HENRIQUE BARBOSA DA SILVA

ESTUDO COMPARADO DO VASO DORSAL PULSÁTIL E TECIDOS ASSOCIADOS EM DIFERENTES ESPÉCIES DE MOSQUITOS

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*.

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SUMÁRIO

RESUMO

SILVA, Henrique Barbosa, M.Sc., Universidade Federal de Viçosa, julho de 2018. **Estudo comparado do vaso dorsal pulsátil e tecidos associados em diferentes espécies de mosquitos**. Orientador: Gustavo Ferreira Martins

O sistema circulatório de insetos é aberto, nele a hemolinfa circula principalmente em decorrência da atividade contrátil do coração. Esse órgão está associado a células pericardiais (CP) e músculos alares (MA). O coração desempenha um importante papel do ponto de vista imunológico, uma vez que hemócitos são bombeados em decorrência da atividade cardíaca. Apesar dessa importância, ainda são escassos os trabalhos sobre a morfologia e ultraestrutura do coração e dos tecidos associados em mosquitos. Por meio de diferentes técnicas de microscopia foi analisado, de maneira comparativa, aspectos do coração, CP e MA em adultos de mosquitos hematófagos *Aedes aegypti*, *Anopheles aquasalis* e *Culex quinquefasciatus* e do fitófago *Toxorhynchites theobaldi*. O coração das espécies analisadas está na região dorsal mediana de todo o abdômen. Eles são formados por fibras musculares circulares que formam um órgão tubular e estriado. As estrias correspondem a miofibrilas. Em todas as espécies analisadas, os núcleos e mitocôndrias estão posicionados na região periférica. Entre a região de transição dos segmentos abdominais 1 a 8 foram observadas óstias incurrentes, enquanto que no cone terminal foi observado uma abertura excurrente. As CP localizam-se nas laterais do coração em regiões correspondentes aos intersegmentos abdominais. O citoplasma dessas células possui inclusões citoplasmáticas que correspondem a lisossomos e vacúolos. Em *An. aquasalis* e em *T. theobaldi*, as CP são mais numerosas e formam um cordão de células, semelhante a um sincício. Em *Ae. aegypti* e *C. quinquefasciatus*, as CP ocorrem aos pares, sendo dois ou quatro pares de CP por região intersegmentar. Os AM são polinucleados e se ligam por meio de suas ramificações à parede do coração. Em *T. theobaldi* os MA se ligam em todas as regiões do coração, enquanto que nos outros mosquitos eles se ligam apenas nas regiões do órgão localizados nas regiões intersegmentais. A morfologia e ultraestrutura do coração é conservada entre as espécies de mosquitos adultos de diferentes gêneros e hábitos alimentares. Entretanto, as espécies apresentam diferenças quanto à morfologia das CP e disposição dos MA, que provavelmente refletem diferenças evolutivas e fisiológicas do sistema circulatório de mosquitos.

ABSTRACT

SILVA, Henrique Barbosa, M.Sc., Universidade Federal de Viçosa, July, 2018. **Comparative study of the pulsatile dorsal vessel and associated tissues in different species of mosquitoes**. Advisor: Gustavo Ferreira Martins.

In the opened circulatory system of insects, the hemolymph circulates mainly through of the contractile activity of a tubular-shaped heart. The heart is associated with pericardial cells (PC) and alary muscles (AM). The heart also plays an important immunological role, since hemocytes are propelled by the organ activity. Despite this importance, works dedicated to the morphology and ultrastructure of the heart and associated tissues in mosquitoes are still scarce. In the present work, the morphology of the heart, PC and AM in adults of hematophagous mosquitoes *Aedes aegypti*, *Anopheles aquasalis* and *Culex quinquefasciatus* and of the phytophagous *Toxorhynchites theobaldi* were compared by means of different microscopy techniques. The hearts of mosquitos are located in the median dorsal region of the entire abdomen. They are formed by circular muscle fibers that form a tubular and striated structure. Nuclei and mitochondria are positioned in the peripheral region of muscle fibers. Paired openings of the heart wall or ostia are located in the intersegmental regions of the abdomen from abdominal segments 1 to 8. These ostia are of the incurrent type, while in the terminal cone an excurrent opening was observed. PC are located on the sides of the heart and the cytoplasm of PC has structures that correspond to lysosomes and vacuoles. PCs are more numerous in *An. aquasalis* and *T. theobaldi* and form a cord of cells, resembling a syncytium. In *Ae. aegypti* and *C. quinquefasciatus*, PC occur in pairs, with two or four PC pairs per intersegmental region. AM are multinucleated and bind through their branches to the heart wall. In *T. theobaldi* AM binds in all regions of the heart, whereas in other mosquitoes they bind only in the intersegmental regions. The morphology and ultrastructure of the heart is conserved among adult mosquito species of different genera and feeding habits. However, the species present differences regarding the morphology of PC and of AM. These differences seems to be related to evolutionary and physiological differences in the circulatory system of mosquitoes.

INTRODUÇÃO E REVISÃO BIBLIOGRÁFICA

I. Mosquitos

Os mosquitos são organismos pertencentes à Ordem Diptera, Subordem Nematocera, e Família Culicidae. Eles ocupam quase toda a região continental do planeta, exceto Antártica, e são conhecidos, principalmente, por serem transmissores de vírus, protozoários e nematelmintos causadores de doenças em seres humanos [1].

A família Culicidae é um clado monofilético com cerca de 3220 espécies distribuídas nas subfamílias Anophelinae e Culicinae [1]. Anophelinae é considerado um grupo primitivo e é formada pelos gêneros *Anopheles*, *Bironella* e *Chagasia* enquanto que Culicinae é composta por 109 gêneros dentre os quais estão os gêneros *Aedes, Culex* e *Toxorynchites* [2].

Os mosquitos são insetos holometábolos que possuem ciclo de vida aquático e terrestre. As larvas e pupas desenvolvem-se na água e os adultos são alados e vivem no ambiente terrestre. O tempo de desenvolvimento varia entre as espécies e depende de características ambientais, como temperatura e disponibilidade de alimento. Os machos e fêmeas de mosquitos adultos se alimentam de soluções açucaradas. Em alguns casos, como o dos mosquitos do gênero *Toxorhychites*, esse tipo de alimentação é suficiente para a produção de ovos. Entretanto, algumas espécies, como as dos gêneros *Aedes*, *Anopheles* e *Culex*, necessitam de uma alimentação de sangue para suprir a produção de ovos. Ao realizar a alimentação com sangue, os mosquitos anautógenos podem transmitir patógenos. Por essa razão, eles são considerados importantes do ponto de vista médico-veterinário [1].

Dentre as espécies transmissoras de patógenos, destaca-se o *Aedes aegypti*, o *Anopheles aquasalis* e o *Culex quinquefasciatus*. O *Ae. aegypti* é um mosquito originário da África Subsariana que se dispersou, principalmente, por meio de navios durantes as grandes navegações durante a colonização de diferentes continentes. Hoje, ele é encontrado na maioria das regiões tropicais e subtropicais do mundo. Possui hábito diurno, é antropofílico e seus principais criadouros são recipientes contendo água limpa e parada [3]. Esse mosquito é responsável pela transmissão de vírus causadores de doenças como dengue, febre amarela e zika [4].

Mosquitos do gênero *Anopheles* são conhecidos por serem vetores das espécies de protozoários do gênero *Plasmodium* causadores de malária. Atualmente, existem cerca de 216 milhões de pessoas ao redor do mundo com essa doença [5]. No Brasil, o principal vetor de malária é o mosquito *Anopheles darlingi* [6]. Entretanto, já foram encontrados na natureza *An. aquasalis* contaminados com o *Plasmodium* [7], porém, ele só é considerado um vetor importante do protozoário quando há aumento da densidade populacional dessa espécie [6, 8].

O *An. aquasalis* é um mosquito zoofílico de hábito crepuscular, que é encontrado em regiões litorâneas do Atlântico (São Paulo até Costa Rica) e no limite sul da costa Pacífica (Equador) [8]. Apesar de não possuir grande importância do ponto de vista epidemiológico, o *An. aquasalis* vem sendo usado como modelo de estudo do gênero *Anopheles*, uma vez que sua criação em laboratório é relativamente fácil [9].

O mosquito *C. quinquefasciatus* é encontrado em ambientes urbanos e rurais de regiões tropicais e subtropicais. Esse mosquito é antropofílico e zoofílico e possui hábito noturno. Seus principais criadouros são locais em que a água é rica em matéria orgânica em decomposição [8, 10]. O *C. quinquefasciatus* é vetor de diferentes patógenos incluindo o nematelminto *Wuchereria bancrofti* que causa filariose brancrofitiana [11], doença que atinge cerca de 120 milhões de pessoas no mundo [12].

O gênero *Toxorhnychites* compreende 89 espécies autógenas que, portanto, não são capazes de transmitir patógenos causadores de doenças [13]. Esses mosquitos, também conhecidos como mosquitos elefantes, são grandes e são encontrados em matas de regiões tropicais e subtropicais [14]. Os criadouros naturais de *Toxorhynchites* são microambientes aquáticos como folhas de bromélia, ocos de árvores e entrenós de bambus. Nesse local os imaturos convivem com larvas de outros culicídeos que servem como alimento para as larvas de *Toxorhynchites* que são predadoras [13]. Por essa razão, considera-se que as espécies de *Toxorhynchites* possam ter um grande papel no controle biológico de outros mosquitos [14].

II.O sistema circulatório de insetos

O sistema circulatório dos insetos é aberto e possui um tubo dorsal que se estende da porção posterior do abdômen até a cabeça. O tubo dorsal é a principal estrutura responsável pelo bombeamento da hemolinfa e é dividido em duas regiões anatômicas: o coração (contrátil), localizado na porção abdominal, e a aorta (não contrátil) que se estende do tórax até a cabeça. Além do coração órgãos pulsáteis acessórios são encontrados nas asas, pernas e antenas. Em conjunto, esse sistema é responsável por propulsionar a hemolinfa pelo corpo do inseto e consequentemente transportar hormônios, nutrientes, hemócitos e excretas [15].

III. Coração

O coração de insetos localiza-se em uma região denominada *sinus* pericárdico. Esta região é limitada pelo diafragma dorsal, formado por tecido conjuntivo e músculos alares (MA) onde se encontram também as células pericardiais (CP). Ele é um órgão tubular e é considerado como o principal responsável pela propulsão da hemolinfa [15].

O coração é formado por fibras musculares ou cardiomiócitos arranjados de forma helicoidal [16, 17] ou anelar [18]. Essas células são caracterizadas por ter sarcômeros, compostos por bandas I e A, delimitados por linhas Z descontínuas, que juntamente às mitocôndrias ocupam praticamente todo o volume celular [17]. Em geral, essas organelas estão presentes em grupos nas regiões periféricas [17] e nas regiões centrais das células [18]. Além disso, as mitocôndrias podem formar estruturas semelhantes a cordões entre as miofibrilas [18] ou serem compactadas em estruturas semelhantes a bolsas voltadas apenas para o lúmen cardíaco conferindo ao declive luminal uma ondulação profunda [19].

O cardiomiócito é pobre em termos de diversidade de organelas. Apenas em larvas de *Calpodes* (Lepidoptera) e em adultos de *Rhodinus* (Hemiptera) foi observada a presença de retículo endoplasmático rugoso, complexo de Golgi e de vesículas contendo material elétron- denso. Essas observações sugerem que nessas espécies o órgão pode secretar proteínas para a hemolinfa [20], porém, isso ainda não foi confirmado.

O coração é revestido tanto na superfície luminal quanto na superfície voltada para hemocele por uma fina lâmina basal, tornando os cardiomiócitos uma única massa funcional. Também é possível observar invaginações da membrana plasmática que é acompanhada pela lâmina basal formando estruturas semelhantes a túbulos T [23, 24, 25, 27]. Especula-se que esses locais são regiões em que o impulso elétrico chega às células e estimula a liberação de cálcio do retículo sarcoplasmático resultando em contração muscular [22].

A posição dos núcleos dos cardiomiócitos varia entre as espécies. Por exemplo, em *Ae. aegypti* eles são observados sempre na periferia celular voltados tanto para a superfície luminal quanto na superfície voltada para a hemocele e com predominância de cromatina descondensada [17]. Já em *Protophormia terraenovae* (Diptera), eles estão presentes apenas voltados para o lúmen do órgão [19].

O coração possui aberturas em pares chamadas de óstias. Elas são encontradas nas regiões intersegmentares e podem ser do tipo incurrente ou excurrente. As incurrentes são aberturas que possuem válvulas que permitem a entrada de hemolinfa durante a diástole, mas previne sua saída durante a sístole. As óstias excurrentes não possuem válvulas e em Diptera abrem-se na porção posterior do coração [15]. Ambas são formadas por cardiomiócitos especializados [23] e nos locais em que estão presentes há uma tendência da parede do coração tornar-se mais fina [18, 19]. Em adultos de *An. gambiae*, cada óstia é formada por válvulas que formam uma protrusão em ângulo em direção ao lúmen do coração, por essa razão são chamadas de lábios ostiais [16]. Além disso, em *Ae. aegypti* foi observado um núcleo em cada uma das células labiais [17].

O número de óstias em cada espécie é variável. Por exemplo, a mosca *P. terraenovae* possui cinco pares de óstias, sendo o primeiro par localizado no limite entre o coração e a aorta, o segundo, terceiro e quarto pares localizados, respectivamente, nos limites do segundo e terceiro, terceiro e quarto, quarto e quinto tergitos. Além disso, o quinto par de óstias está posicionado na porção final do coração em uma região denominada cone terminal [19]. O mosquito *An. gambiae* possui 7 pares de óstias e uma abertura *excurrente* localizada no cone terminal [16, 24], enquanto que em adultos de *D. melonogaster* existem cinco pares de óstias [25].

Nas proximidades das regiões ostiais existe um pequeno espaço denominada região periostial. Mediante a uma infecção, hemócitos circulantes rapidamente acumulam-se nessas regiões e fagocitam os patógenos, contribuindo para a resposta imune de *An. gambiae* [26, 27]. Além disso, durante a jornada do *Plasmodium* na hemocele, parte dos protozoários circulantes é morta nesses locais [26].

IV. Células pericardiais (CP)

As CP são os tipos de nefrócitos mais bem caracterizados dos insetos [15]. Elas estão dispostas nas laterais do coração formando estruturas semelhantes a cordas, cada uma em um dos lados do órgão [21]. A disposição das CP varia entre as espécies. Em *Ae. aegypti* foi possível contabilizar 27 pares de células individualizadas e distribuídas ao longo do coração [17]. Na abelha *Scaptotrigona postica* (Hymenoptera), observou-se que as CP podem estar organizadas tanto individualmente quanto em conjunto nas laterais do coração [18].

A organização morfológica das CP é bastante similar entre as espécies de insetos. Elas são ovoides com o citoplasma repleto de inclusões que quando vistas em microscópio eletrônico de transmissão equivalem a estruturas elétron-densas e elétron-lucentes semelhantes a lisossomos e vacúolos, respetivamente. Em *Myzus persicae* (Hemiptera, Aphidae) as vesículas são classificadas em dois grupos: o das pequenas vesículas que estão localizadas na superfície da célula, próximo do córtex e o das grandes vesículas, presentes no interior celular [28]. Também já foram identificados retículo endoplasmático rugoso, complexo de Golgi, corpos multivesiculares e mitocôndrias distribuídos no citoplasma das CP [23,24,25,26].

Uma lâmina basal fina e granular com carga elétrica negativa reveste as CP. A membrana plasmática dessas células forma invaginações. Em conjunto, a lâmina basal e estas invaginações, contribuem para a formação de canais na periferia das CP [30]. A localização e a presença de invaginações e vesículas são características que condizem com o papel que as CP desempenham na desintoxicação através da filtragem da hemolinfa [15]. A filtragem da hemolinfa inicia-se quando ela atravessa a barreira constituída pela lamina basal, sendo, em seguida, armazenada, por pouco tempo, nas invaginações, ficando disponíveis para a endocitose [30]. Há evidências de que ao serem endocitados, os componentes tóxicos da hemolinfa são transformados em moléculas solúveis dentro de lisossomos [31].

Há exemplos na literatura que mostram o sequestro de substâncias da hemolinfa pelas CP. Por exemplo, larvas de *Hyalophora cecropia* (Lepidoptera) quando foram expostas *ex-vivo* à ferritina, apresentaram essa substância nos vacúolos e invaginações das CP [29]. Resultados semelhantes foram observados em larvas de *Ae. aegypti* alimentadas com o corante carmim acético em pó, que tornaram-se avermelhadas [17].

V. Músculos alares (MA)

O coração é sustentado na hemocele por dois a 12 pares de músculos alares, dependendo da espécie [15]. Nos mosquitos esses músculos ocorrem nos sete segmentos abdominais anteriores formando uma rede, semelhante a uma cesta de basquete, que liga a parede do corpo do inseto diretamente aos dois lados do coração. Próximo ao órgão, os músculos alares se ramificam formando fibras que se prendem à superfície do coração [32].

Em *An. gambiae* foi contabilizado seis pares completos e dois pares incompletos de músculos alares [24]. Eles se originam próximo à junção de tergitos e pleuritos da porção anterior de cada segmento abdominal e dividem-se, inicialmente, em dois grandes ramos os quais formam mais ramificações que por sua vez dão origem a 10-30 miofibras que se ligam diretamente ao órgão. Acredita-se que eles auxiliem abertura e fechamento das óstias e por tracionar a parede do coração quando os músculos cardíacos são relaxados [16].

Em *Periplaneta americana*, as miofibras dos músculos alares estão organizadas em um arranjo difuso, contendo mitocôndrias próximas as discretas bandas Z. Além disso, sabe-se que a disposição dos núcleos dessas células varia, por exemplo em *P. americana* estão localizadas na periferia [33] enquanto que em *Ae. aegypti* concentram-se em regiões próximas as ramificações, sendo mais abundantes em adultos do que em larvas e pupas [17].

OBJETIVO

Comparar a morfologia do coração, das células pericardiais e dos músculos alares entre os mosquitos adultos *Ae. aegypti*, *An. aquasalis*, *C. quinquefasciatus* e *T. theobaldi*.

REFERÊNCIAS BIBLIOGRÁFICAS

1. Mullen GR (Gary R, Durden LA. Medical and veterinary entomology. 2nd edition. San Diego: Elsevier; 2009.

2. Reidenbach KR, Cook S, Bertone MA, Harbach RE, Wiegmann BM, Besansky NJ. Phylogenetic analysis and temporal diversification of mosquitoes (Diptera: Culicidae) based on nuclear genes and morphology. BMC Evol Biol. 2009;9:298. doi:10.1186/1471-2148-9-298.

3. Clemons A, Haugen M, Flannery E, Tomchaney M, Kast K, Jacowski C, et al. *Aedes aegypti*: an emerging model for vector mosquito development. Cold Spring Harb Protoc. 2010:pdb.emo141. http://www.ncbi.nlm.nih.gov/pubmed/20889691. Accessed 12 Jun 2018.

4. Ministério da Sáude B. Dengue, chikungunya, zika. 2018. http://combateaedes.saude.gov.br/pt/. Accessed 12 Jun 2018.

5. WHO | World malaria report 2017. WHO. 2017. http://www.who.int/malaria/publications/world-malaria-report-2017/en/. Accessed 12 Jun 2018.

6. Ministério da Saúde B. Descrição da doença. 2018. http://portalms.saude.gov.br/saude-de-a-z/malaria/descricao-da-doenca. Accessed 12 Jun 2018.

7. Silva AN da, Santos CC, Lacerda RN, Machado RL, Póvoa MM. Susceptibility of *Anopheles aquasalis* and *Anopheles darlingi* to *Plasmodium vivax* VK210 and VK247. Mem Inst Oswaldo Cruz. 2006;101:547–50. doi:10.1590/S0074-02762006000500011.

8. Consoli RAGB, Oliveira RL de. Principais mosquitos de importância sanitária no Brasil. Editora FIOCRUZ; 1994. https://portal.fiocruz.br/livro/principais-mosquitos-de-importancia-sanitaria-no-brasil. Accessed 12 Jun 2018.

9. Da Silva ANM, Dos Santos CCB, Lacerda RNL, Santa Rosa EP, De Souza RTL, Galiza D, et al. Laboratory colonization of *Anopheles aquasalis* (Diptera: Culicidae) in Belém, Pará, Brazil. J Med Entomol. 2006;43:107–9.

http://www.ncbi.nlm.nih.gov/pubmed/16506455. Accessed 4 Aug 2018.

10. Bhattacharya S, Basu P, Sajal Bhattacharya C. The southern house mosquito, *Culex quinquefasciatus*: profile of a smart vector. J Entomol Zool Stud JEZS. 2016;73:73–81.

11. Farajollahi A, Fonseca DM, Kramer LD, Marm Kilpatrick A. "Bird biting" mosquitoes and human disease: a review of the role of *Culex pipiens* complex mosquitoes in epidemiology. Infect Genet Evol. 2011;11:1577–85. doi:10.1016/j.meegid.2011.08.013.

12. WHO | Epidemiology. WHO. 2018. http://www.who.int/lymphatic_filariasis/epidemiology/en/. Accessed 12 Jun 2018.

13. Collins LE, Blackwell A. The biology of *Toxorhynchites* mosquitoes and their potential as biocontrol agents. Biocontrol News Inf. 2000;21:105N–116N.

14. Harbach R. Mosquito taxonomic inventory. 2018. http://mosquito-taxonomic-inventory.info/. Accessed 31 May 2018.

15. Klowden MJ. Physiological systems in insects. 3rd edition. San Diego: Academic Press; 2013.

16. Glenn JD, King JG, Hillyer JF. Structural mechanics of the mosquito heart and its function in bidirectional hemolymph transport. J Exp Biol. 2010;213:541–50. doi:10.1242/jeb.035014.

17. Leódido ACM, Ramalho-Ortigão M, Martins GF. The ultrastructure of the *Aedes aegypti* heart. Arthropod Struct Dev. 2013;42:539–50. doi:10.1016/J.ASD.2013.09.005.

18. Poiani SB, da Cruz-Landim C. Histology and ultrastructure of pericardial cells of *Scaptotrigona postica* Latreille (Hymenoptera, Apidae) in workers and queens of different ages. Micron. 2007;38:766–70. doi:10.1016/j.micron.2006.11.008.

19. Angioy AM, Boassa D, Dulcis D. Functional morphology of the dorsal vessel in the adult fly *Protophormia terraenovae* (diptera, calliphoridae). J Morphol. 1999;240:15–31. doi:10.1002/(SICI)1097-4687(199904)240:1<15::AID-JMOR2>3.0.CO;2-K.

20. Locke M. Secretion by insect heart muscle cells. J Insect Physiol. 1989;35:53–6.

21. Curtis NJ, Ringo JM, Dowse HB. Morphology of the pupal heart, adult heart, and

associated tissues in the fruit fly, *Drosophila melanogaster*. J Morphol. 1999;240:225–35. doi:10.1002/(SICI)1097-4687(199906)240:3<225::AID-JMOR2>3.0.CO;2-V.

22. Sanger JW. Cardiac Fine structure in selected arthropods and molluscs. American Zoologist. 1979;19:9–27. doi:10.2307/3882416.

23. Lehmacher C, Abeln B, Paululat A. The ultrastructure of *Drosophila* heart cells. Arthropod Struct Dev. 2012;41:459–74. doi:10.1016/J.ASD.2012.02.002.

24. Andereck JW, King JG, Hillyer JF. Contraction of the ventral abdomen potentiates extracardiac retrograde hemolymph propulsion in the mosquito hemocoel. PLoS One. 2010;5:e12943. doi:10.1371/journal.pone.0012943.

25. Rotstein B, Paululat A. On the morphology of the *Drosophila* heart. J Cardiovasc Dev Dis. 2016;3:15. doi:10.3390/jcdd3020015.

26. King JG, Hillyer JF. Infection-induced interaction between the mosquito circulatory and immune systems. PLoS Pathog. 2012;8:e1003058. doi:10.1371/journal.ppat.1003058.

27. Sigle LT, Hillyer JF. Mosquito hemocytes preferentially aggregate and phagocytose pathogens in the periostial regions of the heart that experience the most hemolymph flow. Dev Comp Immunol. 2016;55:90–101. doi:10.1016/j.dci.2015.10.018.

28. Bowers B. Coated vesicles in the pericardial cells of the aphid (*Myzus persicae sulz*). Protoplasma. 1964;59:351–67. doi:10.1007/BF01248567.

29. Sanger JW, McCann FV. Fine structure of the pericardial cells of the moth, *Hyalophora cecropia*, and their role in protein uptake. J Insect Physiol. 1968;14:1839–45. doi:10.1016/0022-1910(68)90215-1.

30. Denholm B, Skaer H. Bringing together components of the fly renal system. Curr Opin Genet Dev. 2009;19:526–32. doi:10.1016/j.gde.2009.08.006.

31. Mills RP, King RC. The pericardial cells of *Drosophila melanogaster*. Q J Microsc Sci. 1965;106:261–8. http://www.ncbi.nlm.nih.gov/pubmed/5865262.

32. Martins GF, Ramalho-Ortigão JM, Pimenta PFP. Morphological features of the heart of six mosquito species as revealed by scanning electron microscopy. Int J Trop

Insect Sci. 2011;31:98–102. doi:10.1017/S1742758411000178.

33. Adams ME, Miller T, Thomson WW. Fine structure of the alary muscles of the American cockroach. J Insect Physiol. 1973;19:2199–207. doi:10.1016/0022-1910(73)90135-2.

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ARTIGO

The morphology of the heart is conserved but the morphology of heart-associated tissues varies across different species of adult mosquitoes.

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Abstract

Background – The hemolymph is propelled in the body cavity of insects mainly due to the contractile activity of an abdominal tubular-shaped heart. Therefore, the heart contraction is pivotal in insect homeostasis by performing, for example, the circulation of nutrients, hormones, and hemocytes. The heart is associated with pericardial cells (PC) and alary muscles (AM). Despite this importance, works dedicated to the morphology and ultrastructure of the heart and associated tissues in mosquitos are still scarce. In the present work, the morphology of the heart, PC and AM in adults of hematophagous mosquitos *Anopheles aquasalis*, *Aedes aegypti* and *Culex quinquefasciatus* and of the phytophagous *Toxorhynchites theobaldi* were compared by means of different microscopy techniques.

Results - The hearts of mosquitos are located in the median dorsal region of the entire abdomen. They are formed by circular muscle fibers that form a tubular and striated structure. Nuclei and mitochondria are positioned in the peripheral region of muscle fibers. Paired openings of the heart wall or ostia are located in the intersegmental regions of the abdomen from abdominal segments 1 to 8. These ostia are of the incurrent type, while in the terminal cone an excurrent opening was observed. PC are located on the sides of the heart and the cytoplasm of PC has structures that correspond to lysosomes and vacuoles. PCs are more numerous in *An. aquasalis* and *T. theobaldi* and form a cord of cells, resembling a syncytium. In *Ae. aegypti* and *C. quinquefasciatus*, PC occur in pairs, with two or four PC pairs per intersegmental region. AM are multinucleated and bind through their branches to the heart wall. In *T. theobaldi* AM binds in all regions of the heart, whereas in other mosquitos they bind only in the intersegmental regions.

Conclusions - The morphology and ultrastructure of the heart is conserved among adult mosquito species of different genera and feeding habits. However, the species present differences regarding the morphology of PC and of AM. These differences seems to be related to evolutionary and physiological differences in the circulatory system of mosquitos.

Keywords: mosquitos; heart; pericardial cells; alary muscles.

Background

The circulatory system of the insects is opened and the dorsal vessel is its major component, extending from the posterior portion of the abdomen to the head. The dorsal vessel is divided into two anatomical regions: the heart located in the abdominal portion, and the aorta, that extends from the thorax to the head. The dorsal vessel and accessory pulsatile organs found in the appendages are responsible for propelling the hemolymph through the body of the insect and consequently transporting hormones, nutrients, hemocytes and excreta [1,2].

In mosquitos, the heart is a tubular organ, made up of helically arranged muscle fibers or cardiomyocytes, and is associated with pericardial cells (PC) (or pericardial nephrocytes) and allary muscles (AM) [3,4]. The heart wall contains pairs of lateral openings or ostia, which may be of the incurrent or excurrent type. The incurrent ostia are valves that allow the entry of hemolymph during diastole, but prevent their exit during systole, whereas excurrent ostia do not have valves, and in Diptera, they open in the posterior part of the heart [1]. Both ostia are formed by specialized cardiomyocytes that extends towards heart lumen as ostial lips [5].

The PCs are located on the sides of the heart and through endocytosis maintain the hemolymph composition [6]. Due to this ability and its morphological characteristics, such as the presence of many cytoplasmic vesicles and lysosome-like structures, PCs can act in the filtration and detoxification of hemolymph [1, 4, 7, 8]. The heart is supported by AM and they form a network of branches connecting the wall of the insect's body to both sides of the heart [9]. There is evidence that AM helps to open the ostia allowing hemolymph flow [3, 10].

The heart has been considered an important organ in the context of the distribution of pathogens within the mosquito body cavity, because the propelling of hemolymph by the heart facilitates the arrival of *Plasmodium* in the salivary glands of *Anopheles gambiae* [11]. In addition, the heart plays an important role in immune defense because hemocytes are propelled into the body cavity as a result of cardiac activity or can accumulate on the surface of the organ to phagocyte circulating pathogens such as bacteria and *Plasmodium* [12,13,14]. Furthermore, the cardiac activity can also play role in the thermoregulation during the blood acquisition *by* hematophagous insects. For example, in the kissing-bug *Rhodnius prolixus*, the propelled hemolymph warms up due to the close contact between the aorta and the anterior region of the digestive system containing the blood, which decreases the temperature of the blood before it reaches the abdomen [15]. Despite of the relevance of the heart for insect homeostasis, there are few works about its morphology in mosquitos, and one reason for this is that this organ is small, delicate and hard to manipulate. In the last few years, many advances were achieved considering the heart morphology and functions in mosquitos using the malaria vector *An. gambiae* as a model [3,11,12,13,16], but the literature abounding other mosquito species is scarce [4, 9].

The family Culicidae comprises almost 3,220 species of mosquitos that are included in the subfamilies Anophelinae and Culicinae. The genera *Anopheles* (Anophelinae), *Aedes*, and *Culex* (Culicinae) are famous for being vectors of pathogens of medical and veterinary importance that can be transmitted by females during the blood acquisition. Although hematophagous habit exists in most mosquitos, some species do not require blood for egg production, such as those of the genus *Toxorhynchites* [17]. In spite of the great diversity and dispersion of species of mosquitos, little is known about the morphological and ultrastructural organization of the heart in species of different genera or with different feeding habits. In order to fill this gap, in this work, the morphology and ultrastructure of the heart and associated tissues PC and AM were studied in four adult mosquito species, being three hematophagous, *Anopheles aquasalis* (Anophelinae), *Aedes aegypti* (Culicinae) and *Culex quinquefasciatus* (Culicinae); and one phytophagous species *Toxorhynchites theobaldi* (Culicinae). This work brings a contribution to the existing data regarding the heart and associated tissues of blood-sucking mosquitos of the genera *Aedes*, *Anopheles* and *Culex;* and describes in details the heart and associated tissues of the phytophagous *T. theobaldi*. We have provided a detailed comparison, pointing the similarities and differences of the organ structure across different mosquito species.

Results

Heart

The heart consists of a tube located along the dorsal and medial portion of the abdomen. PC are located laterally to the heart and AM attach to both sides of the organ (Fig. 1 e Fig. 2A). In addition to PC and AM, pericardial hemocytes were observed on the external surface of the organ (Fig. 2B). The hearts of *Ae. aegypti*, *An. aquasalis*, *C. quinquefasciatus* and *T. theobaldi* are similar. For this reason, we used representative images of each morphological and ultrastructural aspect observed. The heart is composed of cardiomyocytes arranged in helicoidal layers (Fig. 2C), forming a tubular organ with a large lumen. Regions with the most dilated lumen correspond to the cardiac chambers that coincide with the intersegmental regions of the abdomen (Fig. 3A).

In all species studied, the cytoplasm of the cardiomyocytes is striated due to the myofibrils that occupy almost all sarcoplasm (Fig. 3B-C). Up to three myofibrils can be seen simultaneously on the heart wall, as seen in *T. theobaldi* (Fig. 3D). Sarcomeres are delimited by discontinuous Z-lines, band A is electron-dense and band I is electron-lucent. Also, for all species studied, a basal lamina surrounds the external and internal surfaces of the heart. It accompanies all invaginations of the plasma membrane of the heart. When the invagination coincides with the Z lines, structures similar to the T tubules are noted (Fig. 3E). Mitochondrial profiles were observed in a large amount at the fiber periphery (Fig. 3C). The cardiomyocyte nuclei are individualized and located in the outer or in the inner periphery from the heart of the species studied here (Fig. 5A, 5D-F) and have predominantly decondensed chromatin, as seen in *An. aquasalis* (Fig. 3F).

In all species, openings or pairs of ostia were observed on the wall of the heart (Fig. 4A-B). In these structures, AM branches are found attached to the heart, as seen in *C. quinquefasciatus* (Fig. 4A). In addition, a pair of nuclei were seen close to the ostia (Fig. 4B). Only in *An. aquasalis*, the ostial cell was observed in TEM (Fig. 4C). It has a nucleus with predominantly decondensed chromatin. It occupies almost all of the fiber together with the myofibrils (Fig. 4D). In order to determine the presence of ostia in all mosquito intersegments and their morphology, we performed cuts of the transition regions of abdominal segments1-2; 2-3; 3-4; 4-5; 5-6; 6-7 and 7-8 ostia. In the heart portions located at these sites, the ostia are classified as incurrent, because they are valvar structures similar to funnels, with the ostial lips. At these lips, the cardiomyocytes are thinner, and it is possible to see two nuclei at the end of each lip. In addition, in *Ae. aegypti* in the final portion of the heart (terminal cone) there is a simple opening, or excurrent ostium (Fig 5A-G).

The width (lumen + heart walls) of the heart is similar in *Ae. aegypti* (28.09 ± 4.00 μm), *An. aquasalis* (15.73 ± 0.37 μm) and *C. quinquefasciatus* (24.33 ± 1.00 μm) ($p>0.05$). However, there is a significant difference between the heart width of *T. theobaldi* (48.17 ± 2.21 μm) and the other species ($p<0.05$) (Fig. 6 A). The thickness of the muscular wall of the heart of *T. theobaldi* (5 ± 0.95 μm) is different of the wall of *C. quinquefasciatus* (2.71 ± 0.7 μm) but is not different of other species ($p<0.05$). The thickness of the heart wall of *Ae. aegypti* is 2.95 ± 0.8 μm and *An. aquasalis* is 3.30 ± 0.31 μm (Fig. 6 B).

Pericardial cells (PC)

Among the species studied, a difference was found in the organization of PC in the intersegmental regions. In this sense, there are four pairs of PC in each intersegmental region (segments 1 to 7) of *Ae. aegypti* [4] and *C. quinquefasciatus* (Fig. 7A) and only two pairs in the last segment (not shown) (Tab. 1). In *An. Aquasalis* and *T. theobaldi*, PCs are also located in the intersegmental regions but are more numerous and are grouped forming a cord of cells in a structure similar to a syncytium that made their counting difficult (Fig. 7B). The other morphological and ultrastructural aspects were similar, for this reason, we used representative images of each of these characteristics.

PC of *Ae. aegypti* and *C. quinquefasciatus* are binucleated (Fig. 7D). However, we did not count the number of nuclei per PC in *An. aquasalis* and *T. theobaldi*, since the PC boundary was not clear (Fig. 7C and 7E). In TEM, it was observed that nuclei of PC have typically decondensed chromatin with evident nucleolus (Fig. 8A). When three nuclei are simultaneously seen in PC of *An. aquasalis*, they are dimorphic in relation to the degree of chromatin condensation, with the chromatin of the two peripheral nuclei more decondensed than the central nucleus (Fig. 8F).

The cytoplasm of PC is full of inclusions (Fig. 7C). These structures are positive for LysoTracker (Fig. 7F) at least in PC of *Ae. aegypti*, confirming that they are acidic organelles related to the cellular digestion process. At TEM, these inclusions are similar to lysosomes and vacuoles (Fig. 8B-C). Mitochondria, multivesicular and multilamellar bodies, and rough endoplasmic reticulum are also seen (Fig. 8C). PC may be physically associated with the heart and AM, but no cell junctions were detected in the contact regions between PC and these muscles, as shown in *C. quinquefasciatus* (Fig. 8D). In all species studied, PCs are enclosed by a basal lamina and have deep invaginations in the plasma membranes, forming extracellular channels (Fig. 8E).

The total area of the PC by intersegment is similar between *Ae. aegypti* (6638.98 ± 1436.29 μm^2) and *C. quinquefasciatus* (6330.03 ± 1268.18 μm^2) and between *An. aquasalis* (16574.75 ± 602.14 μm^2) and *T. theobaldi* (16276.19 ± 782.01 μm^2) ($p>0.05$). However, the PCs of *An. aquasalis* and *T. theobaldi* occupy a larger area in relation to the PCs of *Ae. aegypti* and *C. quinquefasciatus* ($p<0.05$) (Fig. 6 C).

Alary muscles (AM)

The attachment of AM to the heart wall is similar across hematophagous species. AM connect to the heart only in the intersegmental regions in *Ae. aegypti*, *An. aquasalis* and *C. quinquefasciatus,* but in *T. theobaldi*, AM also bind directly to the heart in the segmental regions (Fig. 9A-C) (Tab. 1). Despite this difference, in all species studied the AM attach through their branches to the heart wall, forming a supporting framework for the organ (Fig. 9D-E). In their opposite extremity, AM can be seen connected to the body wall in *T. theobaldi*. In this case, immediately after the attachment point at body wall, the AM form branches, which become more numerous close to the heart (Fig. 9F).

The AM of mosquitos are striated and multinucleated (Fig. 9G) .The myofibrils of AM do not evidence sarcomere details, such as Z-lines, A-band and I-band in TEM. In addition, we noticed the presence of mitochondria, which are distributed in all regions of the sarcoplasm, as shown in *C. quinquefasciatus* (Fig. 8E).

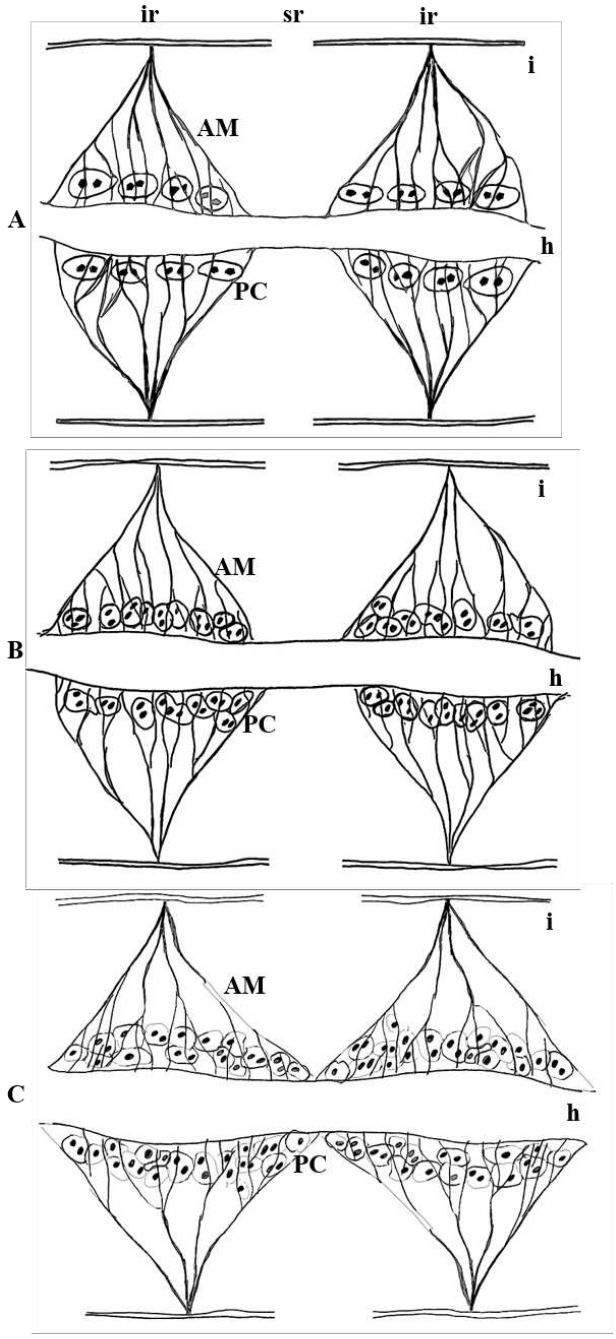

Figure 1. Schematic drawings depicting two typical abdominal intersegmental regions (ir) of the heart (h) with associated tissues (i.e., pericardial cells-PC and allary muscles-AM) of adult mosquitos of *Ae. aegypti* and *C. quinquefasciatus* **(A)**, *An. aquasalis* **(B)** and *T. theobaldi* **(C)** *Ae. aegypti* and *C. quinquefasciatus* have four pairs of PC per intersegmental region (ir) and *An. aquasalis* and *T. theobaldi* they are numerous and grouped. In in **A** and **B**, AM bind only in the ir whereas in *T. theobaldi*, they bind in both ir and sr. i: integument.

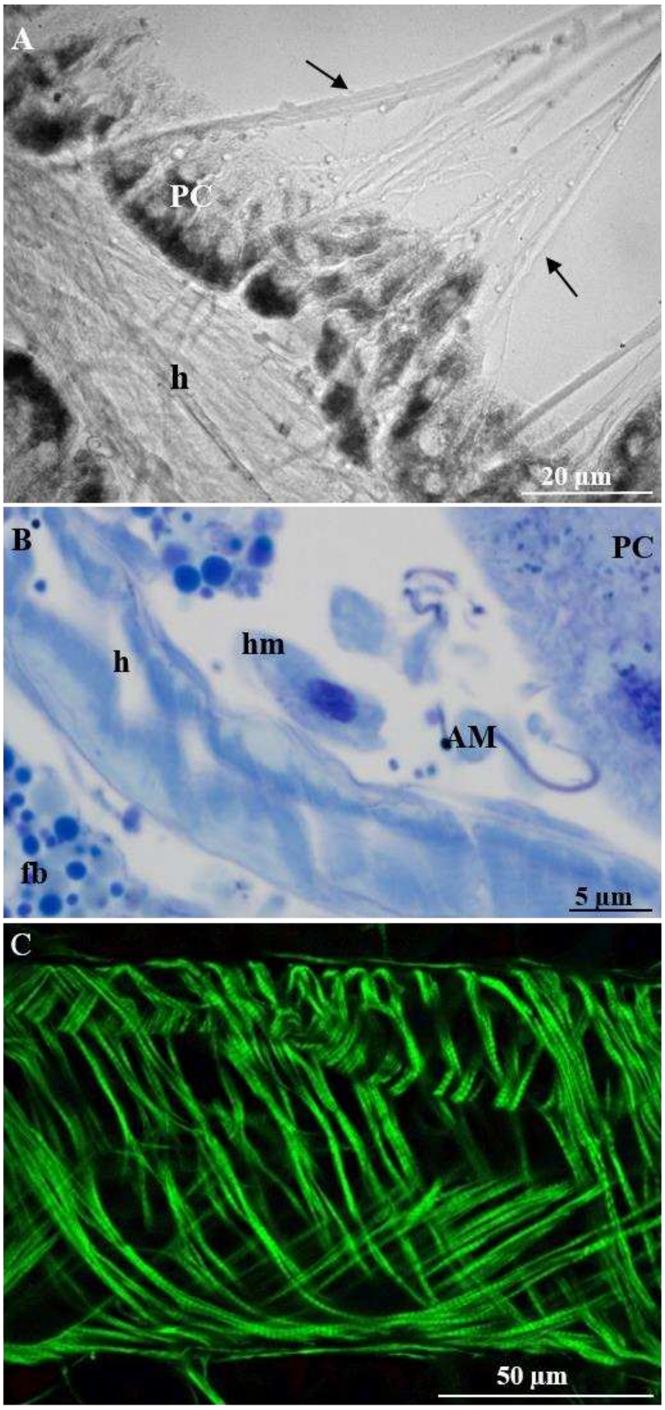

Figure 2. Heart of *T. theobaldi*. **(A)** Whole mounts showing a portion of the heart (h) in the intersegmental region with ramifications of AM (arrows) and pericardial cells (PC) clustered on the sides of the heart (h). **(B)** Histological section of the heart with a hemocyte (hm) on the surface of the heart. **(C)** Portion of heart wall with twisted cardiac musculature (green). fb: fat body.

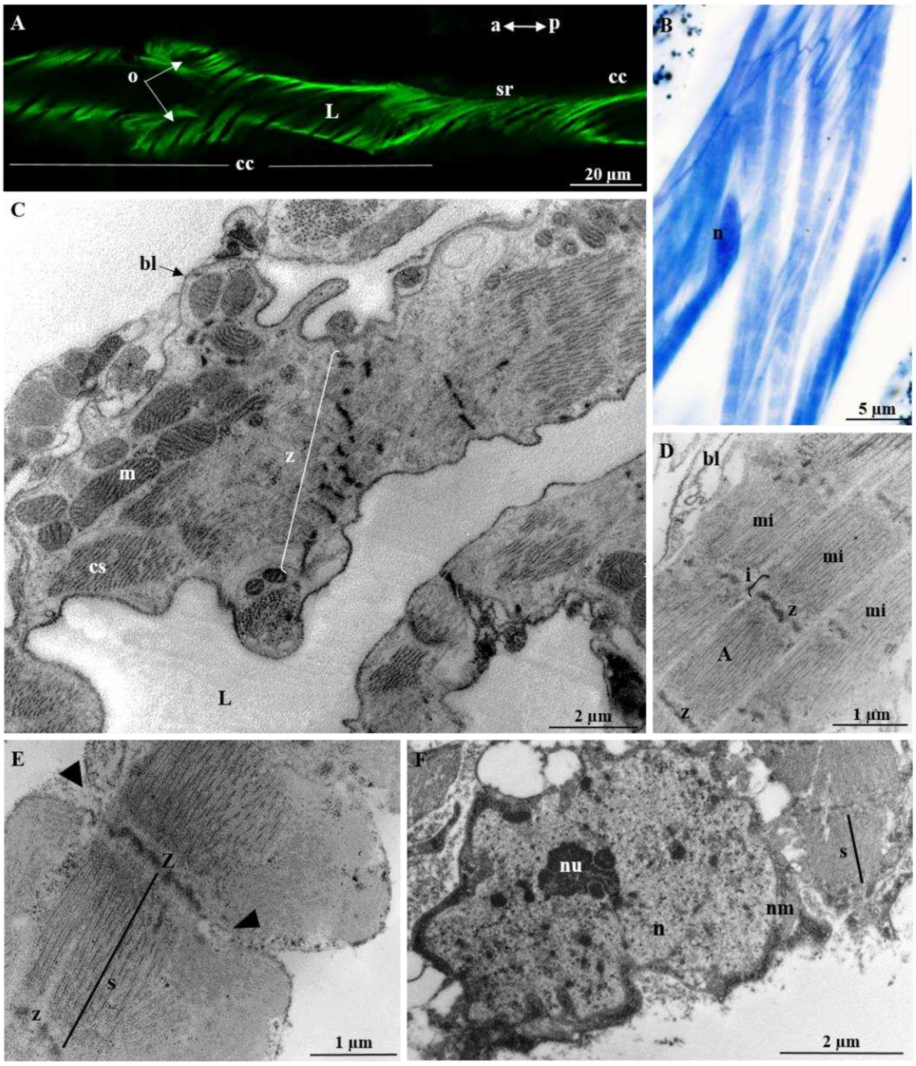

Figure 3. Musculature of the heart of adult mosquitos. **(A)** Heart of *An. aquasalis* with cardiac chambers (cc) and ostia (o). One chamber is separated from another by a less dilated region, positioned at a segmental region (sr) of abdomen. Green: FITC phalloidin-labeled cytoskeleton; a: anterior region and p: posterior region of the body. **(B)** Histological section of the heart of *An. aquasalis* showing striations of cardiomyocytes and cell nucleus (n). **(C)** Heart of *C. quinquefasciatus* showing the basal lamina (bl), externally and internally lining the organ, with mitochondria (m) concentrated in the external periphery of the wall of the heart. cs: L: lumen of the heart. z: discontinuous z-lines. **(D)** Three myofibrils (mi) in *T. theobaldi* cardiomyocytes and sarcomeres with z-stained lines (z), band A (A), band I (I). bl: basal lamina. **(E)** *T. theobaldi* cardiomyocyte with two z lines (z) delimiting the space of a sarcomere (s) and with invaginations that coincide with the z lines, corresponding to T tubules (arrowhead). **(F)** Cardiomyocyte nucleus of *An. aquasalis* with predominantly decondensed chromatin. nm: nuclear membrane, nu: nucleolus and s: sarcomere.

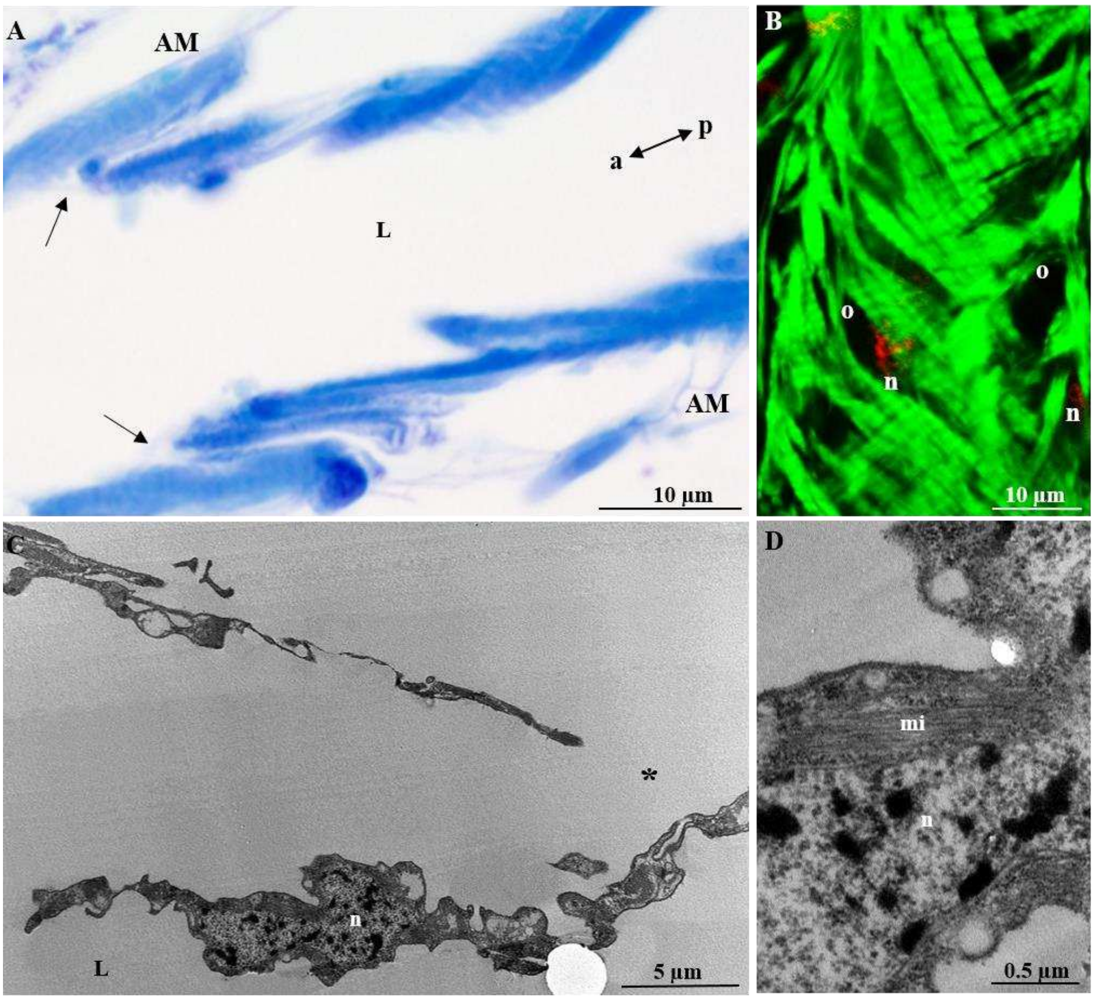

Figure 4. Ostia of the heart of mosquitos. **(A)** Pair of ostia (arrows) in *C. quinquefasciatus* with ramifications of AM associated with the ostial lips. a: anterior region and p: posterior region of the body. **(B)** Heart portion of *C. quinquefasciatus* stained with phalloidin-FITC (green) with a pair of nuclei (n; red) stained with TO-PRO 3 close to the ostia (o). **(C)** Ultrastructure of an ostial lip of *An. aquasalis* showing nucleus (n) with predominantly decondensed chromatin. *: opening to the body cavity; L: heart lumen. **(D)** Detail of the nuclear region of the ostial lip with nucleus (n) and myofibrils (mi).

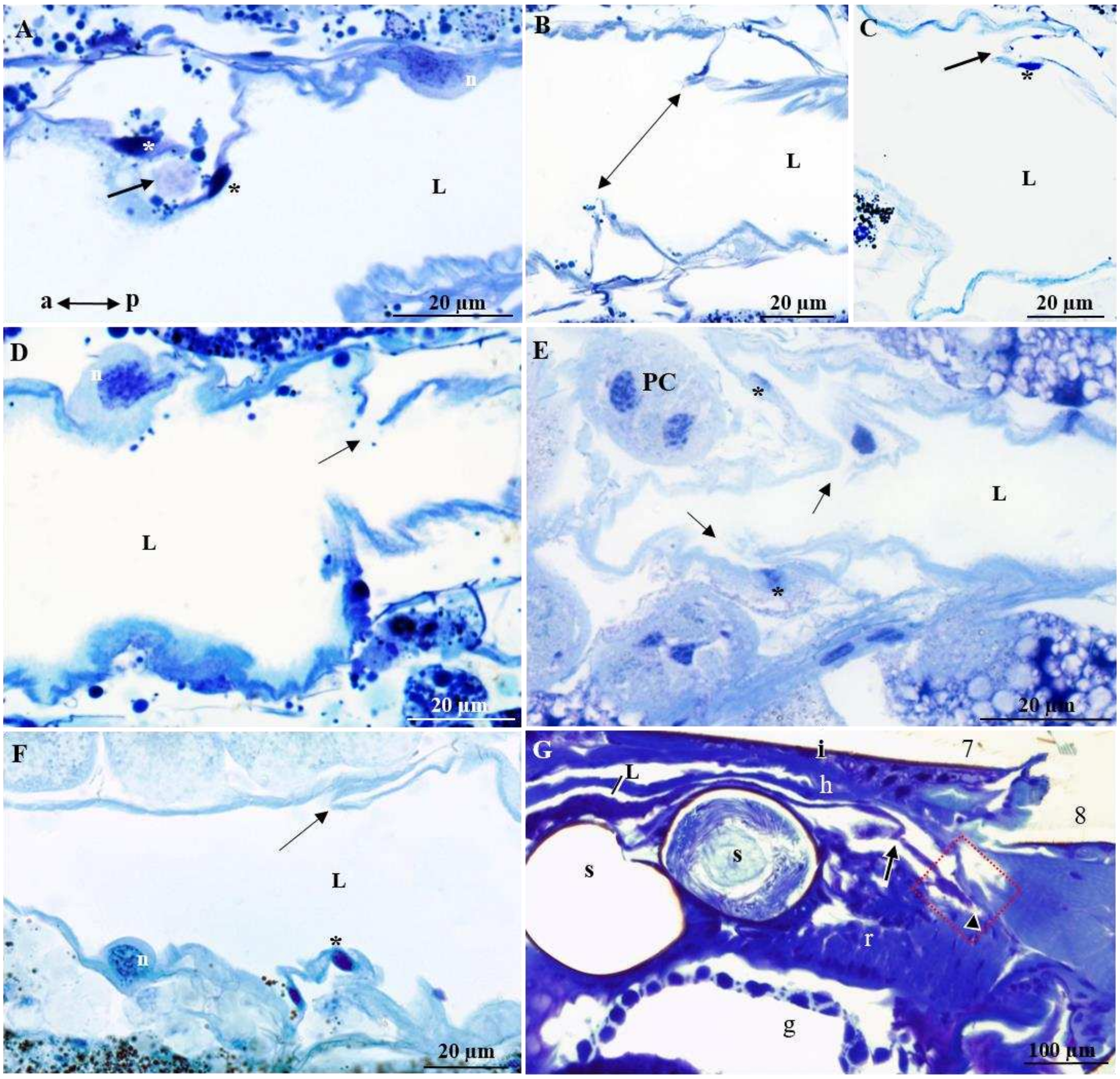

Figure 5. Histological sections of portions the heart of adult mosquitos corresponding to abdominal intersegmental regions showing incurrent ostia found between intersegments 1-2 **(A)**, 2-3 **(B)**, 3-4 **(C)**, and 4-5 **(D)** in *T. theobaldi*, 5-6 **(E)** in *Ae. aegypti*, 6-7 **(F)** in *T. theobaldi* and 7-8 **(G)** in *Ae. aegypti*. Nuclei (n) of cardiomyocytes may be positioned towards the heart lumen (as in A) or outside of the heart (as in D). In **(G)** an excurrent opening (arrowhead) is observed in the terminal cone (red square) of the heart (h) near the region between the abdominal segments 7 and 8. a: anterior region and p: posterior region of the body; g: hind gut; i: integument; L: heart lumen; PC: binucleated pericardial cell; s: spermathecae; r: reproductive system. **(A)**, **(B)**, **(D)**, **(E)** and **(G)** were stained with toluidine blue and **(C)** and **(F)** were stained with methylene blue.

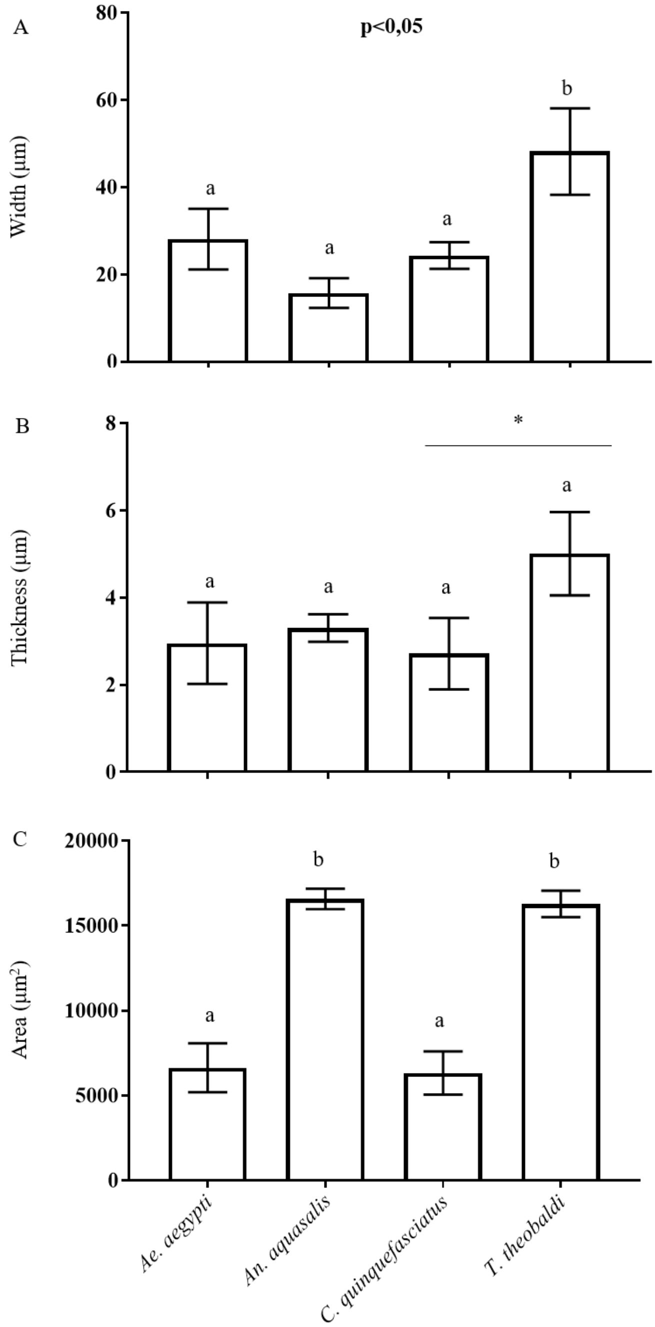

Figure 6. Morphometry of heart and PC of mosquitos. **(A)** Width (lumen + muscular wall) of the heart. **(B)** Thickness of muscular wall of the heart. **(C)** Total area of the PC in intersegmental regions of abdomen. Different letters indicate significant differences. Asterisk (*) indicates the statistical difference found between *C. quinquefasciatus* and *T. theobaldi*, according to the Tukey test (p<0, 05).

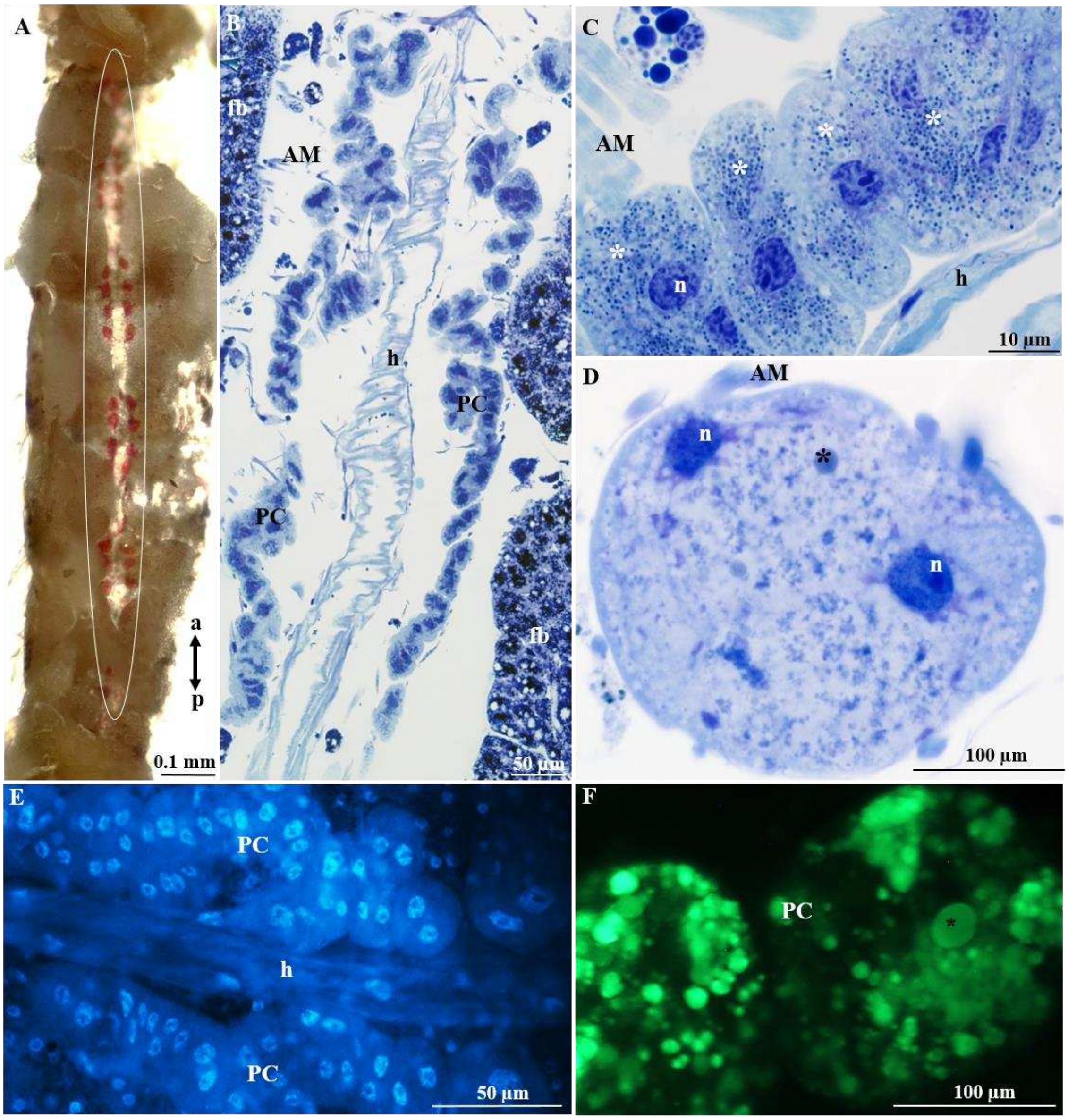

Figure 7. Pericardial cells (PC) of adult mosquitos. **(A)** Internal view of the dorsal abdomen of *C. quinquefasciatus* after the injection of carmine solution. In the pericardial sinus (white line) the heart (h) is associated with pericardial cells (red) in intersegmental (i) regions. a: anterior region and p: posterior region of the body **(B)** PC of *T. theobaldi* forming a cord of cells along the sides of the whole heart (h), resembling a syncytium. AM: alary muscles; fb: fat body. **(C)** PC of *T. theobaldi* with many basophilic cytoplasmic inclusions (*). AM: branches of the alary muscles. h: heart wall. **(D)** Binucleated PC in *C. quinquefasciatus* *: basophilic inclusion; n: nucleus. **(E)** PC of *An. aquasalis*, nucleus stained with DAPI (light blue); h: heart. **(F)** Two PC of *Ae. aegypti* with inclusions positively marked by LysoTracker (green).

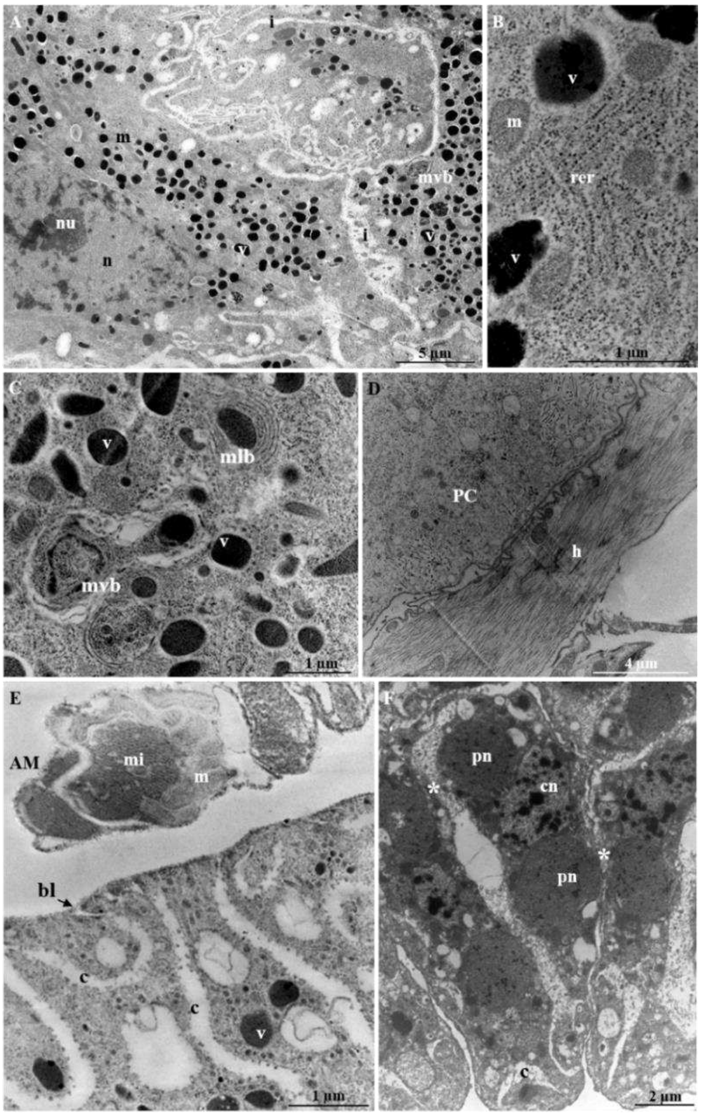

Figure 8. Ultrastructure of pericardial cells (PC) of adult mosquitos. **(A)** Overview of a contact region between PCs with intercellular spaces (i) in *T. theobaldi*. The cytoplasm is filled by several electron-dense vesicles (v), similar to lysosomes. mvb: multivesicular bodies; n: nucleus; nu: nucleolus. **(B** and **C)** Details showing the rough endoplasmic reticulum (rer) and multilamellar and multivesicular bodies (mlb and mvb) in a PC of *T. theobaldi*. v: electron-dense vesicles; m: mitochondria. **(D)** The region of physical contact between a PC and the heart surface (h) of *C. quinquefasciatus* without cell junction. **(E)** The cortex of a PC of *C. quinquefasciatus* with invaginations or canaliculi (c) and electron-denses vesicles (v). bl: basal lamina. AM: ramifications of alary muscles; mi: myofibrils; m: mitochondria. **(F)** PC of *An. aquasalis* with dimorphic nuclei. When there are three nuclei/cell, the central nucleus (cn) has more condensed chromatin than the two peripherals (pn). *: regions of communication between PC. c: cell canaliculus.

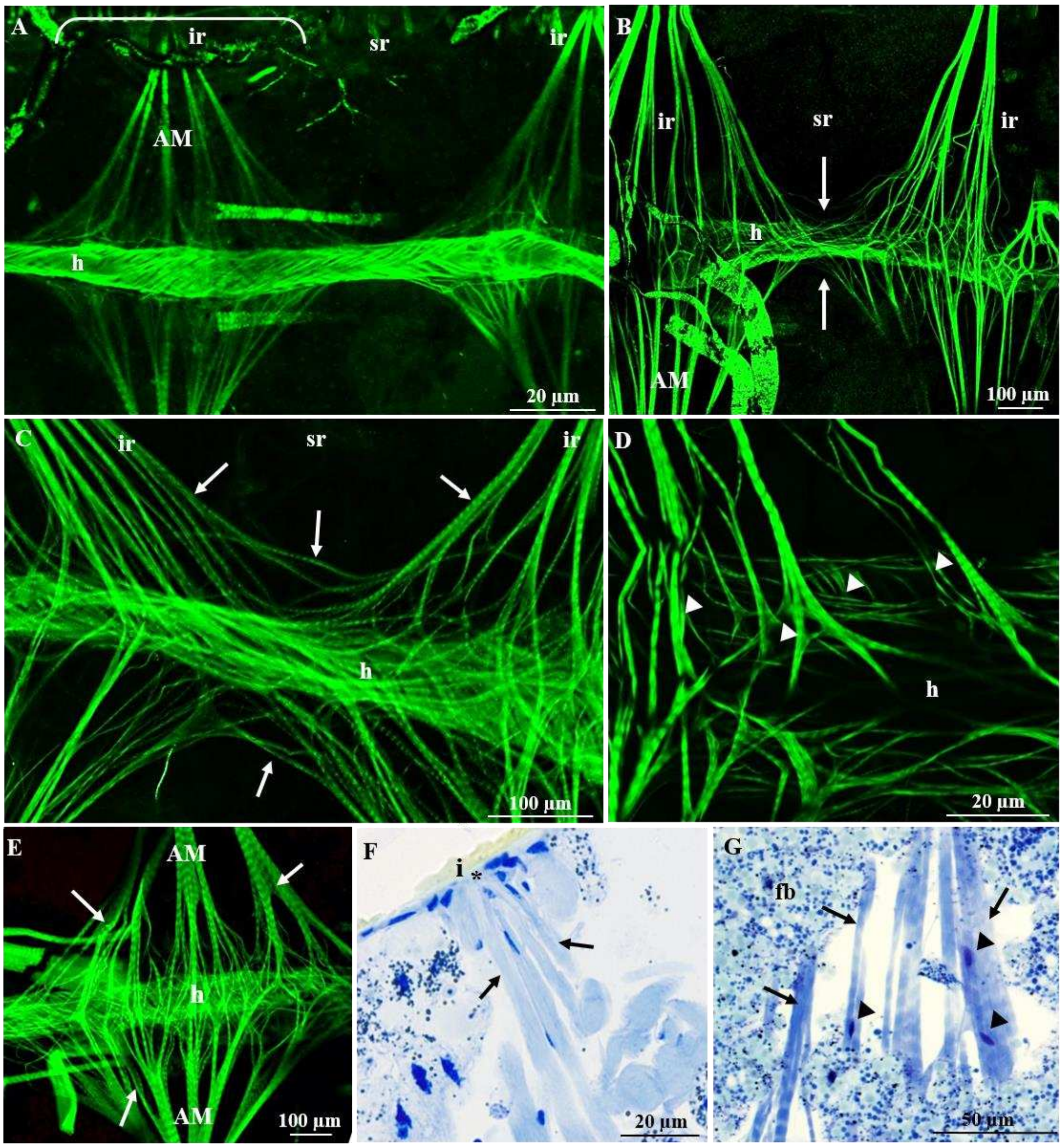

Figure 9. Alary muscles (AM) of mosquitos. **(A)** AM in *An. aquasalis* attached to intersegmental regions (ir) of the heart (h). sr: segmental region. **(B- D)** AM in *T. theobaldi*. **(B)** AM attached to ir and sr. **(C)** Details of the ramifications (arrows) that attached to sr and ir. **(D)** Branches of AM (arrowheads) attaching to the heart (h). **(E)** Intersegmental region of heart (h) with alary muscles (AM) with their branches (arrows) attaching to the heart in *C. quinquefasciatus.* **(F)** Attachment point of AM (*) in the integument (i) in *T. theobaldi*. Immediately after this point, AM ramify (arrows). **(G)** Polynuclear AM of *T. theobaldi*. Arrowheads: nuclei; fb: fat body. Green: FITC phalloidin-labeled cytoskeleton.

Table 1: Summary of the morphological differences of heart-associated tissues in adult females of mosquitos

Species/ Structure	Pericardial cells	Alary muscles
Anopheles aquasalis	clustered cells with no clear separation	Attached to intersegmental regions
Aedes aegypti	4 per intersegmental region, except last intersegment	
Culex quinquefasciatus		
Toxorhynchites theobaldi	clustered cells with no clear separation	Attached to segmental and intersegmental regions

Discussion

The heart of *An. aquasalis*, *Ae. aegypti*, *C. quinquefasciatus* and *T. theobaldi* are associated to PC and AM, and it is located in the dorsal midline of the abdominal cavity. This general organization and position coincide with what has already been described for other insects [7,18,19]. The cardiac fibers or cardiomyocytes are organized in helicoidal layers forming a tube, and this twisted orientation is also described for Diptera [3, 4]. It is assumed that this orientation confers resistance to muscle contraction and hemolymph propulsion [4, 18, 20].

The heart is dilated or has cardiac chambers in the portion located at the intersegmental regions of the abdomen. However, it was not always possible to see them in all the preparations. This may have occurred due to the moment the organ during dissection/fixation that could change the state of muscle contraction. Thus, when hemolymph passes through these chambers, they become more evident because they are full [3]. In the widest regions, the heart of *T. theobaldi* is wider than the other three mosquitos and this can be related to the larger size of this species [21], which can deal with greater relative volumes of hemolymph than the other smaller mosquitos.

As in other insects, the heart is surrounded externally and internally by a basal lamina [18,22]. In addition to isolating the organ from direct contact with hemolymph, the basal lamina contributes to the formation of structures similar to T tubules. These structures may be sites where the electrical impulse reaches the cells and stimulates the release of calcium from the sarcoplasmic reticulum, resulting in muscle contraction [22]. This contraction is important because with the force exerted by it, hemolymph circulates in the anterograde and retrograde direction in the body cavity of the mosquito [3].

During anterograde contraction, hemolymph enters the lumen of the heart through ostia of intersegmental regions [3], which were visualized together with the ostia lips, as described for other Diptera [4,18]. In spite of their conserved location in the intersegmental regions, the number of ostia may vary among species of the order. For example, *Drosophila melanogaster* has five pairs of ostia[18], but *An. gambiae* [12] and all species studied here have seven pairs. Although there are different numbers of ostia in Diptera, they have the same morphology, and due to the presence of valves, they are classified as incurrent ostia, whose format is essential for maintaining the direction of hemolymph flow towards heart lumen [1]. Furthermore, among the mosquitos studied here, a pair of incurrent ostia is found on each intersegmental regions, and an excurrent opening is found in the terminal cone. These data confirm the ostial organization and distribution previously described for *An. gambiae* [16], indicating that in different species of mosquitos, the number of ostia is conserved in adults.

Periostial hemocytes were found in the heart wall in the intersegmental regions. In *Galleria mellonela* (Lepidoptera) infected with bacterium and *An. gambiae* infected with bacterium and *Plasmodium*, these cells rapidly initiate an immune response, including phagocytosis and melanization in the periosteal regions (small spaces near the ostia) [12,13,14]. The presence of pericardial hemocytes in the species studied here suggests that the immune response that occurs near the heart is conserved among different mosquito genera.

The distribution pattern of nuclei and organelles, such as mitochondria; and subcellular structures such as sarcomeres, with their Z lines, A and I-bands, are also similar in the heart of all mosquitos studied here. These data also reinforce the idea that organ structure is conserved among mosquito species [9].

The presence of carmine within PC vesicles, the positivity of these vesicles for Lysotracker, together with their high electron density confirm the participation of PC in the endocytosis and digestion of components taken from hemolymph. It is suggested that extracellular channels, formed through invaginations of the plasma membrane of

PC, facilitate filtration by endocytosis. After filtration through the basal lamina, hemolymph accumulates in the channels, where toxic components are captured, forming vesicles that are delivered to lysosomes for digestion[6, 23]. The cytoplasm of PCs is also plenty of multivesicular and multilamellar bodies that deliver their content to the degradation in the lysosome [24].

The amount of PC varies across the species studied here. There are 54 individualized PC in *Ae. aegypti* and *C. quinquefasciatus* [4, 25]. However, it was not possible to quantify the number of PC in *An. aquasalis* and *T. theobaldi* because they have many agglomerated cells with no clear limit under light microscope. The total area occupied by agglomerates of PC in the intersegments of *An. aquasalis* and in *T. theobaldi* is larger (around 2.5-fold) than the total area of PC in *Ae. aegypti* and *C. quinquefasciatus* whose PCs are few and individualized. It can be speculated that the larger area occupied by the PC of *T. theobaldi* or *An. aquasalis* should enhance the filtration of hemolymph in comparison to *Ae. aegypti* and *C. quinquefasciatus*, but it was not tested yet. The differences in the amount and organization of PC in *T. theobaldi* (Culicidae-Toxorhynchitini) in comparison to the evolutionarily related species *Ae. aegypti* and *C. quinquefasciatus* (Culicidae-Culicini) suggest that during the evolution of these mosquitos, the total area of these cells increased in Toxorhynchitini. This may have been an independent event considering the similar condition (numerous and agglomerated PC with the larger total area) observed in the genus *Anopheles* (Anophelinae-Anophelini) [9, 12, 25].

The AMs originate near the integument and branch out as they approach the heart. The branches of the AMs connect directly to the sides of the organ and harbor the PC. These branches extend along the surface of the heart and form a net similar to a basketball hoop [3,4,9,10]. The physical association of AM and heart promote a tension in the heart wall, creating the cardiac chambers [3]. In addition, we observed that AM branches attach directly to the ostial lips, suggesting that AM assist in the opening and closing of ostia [3]. Unlike other mosquitos, in *T. theobaldi* AM also bind directly to the regions of the heart located at segmental regions of the abdomen. This difference should occur due to the larger size of individuals of this species [21], which would need more AM attached to the heart.

Conclusions

The basic plane of the adult heart is conserved across adult mosquitos of different species and with different feeding habits. Traits such as the organization in helicoidal layers of the cardiac muscle fibers, the presence of ostia and cardiac chambers in all abdominal intersegment, mitochondria located in the periphery of the cells and the presence of T tubules are conserved in *Ae. aegypti*, *An. aquasalis* and *C. quinquefasciatus* (hematophagous species) and in *T. theobaldi* (a phytophagous species). This conserved organization of the heart traits during mosquito speciation is expected considering the importance of the organ for the maintenance of the individual homeostasis that depends on the efficient hemolymph propelling to guarantee the functioning of endocrine system, nutrient supply, detoxification and immune reactions. Differently of the heart structure, morphological differences were found in relation to PC and AM. PC are individualized and less numerous, occupying a smaller total area in *Ae. aegypti* and *C. quinquefasciatus* and do not form agglomeration of cells on the sides of the heart such as in *An. aquasalis* and *T. theobaldi*. Additionally, only in *T. theobaldi* AM bind directly to regions of the heart that are located in the abdominal segments of the mosquito. These morphological differences related to the heart-associated tissues probably reflect evolutionary and physiological differences in the circulatory system of mosquitos that deserve to be studied in detail.

Methods

Mosquitos

Females of *Ae. aegypti* (PPCampos strain, Campos de Goycatazes, Rio de Janeiro, Brazil) were obtained from the colony kept in the insectary (Departamento de Biologia Geral, Universidade Federal de Viçosa-UFV). The insects were maintained under a photoperiod of 12 hours, temperature 25 ± 2°C and 60% ± 5% relative humidity (RH). The larvae were kept in plastic trays containing dechlorinated water and fed with cat food. Female pupae were transferred to plastic cages for subsequent emergence of the adults. Adults with 5 to 10 days post-emergence were separated for dissection. Adult females of *An. aquasalis* with 3 to 10 days post-emergence were obtained from a colony kept in the insectary at Centro de Pesquisas René Rachou (CPqRR, Fiocruz, Minas

Gerais). The insects were raised under a photoperiod of 12 hours, temperature 27 ± 1°C and 70 ± 10% RH.

Immature of *C. quinquefasciatus* were collected with the aid of a plastic sieve in the São Bartolomeu River basin (Viçosa, Minas Gerais, Brazil, 20°45'30"S, 42°52'45"W), a water stream polluted by raw sewage. The individuals were transported to insectary at UFV. Adult females with 5-15 days post-emergence were used. *T. theobaldi* immature were collected in Mata do Paraíso (20°45'14"S, 42°52'55"W) (Viçosa, MG) in dark plastic buckets containing fluvial water, transferred to the insectary and kept individually in plastic pots containing dechlorinated water. The larvae (predators) were fed larvae of *Ae. aegypti* and the pupae transferred to plastic cages. Adult females with 7 to 15 days post-emergence were used. The collection was done under license number 56917-1 of ICMBio (Instituto Chico Mendes de Conservação da Biodiversidade, Ministério do Meio Ambiente). Mosquitos of the four species were fed with 10% sucrose solution.

Adult females of the four species were anesthetized with CO_2 and dissected in PBS (phosphate buffered saline, 0.1 M, pH 7.2). The abdomen was laterally opened with the aid of a microscissor (Petrovich Surgical Instruments, São Paulo) and the visceral organs were removed, and the heart was kept attached to the carcass. Samples for light, confocal and fluorescence microscopies were fixed in Zamboni fixative solution (4% paraformaldehyde and 0.4% picric acid in PBS, pH 7.3) and samples for transmission electron microscopy (TEM) were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3). Samples were kept in the fixatives until the next of sample preparation at 4°C.

Whole mounts

For the initial observation of the heart, whole mounts of the organ of four species were made. The freshly dissected (unfixed) organs were removed from the abdominal carcass of the mosquito, mounted on glass slides and photographed using the Zeiss Primo Star light microscope coupled with the AxioCamER5 camera.

In order to facilitate the observation of PC, a saturated solution containing the powder carmine in PBS was injected about 200 μL in the thorax of five adult females of each species with the aid of the Drummond Nanoject II nanojet (Drummond Scientific

Company, Broomall, PA, USA). The heart with PC was dissected 12 h after injection and photographed using the light microscope.

Light microscopy

Twenty fixed carcasses with the heart of each species were washed in PBS, dehydrated in ascending ethanol series (70% -100%) and embedded in Leica historesin (Heidelberg, Mannheim, Germany). Samples were included in plastic molds, which were stored in a vacuum chamber for polymerization. Histological sections (3-4 μm thick) were obtained with glass knives coupled to the microtome. Sections were stained with toluidine blue or methylene blue solutions and mounted with Eukitt mounting medium (Fluka, St. Louis, MO, USA).

In order to study the intersegmental ostia of hearts separately, abdomen was cut in sequential pairs of segments (totalizing seven pairs: 1-2, 2-3, 3-4, 4-5, 5-6, 6-7, and 7-8) with the aid of microscissors. Stained histological sections of segment pairs of five females of each species were photographed under the fluorescence microscope Olympus BX53 coupled to the Olympus DP73 digital camera.

Confocal laser scanning microscopy (CLSM)

Five fixed carcasses of *Ae. aegypti*, *An. aquasalis*, *C. quinquefasciatus*, and *T. theobaldi* were washed in 0.1% Triton X-100 for one hour. For actin (cytoskeleton) labeling, the samples were incubated in Phalloidin-FITC (Sigma Aldrich, Sigma-Aldrich, Brazil) 1:50 for one hour and 30 min in the dark. After incubation, the samples were washed in PBS and stained with TO-PRO 3 (Invitrogen, Eugene, OR, USA) 1:500 for 30 min for nuclei (DNA) labeling. The samples were again washed and mounted on glass slides with mowiol-based mounting medium. The material was analyzed and photographed under the CLSM Zeiss 510 Meta at the Núcleo de Microscopia e Micoranálise (NMM/UFV).

Fluorescence microscopy

To evidence acidic organelles, about 200 μL of fluorescent green LysoTracker (Life Technologies, Eugene, OR, USA) 1:1000 (1 mM) was injected into the thorax side of ten adults *Ae. aegypti* using a microneedle made from microcapillary coupled to the nanoinjector. After 10 min, the females were dissected, and the heart was removed from the abdomen and mounted on glass slides with mowiol. The samples were visualized and photographed under the fluorescence microscope Olympus BX60 attached to the Olympus QColor 3 camera.

Three fixed dorsal carcasses of *T. theobaldi* and *An. aquasalis* were washed in PBS for 15 min and were incubated with 4'-6-diamidino-2-phenylindole (DAPI) (Biotium, Inc., Hayward, CA, USA) for 30 min in the dark to stain DNA (blue). The samples were washed again, mounted on slides and photographed under the Olympus BX53 microscope.

Transmission electronic microscopy (TEM)

Three fixed dorsal carcasses of each species (except *Ae. aegypti*) were washed in PBS and post-fixed in 1% osmium tetroxide and 0.1M sodium cacodylate buffer (pH 7.2) for two hours in the dark. The samples were washed in PBS, dehydrated in ascending ethanolic series and included in the LRWhite resin (London Resin Company, Berkshire, UK). The polymerization was done in gelatin capsules for 12 hours at 60 °C. Ultrathin sections were contrasted with 2% uranyl acetate and 0.2% lead citrate for 20 min each. Sections were photographed under the Zeis EM 109 microscope (NMM-UFV).

Morphometry

Measurements of heart width (muscle walls + lumen of hearts in intersegmental regions) and thickness of the heart muscle wall were performed in the images of stained histological sections (tangential and longitudinal sections) of four individuals of each species using the Image ProPlus™ program. In order to analyze the width and thickness of the wall of the heart, three and six measurements were performed respectively. The total area occupied by the PC (individually or in cell agglomerates, depending on the species and on both sides of the heart) in the intersegmental regions was also measured using the same software in three individuals per species. For comparison of the measurements among different species, the mean values obtained were submitted to the one-way variance test (ANOVA) and multiple comparisons were made through the Tukey test. Statistical analyzes were performed in the GraphPad Prism 7 program. The results were considered significant when $p<0.05$.

References

1. Klowden MJ. Physiological systems in insects. 3rd edition. San Diego: Academic Press; 2013.

2. Chapman RF, Reginald F, Simpson SJ, Douglas AE, Angela E. The insects : structure and function. 5th edition. Cambridge: Cambridge University Press; 2012.

3. Glenn JD, King JG, Hillyer JF. Structural mechanics of the mosquito heart and its function in bidirectional hemolymph transport. J Exp Biol. 2010;213:541–50. doi:10.1242/jeb.035014.

4. Leódido ACM, Ramalho-Ortigão M, Martins GF. The ultrastructure of the *Aedes aegypti* heart. Arthropod Struct Dev. 2013;42:539–50. doi:10.1016/J.ASD.2013.09.005.

5. Rotstein B, Paululat A. On the morphology of the *Drosophila* heart. J Cardiovasc Dev Dis. 2016;3:15. doi:10.3390/jcdd3020015.

6. Fu Y, Zhu J, Zhang F, Richman A, Zhao Z, Han Z. Comprehensive functional analysis of Rab GTPases in *Drosophila* nephrocytes. Cell Tissue Res. 2017;368:615–27. doi:10.1007/s00441-017-2575-2.

7. Sanger JW, McCann FV. Fine structure of the pericardial cells of the moth, *Hyalophora cecropia*, and their rôle in protein uptake. J Insect Physiol. 1968;14:1839–45. doi:10.1016/0022-1910(68)90215-1.

8. Poiani SB, da Cruz-Landim C. Histology and ultrastructure of pericardial cells of

Scaptotrigona postica Latreille (Hymenoptera, Apidae) in workers and queens of different ages. Micron. 2007;38:766–70. doi:10.1016/j.micron.2006.11.008.

9. Martins GF, Ramalho-Ortigão JM, Pimenta PFP. Morphological features of the heart of six mosquito species as revealed by scanning electron microscopy. Int J Trop Insect Sci. 2011;31:98–102. doi:10.1017/S1742758411000178.

10. Andereck JW, King JG, Hillyer JF. Contraction of the ventral abdomen potentiates Extracardiac Retrograde Hemolymph Propulsion in the Mosquito Hemocoel. PLoS One. 2010;5:e12943. doi:10.1371/journal.pone.0012943.

11. Hillyer JF. Integrated immune and cardiovascular function in Pancrustacea: lessons from the Insects. Integr Comp Biol. 2015;55:843–55. doi:10.1093/icb/icv021.

12. King JG, Hillyer JF. Infection-induced interaction between the mosquito circulatory and immune systems. PLoS Pathog. 2012;8:e1003058. doi:10.1371/journal.ppat.1003058.

13. Sigle LT, Hillyer JF. Mosquito hemocytes preferentially aggregate and phagocytose pathogens in the periostial regions of the heart that experience the most hemolymph flow. Dev Comp Immunol. 2016;55:90–101. doi:10.1016/j.dci.2015.10.018.

14. Pereira MF, Rossi CC, Queiroz MV, Martins GF, Clement I, Bossé JT, et al. *Galleria mellonella* is an effective model to study *Actinobacillus pleuropneumoniae* infection. Microbiology. 2015;161:387–400. doi:10.1099/mic.0.083923-0.

15. Lahondère C, Insausti TC, Paim RM, Luan X, Belev G, Pereira MH, et al. Countercurrent heat exchange and thermoregulation during blood-feeding in kissing bugs. Elife. 2017;6. doi:10.7554/eLife.26107.

16. League GP, Onuh OC, Hillyer JF. Comparative structural and functional analysis of the larval and adult dorsal vessel and its role in hemolymph circulation in the mosquito *Anopheles gambiae*. J Exp Biol. 2015;218:370–80. doi:10.1242/jeb.114942.

17. Mullen GR, Gary R, Durden LA. Medical and veterinary entomology. 2nd edition. San Diego: Elsevier; 2009.

18. Lehmacher C, Abeln B, Paululat A. The ultrastructure of *Drosophila* heart cells. Arthropod Struct Dev. 2012;41:459–74. doi:10.1016/J.ASD.2012.02.002.

19. Wasserthal LT, Wasserthal W. Innervation of heart and alary muscles in *Sphinx ligustri* L. (Lepidoptera). A scanning and transmission electron microscopic study. Cell Tissue Res. 1977;184:467–86. http://www.ncbi.nlm.nih.gov/pubmed/589655.

20. Jones JC. The heart and associated tissues of *Anopheles quadrimaculatus* say (Diptera: Culicidae). J Morphol. 1954;94:71–123. doi:10.1002/jmor.1050940104.

21. Harbach R. Mosquito taxonomic inventory. 2018. http://mosquito-taxonomic-inventory.info/. Accessed 31 May 2018.

22. Sanger JW. Cardiac Fine structure in selected arthropods and molluscs. American Zoologist. 1979;19:9–27. doi:10.2307/3882416.

23. Denholm B, Skaer H. Bringing together components of the fly renal system. Curr Opin Genet Dev. 2009;19:526–32. doi:10.1016/j.gde.2009.08.006.

24. Piper RC, Katzmann DJ. Biogenesis and function of multivesicular bodies. Annu Rev Cell Dev Biol. 2007;23:519–47. doi:10.1146/annurev.cellbio.23.090506.123319.

25. Rajindar P.Nephrocytes in some Culicidae- Diptera. Indian J Entomol. 1944;6:143–7.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HBS e GFM planned the study. HBS e RSMG performed the microscopic analyzes. HBS e GFM wrote the article. GFM contributed reagents. All authors corrected and approved the final manuscript.

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