A GLICONEOGÊNESE COMO UMA VIA METABÓLICA ESTRATÉGICA PARA MANUTENÇÃO ENERGÉTICA EM ARTRÓPODES

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Universidade Estadual do Norte Fluminense - UENF

Campos dos Goytacazes

Abril 2016

TESE DE DOUTORADO

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Resumo

O carrapato *Rhipicephalus microplus* e o mosquito *Aedes aegypti* são hematófagos vetores de doenças de grande importância econômica em países tropicais e subtropicais, principalmente no Brasil. Atualmente, muitos estudos tem sido realizados na busca de alvos que possam ser utilizados para o controle destes vetores. Contudo, ainda não compreendemos muitos aspectos fisiológicos desses artrópodes, principalmente sob condições de estresse. Nesse contexto, o presente trabalho busca investigar os mecanismos regulatórios do metabolismo de carboidratos frente a um desafio nutricional induzido e natural em células BME26 do carrapato *R. microplus* e em ovos quiescêntes do mosquito *A. aegypti*, respectivamente. Interessantemente, a gliconeogênese tem se mostrado como uma via central na manutenção do estado de latência do mosquito, assim como na integridade celular do carrapato, respondendo finamente à disponibilidade de nutrientes, alterando o metabolismo de glicose como um todo. Esses resultados possibilitarão um melhor entendimento da regulação gênica e enzimática do metabolismo de carboidratos em resposta ao desafio nutricional, fazendo-se uma correlação com os dados relativos à fisiologia desses vetores disponíveis na literatura.

Abstract

The tick *Rhipicephalus microplus* and the mosquito *Aedes aegypti* are bloodsucking disease vectors of great economic importance in tropical and subtropical countries, mainly in Brazil. Currently, many studies have been conducted in search of targets to control these vectors. However, still do not understand many physiological aspects of these arthropods, especially under stress conditions. In this context, this study aims to investigate the regulatory mechanisms of the carbohydrate metabolism after induced and natural nutritional challenge in tick *R. microplus* cells line BME26 and *A. aegypti*, mosquito quiescent eggs, respectively. Interestingly, gluconeogenesis has proven to be a central pathway in maintenance of mosquito latency state, as well as the cellular integrity of the tick, finely responding to nutrient availability, altering glucose metabolism as a whole. These results allow a better understanding of genic and enzyme regulation in response to carbohydrate metabolism nutritional challenge, correlating with the data on the physiology of these vectors available in the literature.

1. Introdução

Esta tese foi estruturada utilizando como base dois artigos científicos produzidos pelo nosso grupo de pesquisa, intitulados: “Non-Classical Gluconeogenesis-Dependent Glucose Metabolism in *Rhipicephalus microplus* Embryonic Cell Line BME26” – (Martins et al., 2015) e “Gluconeogenesis is a Key Pathway for Energy Support in *Aedes aegypti* Egg Quiescence” - que está sendo submetido para publicação. Esses estudos, anexados a este documento, tiveram como abordagem o controle do metabolismo de glicose em dois modelos de artrópodes hematófagos: o carrapato *Rhipicephalus microplus* e o mosquito *Aedes aegypti*. Observou-se um refinado controle gênico e enzimático relacionados ao metabolismo energético após um desafio nutricional, seja ele induzido como no primeiro trabalho em células embrionárias de carrapato (Anexo I), ou através de um mecanismo natural de privação nutricional, como a quiescência no segundo trabalho (Anexo II). Os dados apresentados nos anexos I e II, em conjunto com os resultados complementares, nos mostram relações em nível transcricional e enzimático entre as principais enzimas do metabolismo de glicose. Adicionalmente, a quantificação de metabólitos nos permite inferir sobre o percurso tomado pelos substratos através das vias de catabolismo e anabolismo de carboidratos. Todo esse estudo tem ampliado nosso conhecimento a cerca da fisiologia de artrópodes hematófagos, assim como do metabolismo energético. Além do mais, a investigação dos componentes-chave das respectivas vias metabólicas nos fornecem potenciais alvos para o desenvolvimento/aperfeiçoamento de estratégias (vacinas ou drogas) necessárias ao controle desses importantes vetores de doenças. Apesar de esses eventos serem primordialmente estudados em células embrionárias, durante o processo embrionário ou no embrião já formado, aspectos relacionados ao metabolismo de glicose também foram investigados em tecidos e órgãos de artrópodes adultos, antes ou depois da

alimentação sanguínea (resultados complementares). Dentre eles destaca-se o ovário, órgão responsável na formação dos ovócitos.

Os artrópodes depositam todos os nutrientes necessários para o desenvolvimento embrionário nos ovócitos durante a ovogênese, caracterizada pelo aumento no tamanho do ovário (Cherry, 1973; Matova & Cooley, 2001). Neste momento os níveis de glicogênio, lipídios e proteínas aumentam consideravelmente nos ovócitos (Fagotto, 1990; Cherry, 1973 & 1978; Chippendale & Song *et al*., 2006). Portanto, entende-se que no momento da oviposição todos os nutrientes requeridos para sustentar o metabolismo energético durante o desenvolvimento embrionário já foi adicionado ao ovo, podendo assim dar início à embriogênese propriamente dita (Rahn *et al.,* 1974). O processo embrionário em si requer uma alta demanda energética para sustentar a proliferação celular e o desenvolvimento (Thompson & Stewart, 1997). Neste contexto, o metabolismo energético precisa ser finamente regulado uma vez que o ovo constitui um sistema fechado, dependente do conteúdo do vitelo (reserva do ovo composto majoritariamente de proteínas) para o desenvolvimento dos tecidos e para manutenção do gasto energético do embrião (Fagotto, 1990; Yamamoto and Takahashi, 1993; Logullo *et al*., 1998).

Durante a embriogênese do mosquito *Aedes aegypti*, por exemplo, foi observado uma drástica mudança no metabolismo de glicose após a retração da banda germinal (24 horas de desenvolvimento) com aumento no fluxo glicolítico e queda nos níveis de proteína e glicogênio, de modo que a taxa metabólica ao fim da embriogênese (62h de desenvolvimento) encontrava-se elevada (Vital *et al*., 2010). Na presença de água o ovo ao fim da embriogênese eclode, mas na ausência desta o organismo precisa aguardar mesmo com baixas reservas energética e elevadas taxas metabólicas. Neste caso, é necessário que ocorra uma adaptação metabólica para que a larva consiga sobreviver com recursos energéticos escassos até o próximo período sazonal de chuvas (Anexo II). O carrapato

também passa por desafios nutricionais, e dependendo da espécie, isso poderá ocorrer em diferentes fases do ciclo de vida desses animais (Diamant & Strickland, 1965). De qualquer forma, a embriogênese sempre consistirá em um grande desafio metabólico, um momento crítico no ciclo de vida dos artrópodes, no qual o conteúdo restrito de nutrientes deverá ser adequadamente mobilizado, uma vez que todo o processo embrionário nos ovos ocorre desconectado do ambiente materno, diferentemente de mamíferos.

O estudo clássico em metabolismo embrionário de artrópodes nos mostra como as reservas de glicogênio são importantes para sustentar o desenvolvimento do embrião. A mosca da fruta *Drosophila melanogaster*, considerada um organismo modelo no estudo da ovogênese e embriogênese de insetos, acumula glicogênio em folículos ovarianos maduros, sendo a forma predominante de estocagem de carboidratos nos ovos (Gutzeit *et al*., 1993). Nas primeiras etapas da embriogênese de *Drosophila* foi observado que o conteúdo de glicogênio e proteína apresentava uma correlação inversa, ou seja, quando os níveis de proteína totais declinavam, ocorria um acúmulo de glicogênio (Shiomi & Kitazume, 1956; Medina & Vallejo, 1989; Gutzeit *et al*., 1993). Outros estudos revelaram ainda que o conteúdo de glicogênio oscila durante o processo embrionário de *Drosophila*, contudo sendo abundantemente estocado nos momentos tardios da embriogênese (Yamazaki & Nusse, 2002; Yamazaki & Yanagawa, 2003). O nosso grupo, ao estudar a embriogênese do carrapato *Rhipicephalus microplus*, observou um perfil similar com relação ao metabolismo de glicogênio. Na fase inicial da embriogênese do carrapato observou-se a degradação do glicogênio, e já nos momentos finais do processo embrionário essas reservas de glicogênio são ressintetizadas com concomitante queda nos níveis de proteína totais (Moraes *et al*., 2007). Em mosquitos *Aedes fluviatilis* pôde-se também notar um aumento nos níveis de glicogênio ao final da embriogênese, embora os níveis de proteínas não apresentem acentuada queda como observada nos outros modelos (Fernandes et al., 2014). Contudo, é

imperioso notar que essa ressíntese de glicogênio é necessária ao final da embriogênese de muitos artrópodes, inclusive naqueles que entram em um estado hipometabólico como a diapausa ou quiescência, ao término do processo embrionário. Este é o caso da *D. melanogaster,* do *A. aegypti* e de inúmeros outros artrópodes citados no Anexo II.

Nos trabalhos citados como referência nessa tese foram estudas diversas enzimas do metabolismo de carboidratos e em diferentes modelos, contudo sem fornecer uma visão mais ampla da regulação metabólica que integra as principais vias do catabolismo e anabolismo de glicose em artrópodes. O nosso grupo de pesquisa é pioneiro no estudo da regulação dessas vias metabólicas em artrópodes hematófagos vetores de doenças, principalmente na embriogênese, mas não necessariamente restrito a esta fase do ciclo de vida desses animais (resultados complementares). O estudo dessas enzimas e a compreensão das vias às quais pertencem tem como objetivo entender as particularidades da regulação do metabolismo de glicose observadas em artrópodes. Para isso, utilizamos dados disponíveis na literatura para descrever os novos mecanismos, e construir esquemas metabólicos a partir dos dados obtidos, seja realizando desafio nutricional, silenciamento gênico ou inibição química das enzimas do metabolismo de glicose.

2. Revisão Bibliográfica

O filo artrópoda é o maior do reino animal, ocupando um vasto nicho ecológico e habitando em quase todo tipo de ambiente no planeta, seja aquático ou terrestre. Esses organismos apresentam uma diversidade de tamanhos, formas e hábitos de vida, sendo muitas espécies encontradas em ambientes inóspitos às baixas temperaturas ou no calor extremo. Para sobreviver a essas condições adversas os artrópodes sofrem adaptações fisiológicas nas quais certos processos reprodutivos e comportamentais são alterados para garantir sua sobrevivência (Anexo II). Várias dessas adaptações são relatadas na literatura para inúmeros organismos, e dependendo da espécie e suas particularidades podem ser chamadas de torpor, quiescência, diapausa ou hibernação.

Toda essa nomenclatura em última instância designa um estado hipometabólico nos quais a temperatura corporal, o ritmo cardíaco e a respiração podem baixar, além de muitas outras funcionalidades do corpo, poupando assim energia até um período mais favorável à sobrevivência do animal (Anexo II). Em artrópodes esse período de latência é caracterizado por uma interrupção no desenvolvimento, sincronizando períodos de crescimento e reprodução com os períodos de ótima temperatura e suprimentos alimentares adequados (Lees, 1955; Tauber & Tauber, 1976; Denlinger, 1986; Denlinger, 2002). Além da parada no desenvolvimento, podem também ocorrer mudanças fisiológicas espécie-específicas que tipicamente incluem redução do metabolismo, aumento da tolerância ao estresse e aumento na síntese de proteínas que protegem contra uma variedade de intempéries ambientais como seca ou frio extremo (Denlinger, 2002; Lee *et al*., 2002; Clegg, 1965).

Nesta tese utilizamos dois modelos experimentais: o carrapato R. microplus e o mosquito A. aegypti, artrópodes predominantes em regiões tropicais e subtropicais, incluindo o Brasil. O R. microplus tem como principal hospedeiro o bovino, embora seja capaz de

completar, eventualmente, o seu ciclo em búfalos, ovelhas, cavalos e veados (Sonenshine, 1991). Além da espoliação ao couro, causada por reações inflamatórias nos locais de fixação do carrapato, existe ainda um grande prejuízo na produção de leite e carne devido à perda de sangue do animal hospedeiro (Seifert et al., 1968; Sutherst, 1983). Ainda, o R. microplus é um importante vetor de doenças, como a tristeza parasitária bovina, causada por protozoários do gênero Babesia e pela riquétsia do gênero Anaplasma (McCosker, 1981; Young e Morzaria, 1986). Cada fêmea de carrapato pode colocar dois a três mil ovos que eclodem em duas a três semanas, dependendo das condições de temperatura e umidade relativa do ar. A embriogênese do R. microplus é completada após 21 dias e as larvas recém eclodidas não tem poder infestante, pois precisam de um tempo para fortalecimento da cutícula (quatro a seis dias). Após esse período, elas sobem nas hastes dos capins, aguardando a passagem do hospedeiro para parasitá-lo (Figura 1). A fase parasitária inicia-se quando a larva infestante instala-se no hospedeiro e dura em torno de 21 dias. O acasalamento ocorre a partir do 17º dia da infestação, durante o repasto sanguíneo da fêmea (Londt and Artur, 1975). Após a fertilização e ingurgitamento ocorre a queda da fêmea ao solo, onde fará a oviposição e depois morrerá.

Em contrapartida, o mosquito A. aegypti é o vetor de doenças responsável pelo maior número de mortes humanas no Brasil, onde foram registrados 3,2 milhões de casos e mais de 800 mortes somente para dengue nos últimos 5 anos (Ministério da Saúde, 2015). Além da dengue, esse mosquito também pode transmitir chikungunya e o Zica Virus que no final de 2015 alcançou níveis alarmantes, atingindo 18 estados brasileiros (Ministério da Saúde, 2015). Os ovos são postos pela fêmea aderidos a substratos úmidos próximos à superfície da água (Funasa, 2001). Ao fim da embriogênese os ovos eclodem e as larvas passam por quatro estádios distintos até a transformação em pupa. Os mosquitos adultos emergem das pupas na superfície da água (Figura 2). No ambiente terrestre, macho e fêmea adultos

realizam a cópula durante o chamado vôo nupcial, momento no qual os machos depositam seus espermatozóides na fêmea, que os armazenam em uma espermateca até o momento da fertilização, no ato da postura. Tanto o *A. aegypti* quanto o carrapato *R. microplus* possuem fases de resistência que lhes possibilitam uma maior sobrevida no ambiente, garantindo o sucesso reprodutivo e o fechamento de seu ciclo de vida.

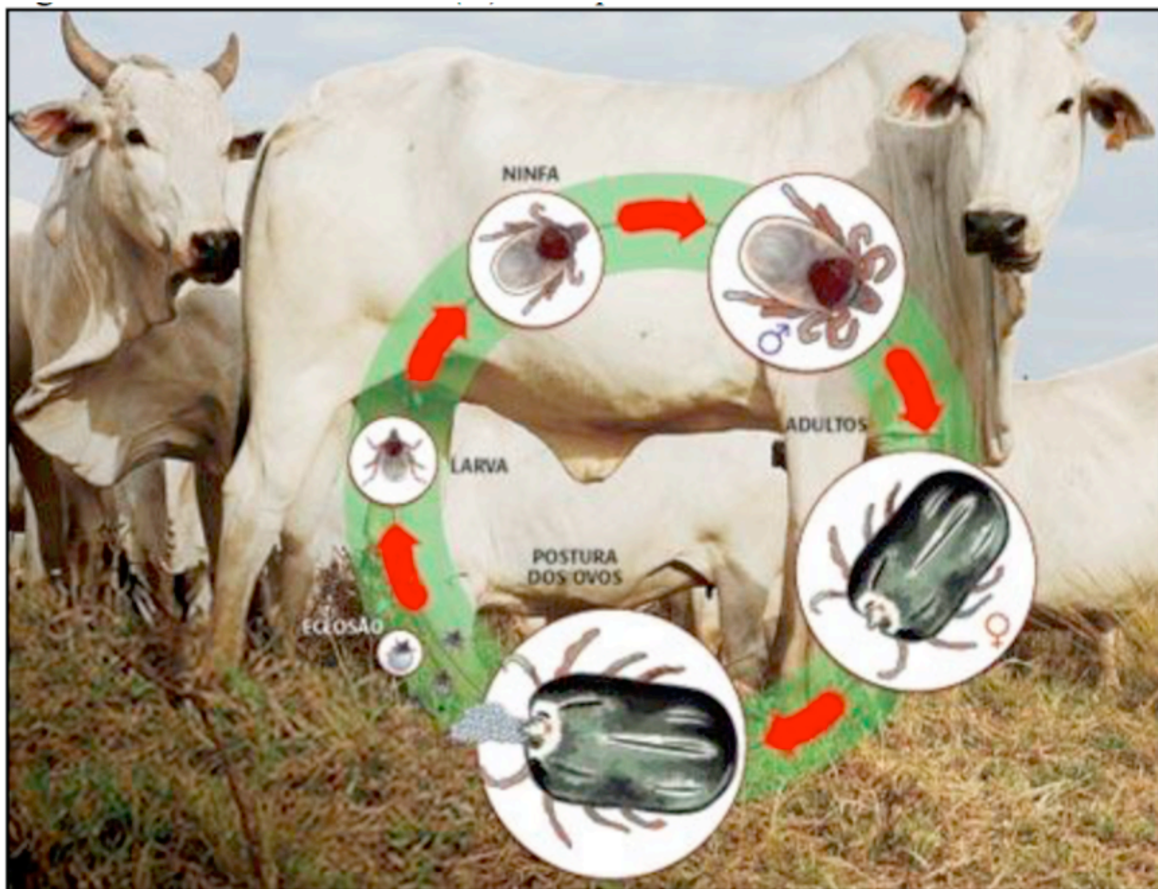

Figura 1: Ciclo de vida do carrapato *Rhipicephalus microplus*.

Até as últimas décadas do século XX as reservas de gordura eram descritas como as mais importantes reservas utilizadas por artrópodes em diapausa (Ludwig 1948; Karnavar and Nair 1969; Boell, 1935; Lees, 1955). Entretanto, hoje se sabe que muitas espécies de artrópodes diferem na utilização relativa de cada classe de reserva energética durante o estágio de latência. O besouro *Leptinotarsa decemlineata*, por exemplo, usa primariamente

as reservas de carboidratos no início da diapausa, mas se o período de latência se prolongar inicia a mobilização das reservas de lipídios (Lefevere *et al*., 1988). Já a mosca *Sarcophaga crassipalpis* deixa as reservas de carboidratos para os momentos finais da diapausa, que serão utilizadas para o despertar (Adedokun & Denlinger, 1985). As reservas de carboidratos representam energia de mobilização rápida como descrito pela literatura clássica, e são fortes candidatos para a manutenção dos momentos iniciais do estágio hipometabólico de inúmeros organismos, incluindo o *A. aegypti* (Anexo II). Ovos do mosquito *A. aegypti* podem permanecer viáveis durante longos períodos de tempo sem contato com a água (Anexo II), estima-se um ano ou mais. A aquisição da resistência ao ressecamento dos ovos de *A. aegypti* está associada com a formação da cutícula serosa, sintetizada em 15 horas de desenvolvimento embrionário (HDE) do mosquito (Rezende *et al*., 2008). Após esse período não há mais passagem de água do meio exterior para o ovo e vice-versa. Nesse contexto, se toda água em torno dos ovos secar após 15HDE, a embriogênese prossegue normalmente sem que nenhum embrião morra. No entanto, os recursos energéticos estão limitados ao fim da embriogênese e a larva do mosquito precisa modular seu metabolismo a fim de sobreviver por longos períodos de seca, ou seja, iniciar seu estado hipometabólico (Anexo II). Como esse estado hipometabólico é induzido por um estímulo (a seca) e não programado para ocorrer, torna-se mais adequado chamá-lo de quiescência (Anexo II). Contudo, a maioria dos artrópodes que entram em estado hipometabólico, o faz no inverno como no caso da mariposa *Bombyx mori*, a mosca *Eurosta solidaginis* e o mosquito *Aedes albopictus* (entre outros - Anexo II), e algumas espécies são classificadas como extremófilos por inúmeros pesquisadores, como é o caso dos insetos ápteros da ordem Grylloblattodea (Schoville *et al*., 2015).

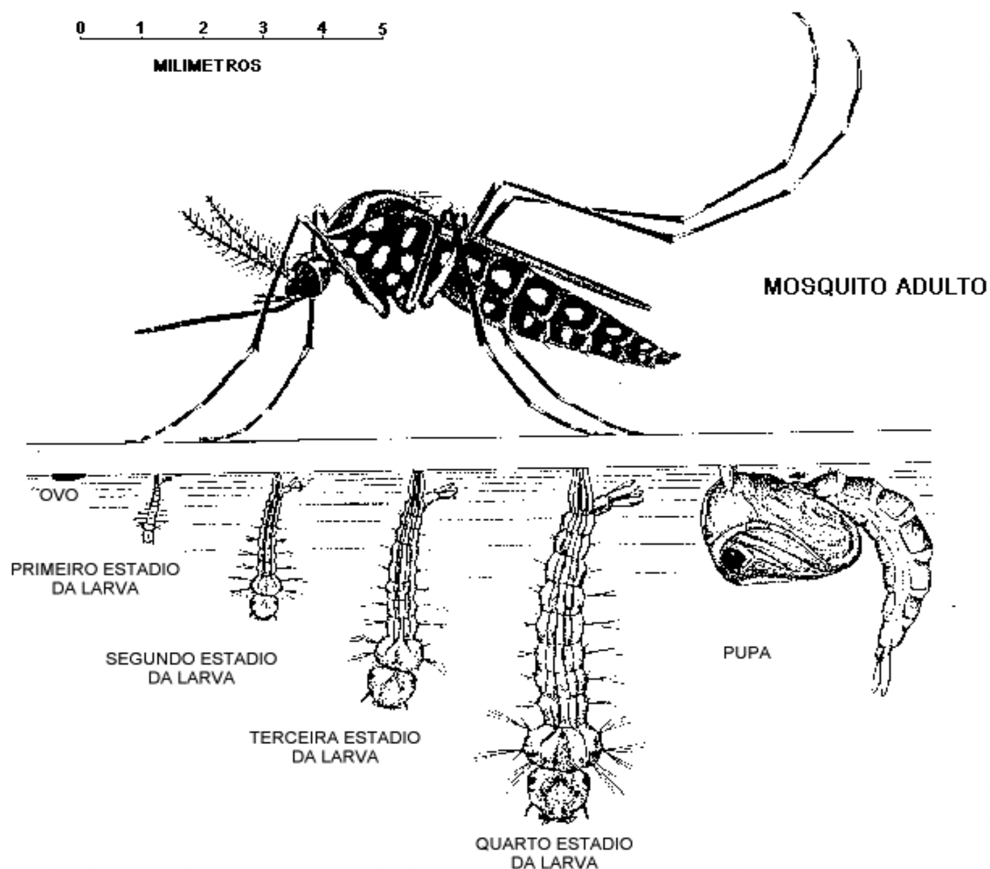

Figura 2: Ciclo de vida do mosquito *Aedes aegypti*.

Estudar a adaptação metabólica desses organismos nos períodos longos de restrição de nutrientes nos ajuda a entender como as enzimas-chave do metabolismo energético e as vias as quais pertencem estão sendo reguladas e assim entender toda a dinâmica de utilização de nutrientes que permitem a esses organismos sobreviver às condições extremas de seus habitats. O desafio nutricional não está restrito apenas ao processo de diapausa ou quiescência propriamente dito (Anexo I). Qualquer condição que limita a disponibilidade de nutrientes pode induzir um programa metabólico com muitas similaridades observadas em estados hipometabólicos naturais. O carrapato *R. microplus*, por exemplo, apesar de não entrar em diapausa ou quiescência, suas larvas podem sobreviver longos períodos de jejum. Após a eclosão, a larva do carrapato procura um hospedeiro para fixar-se, podendo permanecer em jejum no ambiente por mais de 120 dias se as condições climáticas forem

amenas ou até 30 dias para os locais quentes, sobrevivendo apenas com as reservas energéticas provenientes do ovo (Guglielmone *et al*., 2006). No caso dos carrapatos trioxenos (que realizam um ciclo de três hospedeiros), tanto as larvas como as ninfas e adultos são estágios de resistência no ambiente, uma vez que sua sobrevida é dependente das reservas energéticas adquiridas do estágio anterior do ciclo de vida. O adulto é o estágio que por mais tempo consegue sobreviver sem que encontre um hospedeiro, seguido pela ninfa, e por ultimo a larva, que apresenta a menor sobrevida em jejum. Os adultos de *Amblyomma spp*, por exemplo, podem sobreviver em jejum sob condições naturais por 12 a 24 meses, a ninfa por até 12 meses, e as larvas ao redor de 6 meses (Diamant & Strickland, 1965).

Os ovos desses artrópodes também consistem numa fase de resistência, no sentido de que precisam lidar com recursos energéticos limitados, depositados no ovo pela fêmea (Anexo I). Deste modo, as células embrionárias do carrapato *R. microplus* (BME26) constituem um modelo atraente de estudos para desafio nutricional induzido. Essa abordagem possibilitará um melhor entendimento da regulação gênica e enzimática do metabolismo de carboidratos durante o jejum, correlacionando esses dados com os eventos observados na quiescência de *A. aegypti* e outros modelos disponíveis na literatura (Anexo I e II). Nas sessões seguintes serão apresentados resultados complementares e seus respectivos materiais e métodos, agregando informações adicionais aos anexos I e II da presente tese. Além disso, será realizado uma discussão complementar que integrará todos os eventos metabólicos estudados nesses trabalhos que compõe a tese.

3. Metodologia Complementar

3.1 Extração de RNA total de ovos e órgãos de *A. aegypti*

Ovos sincronizados (20mg) de *A. aegypti* de 0h, 5h, 15h, 24h, 48h e 62h foram obtidos como tempo total de embriogênese. Três grupos de fêmeas do mosquito passaram por condições nutricionais diferentes: (1) Fêmeas não-alimentadas (NA), (2) 24 horas após a alimentação de sangue e (3) 48 horas após a alimentação de sangue. Em seguida vários órgãos (cabeça, tórax, abdômen, intestino médio e ovários) desses grupos de fêmeas foram dissecados. O RNA total de todas as amostras foram extraídos com o reagente Trizol ((Invitrogen, Grand Island, NY, USA) de acordo com as instruções do fabricante.

3.2 Quantificação relativa de transcritos de GSK3 e PEPCK por qRT-PCR após a alimentação sanguínea e na embriogênese de *A. aegypti*

Foi realizada uma síntese de cDNA utilizando-se 2µg de RNA total obtidos dos órgãos e ovos de A. aegypti (High-Capacity cDNA Reverse Transcription-M-MLV kit (Takara Biotechnology, Shiga, Japan). A amplificação foi realizada na plataforma de PCR em tempo Real LightCycler 1.5 (Roche, Göttingen, Germany) e a expressão relativa foi determinada pelo programa *Computacional Relative Expression Software Tool-REST* (Pfaffl, 2001). Diluições seriadas do cDNA foram utilizadas para a preparação de curva de calibração para cada par de primer utilizado. Reações com eficiências entre 85 e 100% foram determinadas a partir de curvas de calibração para cada conjunto de primers em reações de 10 µL. As isoformas da PEPCK de A. aegypti estão depositadas no GenBank com as seguinte identificação: Transcript ID AAEL000025-RA, AAEL000006-RA, AAEL000080-RA, citados nesta tese como PEPCK(25), PEPCK(6) e PEPCK(80), respectivamente. O gene ribossomal rp49 de A. aegypti foi utilizado como gene constitutivo (Gentile et al., 2005) e os primers utilizados para a isoforma de cada gene da PEPCK encontram-se no Anexo II.

3.3 Síntese da dupla-fita de RNA (dsRNA) para AKT em células embrionárias BME26 do carrapato *R. microplus*

A síntese de dsRNA de AKT foi realizada segundo a metodologia apresentada no item 3.5 do Anexo I.

3.4 Quantificação relativa de transcritos de AKT e PEPCK em células embrionárias BME26 do carrapato *R. microplus*

A análise transcricional de AKT e PEPCK foi realizada segundo a metodologia apresentada no item 3.7 do Anexo I. Os primers para os respectivos genes são encontrados na Tabela 1 do Anexo I.

4. Resultados Complementares

4.1 Regulação de GSK3 pela AKT a Nível Transcricional

A análise transcricional de GSK3 e PEPCK foi avaliada em células embrionárias do carrapato *R. microplus* após o silenciamento de AKT. A transcrição de GSK3 é significativamente reduzida quando a AKT é silenciada (Figura 3A e B), porém, o mesmo não acontece com a PEPCK. A transcrição do gene da PEPCK permanece inalterada após o silenciamento da AKT (Figura 3C).

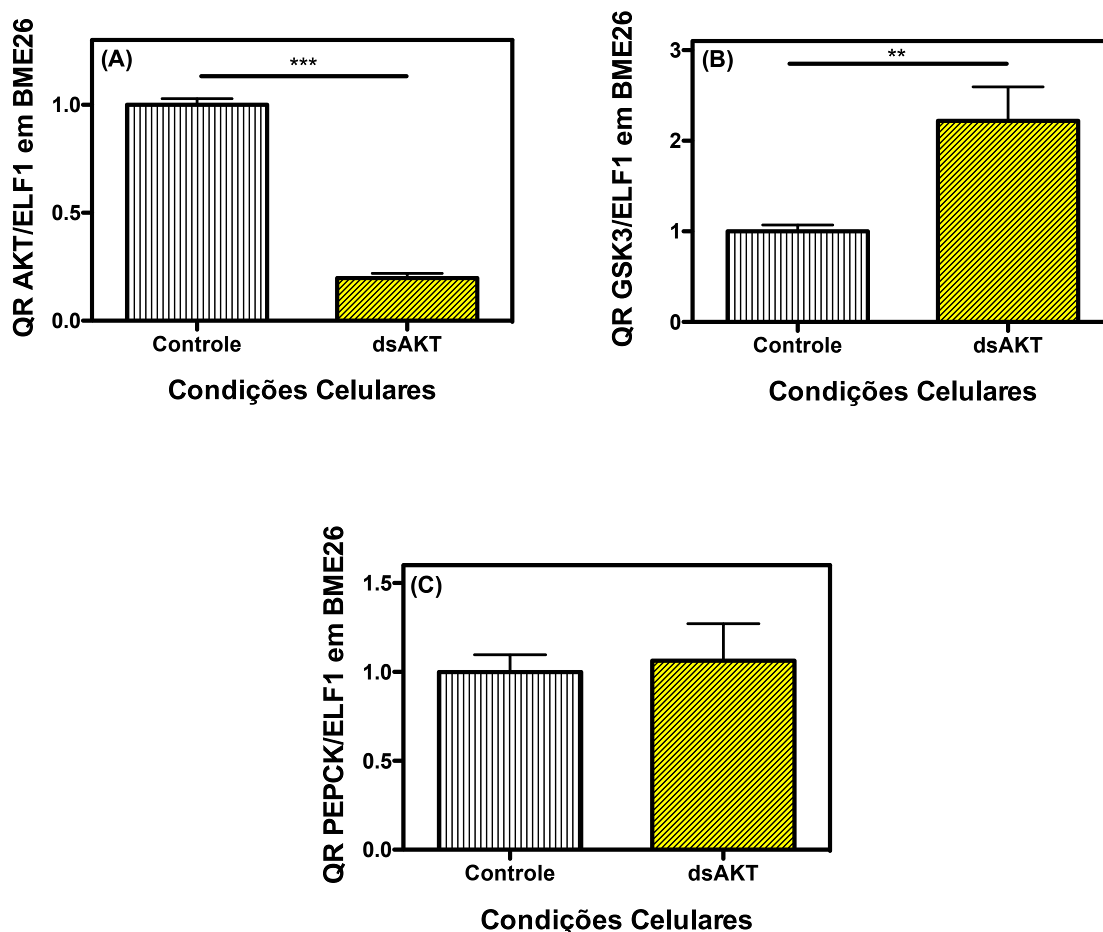

Figura 3: O silenciamento de AKT afeta a transcrição de GSK3, mas não a de PEPCK. A análise transcricional de AKT foi realizada para validar seu silenciamento (A) e analisar a transcrição de GSK3 (B) e PEPCK (C) em células embrionárias BME26 do carrapato *R. microplus*. As barras representam as médias ± erro padrão. ** Representa diferenças significativas entre as amostras e PrD (** $p < 0.001$, Teste T Pareado).

4.2 Expressão relativa das isoformas de duas isoformas de PEPCKs em resposta a alimentação sanguínea do mosquito *A. aegypti*.

A quantidade relativa de transcritos (RNAm) dos genes da PEPCK(80) e (25) de *A. aegypti* foi determinada em diferentes órgãos (cabeça, tórax, abdômen, intestino médio e ovários) de fêmeas de *A. aegypti* em resposta a alimentação sanguínea (Figura 4 e 5). Para a PEPCK(80) podemos observar uma quantidade alta de transcritos nos órgãos dos mosquitos que não foram alimentados com sangue (Figura 4A, B, C e D). Entretanto, após a 24 horas da alimentação sanguínea a transcrição de PEPCK sofre uma abrupta queda quando comparados aos mosquitos não alimentados. Essa transcrição da PEPCK(80) volta a subir após 48 horas da alimentação sanguínea. Surpreendentemente, o ovário foi o único órgão a apresentar um perfil transcricional diferenciado (Figura 4E). A transcrição da PEPCK(80) apresentou-se reduzida em mosquitos não alimentados, e após 24 horas da alimentação sanguínea o nível de transcritos subiu abruptamente, diferentemente do observado para os outros órgãos. Em 48 horas após alimentação observa-se uma redução na transcrição de PEPCK(80).O perfil transcricional da PEPCK(25) assemelha-se ao da PEPCK(80) para a cabeça, tórax, abdômen e intestino (Figura 5A, B, C, D). Novamente no ovário a resposta transcricional é diferenciada em relação aos demais órgãos ou em relação à PEPCK(80) apresentando o mesmo nível de transcrição em cada tratamento, seja não-alimentado, 24 e 48 horas após alimentação sanguínea (Figura 5E).

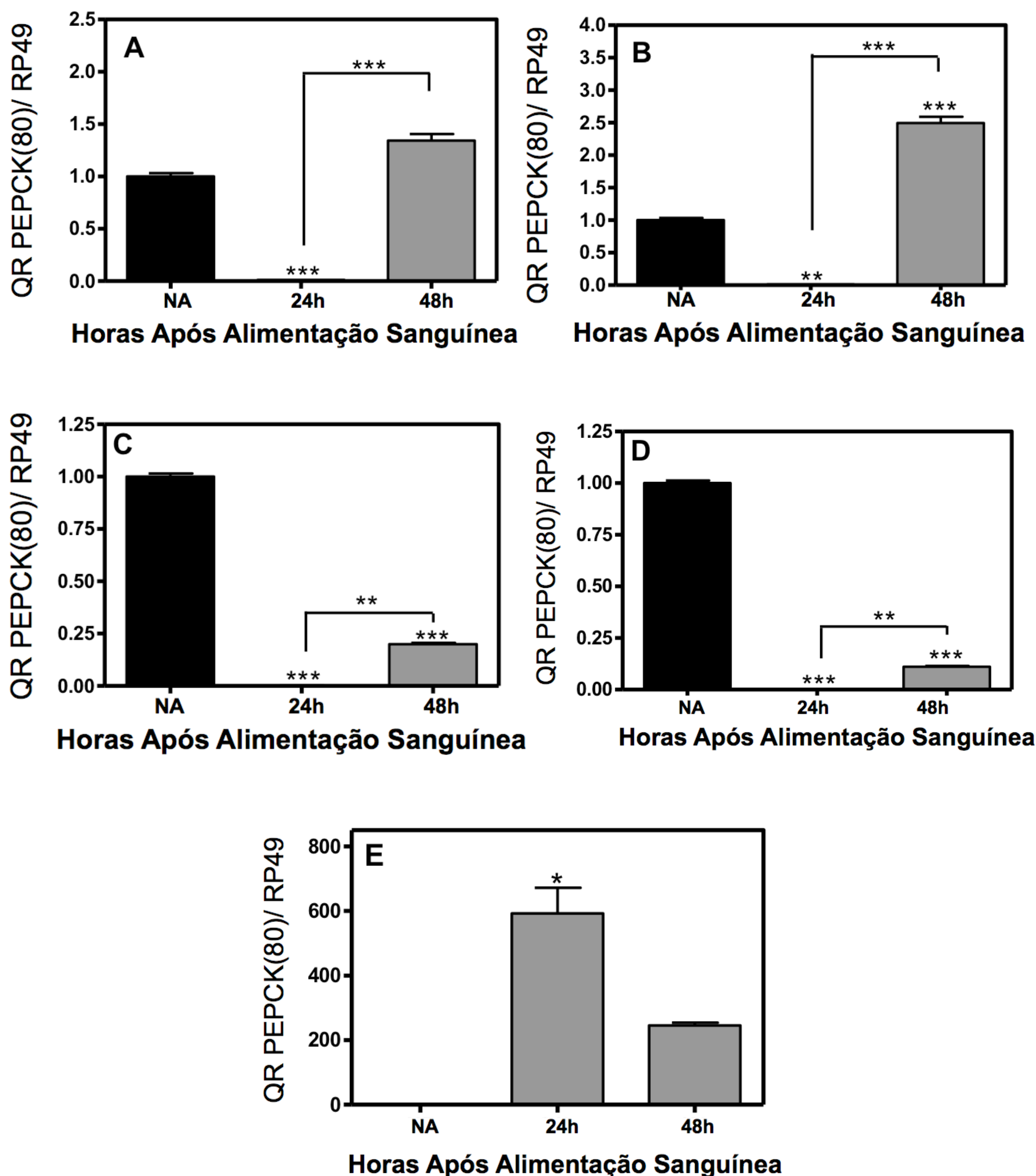

Figura 4: Resposta transcricional da PEPCK(80) é diferenciada em ovários de *A. aegypti* após alimentação sanguínea. A análise transcricional de PEPCK(80) foi realizada em (A) cabeça, (B) Tórax, (C) Abdômem, (D) Intestino e (E) Ovários de *A. aegypti* alimentados com sangue (24 e 48 horas após alimentação sanguínea) e em jejum (NF). As barras representam as médias ± erro padrão. ** Representa diferenças significativas entre as amostras e PrD (One-way ANOVA, P< 0,05 e teste complementar Tukey).

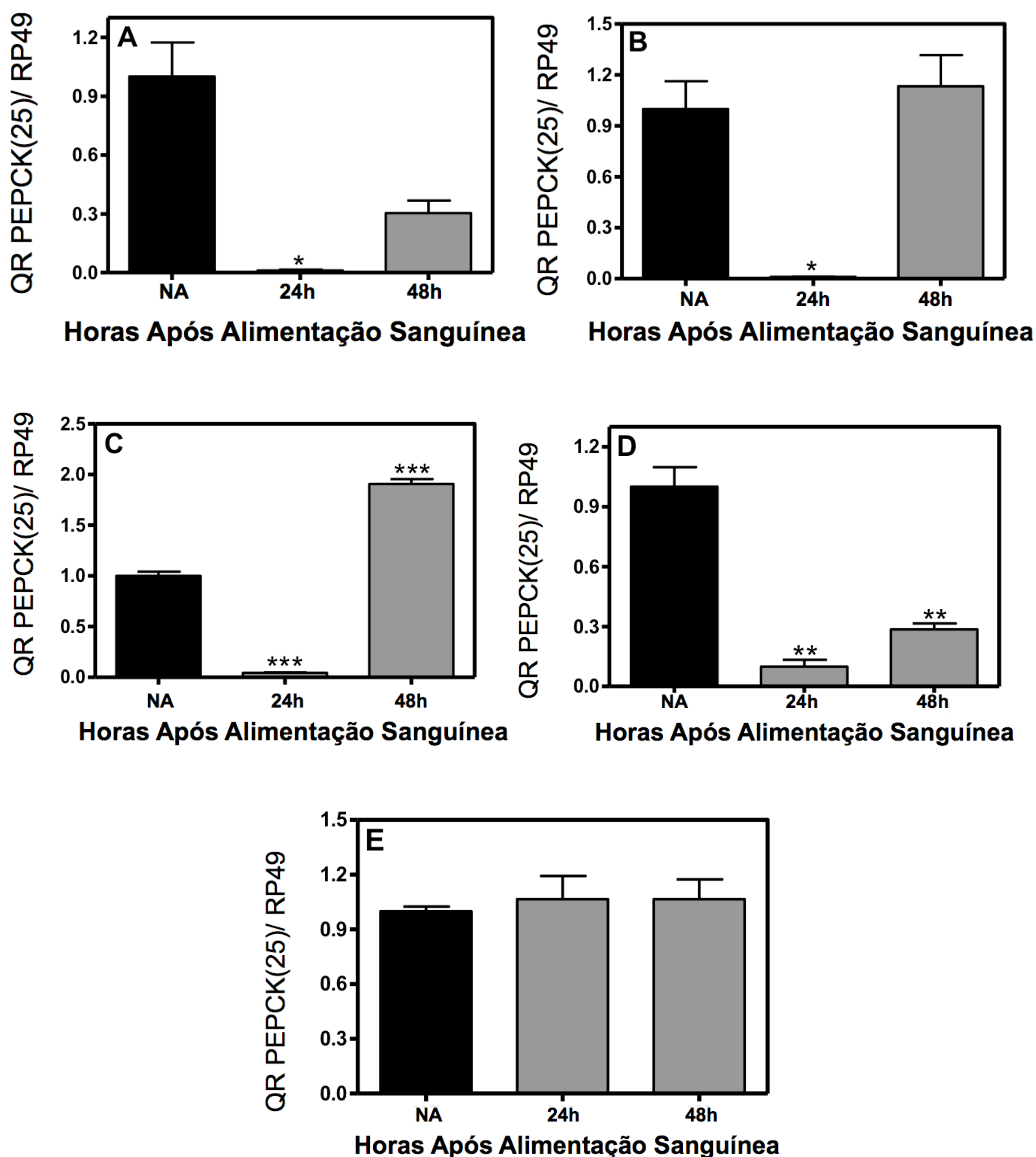

Figura 5: A transcrição de PEPCK(25) não responde à alimentação sanguínea em ovários de *A. aegypti*. A análise transcricional de PEPCK(80) foi realizada em (A) cabeça, (B) Tórax, (C) Abdômem, (D) Intestino e (E) Ovários de *A. aegypti* alimentados com sangue (24 e 48 horas após alimentação sanguínea) e em jejum (NF). As barras representam as médias ± erro padrão. ** Representa diferenças significativas entre as amostras e PrD (One-way ANOVA, P< 0,05 e teste complementar Tukey).

4.3 Os genes da PEPCK(80) e PEPCK(25) correspondem às isoformas citosólica e mitocondrial, respectivamente.

A análise transcricional do gene da PEPCK(80) foi realizada durante toda embriogênese do mosquito *A. aegypti*, mostrando a presença de transcritos em todos os momentos da embriogênese (Figura 6A). De 0 a 24 horas de desenvolvimento não ha diferença estatística na transcrição de PEPCK(80), mas de 24 para 62 horas o nível de transcritos aumentou significativamente. Já a transcrição de PEPCK(25) só foi observada a partir de 15 horas de desenvolvimento, onde obteve o maior número de transcritos (Figura 6B). Em 24 horas de desenvolvimento a transcrição de PEPCK(25) foi reduzida, permanecendo constante até o final da embriogênese.

Um fracionamento celular foi realizado em ovos de A. aegypti de diferentes momentos da embriogênese para obtenção de frações mitocondriais e citosólicas, e a atividade enzimática de PEPCK foi realizada em ambas frações. Na fração citosólica podemos observar um aumento significativo na atividade de PEPCK de 24 para 62 horas de desenvolvimento (Figura 7A). Já na fração mitocondrial observamos um aumento de atividade 5 para 24 horas de desenvolvimento, porém de 24 a 62 horas a atividade de PEPCK permaneceu constante (Figura 7B).

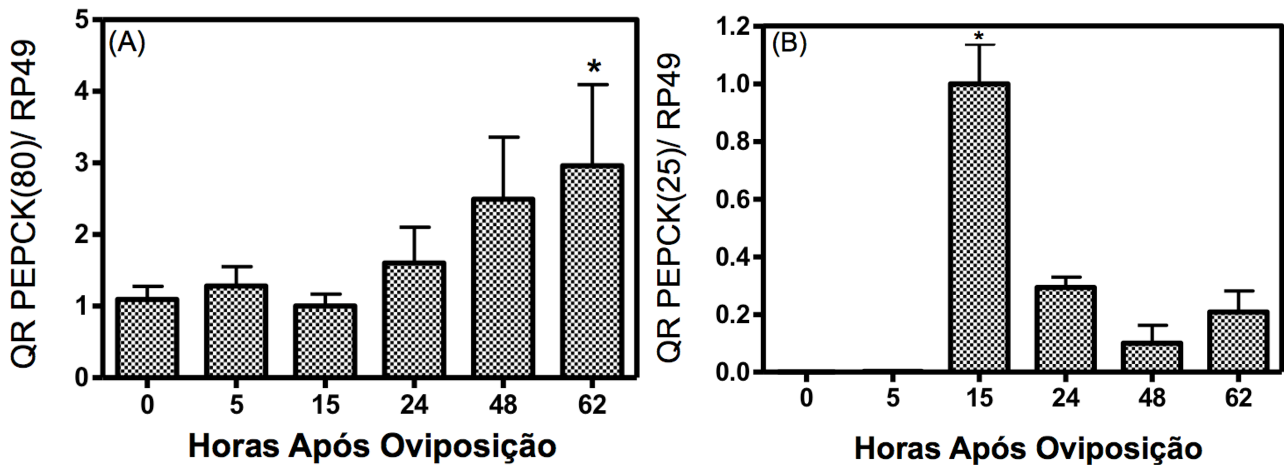

Figura 6: A regulação transcricional das PEPCK ocorre de forma diferenciada ao longo da embriogênese do *A. aegypti*. A análise transcricional de PEPCK(80)(A) e PEPCK(25)(B) foi realizada em ovos de A. aegypti sinconizados com 0, 5, 15, 24, 48, 62 horas após a oviposição. As barras representam as médias ± erro padrão. ** Representa diferenças significativas entre as amostras e PrD (One-way ANOVA, P< 0,05 e teste complementar Tukey).

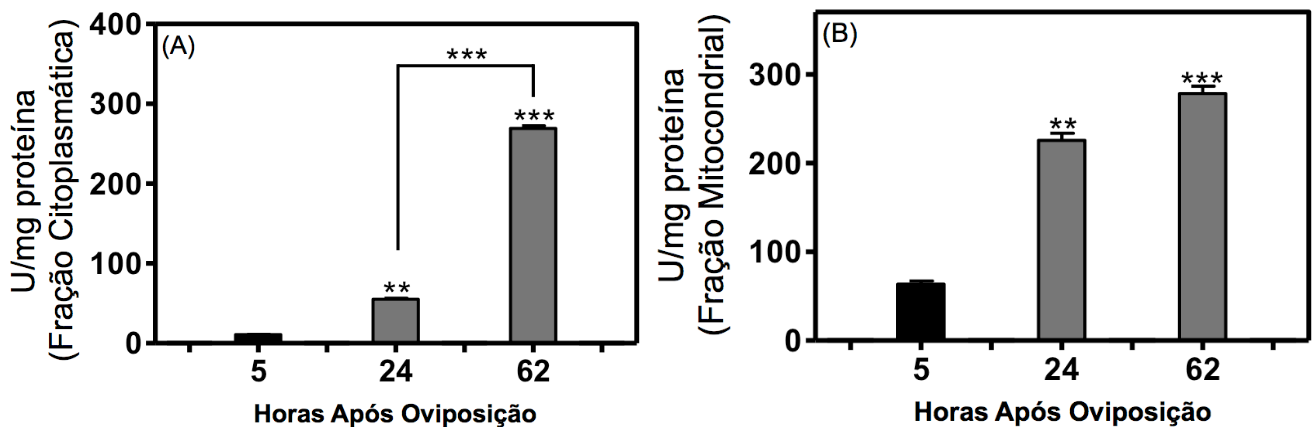

Figura 7: A atividade de PEPCK nas frações citosólica e mitocondrial correspondem à isoforma PEPCK(80) e PEPCK(25), respectivamente. A atividade enzimática da PEPCK foi realizada em ovos de *A. aegypti* sinconizados com 5, 24 e62 horas após a oviposição. As barras representam as médias ± erro padrão. ** Representa diferenças significativas entre as amostras e PrD (One-way ANOVA, P< 0,05 e teste complementar Tukey).

5. Discussão Complementar

Os dois trabalhos apresentados nos anexos aborda os mecanismos de regulação metabólica frente ao desafio nutricional, induzido ou natural. No anexo I, em particular, avaliou-se a resposta transcricional de enzimas reguladoras do catabolismo e anabolismo de carboidratos em células tratadas com diferentes concentrações de glicose. Dentre esses genes, destacam-se a Glicogênio Sintase Quinase (GSK3) e Glicogênio Sintase (GS) que apresentaram correlação gênica (Figura 5 do Anexo I), comprovando a corregulação classicamente descrita para a regulação do metabolismo de glicogênio em mamíferos. Ambas as enzimas estão relacionadas a nível enzimático, porém nesse trabalho mostramos que essa relação também pode ocorrer a nível gênico, uma vez que em elevadas concentrações de glicose na célula a transcrição de GSK3 é reduzida comparada com as condições anteriores. Nesse mesmo momento, a transcrição de GS aumenta, sugerindo uma regulação indireta entre ambas as enzimas a nível gênico. Nesse mesmo trabalho observamos ainda que o silenciamento de GSK3 afeta a transcrição dos genes relacionados à gliconeogênese, sugerindo que a GSK3 pode estar envolvida de alguma forma com essa importante via de ressíntese de glicose (Figura 8 – Anexo I). Uma das principais reguladoras da GSK3 é a proteína quinase B (AKT) que através de fosforilação a inibe. Embora essa regulação seja descrita apenas à nível enzimático, resultados complementares nos mostram uma possível regulação da AKT sobre a GSK3 também a nível gênico em carrapato R. microplus. Após o silenciamento da AKT em células do carrapato, a transcrição de GSK3 aumentou significativamente (Figura 3A e B). No entanto, o silenciamento de AKT não afetou a transcrição de PEPCK como observado no silenciamento da GSK3 (Figura 3C), sugerindo que a regulação da gliconeogênese pode ocorrer através de componentes abaixo da AKT. Outras correlações também foram avaliadas durante todo o estudo, abordando genes do controle glicolítico, da degradação de glicogênio e gliconeogênese. É interessante notar que

de todos os resultados, o mais intrigante foi novamente a transcrição dos genes da gliconeogênese (PEPCK e GP), neste caso após o desafio nutricional (Figura 7 do Anexo II).

A PEPCK é responsável por catalisar a conversão de oxaloacetato em fosfoenolpiruvato, ou seja, uma enzima importante na intersecção da glicólise, gliconeogênese e ciclo de Crebs (Yang *et al*., 2009; Adt *et al*., 2000; Chakravarty *el al*., 2005). Por essa razão a PEPCK ocupa uma importante posição na homeostase energética porque está envolvida na re-esterificação de ácidos graxos, síntese de glicose e na catabolização de substratos do ciclo de Crebs (Yang *et al*., 2009). Está bem estabelecido que mudanças na taxa de transcrição de PEPCK citosólica estão correlacionadas a atividade enzimática desta enzima em fígado e córtex renal de ratos, tecidos que sintetizam glicose (Chakravarty *el al*., 2005). Após anos de estudo da PEPCK, o consenso geral é que alterações na transcrição de PEPCK citosólica regulam a atividade total desta enzima nesses tecidos, e o mesmo é observado para a glicose-6 fosfatase (GP) (Hanson & Reshef, 2003; Yang *et al*., 2009). Portanto, no anexo I ao observar transcrição dessas enzimas correlacionamos com suas respectivas atividades. Porém, em células BME26 tratadas com baixas concentrações de glicose houve um aumento na transcrição de PEPCK e GP ao invés de redução em relação aos demais tratamentos. Já com elevada concentração de glicose ocorre o inverso.

Sob condições fisiológicas normais, quando a o nível de glicose torna-se reduzido, como no jejum, o fluxo gliconeogênico aumenta (Anexo I). Nesse caso, espera-se uma elevada transcrição das enzimas gliconeogênicas quando a disponibilidade de glicose torna-se reduzida. Surpreendentemente, em células BME26 o perfil transcricional observado é inverso (Figura 7 do Anexo I). A PEPCK, além de regular a gliconeogênese, também regula a gliceroneogênese, via necessária à re-esterificação de ácidos graxos livres mantendo a

síntese de triglicerídeos ativa. Em condições onde há excesso de carboidratos o fluxo pela gliceroneogênese pode aumentar conduzindo os substratos da gliconeogênese para glicerol (Anexo I). Neste caso, a elevada transcrição de PEPCK pode representar duas situações. A quantidade elevada de glicose nessas células põe em curso um programa metabólico que favoreça o fluxo pela gliceroneogênese ou a resposta observada não se refere à PEPCK citosólica, e sim à isoforma mitocondrial. Apesar das PEPCKs serem descritas coo tendo a mesma função, estudos recentes mostraram que o silenciamento de PEPCK mitocondrial em órgãos gliconeogênicos de ratos promove a redução de glicose no plasma sanguíneo, a liberação de insulina e triglicerídeos, além de reduzir as reservas de glicogênio hepática. Já o silenciamento da isoformas citosólica não apresenta esses efeitos. De fato, a PEPCK mitocondrial é, aparentemente, negligenciada na maioria dos estudos envolvendo gliconeogênese. Sabe-se classicamente que todos os eucariotos têm uma isoforma citosólica da PEPCK (PEPCK-C) e outra mitocondrial (PEPCK-M). No fígado da maioria dos mamíferos estudados até então, incluindo humanos, 50% da atividade total de PEPCK corresponde a isoformas citosólica e a outra metade à isoforma mitocondrial (Yang *et al*., 2009). Se ambas isoformas estão em compartimentos tão diferentes como citosol e mitocôndria e expostos a condições energéticas e disponibilidade de substratos tão particulares, sua participação no metabolismo geral pode não ser tão redundante como se acredita. Já foi demonstrado que a superexpressão somente do gene da PEPCK-C altera o metabolismo e a fisiologia de forma singular em camundongos, causando aumento de longevidade nos animais, resistência física e biogênese mitocondrial (Hakimi *et al*., 2007). É provável que a PEPCK-M esteja envolvida em uma intersecção do metabolismo energético até então pouco conhecida.

Contudo, todo trabalho desenvolvido pelo nosso grupo tem evidenciado que a gliconeogênese é mais complexa do que a literatura clássica tem nos mostrado. No anexo II

também foi estudado a regulação do metabolismo de carboidratos em resposta à quiescência dos ovos do *A. aegypti*, outro desafio nutricional. Neste caso, observamos que reservas de glicogênio são degradadas para manter os momentos iniciais da quiescência do mosquito, além de serem também recursos essenciais para a eclosão da larva ao fim da quiescência (Figura 5 do Anexo II). A redução na atividade de enzimas reguladoras das vias de catabolismo de glicose como a glicólise e a via das pentoses fosfato junto com acúmulo de G6P e redução no consumo de oxigênio desses ovos em quiescência sugere uma redução na taxa metabólica para manter o período de latência (Figura 1, 2 e 4 do Anexo II). Interessantemente, a PEPCK aumenta em atividade no período de quiescência e pós-quiescência, justamente no momento onde há uma queda significativa na quantidade de proteína do ovo, sugerindo que a degradação de proteína estaria fornecendo aminoácidos como substrato para a gliconeogênese (Figura 6 e 7 do Anexo II). Insetos que entram em diapausa normalmente estocam proteínas antes do período de latência. Este é o caso da broca do milho, *Diatraea grandiosella*, e do besouro da batata, *L. decemlineata*, que estocam aminoácidos em proteínas especializadas nos momentos anteriores à estação desfavorável (Chippendale, 1973; Lefevere, 1988). Durante o inverno, esses insetos degradam essas proteínas e os aminoácidos remanescentes são oxidados na geração de energia ou na produção de outros compostos como a glicose, via gliconeogênese (Anexo II). Na quiescência de *A. aegypti* este mesmo processo pode ocorrer.

A gliconeogênese nos embriões de *A. aegypti* parece ser fundamental para manter a quiescência e auxiliar no despertar da mesma. Como resultado complementar tentamos encontrar as isoformas citosólica e mitocondrial do *A. aegypti*, uma vez que esse mosquito possui seu genoma completo depositado no banco de dados, e mostrar que há uma resposta diferenciada de ambas no metabolismo de glicose. Ao pesquisar no banco de dados, foram encontradas três sequências da PEPCK no genoma de A. aegypti,

identificadas como PEPCK(25), PEPCK(80) e PEPCK(06). A análise transcricional nos mostra uma resposta diferenciada de duas dessas isoformas nos três grupos estudados em A. aegypti: pré-quiescência, quiescência e pós-quiescência (Figura 9 do Anexo II). A PEPCK(06) é o único gene que não apresenta transcrição em nenhuma fase do ciclo de vida estudada (Anexo II), ou seja, embriogênese e órgãos de mosquitos adultos alimentados ou não com sangue. Existe a possibilidade de larvas e pupas de A. aegypti apresentarem transcrição para o gene PEPCK(08), entretanto esses estágios ainda não foram investigados.

Ao avaliar a transcrição da PEPCK(80) podemos sempre observar uma quantidade elevada de transcritos nos órgãos dos mosquitos que não foram alimentados com sangue (Figura 4A, B, C e D). Nos momentos de jejum a elevada transcrição de PEPCK permite o abastecimento de glicose pela via gliconeogênese, como observa-se nos mosquitos não alimentados. Entretanto, após a 24h da alimentação sanguínea a transcrição de PEPCK sofre uma abrupta queda quando comparados aos mosquitos não alimentados, sugerindo que o aporte de glicose reduz a atividade gliconeogênica do mosquito. Após 48 horas da alimentação sanguínea essa transcrição volta a subir uma vez que o intestino já digeriu a maior parte de seu conteúdo, tornando-se necessário um aumento no fluxo gliconeogênico.

O ovário foi o único órgão que apresentou um perfil diferenciado (Figura 4E). O nível de transcritos é reduzido em mosquitos não-alimentados e após alimentação observa-se um acentuado aumento na transcrição. Essa resposta contrária à alimentação sanguínea e ao jejum pode refletir a necessidade do ovário em depositar transcritos dessa isoforma (PEPCK-80) nos ovócitos em desenvolvimento para um processo posterior chamado de transição materno-zigótica, processo de transição que ocorre na embriogênese no qual o embrião deixa de utilizar os produtos depositados pela mãe e sintetiza por si só os

transcritos, proteínas e metabólitos. Após 48h da alimentação sanguínea os transcritos voltam a cair, justamente quando a ovogênese está chegando ao fim. Embora parte dos transcritos de PEPCK(80) esteja sendo depositada nos ovócitos, provavelmente outra parte está destinada ao próprio ovário, garantindo a integridade metabólica desse órgão. Contudo, a PEPCK(25) pode responder de uma forma diferente ao influxo de glicose nos ovários proporcionado pela alimentação sanguínea, uma vez que as PEPCKs podem ter papéis diferentes no metabolismo geral.

O nível de transcritos da isoforma PEPCK(25) apresentam um perfil similar ao observado para a PEPCK(80) para todos os órgãos (Figuras 5A, B, C e D), exceto o ovário que mostra um perfil diferenciado em relação aos demais órgão e à isoforma mostrada anteriormente (PEPCK-80). No ovário a transcrição da PEPCK(25) foi a mesma em ambos tratamentos, sugerindo uma atividade gliconeogênica constante nos ovócitos, seja no jejum ou após alimentação sanguínea (Figura 5E).Sabemos que estamos tratando aqui de duas isoformas diferentes de PEPCK, mesmo que ainda não possamos identificar qual é a mitocondrial e a citosólica. Sendo assim, para avaliar se uma isoforma está sendo depositada nos ovócitos e a outra está apenas atuando no metabolismo do ovário, analisamos o nível de transcrição desses genes ao longo da embriogênese (Figuras 6A e B).

Os dados relativos à embriogênese do *A. aegypti* nos mostra que há presença de transcritos da PEPCK(80) desde o início do processo embrionário (0 hora) do mosquito (Figura 6A). Já os transcritos de PEPCK(25) aparecem apenas em 15 horas do desenvolvimento embrionário (Figura 6B), justamente durante o processo de celurização e biogênese mitocondrial (Rezende *et al*., 2008; Vital *et al*., 2010). Observamos também que a atividade de PEPCK nas frações citosólica e mitocondrial correspondiam ao perfil de transcrição observado para a PEPCK(80) e PEPCK(25), respectivamente (Figuras 7A e B). A

atividade de PEPCK na fração citosólica é significativa entre ovos de 24 e 62 horas de desenvolvimento, assim como ocorre com a transcrição da PEPCK(80). E na fração mitocondrial a atividade é a mesma entre ovos de 24 e 62 horas, sem diferença estatística, assim como observado na transcrição da PEPCK(25). Esses dados em conjunto sugerem que a isoforma PEPCK(80) como a enzima citosólica e a PEPCK(25) como a mitocondrial.

Acredita-se que muitos estudos ainda são necessários para entender melhor como a gliconeogênese e as outras vias do metabolismo de glicose atuam para promover programas metabólicos que permitam o surgimento das fascinantes adaptações fisiológicas observadas em inúmeros organismos. Esta tese também demonstra que o desafio nutricional consiste em uma excelente metodologia para estudo da regulação e integração do metabolismo, fornecendo novos alvos que possam ser utilizados no controle de artrópodes vetores de doenças, no caso dos hematófagos ou até mesmo de pragas agrícolas, no caso dos fitófagos. Todavia, esses trabalhos apresentados aqui, acima de tudo, têm como objetivo agregar novas informações à literatura clássica, que muitas das vezes fixa a conceitos muito tradicionais referentes aos processos celulares necessários à manutenção da vida.

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ANEXO I

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Article

Non-classical gluconeogenesis-dependent glucose metabolism in *Rhipicephalus microplus* embryonic cell line BME26

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Abstract: In this work we evaluated several genes involved in gluconeogenesis, glycolysis and glycogen metabolism, the major pathways for carbohydrate catabolism and anabolism, in the BME26 *Rhipicephalus microplus* embryonic cell line. Genetic and catalytic control of the genes and enzymes associated with these pathways are modulated by alterations in energy resource availability (primarily glucose). BME26 cells in media were investigated using three different glucose concentrations, and changes in the transcription levels of target genes in response to carbohydrate utilization were assessed. The results indicate that several genes, such as glycogen synthase (GS), glycogen synthase kinase 3 (GSK3), phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (GP) displayed mutual gene regulation in response to glucose treatment. Surprisingly, the transcription of gluconeogenic enzymes was found to increase alongside that of glycolytic enzymes, especially pyruvate kinase, under high glucose treatment. In addition, RNAi data from this study revealed that the transcription of gluconeogenic genes in BME26 cells is controlled by GSK-3. Collectively, these results improve our understanding of how glucose metabolism is regulated at the genetic level in tick cells.

Keywords: Metabolism, Gluconeogenesis, Glycolysis, Tick, Gene expression, Glucose

Abbreviations

HK, hexokinase; PK, pyruvate kinase; GS, glycogen synthase; TIM, triose phosphate isomerase; AKT, protein kinase B; GSK3, glycogen synthase kinase 3; PGM, phosphoglucomutase; GDE, glycogen-debranching enzyme; PEPCK, phosphoenolpyruvate carboxykinase; GP, glucose-6 –phosphatase; GK, glucokinase.

1. Introduction

The cattle tick *Rhipicephalus microplus* is an ectoparasite found in tropical and subtropical areas. Its importance in veterinary is due to its ability to transmit pathogens. It causes considerable losses in the cattle industry, with substantial damage to livestock [1]. Together, the economic losses caused by *R. microplus* parasitism and costs associated with its control in Brazil are estimated at US$ 3 billion dollars a year [2].

Currently, few studies have investigated the mechanisms underlying energy metabolism during embryonic development in *R. microplus* or in the BME26 tick cell line [3]. Recent works have provided some insights into the dynamic processes that accompany nutrient utilization during tick embryogenesis [4,5,6].

Embryogenesis has been classically described as an energy-consuming process [7,8]. For oviparous organisms, the embryonic stage is characterized by the mobilization of metabolites of maternal origin for the development of new tissues and organs [9]. Studying the molecules involved in metabolic pathways during embryogenesis could reveal regulatory networks that control metabolism during embryonic development in many organisms. However, despite the recent advancements in molecular information, our understanding of genetic regulatory mechanisms, including that controlling energy metabolism, remains incomplete. In fact, many relevant aspects of metabolism during embryogenesis are not fully studied at present; however, essential pathways, such as those related to carbohydrate metabolism, are likely to be highly conserved among *important disease vectors, including ticks and mites.*

During embryogenesis, before blastoderm formation (a landmark stage of tick embryonic development), glycogen reserves are preferentially mobilized to support the energy-intensive process of embryogenesis [5]. Subsequently, protein degradation and gluconeogenesis intensify, in order to supply the embryo with sufficient glucose to allow glycogen resynthesis. Thus, the use of amino acids as a substrate for gluconeogenesis and the subsequent glycogen resynthesis plays an important role during the stages of *R. microplus* embryogenesis. Glycogen is the main energy source during the early stages of *R. microplus* embryogenesis, and protein degradation increases during late embryogenesis [5]. Protein metabolism depends strongly on the substantial expression and activity of carbohydrate metabolism enzymes. The opposite is true for *Aedes aegypti* mosquitoes, with glycogen and protein levels decreasing 24 hours into embryonic development, with a concomitant increase in the activity of phosphoenolpyruvate carboxykinase (PEPCK), a key gluconeogenic enzyme [10]. Thus, energy

homeostasis is maintained by glycogen and protein mobilization at the end of mosquito embryonic development. However, the molecular mechanisms that regulate this process are poorly understood at present. Previous work by our group investigated the insulin-signaling pathway (ISP) and its possible role during embryogenesis, using the BME26 cell line as a model [3]. Compared with untreated cells, exogenous insulin elevated the cell glycogen content in the absence of fetal calf serum (FCS). Moreover, in the presence of PI3K inhibitors (wortmannin or LY294002), these effects were blocked. These results strongly suggested the presence of an insulin-responsive system in BME26 cells that may correlate with carbohydrate/glycogen metabolism during embryogenesis. GSK3 knockdown in *R. microplus* females resulted in a strong reduction in GSK-3 expression in ovaries, followed by significant reductions in both oviposition and hatching [11]. Moreover, similar effects were observed in females treated with GSK3 inhibitors (alsterpaullone, bromo-indirubin-oxime-6 and indirubin-3-oxime). The appearance of the eggs was also altered after these treatments, suggesting an important role for GSK3 in proper embryonic development. Another recent study reported that monoclonal antibodies for triosephosphate isomerase (TIM) inhibited BME26 cell growth [6], providing further evidence of the importance of glucose metabolism in cell proliferation. However, few studies have addressed the molecular mechanisms that control the expression of genes that are central to energy metabolism. Our previous works focused on distinct protein targets involved in tick energy metabolism, with the aim of improving our understanding of tick physiology.

BME26 cells were initially characterized by Esteves *et al*. [12]. Since then, BME26 cells have been used to examine regulators of glycogen metabolism under experimental conditions [3,12]. The objective of the present study was to investigate the transcriptional profiles of important genes involved in energy metabolism in BME26 cells cultured under three different conditions: i) treated with a high glucose concentration (100 mM); ii) cells treated with a low glucose concentration (without additional glucose); and iii) cells maintained under standard glucose concentration (50 mM) that is used in BME26 maintenance media (control cells). Glycolysis, gluconeogenesis, glycogenolysis and glycogen synthesis pathways have been described in other organisms [4,5,10]. Research about these genes is important for the understanding of genetic causes of flux through these pathways in tick cells, with the aim of further elucidating arthropod physiology. In this regard, the development of tick cell cultures has presented great opportunities for performing experiments under controlled conditions, interfering with metabolic pathways, and understanding the processes that underlie genetic regulatory networks, metabolic fluxes, and the regulation of energy homeostasis. Thus, characterizing the expression patterns of key enzymes in energy metabolism in ticks [4,5,12] may yield new targets for developing novel acaricides and other interventions to control *R. microplus* infestations.

2. Methods

BME26 cell line

Cells were maintained as previously described [12]. The BME26 cells were maintained in Leibovitz's L-15 medium (Gibco BRL) supplemented with amino acids, glucose, mineral salts and vitamins [11]. During medium preparation, glucose was added at two different concentrations: 50 mM (control-the

usual glucose concentration) and 100 mM (high-glucose treatment). The medium was diluted in sterile water (3:1), and further addition of tryptose phosphate broth (10%), fetal calf serum (10%) and penicillin/streptomycin (100 U/mL and 100 µg/mL, respectively) were added. Cells from confluent flasks (25 cm^2) were resuspended in fresh complete medium using a 22-gauge needle with bent tip attached to a 5-mL plastic syringe. The culture density was determined using a Neubauer hemocytometer, and cell viability was determined using the trypan blue exclusion technique (0.4%, Sigma). An aliquot of 1×10^7 viable cells was transferred to 5 mL (final volume) of fresh complete medium and incubated at 34°C for up to two weeks to promote cell proliferation. The medium was replaced weekly to achieve high cell homogeneity. Next, 24-well plates ($5x10^5$ cells/well) were seeded with cells suspended in 500 µL of normal medium, incubated overnight for adhesion. Then, normal medium was completely replaced by either medium without the addition of glucose 50 mM (remaining only with the glucose present in the medium, called low-glucose treatment), with a normal glucose concentration (control), or by a high glucose concentration (high-glucose treatment). In the low-glucose medium, the content of glucose is that present in the medium itself (3.125 mM) (L-15 MEDIUM LEIBOVITZ composition, number L4386-Sigma). All these glucose concentrations were chosen based on the glucose present in the control medium, duplicating this amount or without glucose addition.

Viability assay

Cell viability was determined 24 hours after treatment with different media using MTT assay. Briefly, 50 µL MTT (5 mg/mL in PBS) were added on each well. After 2 hours of incubation at 34ºC, the medium was completely discarded, and 1 mL of acid-isopropyl alcohol (0.15% HCl in isopropyl alcohol) was added to dissolve formazan crystals. The mixture was transferred to 1.5-mL tubes and centrifuged at 6,000 x g for 15 minutes, and the clear supernatant was collected for absorbance measurements at 570 nm in a UVmini-1240 UV-VIS spectrophotometer (Shimadzu, Japan). Unless stated otherwise, the absorbance values of the control treatment were used for normalization (100% viability).

Membrane integrity analysis

BME26 cells were plated (3×10^5 cells/well) onto glass coverslips placed at the bottom of a 24-well plate. The cells were treated with different glucose concentrations. Subsequently, the cells were directly stained by adding Hoechst 33342 (0.4 µg/mL, final concentration) for 5 minutes, followed by staining with propidium iodide (2 µg/mL, final concentration) for further 2 minutes. The loss of membrane integrity was detected in cells stained with Hoechst 33342/Propidium iodide observed with pink nuclei under fluorescence microscope (Nikon 80i). Hoechst 33342 permeable cells were stained with blue nuclei. The incubations were performed at room temperature and in the dark. The well contents were discarded, and the coverslips were washed with PBS before being mounted over glass slides with 5 µL of glycerol. The cells were observed under a fluorescence microscope (model Eclipse 80i, Nikon, Japan), and images were obtained at 400× magnification.

Double-stranded RNA (dsRNA) synthesis

Oligonucleotide primers containing T7 promoter sequence were synthesized for the in vitro transcription and synthesis of dsRNA using RiboMAX^{TM} Express RNAi System Kit (Promega). cDNA from 1-day-old tick eggs was used for dsRNA synthesis. The dsRNA was purified according to the manufacturer's instructions, and its concentration was measured at 260 nm. An aliquot of dsRNA was analyzed by agarose gel electrophoresis to check for any degradation. Double-stranded RmGSK3 RNA was synthesized as described previously [12]. The negative control for the RNAi-induced gene silencing was an unrelated dsRNA designed for *E. coli* β-galactosidase (kindly donated by Professor Marcos H. Sorgine, Instituto de Bioquímica Médica [IBqM], Universidade Federal do Rio de Janeiro [UFRJ]). The sizes of the synthesized double-stranded RNA complexes were 635 bp for dsAKT, 800 bp for dsCN, and 798 bp for dsGSK.

dsRNA delivery into BME26 cells

BME26 cell suspensions were seeded into 24-well plates (5×10^5 cells/well) to a final volume of 500 µL of complete medium. After 24 hours of incubation at 34°C, the culture medium was replaced with 200 µL of fresh medium containing 4.8 µg of dsRNA/well, with gentle mixing. The cells were incubated for an additional 24 hours and were harvested for subsequent processing.

RNA extraction

Total RNA was extracted from the BME26 cells harvested from 24-well plates using Trizol reagent (Invitrogen) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed with the High-Capacity cDNA Reverse Transcription-M-MLV kit (Takara Biotechnology). Amplifications were performed on the LightCycler platform (Roche). Serial dilutions of the cDNA were used to construct a calibration curve. Reaction efficiencies between 85 and 100% were determined from calibration curves for each set of primers in 10 µL reactions. The *R. microplus* elongation factor-alpha gene (Elf1A) [14] was utilized as a reference to normalize the reactions. cDNA from the control BME26 cells was used as a calibrator for the assays. The relative expression of the calibrators was assigned a value of 1 unit. Statistical analyses (means and standard deviation) were performed on data from three independent experiments.

Relative quantification of metabolism genes by real-time PCR

A relative transcriptional analysis [15] was conducted with cDNA as a template for quantitative PCR using the LightCycler 480 II platform (Roche). Serial dilutions of cDNA were used for calibration curve preparation. Reaction efficiencies between 85 and 100% were determined from the calibration curves for each set of primers in 10 µL reactions. The primers used to amplify the targets are listed in Supplementary Table 1. The relative expression was determined using the Cp values from each run and the Relative Expression Software Tool [15].

Table 1.

Gene	Primers used for *R. microplus* genes	amplicon size (bp)	gene used as reference*	GenBank *access number of R. microplus gene*
Hexokinase	CATGGACAAAGAGCTTCAACTGCTC GGAAAGCTCCCTTGACCAGGGTA	150	XM_002412706.1 *Ixodes scapularis*	KF951259
Pyruvate kinase	GGGCAAGAGGGCAAGACAACTG CACGTTGAGCACCTTGGTGATG	141	XM_002407420.1 *Ixodes scapularis*	KF951260
Phophoenolpyruvate carboxykinase	CAAGCAATGAGTGCCTGCCAC ACAGTCTTCCGTTTTCATCTTG	147	XM_002413329.1 *Ixodes scapularis*	KF951261
Glucose-6- phosphatase	GGCAGCCATTTGGTACATCATCC CGACAGGCTGACAATGCACAGG	133	XM_002407091.1 *Ixodes scapularis*	KF951262
Glycogen synthase-6	GCTGGTATCGGGCTGATCCTG GATGCCTCTGTCTCCAGCCTCC	165	XM_002435718.1 *Ixodes scapularis*	KF951264
Phosphoglucomutase	CGGATCTGGGCAAGCTGGG CCGTCGTGACCCTTGATGAGG	151	XM_003695907.1 *Apis florea*	KF951265
Triose phosphate isomerizes	CCTCGCTGCACAAAATTGCTAC TCCGAATGACCCAGTATGACCC	128		EF014474
Protein kinase B	GGCCAAAGCCATTCACCTTCA CCTCCTCACTCGCCAACTTCTC	151		JX648548
Glycogen synthase kinase 3	CCCACACCCGCTATTTATTG TGTGCAGGAGAGCCAGTTTA	113		EF142066
Glycogen-debranching enzyme	ATGCTCAGGATCACGCAGAAGC GTACGTCGGTTGGGAAGGACAAGG	173	XM_002401176.1 *Ixodes scapularis*	

* Access numbers of published DNA sequences used to conduct Blast searches for matching gene transcripts in the *R. microplus* database (unpublished data).

Gene sequences for hexokinase (HK), pyruvate kinase (PK), phosphoenolpyruvate carboxykinase (PEPCK), glucose 6-phosphatase (GP), glycogen synthase (GS), phosphoglucomutase (PGM), triose phosphate isomerase (TIM), protein kinase B (AKT), glycogen synthase kinase 3 (GSK3), glycogen-debranching enzyme (GDE) and ATP citrate lyase (ACL) from different species were retrieved from the GenBank database (Table 1) and used to conduct a BLAST search of an *R. microplus* transcriptome database generated using the Illumina Solexa sequencing platform (unpublished data). The *R. microplus* gene sequences for TIM, AKT and GSK3 were obtained from GenBank. Specific primers for the genes were designed based on these *R. microplus* sequences (see Table I).

Hexokinase (HK) activity

BME26 cell suspensions were seeded into 24-well plates (5×10^5 cells/well) to a final volume of 500 µL of complete medium and lysate with PBS (5 mg/mL). The cell lysate was assayed for HK activity in 20 mM Tris-HCl pH 7.5 containing 6 mM $MgCl_2$, 1 mM ATP, 0.5 mM NAD^+ and 10 mM NaF, and the reaction was started with 2 mM glucose. The glucose 6-phosphate that formed was measured by adding an equal volume of 20 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 1 unit/mL glucose 6-phosphate dehydrogenase from *Leuconostoc mesenteroides* and 0.3 mM β-NAD^+. The production of β-NADH was determined at 340 nm using a molar extinction coefficient of 6.22 M^{-1}, as previously described [16,17].

Pyruvate kinase (PK) activity

Samples were prepared as described in the HK activity section. PK activity was measured in 20 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1 mM ADP, 0.4 mM NADH and 1 unit/mL lactate dehydrogenase, and the reaction was started with 1 mM PEP. The β-NADH consumption was evaluated using a Shimadzu U1240 spectrophotometer at 340 nm, with a molar extinction coefficient of 6.22 M-1, as previously described [16,17].

Oxygen consumption

The total oxygen consumed by BME26 cells after glucose treatments was assayed using a Clark-type electrode (YSI, mod. 5775, Yellow Springs, OH). The calibration process was executed using 100% as complete air-saturated buffer at 28ºC. Measurements were carried out in 1.5 mL 20 mM PBS buffer (pH 7.4) and the rate of oxygen consumption was calculated in mmol O_2/min/mL-cell. A solution containing 1 mM KCN was added to inhibit cytochrome oxidase. The same solution was used as negative control. Three

assays were performed using 1x107 cells/mL for three independent experiments in triplicate.

Statistical analysis

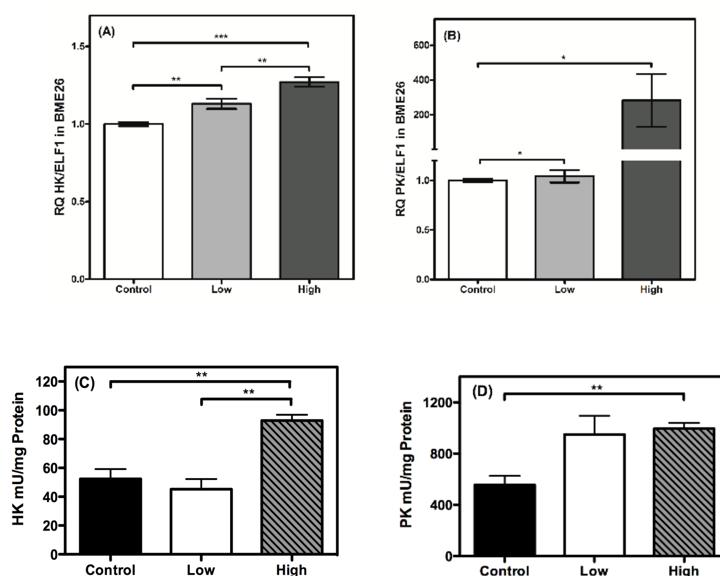
The experiments were performed with 3 independent biological samples in 3 experimental replicates each to obtain an average. All data values were expressed as mean ± S.D. The ANOVA was used to determine significant differences between groups when data were normally distributed. The t-tests were used when comparing data between two groups (Figure 8). The Tukey's test was used to compare data between three groups. Significance was set at $^{*}p < 0.05$; $^{***}p < 0.001$, ANOVA).

3. Results and Discussion

Ticks are adapted to survive under different environmental conditions, including periods of low energy availability, e.g., during starvation [18,19]. These and other studies have suggested that alternative metabolic strategies, some exclusive to ticks, may exist under different environmental conditions. In the present work, the transcription of genes that regulate energy metabolism in *R. microplus* was characterized using the BME26 embryonic cell line cultured in the presence of high or low glucose concentrations. These different glucose concentrations were selected based on the glucose necessary to maintain the cell line under normal condition (50 mM), duplicating this glucose amount (100 mM, called high-glucose cells) or not adding the usual glucose amount (remaining with 3.125 mM, called low-glucose). Alterations in glucose availability affect the expression profiles of genes that encode glycolytic/gluconeogenic enzymes, as well as genes involved in glycogen metabolism, and in catabolic and anabolic pathways. Glycolysis is classically considered the principal pathway for carbohydrate catabolism, and pyruvate kinase (PK) and hexokinase (HK) are key enzymes in regulating this pathway. In the present work, the transcription of PK and HK was significantly increased in cells treated with a high glucose concentration (Figure 1). In mammals, these transcriptional changes were observed in cells after high glucose treatment [20]. In the current case, it is likely that the glycolytic pathway in tick cells accelerates, in response to greater glucose availability in the cytoplasm, because key enzymes for carbohydrate metabolism were highly up-regulated. A large number of genes show high expression in response to high carbohydrate feeding, including PK and HK in cultured hepatocytes [21,22]. Both of

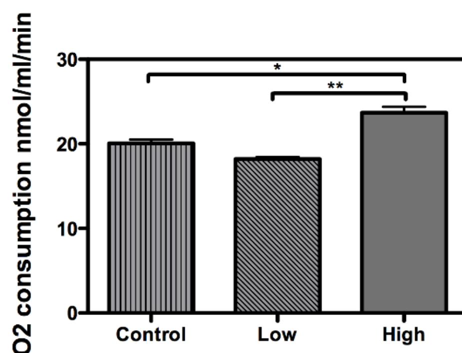
these glycolytic enzymes exhibit increases in the amounts of mRNA and protein, showing a clear sensitivity to glucose levels [23,24]. This hypothesis of sharpest glycolysis is supported by our data, whereby an increase in PK activity was observed in cells under high glucose treatment (Figure 1D), compared with the other conditions. Another glycolytic regulatory limiting step is triose phosphate isomerase (TIM), which also participates in gluconeogenesis. The TIM transcription level remained unchanged under varying glucose treatments relative to the control (data not shown). Because TIM reciprocally regulates both the carbohydrate catabolic and anabolic pathways [25], changes in substrate availability in these pathways probably do not alter TIM transcription, unlike other key enzymes exclusive to the glycolysis pathway, such as HK and PK (Figure 1).

Figure 1. Transcriptional and activities of glycolytic enzymes are glucose concentration-dependent in BME26 cells. Transcriptional analysis of hexokinase (A), pyruvate kinase (B), HK activity (C) and pyruvate kinase activity (D), glycolytic key-enzymes, in embryonic *Rhipicephalus microplus* cells (BME26) in response to glucose treatment. Control - cells maintained with 50 mM of glucose; Low - cell maintained without glucose addition, and High - cells maintained with 100 mM of glucose. The experiment was performed with 3 independent biological samples in 3 experimental replicates each (*p < 0.05; ***p < 0.001, ANOVA).



Interestingly, HK exhibited a higher relative transcription level in the cells treated with a low glucose concentration (Figure 1A), while HK activity remained unaltered during the same treatment (Figure 1C), underlining a differential relationship between transcriptional and enzymatic regulation for this enzyme. HK is responsible for phosphorylating glucose at carbon 6 to generate glucose-6-phosphate, allowing it to be trapped inside the cell and to be channeled into the glycolytic pathway, the pentose phosphate pathway or glycogen synthesis, and relies on a refined allosteric regulation mechanism [26]. Thus, HK activity possibly will present a different response of the transcriptional rate. In the presence of high glucose concentration, HK activity and transcription increased, as observed for PK. Our data also shows a higher oxygen consumption during high glucose treatment (Figure 2), suggesting (in addition with HK and PK) an increased glycolysis specific to this condition, not in low glucose treatment.

Figure 2. Higher oxygen consumption under high-glucose treatment. The oxygen consumption rate was measured in embryonic *Rhipicephalus microplus* cells (BME26) in response to glucose treatment. Control - cells maintained with 50 mM of glucose; Low - cell maintained without glucose addition, and High - cells maintained with 100 mM of glucose. The experiment was performed with 3 independent biological samples in 3 experimental replicates each (*p < 0.05; ***p < 0.001, ANOVA).



A cell viability assay was performed for the three culture conditions (Figure 3). Under low glucose treatment, cell viability was lower than in the control cells. However, cell viability was enhanced under treatment with a high glucose concentration. Mitochondrial hexokinase activity is critical for sustaining constant ADP steady-state cycling, which in turn reduces the membrane potential and consequently decreases mitochondrial ROS formation, as previously described in rat brain cells [27,28]. Thus, glucose supports an increase in HK activity, leading to oxidative stress protection and higher cell survival

[28]. Furthermore, if glucose availability is extremely high, the mitochondrial hexokinase activity decreases when ADP is produced. In this case, ROS production leads to a decrease in cell viability [29]. In this study, an increase in glucose disposal under high glucose condition is likely able to improve the cell's energetic fitness, resulting in higher viability, unlike cells cultured in low glucose levels. Furthermore, the microscopy analysis with propidium iodide did not show loss of membrane integrity, in any treatment (Figure 4).

Figure 3. Glucose availability has an essential role in BME26 cell survival. Cell viability was performed in embryonic *Rhipicephalus microplus* cells (BME26) in response to glucose treatment. Control - cells maintained with 50 mM of glucose; Low - cell maintained without glucose addition and High - cells maintained with 100 mM of glucose. The experiment was performed with 3 independent biological samples in 3 experimental replicates each (*$p < 0.05$; ***$p < 0.001$, ANOVA).

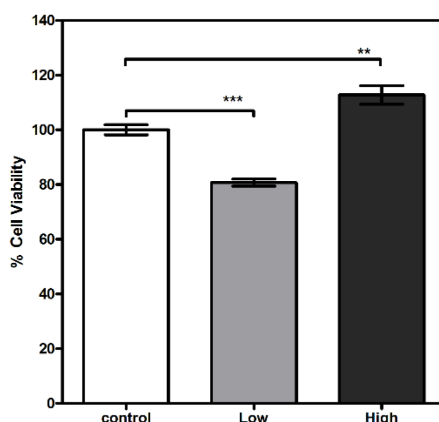
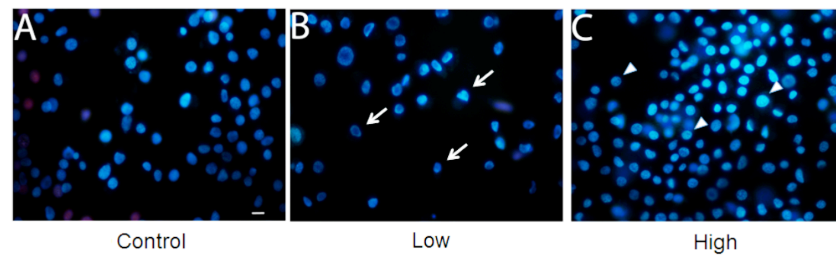


Figure 4. Membrane integrity is unaffected in BME26 cells after glucose treatment. The cells were directly stained by adding Hoechst 33342 and propidium iodide. Glass slides were observed in a fluorescence microscope (model Eclipse 80i, Nikon, Japan), and pictures were obtained at 400× magnification. Control - cells maintained with 50 mM of glucose; Low - cell maintained without glucose addition and High - cells maintained with 100 mM of glucose. The arrows indicate the shape of low-glucose cells and the triangles indicate the rounded shape of high-glucose cells.



The regulation of glycogen synthesis and degradation is a complex process involving several enzymes and control points, and it is highly sensitive to changes in cell energy balance [30,31]. When the insulin-signaling pathway (ISP) is activated in response to elevated glucose, GSK3 activity is reduced and GS phosphorylation (inhibition) is blocked [32,33]. Under this condition, GS remains active and catalyzes glycogen synthesis. In BME26 cells, it was recently demonstrated that ISP was activated in response to exogenous insulin with increased glycogen levels [3], and these effects were cancelled out in the presence of PI3K inhibitors (wortmannin or LY294002). However, a transcriptional analysis of the genes involved in glycogen synthesis and degradation has not been reported in tick cells.

GSK3 transcription was elevated in the cells treated with low glucose concentrations in the present study. This enzyme participates in numerous cellular processes, including the regulation of GS by phosphorylation [32]. Apparently, a higher transcriptional level of GSK3 did not affect GS transcription in the low-glucose cells (Figure 5), reinforcing the reciprocal regulation between both enzymes that occurs at the enzymatic level. However, when the cells were treated with high amounts of glucose, GSK3 transcription was significantly reduced (Figure 5B), accompanied by an increase in GS transcription in cells under similar treatment (Figure 5A). This result may suggest the presence of mutual transcriptional regulation. Metabolic pathways that respond to cell glucose availability usually exhibit mutual transcriptional regulation [34]. In cells stimulated by insulin, AKT induces HK/GK expression [35]. A similar process is observed between PFK2/FBPase2 and HK/GK [36]. Nevertheless, high-glucose treatment can lead to increased levels of glycogen, which is normally stored under these conditions [3]. Therefore, this higher GS transcription could be directly related to an increase in the amount of glucose entering the cell and may not be related to the reduction in GSK3 transcription itself because the supposedly coordinated regulation between both enzymes was not observed in all the glucose treatments. Recently, Abreu *et al*. [37] reported that the GSK3/ATK axis in BME26 cells is involved in glycogen synthesis regulation and cell survival. AKT is a component of ISP, and phosphorylates a wide range of substrates, including GSK3, resulting in GSK3 inhibition [38,39]. AKT transcription did not change 24 hours after the

glucose treatments (data not shown). Collectively, the transcription of these enzymes appears to respond to changes in glucose availability and may share a mutual genetic regulation.

The regulation of glycogen mobilization was assessed by analyzing PGM and GDE transcription. Both enzymes are involved in glycogen polymer degradation. GDE cleaves the α-1,6-glycosidic bond between adjacent glucose molecules in the glycogen polymer, assisting the glycogen phosphorylase and PGM [40,41]. PGM transcription is elevated in low-glucose cells (Figure 6B). This result suggests a type of genetic regulation that increases the transcript level of PGM when glucose availability is low and glycogen mobilization is necessary. On the other hand, the transcription of GDE does not change when the glucose amount is low (Figure 6A). However, when the cells were treated under high-glucose conditions, GDE transcription increased significantly, indicating a differential genetic regulation in relation to PGM.

Figure 5. Glycogen metabolism synthesis has a transcriptional control in BME26 cells. Transcriptional analysis of glycogen synthase (A) and glycogen synthase kinase 3 (B) in embryonic *Rhipicephalus microplus* cells (BME26) in response to glucose treatment. Control - cells maintained with 50 mM of glucose; Low - cell maintained without glucose addition and High - cells maintained with 100 mM of glucose. The experiment was performed with 3 independent biological samples in 3 experimental replicates each (*p < 0.05; ***p < 0.001, ANOVA).

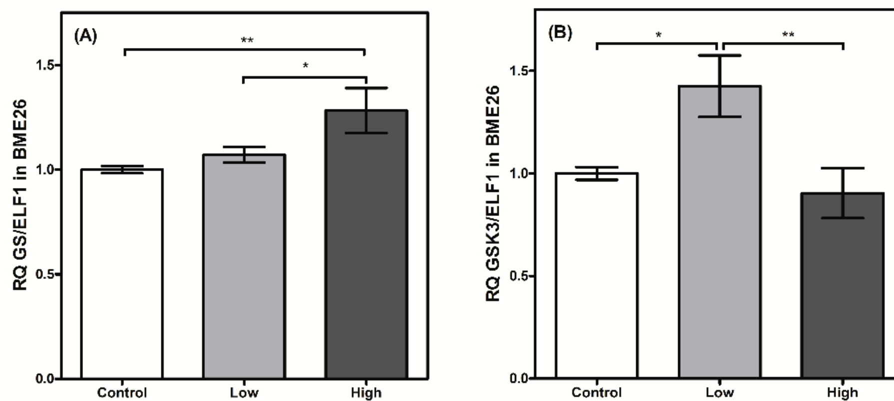
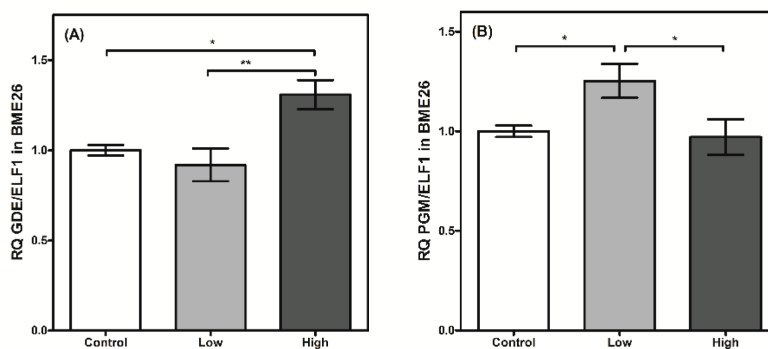


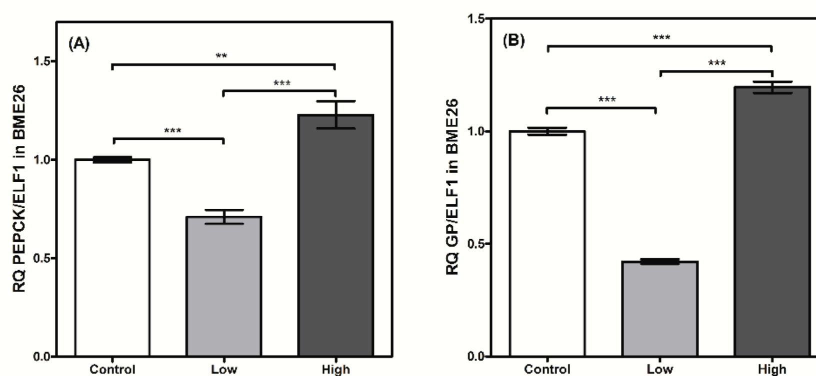
Figure 6. Glycogen degradation has a transcriptional control in BME26 cells. Transcriptional analysis of glycogen debranching enzyme (A) and phosphoglucomutase (B) in embryonic *Rhipicephalus microplus* cells (BME26) in response to glucose treatment. Control - cells maintained with 50 mM of glucose; Low - cell maintained without glucose addition and High - cells maintained with 100 mM of glucose. The experiment was performed with 3 independent biological samples in 3 experimental replicates each (*p < 0.05; ***p < 0.001, ANOVA).



Gluconeogenesis produces glucose from non-glycosidic compounds, and it is an important strategy for the maintenance of cell energy homeostasis. PEPCK and GP are regulatory enzymes that catalyze the initial and final steps of gluconeogenesis, respectively [42,43]. GP removes a phosphate from glucose-6-phosphate to produce free glucose. Both enzymes exhibited similar transcriptional profiles across glucose treatments, showing reduced amounts of transcripts in the low glucose-treated cells (Figure 7). In the high-glucose cells, the opposite was observed, with increased transcription levels of both PEPCK and GP. Under normal physiological conditions, when glucose levels become low, as in starvation, the gluconeogenic flux accelerates [44,45]. In such cases, it is expected that these enzymes will undergo higher transcription when glucose levels are reduced, particularly PEPCK, as an enzyme that is mainly regulated by transcription in mammals. Surprisingly, in BME26 cells, this transcriptional profile was reversed. In cell culture, because there are no groups of specialized cells as seen in vivo, glucose dephosphorylation catalyzed by GP would result in the release of glucose and, consequently, the loss of this metabolite. Cells treated with low glucose may lack carbohydrate reserves [20], and a decrease in GP transcription is necessary to avoid the additional loss of glucose content. On the other hand, GP transcription increases in high-glucose cells. Massillon [46] observed an increase in GP transcription when glucose

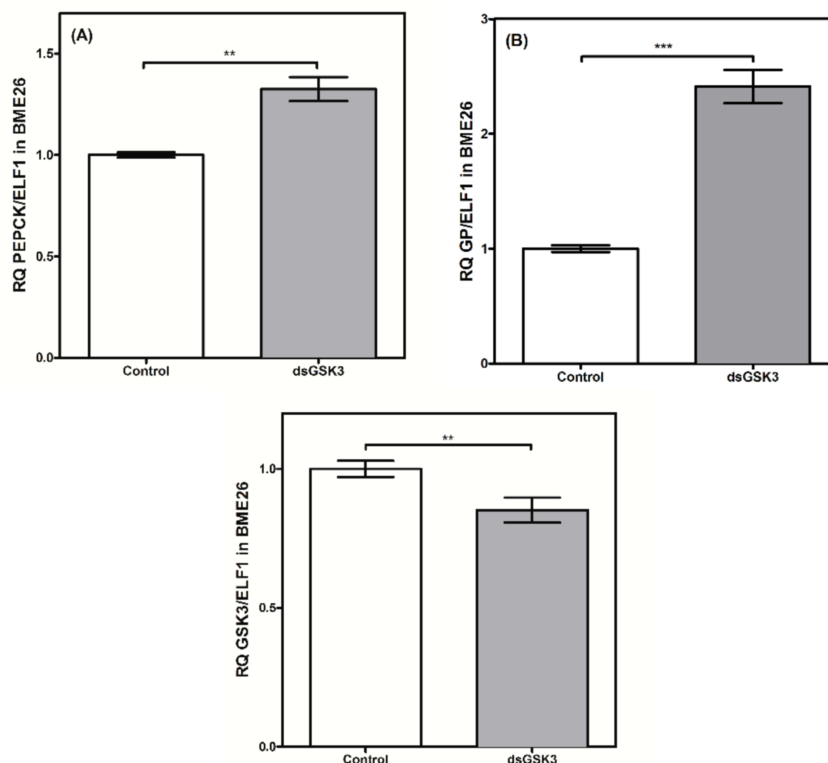
levels were elevated in hepatocyte cell culture, showing a dose-dependent response. The uptake of large amounts of glucose and the consequent phosphorylation of glucose inhibits HK activity as a result of elevated glucose-6-phosphate levels, leading to ROS generation by mitochondria [27]. In the context of cell culture, GP may be necessary to allow the diffusion of excess glucose away to avoid cell damage. PEPCK is also increased at the transcriptional level in high glucose-treated cells (Figure 7A). In addition to regulating gluconeogenesis, PEPCK regulates glyceroneogenesis, a pathway required for free fatty acid re-esterification to maintain an active level of triglyceride synthesis [43,47]. Under high carbohydrate availability, the flux through glyceroneogenesis increases [43]. Due to this phenomenon, we postulated that an increase in PEPCK transcription reflects a condition of high energy availability and possible glyceroneogenesis induction. Such genic profile in high glucose tick cells is very similar to observed in mammalian diabetic cells [48]. Interestingly, the glycolysis presented increased at this same moment, suggesting a deviation of pyruvate produced by glycolysis to gluconeogenesis or glyceroneogenesis (Figure 1). Indeed, a change in cell phenotype characterized by altered morphology was observed following glucose treatments. Cell culture heterogeneity was higher in the low-glucose cells than in control cells.

Figure 7. Gluconeogenic response increase in high glucose concentration in BME26 cells. Transcriptional analysis of PEPCK (A) and glucose-6-phosphatase (B), gluconeogenic key-enzymes, in embryonic *Rhipicephalus microplus* cells (BME26) in response to glucose treatment. Control - cells maintained with 50 mM of glucose; Low - cell maintained without glucose addition and High - cells maintained with 100 mM of glucose. The experiment was performed with 3 independent biological samples in 3 experimental replicates each (*p < 0.05; ***p < 0.001, ANOVA).



These results demonstrate a differential genetic regulation between cell culture and in vivo models. Nevertheless, both gluconeogenesis enzymes were regulated in a coordinated manner, with similar transcriptional profiles. Our group has been investigating the role of GSK3 in a wide range of metabolic processes, including gluconeogenesis. GSK3 knockdown induces increases in PEPCK and GP transcription levels (Figure 8), suggesting an indirect genetic regulation of the gluconeogenesis pathway through GSK3 (data not shown).

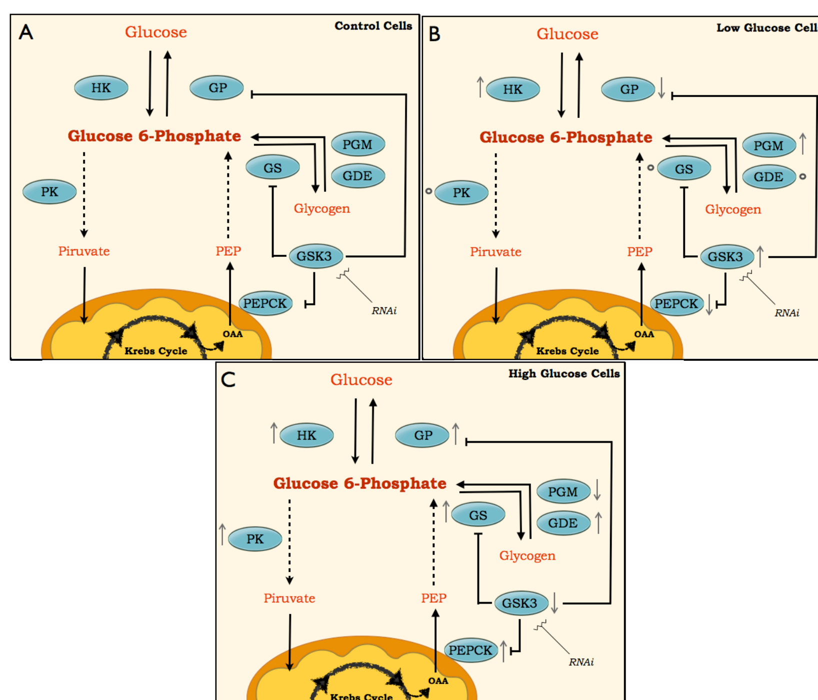
Figure 8. Gluconeogenic enzymes has transcriptional control by GSK3 in BME26 silenced-cells. Transcriptional analysis of PEPCK (A) and glucose-6 phosphatase (B), gluconeogenic key-enzymes, in embryonic *Rhipicephalus microplus* cells (BME26) in response to GSK3 silencing (C). Control - cells maintained with 50 mM of glucose; Low - cell maintained without glucose addition and High - cells maintained with 100 mM of glucose. The experiment was performed with 3 independent biological samples in 3 experimental replicates each (*p < 0.05; ***p < 0.001, ANOVA).



To date, and to our knowledge, no mutual regulation between GSK3 and gluconeogenesis has been reported. Future studies could focus on whether any direct enzymatic regulation

between GSK3 and other gluconeogenic enzymes exists. Figure 9 summarizes all metabolic and molecular changes in the respective treatments. Altogether, these results contribute to the understanding of the mechanisms that control glucose metabolism at the genetic level in the model cell line BME26. Characterizing changes in metabolic enzymes at both the transcriptional and functional levels in response to nutrient availability may lead to the identification of genes that are critical for maintaining the cellular energy balance. Moreover, this research may yield potential antigens for improved anti-tick vaccines or novel targets for acaricide action, which are urgently needed to control the tick vector R. microplus.

Figure 9. Scheme for pathways of glucose metabolism. The scheme is based on enzyme activities, metabolites and molecular changes in BME26 cells after glucose treatments. The enzymes studied are presented inside blue spheres (HK, PK, GS, GSK3, PGM, GDE, PEPCK and GP). Arrows next to the enzymes (↓ ↑) represent the oscillations in enzymatic activity and transcriptional response. Small spheres next enzymes (○) indicate no variations in these enzymes or genes compared with the control. The low-glucose (B) and high-glucose (C) are compared with the control (A).



4. Conclusion

In conclusion, a number of genes involved in the major pathways for carbohydrate catabolism and anabolism were evaluated in a tick embryonic cell line. The results show that glycogen synthase (GS), glycogen synthase kinase 3 (GSK3), phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (GP), exhibited mutual gene regulation in response to glucose treatment. These results improve the understanding of glucose metabolism in ticks.

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I

ANEXO II

Gluconeogenesis is a Key Pathway for Energy Support in *Aedes aegypti* Egg Quiescence

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Abstract

The *Aedes aegypti* mosquito is a medically important vector of viruses that cause yellow fever and dengue, with global impact especially in Africa and Latin America. Aedini mosquitoes, including the *A. aegypti* species, produce eggs that are resistant to desiccation and undergo quiescense to increase survival chances and facilitate re-infestation. Quiescense is a physiological adaptation induced by environmental stimuli and characterized by an interruption in development, which enables the survival of animals under unfavorable environmental conditions. However, metabolism control is essential to support these organisms throughout this process. Carbohydrate metabolism has been proven to be of great importance during arthropod embryogenesis, which changes dramatically, in order to promote growth and differentiation. This study investigates the fundamental aspects of glucose metabolism during the three stages of quiescent *A. aegypti* eggs: pre-quiescence, quiescence and post-quiescence. The activities of regulatory enzymes of carbohydrate metabolism such as pyruvate kinase (PK), hexokinase (HK) and glucose 6-phosphate dehydrogenase were evaluated. We show that these regulatory enzyme activities were reduced in *A. aegypti* mosquito quiescent eggs, suggesting a lower flow of nutrients through glycolytic and pentose phosphate pathways. In contrast, the gluconeogenesis pathway increased during quiescence, due to the mobilization of egg proteins to supply substrates to glucose synthesis. Additionally, the protein amount decreased during this stage, and the glucose levels increased when gluconeogenesis was higher. Glycogen content, major carbohydrate reserve in mosquitoes, was evaluated and was reduced in eggs

during quiescence and post-quiescence, supplying the energy metabolism. We observed that with reactivation of carbohydrate catabolism and an increased flow-through in gluconeogenesis after rehydration of eggs during quiescence, glucose metabolism was apparently essential not only to maintain quiescence, but also for larvae to hatch. Taken together, these results contribute to a better understanding of the molecular mechanisms that regulate a hypometabolic state that ensures higher survival rate and consequently the completion of the life cycle and a key adaptative feature of *A. aegypti* mosquito.

Keywords

Metabolism, Gluconeogenesis, Glycolysis, Mosquito, Diapause, Glucose

Abbreviations

HK: hexokinase; PK: pyruvate kinase; PEPCK: phosphoenolpyruvate carboxykinase; G6PDH: glucose-6-phosphate dehydrogenase; G6P: glucose-6 phosphate; PEP: phosphoenolpyruvate.

1. Introduction

Animals need proper nutrition for growth, survival, and as a source of energy for cellular processes. However, nutrients in the environment change with seasonal climatic transitions. To overcome these unfavorable conditions, some arthropods adopt diapause or quiescence as a survival strategy (Perez and Noriega, 2013). Diapause is a stage characterized by environmentally triggered developmental arrest, which allows animals to survive under unfavorable environmental conditions, synchronizing periods of growth and reproduction only

in optimal conditions (Lees, 1995; Tauber and Tauber, 1976; Denlinger, 1986; Denlinger, 2002). Quiescence is similar to diapause with regard to developmental arrest, but differs in other points. Quiescence is a dormancy state induced in direct response to unfavorable environmental conditions and is immediately terminated upon the return of favorable environmental conditions (Denlinger and Armbruster, 2014). Species-specific physiological changes occur during these two forms of developmental arrest, including decreased metabolism, enhanced stress tolerance and increased protein production. These physiological changes ensure protection against numerous environmental stressors, including dryness, extreme temperatures, and oxidative stress (Denlinger, 2002; Lee et al., 2002; Clegg, 1965).

Multiple organisms are able to start diapause or quiescence, including arthropods with important roles in disease transmission. The impact of the insects on human health as disease vectors or as agricultural pests has prompted the discovery of novel control methods. This work investigates quiescence in the mosquito *Aedes aegypti* and the increased lifespan promoted by this survival strategy. It is also compared with metabolic changes in diapause of numerous widely studied organisms. This mosquito is an important vector for dengue and yellow fever viruses, causing considerable public health impact in tropical and subtropical countries (Gould and Solomon, 2008). The failure of hatching in *Aedes aegypti* eggs that have been deposited above the water line is considered quiescence because development can be prompted immediately upon immersion in water (Perez and Noriega, 2013), not environmentally programmed as in the *Aedes albopictus* where this dormancy is considered diapause because starts in advance of its onset (during the mother's life) and will persist even if the eggs are submerged in water (Poelchau et al., 2013).

One of the hallmarks of a dormancy period is metabolism depression, accompanied by a decrease in biosynthetic activities, including protein synthesis (Wigglesworth, 1957; Kunkel and Williams, 1951), since larvae and adult insects perform diapause or quiescence utilizing energy reserves obtained before the dormancy period (Denlinger, 2002; Hahn and Denlinger, 2007). Moreover, embryogenesis occurs without exogenous nutrient supplies (Stewart and Thompson, 1993), because all nutrients necessary for embryo development are deposited in the eggs during oogenesis, except the oxygen for aerobic metabolism, and water in some species (Rahn *et al*., 1974; Vleck, 1991).

The transmission of dengue is favored by the quiescence of *A. aegypti* eggs (Christophers, 1960; Kliewer, 1961; Clements, 1992), for it improves survival of eggs in environment. The *A. aegypti* females lay their eggs in humid substrates near the water surface (Funasa, 2001). During oviposition, these eggs are permeable to water; however, within around 20 hours of development the eggs acquire a layer that lends resistance to desiccation, the serosal cuticle (Rezende *et al.*, 2008). Despite water resistance, the eggs need appropriate energy metabolism modulation to survive for long periods (Denlinger, 2002), since the embryonic process requires high-energy levels in order to sustain cell proliferation and development and, subsequently, promote egg hatching (Thompson and Stewart, 1997). Metabolic modulation during dormancy pediods varies from species to species, and different organisms mobilize diverse classes of nutrients in order to obtain the energy necessary in the latency period (Adedokun and Denlinger, 1985; Yaginuma *et al*., 1990; Godlewski *et al*., 2001). The beetle *Leptinotarsa decemlineata* uses carbohydrate reserves primarily in the early diapause stage. However, if the latency period extends for too long, the beetle starts to mobilize lipid reserves (Lefevere *et al*., 1988). In contrast, the fly *Sarcophaga crassipalpis* saves carbohydrate reserves for the late

diapause stage, for arousing (Adedokun and Denlinger, 1985). Since carbohydrate reserves are mobilized by various organisms as a way to obtain energy readily, it is a candidate to maintain the early stage of *A. aegypti* quiescence.

Here, key enzymes involved in the gluconeogenic and glycolytic pathways in three eco-physiological phases (pre-quiescence, quiescent, and post-quiescence) are characterized (Kostal, 2006), and it is shown that gluconeogenesis is the most active pathway during *A. aegypti* quiescence. These results can help understand the modulation of energy metabolism in the mosquito quiescent stage and correlates with another dormancy periods as diapause

2. Methods:

2.1 Mosquito maintenance

Aedes aegypti (Rockefeller strain) mosquitos were reared and maintained in the laboratory. Larvae were fed rat food, and adults were fed sucrose solution 10% (w/v). For egg production, female mosquitoes were blood-fed on mice.

In the oviposition assays, females were kept at 28°C in a humidity chamber with a 12 h:12 h (light:dark) cycle, 80% humidity and maintained at 28ºC for a period of 24 and 48 h after a blood meal.

2.2 Synchronous Egg-laying and eggs homogenates

Adult *A. aegypti* females were left to oviposit as described by literature (Rezende et al., 2008), with slight modifications. The egg development was defined to start 30 min after egg laying. Eggs were kept in a humidity chamber at 28°C and 80% humidity until the end of embryogenesis.

A. aegypti eggs were collected under three different conditions: pre-quiescence (PRQ), quiescent (Q) and post-quiescence (PSQ). The pre-quiescence stage (PRQ) eggs included synchronized 62-h eggs (end of embryogenesis) without desiccation. The quiescent stage (Q) eggs were synchronized 62-h eggs that were removed from water and kept dry for 2 weeks. Finally, the post-quiescence stage (PSQ) eggs included quiescent eggs that were rehydrated (soaked in water) for 9 min (mean time for hatching) to interrupt quiescence, and immediately used in the experiments.

Twenty milligrams of synchronized PRQ, Q and PSQ eggs wee used for each group in all experiments. The amount of eggs in 20 mg was the same for each condition (PRQ, Q and PSQ). Egg homogenates were prepared by mechanical homogenization in appropriate buffer according to the protocols used for each enzyme, followed by centrifugation (200 × *g* for 10 min). The supernatant was used to determine enzyme activities and measure the metabolites. To test enzyme activities, a protease inhibitor cocktail was added to egg homogenates containing 1 μM phenylmethylsulfonyl fluoride (PMSF), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 μM pepstatin A, and 10 μM leupeptin. Total protein content was determined according to the Lowry method (Lowry *et al*., 1951), using bovine serum albumin as standard.

2.3 Relative quantification of PEPCK transcripts by RT-qPCR in the A. aegypti eggs Total RNA was extracted from eggs using the Trizol reagent (Life Technologies) according to the manufacturer's instructions. The amount of total RNA was estimated by spectrophotometry at 260/280 nm in a Shimadzu spectrophotometer U1240.

Two micrograms of total RNA were reverse-transcribed using the High-capacity cDNA Reverse Transcription synthesis kit according to the manufacturer's instructions (Life Technologies). Amplification was performed in a real-time PCR LightCycler 1.5 (Roche) that uses a capillary platform. Serial dilutions of cDNA were used to construct the calibration curve for each pair of primers. Primer efficiencies between 85 and 100% were determined from calibration curves for each set of primers in 10-μL reactions. Three distinct sequences of the *A. aegypti* PEPCK genes are deposited in GenBank with the following Transcripts ID AAEL000025-RA, AAEL000006-RA and AAEL000080-RA, referred to as PEPCK (25), PEPCK (6), and PEPCK (80), respectively. Specific primers were designed for each of the three *A. aegypti* PEPCK genes. The primers used for the relative expression determination of specific PEPCK genes were 5`-TTCCGTAGCGGACAACCAAATCC-3' (forward) and 5`-AGCGTGAAGAGTATTAATCAGGGTAGCA-3' (reverse) for PEPCK gene (25) sequence , 5`-GCCAAAGGCCATCTACAACCACC-3` (forward) and 5`-TCGGTGTAGCATCTTCAGTAGTGTGTTG-3' (reverse) for PEPCK gene (6) sequence, and 5`-CCCAAGTCGCAAAACGTGATCC-3' (forward) and 5`-GATGGTGCCTTGGGCCTGTAGAG-3 ' (reverse) for PEPCK gene (80) sequence. Relative expression was determined using the Cp values obtained for each reaction using the Relative Expression Software Tool-REST (Pfaffl, 2001), and primers for the constitutive rp49 gene as an internal reference gene (Gentile *et al*., 2005). Then, the PrQ stage was used as calibrator to the relative PEPCK expression during the stages investigated. The purity of the total RNAs used was pre-tested before use of the respective cDNAs for PEPCK

relative quantification. For this test, we used specific primers for *A. aegypti* PEPCK (25), PEPCK (6), and PEPCK (80) with the Master SYBR Green I kit (Roche), according to the manufacturer's instructions in 10-µL reactions. PCR reactions were performed in real time.

2.4 Phosphoenolpyruvate carboxykinase (PEPCK) activity assay

Egg homogenates (20 mg/volume) from each experimental condition (PRQ, Q, and PSQ) were obtained in extraction buffer containing 100 mM HEPES buffer, pH 7.0. Supernatant aliquots were assayed (in triplicates) in 400 µL of 100 mM HEPES buffer pH 7.0 containing 10 mM $MnSO_4$, 100 mM $KHCO_3$, 2 mM reduced glutathione, 10 mM PEP, 0.2 mM NADH, and 24 units of malate dehydrogenase (Sigma Chemicals). The reaction started by the addition of 10 µL 2.5 mM inosine diphosphate (IDP). The consumption of b-NADH was monitored at 340 nm, and PEPCK activity was determined as described by Petersen (Petersen et al., 2001).

2.5 Determination of glucose content

Eggs were homogenized (20 mg/volume) in 200 mM phosphate buffered saline (PBS) pH 7.4. Glucose content was enzymatically quantified by glucose oxidase (Glucox® enzymatic Kit for glucose determination; Doles, Inc.). After 30 min of incubation at 37°C, samples were read at 510 nm in a Shimadzu U1240 spectrophotometer, according to the manufacturer’s instructions. Free glucose was determined using a standard curve submitted to the same treatment.

2.6 Hexokinase (HK) activity assay

Eggs (20 mg) were homogenized (PRQ, Q, and PSQ) in extraction buffer containing 20 mM Tris-HCl, pH 7.5 $MgCl_2$. Supernatant aliquots (in triplicates) were assayed in 20 mM Tris-HCl pH 7.5 containing 6 mM $MgCl_2$, 1 mM ATP, 0.5 mM NAD^+ and 2 mM glucose. HK catalytic activity was measured by adding *Leuconostoc mesenteroides* glucose 6-phosphate dehydrogenase (Sigma-Aldrich Chemicals) (Worthington Code: ZF or ZFL) dissolved to 1 UI/mL in Tris-$MgCl_2$ buffer described above (Tøien et al., 2011). The production of b-NADH was monitored at 340 nm in a Shimadzu U1240 spectrophotometer using a molar extinction coefficient of 6.22 M^{-1}, as described by Worthington (Worthington, 1998).

2.7 Pyruvate kinase (PK) activity assay

Eggs (20 mg) were homogenized (PRQ, Q and PSQ) in extraction buffer containing 20 mM Tris-HCl pH 7.5 with 5.5 mM $MgCl_2$. Supernatant aliquots (in triplicate) were assayed in 1 mL of 20 mM Tris-HCl pH 7.5 containing 5.5 mM $MgCl_2$, 1 mM ADP, 0.4 mM NADH, 1 unit/mL lactate dehydrogenase and 1 mM phosphoenolpyruvate. Consumption of b-NADH was monitored at 340 nm in a Shimadzu U1240 spectrophotometer using a molar extinction coefficient of 6.22 M^{-1}, as described by Worthington (Worthington, 1998).

2.8 Mosquito eggshell clarification

Eggs obtained from synchronous eggs laying (PRQ, Q and PSQ) were fixed and clarified according to Trpiš (Trpiš, 1973). This technique fixes the embryo and turns the eggshell

transparent. Eggs were viewed under a magnifying glass with 10X magnification for the evaluation of normal embryo morphology.

2.9.1 Determination of glycogen content

Egg (20 mg) homogenates were prepared in 200 mM sodium acetate, pH 4.8, and supernatant aliquots were incubated with 1 unit of a-amyloglucosidase (Sigma Chemicals) for 4 h at 40° C. The newly generated glucose was enzymatically determined by glucose oxidase as described for glucose content determination. Free glucose was subtracted from samples without a-amyloglucosidase. Glycogen content was determined using a standard curve submitted to the same conditions.

2.9.2 Glucose-6-phosphate dehydrogenase (G6PDH) activity Assay

Egg (20 mg) homogenates were homogenized in extraction buffer containing 55 mM Tris-HCl pH 7.8 for each condition (PRD, D and PSD). Supernatant aliquots (in triplicate) were assayed in 1 mL of 55 mM Tris-HCl, pH 7.8 containing 6 mM MgCl2, 100 mM glucose 6-phosphate and 0.5 mM b-NADP+. The reaction was started with sample addition. The formation of b-NADPH was monitored at 340 nm in a Shimadzu U1240 spectrophotometer during 5 min, using a molar extinction coefficient of 6.22 M-1 as described by Worthington (Worthington, 1998).

2.9.3 Determination of Glucose-6-phosphate content

Egg (20 mg) homogenates were prepared in 55 mM Tris-HCl pH 7.8 for each condition (PRD, D and PSD). Supernatant aliquots (in triplicate) were assayed in 1 mL of 55 mM Tris-HCl, pH 7.8 containing 6 mM MgCl2, 100 mM, 0.5 mM b-NADP+ and G6PDH (300U/ml). The reaction was started with sample addition. The formation of b-NADPH was monitored at 340 nm in a Shimadzu U1240 spectrophotometer during 5 min, using a molar extinction coefficient of 6.22 M-1 as described by Worthington (Worthington, 1998).

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2.9.4 Phylogenetic analysis of PEPCK in insects

Aedes aegypti PEPCK protein sequences were used as queries for BLAST searches in other insect genomes. The best protein sequence model was calculated with MEGA6 and maximum likelihood trees were calculated. One hundred bootstraps were used for tree resampling.

2.9.5 Statistical analysis

The experiments were carried out in triplicate. All data values were expressed as mean ± S.D. The ANOVA was used to determine significant differences between groups when data were normally distributed. The Tukey's test was used to compare data between three groups. Significance was set at $*p < 0.05$; $***p < 0.01$, ANOVA).

3. Results

3.1 Glycolytic pathway decreases in A. aegypti diapause eggs

The glycolytic pathway was evaluated in *A. aegypti* pre-quiescence, quiescent, and post-quiescence embryos to determine the enzymatic activity of pyruvate kinase (PK) (Figure 1A) and hexokinase (HK) (Figure 1B). Comparing with the end of embryogenesis (pre-quiescence), the PK and HK activities exhibited a statistically significant decrease during quiescence. In post-quiescence embryos, PK activity returns to a level that is similar to that observed in the pre-quiescence period ($p < 0.05$); however, HK activity is lower than in the previous stages ($p < 0.001$). Glucose 6-phosphate levels were low in pre-quiescence, increased in quiescence, and decreased again in post-quiescence eggs (Figure 2).

3.2 Larvae external morphology after diapause induction

Egg morphology was not affected across quiescence and post-quiescence induction (Figure 3), showing a 2-week quiescence period did not cause visual morphological differences.

3.3 PentosePhosphate pathway decreases in diapause

The activity of G6PDH, a key-enzyme in pentose-phosphate pathway, decreases during the quiescent stage, returning to a level similar to the level observed in the pre-quiescence period (Figure 4). Concomitantly, glycogen levels decreased continually during quiescence (Figure 5).

3.4 Gluconeogenesis increases in diapause and post-diapause eggs

The gluconeogenic pathway was evaluated in *A. aegypti* during pre-quiescence, quiescent, and post-quiescence stages, upon determination of phosphoenolpyruvate carboxykinase (PEPCK) enzymatic activity, total protein content and free glucose levels. Protein level was high in pre-quiescence eggs, and decreased in quiescent and post-quiescence eggs (Figure 6). PEPCK activity was low during pre-quiescence, increasing during quiescence and post-quiescence eggs (Figure 7A). Glucose level also was low in pre-quiescence eggs, and increased in quiescent and post-quiescence eggs (Figure 7B). The same pattern was observed for PEPCK activity. The protein presented an inverse relation with PEPCK activity.

3.5 Phylogenetic and transcriptional analysis of PEPCK genes in the A. aegypti eggs

Three PEPCK genes are present in the *A. aegypti* genome (PEPCK-6, PEPCK-25, and PEPCK-80), which can be grouped with other mosquito PEPCKs, out of different arthropods (Figure 9A). The relative amounts of transcripts (mRNA) of the three *A. aegypti* PEPCK showed differences in the phases of quiescence. PEPCK-80 and PEPCK-25 had higher expression in pre-quiescence and quiescence, respectively (Figure 9B and C).

4.0 Discussion

Carbohydrates are the most abundant biomolecules in living organisms. Insoluble carbohydrate polymers function as structural elements and as protection in bacterial and plant cell walls. Carbohydrates also bind to proteins and lipids covalently, acting as signals to determine intracellular localization and metabolic fate of these molecules. In addition, carbohydrates have a major role as energy reserves, and play a central role in

metabolism in most organisms. Our group previously demonstrated that the germ band retraction is a morphological landmark, which indicates that glucose metabolism undergoes a dramatic alteration, suggesting that carbohydrate metabolism is fundamental to regulate *A. aegypti* mosquito embryogenesis (Vital *et al*., 2010). Therefore, the study of the nutrient utilization dynamics during quiescence complements the prior work performed during *A. aegypti* mosquito embryogenesis.

Quiescence and diapause prolongs lifespan of an insect, which therefore is better prepared to wait until the appropriate stimulus is provided, when it can then complete its dormancy period (Denlinger, 2002; Danks, 1987; Zaslayski, 1988). This strategy ensures the organisms' reproductive success and the completion of its life cycle. Thus, modulation of energy metabolism is essential for insect survival and for reinfestation (Silva and Silva, 1999). Here, we observed that the enzymatic activities of PK and HK, key enzymes in glycolysis, decreased in the quiescent stage (Figures 1A and 1B), suggesting a reduction in glycolysis at this dormancy stage. Thus, this glycolytic reduction may be an evolutionary strategy to reduce overall energy metabolism, and ensure survival of the species. Similar strategies have been reported for other species in nature (Lyman and Chatfield, 1955). Dormancy periods have broad metabolic implications, since ATP demand is reduced during this moments, decreasing the need for production. Additional evidence that lends strength to the hypothesis of metabolic rate reduction in *A. aegypti* mosquito during quiescence is the decreased oxygen consumption, when compared with pre-quiescence eggs (data not shown). To ensure egg survival, energy conservation becomes indispensable, and it is necessary to control the access to nutrients. The reduction of the main energy-producing pathways is observed in

A. aegypti quiescent eggs, strongly suggesting the activation of a particular metabolic program that leads to an induced-dryness hypo-metabolic state. After rehydration of diapause eggs, PK enzymatic activity increases, returning to similar levels to those observed in the pre-quiescence eggs (Figure 1A). Unlike PK, HK enzymatic activity remains low in post-quiescence eggs (Figure 1B). Water ends the quiescent stage in *A. aegypti* eggs, and metabolism returns to normal parameters (Silva and Silva, 1999). Eggs need to be exposed to water for 10 min in order to hatch. This abrupt change in the PK activity profile suggests the input of intermediate metabolites in glycolysis, like glucose-6-phosphate yielded from glycogen degradation (Figure 2), therefore sustaining the reactivation of larval metabolism after exposure to water. A phenotypical analysis of eggs at the three stages was performed, showing no apparent morphological differences (Figure 3).

Environmental signals and energy metabolism regulation that promote arousal in diapause vary between species (Tauber *et al*., 1986; Danks, 1987; Harvey, 1962). Many studies monitored oxygen consumption and reported an increased metabolic rate in insects at the final moments of diapause (Wipking and Viebahn, 1995; Kostal *et al*., 1998; Singtripop *et al*., 2007). However, few studies describe the changes in the central pathways of glucose metabolism in the transition from diapause to awakening or during quiescence. Studies conducted in the 1980's showed that mobilization of carbohydrates is essential for the *Bombyx mori* moth and the *S. crassipalpis* fly to wake from diapause (Adedokun and Denlinger, 1985; Su *et al*., 1994). Nevertheless, these studies require more detailed descriptions of the glucose metabolism regulation during these important dormancy periods.

The amount of glucose 6-phosphate decreases in pre- and post-quiescence (Figure 2) at the same time that PK and G6PDH activities rise (Figure 4). The glycolytic and pentose phosphate pathway may utilize the glucose 6-phosphate produced by HK or by glycogen mobilization. We observed an increase in the amount of G6P during quiescence, possibly due to reduced carbohydrate catabolism in this stage. These results suggest a strong involvement of the pentose phosphate pathway and glycolytic pathway after metabolism reactivation in post-quiescence eggs.

Glycogen is the main form of glucose stored in animal cells and is considered as a primary form of energy in these organisms. In a deficiency in caloric energy intake, the moths *Manduca sexta* and *B. mori* carry out glycolysis by glycogen degradation (Steele, 1985; Danks, 1987). In oviparous animals, carbohydrate reserves may change consistently during the embryonic process, such as observed in the *R. microplus* tick embryogenesis (Moraes *et al*., 2007). During the first 9 days of embryogenesis of this tick, glycogen levels decrease immensely. And, after this period, glycogen re-synthesis occurs, supported by intense gluconeogenesis (Moraes *et al*., 2007). Accordingly, carbohydrate mobilization is a dynamic process, essential for normal embryonic development.

Here, the amount of glycogen in *A. aegypti* eggs was higher in pre-quiescence eggs and decreased in quiescence (Figure 5). After rehydration, glycogen levels in the mosquito eggs continued to decrease, and reached the lowest values. Some insects that perform diapause, like the potato beetle *L. decemlineata* and the fly *Eurosta solidaginis*, normally use carbohydrate reserves primarily in early diapause (Lefevere, 1988; Storey and Storey, 1986). In a similar way, carbohydrate reserves in *A. aegypti* eggs are mobilized during quiescence and arousal (Figure 5).

The PK and G6PDH activities in post-quiescence eggs suggest that phosphorylated glucose can be directed to the glycolytic and pentose phosphate pathway.

To maintain diapause, various insects accumulate essential energy reserves and protein (Danks *et al*., 1987). Like carbohydrate reserves, protein content aids to maintain the energy homeostasis during the dormancy period, as it proceeds. In the present study, we observed that the total protein levels in *A. aegypti* eggs varied among the quiescence stages investigated. The amount of protein increased in pre-quiescence eggs when compared with quiescent eggs (Figure 6). During quiescence, protein levels undergo a significant decrease, suggesting that protein degradation contributes to latency period maintenance in addition to carbohydrates reserves. Insects performing diapause usually store proteins before the latency period for this purpose (Telfer and Kunkel, 1991). The corn borer larvae, *Diatraea grandiosella*, and the potato beetle, *L. decemlineata* store amino acids in specialized proteins before the unfavorable season. When these insects enter diapause during winter, the levels of stored proteins decrease (Chippendale, 1973; Lefevere, 1988). Proteins can be degraded during diapause, and their amino acids may oxidize in order to generate energy or produce other compounds, such as glucose through gluconeogenesis. We believe that in the A. aegypti quiescence a similar process may occur.

Overall, when the glycogen and protein reserves decrease, the rate of gluconeogenesis increases so as to maintain glucose homeostasis. Gluconeogenesis is an antagonistic pathway of glycolysis, and is the central pathway that leads to glucose formation from non-carbohydrate precursors such as lactate, glycerol, and amino acids. Therefore, the enzymatic activity of PEPCK, a key enzyme in gluconeogenesis, was at its lowest in pre-quiescence, when compared with other stages. PEPCK activity increased in quiescent and post-quiescence eggs, suggesting the activation of the gluconeogenic pathway (Figure 7A) in the latency period. When free glucose

was determined, the amount of this metabolite increased concurrently with PEPCK activity. The levels of total protein showed a contrasting profile, compared to PEPCK activity. It is possible that the protein degradation during quiescence may provide amino acids as gluconeogenic substrate for glucose synthesis, the final product of gluconeogenesis (Figure 7B). As stored glycogen is utilized and depleted while quiescence proceeds (Figure 5), glucose resynthesis from other precursors is necessary for the maintenance of the organisms' physiological processes.

The highest PEPCK and PK activity was observed in post-quiescence eggs. (Figures 7A and 1A). PEPCK regulates an anabolic pathway, while PK regulates a catabolic pathway. When both pathways are active at the same time, the energy futile cycle is generated. Futile metabolic cycle is a simultaneous operation of anabolic and catabolic pathways that wastes energy, resulting in the hydrolysis of ATP without resulting work. When these futile cycles occur, a large amount of energy is dissipated as heat. In mammals entering cold-induced hibernation, futile cycles are needed to generate heat and thus maintain body temperature (Toien *et al*., 2011). In *A. aegypti* quiescent eggs, this probably should not be the strategy in place, since quiescence eggs are not induced by low temperatures, so this physiological explanation of this futile cycle for heat production is not clear. However, it is possible that PEPCK activity is restricted to a particular organ or tissue, such as fat body (major gluconeogenic tissue in insects), while PK activity is linked to other organs, catabolizing glucose produced by gluconeogenesis. A schematic representation of these pathways through egg stages is shown in Figure 8 and helps to understand the alterations in metabolism during this phase of life of the insect. The scheme shows that the carbohydrate reserves are high in pre-quiescence, as well as proteins reserves. The glycolytic and pentose pathways are also at higher levels, at this stage. During quiescence, both carbohydrate reserves and protein content are consumed, in order to maintain energy

homeostasis. The glycolytic and pentose pathways decrease in this period. However, gluconeogenesis increases during quiescence and post-quiescence, to produce glucose through amino acids, sustaining the quiescence. In post-quiescence the glycolytic and pentose pathways increase at similar level as observed in pre-quiescence.

The changes in the transcription rate of the PEPCK gene are critical steps in the establishment of the total activity of the enzyme (Chakravarty *et al*., 2005). After years of research on PEPCK, the consensus is that changes in PEPCK transcription regulate the total activity of the enzyme. It is known that eukaryotes have a cytoplasmic and a mitochondrial isoform of this enzyme (Hanson and Reshef, 2003; Yang *et al*., 2009). However, in this study we analyzed three isoforms of the PEPCK gene in the mosquito *A. aegypti* (PEPCK-25 PEPCK-6 and PEPCK-80) and compared with other mosquito PEPCKs (Figure 9A). Interestingly, PEPCK duplications appear to be mosquito-specific, since *Culex quinquefasciatus* and *Aedes aegypti* PEPCKs may be identified as a single group separated from the duplicated PEPCKs in higher diptera, like *Drosophila*. The transcriptional analysis of the three isoforms of the PEPCK gene in the stages of *A. aegypti* eggs was performed in order to observe if PEPCK transcription accompanies the enzymatic activity (gluconeogenesis hypothesis). However, the PEPCK transcription profile shows a higher transcript level of isoform PEPCK-80 in pre-quiescence eggs, compared with quiescence and post-quiescence (Figure 9C). This profile was not similar to that observed for PEPCK activity, since it has the lowest value in pre-quiescence, while the highest activity was observed in post-quiescence (Figure 7A). On the other hand, the isoform of PEPCK-25 showed a high number of transcripts in quiescent eggs (Figure 9B), suggesting that PEPCK activity may be linked with this isoform expression

in this period. The higher transcript level of PEPCK-80 transcripts in pre-quiescence eggs may be explained based on the preparatory period before the organism enters quiescence, necessary for a prompt metabolism response after stimulation triggered with water. In advance of unfavorable periods, some organisms change behavior, storing reserves and preparing for diapause (Denlinger, 2002). The high level of PEPCK transcripts observed in *A. aegypti* pre-quiescence eggs perhaps reflects a preparation period for the latency stage. As observed for other organisms (Watt *et al*, 2013), studies based on enzymatic activity and transcriptional profiles show that PEPCK-25 and PEPCK-80 genes correspond to the mitochondrial and the cytosolic isoforms (unpublished data), respectively. The third gene (PEPCK-6) showed no transcription in any of the stages investigated in the present work. Likewise, in the mosquito *Anopheles gambiae,* fat body usually comprises the accumulation of transcripts that are translated only after the appropriate stimulus, in this case, blood meal (Marinotti, 2006). Thus, PEPCK transcripts would be present at late embryogenesis (pre-quiescence), but would only be reflected later on, in the quiescence and post-quiescence stages, when a greater demand for this enzyme is observed.

Taken together, the results described here provide an overview of metabolism. Despite of two different dormancy process parallels, quiescence and diapause, the metabolic alterations may be similar in some points during both strategies. This work improves the understanding of the glucose modulation during quiescence of *A. aegypti*. Additionally, this study contributes to the identification of new targets in the development of drugs used in control strategies against this species. Currently, alternative control methods interfere in preferential mosquito life cycle phases, such as larvae,

adults, and even the embryo (Chandra, *et al*., 2008; Service, 1983; Raghavendra and Subbarao, 2002). Although this last stage is neglected, further studies on the embryogenesis of arthropods, particularly focused on carbohydrate metabolism, have shed new light on nutrient utilization dynamics, from the formation of the oocyte to the mobilization of reserves embryo formation (Vital *et al*., 2010; Moraes *et al*., 2007; Campos *et al*., 2006). However, no studies have been published describing energy metabolism during quiescence of the mosquito *A. aegypti*. This study contributes with new knowledge about this topic, and shows that glucose metabolism regulation during *A. aegypti* eggs quiescence can be a more dynamic process than previously thought. Clearly, the study of lipid metabolism is also essential to elucidate various cellular processes, including maintenance of quiescence and diapause (Adedokun and Denlinger, 1985; Hahn and Denliger, 2007; Mitchell and Briegel, 1989). Lipids are the main source of energy stored by animals, as intracellular neutral lipid drops in specialized tissues such as adipose tissue in mammals and in the fat body in insects (Teixeira *et al*., 2003). The maintenance of a dormant period requires a species-specific metabolic modulation, in which lipids may have an important role. Yet, further description of glucose metabolism becomes necessary in order to understand the mechanism that sustains the initial phase of *A. aegypti* mosquito eggs during quiescence.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CL directed the project and participated in the coordination and management of the study. RMS performed the laboratory tests and the data analysis and wrote the manuscript. WOV, RNF, MRF, YPM and ISV helped with various aspects of the experiments and manuscript revising. CL provided new analytical reagents and tools. All authors read and approved the final version of manuscript.

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Figures

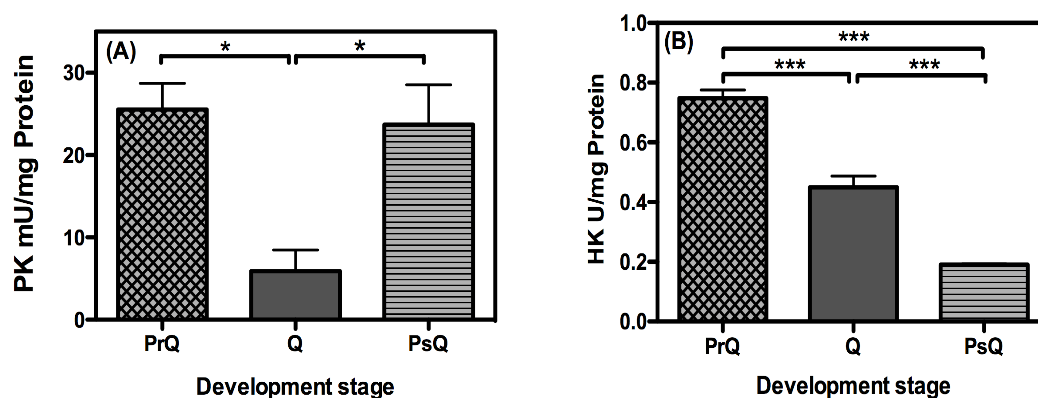

Figure 1: Glycolysis decreases in *A. aegypti* quiescent eggs. The canonical glycolytic pathway decreases in the quiescence stage, as suggested by glycolytic key enzymes, pyruvate kinase activity (A), and hexokinase activity (B), pointing to a hypometabolic program activation, consuming less G6P. Each experiment was replicated three times (*p<0.05; ***p<0.001 ANOVA).

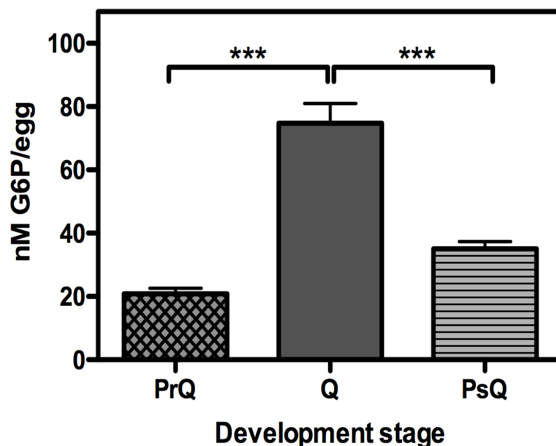

Figure 2: Glucose 6-phosphate content accumulates in *A. aegypti* quiescent **eggs as a consequence of a hypometabolic program.** G6P levels increase nearly 4 times during quiescence due to glycolysis and pentose-phosphate pathway reduction, as well as during glycogen degradation. Glucose concentration was measured in *A. aegypti* pre-quiescence (PrQ), quiescent (Q) and post-quiescence (PsQ) eggs. Each experiment was replicated three times (*p<0.05; ***p<0.001 ANOVA).

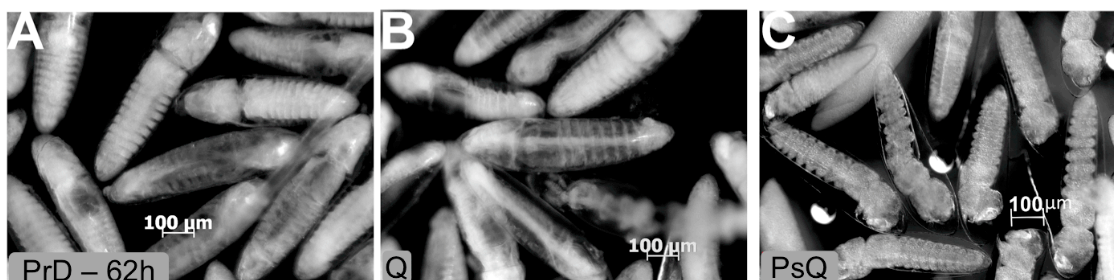

Figure 3: No significant differences in larvae external morphology were observed in *A. aegypti* egg stages. Eggs of different stages (PrQ, Q, and PsQ) were clarified and viewed under a magnifying glass (10x) in order to observe embryo morphology. Egg morphology does not vary consistently between pre-quiescence, quiescent, and post-quiescence eggs, showing that two

weeks of quiescence are not sufficient to alter larva phenotype. Scale bar 100 µm. Each experiment was replicated three times (*p<0.05; ***p<0.001 ANOVA).

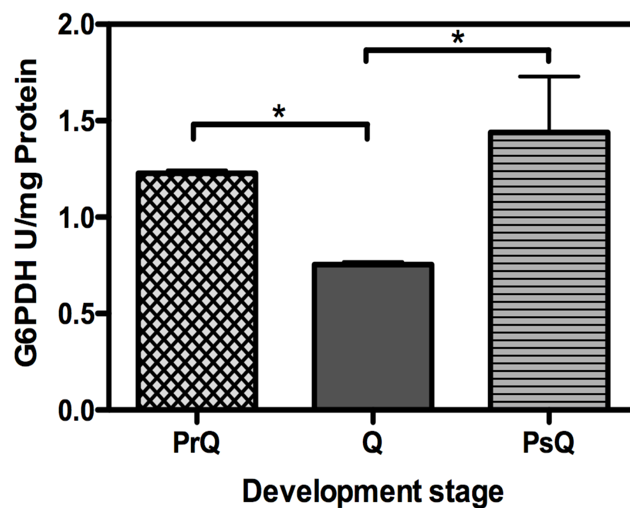

Figure 4: Pentose-phosphate pathway undergoes a decrease when *A. aegypti* eggs enter in quiescence**.** Glucose 6-phosphate dehydrogenase activity, a key enzyme in the pentose-phosphate pathway, decreases by nearly 50% after quiescence induction, as a consequence of a hypometabolic program activation. Each experiment was replicated three times (*p<0.05; ***p<0.001 ANOVA).

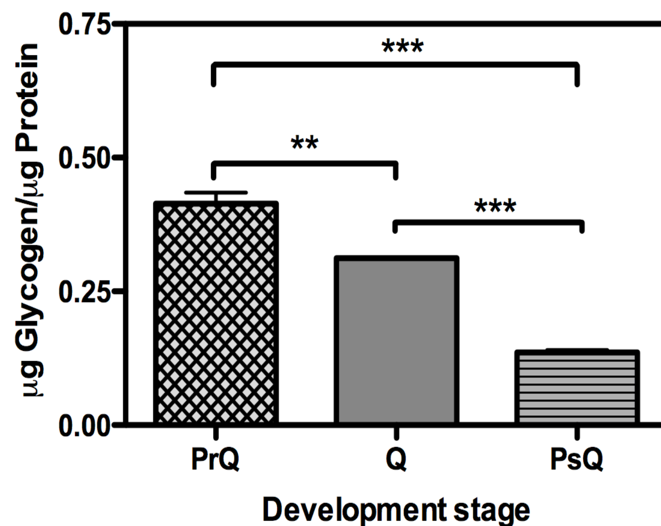

Figure 5: Glycogen degradation is necessary to arouse the *A. aegypti* quiescent **eggs.** Egg homogenates of different egg stages (PRQ, Q, and PSQ) were homogenized and glycogen content was assayed. Glycogen reserves help maintain high-energy demand during post-quiescence, decreasing by more than 50% after water stimulus in these eggs so as to ensure hatching. Each experiment was replicated three times (*p<0.05; ***p<0.001 ANOVA).

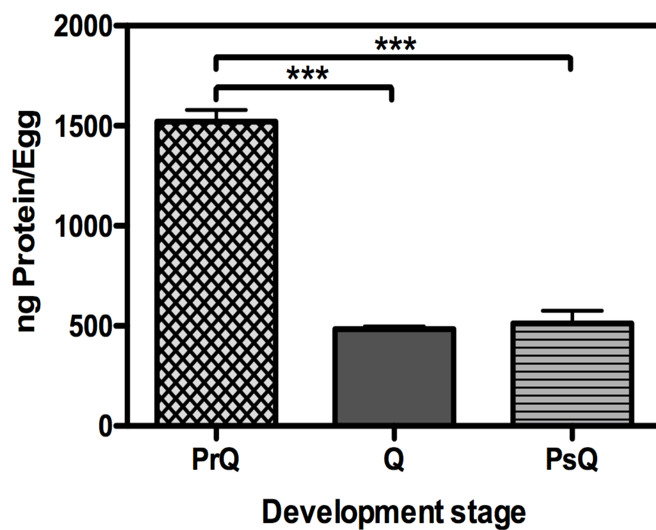

Figure 6: Protein reserves support gluconeogenesis in *A. aegypti* quiescent **and post-**quiescence **eggs.** Protein degradation increases in quiescence (Q) and post-quiescence (PsQ) so as to support gluconeogenesis in these stages. Concentration was measured in *A. aegypti* pre-quiescence (PrQ), quiescent (Q), and post-quiescence (PsD) eggs. Each experiment was replicated three times (*p<0.05; ***p<0.001 ANOVA).

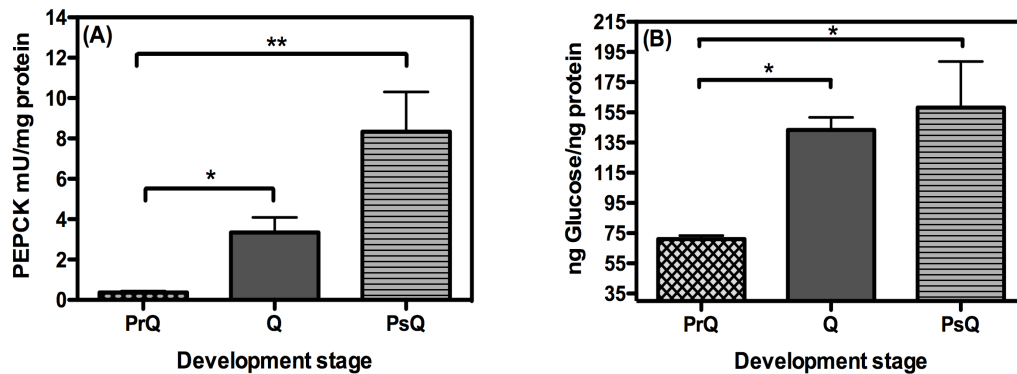

Figure 7: Gluconeogenesis is essential to sustain quiescence **in *A. aegypti* mosquito.** Phosphoenolpyruvate carboxykinase activity (A), a gluconeogenic key enzyme, increases in quiescence and post-quiescence together with the highest glucose content (B), the end product of gluconeogenesis. Each experiment was replicated three times (*p<0.05; ***p<0.001 ANOVA).

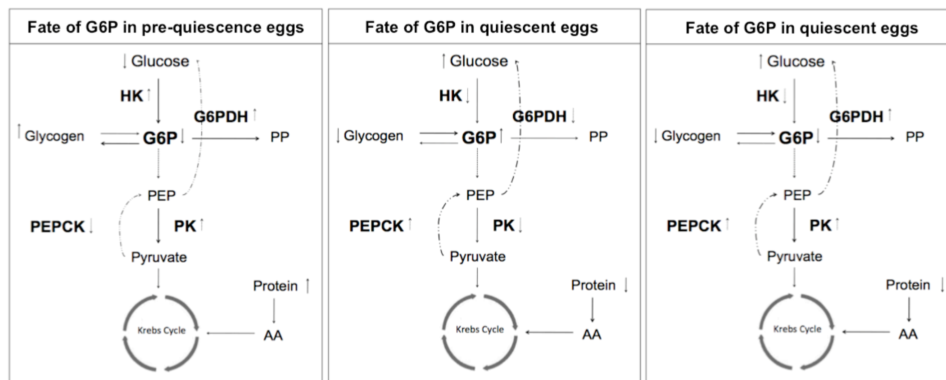

Figure 8: Metabolism regulation in *A. aegypti* pre-quiescence **(PrQ),** quiescent **(Q), and post-**quiescence **(PsQ) eggs.**

The flux through glucose metabolism pathways is represented for each egg stage: *A. aegypti* pre-quiescence (PrQ), quiescent (Q), and post-quiescence (PsQ) eggs.

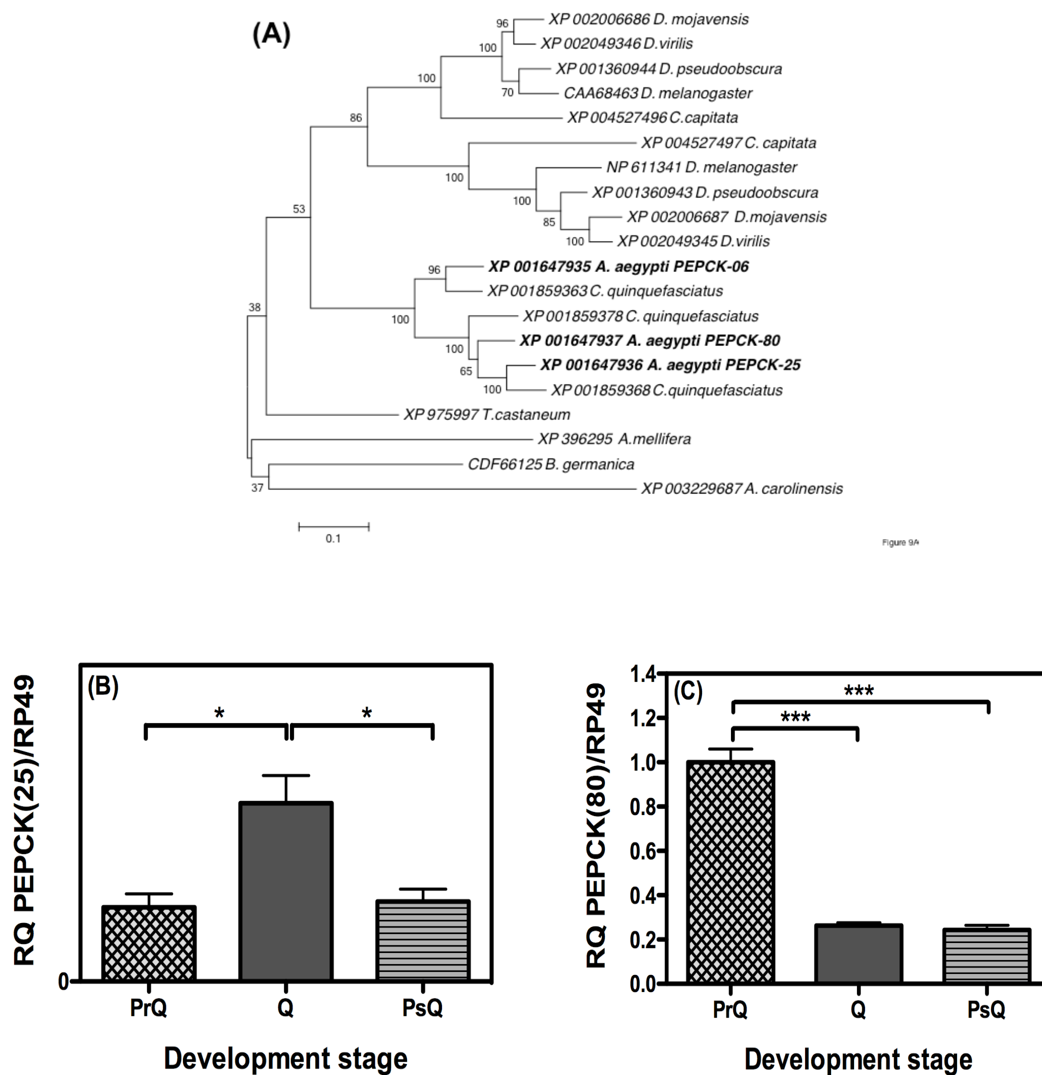

Figure 9: Phylogenetic analysis and transcriptional regulation of PEPCK in *A. aegypti* pre-quiescence **eggs. (A) Phylogenetic analysis (Maximum Likelihood) of PEPCKs in insects.**

Phylogenetic three (A) and transcriptional analysis of PEPCK-25 (B) and PEPCK-80 (C) in *A. aegypti* pre-quiescence (PrQ), quiescent (Q) and post-quiescence (PsQ) eggs. Sequences and respective accession numbers: XP_001647935.1|_phosphoenolpyruvate_carboxykinase_[Aedes_aegypti] PEPCK-006, XP_001647936.1|_phosphoenolpyruvate_carboxykinase_[Aedes_aegypti] PEPCK-25,

XP_001647937.1|_phosphoenolpyruvate_carboxykinase_[Aedes_aegypti] PEPCK-80,
XP_001859363.1|_phosphoenolpyruvate_carboxykinase_[Culex_quinquefasciatus],

XP_001859368.1|_phosphoenolpyruvate_carboxykinase_[Culex_quinquefasciatus],

XP_001859378.1|_phosphoenolpyruvate_carboxykinase_[Culex_quinquefasciatus],

XP_002006686.1|_GI18450_[Drosophila_mojavensis],

XP_002006687.1|_GI18449_[Drosophila_mojavensis],

XP_002049345.1|_GJ21534_[Drosophila_virilis]

XP_002049346.1|_GJ21535_[Drosophila_virilis]

XP_001360944.2|_GA14628_[Drosophila_pseudoobscura_pseudoobscura]

XP_001360943.2|_GA10647_[Drosophila_pseudoobscura_pseudoobscura]

NP_611341.1|_CG10924,_isoform_A_[Drosophila_melanogaster]

XP_396295.3|: phosphoenolpyruvate_carboxykinase_[GTP] [Apis_mellifera]

XP_004527496.1|: phosphoenolpyruvate_carboxykinase_[GTP] Ceratitis_capitata]

XP_004527497.1|: phosphoenolpyruvate_carboxykinase_[GTP] [Ceratitis_capitata]

CDF66125.1| phosphoenolpyruvate_carboxykinase_[Blattella_germanica]

XP_003229687.2: phosphoenolpyruvate_carboxykinase [GTP], mitochondrial [Anolis_carolinensis]

CAA68463.1 unnamed_protein_product_[Drosophila_melanogaster]

XP_975997.1| : phosphoenolpyruvate_carboxykinase_[GTP] [Tribolium_castaneum]