RAQUEL SOARES MAIA GODOY

MORFOFISIOLOGIA DO INTESTINO MÉDIO DE ADULTOS DO MOSQUITO NÃO HEMATÓFAGO Toxorhynchites (Lynchiella) theobaldi (DIPTERA, CULICIDAE)

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Celular e Estrutural, para obtenção do título de Magister Scientiae.

VIÇOSA
MINAS GERAIS - BRASIL
2015

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa

T

G588m
2015

Godoy, Raquel Soares Maia, 1986-
Morfofisiologia do intestino médio de adultos do mosquito não hematófago *Toxorhynchites (Lynchiella) theobaldi* (Diptera, Culicidae) / Raquel Soares Maia Godoy. - Viçosa, MG, 2015.
iv, 69f. : il. (algumas color.) ; 29 cm.

Orientador: Gustavo Ferreira Martins.
Dissertação (mestrado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Culicidae. 2. Intestino. 3. Intestino médio. 4. *Toxorhynchites*. I. Universidade Federal de Viçosa. Departamento de Biologia Animal. Programa de Pós-graduação em Biologia Celular e Estrutural. II. Título.

CDD 22. ed. 595.772

RAQUEL SOARES MAIA GODOY

MORFOFISIOLOGIA DO INTESTINO MÉDIO DE ADULTOS DO MOSQUITO NÃO HEMATÓFAGO *Toxorhynchites (Lynchiella) theobaldi* (DIPTERA, CULICIDAE)

Dissertação apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Celular e Estrutural, para obtenção do título de *Magister Scientiae*.

APROVADA: 29 de julho de 2015.

Caroline Macedo Gonçalves

Caroline Macedo Gonçalves

José Marcelo Ramalho Ortigão

Patrícia Dias Games

Patrícia Dias Games

Gustavo Ferreira Martins

Gustavo Ferreira Martins
(Orientador)

SUMÁRIO

RESUMO

GODOY, Raquel Soares Maia, M.Sc., Universidade Federal de Viçosa, julho de 2015. **Morfofisiologia do intestino médio de adultos do mosquito não hematófago Toxorhynchites (Lynchiella) theobaldi (Diptera, Culicidae).** Orientador: Gustavo Ferreira Martins. Coorientador: Kenner Morais Fernandes.

Na maioria das espécies de mosquitos, as fêmeas precisam se alimentar do sangue de vertebrados para completar o desenvolvimento de seus ovos. No entanto, em mosquitos do gênero Toxorhynchites, os ovos se desenvolvem sem a dieta de sangue, e ambos, fêmeas e machos desse gênero se alimentam exclusivamente de substâncias açucaradas. O intestino médio é um órgão bem conhecido nos mosquitos que se alimentam de sangue, mas pouco se sabe a seu respeito em mosquitos não-hematófagos. No presente trabalho, foi investigada a morfologia detalhada do intestino médio de Toxorhynchites theolbadi utilizando-se métodos histoquímicos e ultraestruturais. O intestino médio de fêmeas e machos adultos de T. theobaldi consiste em um longo e fino do intestino médio anterior (AMG), e um curto e dilatado intestino médio posterior (PMG). O AMG é subdividido em AMG1 (curto, com pregas) e AMG2 (longo, sem pregas). Ramificações neuronais e células enteroendócrinas estão presentes no AMG e PMG, respectivamente. Em comparação com o PMG de mosquitos-fêmeas que se alimentam de sangue, o PMG de T. theobaldi é menor; no entanto, em ambos os mosquitos, o PMG parece ser a principal região de digestão e absorção de alimentos, e secreção de proteínas. As pregas presentes no AMG de T. theobaldi não foram relatadas em outros mosquitos; no entanto, a organização muscular do intestino médio e o controle endócrino do processo digestivo são conservados em ambos T. theobaldi e mosquitos hematófagos.

ABSTRACT

GODOY, Raquel Soares Maia, M.Sc., Universidade Federal de Viçosa, July, 2015.**Midgut of the non-hematophagous mosquito Toxorhynchites theobaldi (Diptera, Culicidae).** Adviser: Gustavo Ferreira Martins. Co-adviser: Kenner Morais Fernandes.

In most mosquito species, the females require a blood-feeding for complete egg development. However, in Toxorhynchites mosquitoes, the eggs develop without blood-feeding, and both females and males exclusively feed on sugary diets. The midgut is a well-understood organ in blood-feeding mosquitoes, but little is known about it in non-blood-feeding ones. In the present study, the detailed morphology of the midgut of Toxorhynchites theobaldi was investigated using histochemical and ultrastructural methods. The midgut of female and male T. theobaldi adults consists of a long, slender anterior midgut (AMG), and a short, dilated posterior midgut (PMG). The AMG is subdivided into AMG1 (short, with folds) and AMG2 (long, without folds). Nerve branches and enteroendocrine cells are present in AMG and PMG, respectively. Compared with the PMG of blood-feeding female mosquitoes, the PMG of T. theobaldi is smaller; however, in both mosquitoes, PMG seems be the main region of food digestion and absorption, and protein secretion. The epithelial folds present in the AMG of T. theobaldi have not been reported in other mosquitoes; however, the midgut muscle organization and endocrine control of the digestion process are conserved in both T. theobaldi and blood-feeding mosquitoes.

1. INTRODUÇÃO

O gênero Toxorhynchites pertence à família Culicidae, subfamília Culicinae, tribo Toxorhynchitini. Apesar de compartilhar várias características simplesiomórficas com os demais membros da família, Toxorhynchites possui hábito alimentar peculiar e modificações adaptativas que o difere dos outros gêneros de culicídeos. No entanto, sua biologia é pouco estudada, o que dificulta o entendimento de suas distinções e o esclarecimento de sua história evolutiva.

Um dos aspectos mais intrigantes da biologia de Toxorhynchites é seu comportamento alimentar cujas fêmeas, diferentemente dos culicídeos em geral, não são hematófagas. Como o tipo de alimentação está intimamente ligado às características morfofuncionais do trato digestivo, o conhecimento desse trato nas espécies do gênero traz informações-chave para o entendimento de sua biologia, auxiliando também na compreensão da morfofisiologia geral dos mosquitos.

1.1. CULICIDAE

O táxon Culicidae é monofilético e engloba todas as espécies de mosquitos. Seus representantes, quando no estágio adulto, são delgados, possuem peças bucais alongadas e longas pernas, além de escamas em grande parte do corpo, tornando-os facilmente reconhecíveis (Harbach e Kitching, 1998). Devido à falta de ferramentas de estudo mais conclusivas, a classificação atual dos membros do táxon Culicidae não reflete inteiramente sua história evolutiva. Portanto, apesar de estar sujeita a modificações, a família Culicidae é atualmente composta por duas subfamílias, Anophelinae e Culicinae (Harbach e Howard, 2007; Reidenbach et al, 2009; WRBU, 2014). Culicinae é a maior subfamília do táxon Culicidae, abrangendo 3059 espécies (Harbach, 2014). Seus representantes são denominados comumente como “mosquitos verdadeiros” e seus membros são considerados mais derivados que os da subfamília Anophelinae (Pawlowski et al, 1996; Besansky e Fahey, 1997; Miller et al, 1997; Harbach e Kitching, 1998; WRBU, 2014).

Os mosquitos apresentam ampla diversidade morfológica, paralela a uma espetacular irradiação em praticamente todo tipo de ambiente, o que proporcionou a

esses animais um enorme sucesso evolutivo (Grimaldi e Engel, 2005). Eles habitam regiões tropicais e temperadas, sendo algumas espécies encontradas até bem além do Círculo Polar Ártico (Foley et al, 2007). No entanto, seu número e diversidade são muito maiores em ambientes de floresta tropical (Harbach e Howard, 2007).

Os mosquitos são normalmente encontrados em ambiente cuja umidade é alta. Algumas espécies vivem a poucos metros do solo, enquanto outras, principalmente as silvestres, vivem no dossel de florestas. O tempo de vôo e a duração da atividade alimentar são geralmente característicos de cada espécie. Em relação ao período de atividade, há culicídeos noturnos, crepusculares, ou ativos durante o dia (Harbach e Howard, 2007).

Culicídeos são insetos holometábolos (de metamorfose completa), pois possuem distintos estágios de desenvolvimento, com seu ciclo de vida incluindo as fases de ovo, larva, pupa e adulto. O estágio de pupa é uma fase quiescente, mas os estágios larvais precisam se alimentar para dar continuidade ao seu desenvolvimento (Wiegmann et al, 2009). As fases imaturas dos mosquitos são encontradas em um amplo espectro de ambientes aquáticos, ocupando principalmente corpos temporários e permanentes de água subterrânea (Harbach e Howard, 2007).

Fêmeas e machos adultos de Culicidae são em geral morfologicamente distintos, principalmente quanto às antenas, peças bucais e genitália. As diferenças são mais visíveis em relação à morfologia das antenas e do aparelho bucal. Fêmeas possuem antenas pilosas, estruturas de perfuração no aparelho bucal e são geralmente mais robustas que os machos, que, por sua vez, têm antenas plumosas e não apresentam probóscide com peças perfurantes (Forattini, 2002).

Todos os mosquitos machos se alimentam exclusivamente de líquidos de plantas, como néctar, mel, sumos de frutas e exudados. No entanto, com exceção dos gêneros Toxorhynchites, Malaya e Topomyia, as fêmeas de mosquitos se alimentam também de sangue de animais vivos, o que faz delas excelentes veículos para transmissão de patógenos, exibindo uma enorme importância médica (Fig. 1) (Harbach e Howard, 2007). Dentre os três gêneros de mosquitos não-hematófagos, Toxorhynchites possui um número muito maior de espécies e ampla distribuição geográfica, tornando-o mais representativo.

O sangue ingerido pelas fêmeas hematófagas é requerido para o desenvolvimento de seus ovos, de forma absoluta (anautogenia) ou facultativa

(autogenia). Fêmeas anautógenas não produzem ovos sem o repasto sanguíneo. Nas fêmeas autógenas, um lote de ovo é desenvolvido após a emergência do estágio adulto, mas alimentações de sangue são necessárias para a produção dos lotes posteriores (Clements, 1992).

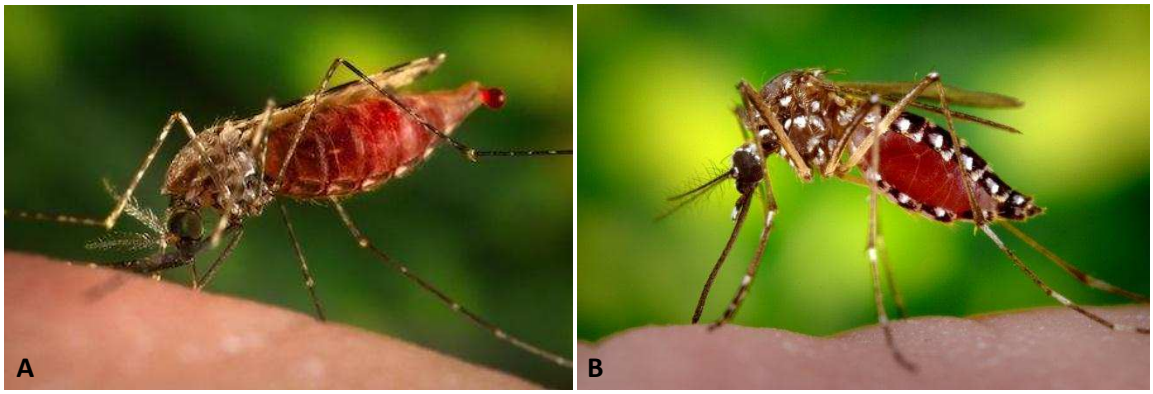


Figura 1: Fêmeas hematófagas adultas de mosquitos realizando repasto sanguíneo. **A:** Fêmea da espécie Anopheles quadriannulatus, representante da subfamília Anophelinae. **B:** Fêmea da espécie Aedes aegypti, representante da família Culicinae. Fonte: https://www.vectorbase.org/.

A hematofagia surgiu independentemente várias vezes no curso da evolução dos artrópodes e muitas adaptações comportamentais, anatômicas e fisiológicas acompanharam essa tendência evolutiva (Justice et al, 2003). Dentre essas adaptações, as mandíbulas dos mosquitos foram modificadas para facilitar o acesso ao sangue; as glândulas salivares passaram a produzir moléculas capazes de bloquear a hemostasia; e o intestino médio foi modificado para neutralizar reações imunológicas mediadas pelo sangue e otimizar a digestão dos componentes sanguíneos (Ribeiro, 1996; Stark e James, 1996). No entanto, o grupo que engloba os mosquitos do gênero Toxorhynchites sofreu novas modificações adaptativas, perdendo a habilidade de se alimentar de sangue (Marquardt, 2005).

1.2. TOXORHYNCHITINI

A subfamília Culicinae é composta por 11 tribos, sendo a Toxorhynchitini uma delas (WRBU, 2014). Toxorhynchitini possui apenas um gênero, Toxorhynchites, que por sua vez engloba 4 subgêneros, Toxorhynchites, Afrorhynchus, Ankylorhynchus e Lynchiella. O subgênero Toxorhynchites ocorre apenas no Velho Mundo; Afrorhynchus é encontrado na África; e Ankylorhynchus e Lynchiella (a qual inclui a espécie em

estudo) são confinados ao Novo Mundo. Ao todo, são reconhecidas 90 espécies do gênero atualmente. Toxorhynchites foi anteriormente encaixado em uma subfamília exclusiva (Toxorhynchitinae) dentro do táxon Culicidae. Hoje, por apresentar maiores semelhanças morfológicas com culicíneos do que com anofelinos, e por se acreditar que a maioria das características distintivas deste gênero são características adaptativas secundárias relacionadas ao grande tamanho e aos hábitos de alimentação das larvas e adultos, Toxorhynchites foi incluído na subfamília Culicinae (WRBU, 2014).

O gênero Toxorhynchites inclui os maiores mosquitos já identificados. Os adultos são facilmente reconhecidos pelo grande tamanho e pela peculiar probóscide dobrada (Fig. 2). O corpo é coberto por escamas iridescentes de cores vivas e os segmentos abdominais posteriores possuem tufos de escamas laterais. As larvas também são grandes e apresentam cores que variam entre o rosa, vermelho e roxo, o que também as torna de fácil identificação (WRBU, 2014).

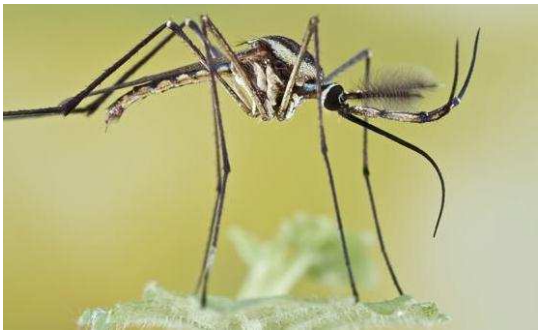


Figura 2: Macho de Toxorhynchites rutilus, representante da tribo Toxorhynchitini (subfamília Culicinae). Fonte: http://bugguide.net/node/view/721549/bgpage

Toxorhynchites é encontrado na maioria das regiões tropicais e também em algumas regiões subtropicais e temperadas do mundo, e os principais tipos de vegetação que eles ocupam são as florestas (Muspratt, 1951). Suas larvas geralmente habitam buracos de árvores e bambus, mas algumas são vistas nas axilas de folhas, em plantas carnívoras, fendas de rochas e recipientes artificiais (Schreiber, 2007).

Os ovos de Toxorhynchites são ovais, brancos ou amarelos e encontram-se flutuando na superfície da água ou logo abaixo desta. O período de incubação é de 40-60 horas e pode variar dependendo da temperatura. A fase larval possui quatro instares, com duração total de desenvolvimento variando de 13-91 dias, dependendo da espécie, temperatura e disponibilidade de alimento. O período pupal ocorre entre 3-12 dias e depende principalmente da temperatura (Steffan e Evenhuis, 1981).

As larvas de Toxorhynchites são predadoras e, por isso, possuem partes bucais modificadas, compostas por 10 ou menos filamentos espessos achatados utilizados para agarrar as presas (WRBU, 2014). Elas se alimentam de invertebrados aquáticos, incluindo larvas de outros mosquitos, sendo úteis em tentativas de controle de culicídeos de importância epidemiológica, como Aedes aegypti, Aedes albopictus e Culex quinquefasciatus (Regis, 1995; Collins e Blackwell, 2000; Shaalan e Canyon, 2009; Nyamah et al, 2011). Algumas dessas tentativas falharam, o que pode ter ocorrido, pois, na elaboração de estratégias de controle biológico, é indispensável que a biologia de ambos espécie-alvo e agente potencial de controle sejam entendidas completamente, o que não é o caso do gênero Toxorhynchites. Além disso, a utilização desses predadores no controle de outras espécies esbarra na dificuldade de criação em laboratório para obtenção de mosquitos em grande quantidade (Steffan e Evenhuis, 1981; Collins e Blackwell, 2000; WRBU, 2014).

Como o gênero não está incluído na lista de "espécies-praga", e não é vetor de doenças por não apresentar comportamento de hematofagia, a biologia geral e a taxonomia de Toxorhynchites têm sido amplamente negligenciadas. Exceções foram descrições da ecologia de algumas espécies, somado a um pequeno número de estudos taxonômicos (Collins e Blackwell, 2000).

Por fim, o peculiar comportamento alimentar das fêmeas de Toxorhynchites somado à falta de estudos sobre sua biologia revela a importância da investigação da morfofisiologia do órgão digestivo de espécies do gênero, a fim de revelar que modificações relacionadas com a dieta podem ter ocorrido durante sua evolução, além de aperfeiçoar o conhecimento geral sobre o sistema digestivo de Culicidae.

1.3. SISTEMA DIGESTIVO DE INSETOS

A estrutura básica do trato digestivo dos insetos consiste em um tubo formado por tecido epitelial de camada única que vai da boca ao ânus (House, 1974, Dow, 1986). Ele é sustentado na cavidade corporal por músculos viscerais extrínsecos, que ao contraírem promovem a dilatação do lúmen intestinal. Músculos viscerais intrínsecos longitudinais e circulares também estão presentes, permitindo que o intestino sofra dilatação e submeta-se a movimentos peristálticos (Bernick et al., 2008). De acordo com sua origem embrionária e função fisiológica, o trato digestivo é dividido em três

regiões principais: intestino anterior, intestino médio e intestino posterior (House, 1974; Chapman, 1985; Dow, 1986).

Invaginações do ectoderma denominadas estomodeu e proctodeu originam os intestinos anterior e posterior, respectivamente. Por serem derivadas de células ectodérmicas, essas porções do intestino são revestidas internamente por cutícula, característica que oferece limitações para sua capacidade de absorção e digestão (Snodgrass, 1935). A cobertura cuticular é secretada pelas próprias células epiteliais dessas regiões e é contínua com a cutícula do revestimento corporal (House, 1974). O intestino médio é a única estrutura do inseto que deriva da endoderme, e, por isso, não possui revestimento cuticular. Os túbulos de Malpighi se inserem entre o intestino médio e o intestino posterior como evaginações ectodérmicas desse último (Chapman, 1985; Lehane e Billingsley, 1996).

A primeira região do intestino anterior é a faringe, que é seguida pelo esôfago. Esse último consiste geralmente em um tubo simples que desemboca no intestino médio, embora em alguns insetos possa estar modificado em um "estômago" ou papo dilatável utilizado para estocar alimento (Klowden, 2007). Em insetos adultos das ordens Diptera e Lepidoptera, o "estômago" existe como um divertículo separado do resto do esôfago por um ducto (Winteringham, 1965; Kathuria, 1972). O alimento composto de açúcares é primeiro estocado no "estômago" e então passa vagarosamente para o intestino médio, enquanto proteínas são enviadas diretamente para o intestino médio (Clements, 1963; Klowden, 2007). A cobertura cuticular do "estômago" limita sua capacidade absortiva, fazendo com que este funcione basicamente como um reservatório alimentar, apesar de permitir a absorção de alguns lipídios. Embora a absorção seja limitada, a digestão pode ocorrer nessa região devido à ação continuada de enzimas das glândulas salivares presentes no bolo alimentar. A parte posterior do intestino anterior é modificada em um proventrículo muscular (cárdia) que funciona como um esfíncter, regulando a passagem do alimento para o intestino médio (House, 1974; Klowden, 2007).

O intestino médio consiste basicamente em um tubo que liga o intestino anterior ao posterior (Silva et al, 2012). Ele contém pelo menos três tipos celulares arranjados em uma camada epitelial única, que são as células colunares (ou digestivas), regenerativas e endócrinas. As células colunares são as mais numerosas, e possuem as bordas que são voltadas para o lúmen repletas de microvilos, aumentando a área de

superfície absortiva e secretora. Elas contêm várias redes de retículo endoplasmático necessárias para a produção de enzimas digestivas e absorvem a maioria dos nutrientes do lúmen intestinal (Chapman, 1985, Lehane e Billingsley, 1996). Essas células estão em constante renovação a partir das células-tronco regenerativas, que por sua vez estão localizadas na região do intestino médio adjacente à hemolinfa. As células regenerativas se dividem assimetricamente e tem a capacidade de se diferenciar tanto em células colunares quanto endócrinas (Chapman, 1985). Essas últimas, por fim, estão em contato com o lúmen e/ou com a hemolinfa, contêm muitos grânulos secretórios citoplasmáticos eletron-densos e são encontradas dispersas no epitélio intestinal geralmente como células simples, embora algumas vezes possam estar organizadas em pequenos grupos (Andriès e Tramu, 1985, Klowden, 2007).

A região anterior do intestino médio pode conter divertículos denominados cecos gástricos, que aumentam a área de superfície do órgão para a absorção e secreção de substâncias e criam um fluxo contracorrente dentro do intestino, resultante de sua absorção diferencial de água (Terra, 1990; Billingsley e Lehane, 1996). Esta é secretada no lúmen pelo intestino posterior, e se movimenta para frente para ser absorvida na região cecal, permitindo que sólidos não digeridos se movimentem de volta para serem finalmente digeridos e absorvidos no intestino médio (Billingsley e Lehane, 1996; Klowden, 2007).

Devido à ausência de cutícula, as células do intestino médio estão constantemente sujeitas a abrasão pelo alimento. Na grande maioria dos insetos, células do intestino médio produzem uma matriz extracelular denominada matriz peritrófica (MP), que consiste em uma estrutura saculiforme composta por microfibrilas de quitina, proteínas e glicoproteínas em uma matriz de proteoglicano (Moskalik et al., 1996; Lehane, 1997). As várias proteínas que suprem a MP são denominadas peritrofinas e possuem domínio de ligação a moléculas de quitina (Dinglasan et al., 2009). Proteínas e precursores de quitina são secretados por células digestivas do intestino médio, e a MP se organiza por intercalação de fibrilas de quitina e peritrofinas (Hegedus et al, 2009; Rose et al, 2014).

Existem dois tipos de MP com base em seus sítios de síntese (Peters, 1992; Lehane, 1997). A MP tipo I é secretada por todas as células digestivas e se condensa para formar a rede que circunda o bolo alimentar. Sua produção pode ocorrer continuamente, no caso da maioria dos insetos não-hematófagos; ou após estímulos do

alimento ao chegar no intestino médio, como ocorre tipicamente em insetos hematófagos. A MP do tipo II é formada sempre continuamente por um anel de células localizado na cárdia (ou válvula estomodeal), que produz um tubo em movimento retrógrado com função de englobar o bolo alimentar. Esse tubo então se estende revestindo o interior dos intestinos médio e posterior (Jacobs-Lorena e Oo, 1996; Lehane, 1997).

Distintos estágios de vida de um mesmo inseto podem produzir MPs de diferentes tipos (Tellam et al, 1999). Todos os tipos de MP contém poros e apresentam permeabilidade a algumas enzimas digestivas e certos produtos da digestão. Essa permeabilidade seletiva cria dois compartimentos; o espaço endoperitrófico, circundado pela MP; e o espaço ectoperitrófico, localizado entre a parede intestinal e a MP. A compartimentalização das enzimas digestivas e seus produtos resultante da presença da MP leva a uma maior eficiência na digestão por manter as enzimas e seus substratos específicos concentrados em um mesmo compartimento (Lehane, 1977; Hegedus et al, 2009).

O intestino médio também age como uma barreira contra patógenos, cuja principal importância está ligada à transmissão de doenças pelos vetores hematófagos (Arias-Goeta et al, 2013). As enzimas digestivas e a MP secretadas nessa porção do intestino oferecem resistência ao desenvolvimento dos parasitas. Além disso, a permeabilidade seletiva da MP pode proteger o inseto contra possíveis toxinas presentes no alimento, impedindo-as de atravessarem a MP e afetarem diretamente as células intestinais (Abraham and Jacobs-Lorena, 2004; Arias-Goeta et al, 2013; Agrawal et al, 2014).

O processo digestivo dos insetos envolve a ação de várias enzimas. A digestão inicial ocorra no espaço endoperitrófico; e as fases intermédiaria e final ocorrem no espaço ectoperitrófico e ao nível das células intestinais, respectivamente (Terra, 1990; Silva et al, 2012). A maior parte do alimento que requer digestão é composta por polímeros, tais como amido, celulose, hemiceluloses e proteínas. Primeiramente, ocorre um descréscimo na massa molecular dos polímeros pela ação das despolimerases (amilases, proteinases, etc) e oligomerases (aminopeptidases), que então formam dímeros e pequenos oligômeros (Terra, 1990). Os dímeros são clivados a monômeros por dimerases (celobiase, maltase e dipeptidases), encerrando o processo digestivo (Silva et al, 2012).

Após a digestão do alimento no intestino médio, o material não digerido segue em direção ao intestino posterior, que é composto por piloro, íleo e reto. O piloro é a válvula de onde os túbulos de Malpighi surgem. O íleo consiste em um tubo estreito seguido pelo reto, que é mais alargado (Klowden, 2007). O intestino posterior, cuja principal função é osmoregulação, é revestido por uma cutícula mais permeável que a do intestino anterior e contém a abertura dos túbulos de Malpighi, que por sua vez funcionam produzindo uma "pré-urina" isosmótica a partir da hemolinfa contendo íons, aminoácidos e metabólitos (Hanrahan et al, 1984). O intestino posterior reabsorve seletivamente água, aminoácidos e alguns íons, produzindo uma urina hiper ou hiposmótica, que é excretada juntamente com o bolo fecal. Esse bolo é formado por produtos não digeridos e materiais descartáveis que passam pelo intestino posterior e perdem água (Phillips et al, 1987, Klowden, 2007).

1.4. INTESTINO MÉDIO DE MOSQUITOS

Somente os mosquitos que se alimentam de sangue estão envolvidos na transmissão de patógenos. Por esse motivo, as pesquisas são mais direcionadas ao seu estudo, sendo grande parte da revisão bibliográfica voltada ao conhecimento sobre sistema digestivo de fêmeas hematófagas.

Alguns estudos com glândulas salivares de mosquitos mostraram que tais estruturas são sexualmente dimórficas nas espécies em que as fêmeas são hematófagas. As glândulas salivares dos machos são menores e não têm, ou possuem pouca atividade relacionada com a ingestão de sangue, como a produção de substâncias antiplaquetárias, anticoagulantes e vasodilatadoras (Ribeiro et al, 1984; Rossignol e Lueders, 1986; Marinotti et al, 1990; Moreira-Ferro et al, 1999). Porém, experimentos utilizando Toxorhynchites revelaram que suas glândulas salivares são sexualmente monomórficas, refletindo o tipo idêntico de dieta do macho e da fêmea (Jariyapan et al, 2004). Tal achado oferece uma pista de que modificações evolutivas importantes ocorreram no trato digestivo desses insetos.

Assim como ocorre na classe Insecta em geral, o intestino médio (Fig. 3) é a porção do trato digestivo de mosquitos responsável por quase a totalidade da digestão do alimento (Billingsley, 1990). Por isso, e por essa ser consequentemente a região mais

propícia a modificações adaptativas relacionadas ao tipo específico de dieta, seu estudo torna-se de maior interesse.

O intestino médio dos mosquitos compreende duas regiões distintas que se diferem morfológica e funcionalmente: intestino médio anterior (IMA) e intestino médio posterior (IMP). O IMA configura-se como um tubo simples relacionado com a digestão/absorção de açúcares (Billingsley, 1990). O IMP está mais envolvido com a digestão de sangue e compreende um saco expansível cujas células são multifuncionais para desempenhar pelo menos três papéis: regulação hídrica pós-repasto sanguíneo; síntese e secreção de enzimas digestivas e componentes da MP; e absorção de nutrientes (Billingsley, 1990).

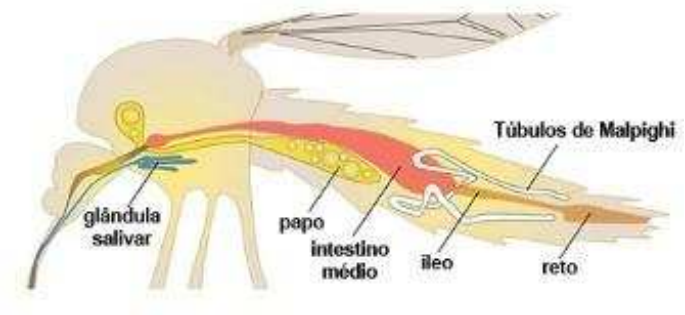


Figura 3: Visão geral do trato digestivo de mosquitos, com destaque para o intestino médio. Modificado de Rebecca et al, 2014.

Quando o alimento é uma solução açucarada, como néctar, ele é estocado primeiramente no "crop" ou "estômago", e vai passando aos poucos para o intestino médio. Se o alimento é sangue, ele vai diretamente para o intestino médio, sendo armazenado no IMP. O principal fator que determina o destino do alimento é sua composição química (Gooding, 1972), apontando para a existência no mosquito de receptores para glicose e/ou para alguma substância presente no sangue. Além disso, sabe-se que o néctar possui inibidores de proteinases, e isso pode ser um dos motivos pelo qual ocorre essa separação espacial do tipo de alimento (Gooding, 1973), essencial no caso das fêmeas, por possuírem ambos os tipos de dietas.

Nas espécies de mosquitos cujas fêmeas são hematófagas, o intestino médio de fêmeas e machos difere quanto ao tamanho e morfologia. As fêmeas possuem região anterior reduzida e região posterior mais desenvolvida que os machos (Christophers, 1960). Essas diferenças provavelmente refletem adaptações relacionadas à dieta.

O epitélio do IMA é responsável pela digestão e absorção de carboidratos (Billingsley e Hecker, 1991; Billingsley, 1990). Nas fêmeas hematófagas, a presença de um plugue de material da matriz peritrófica (MP) no início do IMP impede o envolvimento direto do IMA na digestão de sangue (Perrone e Spielman, 1988; Billingsley e Rudin, 1992). Além disso, glicosidases secretadas pelas células do IMA ajudam a suportar um papel dessa porção do intestino na digestão de carboidratos (Billingsley, 1989). Esta porção é bem suprida por terminações nervosas, ao contrário do IMP, o que colabora com a idéia de que essas duas regiões funcionam independentemente (Hecker, 1977; Billingsley, 1990).

Tanto as células epiteliais do IMA quanto as do IMP possuem características ultraestruturais que apontam para as funções de estocagem, síntese, secreção e absorção de substâncias (Bertram e Bird, 1961; Rudin e Hecker, 1976).

As células que compõem o IMA normalmente possuem uma lâmina basal contínua em contato com a hemolinfa, microvilos densamente empacotados e um labirinto basal bem formado (Hecker et al, 1974; Hecker, 1977). As membranas plasmáticas altamente dobradas dos microvilos (região apical) e labirinto (região basal) são típicas do epitélio absortivo. O retículo endoplasmático rugoso (RER) das células do IMA ocorre como pequenas cisternas simples em Aedes aegypti, e cisternas mais longas e pobremente organizadas em Anopheles gambiae, Anopheles stephensi, e Culex pipiens fatigans. O retículo endoplasmático liso (REL) é encontrado denso e altamente organizado nessa região (Hecker, 1977).

O IMP possui células secretoras apresentando pilhas e espirais de retículo endoplasmático rugoso (RER); aparelho de Golgi numeroso e pobremente organizado; e vesículas secretoras. Células com função de estocagem também foram encontradas nessa região, contendo depósitos de lipídio e glicogênio (Bertram e Bird, 1961; Reinhardt e Hecker, 1973). O glicocálice das células do IMP de Culex tarsalis possuem glicosaminoglicanos e/ou glicoproteínas ácidas, enquanto que as membranas plasmáticas laterais, o labirinto basal e a lâmina basal são altamente aniônicos, consistente com seu papel regulatório (Houk et al, 1986a; Houk et al, 1986b).

Quando o alimento é sangue, as células do IMP submetem-se a uma deformação assim que essa região é preenchida, e o estresse mecânico envolvido é compensado pela

presença de junções intercelulares em suas membranas laterais. Em espécies de Anopheles, as células do IMP possuem apenas desmossomos septados como junções intercelulares (Hecker, 1977) Em culicíneos, são encontradas junções do tipo zonula continua próximas ao ápice celular, e desmossomos nas regiões basais (Reinhardt e Hecker, 1973; Hecker, 1977; Houk e Hardy, 1979).

As células epiteliais do intestino médio de fêmeas adultas de mosquitos produzem MP do tipo I em resposta à ingestão de sangue. Vários papéis foram propostos para essa estrutura, dentre eles o de proteção contra patógenos e proteção física do epitélio; filtração semipermeável de proteínas e enzimas digestivas; barreira de retenção para inibidores de proteinases; e uma camada para prevenir o entupimento dos microvilos por material do lúmen (Marquardt, 2005).

A MP impede que o alimento ingerido fique em contato direto com o epitélio. A formação dessa matriz ocorre diferentemente em culicíneos e anofelinos. Nesses últimos, o material precursor da matriz já está presente em grânulos, que são liberados após a ingestão de sangue (Hecker, 1977). Em culicíneos, a MP é formada "de novo" pelas células do IMP (Rudin e Hecker, 1976).

Para a formação da MP em anofelinos, grânulos de secreção apicais das células digestivas são liberados no lumen do IMP, exclusivamente em resposta ao estresse mecânico durante a alimentação. Esses grânulos são repostos enquanto ocorre a digestão subsequente (Berner et al, 1983; Billingsley e Rudin, 1992). Os seus conteúdos coalescem e então condensam para formar a MP (Berner et al, 1983).

Em culicíneos, algum tempo após a refeição sanguínea, forma-se uma MP discernível mostrando uma estrutura densamente laminada próxima aos microvilos, que por sua vez tornam-se progressivamente menos estruturados e, finalmente, a MP atinge sua estrutura altamente organizada, com numerosas camadas (Perrone e Spielman, 1988).

As células do intestino médio que não estão diretamente envolvidas com a digestão são as regenerativas e endócrinas (Hecker, 1977; Brown et al., 1985). As células regenerativas são caracterizadas por um citoplasma denso contendo poucas organelas diferenciadas, uma quantidade pequena de mitocôndrias, nucléolo não-evidente, além de nenhuma especialização nas membranas celulares apicais e basais

(Clements, 1992). As células endócrinas podem ser distinguidas das demais células do sistema digestivo devido à sua estrutura refinada. Em *A. aegypti*, pelo menos 500 células endócrinas estão presentes por epitélio do IMP, sendo concentradas posteriormente e constituindo o maior órgão endócrino do mosquito (Brown et al., 1985). Pelo menos dois tipos de células endócrinas estão presentes, as células claras e as escuras, e cerca de 25% dessas células são encontradas adjacentes às células regenerativas do epitélio. Dentre esses dois tipos, são encontradas células que atingem e que não atingem a superfície luminal, essas primeiras contendo microvilosidades e em nenhuma delas havendo labirinto basal. Vesículas secretoras, produzidas pelo complexo de Golgi, são geralmente concentradas no terço basal do citoplasma dessas células, e várias formas de corpos lamelares estão também presentes. O conteúdo das vesículas é liberado diretamente na hemolinfa por exocitose (Brown et al., 1985; et al., 1987; Billingsley e Lehane, 1996).

Dois hormônios peptídicos, o neuropeptídeo semelhante ao FMRFamida de moluscos e o hormônio semelhante ao polipeptídio pancreático de vertebrados (PP), foram localizados nas células endócrinas intestinais de algumas espécies de mosquitos. Ambos os hormônios são encontrados em vesículas tanto das células endócrinas claras quanto das escuras, mas nem todas as células apresentam imunoreatividade (Brown et al, 1986; Glanttli et al, 1987). A marcação das células enteroendócrinas com anticorpos anti-FMRFamida é a mais utilizada para auxiliar na sua distinção entre os tipos celulares do epitélio.

Em relação ao processo digestivo enzimático, quando o mosquito ingere sangue, a sua presença no intestino médio estimula a secreção de enzimas digestivas no lúmen intestinal, cujas principais são as proteolíticas (Billingsley, 1991; Terra, 1996). Enzimas proteolíticas (ou proteases) são divididas em endo e exo-peptidades. Endopeptidases são moléculas relativamente pequenas (~25-30 kDa) que clivam grandes complexos proteicos e são capazes de passar através dos poros e espaços da MP. Exopeptidades são enzimas maiores (>100 kDa) geralmente ligadas à membrana plasmática do epitélio intestinal que hidrolisam as regiões terminais de pequenas proteínas e peptídeos (Borges-Veloso et al., 2012; Weidlich et al., 2012).

A principal enzima do processo digestivo dos mosquitos hematófagos é a protease tripsina. Dentro de poucas horas ou dias após a ingestão do sangue, os níveis

de proteases podem aumentar 20 vezes. A síntese da tripsina inicial é aparentemente parte de um sistema único de transdução de sinais e sua síntese geralmente é induzida pela presença de aminoácidos livres encontrados no bolo sanguíneo (Noriega et al, 1999; Marquardt, 2005). Acredita-se que sua função pode ser determinar se há proteína suficiente para suportar o ciclo gonadotrófico. Quando isso ocorre, vias de transdução de sinais ativam a transcrição da tripsina tardia, e de genes que codificam outros tipos de enzimas digestivas para digerir o sangue. Dentre esses outros tipos, as principais são as amino e carboxipeptidades (Marquardt, 2005; Weidlich et al, 2012).

Imediatamente após a emergência das fêmeas adultas de mosquito, o intestino médio ainda não está completamente diferenciado, o que as impede de se alimentarem de sangue (Hecker, 1971). As células do IMP de A. aegypti nesse momento são extremamente irregulares na forma, possuem poucos microvilos e um aparato sintético-secretório precariamente desenvolvido. Dentro de um dia após a emergência, desmossomos se desenvolvem ao longo da margem celular apical, os microvilos formam uma "borda em escova" completa, e o retículo endoplasmático rugoso se desenvolve em pilhas e espirais bem organizadas. Três dias após a emergência, as células do IMP estão maduras formando um epitélio colunar regular e os mosquitos estão prontos para realizar o repasto sanguíneo (Hecker, 1971; Hecker et al, 1974) . Em Anopheles spp., processo de maturação semelhante ocorre, mas não envolve desenvolvimento de desmossomos e inclui síntese de vesículas apicais contendo precursores da MP (Berner et al, 1983; Hecker, 1977). Se o mosquito não realiza o repasto sanguíneo, as células do IMP exibem sinais de processos degenerativos, como aumento das frações de lisossomos e mitocôndrias, e perda drástica da quantidade de retículo endoplasmático (Bauer et al, 1977).

Apesar de todo o conhecimento atual sobre a biologia do intestino médio de mosquitos hematófagos, ainda não há uma descrição morfofisiológica do intestino de nenhuma espécie fitófaga. Estudos envolvendo essas espécies seriam importantes para acrescentar na literatura informações a respeito da biologia geral dos mosquitos, elucidando diferentes adaptações evolutivas relacionadas aos tipos de dietas.

2. REFERÊNCIAS BIBLIOGRÁFICAS

Abraham, E.G. and Jacobs-Lorena, M. Mosquito midgut barriers to malaria parasite development. Insect Biochemistry and Molecular Biology **34**, 667-671 (2004).

Agrawal, S.; Kelkenberg, M.; Begum, K.; Steinfeld, L.; Williams, C.E.; Kramer, K.J.; Beeman, R.W.; Park, Y.; Muthukrishnan, S. and Merzendorfer, H. Two essential peritrophic matrix proteins mediate matrix barrier functions in the insect midgut. Insect Biochemistry and Molecular Biology **49**, 24-34 (2014).

Andriès, J.C. and Tramu, G. Ultrastructural and immunohistochemical study of endocrine cells in the midgut of the cockroach Blaberus craniifer (Insecta, Dictyoptera). Cell and Tissue Research **240**, 323-332 (1985).

Arias-Goeta, C.; Mousson, L.; Rougeon, F. and Failloux, A.B. Dissemination and Transmission of the E1-226V Variant of Chikungunya Virus in Aedes albopictus are Controlled at the Midgut Barrier Level. Plos One **8**, e57548 (2013).

Bauer, P.G.; Rudin, W. and Hecker, H. Ultrastructural changes in midgut cells of female Aedes aegypti L. (Insecta, Diptera) after starvation or sugar diet. Cell Tissue Research **177**, 15-19 (1977).

Berner, R.; Rudin, W. and Hecker, H. Peritrophic membranes and protease activity in the midgut of the malaria mosquito, Anopheles stephensi (Liston) (Insecta: Diptera) under normal and experimental conditions. Journal of Ultrastructure Research **83**, 195-204 (1983).

Bernick, E.P.; Moffett, S.B. and Moffett, D.F. Ultrastructure and morphology of midgut visceral muscle in early pupal Aedes aegypti mosquitoes. Tissue and Cell **40**, 127-141 (2008).

Bertram, D. S. and Bird, R. G. Studies on mosquito-borne viruses in their vectors. I. The normal fine structure of the midgut epithelium of the adult female Aedes aegypti (L.) and the functional significance of its modification following a blood meal. Transactions of the Royal Society of Tropical Medicine and Hygiene **55**, 404-23 (1961).

Besansky, N.J. and Fahey, G.T. Utility of the white gene in estimating phylogenetic relationships among mosquitoes (Diptera: Culicidae). Molecular Biology and Evolution **14**, 442-454 (1997).

Billingsley, P.F. Blood Digestion in the Mosquito, Anopheles stephensi Liston (Diptera: Culicidae): Activity and Distribution of Trypsin, Aminopeptidase, and α-Glucosidase in the Midgut (1991).

Billingsley, P.F. The midgut Ultrastructure of hematophagous insects. Annual Review of Entomology **35**, 219-48 (1990).

Billingsley, P.F. and Hecker, H. Blood Digestion in the Mosquito, Anopheles stephensi Liston (Diptera: Culicidae): Activity and Distribution of Trypsin, Aminopeptidase, and α-Glucosidase in the Midgut. Journal of Medical Entomology **28**, 865-871 (1991).

Billingsley, P.F. and Lehane, M.J. Structure and ultrastructure of the insect midgut. Biology of the Insect Midgut, 3-30 (1996).

Billingsley, P. F. and Rudin, W. The role of the mosquito peritrophic membrane in digestion and malaria infectivity. Journal of Parasitology **78**, 430-40 (1992).

Borges-Veloso, A., Saboia-Vahia, L., Cuervo, P., Pires, R. C., Britto, C., Fernandes, N., d'Avila-Levy, C. M. and De Jesus, .J. B. Proteolytic profiling and comparative analyses of active trypsin-like serine peptidases in preimaginal stages of Culex quinquefasciatus. Parasites and Vectors **5**, 113-123 (2012).

Brown, M. R., Raikhel, A. S. and Lea, A. O. Ultraestructure of midgut endocrine cells in the adult mosquito, Aedes aegypti (Diptera). Tissue and Cell **17**, 709-721 (1985).

Brown, M.R.; Crim, J.W. and Lea, A.O. FMRFamide- and pancreatic polypeptide-like immunoreactivity of endocrine cells in the midgut of a mosquito. Tissue and Cell **18**, 419-428 (1986).

Chapman, R.F. Structure of the digestive system. Comprehensive insect physiology, biochemistry, and pharmacology **4**, 165–211 (1985).

Clements, A. N. The Physiology of Mosquitoes. Oxford: Pergamon. 393 pp. (1963).

Clements, A.N. The Biology of Mosquitoes. Development, Nutrition, and Reproduction. Chapman & Hall, New York and London (1992).

Consoli, R. A. G. B. and Lourenço-de-Oliveira, R. Principais mosquitos de importância sanitária no Brasil. Rio de Janeiro, Editora Fiocruz, 225p (1994).

Collins, L.E. and Blackwell, A. The biology of Toxorhynchites mosquitoes and their potential as biocontrol agents. Biocontrol News and Information **21**, 105-116 (2000).

Dinglasan, R.R., Devenport, M., Florens, L, Johnson, J.R., McHugh, C.A., Donnelly-Doman, M., Carucci, D.J., Yates, J.R. and Jacobs-Lorena, M. The Anopheles gambiae adult midgut peritrophic matrix proteome. Insect Biochemistry and Molecular Biology **39**, 125-134 (2009).

Dow, J.A.T. Insect midgut function. Advances in insect physiology **19**, 87–328 (1986).

Eliason, P. A. Feeding adult mosquitoes on solid sugars. Nature **200** 289 (1963).

Foley, D.H.; Rueda, L.M. and Wikerson RC. Insight into global mosquito biogeography from country species records. Journal of Medical Entomology **44**, 554-567 (2007).

Forattini, O.P. Culicidologia Médica. EDUSP, São Paulo, 864p. (2002).

Glattli, E.; Rudin , W. and Hecker, H. Immunoelectron microscopic demonstration of pancreatic polypeptide in midgut epithelium of hematophagous dipterans. Journal of Histochemistry and Cytochemistry **35**, 891-896 (1987).

Gooding, R.H. Digestive processes of haematophagous insects. I. A review literature. Quaestiones entomologicae **8**, 5-60 (1972).

Gooding, R.H., Cheung, A.C. and Rolseth, B.M. The digestive processes of haematophagous insects. III. Inhibition of trypsin by honey and the possible functions of the oesophageal diverticula of mosquitoes (Diptera). Canadian Entomologist **105**, 433-436 (1973).

Grimaldi, D. and Engel, M.S. Evolution of the Insects. Cambridge: Cambridge University Press (2005).

Hanrahan, J. W., Meredith, J., Phillips, J. E. and Brandys D. Methods for the Study of Transport and Control in Insect Hindgut. Measurement of Ion Transport and Metabolic Rate in Insects in Experimental Entomology 19-67 (1984).

Harbach, R.E. Mosquito Taxonomic Inventory, http://mosquito-taxonomic-inventory.info/, (2014).

Harbach, R.E. and Kitching, I. J., Phylogeny and classification of the Culicidae (Diptera). Systematic Entomology **23**, 327–370 (1998).

Harbach, R.E. and Howard, T.M. Index of currently recognized mosquito species (Diptera: Culicidae). European Mosquito Bulletin **23**, 1-66 (2007).

Hecker, H. Structure and function of midgut epithelial cells in Culicidae mosquitoes (Insect, Diptera). Cell Tissue Research **84**, 321-4 (1977).

Hecker, H.; Brun, R.; Reinhardt, C. and Burri, P.H. Morphometric analysis of the midgut of female Aedes aegypti (L.) (Insect, Diptera) under various physiological conditions. Cell Tissue Research **152**, 1-49 (1974).

Hecker, H.; Freyvogel, T.A.; Briegel, H. and Steiger, R. Ultrastructural differentiation of the midgut epithelium in female Aedes aegypti (L.) (Insecta, Diptera) imagines. Acta Tropica **28**, 80-104 (1971).

Hegedus, D., Erlandson, M., Gillott, C. and Toprak, U. New insights into peritrophic matrix synthesis, architecture, and function. Annual Review of Entomology **54**, 285-302 (2009).

Houk, E.J. and Hardy, J.L. In vivo negative staining of the midgut continuous junction in the mosquito, Culex tarsalis (Diptera : Culicidae). Acta Tropica **36**, 267-75 (1979).

Houk, E.J.; Chiles, R.E. and Hardy, J.L. Unique midgut basal lamina in the mosquito, Aedes dorsalis (Meigen) (Insecta, Diptera). International Journal of Insect Morphology and Embryology **9**,161-164 (1980).

Houk, E.J.; Hardy, J.L. and Chiles, R.E. Permeability of the midgut basal lamina in the mosquito, Culex tarsalis Coquillet (Insecta : Diptera). Acta Tropica **8**, 163-171 (1981).

Houk, E.J., Hardy, J.L. and Chiles, R.E. Histochemical staining of the complex carbohydrates of the midgut of the mosquito, Culex tarsalis Coquillett. Insect Biochemistry **16**, 667-675 (1986a).

Houk, E.J.; Hardy, J.L. and Chiles, R.E. Mesenteronal epithelial cell surface charge of the mosquito, Culex tarsalis Coquillett. Binding of colloidal iron hydroxide, native ferritin and cationized ferritin. Journal of Submicroscopic Cytology and Pathology **18**, 385-96 (1986b).

House, H.L.. Digestion. In The Physiology of Insecta, Academic Press, New York, **5**, 63-117 (1974).

Jacobs-Lorena, M. and Oo, M-M. The peritrophic matrix of insects. In: Beaty BJ, Marquardt WC, editors. The Biology of Disease Vectors. Niwot, Colorado: University Press of Colorado, 318-332 (1996).

Jariyapan, N.; Choochote, W.; Jitpakdi, A. and Bates, P.A. Salivary gland of Toxorhynchites splendens Wiedemann (Diptera: Culicidae): ultrastructural morphology and electrophoretic protein profiles. Journal of Medical Entomology **41**, 569-574 (2004).

Kathuria, O. P. Metamorphic changes in posterior region of foregut of Papilio aristolochae F.(Lepidoptera: Papilioidae). International Journal of Insect Morphology and Embryology **1**, 163-168 (1972).

Klowden, M.J. Physiological Systems in Insects. Second Edition (2007).

Lehane, M.J. Peritrophic matrix structure and function. Annual Review of Entomology **42**, 525-550 (1997).

Lehane, M.J., Billingsley, P.F. The biology of the insect midgut. Chapman & Hall, London (1996).

Marquardt, W.C. Biology of Disease Vectors, 2nd ed. Elsevier Academic Press, Burlington, Massachusetts. Second Edition, 785 p. (2005).

Marinotti, O.; James, A.; Ribeiro, J.M.C. Diet and salivation in female Aedes aegypti mosquitoes. Journal of Insect Physiology **36**, 545-548 (1990).

Miller, B.R.; Crabtree, M.B. and Savage, H.M. Phylogenetic relationships of the Culicomorpha inferred from 18S and 5.8S ribosomal DNA sequences (Diptera: Nematocera). Insect Molecular Biology **6**, 105-114 (1997).

Moreira-Ferro, C.K.; Marinotti, O. and Bijovsky, A.T. Morphological and biochemical analyses of the salivary glands of the malaria vector, Anopheles darlingi. Tissue and Cell **31**, 264-273 (1999).

Moskalyk, L.A. Oo, M.M. and Jacobs-Lorena, M. Peritrophic matrix proteins of Anopheles gambiae and Aedes aegypti. Insect Molecular Biology **5**, 261-268 (1996).

Muspratt, J. The bionomics of an African Megarhinus (Diptera, Culicidae) and its possible use in biological control. Bulletin of Entomological Research **42**, 355-370 (1951).

Noriega, F.G., Colonna, A.E. and Wells, M.A. Increase in the size of the amino acid poll is sufficient to activate translation on early trypsin mRNA in Aedes aegypti midgut. Insect Biochemistry and Molecular Biology **29**, 243-247 (1999).

Nyamah, M.A.; Sulaiman, S. and Omar B. Field observation on the efficacy of Toxorhynchites splendens (Wiedemann) as a biocontrol agent against Aedes albopictus (Skuse) larvae in a cemetery. Tropical Biomedicine **28**, 312-319 (2011).

Pawlowski, J.; Szadziewski, R.; Kmieciak, D.; Fahmi, J. and Bittar, G. Phylogeny of the infraorder Culicomorpha (Diptera: Nematocera) based on 28S RNA gene sequences. Systematic Entomology **21**, 167-178 (1996).

Perrone, J. B. and Spielman, A. Time and site of assembly of the peritrophic membrane of the mosquito Aedes aegypti. Cell and Tissue Research **252**, 473-78 (1988).

Peters, W.. Peritrophic membranes. In: Bradshaw D, Burggren W, Heller HC, Ishii S, Langer H, Neuweiler G, Randall DJ, editors. Zoophysiology, 130, Berlin (1992).

Phillips, J.E., Hanrahan, J., Chamberlin, M. and Thomson B. Mechanisms and Control of Reabsorption in Insect Hindgut. Advances in Insect Physiology **19**, 329-422 (1987).

Regis, L.; Silvafilha, M.H.N.; Deoliveira, C.M.; Rios, E.M.; Dasilva, S.B. and Furtado, A.F. Integrated control measures against Culex quinquefasciatus, the vector of filariasis in Recife. Memórias do Instituto Oswaldo Cruz **90**, 115-119 (1995).

Reidenbach, K.R., Cook, S., Bertone M.A., Harbach, R.E., Wiegmann, B.M. and Besansky, N.J. Phylogenetic analysis and temporal diversification of mosquitoes (Diptera: Culicidae) based on nuclear genes and morphology. BMC Evolutionary Biology **9**, 298 (2009).

Reinhardt, C. and Hecker, H. Structure and function of the basal lamina and cell junctions in the midgut epithelium (stomach) of female Aedes aegypti L. (Insecta, Diptera). Acta Tropica **30**, 13-36 (1973).

Reynolds E.S. The use of lead citrate at high pH as an electron-opaque stain in electron microcopy. Journal of Cell Biology **17**, 208-212 (1963).

Ribeiro, J.M.C.; Sarkis, J.J.F.; Rossignol, P.A. and Spielman, A. Salivary apyrase of Aedes aegypti: Characterization and secretory fate. Comparative Biochemistry and Physiology, 79, 81-86 (1984).

Rose, C.; Belmonte, R.; Armstrong. S.D.; Molyneux, G.; Haines, L.R.; Lehane, M.J.; Wastling, J. and Acosta-Serrano, A. An investigation into the protein composition of the teneral Glossina morsitans morsitans peritrophic matrix. Plos Neglected Tropical Diseases **8**, e2691 (2014).

Rossignol, P.A.; Lueders, A.M. Bacteriolytic factor in the salivary glands of Aedes aegypti. Comparative Biochemistry and Physiology **83**, 819-822 (1986).

Rudin ,W. and Hecker, H. Morphometric comparision of the midgut epithelial cells in male and female Aedes aegypti L. (Insecta, Diptera). Tissue and cell **8**, 459-470 (1976).

Schreiber, E.T. Toxorhynchites. Journal of the American Mosquito Control Association **23**, 129-132 (2007).

Shaalan, E.A.S. and Canyon, D.V. Aquatic insect predators and mosquito control. Tropical Biomedicine **26**, 223-261 (2009).

Silva, C.P.; Lemos, F.J.A. and Silva, J.R. Tópicos avançados em Entomologia Molecular. Instituto Nacional de Ciência e Tecnologia em Entomologia Molecular (2012).

Snodgrass, R.E. Principles of Insect Morphology. New York, NY: McGraw-Hill (1935).

Springer-Verlag.Preuss, U.; Landsberg, G. and Scheidtmann, K.H. Novel mitosis-specific phosphorylation of histone H3 at Thr11mediated by Dlk/ZIP kinase. Nucleic Acids Research **31**, 878-85 (2003).

Steffan, W.A.; Evenhuis, N.L. Biology of Toxorhynchites. Annual Review of Entomology, **26**, 159-181 (1981).

Tellam, R.L.; Wijffels, G. and Willadsen, P. Peritrophic matrix proteins. Insect Biochemistry and Molecular Biology **29**, 87-101 (1999).

Tellam, R.L. and Eisemann, C. Chitin is only a minor component of the peritrophic matrix from larvae of Lucilia cuprina. Insect Biochemistry and Molecular Biology **30**, 1189-2001 (2000).

Terra, W.R. Evolution and function of insect peritrophic membrane. Ciência e Cultura **48**, 317324 (1996).

Terra, W.R. Evolution of digestive systems of insects. Annual Review of Entomology **35**, 181-200 (1990).

Thompson, M.T. Alimentary canal of the mosquito. Proceedings of the Boston Society of Natural History **32**, 145-202 (1905).

Weidlich, S.; Huster, J.;, Hoffmann, H. and Woodring, J. Environmental control of trypsin secretion in the midgut of the two-spotted field cricket, Gryllus bimaculatus. Journal of Insect Physiology **58**, 1477-1484 (2012).

Wiegmann, B.M.; Trautwein, M.D.; Kim, J.W.; Cassel, B.K.; Bertone, M.A.; Winterton S.L. and Yeates, D.K. Single-copy nuclear genes resolve the phylogeny of the holometabolous insects. BMC Biology **7**, 34 (2009).

Winteringham, F.P. Some distinctive features of insect metabolism. Biochemical Society Symposium **25**, 29-37 (1965).

WRBU. The Walter Reed Biosystematics Unit (2014).

3. OBJETIVOS

3.1. OBJETIVOS GERAIS

Analisar e caracterizar a estrutura do intestino médio de mosquitos adultos de Toxorhynchites theobaldi.

3.2. OBJETIVOS ESPECÍFICOS

a) Descrever a morfologia e caracterizar os tipos celulares do intestino médio;

b) Verificar se há diferenças morfológicas entre os epitélios do intestino médio anterior e posterior.

c) Observar se ocorre a produção de matriz peritrófica pelas células intestinais.

d) Comparar a estrutura intestinal do macho e da fêmea.

4. ARTIGO

Midgut of the non-hematophagous mosquito Toxorhynchites theobaldi (Diptera, Culicidae)

Raquel S. M. Godoy, Kenner M. Fernandes, Gustavo F. Martins*

Departamento de Biologia Geral - Universidade Federal de Viçosa, 36570-900 Viçosa, Minas Gerais, Brasil

Email list:

Raquel S. M. Godoy: raquel.godoy@ufv.br

Kenner M. Fernandes: kennerbio@yahoo.com.br

Gustavo F. Martins: gmartins@ufv.br

* Corresponding author: Professor Gustavo Ferreira Martins

Departamento de Biologia Geral - Universidade Federal de Viçosa, 36570-900 Viçosa, Minas Gerais, Brazil

Tel# +553138993492

Fax# +553138992549

Abstract

In most mosquito species, the females require a blood-feeding for complete egg development. However, in Toxorhynchites mosquitoes, the eggs develop without blood-feeding, and both females and males exclusively feed on sugary diets. The midgut is a well-understood organ in blood-feeding mosquitoes, but little is known about it in non-blood-feeding ones. In the present study, the detailed morphology of the midgut of Toxorhynchites theobaldi were investigated using histochemical and ultrastructural methods. The midgut of female and male T. theobaldi adults consists of a long, slender anterior midgut (AMG), and a short, dilated posterior midgut (PMG). The AMG is subdivided into AMG1 (short, with folds) and AMG2 (long, without folds). Nerve branches and enteroendocrine cells are present in AMG and PMG, respectively. Compared with the PMG of blood-feeding female mosquitoes, the PMG of T. theobaldi is smaller; however, in both mosquitoes, PMG seems be the main region of food digestion and absorption, and protein secretion. The epithelial folds present in the AMG of T. theobaldi have not been reported in other mosquitoes; however, the midgut muscle organization and endocrine control of the digestion process are conserved in both T. theobaldi and blood-feeding mosquitoes.

Keywords: Culicidae, digestive system, midgut, Toxorhynchites

Introduction

The family Culicidae (Diptera) is monophyletic and consists of all mosquito species [1], including species of the tribe Toxorhynchitini [2]. This tribe includes a single genus, Toxorhynchites, comprising approximately 93 species [3]. Unlike most mosquitoes, in Toxorhynchites, females are not hematophagous [4,5,6]. Hence, egg development does not depend on blood supply and, as adults, both males and females feed exclusively on nectar, honey, and other sugary substances [3,4,7]. Lack of hematophagy is not an exclusive characteristic of Toxorhynchites and is shared with other genera (e.g., Malaya and Topomyia) in the family Culicidae. Among the non-hematophagous mosquitoes, Toxorhynchites has a greater number of species and wider geographic distribution [8], making this genus more representative.

The midgut is the portion of the digestive tract responsible for digestion of food in mosquitoes [9,10]. In adult mosquitoes, the midgut has two portions, which differ morphologically and functionally: the anterior midgut (AMG) is mainly associated with sugar digestion and absorption [11,12]; and the posterior midgut (PMG), which is an expandable sac whose cells are involved in blood digestion (females exclusively), water regulation, digestive enzyme and peritrophic matrix (PM) component synthesis and secretion, and nutrient absorption [9,13,14].

Unlike the PMG, the AMG of adult mosquitoes is well supplied by nerve endings [13]. However, both AMG and PMG are enclosed externally by circular and longitudinal muscles, which assist in food movement and provide structural integrity [10,15]. The midgut epithelium is adjacent to the muscle fibers, and is predominantly made up of digestive cells. These cells actively participate in nutrients digestion and absorption, with two typical types of cell membrane specializations: microvilli and basal labyrinth [13]. The other cells not directly involved in digestion include endocrine

cells, related to the control of digestive processes through the release of hormones and neuropeptides; and regenerative cells, responsible for the renewal of midgut epithelium [10,13,16].

The midgut in blood-feeding female mosquitoes is the site of blood digestion and the gateway for establishment of various human pathogen, including viruses, protozoa, and nematodes [17-19]. This explains why the midgut is one of the most understood organs in mosquitoes. However, there has been little research on the midgut of non-hematophagous mosquitoes, such as Toxorhynchites. Therefore, in the present study, the midgut morphological and functional characteristics of female and male Toxorhynchites theobaldi were investigated, and the differences between this species and blood-feeding mosquito species were discussed. Additionally, this study will also help in understanding the overall morphophysiology of the Culicidae midgut.

Results

General morphology and histology

The midguts of both female and male T. theobaldi consist of a long, slender AMG, and a smaller, dilated PMG. In both females and males, the AMG is divided into two distinct parts: AMG1, with folds on the surface and located in the thorax; and AMG2, without folds and located in abdomen (Fig. 1a and Sup. Fig. a). The total length of the midgut was 6.1 mm in females and 4.5 mm in males, however, length and width of the different regions of the midgut were proportional between females and males. The length of the AMG corresponded to ~84% of the total midgut length. The length of AMG1 corresponded to a quarter of the total length of the AMG. The width of PMG was higher than AMG1 or AMG2 (Fig. 1b).

In the three regions of the T. theobaldi midgut (AMG1, AMG2, and PMG) there was a single cell layer epithelium with cells displaying brush borders (Figs. 2a, 2g, and 3a). The AMG1 epithelium was continuous with the cardia epithelium (proventriculus or the transition between the foregut and midgut) and had many wrinkles or folds (Figs. 2a and 2d). In AMG2 and PMG, no folds were seen, but undulations occurred in the basal region of the epithelium, where the circular muscles are inserted (Figs. 2g and 3a). In AMG1, digestive cells were approximately of the same height (Fig. 2a), unlike AMG2, where cells exhibited different heights, forming a narrow lumen with an "X" shape when cross-sectioned (Fig. 2f). In the PMG, digestive cells were aligned with the nucleus positioned at the same height (Fig. 3a). The height of the epithelium and the thickness of brush border in each of the three-midgut regions were similar between females and males ($p > 0.05$). Digestive cells in PMG were higher (43.34 µm) and had thicker brush borders (11.31 µm) than AMG1 and AMG2, which had similar measurements (22.78 µm epithelial height and 3.59 µm brush border) (Fig. 1c).

In the three regions of the midgut, digestive cells had elongated or rounded nuclei, and their supranuclear portion was predominantly acidic (Figs. 2c and 3a). The subapical cell region, immediately below the brush border, was negative for the PAS reaction, but rich in proteins. This region was thicker or more evident in PMG (Figs. 2d, 3d-3e). The brush border on all regions of the midgut was positive for the PAS reaction (Figs. 2a, 2f and 3d). Besides digestive cells, other cells were seen in the basal region of the midgut epithelium (Fig. 2c and 3a). These basal cells could correspond to regenerative or enteroendocrine cells. In whole mounts, they had small nuclei with no defined shape, unlike the digestive cells with large, elongated, and regular nuclei (Figs. 3f–g). Externally, the midgut had elongated and large nuclei of muscle cells (Fig. 3h).

The lumen of AMG1 and AMG2 was narrow, sometimes with opposite brush borders very close to each other (Figs. 2a and 2f), while the PMG lumen was large (Fig. 3c). In AMG1 and PMG, structures protruding from the apical side of the digestive cells towards midgut lumen were visualized, characterizing the process of aprocrine secretion. These structures were positive for proteins (Figs. 2d and 3b), and negative for PAS reaction (Supplementary Figs. d-e). In AMG1, these structures were basic, while in PMG, they were acidic (Figs. 2c and 3a). This type of cell process was not seen in AMG2.

In midguts where the food bolus is being transferred to the hindgut, digestive cells of AMG and PMG exhibited intense staining for the PAS reaction. This staining was observed in the apical and basal regions, or only in the basal region of cells (Figs. 2e and 3d).

A PAS-positive material, corresponding to the PM, was also seen in the lumen of midgut, including inside epithelial folds of AMG1 (Figs. 2b, 2g and 3d). This PM was thin and loosely organized (Figs. 2b and 3d). The presence of PM in the lumen of all midgut regions of T. theobaldi were confirmed by WGA-FITC staining (Figs. 4a–f). In the AMG1 (Fig. 4a) and AMG2 (Fig. 4b), the labelling was seen just above the brush border. Differently, the labelling in PMG were more diffused or located in the center of the lumen (Figs. 4c).

By phalloidin-FITC labeling (actin marker), muscle bundles were seen in the midgut of T. theobaldi, forming a network covering the outer wall of the organ. This labeling revealed the arrangement of circular and longitudinal bundles in the three regions of the midgut (Fig. 5a–c). The longitudinal bundles had similar width in the AMG and were apparently thicker in the PMG, whereas the circular bundles were

thicker in AMG1, becoming narrower in the passage between AMG1 and AMG2 (Figs. 5d–f).

The circular bundles were organized orthogonally to the longitudinal ones. Each circular bundle was interconnected to neighbor circular bundles. The interconnection of the circular muscle bundles always occurred at the same position along the midgut length (Figs. 5d-e and 5g).

The longitudinal bundles were parallel and with few branches, seen only in some muscle bundles of AMG1 and PMG (Figs. 5d and 5g). The longitudinal bundles were not all continuous from the beginning to the end of the midgut, with some of them terminating early in the PMG, while others originated from the rear end of the PMG, extending the transition of AMG2 to PMG (Fig. 5f).

Transmission electron microscopy

AMG1

The AMG1 digestive cells had densely-packed microvilli in the apical region and invaginations in the basal region, forming an extensive and sparse labyrinth, which occupied nearly half the cell (Figs. 6a and 6f). The microvilli were thin, tall, numerous, and contained extracellular material with granular aspect on its ends, corresponding to the PM (Fig. 6b).

The digestive cells of AMG1 had many mitochondria and lamellar bodies in the apex (Fig. 6c). Golgi apparatus, small autophagic vacuoles, lamellar, and multilamellar bodies were also seen (Figs. 6d, 6e, and 6g). The basal lamina was compact and continuous, and had undulations and depressions (Figs. 6f and 6h). Muscle cells were located adjacent to the basal lamina (Figs. 6a and 6g).

Regenerative cells were seen in the basal region of the epithelium of AMG1, AMG2, and PMG (Figs. 6h and 7d). These small cells had few organelles and extensive lateral expansions, which establish a connection with the neighboring regenerative cells and the basal lamina. Differentiating regenerative cells had emerging microvilli and basal labyrinths (Fig. 6h).

AMG2

AMG digestive cells had long and slender microvilli (Figs. 7a, inset and 7b). As well as in AMG1, microvesicle-like structures were seen close to the PM, with single or double layers (Fig. 7c). The digestive cell cytoplasm had autophagic vacuoles (Fig. 7b, inset), electrondense lysosome-like structures, and many mitochondria and lamellar bodies concentrated in the apex (Fig. 7b). The basal labyrinth was extensive, but less developed than in AMG1, and the basal lamina was compact and continuous (Fig. 7d).

Enteroendocrine cells were seen in AMG2, and in PMG. In both regions, these cells had electron-lucent nuclei, few mitochondria, and many small electrondense granules. These cells established contact with the basal lamina through extensive cytoplasmic processes (Figs. 7d, 8h, and 8i).

PMG

The microvilli of digestive cells of PMG were thin, numerous, and higher than that of AMG and were also associated to microvesicle-like structures (Fig. 8a, inset). Autophagic vacuoles of various sizes, multilamellar bodies, and Golgi apparatus were also found here (Figs. 8b–f). Digestive cells in the PMG were rich in rough endoplasmic reticulum with their concentric lamellae accumulated in the supranuclear region (Figs. 8f-g). Large vesicles, or inclusion bodies, containing eletrondense structures or a granular material (Fig. 8g, inset) were seen in the apex of the digestive cells. Basal

labyrinth in PMG was less expressive and the basal lamina was thick and compact in some intervals in comparison to AMG (Figs. 8h-i).

Scanning electron microscopy

The midgut topography was similar in T. theobaldi females and males. AMG1 was continuous with the cardia, a dilated structure that connected the esophagus to AMG (Figs. 9a and 9b). As seen in the histological sections, the AMG1 epithelium had folds (Figs. 9a and 9d) that were not seen in AMG2 (Fig. 9e).

Ganglia were located just above the cardia and nerve fibers extended along AMG. Nerves ramified and connected to the longitudinal muscle bundles (Figs. 9b and 9c). Tracheoles were seen on the entire surface of the midgut and were most commonly found in AMG2 and PMG (Figs. 9e and 9f).

In AMG1 and AMG2, only the longitudinal muscle bundles were seen (Figs. 9c–e) under SEM. In AMG1, longitudinal bundles were more widely spaced, but the circular bundles still could not be seen, as they were hidden in the furrows formed between the epithelial folds (Fig. 9c). In AMG2, there were many tracheoles and the longitudinal bundles were very close to each other, hiding the circular bundles (Fig. 9e). In the PMG, the longitudinal bundles were widely spaced, allowing the visualization of circular muscle bundles (Figs. 9f-g).

Cell proliferation

Cell proliferation was not detected in any of the three regions in the midgut of 5- to 10-day-old adult mosquitoes under experiment conditions. However, in the positive control, corresponding to the midguts of A. aegypti (4^{th} larval stage), labeled nuclei were present as expected (Sup. Fig. f).

FMRFamide-like positive cells

The anti-FMRFamide antibodies labeled neurons and endocrine cells in T. theobaldi midgut. The pattern of this labeling was similar in female and male T. theobaldi adults. FMRFamide-like positive ganglions were seen above the cardia and their ramifications were seen overlying more than half of the AMG (Figs. 10a–c).

Enteroendocrine cells (i.e., FMRFamide-like positive cells) were abundant and scattered among the digestive cells of the extreme end of AMG2 (close to PMG) and throughout PMG (Figs. 10d and 10e). The number of enteroendocrine cells was similar in males and females ($p = 0.842$), with approximately 99 cells per midgut.

Discussion

The general morphology of the midgut in female and male T. theobaldi resembled that of the midgut of male mosquitoes whose females are hematophagous. In this regard, similar to these males, the AMG of T. theobaldi was thin and long, while the PMG was enlarged and reduced in size. Different of this, in blood-feeding female mosquitoes, the AMG is short and the PMG is expanded (Sup. Figs. b and c) [13,20-23].

The AMG of T. theobaldi was subdivided into two morphologically distinct regions: AMG1 and AMG2. In other Culicidae (both females and males) this subdivision is not evident (or absent), and the AMG is slender and without folds, similar to AMG2 of T. theobaldi. By being wider than AMG2 and containing folds, AMG1 seems to function as a first site of food digestion. The presence of folds increase the contact surface between the food and the digestive epithelium, and probably reduce the speed in which nutrients pass through the lumen, facilitating the digestion and absorption [24].

The epithelium characteristics of the three midgut regions of T. theobaldi were compatible with the secretory, digestive, absorptive, and nutrient transport functions as reported elsewhere [13,25]. Both AMG1 and PMG seem to be more involved in enzyme secretion and nutrient absorption compared with AMG2. These two regions presented apocrine secretion of proteins and intense labeling for carbohydrates, especially in the basal portion of the digestive cells. However, the acidic character of apocrine secretion in AMG1 versus the basic character of this secretion in PMG indicates that the secreted proteins are probably different in the two regions.

The AMG1 and PMG digestive cells of T. theobaldi showed greater carbohydrates accumulation when food was being transferred to the hindgut. The carbohydrates accumulation, such as glycogen, is common in insect digestive cells during absorption activity [10,26], and in the PMG digestive cells of larval and adult mosquitoes fed with sugar or blood [27-29]. These carbohydrate deposits seen in PMG seem to accumulate because of the digestion process, functioning as energy reserves, or facilitating the subsequent absorption of more carbohydrates [30].

Apocrine secretions are typically released during the digestive process of insects, and it is speculated that this is also related to regions that perform nutrients absorption [31]. Corroborating this, AMG1 and PMG are apparently more involved with the carbohydrate absorption, and are the regions where apocrine secretion occurred.

Another possibly secretory mechanism present in the midgut of T. theobaldi is the microapocrine secretion. The small single and double membrane structures seen across the midgut lumen of T. theobaldi resemble microapocrine secreted vesicles found in the midgut lumen of various insects [31]. Enzymes, such as amylase, and various peritrofins are released into the midgut lumen by this secretory mechanism [32]. The

existence of this type of secretory mechanism is something that needed to be clarified in adults of non-hematophagous mosquitoes.

The abundant rough endoplasmic reticulum (RER) lamellae in the PMG are also found in the PMG of hematophagous females when a blood meal is acquired. The marked presence of these organelles occurs in cells that are specialized in protein secretion [9,13,29]. Accordingly, it is possible to infer that the bloodmeal in blood-feeding female mosquitoes, and the sugar meal in T. theobaldi stimulate intense activity of protein secretion in PMG digestive cells. In addition to the PMG, the AMG1 also had many RER lamellae in the digestive cells, which is probably related to the apocrine secretion of proteins as demonstrated by histochemistry with bromophenol blue.

Autophagic vacuoles were seen in the digestive cells of all regions of T. theobaldi midgut, being larger in size and quantity in PMG. These vacuoles are related to the recycling of membranes that occurs due to endo- and exocytosis during digestion [29]. Large inclusion bodies were also seen in the digestive cells of T. theobaldi, similar to those observed in the PMG of blood-feeding mosquitoes post bloodmeal [16,29]. The function of these inclusion bodies is unknown, but it has been proposed for recycling membranes, along with the autophagic vacuoles [16]. By containing a large amount of autophagic vacuoles and inclusion bodies compared with AMG, PMG digestive cells may be more involved in vesicular transport than AMG digestive cells.

Lamellar bodies were also abundantly found in digestive cells throughout the T. theobaldi midgut. These structures are composed primarily of lipids and proteins, and their biogenesis involves endocytic and/or autophagic pathways [33]. In some vertebrate digestive epithelia, lamellar bodies are secreted to protect cell membranes from digestive enzymes and the abrasion of food flow [34]. However, the function of these organelles in mosquitoes is unknown.

The PAS reaction and WGA staining [36] confirmed the presence of a thin PM throughout the midgut lumen in T. theobaldi. This PM had a granular/loose appearance under TEM. In hematophagous mosquitoes, the PM is of type I [35,36], however, for the non-hematophagous T. theobaldi, the classification of PM (if it is type I or II) is still unclear. In comparison with the PM of T. theobaldi, the PM of hematophagous mosquitoes is thicker, more compact, and is found only in the PMG [29,37-39]. The presence of PM in T. theobaldi midgut indicates that this structure is not only related to blood digestion in Culicidae, but also plays a role in sugar digestion of non-hematophagous species of this family. In the latter case, PM would be important to protect the midgut cells against microorganisms, which are found abundantly in sugar-based foods such as nectar and honey [40].

Cell divisions could not be identified in the midgut of 5- to 10-day-old T. theobaldi adults through antibodies against PH3. The absence of cell division was also reported in adults of A. aegypti (with the exception of newly emerged A. aegypti) and Culex quinquefasciatus [41,42]. The dynamics of stem cells division and differentiation in T. theolbadi midgut need more investigations considering different ages and digestive phases.

The circular muscle bundles of T. theobaldi midgut are interconnected with adjacent circular bundles. This characteristic also occurs in adult female A. aegypti and Anopheles gambiae [15]. In these two hematophagous species, as well as in T. theobaldi, not all PMG longitudinal bundles are continuous. Some bundles emanate from AMG, ending close to the AMG/PMG transition. Additionally, other longitudinal bundles extend from the hindgut towards the PMG surface [15]. These structural similarities between the midgut muscles of T. theobaldi and blood-feeding females indicate the

existence of a common organization of muscle fibers in mosquitoes, even with markedly different feeding habits.

The ganglia associated with the cardia are part of the stomatogastric nervous system of insects [43,44]. FMRFamide-like immunoreactive neurons in T. theobaldi innervate only the anterior portion of the midgut, as in other mosquitoes previously studied [43]. Neuropeptides, such as FMRFamide-like peptides (FLPs), are secreted by neurons and endocrine cells, and supposedly these peptides act in the control of the digestive process [43,45,46]. The physiological roles of these peptides in mosquito midguts are unknown, but in some insects the FLPs look like to be involved in the control of gut motility and secretion of digestive enzymes [47-50].

FMRF-like immunoreactive (enteroendocrine) cells are found in the PMG and in the final portion of AMG2 of T. theobaldi adults, while in A. aegypti adults, these cells are only seen in the PMG [22,42,43,47]. Despite this minor difference in the location of enterodocrine cells, the neuroendocrine control of the digestive process based on FMRFamide-like neuropeptides, in both blood-feeding and in non-blood-feeding mosquitoes, seems to be performed by the ramifications of the stomatogastric nervous system in AMG, and the endocrine cells in PMG. The enteroendocrine cells of T. theobaldi present abundant small, electrondense secretory granules, as well as the enteroendocrine cells described elsewhere [16]. Thus, the presence of these granules is another feature conserved among adult mosquitoes with different diets.

The presence of a midgut with a long AMG and reduced PMG in T. theobaldi adults is likely an adaptation to sugar-rich diets. Other adaptations to this diet include modifications of the stimuli perception [51], as in the morphophysiology of salivary glands [52]. Compared to blood-feeding females, Toxorhynchites amboinensis females lost some types of chemoreceptors in the palps and antennae, such as a putative ionotropic

receptor and various odorant receptors. This simplification of the chemoreceptive repertoire probably resulted from the loss of the blood-feeding habit by Toxorhynchites [51]. Additionally, Toxorhynchites splendens salivary glands lack secretory cavities and are morphologically and biochemically similar between male and female, unlike in blood-feeding species, where these glands are sexually dimorphic, and females synthesize a range of proteins related to blood-feeding habits [52].

Through our observations, we conclude that the midgut features that are similar among T. theobaldi and hematophagous females include: (1) PMG is rich in specialized organelles for protein secretion; (2) the muscular organization in PMG involves sharing of muscle fibers between neighboring muscle bundles; (3) longitudinal muscle bundles are not continuous along the organ; (4) regenerative cell divisions were not detected in aged adults; and (5) FMRF-like immunoreactive cells, including nervous cells and endocrine cells, are located in AMG and in PMG, respectively. However, the differences between the midguts of T. theobaldi and hematophagous females include: (1) AMG in T. theobaldi is subdivided into two anatomically distinct regions, AMG1 and AMG2, while in hematophagous females this subdivision is not evident; (2) AMG is very long and PMG is small in T. theobaldi when compared with hematophagous females; (3) PM is very thin and is observed in the whole midgut of T. theobaldi, but thick, compact, and is synthesized only after a bloodmeal in hematophagous females.

Our results indicate that the morphophysiology of the midgut of the autogenous and sugar-feeding mosquito T. theobaldi is similar in both males and females, unlike blood-feeding mosquito species, where sexual dimorphism is evident. This similarity can be ascribed to both female and male T. theobaldi having the same feeding behavior. Information on protein synthesis by the midgut of these mosquitoes can unravel the differences in morphology and physiology between the midguts of T. theobaldi and

hematophagous mosquitoes; however, to date, nothing is known regarding this in the genus Toxorhynchites. Studies focusing on the enzymatic activity and proteomics of the midgut in Toxorhynchites species are the next steps to improve the understanding of midgut physiology in these insects, providing new insights into the evolutionary adaptations of the family Culicidae related to a sugar-based diet.

Material and methods

Mosquitoes

Immature specimens of T. theobaldi (larvae of different stages and pupae) were collected from Mata do Paraíso (20°45'14"S, 42°52'55"W), Atlantic Forest region of Viçosa, Minas Gerais, Brazil. Larvae and pupae were collected in black plastic buckets (5 L) containing rainwater at ground level, near tree trunks. The specimens were transferred to the insectary of the Departamento de Biologia Geral/UFV, where the larvae were reared individually in transparent glass bottles (200 mL) and fed daily with Aedes aegypti larvae at different stages.

Pupae were transferred to plastic pots containing dechlorinated tap water and kept in cages until the emergence of adults. Adults (5–10 d after emergence) were fed ad libitum with 10% sucrose solution, and dissected. The insects were kept at a temperature of 26 ± 2°C and humidity 60–70%.

Whole mounting and histology

Twenty midguts (10 males and 10 females) were dissected in phosphate buffered saline – PBS pH 7.6 (0.1 M NaCl, 20 mM KH2PO4, and 20 mM Na_2HP_4) and fixed in 2.5% glutaraldehyde solution (sucrose/cacodylate buffer 0.1 M pH 7.2) for 24 h. The fixed midguts were cut with using microscissors in three distinct regions: AMG1

(initial portion of the AMG), AMG2 (posterior portion of the AMG), and PMG. The samples were washed with distilled water, dehydrated in ascending ethanol series (70, 80, 90, 95 and 100%) and embedded in 2-hydroxyethyl methacrylate historesin (Leica Microsystems Heidelberg Mannhein, Germany). Serial sections of 2-µm thickness were obtained using an automatic microtome with glass knives.

The sections were subjected to different staining methods. As conventional staining methods, hematoxylin-eosin (HE) or toluidine blue staining protocols were used according to standard routine laboratory procedures. For histochemistry, the periodic acid Schiff (PAS) reaction [53] was employed for neutral glycoproteins, neutral carbohydrates and glycogen detection; and bromophenol blue [54] for total protein detection. After staining and drying, the slides were mounted using Eukitt (Fluka, St. Louis, MO, USA) mounting medium, analyzed, and photographed under an Olympus BX53 microscope, coupled with an Olympus DP 73 digital camera of the Laboratório de Sistemática Molecular, Departamento de Biologia Animal/UFV.

The length and width of AMG1, AMG2, and PMG of 20 insects (10 females and 10 males) were measured. The total organ length was obtained by adding the length measurements of AMG and PMG. The height of the epithelium (i.e., digestive cells), and brush border were measured separately in AMG1, AMG2, and PMG. The measurements were performed only at regular epithelia, with no cytoplasmic protrusions, and with muscular layer and brush border evident. Measurements were performed using the Image Pro-Plus 4.5 software (Media Cybernetics) in light micrographs of six females and six males.

The midgut cell organization was also analyzed in whole mounts. For this, 10 fixed and washed midguts were stained using diamidino-2-phenylindole (DAPI) (Biotium, Inc., Hayward, CA, USA) for 30 min and mounted with Mowiol solution

(Sigma-Aldrich Brasil Ltda, São Paulo, SP, Brazil). The slide preparations were photographed with a epifluorescence microscope (see above).

To examine the midgut muscle organization, 10 midguts (5 males and 5 females) were dissected and fixed for 2 h in Zamboni's solution, rinsed thrice for 30 min in PBS/Triton X-100 1% (PBST); and incubated for 40 min in phalloidin conjugated with fluorescein isothiocyanate (FITC) diluted in distilled water (1:500) (Sigma Aldrich, Sigma-Aldrich Brasil Ltda).Then, the midguts were washed in PBS three times for 5 min, mounted on slides with Mowiol solution, and examined under a confocal laser scanning microscope (CLSM) Zeiss 510 Meta at the Núcleo de Microscopia e Microanálise (NMM) UFV.

WGA staining

To detect glycoconjugates and polysaccharides containing β-1-4 N-acetyl-glucosamine residues, midgut (4 males and 4 females) sections of 2-µm thickness were washed in PBS two times for 10 min and incubated with for 30 min with 10 µg/ml FITC-labeled lectin (WGA-FITC, Sigma Aldrich, #L4895, Israel) diluted in PBS. Sections were washed 3 times for 2 min, mounted with Mowiol solution and photographed under fluorescence microscope.

Transmission electron microscopy (TEM)

Fragments of fixed midgut regions (AMG1, AMG2, and PMG, from 3 males and 3 females) were washed in 0.1 M cacodylate buffer pH 7.2; and post-fixed for 1 h in 1% osmium tetroxide/0.1 M cacodylate buffer pH 7.2. The samples were washed three times in distilled water and counterstained for 2 h in an aqueous solution of 3% uranyl acetate. After further washing in distilled water, samples were dehydrated in ascending

ethanol series; pre-infiltrated in LRWhite resin (London Resin Company Ltd, Berkshire, England) and 100% ethanol (ethanol/resin 2:1, 1:1, and 1:2); and embedded in pure resin for subsequent polymerization at 60°C for 48 h. Ultrathin sections (70–90 nm) were analyzed and photographed under a TEM Zeiss EM 109 at NMM/UFV.

Scanning electron microscopy (SEM)

Whole midguts (3 males and 3 females) were fixed and post-fixed as describe above. After washing in PBS, samples were dehydrated in ascending ethanol series, critical point dried using CO_2 and sputter coated with gold. Samples were analyzed and photographed under a SEM LEO 1430VP at NMM/UFV.

Immunofluorescence

To identify dividing cells in the midgut, a primary antibody against the nuclear protein phospho-histone H3 (PH3) (Cell Signaling Technology, Inc., Beverly, MA, U.S.A.) was used [42]. Midguts of 10 males and 10 females were fixed in Zamboni's fixative, washed three times for 30 min each in PBS/1% Triton X-100 (PBST) and incubated for 24 h at 4°C in anti-PH3 primary antibody (Cell Signaling) (1:100) in 1% PBST. The samples were washed three times and incubated with a secondary antibody conjugated with FITC (Sigma) (1:500) in PBS for 24 h at 4°C, followed by three washes of 10 min each.

For detection of cells expressing neuropeptides FMRFamide (neurons and endocrine cells), after fixation, 20 midguts (10 females and 10 males) were washed and incubated for 24 h at 4°C with anti-FMRFamide primary antibody (Peninsula Laboratories, Inc., San Carlos, CA, U.S.A.) (1:400) in 1% PBST. Samples were washed, incubated with the secondary antibody and washed again, as described above.

For both in situ identification of proteins (H3 and FMRF), the cell nuclei were stained with TO-PRO-3 Iodide (Life Technologies) for 30 min, washed, mounted, and analyzed under CLSM (2.2). The total number of FMRFamide-positive cells in the midgut was determined for each insect manually using the z-stack tool of multiple confocal planes.

Controls

Midguts from six fourth instar larvae (L4) of A. aegypti were used as a positive control for PH3 identification [42]. As negative controls for the two proteins (H3 and FMRF) above, midguts of male and female (n = 3, each) T. theobaldi were treated as described in Immunofluorescence item, but without primary antibodies. As negative controls for WGA-FITC staining, histological sections of midguts of 4 individuals (2 males and 2 females) were mounted with Mowiol solution and observed under fluorescent microscope.

Statistical analysis

Measurements of midguts and cell counts were subjected to analysis of variance (ANOVA) for variables with normal distribution, and to the Kruskal-Wallis test when non-normal distribution was found. Results were deemed significant when $p < 0.05$. Standard deviations (SD) were calculated using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, California, USA), and the data were expressed as replicate means.

Competing financial interests

The authors declare no competing financial interests.

Acknowledgements

This work is supported financially by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior/PVE 88881.030429/2013-01) and Fapemig (Fundação de Amparo à Pesquisa do Estado de Minas Gerais), and technically by Núcleo de Microscopia e Microanálise (NMM-UFV).

References

1. Harbach, R. E. Mosquito taxonomic inventory. (1988) Available at: http://mosquito-taxonomic-inventory.info/. (Acessed: 4th May 2015)
2. Reidenbach, K. R. et al. Phylogenetic analysis and temporal diversification of mosquitoes (Diptera: Culicidae) based on nuclear genes and morphology. BMC Evol. Biol. **9**, 298 (2009).
3. Schreiber, E. T. Toxorhynchites. Am. Mosq. Control Assoc. **23**, 129-132 (2007).
4. Steffan, W. A. & Evenhuis, N.L. Biology of Toxorhynchites. Ann. Rev. Entomol. **26**, 159-181 (1981).
5. Trimble, R. M. Potential of a temperate zone Toxorhynchites for the biological control of tropical container-breeding mosquitoes. Mosq. News. **43**, 71-73 (1983).
6. Collins, L. E. & Blackwell, A. The biology of Toxorhynchites mosquitoes and their potential as biocontrol agents. Biocontrol News Info. **21**, 105-116 (2000).
7. Heinemann, S. J., Aitken, T. H. G. & Belkin, J. N. Collection records of the project "Mosquitoes of middle America" 14. Trinidad and Tobago. Mosq. Syst. **12**, 179-289 (1980).
8. Clements, A. N. 1999. The biology of mosquitoes: sensory reception and behaviour. Wallinford (United Kingdom): CABI.
9. Billingsley, P. F. The midgut ultrastructure of hematophagous insects. Ann. Rev.

Entomol. **35**, 219-48 (1990).

10. Billingsley, P. F. & Lehane, M. J. Structure and ultrastructure of the insect midgut in Biology of the Insect Midgut (eds Lehane, M. J. & Billingsley, P. F.) 3-30 (Springer Netherlands , 1996).
11. Perrone, J. B. & Spielman, A. 1988. Time and site of assembly of the peritrophic membrane of the mosquito Aedes aegypti. Cell Tissue Res. **252**, 473-78.
12. Billingsley, P. F. & Rudin, W. The role of the mosquito peritrophic membrane in digestion and malaria infectivity. J. Parasitol. **78**, 430-40 (1992).
13. Hecker, H. Structure and function of midgut epithelial cells in Culicidae mosquitoes (Insect, Diptera). Cell Tissue Res. **1**, 321-341 (1977).
14. Billingsley, P. F. & Hecker, H. Blood digestion in the mosquito Anopheles stephensi Liston (Diptera: Culicidae); activity and distribution of trypsin aminopeptidase and α-glucosidase in the midgut. J. Med. Entomol. **28**, 865-871 (1991).
15. Park, S. S. & Shahabuddin, M. Structural organization of posterior midgut muscles in mosquitoes, Aedes aegypti and Anopheles gambiae. J. Struct. Biol. **129**, 30-37 (2000).
16. Brown, M. R, Raikhel, A.S. & Lea, A. O. Ultrastructure of midgut endocrine cells in the adult mosquito Aedes aegypti. Tissue Cell. **17**, 709-721 (1985).
17. Baton, L. A. & Ranford-Cartwright, L. C. Plasmodium falciparum ookinete invasion of the midgut epithelium of Anopheles stephensi is consistent with the time bomb model. Parasitology **129**, 663-676 (2004).
18. Salazar, M. I., Richardson, J. H., Sánchez-Vargas, I., Olson, K. E. & Beaty, B. J. Dengue virus type 2: replication and tropisms in orally infected Aedes aegypti mosquitoes. BMC Microbiol. **7**, 9 (2007).
19. Michalski, M. L., Erickson, S. M., Bartholomay, L, C. & Christensen, B. M. Midgut barrier imparts selective resistance to filarial worm infection in Culex pipiens pipiens.

PLoS Negl. Trop. Dis. **4**, e875 (2010).

20. Christophers, S. R. 1960. Aedes aegypti: its life history, bionomics, and structure. London, Cambridge Press.
21. Houk, E. J. Midgut ultrastructure of Culex tarsalis (Diptera, Culicidae) before and after a bloodmeal. Tissue Cell. **9**, 103-118 (1977).
22. Veenstra, J. A., Lau, G. W., Agricola, H. J. & Petzel, D. H. Immunohistological localization of regulatory peptides in the midgut of the female mosquito Aedes aegypti. Histochem. Cell Biol. **104**, 337-347 (1995).
23. Bernick, E. P., Moffett, S. B. & Moffett, D. F. Ultrastructure and morphology of midgut visceral muscle in early pupal Aedes aegypti mosquitoes. Tissue Cell. **40**, 127-141 (2008).
24. Shelomi, M., Hirofumi, W. & Arakawa, G. Endogenous cellulase enzymes in the stick insect (Phasmatodea) gut. J. Insect Physiol. **60**, 25-30 (2014).
25. Rudin, W. & Hecker, H. Functional morphology of the midgut of Aedes aegypti L. (Insecta, Diptera) during blood digestion. Cell Tissue Res. **200**, 193-203 (1979).
26. Silva-Olivares, A. et al. Ultrastructural study of the midgut and hindgut in eight species of the genus Dendroctonus Erichson (Coleoptera: Scolytidae). Ann. Entomol. Soc. Am. **96**, 883-900 (2003).
27. Wigglesworth, V. B. The storage of protein, fat, glycogen and uric acid in the fat body and other tissues of mosquito larvae. J. Exp. Biol. **19**, 56-77 (1942).
28. Schneider, W., Rudin, H. & Hecker, H. Absorption and transport of radioactive tracers in the midgut of the malaria mosquito Anopheles stephensi. J. Ultrastruct. Mol. Struct. Res. **97**, 50-63 (1986).
29. Okuda, K., Caroci, A. S., Ribolla, P. E. M., Bianchi, A. G. & Bijovsky, A. T. Functional morphology of adult female Culex quinquefasciatus midgut during blood

digestion. Tissue Cell. **34**, 210-219 (2002).

30. Chapman, R. F. Alimentary canal, digestion and absorption in The insects: structure and function (Eds Simpson, S. J. & Douglas, A. E.) 72-73 (Cambridge University Press, 2013).
31. Cristofoletti, P. T., Ribeiro, A. F. & Terra, W. R. Apocrine secretion of amylase and exocytosis of trypsin along the midgut of Tenebrio molitor larvae. J. Insect Physiol. **47**, 143-155 (2001).
32. Bolognesi, R., Ribeiro, A. F., Terra, W. R. & Ferreira, C. The peritrophic membrane of Spodoptera frugiperda: secretion of peritrophins and role in immobilization and recycling digestive enzymes. Arch. Insect Biochem Physiol. **47**, 62-75 (2001).
33. Lajoie, P., Ginette, G., Dennis, J. W. & Nabi, I. R. The lipid composition of autophagic vacuoles regulates expression of multilamellar bodies. J. Cell Sci. **118**, 1991-2003 (2005).
34. Schmitz, G. & Muller, G. Structure and function of lamellar bodies, lipid-protein complexes involved in storage and secretion of cellular lipids. J. Lipid Res. **32**, 1539-1570 (1991).
35. Waterhouse, D. F. Digestion in insects. Ann. Rev. Entomol. **2**, 1-18 (1957).
36. Hegedus, D., Erlandson, M., Gillott, C. & Toprak, U. New insights into peritrophic matrix synthesis, architecture, and function. Ann. Rev. Entomol. **54**, 285-302 (2008).
37. Villalon, J. M., Ghosh, A. & Jacobs-Lorena M. The peritrophic matrix limits the rate of digestion in adult Anopheles stephensi and Aedes aegypti mosquitoes. J. Insect Physiol. **49**, 891-895 (2003).
38. Moncayo, A. C., Lerdthusnee, K., Leon, R., Robich, R. M. & Romoser, W. S. Meconial peritrophic matrix structure, formation, and meconial degeneration in mosquito pupae/pharate adults: histological and ultrastructural aspects. J. Med.

Entomol. **42**, 939-944 (2005).

39. Kato, N. et al. Evaluation of the function of a type I peritrophic matrix as a physical barrier for midgut epithelium invasion by mosquito-borne pathogens in Aedes aegypti. Vector-Borne Zoonotic Dis. **8**, 701-712 (2008).
40. Olaitan, B., Adeleke, O. E. & Ola, I. O. Honey: a reservoir for microorganisms and an inhibitory agent for microbes. Afr. Health Sci. **7**, 159-165 (2007).
41. Okuda, K. et al. Cell death and regeneration in the midgut of the mosquito, Culex quinquefasciatus. J. Insect Physiol. **53**, 1307-1315 (2007).
42. Fernandes, K. M., Neves, C. A., Serrão, J. E. & Martins, G. F. Aedes aegypti midgut remodeling during metamorphosis. Parasitol. Int. **63**, 506-512 (2014).
43. Brown, M. R. & Lea, A. O. FMRFamide- and adipokinetic hormone-like immunoreactivity in the nervous system of the mosquito, Aedes aegypti. J. Comp. Neurol. **270**, 606-614 (1988).
44. Hartenstein, V. Development of the insect stomatogastric nervous system. Trends Neurosci. **20**, 421-427 (1988).
45. Lange, A. B. & Orchard, I. The effects of SchistoFLRFamide on contractions of locust midgut. Peptides **19**, 459-467 (1998).
46. Dockray, G. J. The expanding family of -RFamide peptides and their effects on feeding behavior. Exp. Physiol. **89**, 229-235 (2004).
47. Moffett, S. B. & Moffett, D. F. Comparison of immonoreactivity to serotonin, FMRFamide and SCPb in the gut and visceral nervous system of larvae, pupae and adults of the yellow fever mosquito Aedes aegypti. J. Insect Sci. **5**, 1-20 (2005).
48 Zudaire, E., Simpson, S. J. & Montuenga, L. M. Effects of food nutrient content, insect age and stage in the feeding cycle on the FMRFamide immunoreactivity of diffuse endocrine cells in the locust gut. J. Exp. Biol. **201**, 2971-2979 (1998).

49 Peeff, M. N., Orchard, I. & Lange, A. B. The effects of FMRFamide-related peptides on an insect (Locusta migratoria) visceral muscle. J. Insect Physiol. **39**, 207-215 (1993).

50 Fujisawa, Y., Shimoda, M., Kiguchi, K., Ichikawa, T. & Fujita, N. The inhibitory effect of a neuropeptide, Manduca FLRFamide, on the midgut activity of the sphingid moth Agrilus convolvuli. Zool. Sci. **10**, 773-777 (1993).

51. Zhou, X., Rinker, D. C., Pitts, R. J., Rokas, A. & Zwiebel, L. J. Divergent and conserved elements comprise the chemoreceptive repertoire of the non-blood-feeding mosquito Toxorhynchites amboinensis. Genome Biol. Evol. **6**, 2883-2896 (2014).

52. Jariyapan, N., Choochote, W., Jitpakdi, A. & Bates, P. A. Salivary gland of Toxorhynchites splendens Wiedemann (Diptera: Culicidae): ultrastructural morphology and electrophoretic protein profiles. J. Med. Entomol. **41**, 569-574 (2004).

53. Bancroft, J. D. & Gamble, M. in Theory and practice of histological techniques (Eds Bancroft, J. D. & Gamble) 744 (Churchill Livingstone, 2007).

54. Pearse, A. G. E. in Histochemistry: Theoretical and applied (Ed. Pearse, A. G. E) 624 (Churchill Livingstone, 1968).

Author's contributions

Designed the experiments: RSMG and GFM. Performed the experiments and analyzed the data: RSMG, KMF and GFM. Wrote and reviewed the paper: RSMG and GFM. Contributed reagents: GFM.

FIGURES

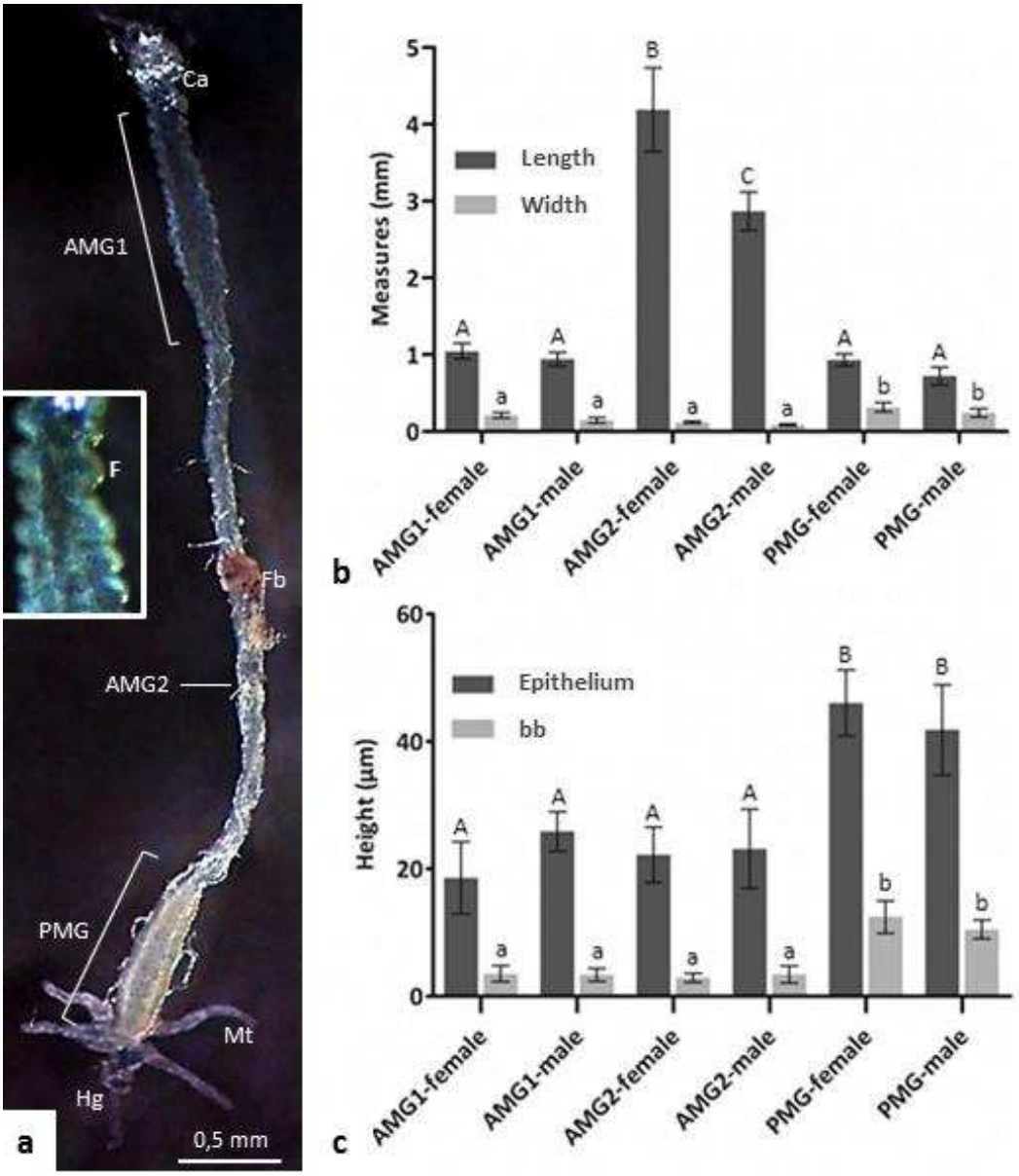

Ca
AMG1
F
Fb
AMG2
PMG
Mt
Hg
0,5 mm
a
b
Measures (mm)
Length
Width
AMG1-female
AMG1-male
AMG2-female
AMG2-male
PMG-female
PMG-male
c
Height (µm)
Epithelium
bb

Figure 1: a: Midgut of Toxorhynchites *theobaldi* adult female depicting the anterior midgut (AMG) subdivided in AMG1 (short and with folds) and AMG2 (long and without folds); and a wide and short posterior midgut (PMG). Fb: fat body. Inset: Portion of AMG1 with epithelial folds (F). **b:** The length and width of the different regions of the midgut are proportional among females and males ($p > 0.05$). The length of the AMG (AMG1 and AMG2) corresponds to ~84% of the total length of the midgut. **c:** The heights of the epithelium and the brush border (bb) for each of the three regions of the midgut did not differ between males and females. Bars with the same letter did not differ statistically according to the ANOVA ($p < 5\%$). AMG1: anterior midgut 1; AMG2: anterior midgut 2; PMG: posterior midgut; Ca: cardia; Mt: Malpighian tubule; Hg: hindgut.

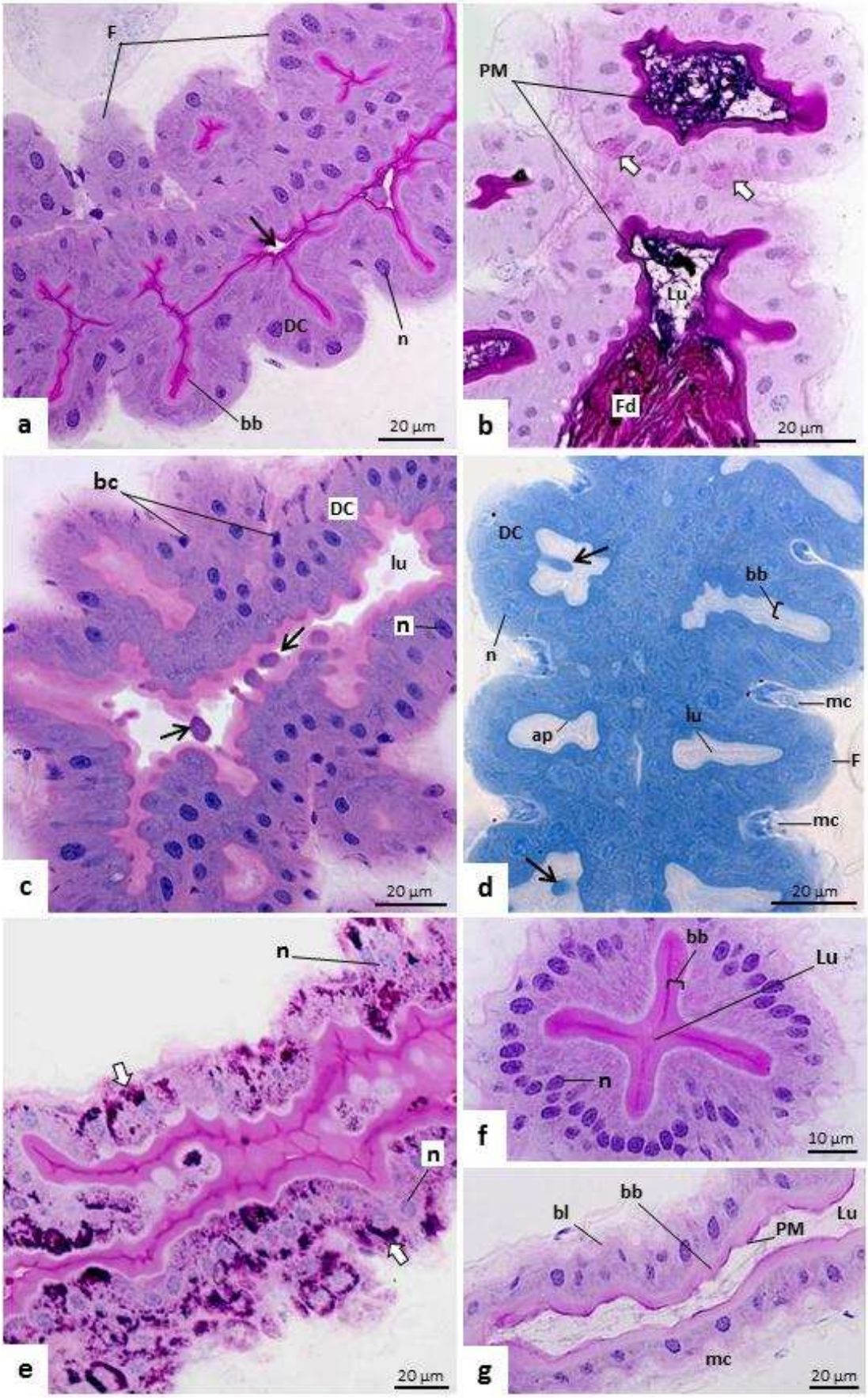

F
DC
n
bb
a
20 µm
PM
Lu
Fd
b
20 µm
bc
DC
lu
n
c
20 µm
DC
bb
n
mc
lu
ap
F
mc
d
20 µm
n
n
e
20 µm
bb
Lu
n
f
10 µm
bb
bl
Lu
PM
mc
g
20 µm

Figure 2: Histological sections of anterior midgut (AMG) of adult T. theobaldi stained using periodic acid Schiff (PAS) reaction followed by counterstaining with hematoxylin **[a**, **b**, **e**, **f**, and **g]**, hematoxylin and eosin **[c]** or bromophenol blue **[d]. a:** AMG1 of a male with folds (F), and PAS-positive brush border (bb). **b:** AMG1 of a female containing food (Fd) and a PAS-positive peritrophic matrix (PM) in the lumen (Lu). Some digestive cells present PAS-positivity (arrows) in their basal region. **c:** AMG1 of a female with digestive cells releasing apocrine secretion of acidic character (arrows) into the lumen (Lu). Non-digestive or basal cells (bc) are seen at the basal region of the epithelium. **d:** AMG1 of a female presenting apical extrusions typical of apocrine secretion, rich in proteins (arrows). The apical portion of the cells, underneath the brush border, contains a thin layer intensely stained for proteins (ap). Muscle cells (mc) are seen between epithelial folds (F). **e:** AMG1 of a male showing intense labeling for carbohydrates (arrows) in the digestive cells. **f:** Cross-section of AMG2 of a female showing cells of different sizes and a X-shaped narrow lumen. **g:** AMG2 of a male with an undulated basal lamina (bl) where muscle cells (mc) are inserted. The peritrophic matrix (PM) is thin, PAS-positive and is located next to the brush border (bb). Lu: midgut lumen.

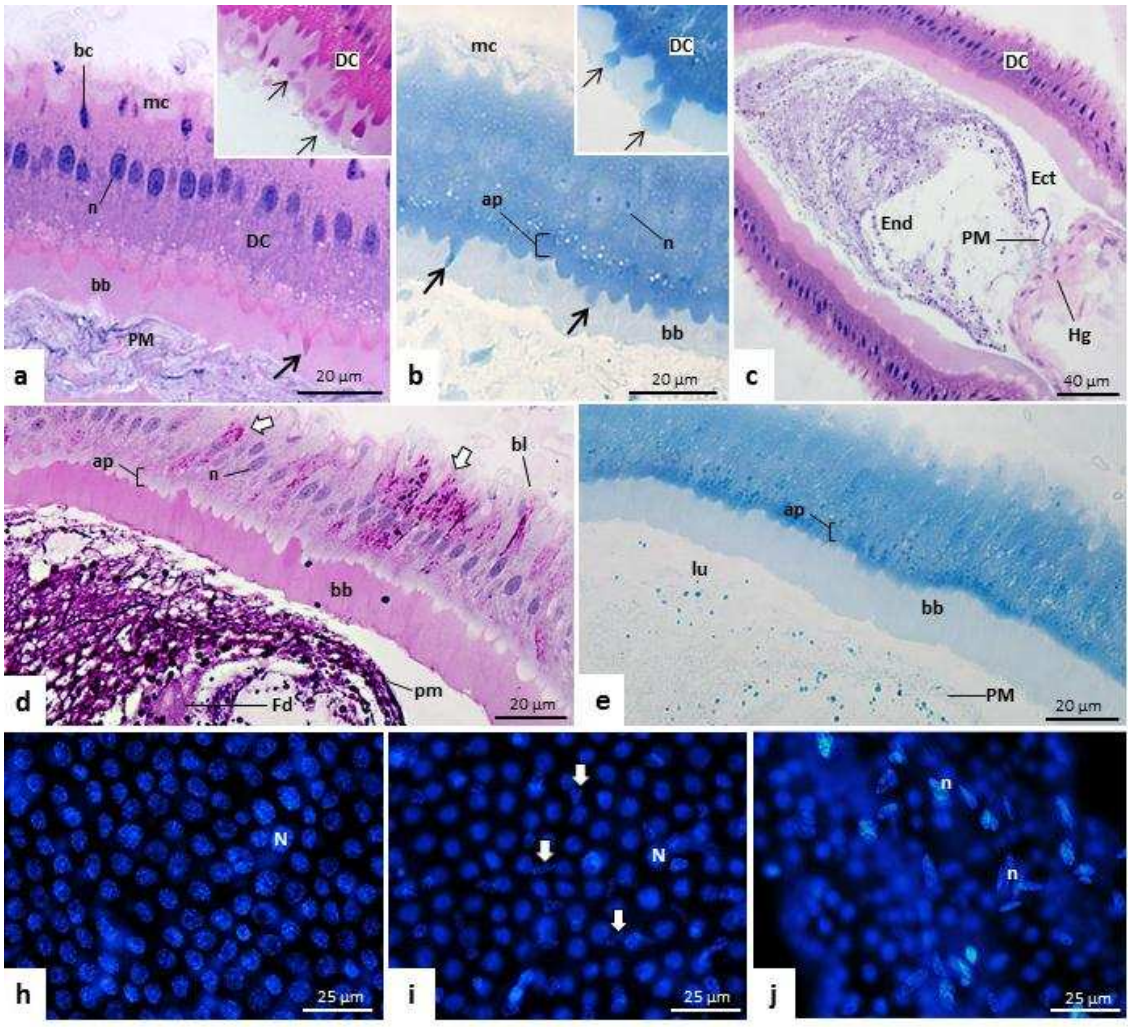

bc
mc
n
DC
bb
PM
20 µm
a
DC
mc
ap
n
bb
20 µm
b
DC
Ect
End
PM
Hg
40 µm
c
ap
n
bl
bb
pm
Fd
20 µm
d
ap
lu
bb
PM
20 µm
e
N
25 µm
h
N
25 µm
i
n
n
25 µm
j

Figure 3: Histological sections of posterior midgut (PMG) of Toxorhynchites theobaldi adults stained with hematoxylin and eosin **[a** and **c]**, periodic acid Schiff (PAS) reaction followed by hematoxylin staining **[d]**, and bromophenol blue **[b**, **e]**. **a:** Epithelium of a male showing cell apex projections typical of apocrine secretion (arrow) and stained for basic substances. Basal cells (bc) are found throughout the epithelium, near muscle cells (mc). n: nucleus of the digestive cell; PM: peritrophic matrix. **b:** PMG of a male with the cell projections (arrows). The cell apical region (ap), including the projections, are intensely marked for proteins. bb: brush border; mc: muscle cells. Insets a and b: detailed views of digestive cells (DC) with apical extrusions (arrows) towards midgut lumen, in a process of apocrine secretion. **c:** Endoperitrophic (En) and ectoperitrophic (Ec) spaces separated by peritrophic matrix (PM) in a female. DC: digestive cells; Hg: hindgut. **d:** Epithelium of a female with the basal region of the digestive cells with intense staining for carbohydrates (arrows), and with the apical region (ap) negative for carbohydrates. Food (Fd) and peritrophic matrix (PM) are intensely stained, while the apex of the digestive cells (c), the brush border (bb) and the basal lamina (bl) are less stained. n: nucleus of the digestive cell. **e:** Epithelium of a female with a thick apical region (ap) rich in proteins, while the peritrophic matrix (PM) and brush border (bb) are weakly stained. **[f-h]** Whole mounts of midgut stained with diamidino-2-phenylindole (DAPI). **f:** Nuclei of digestive cells (N) of PMG of a female. **g:** Basal cell nuclei (arrows) of the PMG of a female, similar to those observed in AMG1 and 2. The epithelium of the three midgut regions contains non-digestive cells (regenerative or enteroendocrine) with small and irregular nuclei. **h:** Fusiform nuclei of muscle cells (n) of an AMG1 of a male.

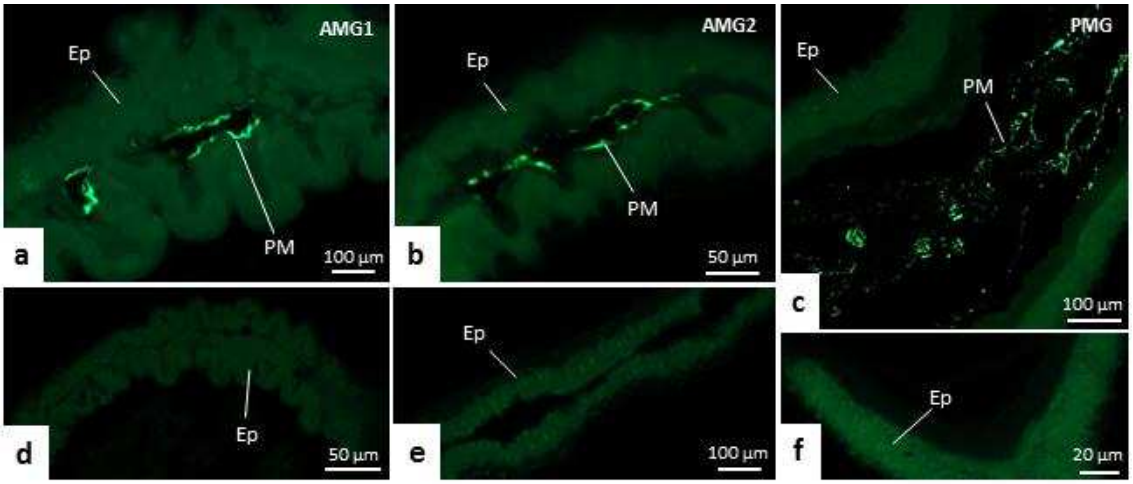


Figure 4: Histological sections of the midgut of adult males of Toxorhynchites theobaldi stained with WGA-FITC **[A-C]** and negative controls **[D-F]**. AMG1: anterior midgut 1; AMG2 anterior midgut 2; PMG: posterior midgut. ep: midgut epithelium.

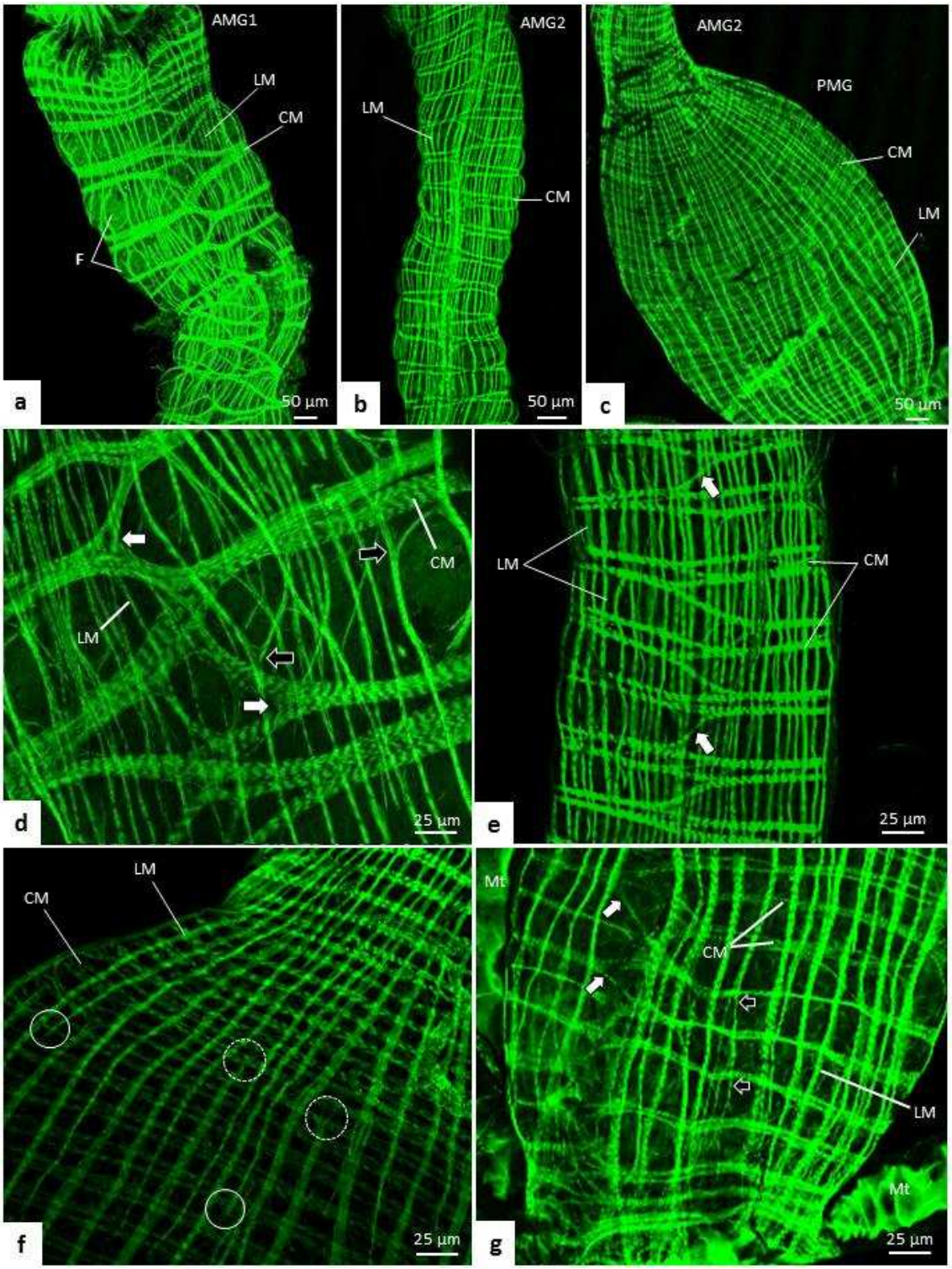

AMG1
LM
CM
F
50 µm
a
AMG2
LM
CM
50 µm
b
AMG2
PMG
CM
LM
50 µm
c
LM
CM
25 µm
d
LM
CM
25 µm
e
CM
LM
25 µm
f
Mt
CM
LM
Mt
25 µm
g

Figure 5: Organization of longitudinal (LM) and circular (CM) muscle bundles of the midgut of Toxorhynchites theobaldi adults stained with phalloidin-FITC. **[a, b** and **c]** anterior midgut 1 (AMG1), anterior midgut 2 (AMG2) and posterior midgut (PMG) of female, respectively. F: epithelial fold. **d:** Portion of AMG1 of a female with circular muscle bundles, which are thick, bifurcated (white arrow), and interconnected with neighboring bundles. Some ramifications are also seen in the longitudinal muscle bundles (black arrows). **e:** Portion of AMG2 with circular muscle bundles (CM) forming interconnected rings between neighboring rings through bifurcations (arrows) in a repeated pattern. The longitudinal muscle bundles (LM) are continuous and without ramifications. **f:** Initial region of the PMG of a female. The longitudinal muscles have some bundles with free ends at the beginning of the PMG. Some of these discontinuous bundles arise from AMG2 (continuous circle) and others from the hindgut (doted circles). **g:** PMG of a female with circular muscle bundles (CM) with bifurcations (white arrows). Close to the insertion of the Malpighian tubules (Mt), the longitudinal muscles (LM) branch (black arrows).

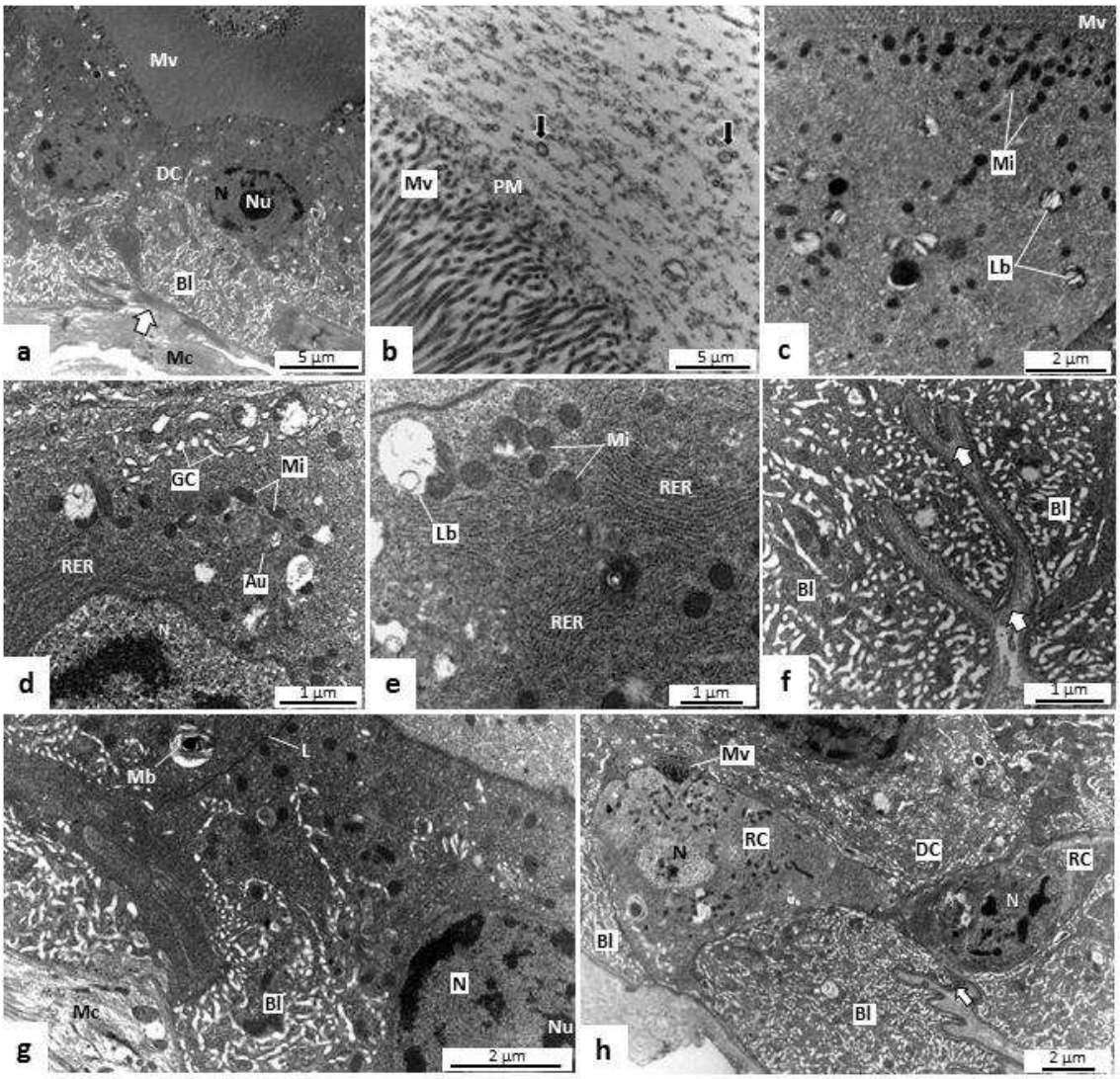

a
Mv
DC
N
Nu
Bl
Mc
5 µm
b
Mv
PM
5 µm
c
Mv
Mi
Lb
2 µm
d
GC
Mi
RER
Au
N
1 µm
e
Mi
RER
Lb
RER
1 µm
f
Bl
Bl
1 µm
g
Mb
L
Bl
N
Mc
Nu
2 µm
h
Mv
N
RC
DC
RC
N
Bl
Bl
2 µm

Figure 6: Transmission electron micrographs (TEMs) of anterior midgut 1 (AMG1) of Toxorhynchites theobaldi adults. **a:** Digestive cells (DC) with tall microvilli (Mv) and a well-developed basal labyrinth (Bl) in a male. Mc: muscle cells close to the basal lamina (arrow). **b:** Peritrophic matrix (PM) with a granular appearance and structures resembling microvesicles (arrows) are seen close to the microvilli (Mv) of a female. **c:** Apical portion of digestive cell rich in mitochondria (Mi) and lamellar bodies (Lb). Mv: microvilli. **d:** Golgi apparatus (GC), rough endoplasmic reticulum (RER), mitochondria (Mi) and autophagyc vacuole (Au) on digestive cell of a male. N: nucleus. **e:** Lamellae of rough endoplasmic reticulum (RER), and lamellar bodies (Lb) in digestive cell of a male. **f:** Basal labyrinth (Bl) and basal lamina (arrow) with branches in a digestive cell of a male. **g:** Digestive cell with multilamellar bodies (Mb) in a male. Bl: basal labyrinth; L: cell limit; Mc: muscle cell; N: nucleus. **h:** Two regenerative cells (RC) in the region of the basal labyrinth (Bl) of digestive cells (DC) in a male. One of the regenerative cells (left) is in the differentiation process, with primordial microvilli (Mv) and basal labyrinth (Bl). N: nucleus.

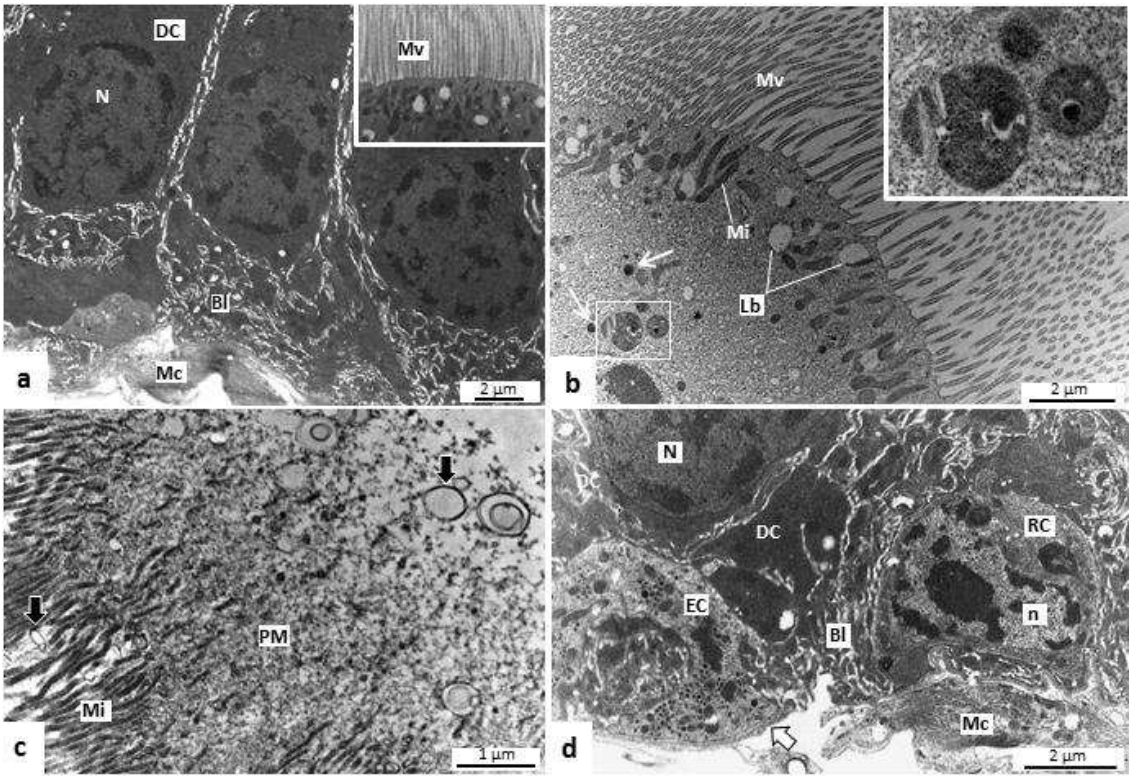


Figure 7: Transmission electron micrographs (TEMs) of the anterior midgut 2 (AMG2) of Toxorhynchites theobaldi adults. **a:** Digestive cells (DC) with thin and densely packed microvilli (Mv, inset), and well-developed basal labyrinth (Bl) in a female. Mc: muscle cell. **b:** Apical portion of a digestive cell with mitochondria (Mi), lysosome-like structures (arrows), lamellar bodies (Lb), and structures resembling autophagic vacuoles (inset). **c:** Structures resembling microapocrine vesicles (arrows) close to the microvilli (Mi) digestive cell and released products (arrows) of these vesicles in the lumen towards the peritrophic matrix (PM) in a female. **d:** Enteroendocrine cell (EC) in contact with the basal membrane close to a regenerative cell (RC) in a male. Arrow: basal lamina; Mc: muscle cell; N and n: nuclei of digestive cell and regenerative cell, respectively.

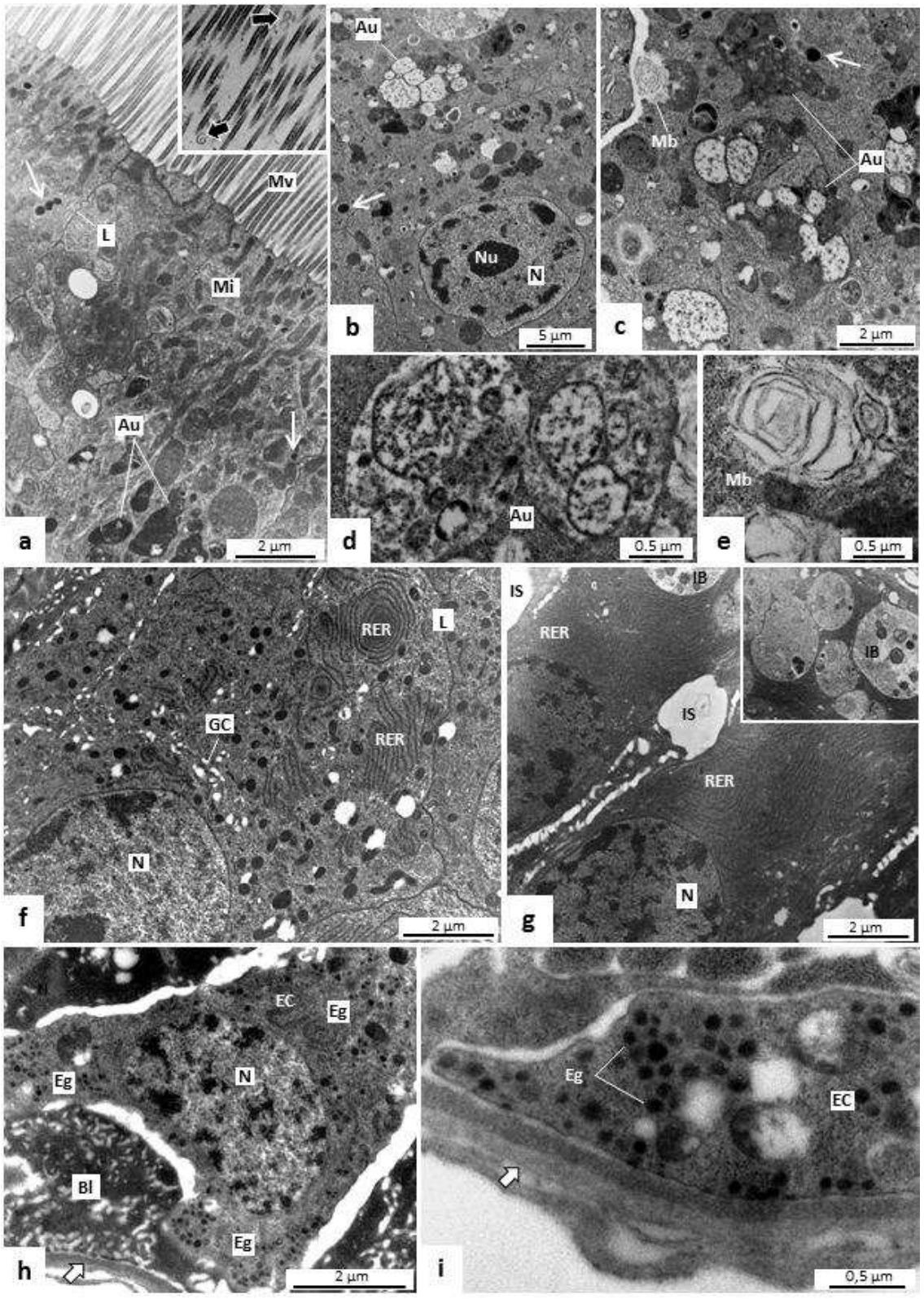

a
Mv
L
Mi
Au
2 µm
b
Au
Nu
N
5 µm
c
Mb
Au
2 µm
d
Au
0.5 µm
e
Mb
0.5 µm
f
RER
L
GC
RER
N
2 µm
g
IS
IB
RER
IB
IS
RER
N
2 µm
h
EC
Eg
Eg
N
Bl
Eg
2 µm
i
Eg
EC
0,5 µm

Figure 8: Transmission electron micrographs (TEMs) of the posterior midgut (PMG) of Toxorhynchites theobaldi adults. **a:** Apex of digestive cell with slender and densely clustered microvilli (Mv) in male. Under the microvilli, there are many mitochondria (Mi), autophagic vacuoles (Au) and lysosome-like structures (arrows). Inset: structures resembling microapocrine vesicles (arrows) close to microvilli; L: cell limit. **b:** Digestive cells with many autophagic vacuoles (Au), lysosome-like structures (arrows) in a female. **c:** Autophagic vacuole (Au) in a digestive cell of a female, and a multilamellar body (Mb) released into the intercellular space. **d** and **e**: Autophagic vacuoles (Au) and multilamellar body (Mb). **f:** Supranuclear portion of a digestive cell with numerous lamellae of rough endoplasmic reticulum (RER) and Golgi apparatus (GC) in a male. **g:** Digestive cell filled with rough endoplasmic reticulum (RER) and inclusion bodies (IB, inset). IS: intercellular space; N- nucleus. **h:** Enteroendocrine cell (EC) contact the basal lamina (arrow) with cytoplasm rich in small electrondense granules (Eg) in a female. **i:** Details of electrondense granules (Eg) of enteroendocrine cell (EC) in a female. Arrow: basal lamina.

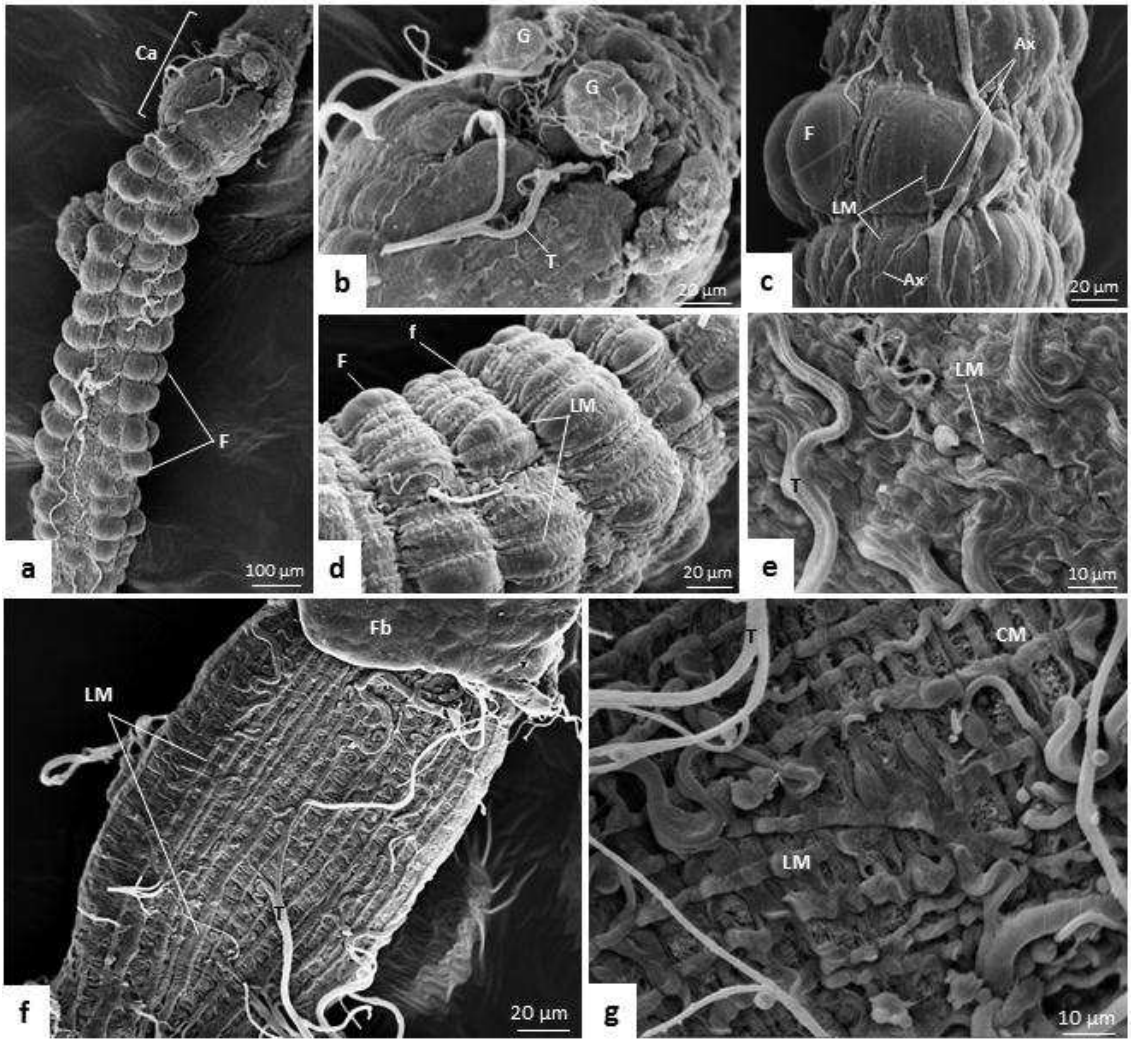


Figure 9: Scanning electron micrographs (SEMs) of the midgut of Toxorhynchites theobaldi adults. **a:** Outer surface of anterior midgut 1 (AMG1) with folds (F) in a female. The cardia (Ca) is positioned between the foregut and midgut. **b:** Ganglia (G) in AMG1 of a female. T: trachea. **c:** Longitudinal muscles (LM), and axons (Ax) in the AMG1 of a female. F: epithelial fold. **d:** AMG1 with folds, showing the furrows (f) and the longitudinal muscles (LM). **e:** Longitudinal muscles (LM) and tracheoles (T) in anterior midgut 2 (AMG2) of a male. **f:** PMG with longitudinal muscles (LM) in a female. Fb: fat body; T: trachea. **g:** Posterior midgut (PMG) depicting the circular muscles (CM) below the longitudinal muscles (LM). T: trachea.

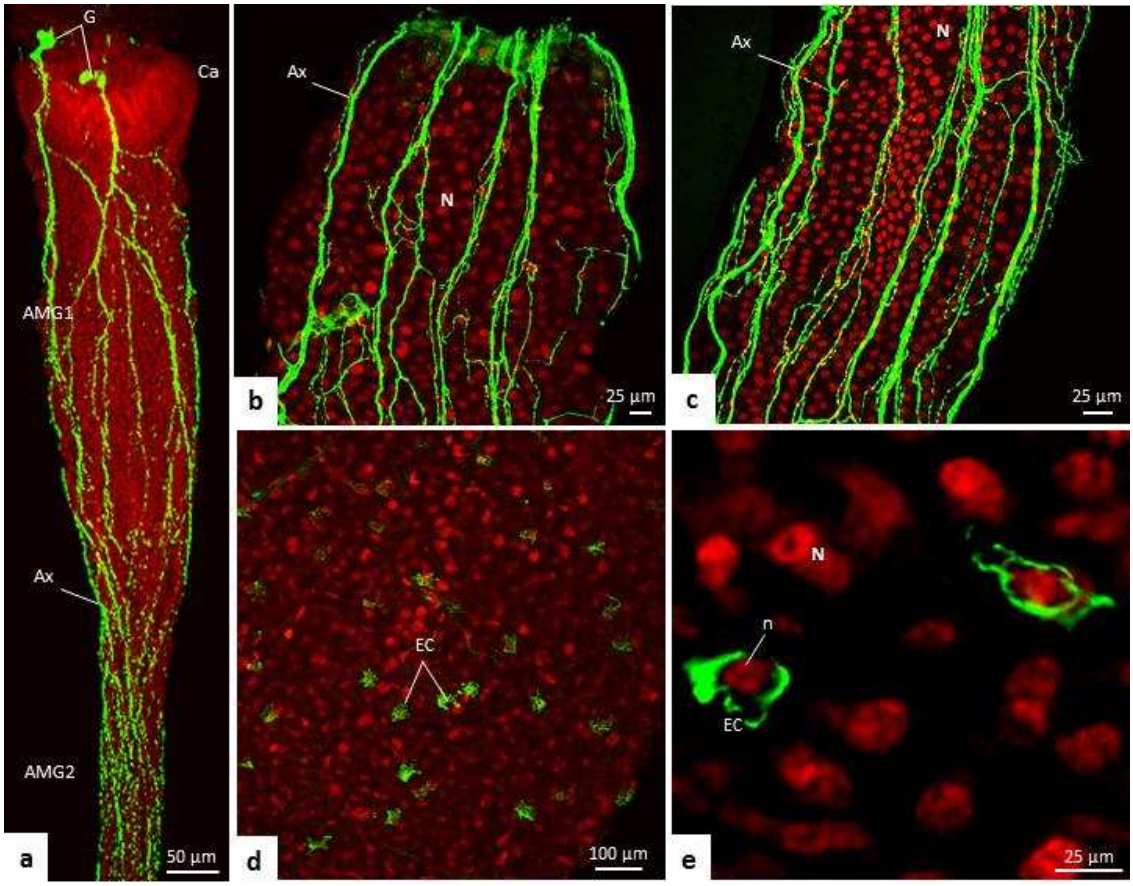
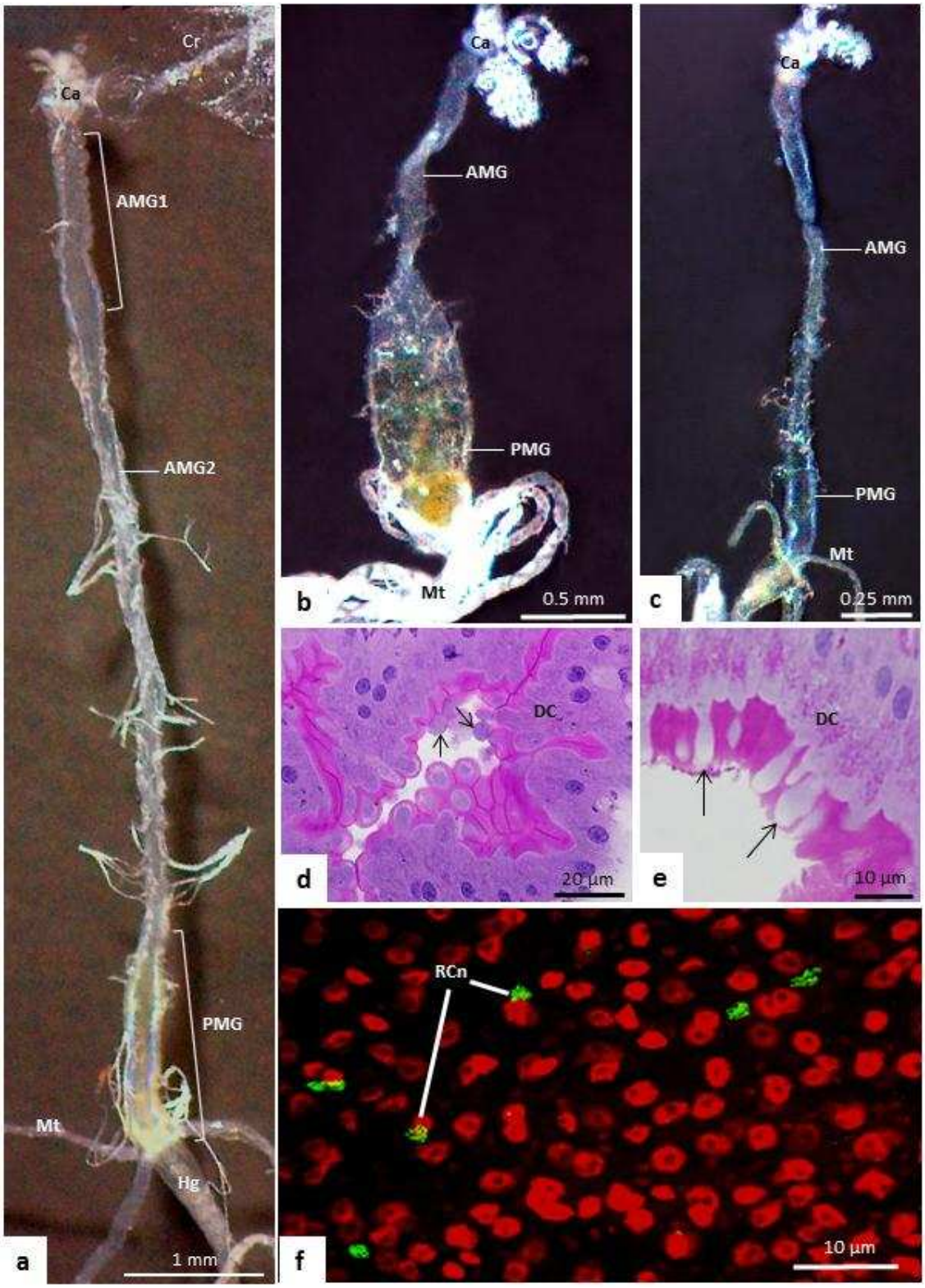


Figure 10: Immunofluorescence for neuropeptide FMRFamide (green) in the midgut of a female Toxorhynchites theobaldi adult. The nuclei of the digestive (N) or endocrine (n) cells are marked with TO-PRO-3 Iodide (red). **a:** Anterior midgut 1 (AMG1) and anterior part of the anterior midgut 2 (AMG2). Ganglia (G) are located on the cardia, and axons (Ax) are seen along the AMG. **b** and **c:** Axons (green) in AMG1 and AMG2, respectively. **d** and **e:** FMRFamide enteroendocrine cells (EC) in posterior midgut (PMG). N and n: nuclei of digestive and enteroendocrine cells, respectively.


Cr
Ca
AMG1
AMG2
PMG
Mt
Hg
a
1 mm
Ca
AMG
PMG
Mt
b
0.5 mm
Ca
AMG
PMG
Mt
c
0.25 mm
DC
d
20 µm
DC
e
10 µm
RCn
f
10 µm

Supplementary figure 1: a: Midgut of a male Toxorhynchites theobaldi showing the anterior midgut (AMG) subdivided into AMG1 (short and wide) and AMG2 (long and slender), and a small and dilated posterior midgut (PMG). **b** and **c:** Unfixed midguts of adult female and male A. aegypti (PPCampos strain), respectively, depicting AMG and PMG. Ca: cardia; Cr: crop; Mt: Malpighian tubules; Hg: hindgut. **d** and **e:** Sections of AMG1 of female and PMG of male, respectively. Cell apexes (arrows) are negative for PAS reaction and project towards midgut lumen, resembling the process of apocrine secretion. DC: digestive cells. **f:** Nuclei of regenerative cells (RCn) positive for phospho-histone H3 (green) in the midgut of fourth larva of A. aegypti. Individuals were obtained from a colony of the insectary of Departamento de Biologia Geral/UFV, and were dissected and stained as described in Materials and Methods (Immunofluorescence) [42].

5. CONCLUSÕES

Nossos resultados mostram que a morfofisiologia do intestino médio de mosquitos T. theobaldi é praticamente idêntica em fêmeas e machos, ao contrário das espécies de mosquitos cuja fêmea é hematófaga, onde o dimorfismo sexual é evidente no órgão. Essa semelhança pode ser atribuída ao fato de ambos machos e fêmeas de T. theobaldi possuírem a mesma dieta.

O epitélio digestivo do intestino médio de T. theobaldi é constituído basicamente por três tipos celulares distintos, as células digestivas (grande maioria), enteroendócrinas e regenerativas, como encontrado para as demais espécies de mosquitos. Cada um desses tipos celulares possui características morfológicas típicas que as tornam facilmente distinguíveis.

Diferentemente do padrão anatômico estabelecido para mosquitos, o intestino médio de T. theobaldi possui uma região anatomicamente e funcionalmente diferenciada no IMA. Apesar disso, a típica compartimentalização do órgão em região anterior e posterior é bem clara, assim como ocorre nos demais mosquitos. Tal compartimentalização, ao que tudo indica, reflete grandes diferenças funcionais no processo digestivo.

Surpreendentemente, as células do epitélio digestivo de T. theobaldi sintetizam matriz peritrófica em torno do alimento, mesmo este sendo constituído apenas de açúcares.

As características do intestino médio que são semelhantes entre T. theobaldi e as fêmeas hematófagas são: (1) O IMP é rico em organelas especializadas na secreção de proteínas; (2) a organização da musculatura do IMP envolve compartilhamento de fibras entre os feixes musculares vizinhos; (3) os feixes musculares longitudinais não são contínuos ao longo do órgão; (4) divisões das células regenerativas não foram

detectadas em adultos, com exceção dos recém-emergidos; e (5) Células imunorreativas para o neuropeptídeo FMRF, tanto neurônios quanto células endócrinas, estão localizadas no IMA e IMG, respectivamente. Contrariamente, as diferenças entre os intestinos médios de T. theolbaldi e das fêmeas hematófagas incluem: (1) o IMA em T. theobaldi é subdividido em duas regiões anatomicamente distintas, IMA1 e IMA2, enquanto nas fêmeas hematófagas essa subdivisão não é evidente; (2) o IMA é muito longo e o IMP é pouco desenvolvido em T. theobaldi em comparação com as fêmeas hematófagas; (3) a MP é muito fina e está presente em todo o intestino médio de T. theobaldi, mas é espessa, compacta, e produzida apenas no IMP após a ingestão de sangue nas fêmeas hematófagas.

Por fim, informações sobre o conjunto de proteínas sintetizadas pelo intestino médio de T. theobaldi podem melhorar o entendimento das suas diferenças morfofisiológicas em relação aos mosquitos hematófagos, mas ainda nada a respeito disso é conhecido em Toxorhynchites. Estudos considerando a atividade enzimática e a proteômica do intestino médio de espécies desse gênero serão os próximos passos para melhorar o entendimento da fisiologia do órgão nesses insetos, fornecendo novos insights a respeito das adaptações evolutivas da família Culicidae relacionadas à dieta de carboidratos.