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Litiele Cezar da Cruz

CONSUMO DE MEL FORNECE PROTEÇÃO CONTRA ALTERAÇÕES MOTORAS E MOLECULARES INDUZIDAS POR HIPÓXIA/REPERFUSÃO EM *Drosophila melanogaster*

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Orientador: Prof. Dr. Jeferson Luis Franco
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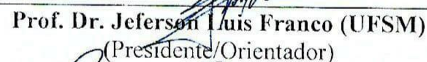

Prof. Dr. Jeferson Luis Franco (UFSM)
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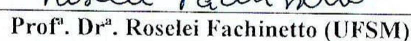

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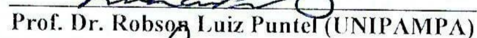

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“Happiness is only real when shared.”

Christopher McCandless

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RESUMO

CONSUMO DE MEL FORNECE PROTEÇÃO CONTRA ALTERAÇÕES MOTORAS E MOLECULARES INDUZIDAS POR HIPÓXIA/REPERFUSÃO EM *Drosophila melanogaster*

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Orientador: Prof. Dr. Jeferson Luis Franco

O mel é um produto complexo produzido por abelhas melíferas a partir do néctar e bastante utilizado pelas suas propriedades edulcorantes, bem como pelos seus benefícios para a saúde humana. O mel apresenta atividades anti-inflamatória, hipoglicêmica e antioxidante. A hipóxia seguida de reperfusão é uma condição desencadeada em diversas patologias e pode culminar com danos teciduais de cunho oxidativo. A mosca-da-fruta, *Drosophila melanogaster*, possui características tolerantes a hipóxia e homologia com animais vertebrados de vias envolvidas na cascata de respostas celulares à hipoxia/reperfusão. Considerando tais aspectos, o objetivo principal desse trabalho foi avaliar os efeitos do mel produzido no Bioma Pampa Brasileiro sobre as alterações teciduais/moleculares induzidas por hipóxia/reperfusão em *Drosophila melanogaster*, com ênfase em estresse oxidativo. Moscas selvagens foram tratadas com 10% de mel por 3 dias e posteriormente submetidas à hipóxia (3hs). Após reperfusão de oxigênio, foram avaliados parâmetros de sobrevivência e comportamento; parâmetros de estresse oxidativo, bem como, a expressão de mRNA de genes envolvidos em vias metabólicas, inflamatórias e morte celular. Os resultados demonstraram que o mel testado é constituído de compostos com atividade antioxidante, que foi reconhecida tanto *in vitro* como *in vivo*. A hipóxia/reperfusão induziu um aumento na expressão do mRNA de Sima (HIF-1), LDH, NFκB, Nrf2, HOX, AKT-1, INR, dILP2, dILP5 e HSP27. As moscas submetidas a hipoxia/reperfusão também apresentaram expressão diminuída do mRNA do EWG e Opa-1. O mel foi efetivo em restabelecer a expressão alterada de Opa-1, gene relacionado com fusão mitocondrial. Além disso, o tratamento com mel reduziu as alterações induzidas por hipóxia/reperfusão sobre os parâmetros de sobrevivência, déficits locomotores, alteração da posição das asas, produção de espécies reativas de oxigênio (EROs), atividades da GST, caspases e complexo I da cadeia respiratória mitocondrial. Os resultados obtidos aqui mostram, pela primeira vez, os efeitos benéficos de mel contra alterações induzidas por hipóxia/reperfusão em *Drosophila melanogaster*. Este estudo evidenciou que o mel do Bioma Pampa Brasileiro tem além de um alto nível de qualidade, uma atividade antioxidante significativa *in vivo*, a qual pode estar relacionada com seus efeitos benéficos contra hipoxia/reperfusão. Com base nos resultados, considera-se ainda tanto o mel como a *Drosophila melanogaster* alvos promissores para estudos voltados para doenças associadas com condições de estresse e hipoxia/reperfusão.

Palavras-chave: mel, antioxidante, hipoxia/reperfusão, *Drosophila melanogaster,* estresse oxidativo.

ABSTRACT

HONEY CONSUMPTION PROVIDES PROTECTION AGAINST MOTOR AND MOLECULAR CHANGES INDUCED BY HYPOXIA / REPERFUSION IN Drosophila melanogaster

Author: Litiele Cezar da Cruz
Advisor: Prof. Dr. Jeferson Luis Franco

Honey is a complex product produced by honeybees from nectar, being widely used for its sweetening properties, as well as for its benefits to human health. Honey presents anti-inflammatory, hypoglycemic and antioxidant properties. Hypoxia followed by reperfusion is a condition triggered in several pathologies and can culminate with oxidative tissue damage. The fruit-fly, *Drosophila melanogaster*, has hypoxia tolerant characteristics and homology with vertebrate from pathways involved in the cascade of cellular responses for hypoxia/reperfusion. Considering these aspects, the main objective of this work was to evaluate the possible protective effect of honey produced in the Brazilian Pampa Biome on changes induced by hypoxia/reperfusion in *Drosophila melanogaster*, with emphasis on oxidative stress. Wild flies were treated with a diet containing 10% honey for 3 days and subsequently submitted to hypoxia conditions (3hs). After reperfusion of oxygen, parameters of survival, behavior, oxidative stress as well as mRNA expression of genes related with metabolic, inflammation and death pathways were evaluated. The results demonstrated that the honey tested is composed of compounds with antioxidant activity that were recognized both *in vitro* and *in vivo*. Hypoxia/reperfusion induced an increase in the regulation of mRNA expression of Sima (HIF-1), NFκB, Nrf2, HOX, AKT-1, INR, dILP2, dILP5 and HSP27. Indeed, flies submitted to hypoxia/reperfusion has a expression decreased of EWG and Opa-1. Honey was able to reestablish the expression of Opa-1, a gene related to mitochondrial fusion. Honey treatment also reduced the changes induced by hypoxia/reperfusion in parameters of survival, locomotor deficits, wing position, reactive oxygen species (ROS) production, GST, caspases and mitochondrial complex I activities. The results obtained here show, for the first time, the beneficial effects of honey against changes induced by hypoxia/reperfusion in *Drosophila melanogaster*. This study evidenced that that the honey from the Brazilian Pampa Biome has a high quality and a significant antioxidant activity *in vivo*, which can be related with its beneficial effects against hypoxia/reperfusion. In addition, we consider the honey as well as *Drosophila melanogaster* promising targets for studying pathologies associated with oxidative stress and hypoxia/reperfusion.

Keywords: honey, antioxidant, hypoxia/reperfusion, *Drosophila melanogaster,* oxidative stress.

LISTA DE FIGURAS

Figura 1 - Bioma Pampa... 12
Figura 2 - Fotos representativas de mel e *Apis mellifera*... 14
Figura 3 - Vias de produção/estabilização de espécies reativas de oxigênio na célula............ 19
Figura 4 - Exemplos de fontes comuns de espécies reativas 20
Figura 5 - O núcleo da maquinaria apoptótica em D. melanogaster.. 24
Figura 6 - Frequência de publicação de artigos que tratam de lesão por reperfusão............... 25
Figura 7 - Ciclo de vida de *Drosophila melanogaster*.. 30

LISTA DE TABELAS

Tabela 1 - Limites permitidos pela legislação Brasileira de parâmetros físico-químicos do mel produzido por *Apis mellifera* 16
Tabela 2 - Classes principais de compostos fenólicos em plantas.. 21
Tabela 3 - Condições clínicas associadas à lesão por isquemia-reperfusão............................ 29

SUMÁRIO

1. INTRODUÇÃO ... 12
1.1 BIOMA PAMPA BRASILEIRO ... 12
1.2 MEL ... 13
1.2.1 Qualidade do mel ... 14
1.2.2 Propriedades do mel ... 16
1.3 ESTRESSE OXIDATIVO ... 18
1.3.1 Estresse oxidativo x antioxidantes ... 20
1.3.2 Estresse oxidativo x condições de estresse ... 22
1.4 HIPÓXIA/ REPERFUSÃO ... 24
1.4.1 Hipóxia/reperfusão x eventos oxidativos ... 26
1.4.2 Hipóxia/reperfusão x situações fisiológicas e patológicas ... 27
1.5 *Drosophila melanogaster* ... 29
2. OBJETIVOS ... 32
3. RESULTADOS ... 33
3.1 ARTIGO ... 33
3.2 MANUSCRITO ... 47
4. DISCUSSÃO GERAL ... 68
5. CONCLUSÕES ... 72
6. PERSPECTIVAS ... 73
7. REFERÊNCIAS ... 74

APRESENTAÇÃO

No item **INTRODUÇÃO**, consta uma revisão bibliográfica sobre os temas trabalhados nesta tese. A metodologia realizada e os resultados obtidos que fazem parte desta tese estão apresentados sob a forma de e artigo e manuscrito, que se encontra no item **ARTIGO e MANUSCRITO**. No item **DISCUSSÃO GERAL**, é apresentado uma discussão do somatório dos resultados presentes na sessão "artigo" e " manuscrito". O item **CONCLUSÕES**, encontrado no final desta tese, apresenta interpretações e comentários gerais sobre os resultados do artigo e manuscrito presentes neste trabalho. Em **PERSPECTIVAS** é apontado possíveis trabalhos futuros para uma continuidade a partir dos resultados da tese. As **REFERÊNCIAS** referem-se somente às citações que aparecem nos itens **INTRODUÇÃO e DISCUSSÃO** desta Tese.

1. INTRODUÇÃO

1.1 BIOMA PAMPA BRASILEIRO

O Brasil possui seis biomas terrestres: Amazônia, Mata Atlântica, Caatinga, Cerrado, Pantanal e Pampa (ROESCH *et al*., 2009). O Bioma Pampa ocupa uma área compartilhada por Brasil, Argentina e Uruguai no sul da América do Sul (figura 1) que apresenta características distintas de vegetação, clima e solo, tornando-se um ecossistema único no planeta. Caracteriza-se por uma vegetação típica de campo nativo, com formações arbustivas e arbóreas dispersas. O Bioma Pampa Brasileiro, ocupa uma área de 176,496 km², representando cerca de 2,07% do território nacional. Este está localizado entre as latitudes 28º 00' S e 34º 00' S e longitudes 49º 30' W e 58º 00' W, ocupando uma área de 63% do estado do Rio Grande do Sul (LUPATINI *et al*., 2013).

O Brasil é um dos maiores produtores de mel, com exportação para quase todos os continentes, incluindo a Europa. O estado do Rio Grande do Sul é o maior produtor de mel do Brasil (IBGE, 2011), responsável por 6,985 toneladas da produção total do país (41,578 t). Em 2014 foi realizada a primeira caracterização físico-química dos méis da região do Bioma Pampa Brasileiro (Gaúcho), os quais foram classificados como produtos de altíssima qualidade (CRUZ *et al*., 2014).

Figura 1 - Bioma Pampa.

Fonte: MARTINO, 2004.

1.2 MEL

O mel é um produto alimentício de mistura complexa produzido por abelhas melíferas a partir do néctar (méis florais) e exsudados de plantas (méis de melato) ou ainda pode ser proveniente das excreções de insetos sugadores que se alimentam de partes vivas das plantas. Este alimento é definido como um produto de cor variável, aspecto viscoso e aroma agradável, consumido mundialmente devido suas propriedades adoçantes e terapêuticas (ALJADI & KAMARUDDIN, 2004; CRANE, 1975).

O mel é constituído principalmente por açúcares, como monossacarídeos e dissacarídeos, bem como ácidos orgânicos, vitaminas, compostos derivados de carotenóides, aminoácidos, proteínas, oligo-elementos, flavonóides e outros compostos fenólicos (EREJUWA *et al.*, 2012). O mel floral é produzido a partir da transformação dos açúcares do néctar, o qual é recolhido pelas abelhas coletoras e alocado em suas vesículas melíferas, onde recebe a adição de enzimas (diastases -α-amilases, as invertases -α-glicosidades e glicose oxidase) produzidas nas glândulas hipofaríngeas e presentes na saliva das abelhas. Na colmeia o composto é regurgitado para outras abelhas sofrendo nova adição enzimática; e então armazenado em uma célula do favo (CRANE, 1980). A adição enzimática é fundamental para elaboração, maturação e qualidade do produto final. Posteriormente, o composto armazenado no favo sofrerá evaporação para formação do produto final, o mel.

Méis florais podem ser classificados de acordo com sua origem floral. Quando o mel é produzido a partir de diversas fontes florais pode ser chamado de mel multifloral ou polifloral, e quando a fonte de néctar possui uma porcentagem majoritária de uma única espécie de planta é caracterizado como monofloral. Para esta determinação é comumente analisada a origem polínica presente no mel. As abelhas ao coletar o néctar podem ingerir conteúdo de pólen, assim como, carregar os grãos de pólen ao favo por via dos pêlos do seu corpo (BARTH, 2005; CRANE, 1980).

Apesar da diversidade de espécies de abelhas existentes, devido à sua domesticação antiga e por ser originária dos principais países consumidores a *Apis mellifera* é considerada a principal abelha melífera para produção de méis florais (CRANE, 1975). *Apis mellifera* também é conhecido como: abelha-alemã, abelha-comum, abelha-da-europa, abelha-de-mel, abelha-doméstica, abelha-do-reino, abelha-escura, abelha-europa e abelha-preta. A espécie *Apis mellifera* faz parte do filo Arthropoda, classe Insecta, ordem Hymenoptera, família

Apidae e gênero Apis. As abelhas melíferas acompanham a humanidade durante toda sua história e ao longo da evolução vem se especializando para produzir um produto cada vez mais complexo, e isto reflete uma composição variada e um efeito sinérgico entre os compostos.

Figura 2 - Fotos representativas de mel e abelha *Apis mellifera*.

Fonte*: Foto*: The Honey Association ©; *Foto abelha:* Marcello Rapisardi.

1.2.1 Qualidade do mel

A composição de cada mel é influenciada por uma série de fatores, incluindo a origem geográfica, fontes botânicas de néctar, ambiente e as condições climáticas, bem como manuseio e processamento de técnicas apícolas (WANG & LI, 2011). Neste sentido, existem limites determinados pela legislação para padrões físico-químicos do mel (BRASIL, 2000). A legislação Brasileira possui um regulamento técnico para fixação de identidade e qualidade do mel, no qual os limites (tabela 1) e métodos são preconizados pela *Códex Alimentarius Commission* (CAC, 2001) e o *Association of Official Analytical Chemists* (AOAC, 1990).

Apesar do mel ser um produto natural, a produção inadequada, como também adulteração da sua composição pode torná-lo um produto de baixa qualidade, comprometendo suas propriedades terapêuticas (ANANIAS, 2010; COUTO & COUTO, 2006). Dessa forma,

torna-se importante caracterizar a identidade e qualidade de méis de diferentes regiões do mundo, assim como, o seu conteúdo fenólico, o qual tem sido relacionado com a capacidade antioxidante de méis de diferentes regiões (FERREIRA *et al*., 2009; ZALIBERA *et al*., 2008). Neste sentido, um recente trabalho voltado para a caracterização de parâmetros de qualidade e atividade antioxidante de méis do Bioma Pampa Brasileiro, apontou uma alta qualidade e atividade antioxidante para os mesmos (CRUZ *et al*., 2014).

As avaliações dos parâmetros físico-químicos basicamente indicam requisitos de maturação e qualidade do mel. A umidade do mel está relacionada com a maturidade, ou seja, para ser considerado "maduro" e garantir uma ótima durabilidade e qualidade, o mel deve conter uma umidade máxima de 20%. Este valor máximo de umidade também é um padrão de garantia que o mel não sofrerá fermentação (CAVIA, *et al*., 2002; ISENGARD, 2001). Dentro dos 80 % de compostos sólidos do mel, os monossacarídeos (glicose e frutose) compõem a maior parte. As análises dos açúcares, também estão relacionadas com a maturidade do mel, o mel é considerado "verde" quando o amido e sacarose não sofreram degradação o suficiente pelas enzimas (SANTOS *et al*., 2010). A análise de sacarose aparente também está relacionada com adulteração, devido adição de açúcar comercial no mel. A presença de acidez livre deve-se a diversos fatores que são ocasionados pela variação de ácidos orgânicos presentes no mel. A acidez livre é um parâmetro importante de qualidade, pois além de contribuir amplamente para o sabor característico deste alimento, influencia a estabilidade, as reações químicas e as suas propriedades antibacterianas e antioxidantes (ISENGARD, 2001).

O teor de sólidos insolúveis em água em méis é considerado um controle de qualidade como índice de pureza (SANTOS *et al*., 2010). Com análise de minerais (cinzas) é possível determinar algumas irregularidades, como por exemplo, a falta de higiene e a não decantação e/ou filtração no final do processo de retirada do mel pelo apicultor. Hidroximetilfurfural (HMF) é um aldeído, produto da degradação da frutose no mel, e este é um importante indicador da qualidade do mel (SPANO *et al*., 2006). O HMF é produzido naturalmente durante o processo de envelhecimento do mel (BASTOS *et al*., 2002), ou quando há adulteração como, por exemplo, super-aquecimento, adição de açúcares comerciais e/ou mudanças de pH.

Tabela 1 - Limites permitidos pela legislação Brasileira de parâmetros físico-químicos do mel produzido por *Apis melífera.*

Parâmetros	**Limites**
Umidade (%)	20 (Máximo)
Sólidos Insolúveis (%)	0,1 (Máximo)
Minerais (cinzas) (%)	0,6 (Máximo)
Acidez livre (mEq/Kg)	50 (Máximo)
Açucares redutores (%)	65 (Mínimo)
Sacarose aparente (%)	6 (Máximo)
HMF (mg/Kg)	60 (Máximo)

Fonte: CRUZ *et al.*, 2014.

1.2.2 Propriedades do mel

A cicatrização de feridas foi provavelmente a primeira condição que fez uso do mel para a saúde humana. Nas escrituras humanas mais antigas da Suméria, que datam de 2000 a.C., receitas médicas recomendavam o uso de mel para tratar estados de feridas (JULL *et al.*, 2008).

Existe uma passagem no Alcorão em relação aos poderes curativos do mel, onde Salomão elogia as virtudes de suas propriedades: "Construí as vossas colmeias nas montanhas, nas árvores e nas habitações (dos homens). Alimentai-vos de toda a classe de frutos e segui, humildemente, pelas sendas traçadas por vosso Senhor! Do abdômen delas sai um líquido de variegadas cores que constitui cura para os humanos. Nisto há sinal para os que refletem (Alcorão 16:68/69) ". Os gregos antigos consideravam o mel como medicina e acreditavam que se o mel de abelha fosse tomado regularmente a vida humana poderia ser prolongada. Os primeiros pensadores, como Homero, Pitágoras, Ovídio, Demócrito, Hipócrates e Aristóteles, mencionavam que as pessoas deveriam comer mel para preservar sua saúde e vigor (JULL *et al*; 2008).

Na literatura atual existem inúmeras pesquisas que comprovam os antigos relatos sobre os efeitos benéficos do mel à saúde. Entre algumas dessas pesquisas, podemos citar os efeitos cicatrizantes (CHAUDHARY *et al.*, 2015), gastroprotetor (GHARZOULI *et al.*,

2002), hepatoprotetor (AL-WAILI *et al.*, 2006), reprodutivo (MOHAMED *et al.*, 2012), anti-hipertensivo (AL-WAILI, 2003), anti-inflamatório (KASSIM *et al.*, 2010), antifúngico, antibacteriano (EREJUWA *et al.*, 2012), hipoglicêmico (ABDULRHMAN *et al.*, 2013; CRUZ *et al.*, 2014; EREJUWA *et al.*, 2010a), e efeitos antioxidantes (CRUZ *et al.*, 2014; EREJUWA *et al.*, 2010 a e b) entre outros.

Muitos estudos descrevem a efetividade do mel na limpeza rápida de feridas infectadas, além da ação anti-inflamatória e cicatrizante. Foi apontado que os principais aspectos a estas atividades estão relacionados com a alta concentração de açúcares (efeito osmótico), produção de peróxido de hidrogênio gerado pelo sistema enzimático de glicose oxidase como também pela presença de substâncias fitoquímicas, como os ácidos fenólicos (EREJUWA *et al.*, 2012; MOLAN, 1992). O alto teor de açúcar no mel também é apontado como principal fator de proteção em modelos de danos gástricos induzidos por etanol, indometacina e ácido acetilsalicílico (GHARZOULI *et al.*, 2002). Porém, mesmo com a variedade de pesquisas em relação aos efeitos anti-antimicrobiano, anti-inflamatório e cicatrizante de méis de diferentes origens, detalhes sobre os mecanismos de ação e o principal componente do mel responsável por essas propriedades vitais é ainda desconhecido, devido a complexa composição do mel. Contudo, os resultados das pesquisas até o momento indicam que esses efeitos estão principalmente relacionados com a presença compostos fenólicos (HADAGALI, 2014).

Com relação aos efeitos anti-hiperglicêmicos, Abdulrhman *et al.* (2013), conduziu um estudo com indivíduos diabéticos (tipo 1), onde comparou a ingestão de concentrações idênticas de mel, glicose e sacarose. Os resultados deste estudo demonstraram que tanto os controles (não diabéticos) como os diabéticos tiveram uma redução significativa nos níveis de glicose após a dieta com mel. Erejuwa *et al.* (2010 a) também obteve resultados similares em ratos anos antes. Cruz *et al.* (2014), obteve resultados similares em análise de glicose total em mosca-da-fruta. Abdulrhman *et al.* (2013) aborda em seu estudo a possível capacidade do mel em estimular as células beta produtoras de insulina em humanos.

Muitos dos problemas de saúde citados acima, como inflamação, hipertensão e hiperglicemia tendem a desencadear estresse oxidativo. Nesse sentido, pesquisas vêem demonstrando que antioxidantes ajudam a reverter ou amenizar tal condição (EREJUWA *et al.*, 2012). Com relação ao mel, pode-se dizer que a presença de antioxidantes está conectada com muito dos seus benefícios a saúde humana. Jubri *et al.* (2013) em um recente estudo usando ratos de meia-idade, mostrou que a suplementação com mel de Manuka (uma espécie

de planta presente na Nova Zelândia e Austrália) reduziu o nível de danos no DNA, níveis de formação de malondialdeído (marcador de dano oxidativo) e atividade das enzimas antioxidantes como a glutationa peroxidase e catalase no fígado quando comparado com ratos controles (sem suplementação de mel). Os mesmos resultados foram encontrados para mel de Gelam (espécie de planta) na Malásia (YAO *et al*., 2011). Estes efeitos antioxidantes foram positivamente relacionados com teores de compostos fenólicos presentes nas amostras utilizadas. Em outro trabalho, com cultura de células pancreáticas de hamster em condições de hiperglicemia, com estresse oxidativo induzido por glicose (glicotoxicidade), a suplementação com mel diminuiu a produção de espécies reativas de oxigênio (BATUMALAIE *et al*., 2014). Erejuwa *et al*. (2010 a e b) sugere que o efeito hipoglicêmico do mel poderia estar relacionado ao seu efeito antioxidante sobre o pâncreas.

1.3 ESTRESSE OXIDATIVO

Nas últimas décadas, têm se evidenciado um aumento na prevalência mundial de doenças degenerativas ou crônicas, como diabetes mellitus, complicações cerebrais e cardiovasculares, câncer e doenças neurodegenerativas (ALBRIGHT, 2008). Evidências apontam no papel do estresse oxidativo na patogênese e/ou complicações destas doenças (KADENBACH *et al.,* 2009). O estresse oxidativo é definido como um desequilíbrio entre agentes oxidantes (espécies reativas) e antioxidantes, que pode culminar com danos às biomoléculas, como lipídios, proteínas e DNA, comprometendo assim funções fisiológicas vitais (SIES, 1991).

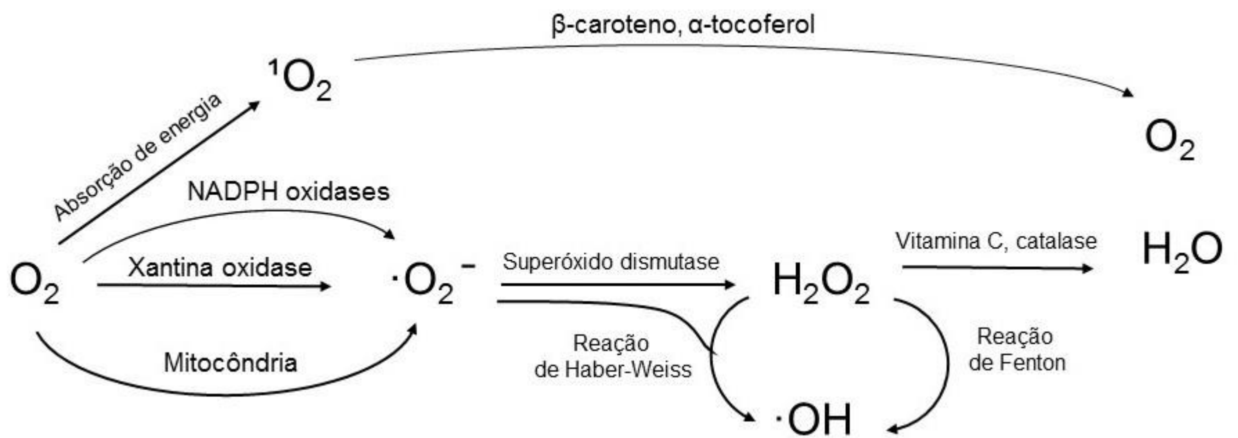
Espécies moleculares mais estáveis têm os elétrons em seu orbital exterior, dispostos em pares. Cada elétron deste par tem uma rotação oposta, o que é importante para estabilizar as moléculas. Um radical livre é uma molécula com um ou mais elétrons desemparelhados no seu orbital, o que torna esta espécie molecular muito instável e tende a reagir com outras moléculas para emparelhar o elétron e, assim, gerar espécie mais estável (ADLY, 2010).

Espécies reativas de oxigênio (ERO), como também, espécies reativas de nitrogênio (ERN) são produzidas constantemente nos sistemas celulares (figura 3). Porém nem todas as espécies reativas são radicais livres. Existem um grupo de espécies reativas não-radicais, como é o caso de espécies reativas de oxigênio (EROs): peroxido de hidrogênio (H_2O_2), ácido

hipocloroso (HOCl), ácido hipobromoso (HOBr), ozônio (O_3) e oxigênio singlete ($^1O_2$) e espécies reativas de nitrogênio (ERNs): dióxido de nitrogênio (NO2), trióxido de dinitrogênio (N_2O_3), tetróxido de dinitrogênio (N_2O_4), peroxinitrito ($OONO^-$), ácido peroxinitroso (ONOOH), anion nitroxil ($NO{\cdot}NO^-$), cátion nitroxil (NO) e alquil peroxinitrito (ROONO).

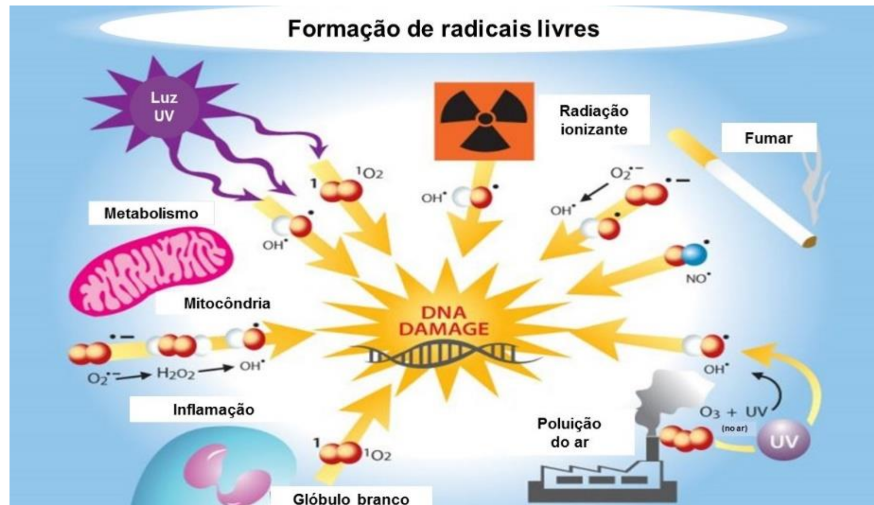
Entre os radicais de EROs destaca-se o radical ânion superóxido ($O_2^{\bullet-}$), radical hidroxila ($OH^{\bullet}$), radical peroxil (RO_2^{-}), radical alquila (RO^{-}) e o radical hidroperoxila (HO_2^{-}). Nos radicais de ERNs estão o óxido nítrico (NO) e o ácido nitroso (NHO_2) (ADLY, 2010; HALLIWELL & GUTTERIDGE, 2007; SIES, 1991). Estas substâncias podem ser produzidas de diversas formas (figura 3 e 4), como por exemplo, durante disfunções mitocôndrias, como resultado de reações de auto-oxidação de biomoléculas instáveis, de inflamação induzida por bactérias invasoras, exposição à radiação UV, contaminação por metais pesados, pesticidas, entre outras (HALLIWELL, 2011; SIES, 1991).

Figura 3 - Vias de produção/estabilização de espécies reativas de oxigênio na célula. Superóxido ($O_2^{\bullet-}$) pode ser formado através de reações químicas catalisada por enzimas, em seguida, pode sofrer reação pela superóxido dismutase (SOD), formando peróxido de hidrogênio (H_2O_2), que por sua vez pode ser eliminado pela ação da catalase ou outros antioxidantes, ou na presença de ferro reduzido sofrer reação de Fenton, produzindo o radical hidroxila (OH•). O_2 pode absorver energia e formar oxigênio singlete (^{1}O2) que pode ser eliminado por compostos antioxidantes.



Fonte: YAN *et al*., 2013.

Figura 4 - Exemplos de fontes comuns de espécies reativas.



Fonte: CADENAS & DAVIES, 2000.

1.3.1 Estresse oxidativo x antioxidantes

A capacidade das células em neutralizar espécies reativas é em grande parte atribuída à eficiência do sistema de defesa antioxidante celular (HALLIWELL, 2011; HALLIWELL & GUTTERIDGE, 2007). Esta rede de defesa antioxidante é constituída de antioxidantes endógenos e exógenos. Os antioxidantes endógenos compreendem uma linha de defesa enzimática, e incluem as enzimas superóxido dismutase (SOD), catalase (CAT), glutationa peroxidase (GPx) e tioredoxina redutase (Trx) que catalisam uma cascata complexa de reações para converter espécies reativas em moléculas mais estáveis, como água e O2 (figura 3). As formas não-enzimáticas incluem diversas moléculas, entre elas: a glutationa (GSH), vitaminas C e E (HALLIWELL & GUTTERIDGE, 2007). Os antioxidantes exógenos compreendem os micronutrientes e outros antioxidantes provenientes principalmente da dieta (HALLIWELL & GUTTERIDGE, 2007; SIES, 1991). Nesta classe destacam-se os compostos fenólicos. Em conjunto, os sistemas antioxidantes enzimáticos e não-enzimáticos são necessários para manter a vida, mantendo um delicado equilíbrio redox intracelular e minimizando indesejáveis danos celulares causados por espécies reativas (RAHAL *et al*., 2014).

A regulação da maquinaria celular antioxidante conta com a participação de diversas moléculas e vias de sinalização. Neste papel destaca-se a ativação do fator de transcrição Nrf2 (fator nuclear eritróide 2 relacionado ao fator 2: via sinalização de elemento de resposta

antioxidante (ARE)), que controla a expressão de genes de proteínas antioxidantes envolvidas na desintoxicação e eliminação de oxidantes reativos. Nrf2 é mantido no citoplasma ligado à uma proteína repressora KEAP1 (proteína kelch 1 associada a ECH) que sinaliza a degradação do Nrf2 via ubiquitinização. Condições de estresse oxidativo ou desequilíbrio eletrolítico causam inibição do KEAP1. Quando Nrf2 não é ubiquitinizado desloca-se para o núcleo e se combina a proteína Maf, ligando-se então ao elemento de resposta antioxidante (ARE) na região promotora de muitos genes antioxidantes e iniciando a transcrição (NGUYEN *et al*., 2009). Esse mecanismo é evolutivamente conservado entre os animais, e é bastante utilizado para marcação de resposta antioxidante frente a condições de estresse. Em condições normais, o Nrf2 é mantido no citoplasma pela ação de um conjunto de proteínas que o degradam rapidamente. Componentes derivados de alimentos naturais têm recebido uma grande atenção nas últimas duas décadas, devido as atividades biológicas como, anti-inflamatório, antioxidante e potencial anti-apoptótico (RAHAL *et al*., 2014). Os flavonóides e outros compostos fenólicos compreendem um grande grupo heterogêneo de derivados metabolitos de frutas, vegetais e ervas (Tabela 2). Estas moléculas exercem um efeito positivo sobre a saúde humana devido às suas capacidades de neutralizar espécies reativas.

Tabela 2 - Classes principais de compostos fenólicos em Plantas.

Classes e sub - classes	Exemplos de compostos específicos
Compostos não-flavonoides	
Ácidos fenólicos	
Ácidos Benzoicos	Ácido gálico; ácido protocatecuico; ácido *p*-hidroxibenzoico
Ácidos hidroxicinâmicos	Ácidos *p*-cumárico; ácido cafeico; ácido ferúlico; ácido sinápico
Taninos hidrolisáveis	Pentagaloilglicose
Estilbenos	Resveratrol
Lignanas	Secoisolariciresinol, matairesinol, lariciresinol, pinoresinol
Compostos Flavonoides	
Flavonóis	Canferol, quercetina, miricetina
Flavonas	Apigenina, luteolina
Flavanones	Naringenina, hesperetina
Flavanóis	Catequinas, galocatequina
Antocianidinas	Pelargonidina, cianidina, malvidina
Taninos condensados ou proantocianinas	Procianidina trimérica, prodelfinidina
Isoflavonas	Daidzeína, genisteína, gliciteína

Fonte: FARAH & DONANGELO, 2006.

1.3.2 Estresse oxidativo x condições de estresse

Evidências crescentes mostram o envolvimento do estresse oxidativo em diversas patologias. Por exemplo, a oxidação da LDL é considerada um dos principais eventos desencadeadores do processo de aterogênese (RAHAL *et al.*, 2014), principal causa de morbidade e mortalidade associadas ao Diabetes mellitus. Neste contexto, alguns estudos dugerem que a geração de radicais livres é em grande parte devido à hiperglicemia, o que agrava ainda mais o desenvolvimento das complicações tardias associadas ao diabetes (ADLY, 2010; RAHAL *et al.*, 2014).

O sistema nervoso central (SNC) é extremamente sensível aos danos dos radicais livres, uma vez que sua capacidade antioxidante total é relativamente pequena. Muitos estudos têm demonstrado o envolvimento do estresse oxidativo em doenças neurodegenerativas como a Doença de Alzheimer e Doença de Parkinson (ALBRIGHT, 2008).

O estresse oxidativo também desempenha um papel na inflamação, acelera o envelhecimento e contribui para uma grande variedade de outras doenças, como por exemplo, cancro, cataratas, doenças inflamatórias do intestino, artrite reumatóide, doenças respiratórias, doenças auto-imunes, doenças hepáticas, doenças renais, doenças da pele, entre outras (GALLI *et al.*, 2005).

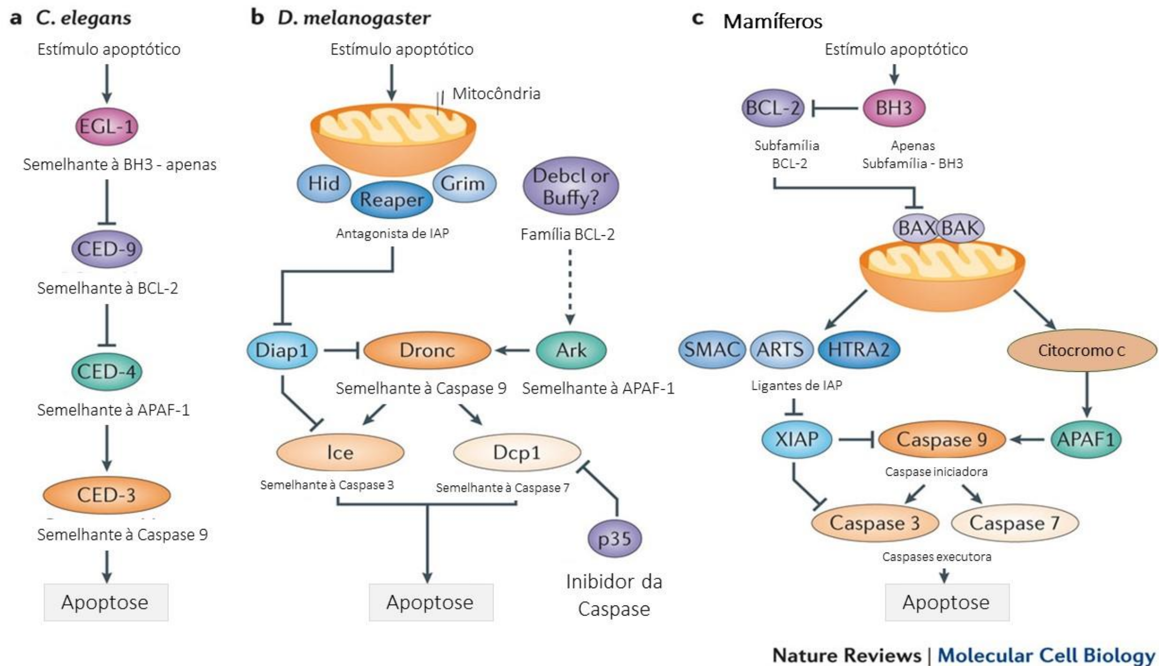
A relação entre espécies reativas e função imune do corpo está bem estabelecida. O mecanismo de defesa imunológica depende dos efeitos de oxidantes, os quais desempenham um papel fundamental na morte de patógenos. O sistema oxidante enzimático, que desempenha papel crucial nesta função, inclui a ação de várias enzimas, entre elas: a nicotinamida adenina dinucleótido fosfato (NADPH) oxidase que produz superóxido, o qual posteriormente este é convertido em peróxido de hidrogênio. Peroxidases, como a mieloperoxidase (MPO), utilizam o peróxido de hidrogênio como substrato para formar ácido hiploroso (HOCl), um potente oxidante contra patógenos (MEOTTI *et al.*, 2011).

A hipóxia é uma condição também conhecida por estimular a produção mitocondrial de espécies reativas de oxigênio (mEROS) (LIU *et al.*, 2002). A hipóxia e a reoxigenação causam também uma desorganização reversível da ATPase e da arquitetura da membrana mitocondrial.

Os danos causados pelo estresse oxidativo podem variar desde uma lesão tecidual até à morte celular, que pode ocorrer essencialmente por necrose e apoptose. Na necrose ocorre perda da permeabilidade celular, ocorrendo liberação de toxinas para o meio extracelular. É sempre um processo patológico por provocar lesão e morte generalizada do tecido. A apoptose é uma forma altamente regulada de morte celular, definida por características morfológicas e bioquímicas distintas. É uma série coordenada de eventos e desempenha um papel importante na manutenção da homeostase dos tecidos e controle da resposta imunitária (ADLY, 2010; GUETEENS *et al*., 2002).

Os mecanismos apoptóticos são bastante conservados entre diferentes grupos animais (figura 5). Em *D. melanosgaster*, o processo se dá pela ativação de caspases, que são uma classe de proteases de cisteína expressas como zimogênios inativos em quase todas as células. *D. melanogaster* tem 7 caspases (FUCHS & STELLER, 2015). Sinais pró-apoptóticos, como sinais de desenvolvimento, radiação, e várias formas de estresse e lesão celular podem induzir apoptose pela ativação transcripcional da família de proteínas Reaper, Hid e Grim. Estas induzem a apoptose por ligação a um inibidor das proteínas de apoptose (IAPs), que por sua vez, quando não inibido, inibe as caspases. As caspases são divididas basicamente em duas categorias: a caspase iniciadora Dronc (caspase 9) e as caspases executoras Drice (caspase 3) e DCP1 (caspase 7). Com ativação das caspases, é permitido que Ark (factor 1 ativador da protease apoptótica -APAF1) interaja com a Dronc, produzindo o apoptosoma, que eficientemente ativa as caspases executoras. A proteína p35 é um inibidor especifico de caspases executoras. A ativação da apoptose pode ser regulada pelos membros da família BCL-2, Debcl e Buffy (FUCHS & STELLER, 2015). Embora muitas caspases tenham papéis cruciais na apoptose, estas proteínas também têm funções não-apoptóticas em vários processos, incluindo imunidade, remodelação celular, aprendizagem, memória e diferenciação.

Figura 5 - O núcleo da maquinaria apoptótica em *D. melanogaster*.



Fonte: FUCHS & STELLER, 2015.

1.4 HIPÓXIA/ REPERFUSÃO

A hipóxia caracteriza-se por ser uma condição tecidual de baixo teor de oxigênio, cuja ocorrência pode ser atribuída a diversos fatores. Pode ocorrer por alteração em qualquer mecanismo de transporte de oxigênio, como obstrução física do fluxo sanguíneo, anemia ou permanência em áreas com concentrações baixas de oxigênio (SOLAINI *et al*., 2010; VIGNE *et al*., 2009).

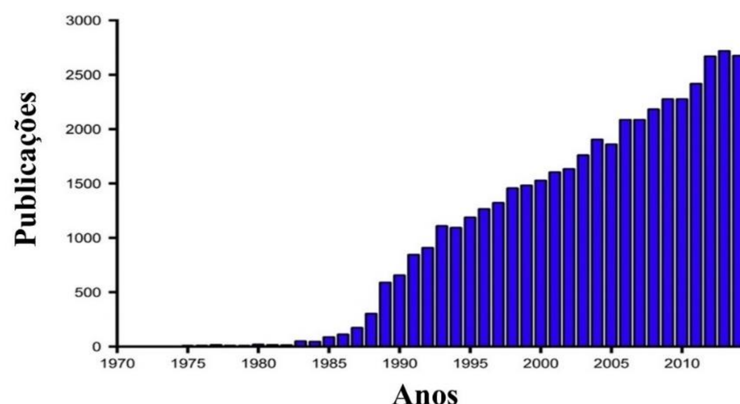
Durante a hipóxia as reservas de ATP são rapidamente depletadas, pela interrupção da fosforilação oxidativa nas mitocôndrias. A ausência de energia leva a uma série de mudanças metabólicas e morfológicas nas células. Inicialmente ocorre uma perda da homeostase iônica da célula: aumento do influxo de Ca^{2+} intracelular, provavelmente por alteração da permeabilidade de membrana; diminuição do pH celular, pelo acúmulo de lactato (GRANGER & KVIETYS, 2015). Estas mudanças podem promover morte celular por mecanismos necróticos, necroptóticos, apoptóticos e autofágicos (KALOGERIS *et al*., 2012). Os distúrbios metabólicos que ocorrem durante hipóxia são muito bem estabelecidos, porém

evidências clínicas e experimentais demonstram que os principais eventos que levam às disfunções celulares e teciduais relacionam-se com a subsequente reperfusão.

A lesão de reperfusão é um termo usado para descrever as alterações funcionais e estruturais que se tornam aparentes durante o restabelecimento do fluxo de oxigênio após um período hipóxico. Dessa maneira, há uma crescente investigação nas áreas de pesquisas em relação aos danos causados por reperfusão (figura 6). A reperfusão ou reoxigenação de tecidos hipóxicos pode induzir a uma rápida remodelação metabólica, reprogramação mitocondrial seguida de alta produção de espécies reativas de oxigênio, reorganização dos fluxos iónicos através da membrana plasmática, inflamação e consequentemente morte celular (BIDDLESTONE *et al*., 2015; SOLAINI *et al*., 2010; VIGNE *et al*., 2009). Embora uma variedade de mecanismos moleculares tenham sido propostos para explicar tais eventos; o excesso de EROS recebe maior atenção, como um fator crítico na gênese da lesão de reperfusão. Entre as potenciais fontes de EROS produzidas durantes a reperfusão descritas até agora, estão as mitocôndrias, e as enzimas xantina oxidase, a NADPH oxidase (Nox), e a óxido nítrico sintase (GRANGER & KVIETYS, 2015).

A determinação dos danos causados por hipóxia seguido de reperfusão correspondem ao somatório de ambos os processos. Para tanto, para determinar a resposta dessas lesões, deve-se considerar uma série de fatores, como por exemplo: tipo de hipóxia (generalizada ou local), tempo (intermitente ou constante), intensidade e duração da hipóxia, tecido específico atingidos e as diferenças entre organismos com diferente sensibilidade à hipóxia (ZHAO *et al*., 2011).

Figura 6 - Frequência de publicação de artigos que tratam de lesão por reperfusão. De 1970 até 2014. Baseado na pesquisa PubMed usando o termo de pesquisa "Danos de isquemia-reperfusão" ou "lesão de reperfusão".



Fonte: GRANGER & KVIETYS, 2015.

1.4.1 Hipóxia/reperfusão x eventos oxidativos

Durante o processo de Hipóxia/reperfusão ocorre uma série de mudanças celulares/teciduais como mencionado acima, que se refletem nos danos ao organismo. Entre estas destaca-se o papel das mitocôndrias. As mitocôndrias consomem a maior quantidade (cerca de 85-90%) de oxigênio nas células para manter a fosforilação oxidativa, que é a principal via metabólica para a produção de ATP (SOLAINI *et al.*, 2010). Os danos mitocondriais induzidos por hipóxia/reperfusão podem se dar pela perda do potencial de membrana, desordem da cadeia de transporte de elétrons (CTE), produção excessiva de espécies reativas de oxigênio, podendo assim conduzir a morte celular (DHAR-MASCARENO *et al.*, 2005).

Uma resposta do organismo aos efeitos da hipóxia é a ativação de mecanismos de adaptação para a sobrevivência, sendo o condutor principal o fator induzível por hipoxia 1 (HIF-1). Os HIFs são heterodímeros compostos de uma subunidade α controlada por oxigênio e uma subunidade β insensível ao oxigênio (BANDARRA *et al.*, 2014). Esse fator de transcrição regula a expressão de mais de 100 genes-alvo envolvidos tanto em funções fisiológicas quanto patológicas em resposta à hipóxia. Por exemplo, o HIF-1 induz a expressão de genes de via glicolítica, devido o aumento da glicólise ser a principal via de obtenção de energia quando os níveis baixos de oxigênio não suportam a fosforilação oxidativa (CRUZ *et al.*, 2016). Existem diferentes reguladores de HIF-1. Por exemplo, autores sugerem que EROs desempenham um papel importante na ativação do HIF-1 (KIETZMANN *et al.*, 2005). Outros autores também mostram o NFkB como um regulador do HIF-1 (VAN UDEN *et al*, 2008). NFkB (factor nuclear kappa B) é um complexo proteico que está envolvido em resposta celulares a estímulos como o estresse oxidativo e infecções (BANDARRA *et al.*, 2014).

O fator respiratório nuclear 1 (NRF-1) é um fator de transcrição de humanos que regula a expressão de genes necessários para a função respiratória mitocondrial, sendo um importante regulador da mitobiogênese, como também exerce papel na regulação do crescimento celular e proteção contra estresse oxidativo (ICREVERZI et al., 2012; RAI *et al.*, 2014; WANG *et al.,* 2016). Em recente publicação foi demonstrado que o NRF-1 regula a expressão de HIF-1 sobre condições de hipóxia, sugerindo que esta proteína também está envolvida em respostas de processos hipóxicos (WANG *et al.*, 2016).

O homólogo do NRF-1 em *Drosophila melanogaster* é chamado de Erect wing (EWG), o qual tem importante função no desenvolvimento neuronal e muscular da mosca (RAI *et al*., 2010). Nos neurônios, o EWG regula o crescimento sináptico em junções neuromusculares no período de terceiro estágio larval, e nos músculos, regula a formação dos músculos indireto de voo durante o estágio de pupa. No entanto, pouco se sabe sobre sua função no estágio adulto da mosca-da-fruta. Até o momento não se tem informações sobre a interação do EWG com as respostas de sinalização em condições de hipóxia.

1.4.2 Hipóxia/reperfusão x situações fisiológicas e patológicas

Hipóxia e reperfusão podem ocorrer tanto em condições fisiológicas quanto patológicas. Por exemplo, a hipóxia, ocorre durante o desenvolvimento embrionário e desempenha função importante durante o processo de angiogênese (KROCK *et al*., 2011). A hipóxia também está associada com problemas clínicos importantes, como doenças inflamatórias, distúrbios isquêmicos (cerebrais ou cardiovasculares), tumores, complicações do diabetes, hipertensão pulmonar, complicações obstétricas/perinatais e transplante de órgãos (SOLAINI *et al*., 2010; ZHAO *et al*., 2011).

A hipóxia promove o crescimento dos vasos através da regulação positiva de várias vias pro-angiogênicas que medeiam aspectos chave da biologia celular endotelial e vascular. A via HIF-1 (fator induzivel por hipóxia) é atualmente vista como um regulador principal da angiogênese. HIF-1 pode proporcionar um benefício terapêutico em diversas patologias, incluindo cancro, doença cardíaca isquémica, doença arterial periférica, cicatrização de feridas e doenças neovasculares oculares (KROCK *et al*., 2011). Estudos mostram também que a hipóxia influencia em aspectos adicionais da angiogênese, como padrões de vasos, maturação e função (KROCK *et al*., 2011). Assim como outros estudos suportam a hipótese de que a hipóxia impulsiona a angiogênese tumoral. Basicamente o tecido tumoral sem vascularização torna-se hipóxico; estimulando a neoangiogênese para melhorar o influxo de oxigênio, uma vez que os tumores devem desenvolver mecanismos para atender às crescentes demandas metabólicas, e dessa maneira, aumentando as possibilidades de ocorrer neoplasia (MOELLER, 2004).

Existe uma variedade de condições patológicas onde os tecidos afetados exibem uma baixa tensão de oxigênio. Por exemplo, a hipóxia é um fator importante onde existem componentes inflamatórios, como na doença inflamatória intestinal crônica, artrite reumatóide e lesão por isquemia/reperfusão. Importante, as consequências da hipóxia também são consideradas para o tratamento do cancro. Além disso, a hipóxia deve ser considerada como um fator em doenças onde as tensões de oxigênio são provavelmente insuficientes devido à má função respiratória, como fibrose cística e bronquite crônica, bem como a doenças proliferativas benignas como a psoríase, vasculopatias diabéticas e epilepsia (AIRLEY *et al*., 2000).

A isquemia se caracteriza como falta de fornecimento sanguíneo para um tecido. O sangue fornece além do oxigênio, nutrientes às células. Dessa forma a isquemia é resultante da falta de glicose e de oxigenação nas células. Nesse sentido, as pesquisas sobre os danos causados pelas condições de hipóxia se concentram em grande parte em estudos isquêmicos (KALOGERIS *et al*., 2012). O crescente número de estudos de isquemia-reperfusão nos últimos anos é provavelmente um reflexo de vários fatores, incluindo a implicação deste mecanismo de lesão tecidual em uma crescente lista de órgãos (tabela 3).

Existe atualmente uma variedade de modelos experimentais para os estudos de isquemia-reperfusão. Estes modelos variam desde modelos *in vitro* a roedores (GRANGER & KVIETYS, 2015; KALOGERIS *et al*., 2012). Recentes pesquisas têm demonstrado o uso da mosca-da-fruta como um ótimo modelo para estudos de hipóxia. Apesar do modelo não se caracterizar como isquêmico, devido a mosca possuir um sistema circulatório aberto, o modelo é valido por vários outros motivos, como resistência a condições de hipóxia e avaliação de danos totais do organismo (ZHAO & HADDAD, 2011).

Tabela 3 - Condições clínicas associadas à lesão por isquemia-reperfusão.

Envolvimento de órgão único	
Órgão	Condição
Sistema nervoso central	Acidente vascular cerebral (AVC) Encefalopatia hipóxica-isquêmica perinatal Doença de Alzheimer
Coração	Síndrome coronária aguda
Articulações	Osteoartrite
Trato gastrointestinal	Úlcera gástrica Enterocolite necrosante neonatal Vólvulo
Rim	Lesão renal aguda
Envolvimento de múltiplos órgãos	
Condição	*Manifestações*
Hemorragia e ressuscitação	Insuficiência de múltiplos órgãos
Doença falciforme	Síndrome torácica aguda, priapismo
Apnéia do sono	Dano oxidativo, hipertensão
Procedimentos médicos ou cirúrgicos	
Condição	*Manifestações*
Terapia trombolítica	AVC, infarto do miocárdio
Transplante de órgão	Insuficiência aguda do enxerto
Circulação extracorpórea	Dano pulmonar, insuficiência cardíaca
Angioplastia coronária	Insuficiência cardíaca
Liberação do torniquete arterial	Dano tecidual local
Cirurgia aórtica toracoabdominal	Dano na medula espinhal
Descompressão da síndrome compartimental	Edema de membro, dano muscular
Torção testicular ou ovariana	Infertilidade

Fonte: GRANGER & KVIETYS, 2015.

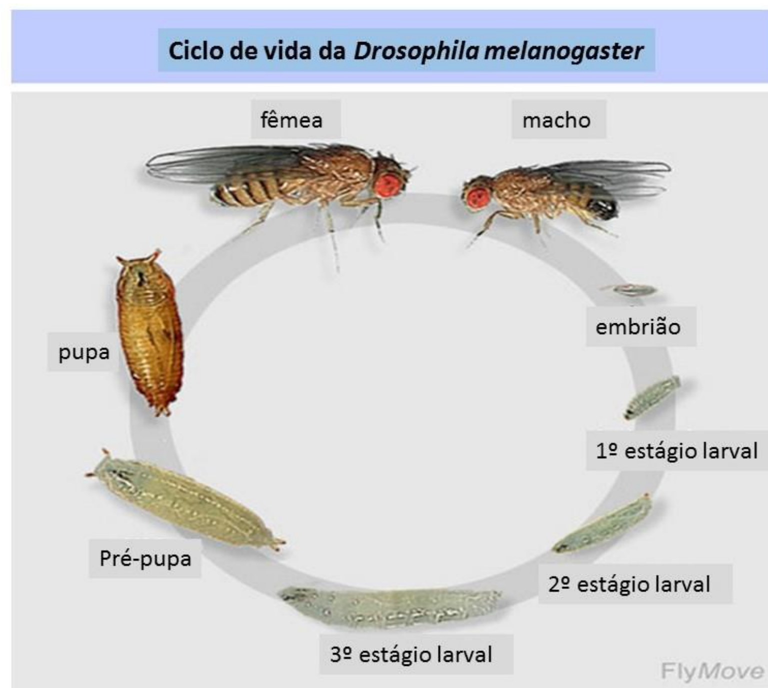
1.5 *Drosophila melanogaster*

A mosca da fruta, *Drosophila melanogaster* (figura 7), destaca-se como um organismo de grande importância para estudos de doenças humanas e pesquisas toxicológicas (SIDDIQUE *et al*., 2005). O modelo tem sido utilizado devido às vantagens advindas de seu curto ciclo biológico, rápido desenvolvimento e fácil manipulação. Outra vantagem é a ausência de mitose celular nas moscas em fase adulta. Isto torna possível a determinação dos danos causados por um xenobiótico ao longo do tempo e a viabilidade celular (JIMENEZ-DEL-RIO *et al*., 2010).

D. melanogaster assim como alguns artrópodes, tem um ciclo de vida de quatro estágios: ovo, larva (3 estágios larvais) pupa e mosca (figura 7). Uma vez fertilizado, o

embrião desenvolve-se no ovo durante cerca de um dia antes da eclosão como larva. A larva come e cresce ao longo de cinco dias até se tornar pupa e sofrer metamorfose durante quatro dias para se tornar mosca adulta. O desenvolvimento pode ser influenciado por alguns fatores, como por exemplo condições de estresse ou mudanças na temperatura.

Figura 7 - Ciclo de vida de *Drosophila melanogaster*.



Fonte: FlyMove.

Durante mais de 100 anos, a *Drosophila* tem sido utilizada como um organismo modelo em análises genéticas. Na verdade grande parte do nosso conhecimento atual sobre genes, desenvolvimento e interações genéticas se origina do trabalho com esta espécie (JENNINGS, 2011). Por exemplo, Thomas Hunt Morgan, considerado o “Pai” da pesquisa com *Drosophila* refinou a teoria de herança proposta por Gregor Mendel, definindo genes muito antes do DNA ser considerado material genético.

Embora os seres humanos e *D. melanogaster* estejam distantemente relacionados evolutivamente, o sequenciamento completo do genoma de *D. melanogaster* revelou homologia em torno 70% de genes relacionados com doenças de seres humanos, o que tornou o organismo um excelente modelo para “screening” de drogas para o tratamento de doenças.

D. melanogaster tem sido usado como um organismo modelo para estudar uma gama diversificada de processos biológicos, incluindo genética e herança, desenvolvimento embrionário, aprendizagem, comportamento e envelhecimento (ZHAO & HADDAD, 2011). Além disso, a mosca-da-fruta vem sendo usada com bastante precisão em pesquisas voltadas para investigar o papel protetor de produtos naturais contra danos de cunho oxidativo (BONILLA *et al*., 2011; JIMENEZ-DEL-RIO *et al*., 2010; ORTEGA-ARELLANO *et al*., 2011).

Mais recentemente, pesquisas demonstraram a utilização da *D. melanogaster* em estudos de hipóxia/reperfusão que frequentemente ocorrem em estados de doenças (ZHAO & HADDAD, 2011). As vantagens para a utilização desse modelo em restrições de oxigênio é a velocidade relativa com a qual se pode realizar tais análises e a disponibilidade de ferramentas e marcadores genéticos. *D. melanogaster* possui vias metabólicas e genes envolvidos em resposta à hipóxia homólogos aos mamíferos. Outra vantagem do modelo é sua forte resistência a flutuações de oxigênio. Acredita-se que essa resistência tenha sido adquirida durante a evolução em sua fase larval, fase onde esta pode se encontrar em lugares com baixa concentração de oxigênio, como no interior de frutos**.** Esta característica possibilita a investigação dos danos celulares em diferentes padrões de hipóxia, como por exemplo, tempo e intensidade de exposição. Outra vantagem é avaliação dos danos no sistema de organismo inteiro, ou seja, avaliar as consequências e os inúmeros danos causados por hipóxia em todo o organismo do animal (FEALA *et al*., 2009; KLOK & HARRISON, 2009; ZHAO & HADDAD, 2011; ZHOU & HADDAD, 2013).

Com base na relação existente entre hipóxia e danos oxidativos, e considerando a qualidade e potencial terapêutico do mel do Bioma Pampa Brasileiro, nós desenvolvemos um trabalho para avaliar o efeito do mel sobre os insultos causados por hipóxia/reperfusão usando *Drosophila melanogaster* como organismo alvo.

2. OBJETIVOS

2.1 OBJETIVO GERAL

Avaliar o efeito do mel contra os danos induzidos por hipóxia/reperfusão em *Drosophila melanogaster.*

2.2 OBJETIVOS ESPECÍFICOS

2.2.1 Determinar as características: físico-químicas, conteúdo fenólico total e atividade antioxidante *in vitro*, identificação e quantificação de compostos fenólicos do mel pertencente ao Bioma Pampa Brasileiro;

Em *Drosophila melanogaster* submetidas à hipóxia/reperfusão, investigar os efeitos do mel sobre:

2.2.2 Percentagem de sobrevivência, mudanças fenotípicas e alterações motoras;

2.2.3 Parâmetros de estresse oxidativo: produção de EROs, atividade de enzimas antioxidantes e função mitocondrial;

2.2.4 Expressão de mRNA de genes envolvidos em vias metabólicas, inflamatória, de morte celular e regulação redox;

2.2.5 Investigar o efeito de compostos fenólicos majoritários presentes no mel sobre a sobrevivência e alterações motoras e fenotípicas em *Drosophila melanogaster* submetidas à hipóxia/reperfusão.

3. RESULTADOS

3.1 ARTIGO

Neurochem Res
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ORIGINAL PAPER

Brazilian Pampa Biome Honey Protects Against Mortality, Locomotor Deficits and Oxidative Stress Induced by Hypoxia/Reperfusion in Adult *Drosophila melanogaster*

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Abstract We aimed to investigate the potential beneficial effects of the Brazilian Pampa biome honey in a Drosophila-based hypoxia model. Adult flies were reared in standard medium in the presence or absence of honey (at a final concentration of 10 % in medium). Then, control flies (4 % sucrose in medium) and honey-treated flies were submitted to hypoxia. Subsequently, flies were analyzed for mortality, neurolocomotor behavior (negative geotaxis), mitochondrial/oxidative stress parameters and expression of hypoxia/stress related genes by RT-qPCR. The HPLC analysis revealed the presence of phenolics and flavonoids in the studied honey. Caffeic acid was the major compound followed by *p*-coumaric acid and kaempferol. The presence of such compounds was correlated with a substantial antioxidant activity in vitro. Flies subjected to hypoxia presented marked mortality, locomotor deficits and changes in oxidative stress and mitochondrial activity parameters. Honey treatment was able to completely block mortality and locomotor phenotypes. In addition, honey was able to reverse ROS production and hypoxia-induced changes in mitochondrial complex I and II activity. Hypoxia also induced an up-regulation in mRNA expression of Sima (HIF-1), NFκβ, NRF2, HOX, AKT-1, InR, dILP2, dILP5 and HSP27. Honey treatment was not able to modulate changes in the tested genes, indicating that its protective effects involve additional mechanisms other than transcriptional activity of hypoxia-driven adaptive responses in flies. Our results demonstrated, for the first time, the beneficial effects of honey against the deleterious effects of hypoxia/reperfusion processes in a complex organism.

Keywords Hypoxia · Honey · Antioxidant · *Drosophila* · Oxidative stress · Gene expression

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Introduction

Honey is a complex mixture produced by honeybees from the nectar and is widely consumed as a sweetener as well as for its therapeutic properties [1]. Honey is mostly constituted by sugars; however; organic acids, vitamins, carotenoid-derived compounds, amino acids, proteins, trace elements, phenolic compounds and flavonoids are also present [2]. The composition of honey is influenced by a number of factors including geographical origin, botanical sources of nectar, environmental and climatic conditions, handling and processing techniques [3]. For instance, phenolic content in honeys from different regions has been correlated with differential antioxidant capacity of honey samples [4]. Thus, it becomes important to characterize the identity, quality and health benefits of honeys from distinct regions around the globe. In a previous study, we characterized Brazilian Pampa biome honeys and demonstrated its capacity in counteracting oxidative stress induced by iron and paraquat in a Drosophila model [5]. It is shown from previous reports that honey can deliver health benefits such as antihypertensive, reproductive, anti-inflammatory, anti-tumor, immunomodulatory, vasodilative, anti-fungal, antibacterial [6], hypoglycemic [7, 8], and antioxidant

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effects [1, 6, 8]. Recent studies have shown that natural products such as polyphenols are able to block oxidative stress induced by redox-active chemicals in Drosophila models of neurological conditions, including Parkinson's disease [9, 10]. However, studies on the protective effects of honey against the deleterious effects of hypoxia/reperfusion are scarce.

The fruit fly, *Drosophila melanogaster*, is a model-organism that has been widely used due to advantages arising from its short life cycle, rapid development and easy handling, making them ideal complex organisms for in vivo bioassays [9, 11]. Although humans and flies are only distantly evolutionarily related, almost 75 % of disease-related genes in humans have functional orthologs in the fly, making it a reasonable model system for human diseases. Drosophila have been also proving to be a powerful model system for the study of development and functioning of nervous system as well as for the investigation of cellular/molecular mode of action of environmental toxicants [11].

In the present study we asked whether Brazilian Pampa Biome honey, a powerful natural antioxidant would deliver a protective effect against the deleterious effects induced by hypoxia in a fruit-fly *Drosophila melanogaster* model.

Experimental Procedure

Chemicals

Sodium bisulfate (71657), copper (ii) sulfate (C1297), potassium sodium tartrate tetrahydrate (217255), tannic acid (403040), Folin-Ciocalteu (F9252), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS; A1888), sodium acetate (S2289), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES; H3375), sucrose (S5016), reduced glutathione (GSH; G4251-5G), tetramethylethylenediamine (TEMED; T9281), quercetin (Q4951), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; M2128-1G), 5,5-dithiobis (2-nitrobenzoic acid) DTNB (D8130), acetylthiocholine iodide (A5751), 1-chloro, 2,4-dinitrobenzene (CDNB; 237329), 2′,7′-dichlorofluorescein diacetate (DCFH-DA; 35845), D-manitol (M9647), K2KO4P (1110216), KH2PO4 (P0662), HEPES (Titration; H3375), Albumin from bovine serum (BSA; A6003), resazurin sodium salt (R7017), Triton X-100 (T8532) were obtained from Sigma-Aldrich. SYBR Select Master Mix Applied (4472908) from Biosystems by Life Technologies, DNAse I Amplification Grade—Invitrogen (18068-15) by Life Technologies and iScript cDNA Synthesis kit (1708891) from Biorad. All other chemicals and reagents used here were of the highest analytical grade.

Honey Sampling, Physicochemical and HPLC Analysis

The honey sample from *Apis mellifera* was courtesy of producers/researchers of the Universidade Federal do Pampa from São Gabriel in the state of Rio Grande do Sul, Brazil. Honey was harvested in March 2015 and identified as multifloral-wild flowers. Honey sample was stored at room temperature (20–25 °C) in plastic containers until analysis. The methods used for determination of quality parameters were according to Brazilian regulation. The methods advocated by this legislation follow the *Codex Alimentarius* Commission and the Association of Official Analytical Chemists. In this paper we evaluated the following quality parameters: moisture, reducing sugars, apparent sucrose and hydroxymethylfurfural (HMF), according to described by Cruz et al. [5].

HPLC–DAD

HPLC was performed using a Phenomenex C18 analytical column (4.6 mm × 250 mm, 5 μm particle size). Gradients were generated using 0.05 % formic acid in water and methanol, according with Gheldof et al. [12]. The flow rate was 0.6 mL/min and the injected volume was 50 μL. The honey sample was diluted in methanol/water (1:4, v/v) and was filtered through 0.45 μm membrane filter (Millipore) prior to use. Stock solutions of standards references were prepared in the HPLC mobile phase at a concentration range of 0.050–0.500 mg/mL. Quantifications were carried out by integration of the peaks using the external standard method, at 270 nm for gallic acid; 325 nm for chlorogenic, caffeic, *p*-coumaric, ferulic and rosmarinic acids; and 366 nm for quercetin, chrysin, apigenin and kaempferol. The chromatography peaks were confirmed by comparing its retention time with those of reference standards and by DAD spectra (200–600 nm). All chromatography operations were carried out at room temperature and in triplicate. LOD and LOQ were calculated based on the standard deviation of the responses and the slope using three independent analytical curves, as defined by Boligon et al. [13].

Analysis of Antioxidant Properties

The analysis of antioxidant properties in vitro were performed spectrophotometrically in 96 well plates using the EnSpire® multimode plate reader (PerkinElmer, USA).

Total Phenolics

Phenolic compounds from honey samples were detected by the Folin–Ciocalteu method [14] with minor modifications [5]. Briefly, 4 μL honey solution (0.1 g/mL) was mixed

with 35 μL 1 N Folin–Ciocalteu's reagent. After 3 min, 70 μL 15 % Na_2CO_3 solution was added to the mixture and adjusted to 284 μL with distilled water. The mixture was kept in the dark for 2 h, after which the absorbance was read at 760 nm. Gallic acid was used as standard (10–300 μg/mL). The results were expressed as mg of gallic acid equivalents (GAEs) per 100 g honey.

DPPH$^{\bullet}$ Radical Scavenging Assay

The scavenging activity towards 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) radical was evaluated according to the method of Baltrušaitytė et al. [15] with minor modifications. In brief, 100 μL of $DPPH^{\bullet}$ (300 μM) diluted in ethanol was mixed with honey (0.1 g/mL) in a 96 wells microtitre plate. The final volume of each well was adjusted to 300 μL with ethanol. Ascorbic acid was used as a positive control. The absorbance was determined at 517 nm after 45 min incubation. The results were expressed as mg of ascorbic acid equivalents (AAEs) per 100 g of honey.

ABTS$^{\bullet}$+ Radical Scavenging Assay

The antioxidant activity of HEPG in the reaction with $ABTS^{\bullet}+$ radical was determined according to the method of Baltrušaitytėet et al. [15] with some modifications. Firstly, $ABTS^{\bullet}+$ radical solution was generated by oxidation 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt stock solution (7 mM) with 2.5 mM potassium persulfate ($K_2S_2O_8$). 200 μL of $ABTS^{\bullet}+$ solution was mixed with 10 μL of honey (0.1 g/mL) in a microplate and the decrease in the absorbance was measured after 10 min. Ascorbic acid was used as a positive control. The results were expressed as mg of ascorbic acid equivalents (AAEs) per 100 g of honey.

Ferric Reducing Antioxidant Power (FRAP)

The ferric ion reducing capacity of honey was assayed according to the original method of Benzie and Strain [16], adjusted to analysis of honey samples. Briefly, 9 μL of honey sample (0.1 g/mL) were mixed with of 270 μL of freshly prepared FRAP reagent. The FRAP reagent was prepared by mixing 2.5 mL of 0.3 M acetate buffer pH 3.6, with 250 μL of 10 mM 2,4,6-Tris(2-pyridyl)-*s*-triazine (TPTZ) solution and 250 μL of $FeCl_3 \cdot 6H_2O$. The mixture was shaken and left in a water bath for 30 min and the absorbance readings were taken at 595 nm. Ammonium iron(II) sulfate hexahydrate was used to calculate the standard curve (100–2000 μM). The reducing ability of honey was expressed as μM of Fe(II) equivalent/100 g honey.

Drosophila Stock

Drosophila melanogaster (Harwich strain) was obtained from the National Species Stock Center, Bowling Green, OH. The flies were reared in glass vials containing Bloomington standard cornmeal Drosophila medium [5] in a constant temperature and humidity (20 °C ± 1; 60 % relative humidity). All experiments were performed with the same strain, using adult flies with 1–3 days old.

Flies Treatment and Exposure to Hypoxia

Flies (1–3 days old) were maintained on standard agar medium (1 % agar, 0.5 % skim milk powder, 0.25 % yeast, 0.08 % nipagin, 4 % sucrose) for 3 days. The Control group was kept on standard medium only. The honey group was maintained in standard medium (less sucrose) supplemented with 10 % honey. After 3 days, both groups were subjected to hypoxia condition for 3 h. The *D. melanogaster* hypoxia model was performed according to previous protocol [17], with some alterations. Flies were placed in a glass chamber with two openings, one of which was connected to a nitrogen cylinder and the other kept in contact with air space for oxygen output. The hypoxia in the flies was produced by continuously infusion of N_2 gas into the air of chamber, resulting in an atmosphere of 3–4 % oxygen at 25 °C during 3 h. In pilot studies (data not shown), which were based on a previous literature report [18], we adapted our model and defined adequate exposure time and oxygen intensity in order to observe behavioral and biochemical changes. An oximeter (AZ-8403) was used to monitoring the levels of oxygen during hypoxia trials. After the hypoxia condition, flies were exposed to oxygen reperfusion ($\cong$21 %). After visual observation of complete flight recovery (around 2 h after reperfusion) flies were used for the behavioral and biochemical assays.

Lifespan and Locomotor Assay

For lifespan experiments, 40 sets of 20 female flies were assayed for each experimental group (n = 40). After hypoxia flies were kept in agar medium according described above for each experimental group and mortality was recorded daily. Flies medium was also replaced every day. Results were expressed as percentage of surviving flies. The neurolocomotor deficits were evaluated by negative geotaxis assay based on previous study [9]. After hypoxic exposure (post-flight recovery $\cong$5 and 24 h) flies were transferred to test tubes marked at 5 cm height. The flies were gently tapped to the bottom of the tube and the number of flies able to climb 5 cm after 6 s was recorded at 1-min intervals. Each experiment was repeated thrice. The

climbing performance index (PI) was calculated according to the following equation: $1/2[(n\text{tot} + n\text{top} - n\text{bot})/n\text{tot}]$, where $n\text{top}$ = numbers of flies at the top, $n\text{bot}$ = at the bottom, and $n\text{tot}$ = total number of flies. Results were expressed as percentage of control. In *D. melanogaster*, mortality rates and locomotor behavior are known to be affected by gender [19]. Therefore, we used female flies for assessment of mortality and locomotor behavior, in order to avoid misleading results. For biochemical analysis, both sexes were used.

Enzyme Assays

Antioxidant enzyme activities were performed in an Agilent Cary 60 UV/VIS spectrophotometer with an 18 cell holder accessory coupled to a Peltier Water System temperature controller. A number of 40 flies were separated and homogenized in 500 µl of 100 mM potassium phosphate buffer (pH 7.0). The homogenates were centrifuged at 20,000*g* for 30 min at 4 °C and the supernatants collected for antioxidant enzymes assay. The total protein was determined by the method of Bradford [20], using bovine serum albumin as standard. Enzyme activity was calculated as mU/mg total protein and expressed as percentage of control. Glutathione transferase activity (GST) was assayed following the procedure of Habig and Jakoby [21] using 1-chloro 2,4-dinitrobenzene (CDNB) as substrate. The assay is based on the formation of the conjugated complex of CDNB and GSH at 340 nm. The reaction was conducted in a mix consisting of 0.1 M potassium phosphate buffer pH 7.0, 1 mM EDTA, 1 mM GSH, and 2.5 mM CDNB. Catalase activity was assayed following the clearance of H_2O_2 at 240 nm in a reaction media containing 0.05 M potassium phosphate buffer pH 7.0, 0.5 mM EDTA, 10 mM H_2O_2, 0.012 % TRITON X100 according to the procedure of Aebi [22]. Superoxide dismutase (SOD), activity was based on the decrease in cytochrome *c* reduction [23]. The acetylcholinesterase activity was assayed following the procedure of Ellman et al. [24]. The system consisted of 120 µL of distilled water, 20 µL of 0.1 M potassium phosphate buffer pH 8.0, 20 µL of 10 mM DTNB, 20 µL of sample and 20 µL of 8 mM acetylthiocholine as initiator. There reaction was monitored for 5 min at 412 nm using a Spectra Max plate reader (molecular devices). Thioredoxin reductase (TrxR) activity was measured based on the method of Holmgren and Bjornstedt [25]. Caspase 3/7 (DEVDase) activity was measured with commercial kits in fly homogenates according to manufacturer instructions (Promega Apo-ONE® homogeneous caspase 3/7 assay). The assay is based on the specific protease activity (caspase) of samples towards a profluorescent substrate provided by the kit. Fly homogenates were prepared by homogenizing flies in HEPES buffer pH 7.0 followed by centrifugation at 20,000*g* for 30 min at 4 °C. Samples were incubated during 30 min with substrate in a 1:1 ratio and fluorescence was captured in a plate reader ($499_{ex}/521_{em}$ nm). The amount of fluorescent product generated is proportional to the amount of caspase 3/7 cleavage activity presented in samples. Similarly, caspase 9 activity was measured with commercial kits in fly homogenates according to manufacturer instructions (Promega Caspase-Glo® 9 assay).

Isolation of Mitochondria and Mitochondrial Complexes Activity Assay

For mitochondria isolation, 80 flies were homogenized in 1 mL of solution containing 250 mM sucrose, 2 mM EGTA, 0,1 % bovine serum albumin, 5 mM Tris (pH 7.4). The homogenate was centrifuged at 1000*g* for 3 min, and the supernatant centrifuged at 12,000*g* for 10 min to yield a mitochondria pellet which was resuspended in 100 mM potassium phosphate buffer (pH 7.4). Mitochondria were disrupted by thrice freezing and thawing cycles [26]. The activities of complexes I and II were determined spectrophotometrically with mitochondrial membranes suspended as previously described [27] with minor modifications, as follows.

NADH: Ubiquinone Oxidoreductase (Complex I) Activity

Complex I was assayed using mitochondrial membranes in 100 mM potassium phosphate buffer (pH 7.4). The reaction was started by adding NADH to a final concentration of 100 µM. The enzymatic activity was determined, following the decrease in absorbance at 340 nm during 180 s corrected by total protein. Rotenone (100 µM) was used as inhibitor. Activity data were calculated as decreases in NADH absorbance at 340 nm and expressed as percentage of control.

Succinate Dehydrogenase (Complex II) Activity

Complex II activity was assayed using mitochondrial membranes incubated with 7.5 mM succinate as substrate, 75 µg/mL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and 100 mM potassium phosphate buffer (pH 7.4) to complete the reaction medium. Malonate (15 mM) was used as inhibitor. After 45 min, dimethyl sulfoxide (DMSO) was added to reaction and the absorbance was determined at 570 nm. Activity data were calculated as increases in the absorbance (MTT reduction) corrected by total protein and expressed as percentage of control.

ROS Production

Reactive species were determined in flies mitochondrial fraction by 2′,7′-dichlorofluorescein diacetate (DCFDA) oxidation as a general index of ROS production following [28]. The fluorescence emission of DCF resulting from DCF-DA oxidation was monitored at regular intervals at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The rate of DCF formation was calculated as a percentage of the DCF formation in relation to the sucrose-treated control group and values were normalized by protein concentration.

Quantitative Real-Time qRT-PCR and Gene Expression Analysis

Approximately 1 μg of total RNA from 20 flies was extracted using the Trizol Reagent (Invitrogen) accordingly to the manufacturer's suggested protocol. After quantification, total RNA was treated with DNase I (DNAse I Amplification Grade—Invitrogen, NY, USA) and cDNA was synthesized with iScript cDNA Synthesis Kit and random primers 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 4), and 0.2× SYBR Green I (molecular probes) using a 7500 real time PCR system (Applied Biosystems, NY, USA).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, USA), and the CT (cycle threshold) value for each sample was calculated and reported using the $2^{-\Delta\Delta CT}$ method [29]. The GPDH and tubulin (data not shown) gene was used as endogenous reference genes presenting no alteration in response to treatments. 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 4). The ΔCT mean value obtained from the control group of each gene was used to calculate the ΔΔCT of the respective gene ($2^{-\Delta\Delta CT}$).

Statistical Analysis

Normality and homogeneity of data was analyzed using Kolmogorov–Smirnov and Bartlett's tests, respectively. All data were expressed as mean ± standard error of mean (SEM) or standard deviation (SD). Statistical analysis was performed by one-way ANOVA followed by Tukey's post hoc test. The survival assessment during 7 days was analyzed using a 4 × 8 repeated measures ANOVA (groups × time as factors), followed by Bonferroni's post hoc test to express the differences in relation to control. Statistics were performed using the GraphPad Prism (version 5) software. Differences were considered significant at the $p < 0.05$ level.

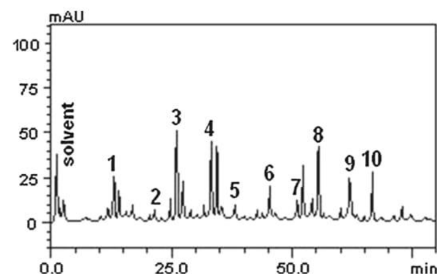
Results

Phenolic Compounds and Flavonoids in Honey

The results of HPLC determination of phenolic compounds and flavonoids in Brazilian Pampa biome honey are

Table 1 Determination of phenolic compounds and flavonoids present in honey by HPLC

Compounds	Honey (mg/g)	LOD (μg/mL)	LOQ (μg/mL)
Gallic acid	3.67 ± 0.04 a	0.027	0.087
Chlorogenic acid	0.25 ± 0.01 b	0.009	0.029
Caffeic acid	7.08 ± 0.01 c	0.013	0.042
p-Coumaric acid	5.93 ± 0.02 d	0.024	0.078
Ferulic acid	0.28 ± 0.01 b	0.021	0.069
Rosmarinic acid	2.61 ± 0.03 e	0.008	0.027
Quercetin	1.37 ± 0.04 f	0.016	0.053
Kaempferol	5.89 ± 0.01 d	0.025	0.082
Apigenin	3.54 ± 0.01 a	0.017	0.058
Chrysin	3.68 ± 0.02 a	0.012	0.041



Different letters represent statistically significant differences at $p < 0.05$ level. 1—gallic acid; 2—chlorogenic acid; 3—caffeic acid; 4—*p*-coumaric acid; 5—ferulic acid; 6—rosmarinic acid; 7—quercetin, 8—kaempferol; 9—apigenin and 10—chrysin

summarized in Table 1. According to this results, caffeic acid (7.08 ± 0.01 mg/g), following by *p*-coumaric acid (5.93 ± 0.02 mg/g) and kaempferol (5.89 ± 0.01 mg/g) seems to be the most abundant phenolic compounds present in this sample. The honey sample contains other minor compounds in addition to gallic acid (retention time t_R = 12.63 min, peak 1), chlorogenic acid (t_R = 20.96 min, peak 2), caffeic acid (t_R = 25.17 min, peak 3), *p*-coumaric acid (t_R = 33.08 min, peak 4), ferulic acid (t_R = 38.11 min, peak 5), rosmarinic acid (t_R = 44.93 min, peak 6), quercetin (t_R = 50.12 min, peak 7), kaempferol (t_R = 55.16 min, peak 8), apigenin (t_R = 62.37 min, peak 9) and chrysin (t_R = 65.04 min, peak 10; Table 1).

Phytochemical Analysis and Antioxidant Properties of Honey

The results from the analysis of identity and quality parameters of Brazilian Pampa biome honey sample are summarized in Table 2. For this sample, the following parameters were found: moisture: 17.6 ± 0.00 %; reducing sugars: 80.32 ± 0.46; apparent sucrose: 4.80 ± 1.54. The presence of hydroximethylfurfural (HMF) was not detected by the method used.

Regarding in vitro antioxidant properties, results are summarized in Table 3. The total phenolic content was 57.77 ± 2.203 mg of GAE/100 g and the values to ferric reducing antioxidant power (FRAP) of the honey tested was 342 ± 3.63 µM of Fe(II)/100 g. DPPH$^\bullet$ and ABTS$^\bullet$+ radical scavenging capacity was 78.4 ± 30.14 µM of AAE/100 g and 94.81 ± 17.06 µM of AAE/100 g, respectively (Table 4).

Lifespan and Locomotor Behavior Tests

According to results, honey caused a significant protection against mortality induced by hypoxia (Fig. 1). A daily observation up to 7 days was estimated. In the first day post-hypoxia the following results regarding percent of flies survival (mean ± SE) were observed: control: 96.48 ± 0.66; honey: 97.17 ± 0.51; control-hypoxia: 85.99 ± 2.08; honey-hypoxia: 89.13 ± 2.12. At the second day: control: 88.94 ± 1.80; honey: 91.68 ± 1.45; control-hypoxia: 64.01 ± 4.84; honey-hypoxia: 84.29 ± 2.34. Third day: control: 74.02 ± 4.21; honey: 85.90 ± 2.46; control-hypoxia: 49.76 ± 5.81; honey-hypoxia: 75.93 ± 2.65. Fourth day: control: 69.94 ± 5.56; honey: 84.02 ± 2.96; control-hypoxia: 43.88 ± 6.71; honey-hypoxia: 71.91 ± 3.30. Fifth day: control: 61.80 ± 5.70; honey: 81.52 ± 3.09; control-hypoxia: 38.05 ± 6.10; honey-hypoxia: 64.61 ± 3.00. Sixth day: control: 52.19 ± 6.75; honey: 76.28 ± 3.52; control-hypoxia: 31.69 ± 5.69; Honey-hypoxia: 57.40 ± 3.07. Seventh day: Control: 45.87 ± 7.36; Honey: 69.99 ± 4.49; Control-hypoxia: 24.14 ± 5.13; Honey-hypoxia: 50.81 ± 3.45. According to the results the hypoxia condition tested in this study caused significant increase in mortality of flies when compared to controls (without hypoxia), and honey was able to avoid this effect (Fig. 1).

Similarly, a significant locomotor deficit was observed in *D. melanogaster* exposed to hypoxia conditions, expressed by a decrease in percentage of flies climbing capacity. After 5 h of re-oxygenation, flies were submitted to the negative geotaxis test and percentage of climbing activity (mean ± SE) was: control: 100.00 ± 3.40; honey: 99.96 ± 3.46; control-hypoxia: 48.17 ± 10.93; honey-hypoxia: 91.20 ± 2.46 (Fig. 2a). This parameter was also evaluated after 24 h post-hypoxia: control: 100.00 ± 8.61; honey: 110.75 ± 3.71; control-hypoxia: 71.40 ± 7.38; honey-hypoxia: 107.66 ± 5.74 (Fig. 2b).

DCFDA Oxidation and Enzymatic Activities

The oxidation of the fluorescent dye DCFDA was used as a general index of ROS formation. Results were expressed as percentage (mean ± SE) of fluorescence intensity in relation to the control group: control: 100.00 ± 6,61; honey: 102.05 ± 11.97; control-hypoxia: 151.80 ± 16.27; honey-hypoxia: 97.84 ± 7.08 (Fig. 3). Flies exposed to hypoxia condition presented a significant increase in DCF signal. Honey treatment was able to completely protect flies against hypoxia induced increase of ROS production.

The increase of GST activity in flies under hypoxic conditions was fully blocked in honey-treated flies (Fig. 4a). The results were expressed as percentage of control (mean ± SE): control: 100.00 ± 5.20; honey: 97.14 ± 6.04; control-hypoxia: 125.12 ± 3.95; honey-hypoxia: 102.44 ± 2.54. CAT, SOD, TrxR and AchE activities were not changed between groups (Fig. 4b–e). In parallel, significant increases in caspases 3 and 9 activities were observed in flies under hypoxia (Fig. 4f, g). This effect was abolished in honey-treated flies. The results of caspase 3 activity were expressed as percentage of control (mean ± SE): control: 100.00 ± 0.64; honey: 104.19 ±

Table 2 Physicochemical analysis of Brazilian Pampa biome honey

	Moisture (%)	Reducing sugars (%)	Apparent sucrose (%)	HMF (mg/kg)
Honey	17.6 ± 0.00	80.32 ± 0.46	4.80 ± 1.54	ND

Data are expressed as mean ± SD

ND not detected

Table 3 Brazilian Pampa biome honey in vitro antioxidant properties

	Phenols (mg of GAE/100 g)	FRAP (μM of Fe(II)/100 g)	DPPH (μM of AAE/100 g)	ABTS (μM of AAE/100 g)
Honey	57.77 ± 2.2	342 ± 3.6	78.4 ± 30.1	94.81 ± 17.0

Data are expressed as mean ± SD

GAE gallic acid equivalent, *AAE* ascorbic acid equivalent

Table 4 Sequences of primers used for gene expression analysis by RTqPCR

Primer sequence	Forward	Reverse
GPDH	5′-ATGGAGATGATTCGCTTCGT	5′-GCTCCTCAATGGTTTTTCCA
Sima (HIF -1)	5′-GATGAGGATCACCATCGCTT	5′-ACAAGACCAACAGGAATCCG
NFκβ	5′-TGTGCTTTCTCTTGCCCTTT	5′-CCGCAGAAACCAGAGAGTTC
HSP27	5′-AAAGATGGCTTCCAGGTGTG	5′-CCCTTGGGCAGGGTATACTT
NRF2	5′-CGTGTTGTTACCCTCGGACT	5′-AGCGCATCTCGAACAAGTTT
HOX	5′-AAACTAAGGCGCGTTTTCAA	5′-GAGGGCCAGCTTCCTAAGAT
AKT-1	5′-ATAGCAGCGGCGTTAAGAAA	5′-CCACCTCGTCCTTTTGGATA
InR	5′-CTGGTGGTGCTGACAGAGAA	5′-GCAGCTGACAACTGGCATTA
dILP2	5′-ATCCCGTGATTCCACACAAG	5′-GCGGTTCCGATATCGAGTTA
dILP 5	5′-GCCTTGATGGACATGCTGA	5′-CATAATCGAATAGGCCCAAGG
FOXO	5′-CCGTAGCTCATCTCCTGCTG	5′-AAGGACAATGCCTGGAGGTG

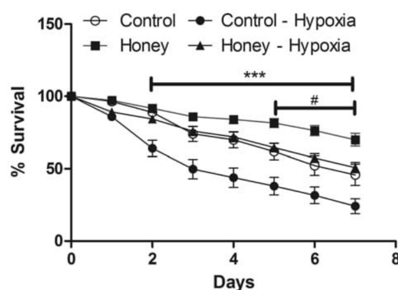


Fig. 1 Effect of hypoxia and honey on *Drosophila* survivorship. Flies were treated in standard medium over the course of the treatment schedule. The control group received standard medium only; honey group was administered a 10 % honey solution (w/w), control-hypoxia and honey-hypoxia groups were treated as described in "Experimental Procedure" section. Survivorship was followed during 7 days. Results are expressed as mean ± SEM of the percentage of survived flies for each experimental group. ***$p < 0.001$ control-hypoxia compared to control; #$p < 0.01$ honey compared to control

3.97; control-hypoxia: 138.25 ± 4.58; honey-hypoxia: 114.66 ± 5.78 (Fig. 4f). In turn, caspase 9 results were: control: 100.00 ± 2.92; honey: 117.00 ± 3.62; control-hypoxia: 156.46 ± 7.04; honey-hypoxia 121.14 ± 9.42 (Fig. 4g).

Mitochondrial Complexes Activity

As observed in Fig. 5a, b, flies under hypoxia showed an increase in both mitochondrial complexes I and II activity. The pre-treatment with honey was able to block these effects. The results were expressed as percentage of control (mean ± SE). The complex I activity was: control: 100.00 ± 8.31; honey: 92.72 ± 12.81; control-hypoxia: 207.45 ± 14.79; honey-hypoxia: 144.43 ± 11.57 (Fig. 5a). For complex II: control: 96.19 ± 4.98; honey: 101.86 ± 5.24; control-hypoxia: 131.00 ± 6.82; honey-hypoxia: 109.53 ± 4.41 (Fig. 5b).

Gene Expression Analysis

The analysis of gene expression in flies under hypoxia/reperfusion process by Quantitative Real-Time qRT-PCR revealed significant changes in the expression of hypoxia/stress related genes (Fig. 6a–j). The Sima (HIF-1), NRF2, HOX, NFκβ, HSP27, dILP 2, dILP5, InR and AKT-1 genes were significantly up-regulated in flies exposed to hypoxia. Flies treated with honey showed no significant differences when compared to those exposed to hypoxia (Fig. 6a–j). The FOXO gene expression did not differ between experimental groups.

Discussion

In a previous study we have reported a marked antioxidant and protective effect of Brazilian Pampa biome honey against oxidative stress in a Drosophila model of Parkinsonism [5]. In the present investigation, it was shown the protective effects of Brazilian Pampa biome honey against the deleterious outcomes of hypoxia in the fly model. This

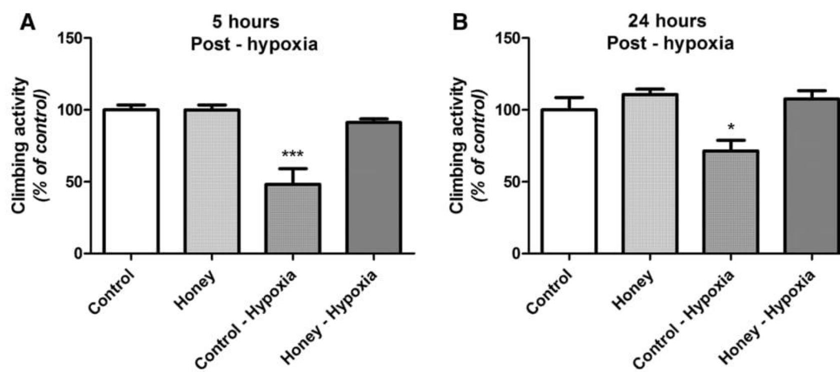


Fig. 2 Effect of hypoxia and honey on *Drosophila* negative geotaxis behavior. Neurolocomotor activity was assessed as negative geotaxis behavior (climbing activity). Results are expressed as mean ± SEM of the percentage of flies able to climb a marked glass tube (5 cm/6 s). **a** Climbing activity at 5 h post-hypoxia. **b** Climbing activity at 24 h post-hypoxia. $*p < 0.05$; $***p < 0.001$ compared to control

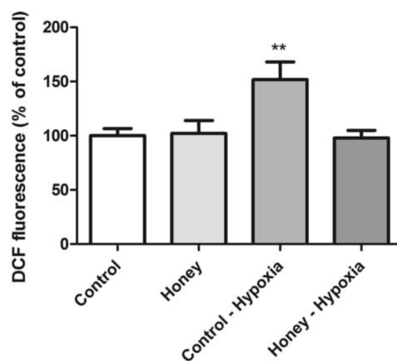


Fig. 3 Effect of hypoxia and honey on *Drosophila* ROS formation. 2′,7′-dichlorofluorescein diacetate (DCFDA) oxidation was used as a general index of reactive oxygen species (ROS) production. Results are expressed as mean ± SEM of the percentage of arbitrary fluorescence units emitted followed by DCFDA oxidation in flies' samples. $**p < 0.01$ compared to control

included a rescue of mortality and neurolocomotor performance caused by honey in flies exposed to hypoxia/reperfusion. Oxidative stress markers and biochemical alterations induced by hypoxic conditions in flies were also ameliorated with honey treatment. At least in our knowledge, this is the first report showing the protective effects of honey against hypoxia in vivo.

Regarding the honey sample tested here, the quality and antioxidant capacity parameters were on the highest standards according to international requirements [30]. The honey ability of free radical scavenging (DPPH and ABTS) and ferric reducing power (FRAP) was demonstrated concomitantly to a protective effect against hypoxia-induced damage (in vivo). Honey treatment was able to block several changes induced by hypoxia in flies, including alterations in lifespan, locomotor ability, ROS production, antioxidant enzymes and caspase activity. Such an effect may be attributed to the presence of significant amounts of phenolic compounds and flavonoids presented on the honey sample tested.

According to literature reports, the phytochemical compounds found in our honey sample, have been exploited against hypoxia-induced damage. Gallic acid [31], quercetin [32], caffeic acid derivatives [33] and *p*-coumaric acid [34] presented a significant neuroprotective effect and decreased oxidative damage induced by hypoxia. Apigenin [35], kaempferol [36], chrysin [37], chlorogenic acid [38] have been shown as preventive agents against hypoxia conditions. Ferulic acid was shown to protect neurons [39] and rosmarinic acid protects cardiomyocytes and hepatocytes against hypoxia-induced injury [40, 41]. Interestingly, a recent study reported important findings on the honey potential as an alternative therapeutic for wound healing during hypoxic condition [42]. However, for the first time, we are demonstrating the protective effects of honey against hypoxia/reperfusion-damage in a complex "whole organism".

Hypoxia refers to a low oxygen condition and disruption of oxygen homeostasis. It may occur during both physiological and pathological conditions, including for instance, ischemic heart disease, cerebral ischemia, complications of diabetes, pulmonary hypertension and obstetrical/perinatal complications [43]. Reperfusion or re-oxygenation of hypoxic tissues may induce metabolic remodeling, mitochondrial reprogramming followed by massive production of reactive oxygen species, inflammation and consequently cell death [44–46]. Determining the responses triggered by hypoxia/reperfusion is somewhat complex task, since the extent of damage (whether it was a generalized or focal hypoxia), intensity, time frame (intermittent or constant hypoxia), tissues and organisms-specific differences may interfere in the sensitivity to hypoxia [43].

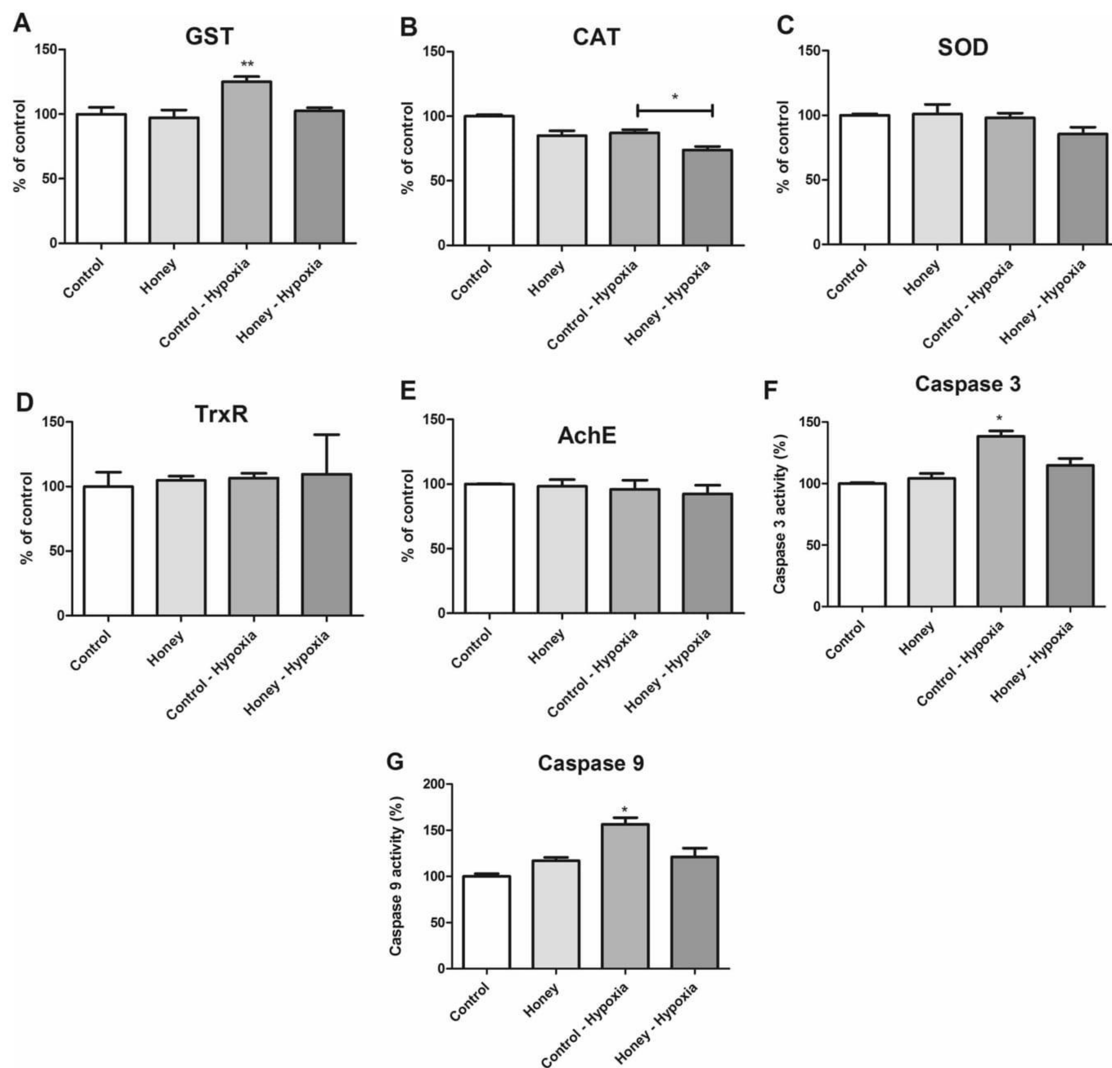


Fig. 4 Effect of hypoxia and honey on *Drosophila* antioxidant enzymes, acetylcholinesterase and caspase activity. Enzyme activity was determined as described in "Experimental Procedure" section. Results are expressed as mean ± SE of the percentage of enzyme activity. $*p < 0.05$ and $**p < 0.01$ compared to control

In this study we used *D. melanogaster* as a model organism, because in addition to sharing many hypoxia response genes with humans, flies have an innate tolerance to extreme fluctuations in oxygen levels making them much more tolerant to hypoxia/reperfusion than humans [47, 51]. Considering that the complex mechanisms of global hypoxic adaptation and resistance are not completely understood [44] and that previous studies have successfully employed the fruit-fly as a model organisms for testing natural antioxidants [48], the *Drosophila* model may represent an important tool in the identifying of novel mechanisms and therapeutics against hypoxia related diseases.

Flies post-hypoxic conditions demonstrated an increased mortality rate as compared to controls. The increased mortality reached significant levels at periods as long as 48 h, continuing to increase up to 7 days after hypoxia (Fig. 1). Treatment of flies with honey induced a complete reversal on the hypoxia-induced loss of survival. Markedly, honey-treated flies presented amelioration in the survival rate even when compared to the untreated controls, indicating the beneficial

effects of honey on the flies' longevity. We also verified by the negative geotaxis assay, a complex neurolocomotor behavior [9] that exposure to hypoxia caused significant impairment in locomotor performance. Such an effect was completely reversed in honey-treated flies after 5–24 h of the hypoxia episode. These effects may be attributed to the honey complex nutritional composition, which besides sugars also contains vitamins, amino acids, minerals and phenolic compounds such as flavonoids. In an elegant study by Jimenez-Del-Rio et al. [9], it was shown the protective effects of polyphenols on survival and locomotor activity of Drosophila exposed to pro-oxidants such as iron and paraquat. Natural extracts have also been reported to provide neuroprotection against oxidative stress inducers [45]. It has been also reported that glucose presents an ability to improve life span and locomotor performance in a Parkinson's disease Drosophila model [10]. Taking into consideration that honey is rich in reducing sugars such as glucose as well as polyphenols [5], one could suppose that the protective effects of honey may be directly correlated to its antioxidant compounds, which may end up contributing with a greater survival and fast recovery of flies post-hypoxia.

The susceptibility of the organism to hypoxia depends on several cellular mechanisms, one of them being the activation of caspases. These proteases play an important role in the initiation (caspase 9) and execution (caspase 3) of apoptosis [49]. A significant increase in caspase 3 and 9 (Fig. 4f, g) activity was observed in hypoxia-exposed flies. In contrast, honey treatment abolished the increase in caspase activity elicited by hypoxia, demonstrating the participation of anti-apoptotic pathways in the protective effect of honey against hypoxia in Drosophila.

Hypoxia-inducible transcription factor (HIF-1) or Sima, the homologue in Drosophila, is shown to regulate the expression of over 100 target genes involved in both physiological functions and hypoxia response. HIF-1 driven hypoxic gene expression changes is thought to work as an adaptive response in cells exposed to a reduced-oxygen environment. For instance, HIF-1 induces the expression of glycolytic genes, because it is presumed that increased glycolysis is necessary to produce energy when low oxygen will not support oxidative phosphorylation at mitochondrial level [50]. Herein, we observed an increase of HIF-1 as also glycolytic gene expression, as Drosophila insulin-like peptides (dILP2 and 5), insulin-like receptor (InR) and AKT under hypoxic conditions. Treatment of flies with honey had no significant effect on hypoxia induced gene expression of targets above mentioned. Vigne et al. [47] reported that poor diets protect flies against anoxia/reoxygenation injuries. Hypoxic and anoxic tolerances can be dissociated and probably involve different mechanisms, but they suggest that hypoglycemic diets induce a state of "metabolic depression". It is of interest to note that vertebrates, such as frogs and turtles, and hibernating mammals switch to a hypometabolic state under hypoxic conditions. Honey, in turn, has hypoglycemic properties [5, 7, 8], a fact that could be related to a major remodeling of energy metabolism in a non-transcriptional manner.

Fig. 6 Effect of hypoxia and honey on *Drosophila* gene expression. ▶ Results are expressed as mean ± SE of the relative mRNA levels. $*p < 0.05$; $**p < 0.01$ and $***p < 0.001$ compared to control

In addition to stimulate glycolytic genes, HIF-1 also actively modulates mitochondrial function during hypoxia [50]. Previous studies have shown that a hypoxia-induced mitochondrial adaptation involves energy regulation, generation of reactive oxygen species (ROS), and apoptosis [51]. The observed increased activity of complex I in flies subjected to hypoxia could reflect an adaptive mechanism that potentially maximizes the aerobic respiration efficiency under limited oxygen availability or a consequence to the re-oxygenation process in order to meet hypoxia-depleted energy demands [51]. The increased complex II activity during hypoxia is already known as a compensatory mechanism towards activation of mitochondrial respiratory chain and a mechanism of critical adaptation to

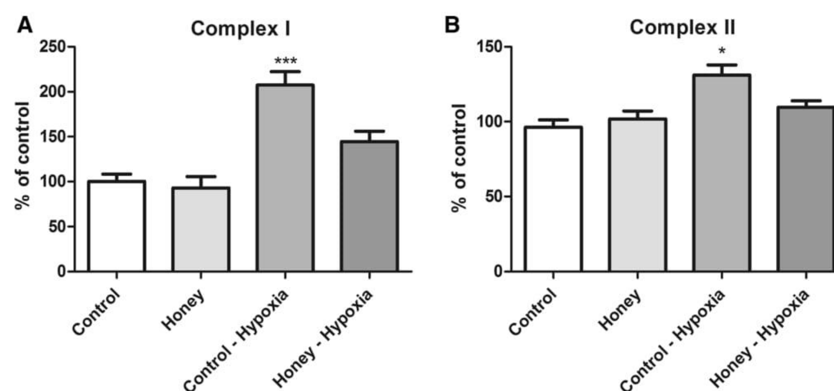
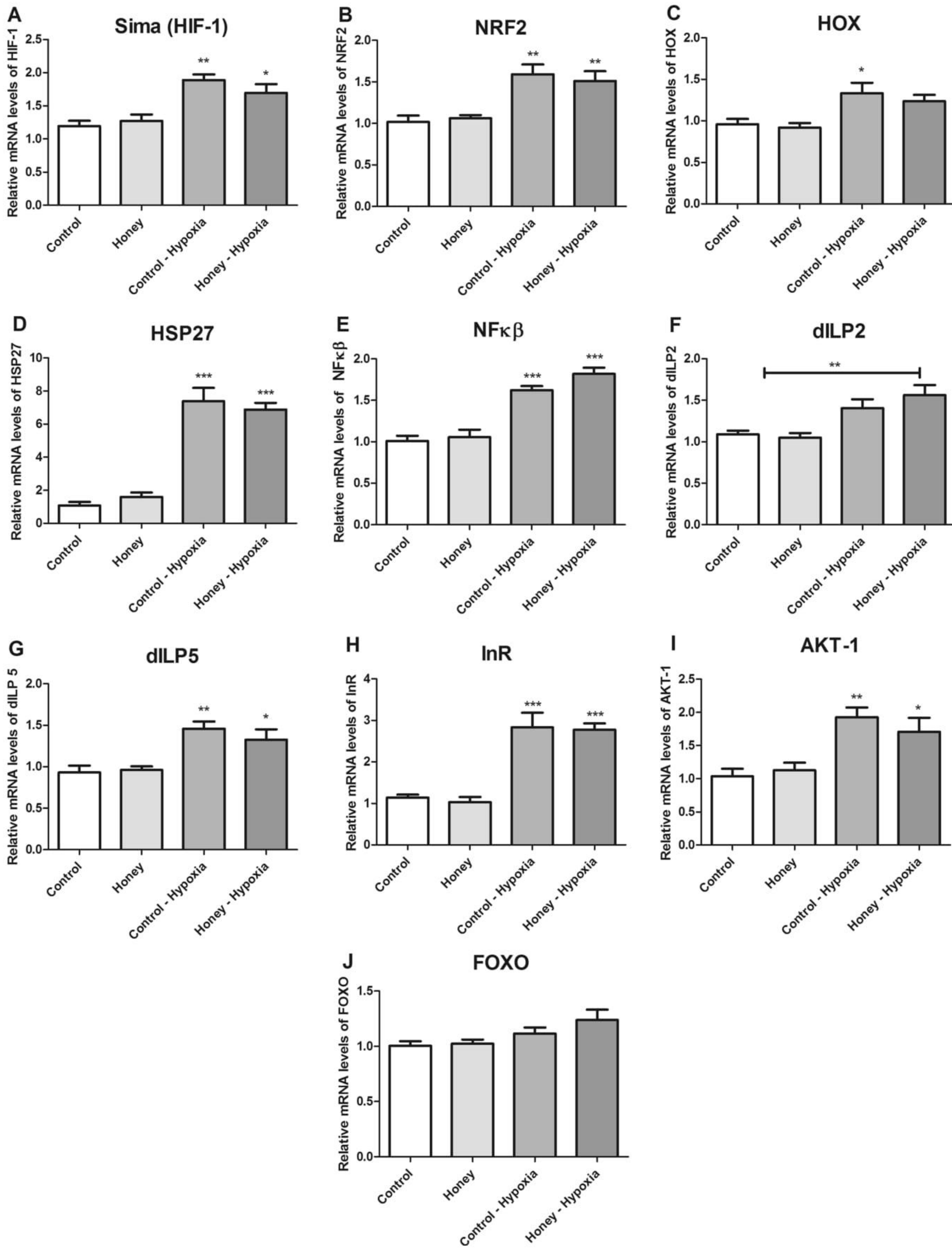


Fig. 5 Effect of hypoxia and honey on *Drosophila* mitochondrial respiratory chain activity. **a** Complex I and **b** complex II activity. Results are expressed as mean ± SEM of the percentage of enzyme activity. $*p < 0.05$ and $***p < 0.001$ compared to control


A
Sima (HIF-1)
Relative mRNA levels of HIF-1
B
NRF2
Relative mRNA levels of NRF2
C
HOX
Relative mRNA levels of HOX
D
HSP27
Relative mRNA levels of HSP27
E
NFκβ
Relative mRNA levels of NFκβ
F
dILP2
Relative mRNA levels of dILP2
G
dILP5
Relative mRNA levels of dILP 5
H
InR
Relative mRNA levels of InR
I
AKT-1
Relative mRNA levels of AKT-1
J
FOXO
Relative mRNA levels of FOXO
Control
Honey
Control - Hypoxia
Honey - Hypoxia

hypoxia [52]. We speculate that the observed blocking effect of honey on the increase of complex I and II activity during hypoxia was based on concomitant metabolic remodeling due to honeys hypoglycemic and antioxidant capacities.

As a consequence of adaptation to hypoxia by increases of mitochondrial respiratory chain complexes, generation of reactive oxygen species may take place [51]. In turn, adaptation to oxidative stress conditions is regulated by activation of Nrf2/ARE pathway [53]. The ROS/xenobiotics induced alterations in the cellular redox state constitute an important signal to promote adaptive responses mediated by Nrf2 [53]. The Nrf2 nuclear translocation and subsequent binding to DNA sequence known as the "antioxidant response element (ARE)" may be triggered by dissociation from the inhibitory protein Keap1 as well as by phosphorylation of serine residues at the Nrf2 protein by upstream protein kinases [53]. Among proteins that are usually involved in response to oxidative stress-driven Nrf2 activation, the NAD(P)H dehydrogenase, quinone 1 oxidoreductase (NQO-1), glutamate cysteine ligase (GCL), GST and CAT plays central role [54]. In our model, hypoxia-induced ROS formation was concomitant with increases in Nrf2 mRNA and GST activity, evidencing an activation of the Nrf2 signaling pathway. Importantly, we found that honey treatment was able to block the increased GST enzyme activity and ROS production, measured as DCF oxidation. Although the DCF oxidation is not able to discriminate between different reactive species, this assay has been widely used to assess ROS overproduction [55]. In the recent years, many flaws regarding this method have been raised, mainly related to the fact that fluorescence signals derived from DCF oxidation may occur unspecifically via Fenton-type reactions or in the presence of cytochrome c, resulting in masked results [55]. However, considering that upregulation of Nrf2 signaling pathway is a well known phenomenon related to ROS overproduction, our results point to oxidative stress as an important mechanism during hypoxia adaptation in the Drosophila model. In addition, our data suggest that antioxidant compounds presented in honey might be responsible for its protective role against oxidative stress-induced by hypoxia in flies.

The physiological changes that take place during hypoxia activate multiple stress response pathways including up-regulation and activation of Heat shock proteins (HSPs). Hypoxia exposure and the physiological stress imposed is a sufficient stimulus to promote HSP induction and overexpression and seems to be, at least in part, explained by enhanced ROS production [56] as also an early marker of injury [57]. The marked increase in HSP27 gene expression suggest a role of this chaperone in supporting flies to adapt and survive under low oxygen condition, as previously reported [43, 56, 57].

It is known that nuclear factor κβ (NF-κβ) regulates the hypoxia-inducible factor (HIF) system, since hypoxia and inflammation are intimately linked [58]. Other authors have recently reported that HIF-1α has an important role in regulating NFκB [59]. In this way, our results corroborate with literature as an up-regulation of NFκB in flies exposed to hypoxia/reperfusion was observed. However, additional studies are required to better clarify the role of NFκβ pathway and inflammation in Drosophila.

In conclusion, our results demonstrated, for the first time, the beneficial effects of honey against the deleterious effects of hypoxia/reperfusion processes in a whole organism, indicating its potential therapeutic use in the treatment of dysfunctions with pathologies involving low oxygen levels. As a potential mechanism involved in such an effect, the antioxidant activity of honey, related to phenolic compounds in its constitution may play central role. Our study also highlights the use of *D. melanogaster* as a viable in vivo model for studies on hypoxia/reperfusion and screening of natural antioxidants.

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Compliance with Ethical Standards

Conflict of interest Authors declare no conflict of interest.

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3.2 MANUSCRITO

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Honey protects against wings posture error and molecular changes related to mitochondrial pathways induced by Hypoxia/Reperfusion in adult *Drosophila melanogaster*

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ABSTRACT

In a previous study we have demonstrated the protective effect of Brazilian Pampa biome honey against pro-oxidative effects of hypoxia in adult *Drosophila melanogaster*. In this study, we conducted an investigation to evaluate the effects of honey and its major phenolic compounds on changes related to complex phenotypes (erected wing posture) and mitochondrial aspects induced by hypoxia/reperfusion in flies. Flies were pre-treated for 3 days with 10% honey and different concentrations of caffeic acid and p-coumaric acid and then submitted to hypoxia for 3h. As results, flies exposed to hypoxia/reperfusion acquired a permanent erected wings posture and this aspect was closely correlated with mortality. Differently from honey, caffeic and p-coumaric acids displayed no protection against erected wings and locomotor damage caused by hypoxia. Honey treatment decreased the frequency of flies with this complex phenotype. This phenomenon is being reported for the first time, and could be associated with down regulation of the Erect Wing gene found in flies exposed to hypoxic conditions. In addition, hypoxia/reperfusion drastically reduced the O_2 fluxes in flies mitochondria and honey protected against decrease of the Opa1 gene expression.

Keywords: honey, antioxidants, erected wings posture, EWG, OPA-1.

1. Introduction

Honey is a complex food produced by honeybees from the nectar and mostly constituted by sugars; however; organic acids, vitamins, phenolic compounds, flavonoids and many other compounds are also present (Erejuwa et al., 2010). Previous reports shows that honey can deliver health benefits, in addition to its well-known antioxidant effects, honey has properties anti-inflammatory, anti-tumor, anti-fungal, antibacterial, and hypoglycemic (Erejuwa et al., 2010 and 2012; Rodriguez et al., 2012; Abdulrhman et al., 2013, Cruz et al., 2014 and 2016). Recently, we characterized Brazilian Pampa Biome honeys and demonstrated its high quality and capacity in counteracting oxidative stress induced by different conditions in fruit-fly model (Cruz et al., 2014; Cruz et al., 2016). In a recent study, we have shown for the first time that treatment of flies with honey blocked mortality and locomotor deficits, reversed ROS production and changes in mitochondrial activity induced by hypoxia/reperfusion (Cruz et al., 2016).

Hypoxia refers to a low oxygen condition and may occur during both physiological and pathological conditions. Ischemic heart disease, cerebral ischemia, complications of diabetes, pulmonary hypertension and obstetrical/perinatal complications are common cases of oxygen disturbance (Zhao et al., 2011; Solaini et al., 2010). Re-oxygenation of hypoxic tissues may induce metabolic remodeling, mitochondrial reprogramming followed by massive production of reactive oxygen species, inflammation and consequently cell death (Vigne et al., 2009; Biddlestone et al., 2015; Solaini et al., 2010).

The susceptibility of the organism to hypoxia depends on several cellular mechanisms. A response of the organism to the effects of hypoxia is to activate mechanisms of adaptation for survival, being conducted by hypoxia-inducible transcription factor (HIF-1). In this context, HIF-1 or Sima, the homologue in Drosophila, induces the expression of several target genes, for instance, glycolytic, mitochondrial function and inflammatory pathway genes (Bandarra et al., 2014). Authors suggest that reactive oxygen species (ROS) play a role in HIF-1 activation (Kietzmann et al., 2005), other authors show NFkB as a HIF-1 regulator (Van Uden et al, 2008); showing that there are different relations in the regulation of HIF-1, which so far is not well understood.

During the process of hypoxia/reperfusion a series of changes as mentioned above occurs, which in sum reflect the damage to the organism, and the role of mitochondria stands out. The mitochondrial damage induced by hypoxia/reperfusion can be due to the loss of

membrane potential, electron transport chain disorder, excessive production of reactive oxygen species and structural remodeling (Solaini et al., 2010).

Mitochondrial fission and fusion play critical roles in maintaining functional mitochondria when cells are exposure to metabolic or environmental stresses. Mitochondrial fusion acts on mitigating stress by mixing the contents of partially damaged mitochondria as a form of complementation (Youle and Bliek, 2012). In recent work was demonstrated a connection between release of cyto c from mitochondria in hypoxia condition and disruption of the mitochondrial fusion (Sanderson et al., 2015a). Recently, it was shown that deletion of Erect Wing (EWG) gene decreased OPA1-like protein expression (necessary for mitochondrial fusion) in *Drosophila melanogaster* (Rai et al., 2014).

Nuclear respiratory factor 1 (NRF-1) is a human transcription factor that regulates the expression of genes necessary for mitochondrial respiratory function, being an important regulator of mitobiogenesis, as well as regulating cell growth and protection against oxidative stress (Icreverzi et al., 2012; Wang et al., 2016; Rai et al., 2014.). Erect Wing (EWG), the NRF-1 homologous in *D. melanosgater* is required for both wing muscle and neuronal development (Rai et al., 2014*).* However, is unknown the influence of EWG on mitochondrial functions in flies.

Despite the complex molecular mechanisms of hypoxia /reperfusion injury are still not well understood, studies have successfully demonstrated the liability of the fruit-fly as a model organisms for hypoxia/reperfusion research. In addition to sharing many hypoxia response genes with humans, flies have an innate tolerance to extreme fluctuations in oxygen levels making them much more tolerant to hypoxia/reperfusion than humans. In addition, the Drosophila model provides a "whole body" tool for the identification of new hypoxia/reperfusion related molecular targets and interactions (Feala et al., 2009; Cruz et al., 2016)**.**

Thus, in this work, we investigated the effects of hypoxia/reperfusion on changes related to flies wings posture and mitochondrial aspects and also the protective effects of honey and its major isolated compounds against hypoxia-induced impairments in *D. melanogaster*.

2. Material and methods

2.1 Chemicals

Caffeic acid (C0625), p-Coumaric acid (C9008), Sucrose (S5016), HEPES (Titration; H3375), Albumin from bovine serum -BSA (A6003), Ethylenediaminetetraacetic acid – EDTA (EDS), Ethylene glycol-bis(2 aminoethylether)-N,N,N′,N′-tetraacetic acid – EGTA (E3889), Glycerol (G5516), β-Mercaptoethanol (M3701), L-Malate (374318), Sodium pyruvate (P2256), L-Proline (P0380), Adenosine 5′-diphosphate (01905), Cytochrome c from equine heart (C2506), Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone – FCCP (C2920) were obtained from Sigma-Aldrich. SYBR Select Master Mix Applied (4472908) from Biosystems by Life Technologies, Trizol Reagent (15596026) and DNAse I Amplification Grade - Invitrogen (18068-15) by Life Technologies and iScript cDNA Synthesis kit (1708891) from Biorad. All other chemicals and reagents used here were of the highestanalytical grade.

2.2 Drosophila Stock and Honey Sampling

Drosophila melanogaster (Harwich strain) was obtained from the National Species Stock Center, Bowling Green, OH. The flies were reared in glass vials containing Bloomington standard cornmeal Drosophila medium (Cruz et al., 2014) in a constant temperature and humidity (20 °C ± 1; 60 %relative humidity). All experiments were performed with the same strain, using adult flies with 1–3 days old.

The honey sample from *Apis mellifera* was courtesy of producers/researchers of the Universidade Federal do Pampa from São Gabriel in the state of Rio Grande do Sul, Brazil. Honey was harvested in March 2015 and identified as multifloral-wild flowers. Honey sample was stored at room temperature (20–25 °C) in plastic containers until analysis.This sample has already been characterized previously, it has highest quality and antioxidant properties (see Cruz et al., 2016).

2.3 Treatment and Exposure to Hypoxia/reperfusion

Flies (1–3 days old) were maintained on filter paper impregnated with a solution of 4% sucrose (Control), Caffeic acid and p-Coumaric acid solution (1 and 3 mg/ml -dissolved in control solution) and 10% honey for 3 days. The hypoxia-exposure model was performed according Cruz et al. (2016) using nitrogen to oxygen output, resulting in an atmosphere of 3–4 % oxygen at 25 °C during 3 h. An oximeter (AZ- 8403) was used to monitoring the levels of oxygen during hypoxia trials. After the hypoxia condition, flies were exposed to oxygen

reperfusion (21%). After visual observation of complete flight recovery (around 2-3 h after reperfusion) flies were used for the behavioral and biochemical assays.

2.4 Locomotor Assay and Wing posture evaluation

The flies were pre-treated on filter paper with honey and isolated phenolic compounds (Caffeic acid and p-Coumaric acid) present in greater quantities in this honey sample (see Cruz et al., 2016). We used a concentration similar to that found in 10% honey (1 mg/ml). 3 mg/ml was considered as a high concentration. After hypoxia treatment flies were kept in filter paper impregnated with corresponding solution according described above for each experimental group. For behavioral analysis, each experimental group were evaluated by negative geotaxis assay based on previous study (Jimenez-Del-Rio et al., 2010). After hypoxic exposure (5 and 24 h) flies were transferred to test tubes marked at 5 cm height and gently tapped to the bottom of the tube and the number of flies able to climb 5 cm after 6 s was recorded at 1-min intervals. Each experiment was repeated thrice. Results were expressed as percentage of control.

The frequencies of flies with erected wings posture was recorded daily after hypoxia treatment. For this purpose, the number of flies with erected and normal wings of each flask and group treated was counted.

2.5 Quantitative Real-Time qRT-PCR and Gene Expression Analysis

Approximately 1 µg of total RNA from 25 young flies was extracted using the Trizol Reagent (Invitrogen) accordingly to the manufacturer's suggested protocol. After quantification, total RNA was treated with DNase I (Invitrogen, NY) and cDNA was synthesized with iScript cDNA Synthesis Kit and random primers again accordingly to the manufacturer's suggested protocol (Invitrogen, NY). Quantitative real-time polymerase chain reaction was performed in 20 µL reaction volumes containing 1x PCR Buffer, 200 µM of dNTPs, of 0.18 µM each primer (described in Table 2), 3 mM MgCl2, 0,1 U platinum 0.1U Taq DNA polymerase (Invitrogen, NY), SYBR Green I (molecular probes) and using a StepOnePlus real time PCR systems (Applied Biosystems, NY). All samples were analyzed as technical and biological triplicates with a control. SYBR fluorescence was analyzed by StepOne software version 2.0 (Applied Biosystems, NY), and the CT (cycle threshold) value for each sample was calculated and reported using the $2^{-\Delta\Delta CT}$ method. The GPDH genes were used as endogenous reference genes presenting no alteration in response to the treatment. For

each well, analyzed in quadruplicate, a ΔCT value was obtained by subtracting the GPDH ΔCT value from the CT value of the interest gene. The ΔCT mean value obtained from the control group of each gene was used to calculate the ΔΔCT of the respective gene ($2^{-\Delta\Delta CT}$) (Livak and Schmittgen, 2001).

2.6 Isolation of Mitochondria and Mitochondrial and High-resolution respirometry

For mitochondria isolation, 50 flies were homogenized in 1 mL of isolation buffer containing 250 mM sucrose, 2 mM EGTA, 0,1 % bovine serum albumin (BSA), 5 mM Tris (pH 7.4). The homogenate was filtered and centrifuged at 200 g for 5 min at 4°C, and the supernatant centrifuged at 9.000g for 10 min at 4°C to yield a mitochondria pellet which was resuspended in 100 µL of isolation buffer without BSA (Miwa et al., 2003).

For respirometry analysis was used the methodology described by Pichaud et al. (2013) using the Oxygraph-2 K respirometer (Oroboros Instruments, Innsbruck, Austria). Without presence of oxygen measurements were taken after sodium dithionite addition and oxygen electrodes of the respirometer were calibrated. Pyruvate, L-proline and malate (10 mM each) were injected into the oxygraph chambers and 0.05 µg/ml of isolated mitochondria were then injected. After, oxygen was injected into the chambers. Injection of ADP (5 mM) allowed to reach a state of OXPHOS (Oxidative phosphorylation) for complex I (CI-OXPHOS). Subsequent injection of cytochrome c (15 µM) allowed evaluation of the functional integrity of the outer mitochondrial membrane (CIc-OXPHOS). After, Succinate (CII- complex II). Sequentially was injecting FCCP to stimulate uncoupled respiration for complex I and as a measure of electron transport system capacity (CIc + CII ETS). Respiratory control ratios (RCR = CI-OXPHOS/CI-LEAK) was used as quality control of mitochondria. All results were expressed as means of respiration rates expressed in pmol of oxygen consumed per second per mg mitochondrial protein.

2.7 Statistical analysis

Statistical analysis was performed by one-way or two-way ANOVA followed by Bonferroni's *post hoc* test, using the GraphPad Prism (version 6) software, for this statistical analysis all data were expressed as mean ± standard error of mean (SEM). In the evaluation of erected wings frequencies and analysis of correlation was used Chi-Square test with Yates's correction and Pearson's correlation coefficient, respectively. Differences were considered to be significant at the $P < 0.05$ level.

3. Results

3.1 Locomotor behavior

Regarding the behavioral analysis, hypoxia/reperfusion induces significant impairments in flies' locomotor ability at 5 and 24 hours (figure 1). Honey treatment completely blocked hypoxia-induced flie's locomotor deficit and p-Coumaric acid (3mg/ml) partially blocked this phenomenon at 5h (figure 1A). After 96h, both honey and p-coumaric acid concentrations were able to block hypoxia-induced locomotor deficit (figure 1B).

3.2 Erected wings posture analysis

An intriguing observation was the fact that flies submitted to hypoxia/reperfusion showed and increased frequency of subjects with an erected wings posture (figure 2B). All groups exposed to hypoxia conditions presented this characteristic. However, flies treated with honey showed a lower frequency of flies with erected wings compared to control (table 1). Isolated compounds caffeic acid and p-coumaric acid were not able to block the appearance of flies with erected wings phenotype (table 1). Another characteristic observed was the increasing mortality of the flies with the erect wings over the time. Interestingly, the correlation analysis between the number of surviving flies and the number of flies with erected wings was significantly positive (figure 2). In this way, it is suggesting that probably the death of the flies is related to the acquired characteristic of erected wings.

3.3 Gene Expression

The analysis of gene expression in flies under hypoxia/reperfusion revealed significant changes in the expression the EWG, LDH, and dIAP gene compared with control (Figure 3). Honey treatment blocked the hypoxic alterations in the Opa-1 gene expression (figure 3F). No significant changes were observed in gene expression in flies treated with both caffeic acid and p-Coumaric acid (data not shown).

3. 4 Respirometry analysis

Respiratory control ratios (RCRs) were calculated for complex I as CI-OXPHOS/CI-LEAK to determine the quality and functionality of mitochondria. RCRs above 3 indicate well-coupled respiration (Gnaiger, 2009). RCRs the control group were above 3 (22.10 ± 2.10), but the hypoxia and honey+hypoxia group had a low and varied values with 4.48 ± 2.57 and 6.52 ± 7.21 respectively. For the all different steps analyses (CI-OXPHOS, CIc-

OXPHOS, CIc + CII-OXPHOS, CIc + CII-ETS) both hypoxia and honey + hypoxia had a dramatic decrease in O_2 fluxes measured compared with control (figure 4).

4. Discussion

Honey is consumed worldwide for its benefits to health and nutrition. The characterization of honey's biological effects helps to a better understanding of its properties and mechanisms towards human health. In previous studies, we showed the high quality and antioxidant activity of Brazilian Pampa Biome Honey (Cruz et al., 2014 and 2016). In this work, we looked after questions that were left open. First, we investigated whether major phenolic compounds present in honey would provide similar results as those obtained for honey. We also searched for potential mechanisms by which honey protects against hypoxia-induced erected wing posture, an intriguing phenomenon observable in flies under hypoxic conditions.

Caffeic acid and p-coumaric acid are the major phenolic compounds found in Brazilian Pampa Biome honey (Cruz et al., 2016). Both phenolics have been shown to present antioxidant and protective effects in different models of disease (Wei et al., 2004; Guven et al., 2015), therefore, we tested them under our experimental conditions of hypoxia in flies. Honey showed a better and faster recovery post-hypoxia exposure compared to the isolated compounds. In addition, isolated compounds alone did not reverse hypoxia-induced molecular changes as did honey itself (data not shown). For that reason, the use of honey isolated compounds as putative therapeutic agents could not be proven here which in turns elicited the beneficial outcomes of honey, a complex mixture of compounds with benefits to health (Ajibola et al., 2012; Erejuwa et al., 2012; Rodrigues et al., 2012).

Recently, for the first time in literature, we reported honey effects against damage induced by hypoxia/reperfusion in *D. melanogaster* (Cruz et al., 2016). Herein, we turned attention to changes on flies' wings posture after submission to hypoxia/reperfusion. It was observed that flies under hypoxia presented an increased frequency of subjects with an erected wings posture, a fact that was positively correlated with flies' mortality after hypoxia. At least in our knowledge, little is known about this behavior literature. This observation led us to interpret such a phenomenon as a consequence of tissue damage leading to locomotor disability, commonly observable in patients after hypoxia episodes (Starosta et al., 2016). We showed here the ability of honey to decrease the frequency of erected wings in flies post-

hypoxia. This data could be connected to the protective effect of honey on locomotor activity. Next, we searched for potential mechanisms by which hypoxia and honey interplay towards the erected wings phenotype.

Erect Wing (EWG) gene in *Drosophila melanogaster*, encodes a vital function important for the development the both nervous system and the indirect flight muscles. Mutations (depletion) of the EWG induces breaks in the central nervous system and missing or reduced indirect flight muscles associated with erect wing posture and adult hypoactivity (DeSimone et al., 1993 and 1996). In this way, we evaluated the expression of the EWG gene, and interestingly, a significant decrease of EWG mRNA was found in hypoxia-submitted flies. This data suggests that hypoxia/reperfusion down regulates EWG, which may be directly related to the observable erected wing phenotype. All studies conducted in literature regarding depletion of the EWG gene are in the fly larval phase, which leads to a blockage of development to adult stage (DeSimone et al., 1996). In this context, our results demonstrate an important role for EWG gene in adult flies in which we speculate a putative role in protection and maintenance of flight muscle and correct wing posture.

Mitochondria are the predominant oxygen-consuming organelles to generate ATP through oxidative phosphorylation and to limited oxygen supply play an important role in the functional adaptation to hypoxia (Dhar-Mascareño et al., 2005). Previously, we observed that honey recovered changes in the activity of the mitochondrial complex I in flies exposed to hypoxia (Cruz et al., 2016). For further investigation of mitochondrial changes, we analyzed the expression of related genes. The increased expression of lactate dehydrogenase (LDH) indirectly indicates a shift to anaerobic respiration after reperfusion. This leads us to believe that hypoxia-induced mitochondrial dysfunction could be occurring (Dhar-Mascareño et al., 2005). We have observed that honey protected against a decrease in the expression of Optic atrophy 1 (Opa 1) induced by hypoxia/reperfusion. This protein is required for mitochondrial fusion (Rai et al., 2014). No changes were observed in the Dynamin-related protein (Drp) expression, a required protein for mitochondrial fission. Decrease of mitochondrial fusion under hypoxic conditions represents a mitochondrial adaptation that can be connected to apoptotic signaling (Youle and Bliek, 2012; Sanderson et al., 2015a).

Perkins et al. (2012) demonstrated that hypoxia-adapted flies plays mitochondria ultrastructural modifications and this could be an important adaptive mechanism in preserving muscle function under chronic hypoxic conditions. In addition to this, it has been shown that EWG controls mitochondrial fusion during muscle growth and maintenance by regulation of

the Opa-1 (Rai et al., 2014) and a more recent publication showed that NRF-1 (EWG homologous) regulates the expression of HIF-1 on hypoxia *in vitro (*Wang et al., 2016). Thus, despite the few studies available in literature, it is clear the existence of interactions between hypoxia, mitochondrial functions, motor damage and EWG expression in Drosophila. Unquestionably, hypoxia/reperfusion altered mitochondrial oxygen fluxes. Treatment with honey was not able to reverse respiratory capacity of mitochondria damaged after hypoxia. Thus, we believe that the protective effect of honey could be on the structural adaptation of mitochondria, as observed for the OPA-1 expression, which regulates in mitochondrial fusion and helps mitigating stress (Youle and Bliek, 2012; Sanderson and Kumar, 2015b). In addition, independent mechanism might occur in parallel, such as activation of anti-apoptotic and antioxidant signaling pathways.

Oxidative stress signaling pathways may converge to programmed cell death responses. The Drosophila Reaper-family proteins induce apoptosis by binding to antagonizing inhibitor of apoptosis proteins – dIAPs, which in turn inhibit caspases (Drice – caspase 3 and Dronc- caspase 9) (Steller, 2008) . In flies, as in other organisms, the balance of anti and pro-apoptotic factors determines the cell fate between cell death and survival (Clavier et al., 2016). The observed increased expression of dIAP in flies post-hypoxia found here may be interpreted as an anti-apoptotic adaptive response against deleterious effects of hypoxic condition. Even though dIAP acts by inhibiting apoptosis, other factors might be involve in the overall process, which in fact, correlates with our previous study in which the activity of caspases 3 and 9 were increased after hypoxia, a phenomenon that was blocked by honey treatment (Cruz et al., 2016). Similarly, in the present study we found that honey was able to partially, abolish the dIAP response under hypoxia. In Drosophila, apoptosis is strictly regulated at mitochondrial level and flies anti and pro-apoptotic factors are tightly connected to ROS production by mitochondria (Clavier et al., 2016). Taking this into consideration, the antioxidant properties of honey might be directly related to its anti-apoptotic effect in flies under hypoxia.

In summary, our results demonstrate that hypoxia/reperfusion in fruit-fly induces an erected wings posture phenotype. This phenomenon might be associated with EWG gene expression, as well as structural and respiratory function changes of the mitochondria. Isolated compounds from honey did not show substantial improvements on locomotor ability and wing posture change of flies submitted to hypoxia, when compared to honey, a fact that

elicits the benefits of honey's complex mixture to health. As potential mechanisms, it is suggested an interaction between locomotor activity, wings posture and EWG - OPA-1 genes in honeys protective effects during hypoxia, which possibly involves antioxidant and anti-apoptotic mechanisms. In addition, the erected wings phenotype seems to be an interesting model for investigation of hypoxia-induced tissue damage effects. Further investigations are required to determine the exact mechanism of honey protection against the different hypoxia/reperfusion injury in *Drosophila melanogaster*.

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Figure legends

Figure 1. Effect of hypoxia/reperfusion in negative geotaxis of flies treated with Caffeic acid, p-Coumaric acid or Honey. Behavior test were done at 5 (A) and 24 (B) hours after reoxygenation. The control group was administered 4% sucrose; 1 and 3 mg/ml of caffeic acid (CA1 and CA3), the same for p-Coumaric acid (CoA1 and CoA 3) and 10 % honey solution. Flies were treated 3 days before hypoxia exposure. Results are expressed as mean ± SEM of the percentage of flies able to climbling 5cm in 6 seconds. *$p<0.05$, ** $p<0.01$ and *** $p<0.001$ compared to control. “H” is abbreviation of “Hypoxia”.

Figure 2. Correlation analysis between the number of flies with erected wings posture and flies survival. (A) This correlation equates to the 4 days of observation of all groups under hypoxia. The results show that when survival decreases (over the days), the number of flies with erected wings decreases, suggesting that the mortality is related to the erected wings posture effect. *** $p<0.001$. (B) Representative photo of flies with erect wings posture (black arrow) and flies with normal wing (dotted arrow).

Figure 3. Effect of hypoxia/reperfusion and honey on Drosophila gene expression. Results are expressed as mean ± SE of the relative mRNA levels. *$p<0.05$; **$p<0.01$ and ***$p<0.001$ compared to control.

Figure 4. Effects of hypoxia/reperfusion and honey on mitochondrial respiration (O2 fluxes) in *D. melanogaster*. Results are expressed as pmol O2 consumed per second per mg of mitochondrial protein. Mitochondrial respiration were measured in presence of pyruvate + malate + L-proline + ADP (CI-OXPHOS), + cytochrome c (CIc-OXPHOS), + succinate (CIc+CII - OXPHOS), + FCCP (CIc+CII - ETS). ***$p<0.001$ compared to control.

Fig 1

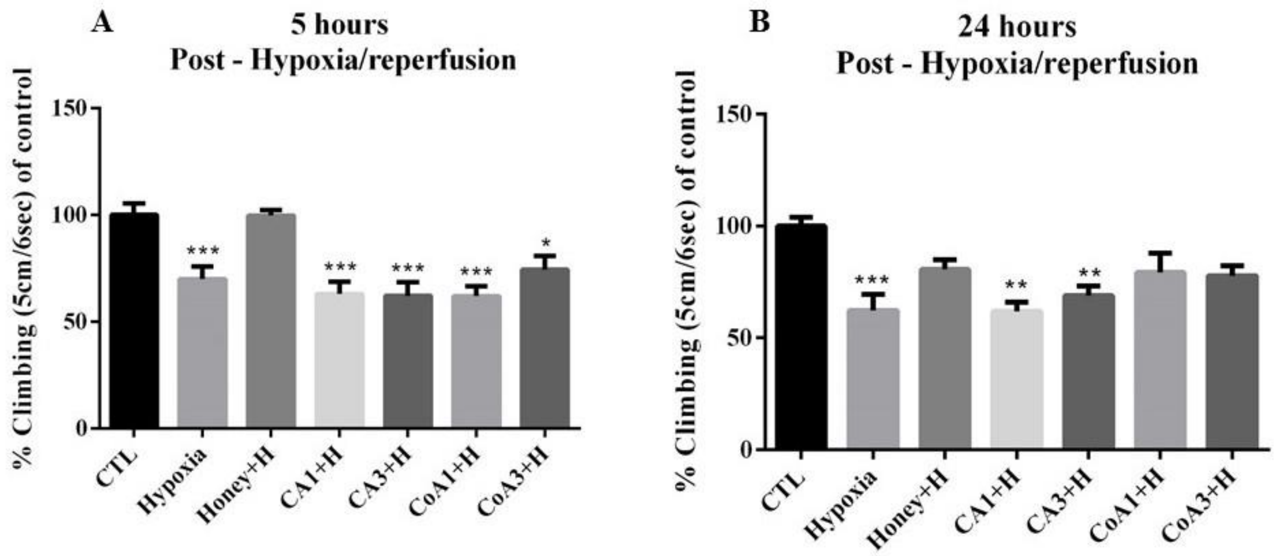
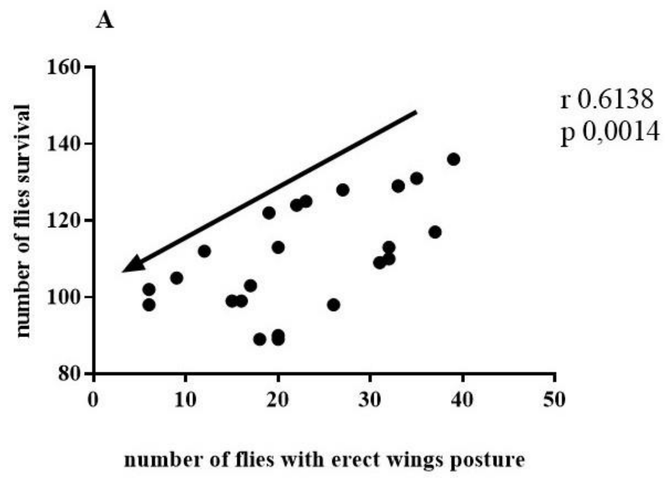


Fig 2

A



B

Fig. 3

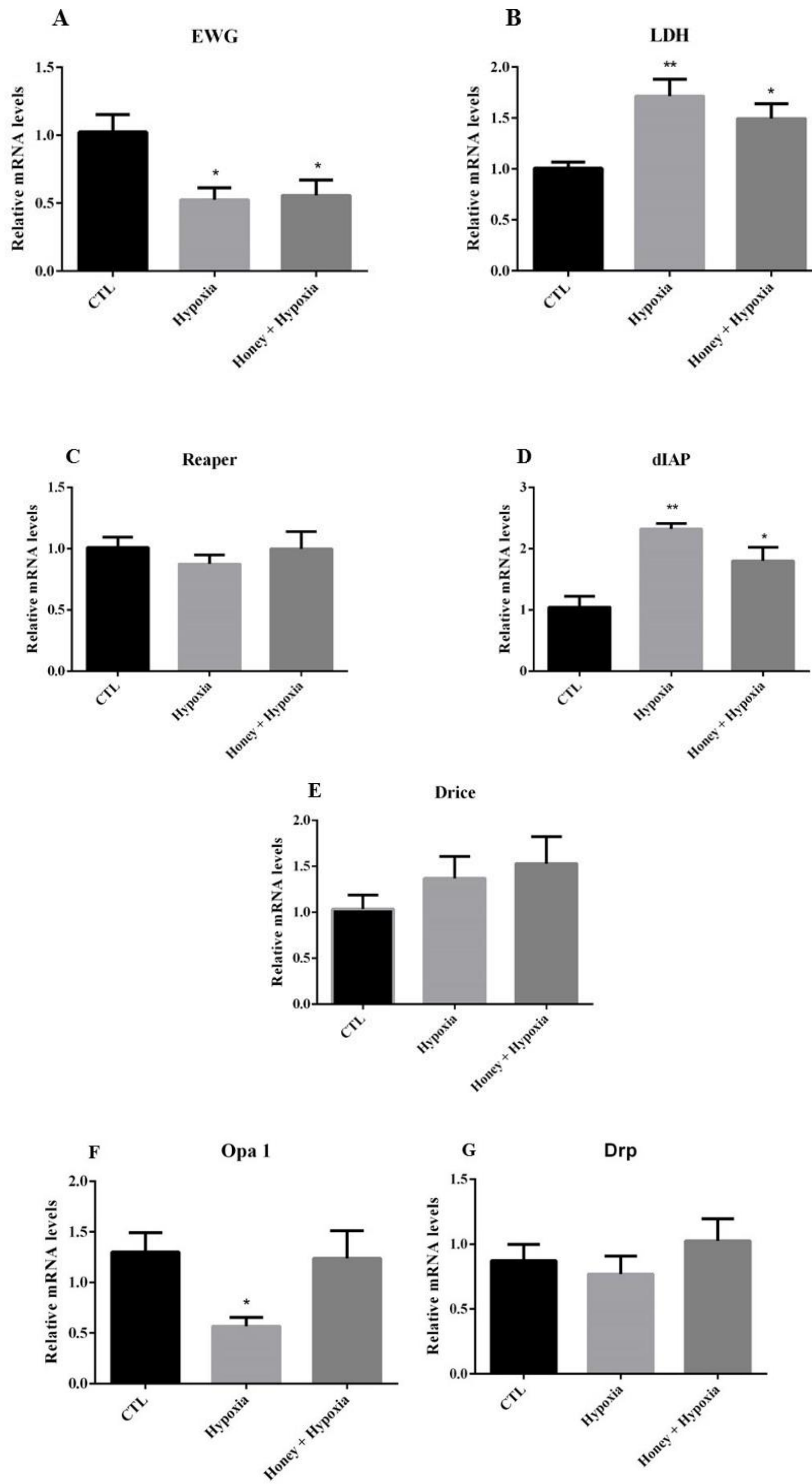


Fig 4

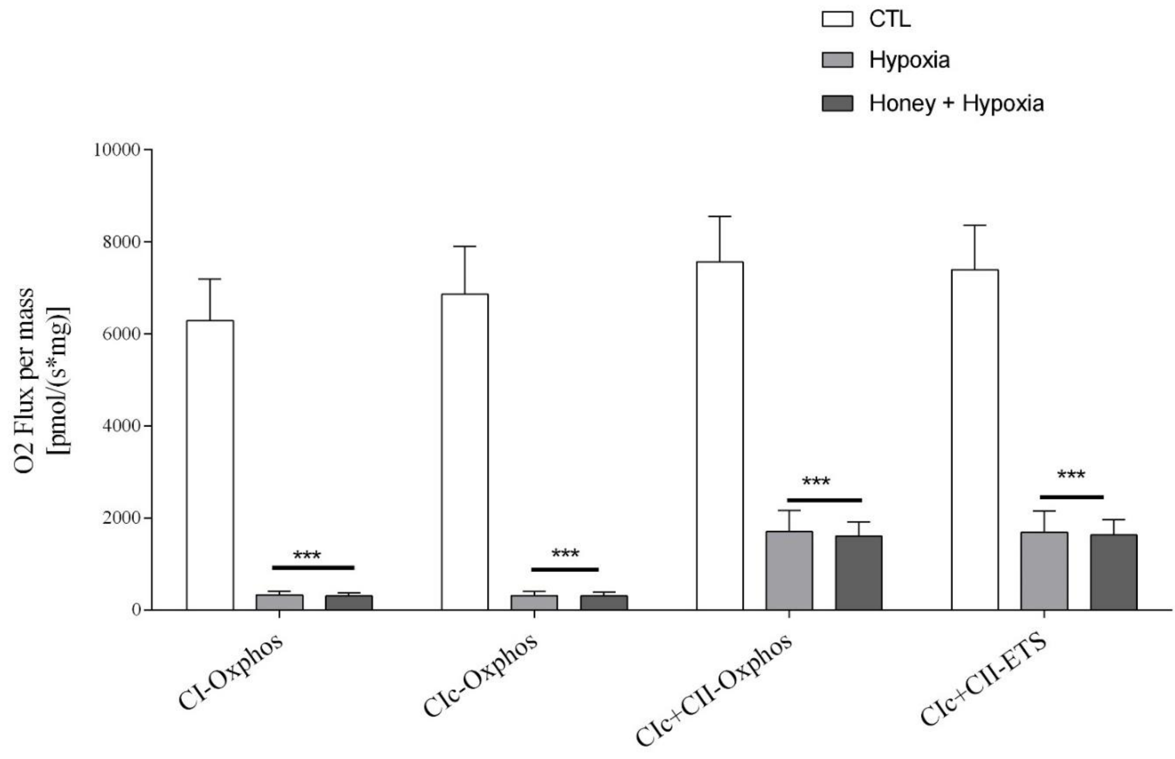


Table 1. Frequency analysis of flies with erected wing posture in different treatment groups after hypoxia/reperfusion. 24 hours after reoxygenation, was counted the number of flies with alteration in the wing. The control group was administered 4% sucrose; 1 and 3 mg/ml of caffeic acid (CA1 and CA3), the same for p-Coumaric acid (CoA1 and CoA 3) and 10 % honey solution. Flies was treated 3 days before hypoxia exposure. Results are expressed as χ^2. *p<0.05 , ** p<0.01 and *** p<0.001 compared to control. "H" is abbreviation of "Hypoxia".

	Erect wings posture	Normal wings posture	Comparison	$\chi 2$	P
CTL-H.	26	111			
Honey + H	12	128	CTL-H.	5.487	**0.019**
CA1 + H	27	131	CTL-H.	0.072	0.787
CA3 + H	39	114	CTL-H.	1.408	0.235
CoA1 + H	37	119	CTL-H.	0.710	0.399
CoA3 + H	20	130	CTL-H.	1.302	0.253

Table 2. Sequences of primers used for gene expression analysis by RTqPCR.

Primer Sequence	Forward	Reverse
EWG	ACGAACAGCGATGGAACAGT	TGCTTAGCAGAGTGGCATCC
LDH	AAGTGGAACGAGCTGCACAA	GTCACACCGTTGGCATTGAG
Diap-1	CCCCAGTATCCCGAATACGC	TCTGTTTCAGGTTCCTCGGC
Reaper	TGGCATTCTACATACCCGATCA	CCAGGAATCTCCACTGTGACT
Drice	GTCGCAGAATCACAGCGATA	GGCAGGCCTGTATGAAGAAC
Opa-1	ACAGTCAGATTCCTTCAACACGA	GAATTCCTGCTGCAACGCTT
Drp	CCAAGTGCATGAGCAGAACC	TGCCGGCGGTAAAATGTTTG

4. DISCUSSÃO GERAL

Em um estudo anterior, nosso grupo de pesquisa havia relatado a qualidade do mel do Bioma Pampa Brasileiro e seu efeito antioxidante *in vitro*, e *in vivo* (CRUZ *et al*. 2014). Neste trabalho, o mel produzido nesta região exibiu atividade antioxidante nos testes de eliminação de radicais livres (DPPH e ABTS) e um poder antioxidante através da redução de ferro (FRAP). Essas respostas antioxidantes *in vitro* refletiram-se na rica composição de compostos fenólicos presente no mel, destacando-se altas concentrações de ácido cafeico e ácido p-cumárico. De forma geral, foi constatado que o mel exerceu efeito protetor contra danos induzidos por hipóxia/reperfusão em *Drosophila melanogaster,* e que tais efeitos podem estar relacionados com a composição fenólica do produto.

A hipóxia se caracteriza por condições de baixo oxigênio e pode ocorrer em diversas situações. A reoxigenação ou reperfusão de um tecido hipóxico pode causar uma série de danos celulares, devido à massiva produção de EROs durante este processo (SOLAINI *et al*., 2010; VIGNE *et al*., 2009). Considerando todas as variáveis de uma hipóxia seguida de reperfusão (tempo, intensidade e duração), escolhemos a mosca-da-fruta, *Drosophila melanogaster,* como organismo alternativo para avaliação das respostas celulares/moleculares/comportamentais alteradas pela condição e moduladas pelo mel. Para as análises *in vivo*, as moscas foram tratadas com 10% de mel dissolvido em meio de ágar ou embebecido em papel filtro. O meio de ágar fornece a possibilidade de avaliações a longo prazo, assim como, para analises de suplementação. Já a exposição em papel filtro é mais efetiva para analises a curto prazo e uso de compostos em sua forma isolada, diminuindo as possibilidades de interações com outros compostos.

A exposição das moscas aos episódios de hipóxia/reperfusão aumentou a expressão gênica de moléculas alvos de diversas vias, como por exemplo, genes relacionados com via metabólica (dILP 2, dILP 5, InR e AKT), respiração anaeróbica (LDH e HIF-1) e estresse oxidativo (Nrf2, HOX, HSP27, NFκB e HIF-1). O fator de transcrição induzível por hipóxia (HIF-1) ou Sima (homólogo em *Drosophila)*, regula a expressão de genes-alvos envolvidos na sobrevivência do organismo ou tecido durante a hipóxia. Este gene é ativado na ausência do oxigênio, porém também pode ser regulado por outros fatores, como o estresse oxidativo. Observamos um aumento na expressão do mRNA de HIF-1 (Sima) mesmo após

reoxigenação, resultado que além de efetivar o modelo de hipóxia, mostra um efeito remanescente da indução durante a hipóxia.

No modelo de hipóxia/reperfusão em *D. melanogaster*, foi possível observar aumento da produção de EROs e atividade aumentada da enzima GST, dados que indicam ativação da via de sinalização mediada pelo Nrf2. O mel diminuiu a produção de EROs e bloqueou o aumento sobre a atividade da GST, uma importante enzima antioxidante, alterados sob condições de hipóxia. Considerando que a via de sinalização desencadeada pelo Nrf2 pode ser modulada por EROs, nossos resultados apontam o estresse oxidativo como um fenômeno importante durante a hipóxia/reperfusão em *Drosophila*. Além disso, o conjunto de resultados sugerem que os compostos antioxidantes presentes no mel podem ser as principais moléculas responsáveis pelo seu efeito nas moscas submetidas a hipoxia/reperfusão.

Mostramos que o tratamento com o mel aumentou a sobrevivência das moscas nos dias seguintes após exposição à hipóxia (meio de ágar), e que o mel forneceu uma rápida recuperação de danos locomotores causados por hipóxia/reperfusão. Em analises onde testamos os efeitos protetores de compostos fenólicos presentes do mel em forma isolada, não foi observado uma proteção contra as alterações locomotoras induzidos por hipóxia/reperfusão. Para esse tratamento utilizamos compostos fenólicos majoritários do mel, os ácidos cafeico e p-cumárico, tanto em concentrações similares aquelas encontradas no mel, como concentrações mais altas. Consideramos dessa forma, entre outras observações, que os efeitos protetores do mel convergem de sua complexa composição nutricional de forma totalitária, ou seja, de uma ação sinérgica entre vários compostos.

Interessantemente após exposição a hipóxia/reperfusão, observamos alterações na posição das asas das moscas, para uma forma permanente de asa erguida. Constatamos que este fenômeno no fenótipo das asas conduziu a perda da habilidade de voo e o tratamento com o mel diminuiu a frequência desse fenômeno. Acreditamos que esses efeitos protetores também estejam vinculados a presença de compostos fenólicos no mel e suas propriedades antioxidantes (JIMENEZ-DEL-RIO *et al.*, 2010). Também mostramos que esta característica de postura das asas está correlacionada com o número de mortes entre as moscas expostas a hipóxia/reperfusão. Investigando sobre esse fenômeno nunca antes correlacionado com os efeitos deletérios de exposição hipóxica, descobrimos a interação desta característica com a expressão do gene Erect Wing (EWG), um fator de transcrição com importante função durante o desenvolvimento do músculo da asa e do sistema nervoso central da mosca-da-fruta.

A ausência dessa proteína na mosca acarreta em danos no sistema nervoso, e consequentemente em perda da comunicação neuromuscular e hipoatividade relacionada a postura erguida da asa (DESIMONE *et al.*, 1993). Nas análises de expressão gênica, observamos que hipóxia/reperfusão induziu uma significativa diminuição da expressão de EWG, o que possivelmente está relacionado com o fenômeno observado das asas erguidas nas moscas.

EWG regula um gene de importância mitocondrial. Foi mostrado que a depleção de EWG conduz a regulação negativa de Opa-1, proteína necessária para fusão mitocondrial (RAI *et al.*, 2014). Essa regulação também está associada com desorganização muscular. Esses dados corroboram com nossos resultados, e acrescentamos que o tratamento com o mel foi capaz de reverter as mudanças na expressão do gene Opa-1. O efeito protetor do mel também foi observado sobre a atividade do complexo I da mitocôndria. As disfunções mitocondriais induzidas por reperfusão são bastante elucidadas (GRANGER & KVIETYS, 2015) e parecem ser o principal mecanismo de estresse oxidativo e morte celular nessas condições. Nesse sentido, observamos que a exposição hipóxica seguida de reperfusão provocou uma alteração na respiração, pela diminuição do fluxo de oxigênio, mudança na atividade do complexo I e na fusão mitocondrial. Supomos que os efeitos protetores do mel sobre algumas destas alterações ocorra pela regulação das adaptações estruturais das mitocôndrias, uma vez que, estudos mostram que moscas adaptadas a ambientes hipóxicos aumentam sua área de cristas mitocondriais, conduzindo um aumento dos complexos proteicos da cadeia transportadoras de elétrons. Essa adaptação ocorre no sentido de maximizar a maquinaria para manter uma produção energética suficiente para a sobrevivência da mosca (PERKINS *et al.*, 2012). A fusão mitocondrial pode ocorrer no sentido de minimizar os danos oxidativos produzidos por qualquer situação de estresse (YOULE & BLIEK, 2012).

A morte celular programada ocorre em resposta a diferentes sinais celulares, como o aumento do estresse oxidativo. Observamos que a suplementação com mel foi eficaz em diminuir a atividade de caspases iniciadora e executora de apoptose celular. Este efeito em condições de tratamento em papel filtro mostrou causar um aumento da expressão gênica de proteína inibidora de apoptose (dIAP). Este fato é condizente com as respostas primarias das moscas em condições de ativação de vias apoptóticas. A mosca por ser um organismo de pequeno porte, primeiramente resiste à ativação da via de apoptose (STELLER, 2008). Dessa

maneira só observamos aumento da apoptose em passos a jusantes, com a atividade enzimática das caspases, onde o mel atua diminuindo tal atividade.

De forma geral, nossos resultados demonstraram, pela primeira vez, os efeitos benéficos do mel contra danos induzidos por hipóxia/reperfusão em um organismo inteiro, indicando seu potencial uso terapêutico no tratamento de disfunções relacionadas com hipóxia e lesões por reperfusão. Consideramos a propriedade antioxidante do mel, oriunda da sua complexa composição nutricional, o principal mecanismo envolvido nos seus efeitos protetores. Nosso estudo também destaca a *D. melanogaster* como um organismo modelo efetivo para investigações de parâmetros bioquímicos/toxicológicos/farmacológicos e de doenças relacionadas com hipóxia/reperfusão.

5. CONCLUSÕES

De acordo com os resultados apresentados nesta tese pode-se concluir que:

- A qualidade do mel do Bioma Pampa Brasileiro se encontra dentro dos parâmetros exigidos pela legislação;

- O mel produzido nesta região apresentou atividade antioxidante, a qual foi verificada *in vitro* e *in vivo* por diminuir a produção exacerbada de EROs induzida por hipóxia/reperfusão em *Drosophila melanogaster*;

- O Modelo de hipóxia/reperfusão promoveu alteração na postura das asas de *Drosophila melanogaster*; condição que foi atenuada pelo tratamento com o mel;

- O mel do Bioma Pampa aumentou a taxa de sobrevivência e restaurou a atividade locomotora das moscas submetidas a hipóxia/reperfusão;

- O mel protegeu as moscas contra as alterações na atividade enzimática de GST e caspases e danos mitocondriais induzidos pela hipóxia/reperfusão;

- Este estudo mostra pela primeira vez na literatura os efeitos protetores do mel sobre danos causados por hipóxia/reperfusão em *Drosophila melanogaster*;

- O conjunto de dados aponta o mel como um produto natural seguro e promissor como alternativa terapêutica em pesquisas com doenças humanas, principalmente aquelas de cunho oxidativo.

6. PERSPECTIVAS

- A partir dos resultados nesta tese, temos como possíveis trabalhos futuros conduzir maiores investigações em relação ao fenômeno de asa erguida encontrado em moscas expostas a hipóxia/reperfusão.
    - Avaliar os mecanismos que conduzem esse fenômeno, assim como sua relação com o estresse oxidativo, uma vez que o mel atenuou essa alteração;
    - Avaliar a correlação desse fenômeno quanto aspectos musculares e neurológicos;
    - Analisar a relação da expressão de NRF-1 com distúrbios motores isquêmicos em mamíferos.

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