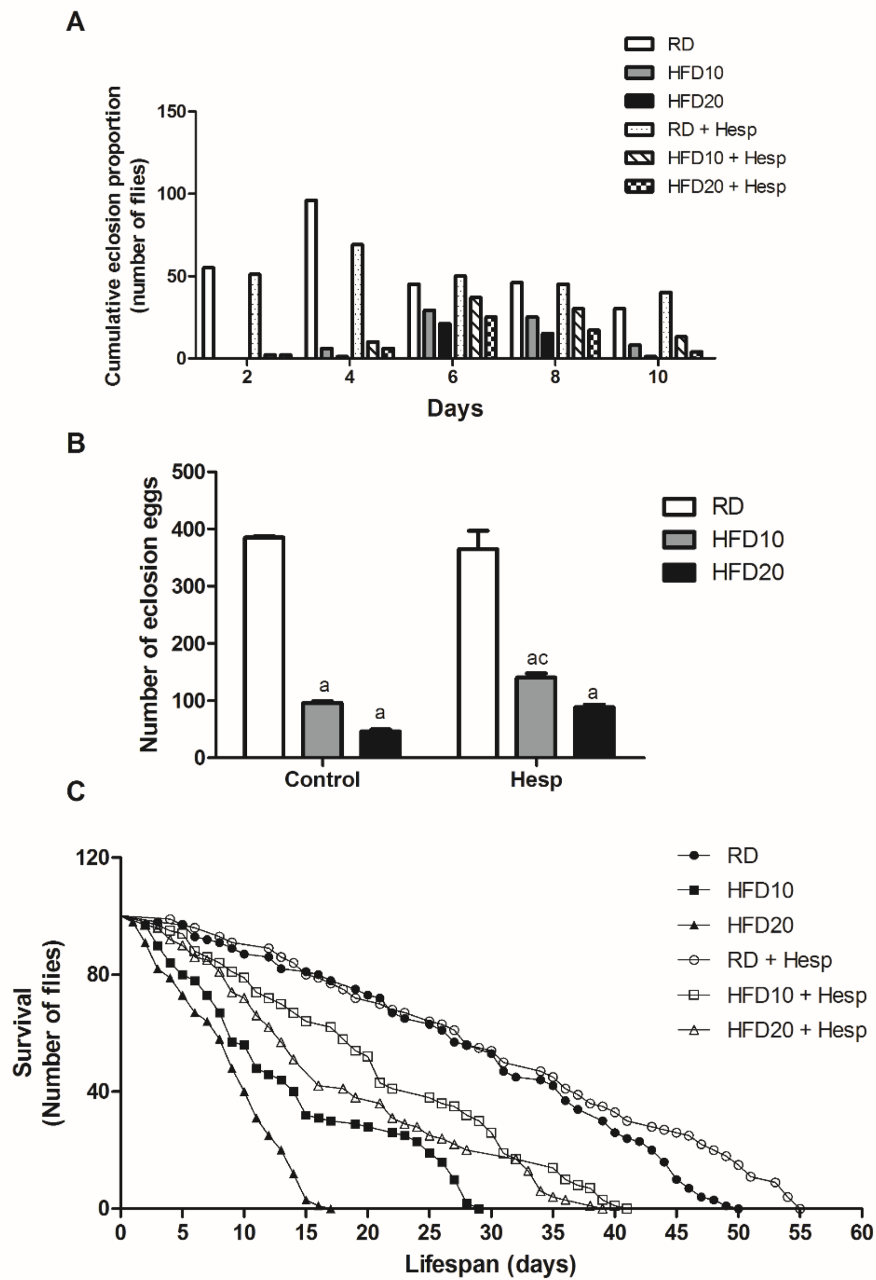


Figure 2. Hesperidin improves the cumulative eclosion eggs and lifespan of *D. melanogaster* treated with HFD. Wild type *Drosophila melanogaster* (strain Harwich: both sexes) were placed on regular diet (RD) or high-fat diet (HFD) with virgin coconut oil with or without hesperidin (10µM) under controlled conditions. Three sets of ten flies (seven females and three males) in each treatment (exposed to RD and HFD for reproduction). After seven days, the progenitors flies were removed and, daily the eggs were monitored until the eclosion. (A) The data expresses the cumulative eclosion proportion in number of flies every two days. (B) Number total of eclosion eggs. (C) Lifespan: the flies were counted daily until there were no more flies alive. [a] $p < 0.05$ and different when compared with your respective control; [b] $p < 0.05$ and different when compared with RD group; [c] $p < 0.05$ and different when compared with HFD10 group; [d] $p < 0.05$ and different when compared with HFD20 group.

3.3 Hesperidin combined with HFD normalizes the levels of triglycerides and glucose of progenitors and descendants flies

According to Van der Horst (2003), the flies store energy predominantly as triglycerides in the body fat. Hence, the triglyceride levels in progenitors and descendants flies of treatments were measured. In fact, when the flies were treated with HFD (both progenitors as descendants in major concentrations) the triglyceride levels were significantly increased. However, when hesperidin was combined with high-fat diet normalized triglyceride levels in both treatments compared to the control group (Figure 3A and 3B).

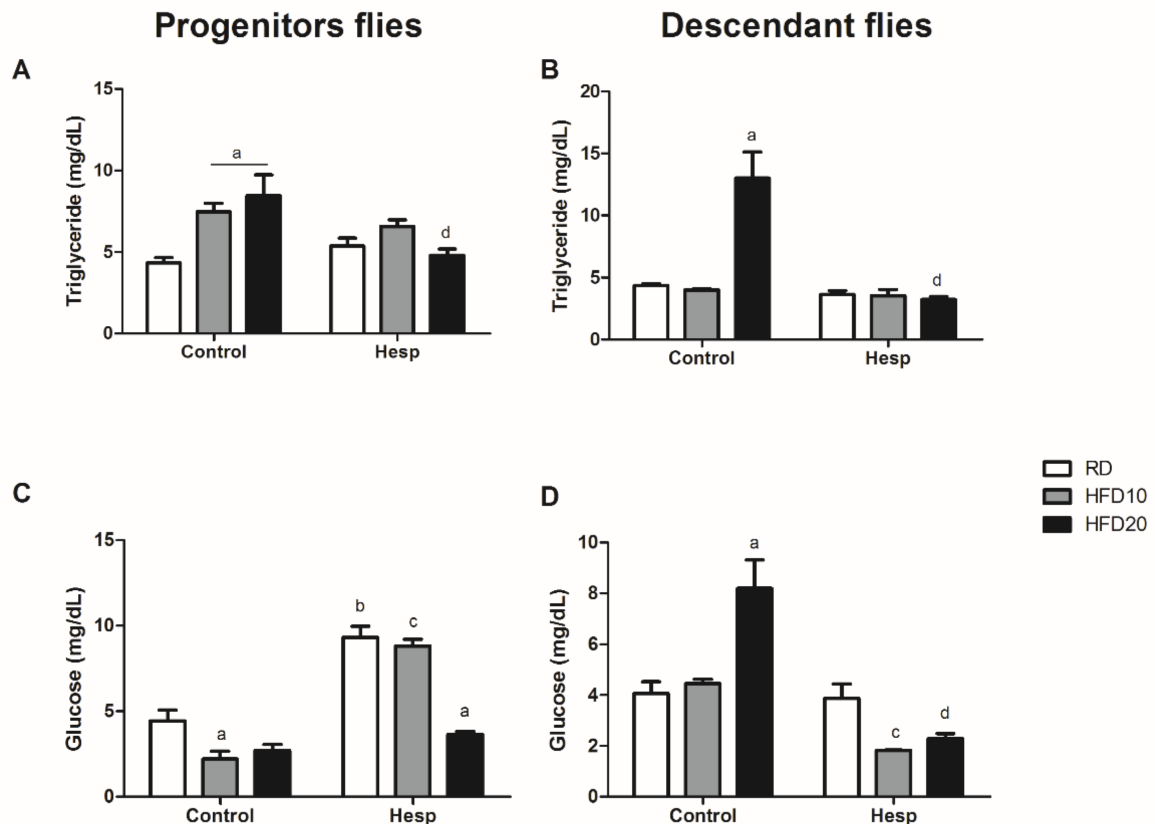


Figure 3: Effects of exposure to hesperidin front of a high fat diet (HFD) on triglyceride levels and glucose levels in progenitors and descendants flies of *D. melanogaster*. After seven days on the diets, three sets of twenty progenitors flies of each diet condition were used for triglyceride and glucose measurements. The medium were kept without progenitors flies to the eclosion of offspring flies. After eclosion, the offspring were taken to an RD and subsequent analysis. (A) and (B) The data expresses triglycerides levels in whole flies homogenate expressed in mg/dL. (C) and (D) Glucose levels in flies homogenate expressed in mg/dL. [a] $p < 0.05$ and different when compared with your respective control; [b] $p < 0.05$ and different when compared with RD group; [c] $p < 0.05$ and different when compared with HFD10 group; [d] $p < 0.05$ and different when compared with HFD20 group.

To assess the possibility that HFD might affect glucose levels, glucose levels were measured in whole body of adult flies treated with HFD10, HFD20 and hesperidin as well the descendants flies staying in RD and HFD during the embryonic development period. Next was asked whether the hesperidin is able to normalize glucose levels of progenitors or descendants flies that have developed in HFD? In progenitors flies hesperidin caused an increase in the group given RD with compound and in the group that received minor concentration of coconut oil (HFD10) when compared to control group. Moreover, the glucose levels when measured in descendants exposed to a RD, HFD or hesperidin during embryonic development period demonstrated a significant decrease when compared to HFD10 and HFD20.

3.4 Locomotor performance and acetylcholinesterase activity

Exposure of progenitors flies to HFD showed changes of climbing behavior performance by negative geotaxis. This effect was abolished by the treatment with hesperidin, once progenitors flies from this group had better performance climbing (Figure 4A). Regarding the descendants flies exposed to HFD during embryonic development period they demonstrated a tendency to increase the climbing time in the group HFD10 and also a significant increase in the group HFD20. When hesperidin was added in the diet (RD and HFD10) this impairment in locomotor performance was repaired when compared to control group but this result was not observed in the group HFD20 (Figure 4B).

For Kim and Lee (2013), the acetylcholine is the primary excitatory neurotransmitter in the central nervous system in parallel with the behavioral parameter and learning/memory. Wherefore, we evaluated acetylcholinesterase activity. As shown in figure 4C, the HFD decreases the acetylcholinesterase activity. This effect was annulled when the flies were treated with RD more hesperidin and HFD20 more hesperidin. Additionally, it was also determined the activity of acetylcholinesterase (Ache) in the descendants flies revealing only a significant result in the group receiving HFD10 more hesperidin (Figure 4D).

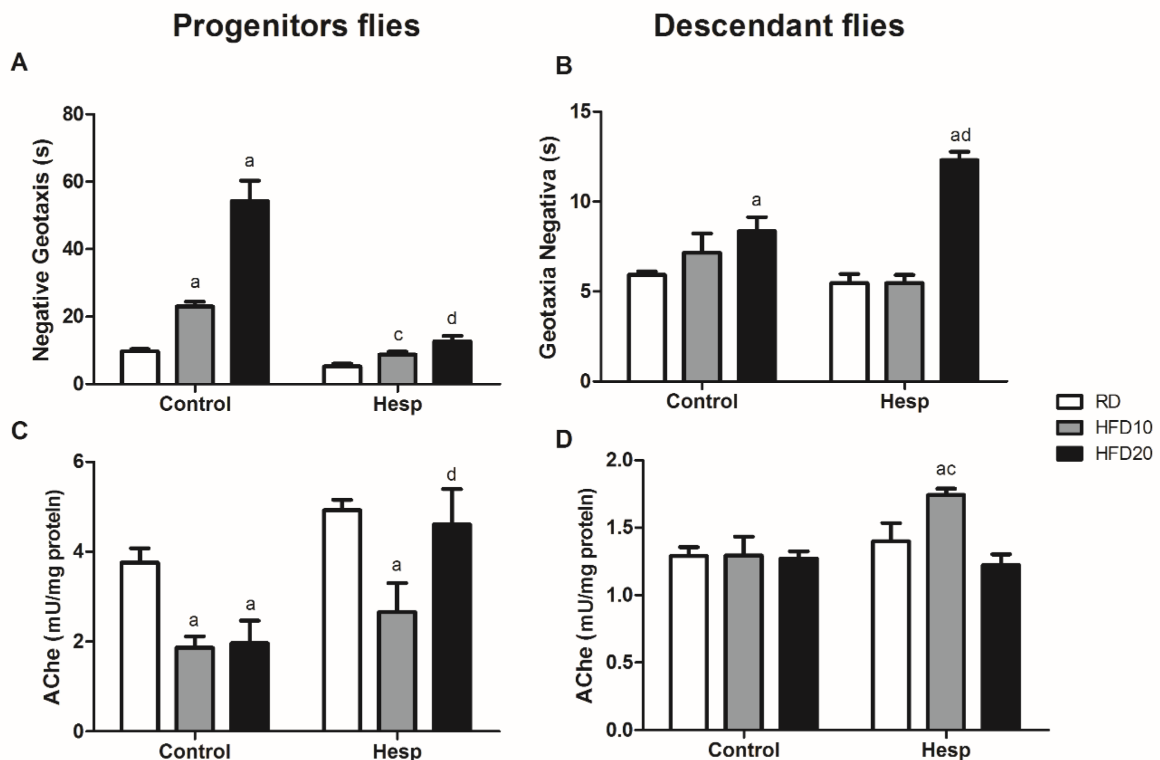


Figure 4: Effects of the exposure to Hesperidin under the influence to a high-fat diet on in *D. melanogaster* **exposed during embryonic development period and during adult life on locomotor performance and acetylcholinesterase (AChe) activity.** Flies (both sexes) were exposed to RD or HFD with or without hesperidin (10µM) during embryonic development period and during adult life. (A) and (B) Locomotor ability of flies was analyzed by negative geotaxis. Three sets of ten flies were used for each diet condition. Results are expressed as mean of time spent to reach 8 cm in a glass tube ± SE of three independent experiments. (C) and (D) The data shows the AChe activity in progenitors and descendant flies homogenate expressed as mean (mU/mg protein) ± standard deviation. Three sets of twenty flies were analyzed for each diet condition. [a] $p< 0.05$ and different when compared with your their respective control; [b] $p< 0.05$ and different when compared with RD group; [c] $p< 0.05$ and different when compared with HFD10 group; [d] $p< 0.05$ and different when compared with HFD20 group.

3.5 Oxidative stress and antioxidants defensive activity

The treated of flies with HFD had a significant increase in the production of reactive species measured by the oxidation of DCFDA and lipid peroxidation and its product malondialdehyde (MDA) in treatment of progenitors flies compared to the control group. The co-exposure to HFD and hesperidin was able to reduce MDA levels and DCFDA oxidation (Figures 5A and 5C). The evaluation of oxidative stress parameters in flies descendants exposed to RD and HFD with or without co-exposure to hesperidin during the embryonic development period revealed a significant decrease in oxidation of DCFDA only in the

hesperidin group (Figure 5B). In contrast, the lipid peroxidation shows a significant decrease in HFD20 group more hesperidin (Figure 5D).

The activity of the antioxidant enzymes CAT and SOD were determined after seven days of treatment with RD and HFD of progenitors flies and in descendants flies exposed to RD and HFD during the embryonic development period. After seven days of treatment, the flies that received HFD20 demonstrated a significant increase in the activity of both catalase (CAT) and superoxide dismutase (SOD) enzymes. This result was sustained by co-exposure of hesperidin (Figures 5E and 5G). However, the results of descendant flies did not show a significant difference in activity of the antioxidant enzyme superoxide dismutase (SOD) (Figure 5H). However, the flies that received HFD20 during the embryonic development period demonstrated a significant decrease in the activity of the antioxidant enzyme catalase (CAT) what was sustained by co-exposure with hesperidin (Figure 5F).

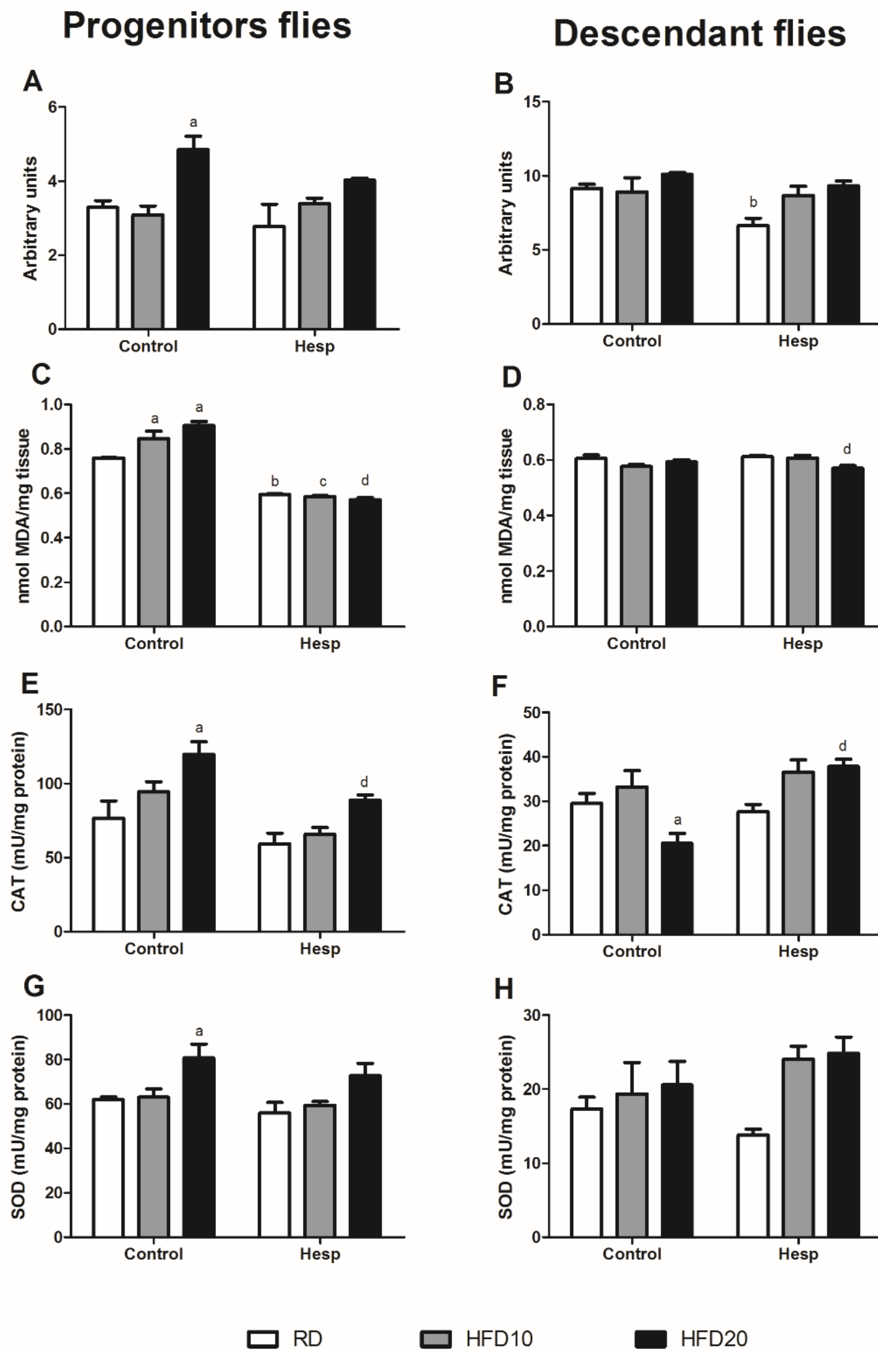


Figure 5: Effects of the exposure to Hesperidin under the influence to a high-fat diet on in *D. melanogaster* exposed during embryonic development period and during adult life on oxidative stress parameters. Flies (both sexes) were exposed to RD or HFD with or without hesperidin (10µM) during embryonic development period and during adult life. Flies were homogenized and the supernatant was used for various analyses of stress markers and the activity of antioxidant enzymes. (A) and (B) Shows the DCF-DA intensity of fluorescence in total flies homogenate. (C) and (D) End products of lipid peroxidation determined by TBARS assay in total flies homogenate, expressed in MDA nmoles/mg of protein (E) and (F) Catalase (CAT) activity in total flies homogenate. (G) and (H) Superoxide dismutase (SOD) activity in total flies homogenate. For both analyzes, three sets of twenty flies were analyzed for each diet condition. Data are expressed as a mean ± standard deviation in mU/mg protein. [a] $p < 0.05$ and different when compared with your respective control; [b] $p < 0.05$ and different when compared with RD group; [c] $p < 0.05$ and different when compared with HFD10 group; [d] $p < 0.05$ and different when compared with HFD20 group.

3.6 MTT reduction in enriched mitochondrial fraction and resazurin reduction

Exposure to HFD did not cause resazurin reduction in either progenitors flies as nor their descendants. However, when hesperidin was added in HFD, this compound was able to increase this resazurin reduction in the flies (Figure 6A and 6B). In addition, it showed that HFD causes a significant decrease of MTT reduction on mitochondria in both progenitors flies and their descendants. This effect was abolished by the hesperidin treatment in progenitors flies (Figures 6C). Yet in treatment of descendants, only the co-exposition with hesperidin in HFD20 caused a significant decrease in MTT reduction (Figure 6D).

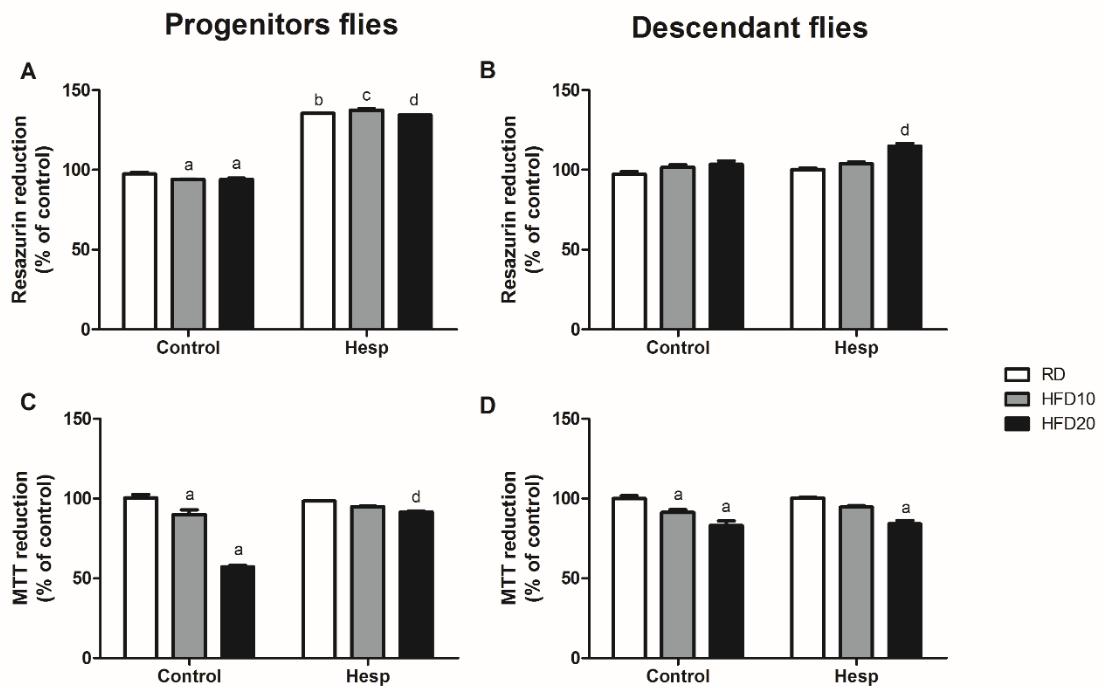


Figure 6: Effects of the exposure to Hesperidin under the influence to a high-fat diet on in *D. melanogaster* **exposed during embryonic development period and during adult life on MTT reduction in mitochondrial enriched fraction and resazurin reduction.** Flies (both sexes) were exposed to RD or HFD with or without hesperidin (10µM) during embryonic development period and during adult life. Flies were homogenized and the supernatant was used for various analyses of stress markers and the activity of antioxidant enzymes. (A) and (B) resazurin reduction both progenitors flies as their descendants. (C) and (D) MTT reduction on mitochondria in both progenitors flies as their descendants (approximately 60 mg fly tissue were analyzed for each diet condition). Both graphs expresses the results as a percentage (%) in relation to the RD group (mean ± standard deviation). [a] $p < 0.05$ and different when compared with your respective control; [b] $p < 0.05$ and different when compared with RD group; [c] $p < 0.05$ and different when compared with HFD10 group; [d] $p < 0.05$ and different when compared with HFD20 group.

3.7 Analyses of Acetyl-Coenzyme A Synthetase (ACeCS 1) and Acyl-Coenzyme Synthetase (ACSL 1)

The treated of progenitors flies with HFD10 and HFD20 had a significant increase in ACeCS1 content when comparing to control group. The co-exposure to HFD and hesperidin was able Increase levels of enzyme expression (Figure 7A). The treated of descendants flies with HFD10 and HFD20 had a significant decrease in ACeCS1 content when comparing to control group. The co-exposure to HFD and hesperidin was effective in increasing levels of enzyme expression (Figure 7A).

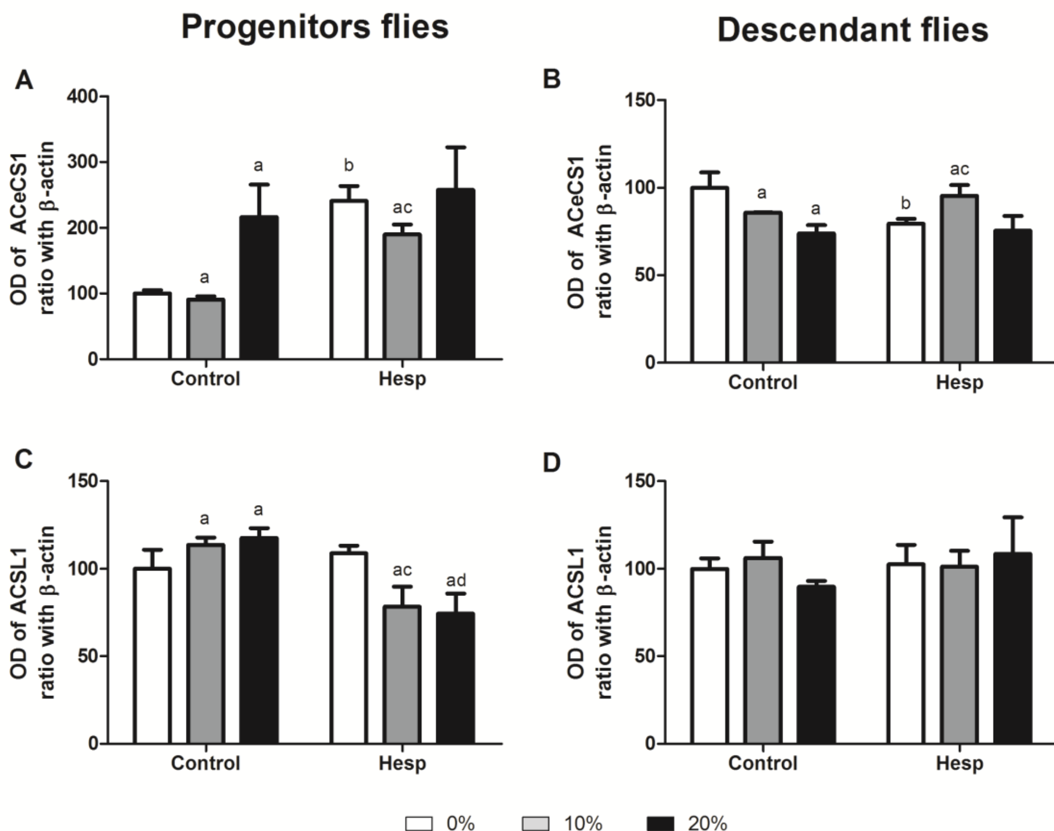


Figure 7: Expression levels of enzymes ACSL1 and ACeCS1 in response to the treatment of D. melanogaster with a regular diet (RD) and a high-fat diet (HFD) in progenitors and descendants flies. After feeding the progenitors flies for seven days with different diets or treatment during development period the flies descendants e after eclosion, they were homogenized and the proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. We quantified total content of proteins using specific antibodies. (A) and (B) is a Western Blot showing expression levels of Acetyl-CoA Synthetase (ACeCS1) with respective contents of β-actin in both progenitors flies as their descendants. (C) and (D) is a Western Blot showing expression levels of Acyl-CoA Synthetase (ACSL1) with respective contents of β-actin in both progenitors flies as their descendants. [a] $p < 0.05$ and different when compared with your respective control; [b] $p < 0.05$ and different when compared with RD group; [c] $p < 0.05$ and different when compared with HFD10 group; [d] $p < 0.05$ and different when compared with HFD20 group.

3.8 Quantitative Real-Time PCR (QRT-PCR) analysis of HSP83, SOD, CAT and *dilp6* mRNA in flies

The data reveals a significant increase in HSP83 expression of mRNA levels in progenitors flies that received HFD, during seven days, in the diets. The co-exposure with hesperidin decreased HSP83 expression of mRNA levels, when compared at RD group (Figure 8E). In the groups of progenitors flies, the statistical tests did not reveal significant difference in SOD and CAT mRNA levels but, when co-exposed with hesperidin, demonstrated an increase of the SOD mRNA levels and decreased CAT mRNA levels (Figures 8A and 8C). Furthermore, the flies exposed to both concentrations tested of HFD during embryonic development demonstrated significant decrease in SOD and CAT expression of mRNA levels which have not been constant with the addition of hesperidin (Figures 8B and 8D). Besides, a significant increase in the HSP83 mRNA levels was observed when added 10% of coconut oil in embryonic development of flies and has not been constant with the addition of hesperidin (Figure 8F). The HFD20 plus hesperidin group demonstrated a significant increase in dilp6 expression of mRNA levels in progenitors flies (Figures 8G). Besides, when descendants flies were developed in HFD20 the dilp6 expression of mRNA levels were high. But, co-exposition HFD with hesperidin showed to be effective in reducing dilp6 expression of mRNA levels (Figure 8H).

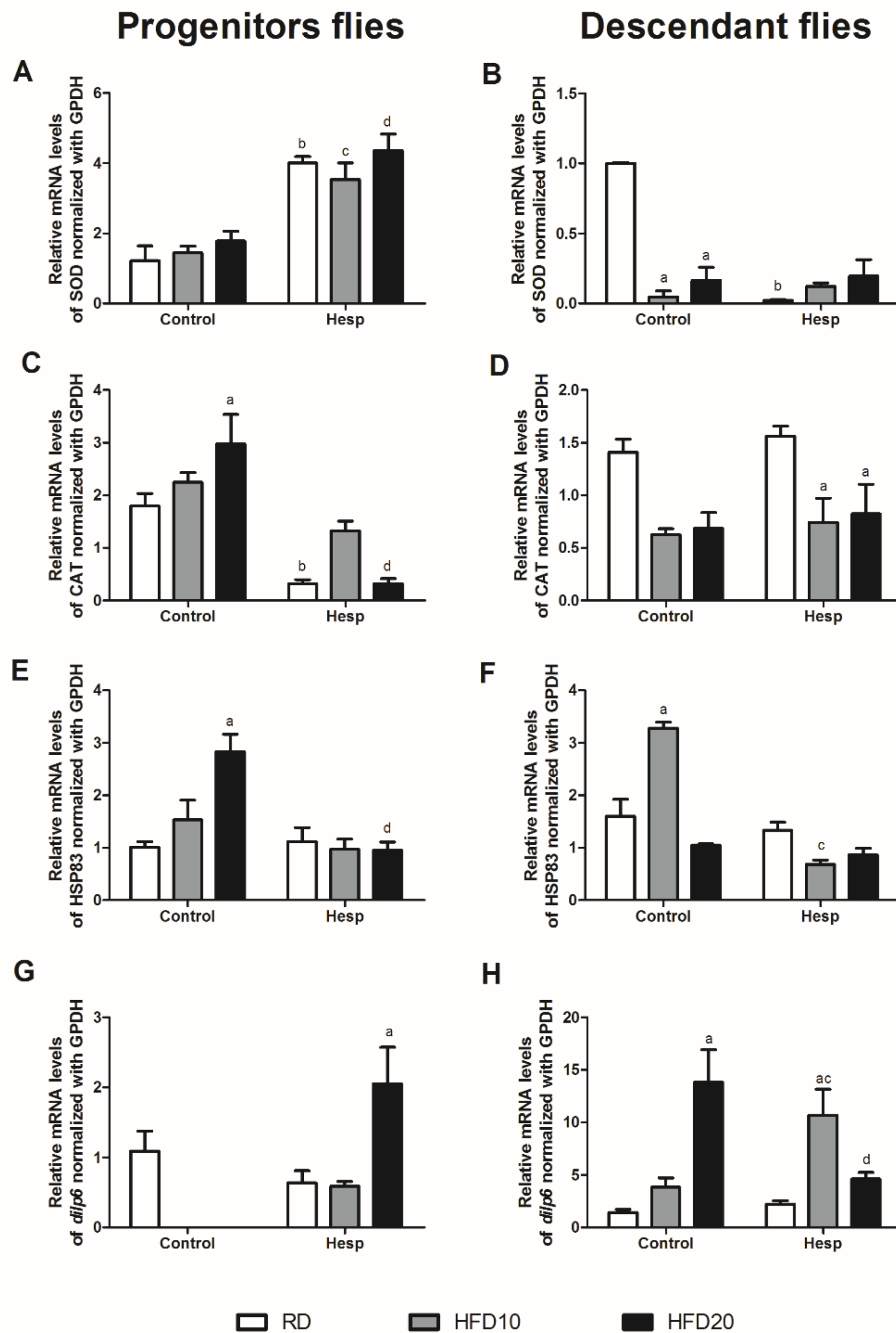


Figure 8: Effects of the exposure to Hesperidin under the influence to a high-fat diet in *D. melanogaster* **exposed during embryonic development period and during adult life on quantitative real time PCR (qRT-PCR) analysis of dilp6, Hsp83, SOD and CAT mRNA.** Flies (both sexes) were exposed to RD or HFD with or without hesperidin (10µM) during embryonic development period and during adult life. Flies were homogenized and the supernatant were used qRT-PCR to quantify levels of mRNA of each diet after exposure. The data were normalized against GPDH transcript levels and each bar represents the mean ± SEM. Three sets of twenty flies were analyzed for each diet condition. [a] $p < 0.05$ and different when compared with your respective control; [b] $p < 0.05$ and different when compared with RD group; [c] $p < 0.05$ and different when compared with HFD10 group; [d] $p < 0.05$ and different when compared with HFD20 group.

4. DISCUSSION

In the previous studies flies which were fed seven days with a high fat diet by adding coconut oil (HFD10 and HFD20) had reduced locomotor performance, metabolic alterations, to which oxidative stress situation might be involved in shortening the lifespan of flies (Paula et al., 2016). In addition, childhood obesity is closely linked to the parents' obesity, analysis was taken from the flies that were descended from parents who received HFD for seven days, and the conclusion is that these flies may develop metabolic dysfunctions due to insulin resistance, causing slow oxidative insults involved in the shortening of the useful life (Paula et al., XXXX). In view of the previous results, there was a need to combine this public health problem, which is maternal and child obesity with the use of alternative medicine methods, since it is increasingly used for health care (Georgiev, 2014).

Flavonoids are a group of natural compounds derived from plant origin with a variety of biological effects in numerous animals cell systems, *in vitro* as well as *in vivo* (El-Sayed et al., 2007). Hesperidin is one of the most abundant natural flavonoids, present in a large number of fruits and vegetables (Garg et al., 2001). Some authors reported that hesperidin prevents oxidant injury and cell death by several mechanisms, such as scavenging oxygen radicals and protecting against lipid peroxidation (Miller and Rice-Evans, 1997; Jung et al., 2003; El-Sayed et al., 2007). Therefore, antioxidant properties demonstrated by hesperidin, may represent a potential protective agent of the oxidative stress damage caused by obesity.

The aim of the present study was to investigate the protective effects of hesperidin on changes caused by HFD induced by the addition of coconut oil (HFD10 and HFD20) in *Drosophila melanogaster* model. From there, Hesp (at the tested concentration of 10μM) is shown to be effective in reducing some parameters that causes the metabolic changes in flies that received HFD. Coconut oil at both concentrations tested (HFD10 and HFD20) caused changes in triglyceride and glucose levels, both progenitor flies and their descendant flies. When these flies were treated concomitantly with the flavonoid hesperidin, triglyceride levels returned at the control level in both groups. Decrease may be directly linked an inhibition of the process of lipogenesis and induction of β-oxidation which has already been observed in rat models that received HFD and hesperidin by Mitsuzumi et al. (2011). In addition, other studies have shown that in combinations of HFD, flavonoids such as hesperidin, when administered, do not affect lipid parameters (López-Munhoz et al., 2015).

Considering that the HFD model is well described in the literature, it plays a key role in influencing glucose metabolism, increasing lactate and pyruvate levels in mammals, as described by Toye et al. (2007) and Newgard et al. (2009) and in flies by Paula et al. (2016) were measured glucose levels in the study in order to describe the influence of hesperidin on this parameter. The addition of coconut oil in the diet of progenitor flies caused a decrease in glucose levels, that may be altered due to the increase in the availability of fats in the diet, altering mitochondrial oxidative metabolism (Paula et al., 2016). However, when adding the flavonoid hesperidin in RD, it alone increased the glucose levels of these flies. When hesperidin was added concomitantly to the concentrations of coconut oil in parent flies, only the lowest concentration tested (10%) showed a significant increase in glucose levels. The supposition is that the increase in these levels may be related to the glycosidic formation of hesperidin, since hesperidin is formed by a molecule of hesperitin plus a disaccharide (Evans, 1996). When the hesperidin molecule is ingested, a break occurs dividing this molecule into hesperitin and sugar, which may be directly related to the increased glucose levels observed in the study (Garg et al., 2001). In descending flies, hesperidin, when available alone in DR, maintains normal glucose levels compared to the control group. However, when given together with HFD, it further decreases these glucose levels. From this it is the belief that the significant decrease is due to the property of the antioxidant compounds when combined with diet may provide protection against the early stage of diabetes mellitus and the development of complications as reported by Jung et al. (2004).

In progenitor flies, since glucose is not sufficient, it is noticed that HFD is being used for energy production and, consequently, generating ketone bodies and acetate. Therefore, the enzymes Acyl-CoA Synthetase and Acetyl-CoA Synthetase were demonstrated at high levels when compared to the control group. Moreover, the addition of hesperidin to these tested groups demonstrated a tendency to decrease the levels of these enzymes. When taking analysis of the levels of these same enzymes present in fatty acid metabolism in descendants flies, it can be shown that these levels remained stable in both flies that received a RD or a HFD supplemented or not with hesperidin. In short this result is due to an adaptation in the metabolism of these animals during their development period, since similar results were also observed by Phillips et al. (2010).

Hesperidin is a flavonoid that demonstrates an antioxidant role well described (Ahmad et al.,2012; Yang et al., 2012; Kuntic et al., 2014; Pari et al., 2015) and that HFD is responsible for promoting oxidative stress due to the increase of acid degradation, some parameters were tested in order to verify the performance of hesperidin in our model. The

increase in mitochondrial RS production caused by high fat consumption by HFD that generated oxidative stress in the flies was significantly decreased with the addition of hesperidin in the diets. Hesperidin alone has been shown to be effective in both progenitor and offspring flies and thus reduce RS production, confirming the idea that this flavonoid possesses the property of eliminating free radicals by protecting cells from oxidative stress as seen in studies with other models by Filho et al. (2013) and Antunes et al. (2014). In addition, HFD also caused an increase in lipid peroxidation of progenitor flies, not altering this parameter in the downward flies. Supplementation with hesperidin in all tested groups was able to decrease the induction of lipid peroxidation in the flies. However, the study confirms that hesperidin has been shown to be an antioxidant that also possesses the property of stabilizing biomembranes and thus avoiding cellular damage as also reported by Antunes et al. (2014). The increased oxidative stress caused by HFD led to flies developing a compensatory response to the damage such as modulation in the activity and expression of antioxidant enzymes (Paula et al., 2016). However, as the oxidative stress parameters decreased in the hesperidin-treated groups, activity and expression of antioxidant enzymes (SOD and CAT) was also decreased, confirming a compensatory response and antioxidant role of compound for the flies.

The cellular stimulus caused by obesity generated by HFD demonstrated a decrease in metabolic activity confirming the state of stress and, consequently, a significant decrease in the cell and mitochondrial viability. Hesperidin was able to reverse this effect and maintained the cell and mitochondrial viability at the control level, corroborating with its activity in helping to reduce harmful processes to the cell. Furthermore, with this decrease the expression of heat shock proteins also remained unchanged, since the damages were decreased. Taken together, the above findings suggested that hesperidin augmented cellular antioxidant defense corroborating with results demonstrated by Kalpana et al. (2009) and Chen et al. (2010). These authors demonstrated that hesperidin has potential as a therapeutic agent in the treatment of oxidative stress-related in cellular damage and can protect cellular membrane from free radical induced oxidative damage.

Considering that the reduction of antioxidant defenses and the development of oxidative stress caused by HFD causes a shortening in the life time of the progenitor flies, hesperidin treatment was able to increase the shelf life and also the fertility of these flies since the eggs of flies that were co-treated with hesperidin during the seven days of HFD were developed in greater number of those who did not receive doses of flavonoid. As described by Buckshee et al. (1997) and Garg et al. (2001) flavonoids can be used during pregnancy as they

do not affect fetal development, weight, growth and nutrition. Therefore, in our study, hesperidin showed no harmful effects on the descendant flies, even if they were developed in a HFD, in addition to keeping the eclosion rate relatively normal, the offspring that had hatched maintained a lifetime comparable to the control flies. In summary, the study has revealed an efficient role of hesperidin in the treatment of obese flies that have received HFD or developed in a HFD demonstrating that this flavonoid acts as a protective agent through analyzes of metabolic alterations, oxidative stress and protein signaling.

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5 DISCUSSÃO

A obesidade se tornou uma prioridade de saúde pública dada a sua crescente epidemia e suas vastas consequências à saúde (Canella, 2014). Sabendo-se que a obesidade é caracterizada pelo acúmulo de gordura corporal, este acúmulo acarreta no desenvolvimento de diversas doenças podendo diminuir a expectativa de vida de indivíduos obesos (Kahn et al., 2006; Labounty et al., 2013; Bastien et al., 2014; Chandra et al., 2014; Ko et al., 2014; Previs et al., 2014; James et al., 2015; Xiang e Na, 2015). Neste estudo, priorizamos estudar um modelo de obesidade alternativo, para o qual utilizamos duas concentrações de óleo de coco (10% e 20%) a fim de gerar uma DRG no modelo de estudo de *D. melanogaster.* Além disso, a maioria das pesquisas relacionadas à obesidade são realizadas em indivíduos adultos e, em nosso modelo escolhido, não há estudos que relatem a causa da obesidade para indivíduos descendentes de pais obesos. Junto à isso, e a busca por medicinas alternativas e não medicamentosas para o tratamento das alterações causadas pelo excesso de gordura corporal impulsionou para que fosse testado o flavonóide Hesperidina que é um conhecido composto bioativo promissor (Georgiev, 2014).

Nossos resultados mostraram que a dieta rica em gordura por adição de óleo de coco (10% e 20%) em modelo de Drosophila mostrou ser eficaz, uma vez que modulou o metabolismo energético destes animais o que também foi observado por Heinrichsen e Haddad (2012). Tendo em vista estas alterações em moscas adultas, foi de extrema importância a análise das moscas descendentes já que a relação entre síndrome metabólica, diabetes mellitus tipo II e obesidade é bem estabelecida em adultos e em proles ainda precisa ser esclarecida (Crespo et al., 2007). Nossos dados revelaram que as moscas descendentes podem desenvolver alterações como insultos oxidativos lentos, resistência à insulina e encurtamento de vida útil. Contudo, o tratamento concomitante com a Hesperidina representou uma ótima alternativa para minimizar os efeitos deletérios causados pelo excesso de gordura saturada consumida na dieta.

Considerando uma alta disponibilidade de gordura na dieta, e a afirmação de obtenção de indivíduos obesos, os níveis de TGC e glicose mantiveram-se alterados em ambos os grupos testados (moscas progenitoras e descendentes). Quando estas moscas foram concomitantemente tratadas com Hesp estes níveis de TGC apresentaram valores compatíveis com os apresentados pelos grupos controles, resultado este compatível com estudos que utilizaram DRG, Hesp em ratos (Mitsuzumi et al., 2011). Ainda para Mitsuzumi et al. (2011)

a explicação para isso pode pode estar diretamente ligada à um processo de inibição da lipogênese e uma indução característica de β-oxidação não afetando os parâmetros lipídicos (López-Munhoz et al., 2015).

Os níveis de glicose de moscas progenitoras que receberam DRG diminuiu o que pode ser parcialmente explicado devido ao aumento da disponibilidade de gorduras na dieta, alterando o metabolismo oxidativo mitocondrial. Já quando adicionado a Hesp, apenas na DRG 10% foi observado um aumento significativo nos níveis de glicose. Para explicar este aumento, a Hesp sozinha também foi capaz de aumentar os níveis de glicose que se deve à estrutura da molécula apresentar um açúcar (Hesperidina = Hesperitina + dissacarídeo) (Evans, 1996). Já nos grupos de moscas descendentes, quando se desenvolveram em uma DRG demonstraram um aumento significativo nos níveis de glicose e, quando a Hesp foi tratada concomitantemente estes níveis foram reduzidos à nível de controle. Esta alteração se deve a propriedade da Hesp proteger contra um possível estágio inicial de diabete mellitus por indivíduos em estágio inicial da vida (Jung et al., 2004). Desta forma, resolvemos investigar os eventos de sinalização insulina / insulina (IIS) que em *D. melanogaster* são notavelmente conservados, e para isso analisamos a expressão de um dos peptídeos semelhantes à insulina (Dilp6). Nossos resultados revelaram que moscas descendentes que receberam DRG a expressão de Dilp 6 aumentou que pode estar diretamente relacionado com elevados níveis de glicose gerando uma insulina modificada / IGF sinalização, a fim de prolongar a vida útil, também relatado em estudos de Kannan e Fridell (2013) em exemplares adultos.

A maior ingestão e disponibilidade de gorduras saturadas na dieta (DRG), ligada à diminuição dos níveis de glicose é uma das principais causas da produção excessiva de ER, devido à saturação da cadeia de transporte de elétrons (Rupérez et al., 2014). Além dessa mobilização de gordura estar sendo utilizada para geração de energia em nosso modelo de moscas progenitoras, pode ser a principal causa de geração e produção de corpos cetônicos, acetato e ER devido ao aumento das enzimas acyl-CoA Sintase e acetyl-CoA Sintetase e marcadores de estresse oxidativo. Porém, quando foi adicionado concomitantemente a Hesp à esses tratamentos estes níveis de expressão das enzimas Acyl-CoA Synthase e Acetyl-CoA Synthetase foram diminuídos ou mantidos estáveis em relação ao seu grupo controle devido há uma possível redução da oxidação de ácidos graxos. Ao analisar os níveis destas mesmas enzimas presentes no metabolismo de ácidos graxos em moscas descendentes, pode-se demonstrar que estes níveis permaneceram estáveis em ambas as moscas que receberam uma dieta normal ou DRG suplementado ou não com hesperidina. Em suma, este resultado é devido a uma adaptação no metabolismo destes animais durante o seu período de

desenvolvimento, uma vez que resultados semelhantes foram também observados por Phillips et al. (2010).

A Hesp desempenha um papel antioxidante já bem descrito (Georgiev, 2014). A DRG gerou um aumento na produção de ER e, consequentemente, uma situação de estresse oxidativo que foi revertido com o tratamento com a Hesp. Através deste resultado confirmamos a propriedade antioxidante da Hesp de eliminar RL e protegendo as células do estresse oxidativo como visto em estudos com outros modelos de Filho et al . (2013) e Antunes et al. (2014). Além disso, a DRG também causou um aumento na peroxidação lipídica de moscas progenitoras, não alterando este parâmetro nas moscas descendentes. A suplementação com hesperidina em todos os grupos testados foi capaz de diminuir a indução de peroxidação lipídica nas moscas estabilizando assim as biomembranas evitando dano celular. Como maneira de compensação ao estresse oxidativo, as moscas que receberam DRG modulando a atividade das enzimas antioxidantes SOD e CAT. A medida que os parâmetros de estresse diminuíram com a adição da Hesp na dieta, a expressão destas enzimas também diminuíram.

O estímulo celular causado pela obesidade gerada pela DRG demonstrou diminuição na atividade metabólica, confirmando o estado de estresse e, consequentemente, uma diminuição significativa na viabilidade celular e mitocondrial. A Hesp foi capaz de reverter esse efeito e manteve a viabilidade celular e mitocondrial no nível de controle, corroborando com sua atividade em ajudar a reduzir os processos nocivos para a célula. Além disso, com esta diminuição a expressão de proteínas de choque térmico também permaneceu inalterada, uma vez que os danos foram diminuídos. Tomadas em conjunto, as conclusões acima sugeriram que a hesperidina aumentou a defesa antioxidante celular corroborando com os resultados demonstrados por Kalpana et al. (2009) e Chen et al. (2010). Esses autores demonstraram que a hesperidina tem potencial como agente terapêutico no tratamento do estresse oxidativo relacionado ao dano celular e pode proteger a membrana celular dos danos oxidativos induzidos por radicais livres.

Considerando que a redução das defesas antioxidantes, o desenvolvimento de estresse oxidativo e o aumento da produção de corpos cetônicos causado por DRG provoca um encurtamento no tempo de vida das moscas progenitoras e, o tratamento com hesperidina foi capaz de aumentar a vida útil e também a fertilidade dessas moscas. Os flavonoides além de representarem uma ferramente útil como medicina alternativa, também pode ser utilizado durante a gravidez sem causar prejuízos tanto para a mãe quanto para o filho (Buckshee et al.,1997 e Garg et al. 2001). Portanto, nosso estudo concluiu que a Hesp demonstrou um papel

eficiente no tratamento de moscas obesas tanto em moscas progenitoras, como em moscas descendentes, atuando como um agente protetor das alterações metabólicas, estresse oxidativo e sinalização de proteínas de indivíduos que receberam uma DRG.

Além disso, DRG são caracterizadas por causar déficits locomotores devido à diminuição dos níveis protéicos e, consequentemente, a redução no crescimento muscular em modelos de ratos (Heinrichsen et al., 2014). Devido à isso, em nosso modelo também observamos prejuízos locomotores causados pela DRG alterando a capacidade de geotaxia negativa das moscas e diminuindo a atividade da enzima acetilcolinesterase alterando a motilidade deste inseto (Fournier et al., 1993). A Hesp por sua vez, melhorou este desempenho locomotor de moscas progenitoras que receberam DRG e não alterou em nenhum dos grupos de moscas descendentes, bem como para a atividade de AChe. Segundo Li et al. (2015) esta melhora na eficiência locomotora pode estar ligada ao efeito neuroprotetor da Hesp também demonstrado em ratos (Justin Thenmozhi et al., 2015).

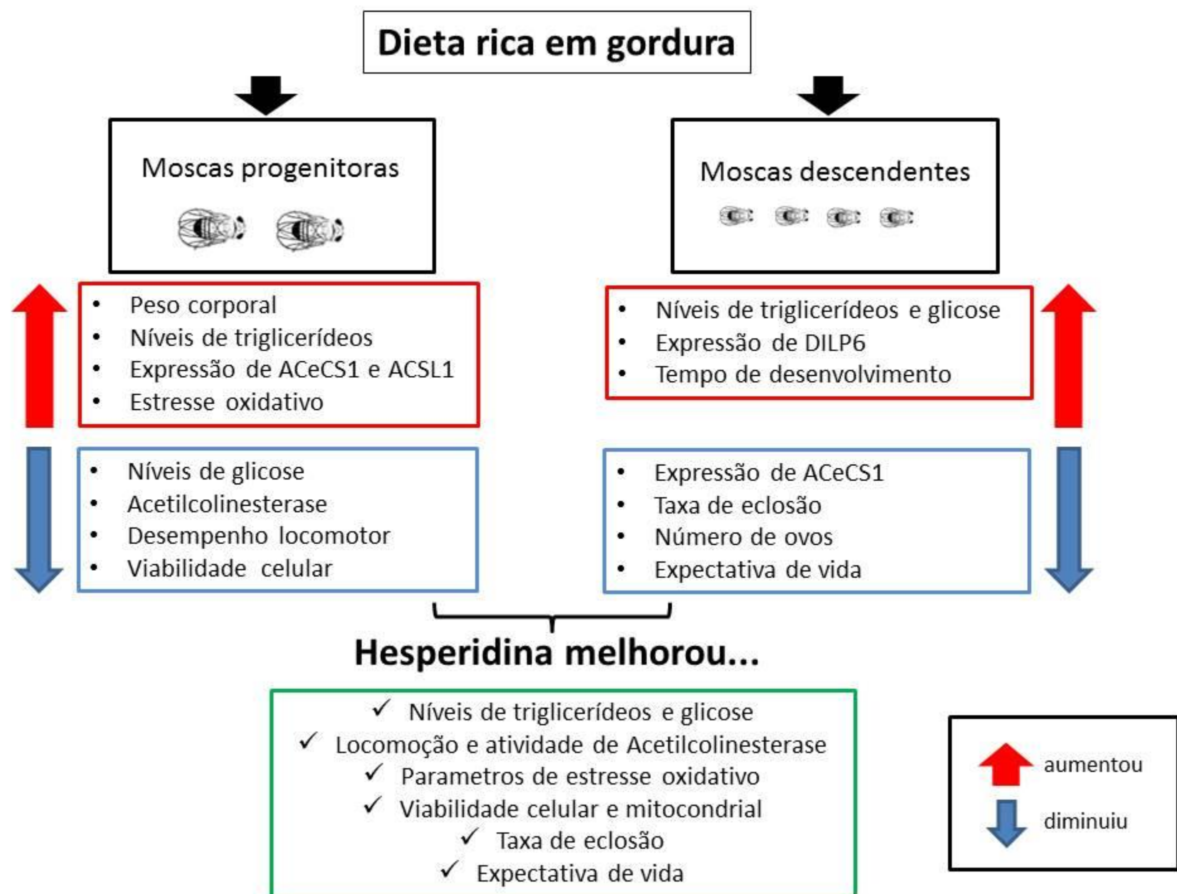
Considerando que a ingestão de uma DRG pelas moscas ocasionou uma redução nas defesas antioxidantes e desencadeou um quadro de estresse oxidativo, ligado à isso está o encurtamento no tempo de vida das moscas progenitoras. Todavia, o co-tratamento com Hesp foi capaz de aumentar este tempo de vida útil e também melhorou a fertilidade destas moscas. Além disso, as moscas descendentes teve sua expectativa de vida encurtada quando se desenvolveram na DRG e, o co-tratamento com a Hesp demonstrou a capacidade de manter este tempo de vida normal em relação ao grupo controle. Este prejuízo causado pela DRG em nosso modelo se dá pelo dano celular causado, o que gerou estresse oxidativo tornando os animais mais susceptíveis à insultos, todavia, a Hesp demonstrou ser um comporto antioxidante ajudando a melhorar esta expectativa de vida.

Assim sendo, nosso estudo representa uma nova abordagem dos efeitos causados pela DRG, bem como o potencial efeito terapêutico do bioflavonóide Hesperidina ampliando assim o entendimento sobre os principais focos e vias metabólicas modificadas utilizando um modelo de estudo alternativo, demonstrando a capacidade deste em outras áreas de estudos que não sejam somente genéticos.

6 CONCLUSÃO

A partir dos resultados aqui apresentados, podemos concluir que:

Figura 8 – Fluxograma esquemático mostrando as principais conclusões deste trabalho



Fonte: Arquivo próprio (2017).

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