**UNIVERSIDADE FEDERAL DE VIÇOSA**

# Morfologia do sistema reprodutor masculino e dos espermatozoides de três espécies de mosquitos (Diptera: Culicidae)

Henrique Barbosa da Silva
*Doctor Scientiae*

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**HENRIQUE BARBOSA DA SILVA**

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Tese 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 *Doctor Scientiae*.

Orientador: Jose Lino Neto

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Assentimento:

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Jose Lino Neto
Orientador

Dedico este trabalho aos meus pais.

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## RESUMO

SILVA, Henrique Barbosa da, D.Sc., Universidade Federal de Viçosa, janeiro de 2025. **Morfologia do sistema reprodutor masculino e dos espermatozoides de três espécies de mosquitos (Diptera: Culicidae)**. Orientador: Jose Lino Neto.

Várias espécies de mosquitos (Diptera: Culicidae) são vetores de patógenos que causam doenças em humanos. Devido à sua importância médica, essas espécies se tornam alvo de pesquisas, incluindo estudos sobre reprodução. Embora alguns pesquisadores já tenham conduzido investigações, ainda existem lacunas sobre a morfologia do sistema reprodutor masculino (SRM) e dos espermatozoides para a maioria das espécies de mosquitos. Este estudo apresenta uma análise sobre a morfologia tecidual do SRM e dos espermatozoides de *Anopheles darlingi* (Anophelinae), *Aedes aegypti* e *Lutzia bigoti* (Culicinae). O SRM de *Ae. aegypti* e *L. bigoti* consistiu em um par de testículos unifoliculares e compactamente espiralizados, ductos deferentes, vesículas seminais, um ducto ejaculatório e um par de glândulas acessórias. Sugere-se que o arranjo espiralado dos testículos os torna maiores e alongados, ocupando menos espaço e reduzindo a superfície de contato. Possivelmente essa estratégia está relacionada ao grande volume de gametas produzidos por essas espécies. Os espermatozoides possuem o formato filiforme e mediram, em média, 335 µm e 219 µm em comprimento, respectivamente. A ultraestrutura dessas células de *Ae. aegypti* revelou uma organização atípica do axonema, com um arranjo de microtúbulos do tipo 9 + 9+ ‘1’ (nove microtúbulos externos, nove duplas periféricas e um elemento central). O SRM de *An. darlingi* caracterizou-se por um par de testículos unifoliculares não espiralizados com espermatogênese radial, ductos deferentes, um ducto ejaculatório muscular e um par de glândulas acessórias. O comprimento dos espermatozoides variou de 92 a 246 µm. Observa-se que o SRM de mosquitos representando a subfamília Culicinae é similar entre as espécies, mas diferenciando-se de *An. darlingi*, representante da subfamília Anophelinae. Propõe-se que essas diferenças sejam marcas de cada subfamília. Por fim, sugere-se que a variação intraespecífica do comprimento dos espermatozoides de *An. darlingi* esteja relacionada ao comportamento monândrico das fêmeas.



Palavras-chave: mosquito; reprodução; testículos; glândulas acessórias; vesículas seminais; ductos deferentes; ducto ejaculatório; espermatozoides

## ABSTRACT

SILVA, Henrique Barbosa da, D.Sc., Universidade Federal de Viçosa, January, 2025. Morphology of the male reproductive system and spermatozoa of three mosquito species (Diptera: Culicidae)
. Adviser: Jose Lino Neto.

Several mosquito species (Diptera: Culicidae) act as vectors for pathogens that cause human diseases. Due to their medical significance, these species have become the focus of numerous studies, including research on reproduction. Although some investigations have been conducted, there remain significant gaps in understanding the morphology of the male reproductive system (MRS) and spermatozoa in most mosquito species. This study provides an analysis of the tissue morphology of the MRS and spermatozoa in *Anopheles darlingi* (Anophelinae), *Aedes aegypti*, and *Lutzia bigoti* (Culicinae). The MRS of *Ae. aegypti* and *L. bigoti* consisted of a pair of unifollicular and spiraled testes, deferent ducts, seminal vesicles, an ejaculatory duct, and a pair of accessory glands. It is suggested that the spiral arrangement of the testes makes them larger and elongated, occupying less space and reducing the contact surface. This strategy is possibly related to the large volume of gametes produced by these species. The spermatozoa are filiform in shape, measuring an average of 335 µm and 219 µm in length, respectively. Ultrastructural analysis of *Ae. aegypti* spermatozoa revealed an atypical axonemal organization, characterized by a 9 + 9 + '1' microtubule arrangement (nine outer microtubules, nine peripheral doublets, and a central element). The MRS of *An. darlingi* is characterized by a pair of non-spiraled, unifollicular testes with radial spermatogenesis, deferent ducts, a muscular ejaculatory duct, and a pair of accessory glands. Spermatozoa size ranges from 92 to 246 µm. It was observed that the MRS of mosquitoes within the Culicinae subfamily is morphologically similar across species, yet distinct from that of *An. darlingi*, a representative of the Anophelinae subfamily. It is proposed that these differences are hallmarks of each subfamily. Additionally, sperm length varied significantly among the three species studied. Finally, the intraspecific variation in spermatozoa length in *An. darlingi* is suggested to be associated with the monandrous behavior of its females.



Keywords: mosquito; reproduction; testes; accessory glands; seminal vesicles; deferent ducts; ejaculatory duct; spermatozoa

**SUMÁRIO**

# 1. INTRODUÇÃO

## 1.1- Mosquitos (Diptera: Culicidae)

Os mosquitos pertencem à família Culicidae Meigen, 1818, a qual constitui um táxon monofilético. Eles são encontrados em praticamente toda a superfície terrestre, exceto na Antártida, e são conhecidos, principalmente, por sua importância médica (Carvalho; Moreira, 2017; Foster; Walker, 2019; Franklinos *et al.*, 2019; Harbach, 2007; Reinert; Harbach, 2024; Harbach; Kitching, 2004; Souza-Neto; Powell; Bonizzoni, 2019). Até o momento, foram descritas 3.726 espécies, distribuídas em 113 gêneros classificados em três subfamílias: Anophelinae Grassi, 1900, Burmaculicinae Borkent e Grimaldi, 2016 e Culicinae Meigen, 1818 (Harbach, 2007; Harbach, 2024). Burmaculicinae engloba apenas culicídeos fósseis (Harbach, 2024). A subfamília Anophelinae inclui 521 espécies que podem ser facilmente reconhecidas, pois o corpo dos adultos quando pousam forma um ângulo de 30-45º em relação à superfície (Foster; Walker, 2002; Harbach, 2024). Essa subfamília é considerada basal em relação aos demais Culicidae atuais e é composta pelos gêneros *Anopheles* Meigen, 1818; *Bironella* Theobald, 1905 e *Chagasia* Cruz, 1906 (Harbach, 2007; Reidenbach et al., 2009; Harbach, 2024).

São conhecidas formalmente 512 espécies de *Anopheles* encontradas em áreas temperadas, subtropicais e tropicais (Harbach, 2024). Dentre elas está o *Anopheles darlingi* Root, 1926 que é considerada a principal espécie transmissora de malária no Brasil (Consoli; Oliveira, 1994). Ela é encontrada em regiões de baixa altitudes, associados, principalmente, a florestas. Sua distribuição geográfica inclui, praticamente, toda a região amazônica. É considerada uma espécie antropofílica (preferência em picar o ser humano), endofílica (se alimenta no intra-domícilio) e com atividade crepuscular (Consoli; Oliveira, 1994; Deane, 1948; Magris *et al.*, 2007).

A subfamília Culicinae inclui 3.201 espécies distribuídas em 110 gêneros, dentre as quais encontram-se o *Aedes* Meigen, 1818 e *Lutzia* Theobald, 1903 (Harbach, 2024). O *A*edes *aegypti* Linnaeus, 1762 é considerado um dos principais vetores de arbovírus para os seres humanos (Nene et al., 2007). É uma espécie antropofílica e cosmopolita (ampla distribuição geográfica), encontrada, principalmente, em regiões tropicais e subtropicais, onde as condições climáticas e situações precárias de

infraestrutura urbana e de saneamento básico permitem o seu desenvolvimento. Esses mosquitos possuem hábito diurno, sendo mais ativos durante o amanhecer e o entardecer. Porém, as fêmeas são oportunistas e podem picar também durante a noite (Carvalho; Moreira, 2017; Consoli; Oliveira, 1994).

O mosquito *Lutzia bigoti* Bellardi, 1862 não é considerado vetor de patógenos (Harbach, 2024) e há poucos relatos sobre ele na literatura científica (Albergaria; Araújo; Martins, 2024; Rocha; Martins, 2024). Sabe-se que suas larvas são predadoras vorazes de larvas de outros mosquitos (Lopes, 1999). Muito pouco se sabe sobre o comportamento e modo de vida dos adultos (Harbach,2024).

## 1.2- O sistema reprodutor masculino (SRM) de mosquitos

Para muitas espécies de insetos, o SRM é composto por um par de testículos, ductos deferentes, vesículas seminais, ducto ejaculatório e glândulas acessórias (Figura 1). Nos testículos, ocorre a formação de espermatozoides que, após serem produzidos, migram, através dos ductos deferentes, para as vesículas seminais, onde ficam armazenados até a cópula. As glândulas acessórias produzem um meio fluido que é liberado junto aos espermatozoides. O ducto ejaculatório é uma estrutura formada pela junção mediana dos ductos deferentes e se abre no gonóporo. O ducto ejaculatório libera todas as substâncias produzidas pelo SRM (Chapman, 1998; Klowden, 2013).

A maior parte dos estudos sobre o SRM de mosquitos datam de aproximadamente 60 anos atrás (Christophers, 1960; Hodapp; Jones, 1961; Snodgrass, 1959). Snodgrass (1959) forneceu uma descrição geral do SRM de mosquitos. Segundo ele, o desenvolvimento do sistema inicia-se na fase larval que ainda é muito rudimentar. Nos adultos o SRM é composto por um par de testículos, ductos deferentes, vesículas seminais, ducto ejaculatório e glândulas acessórias. Os testículos possuem um único folículo com um formato de pera. Na região superior deles há uma massa de células indiferenciadas, enquanto no restante do órgão nota-se a presença de células germinativas em diversos estágios da espermatogênese (Snodgrass, 1959).

Posteriormente, Christophers (1960) realizou análises dos sistemas corporais de *Ae. aegypti* destacando o SRM de larvas e adultos. Nas larvas os testículos estão

localizados no sexto segmento abdominal, revestidos por uma camada de corpo gorduroso não pigmentada. Um fio mesodérmico parte de cada um dos testículos e na base do SRM existem duas massas celulares com cavidades centrais que se tornam o ducto deferente. Nos adultos, o SRM possui aspecto similar ao descrito por Snodgrass (1959), porém as vesículas seminais foram identificadas como um ducto ejaculatório armazenador de espermatozoides (Christophers, 1960).

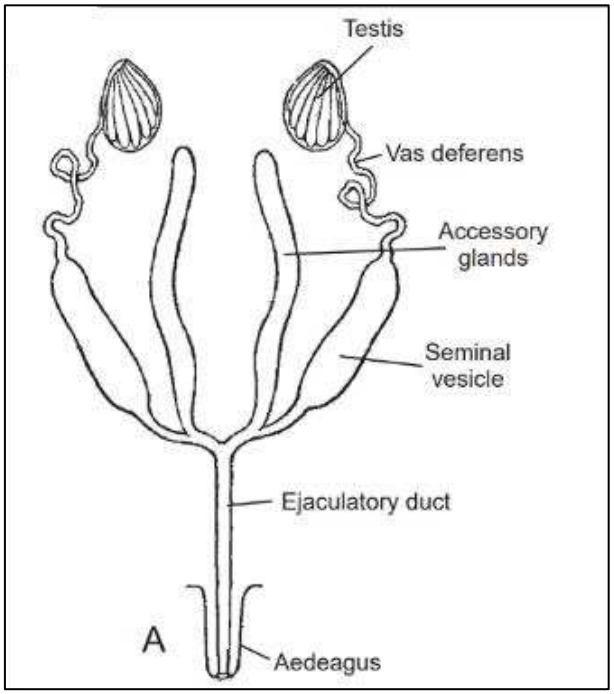


***Figura 1.*** *Morfologia geral do sistema reprodutor masculino de insetos (Klowden, 2013).*

Em outra pesquisa, foi observada a anatomia do SRM de *Ae. aegypti*, confirmando as estruturas descritas por Snodgrass (1959). Esse estudo comparou o SRM de *Ae. aegypti* ao de outros mosquitos (*Anopheles gambiae* Giles, 1902; *Anopheles quadrimaculatus* Say, 1824*; Culex pipiens* Linnaeus, 1758; *Culex quinquefasciatus* Say, 1823; *Culex tritaeniorhynchus* Giles, 1901; *Haemagogus equinus* Theobald, 1903; *Psorophora howardii* Coquillett, 1901), evidenciando que o sistema é formado por um par de testículos, ductos deferentes, vesículas seminais e glândulas acessórias, diferindo, principalmente, em relação ao formato dos órgãos e/ou extensão deles (Hodapp; Jones, 1961).

A montagem-total do sistema reprodutor masculino (SRM) de três espécies de *Anopheles* (*An. gambiae*, *Anopheles stephensi* Liston, 1901, e *Anopheles culicifacies*

Giles, 1901) foi utilizada na tentativa de prever o *status* de acasalamento delas. Foi sugerido que o número de cistos testiculares diminui após múltiplos eventos de acasalamento e que é possível observar um halo esbranquiçado ao redor das glândulas acessórias, assim como um reservatório de espermatozoides vazio (uma região com aspecto estriado localizada nas porções inferiores dos testículos). Em um teste cego, o método demonstrou-se eficaz para *An. stephensi* e *An. culicifacies*, mas falhou para *An. gambiae* (Huho et al., 2006; Mahmood; Reisen, 1982, 1994).

Embora os dados histológicos do SRM de mosquitos sejam escassos, pesquisas utilizando o *Ae. aegypti* trouxeram avanços significativos. Por exemplo, foi distinguido dois tipos de células nas glândulas acessórias. As células anteriores não se coram com azure B e ocupam, aproximadamente, 2/3 das glândulas. Já as células posteriores se coram com o azure B. Além disso, foi constatado que após copular o volume glandular é reduzido pela metade, mas é novamente aumentado após alguns dias (Dapples; Foster; Lea, 1974). Outro estudo, mostrou a presença do Dengue Vírus 2 nas glândulas acessórias e no epitélio da vesícula seminal de *Ae. aegypti*, sugerindo uma relação entre os fluidos das glândulas contaminadas e a transmissão venérea do vírus (Tu; Chen; Hou, 1998). Apesar dessas contribuições, ainda há muito o que avançar, sobretudo, a respeito da organização tecidual do SRM desse importante grupo de insetos.

## 1.3- Espermatozoides de mosquitos

Os espermatozoides são células que sofrem intensa pressão seletiva, resultando em rápida diversificação (Dallai, 2014). Eles são células muito diversas nos diferentes grupos de insetos, mas conservadas dentro das espécies podendo ser utilizados na filogenia e taxonomia (Barcellos et al., 2015; Dallai; Gottardo; Beutel, 2016; Fitzpatrick; Kahrl; Snook, 2022; Pereira; Lino-Neto; Do Prado, 2008).

Sabe-se que os gametas masculinos de mosquitos são longos e filiformes (Krafsur; Jones, 1967). Surpreendentemente, a plataforma SpremTree (https://spermtree.org/database/), uma base de dados que atualmente reúne registros morfométricos dos gametas masculinos de 5676 espécies em 27 filos animais, não possui nenhuma informação sobre os espermatozoides de mosquitos (Fitzpatrick; Kahrl; Snook, 2022). Entretanto, algumas medidas já foram descritas. Por exemplo, no estudo de Christophers (1959) foi apontado que o tamanho da cabeça dos

espermatozoides de *Ae. aegypti* varia entre 40-45 µm, enquanto a cauda possui cerca de 200 µm. Em um estudo anterior, foi identificado que os espermatozoides de *Ae. aegypti* possuem entre 250 e 300 µm (Klowden; Chambers, 2004). Adicionalmente, nos espermatozoides de *Ae. aegypti* não se observou acrossomo (Krafsur; Jones, 1967), enquanto em *Aedes mariae* Sergent e Sergent, 1903 essa estrutura mede 0,12 µm de comprimento e 0,2 µm de largura (Jamieson, 1999).

Mais recentemente, foi identificado polimorfismo espermático em *An. gambiae*, *An. darlingi* e *Anopheles quadriannulatus* Theobald, 1911 (Klowden; Chambers, 2004; Voordouw; Koella; Hurd, 2008). Esse fenômeno está relacionado a presença de morfotipos diferentes de espermatozoides (Simmons, 2001; Ward, 1998). Nesse estudo, os autores realizaram as análises baseado em espermatozoides coletados dos testículos de 3-5 indivíduos e após medições os categorizaram em grupos de 50 µm de intervalo. Foi sugerido que o polimorfismo seja uma característica que marca a existência de um complexo de espécies, mas essa hipótese ainda não foi testada (Klowden; Chambers, 2004).

A espermatogênese em *Aedes* inicia-se na fase larval tardia (L4) (Mukherjee; Rees, 1969). Em Ae. aegypti as espermatogônias ocupam a região anterior dos testículos e são caracterizadas como células esféricas, cujo núcleo ocupa praticamente todo o citoplasma. Os espermatócitos I e II são difíceis de distinguir entre si, mas ambos possuem um formato arredondado e núcleos menores que os das espermatogônias. As espermátides iniciais são células arredondas com núcleo esférico e nebenkern bem pequeno (estrutura resultante da fusão de mitocôndrias que posteriormente originará os derivados mitocondriais). As espermátides passam por uma série de mudanças conformacionais, incluindo o alongamento da célula e o afilamento do núcleo, resultando em espermatozoides maduros (Krafsur; Jones, 1967).

A ultraestrutura espermática foi investigada para algumas espécies de mosquitos. Utilizando microscopia eletrônica de transmissão, e baseado na quantidade e diâmetro dos microtúbulos, foram identificados quatro tipos de manchette (estrutura composta por microtúbulos ao redor do núcleo das espermátides que auxiliam no alongamento nuclear) nos gêneros *Aedes*, *Anopheles*, *Culex* Linnaeus, 1758 e *Toxorhynchites* Theobald, 1901 (Ndiaye; Mattei; Thiaw, 1997a). Outro estudo mostrou que existem modificações da parede testicular e do acrossomo

dos espermatozoides na região baixa do testículo, nas vesículas seminais e na espermateca das fêmeas (Nydiae; Mattei; Thiaw, 1997b). Além disso, a análise da ultraestrutura dos espermatozoides de *Culex* sp., *Aedes canadensis* Theobald, 1901 e *Toxorhynchites* identificou que o axonema possui um padrão não usual de microtúbulos, denominado 9 + 9 + '1' (nove microtúbulos simples externos, nove duplas de microtúbulos e um único elemento central que não é um microtúbulo) que inicialmente foi sugerida como sinapomorfia da família Culicidae (Phillips, 1969. Justine; Mattei, 1988), mas que já foi identificado também em Bibionidae (Dallai et al. 1993). Apesar dessas descobertas, ainda há muito a ser elucidado sobre os espermatozoides de mosquitos, especialmente no que diz respeito à diversidade deles.

## 2. OBJETIVOS

Esta tese tem por objetivo geral prover informações sobre o SRM e espermatozoides de mosquitos, buscando preencher lacunas e sanar inconsistências existentes na literatura.

### 2.1- Objetivos específicos

- Descrever o SRM de adultos de *L. bigoti* e realizar análise morfométrica de seus espermatozoides.
- Caracterizar a estrutura do SRM de *Ae. aegypti* nas fases de larva tardia (L4), pupa e adultos.
- Analisar as dimensões dos espermatozoides de *Ae. aegypti*, além de estudar sua ultraestrutura.
- Descrever o SRM de *An. darlingi* e avaliar a aplicação do conceito de polimorfismo espermático a essa espécie.

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## 4. CAPÍTULO 1: Insights into the male reproductive system and spermatozoa of *Lutzia bigoti* (Diptera: Culicidae)

Neste capítulo foi realizada a análise da organização morfológica do SRM e dos espermatozoides de *L. bigoti*. Embora essa espécie não tenha relevância médica e, portanto, não tenha sido amplamente estudada, a análise se justifica pela necessidade de aprofundar o conhecimento sobre as espécies de mosquitos. Essas informações podem contribuir significativamente para o entendimento da história evolutiva do grupo.

SILVA, H. B.; BARBOSA, R. C.; COSTA, D. A. et al. Insights into the male reproductive system and spermatozoa of *Lutzia bigoti* (Diptera: Culicidae). *Zoomorphology*, v. 143, p. 107–116, 2024. Disponível em: https://doi.org/10.1007/s00435-024-00643-w.

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RESEARCH

# Insights into the male reproductive system and spermatozoa of *Lutzia bigoti* (Diptera: Culicidae)

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**Abstract**
Spermatozoa and the male reproductive system (MRS) present great variability, featuring morphological characters that could be used in the taxonomy of different insect groups. Despite their importance, such data are scarce for mosquitoes, and their potential application in taxonomy has been neglected. In this study, we describe the morphology of the MRS and spermatozoa of the mosquito *Lutzia bigoti* Bellardi, 1862. The MRS consists of a pair of testes, deferent ducts, seminal vesicles, accessory glands, and an ejaculatory duct. The testes have only a single well-compacted follicle. The deferent ducts originate at the base of each testis, with the first portion resembling a goblet, and present an enlargement that forms the seminal vesicles in the posterior region. Subsequently, the ducts merge with the respective ducts of the accessory glands and open into the ejaculatory duct. The spermatozoa of *L. bigoti* are characterized as filiform and long, measuring around 220 µm, providing a potential distinguishing feature of this species. We hypothesize that the spiral organization of the testes may confer advantages in spermatozoa production. Similar to other mosquitoes, the MRS organization plan of *L. bigoti* conforms to type B. In addition, we also suggest that Culicinae species possibly share juxtaposed vesicles. This work paved the way for future analyses exploring of reproductive biology of *Lutzia*, including ultrastructure examinations and taxonomic investigations in the Culicidae family.

**Keywords** Spiraled follicle · Goblet of deferent duct · Mosquito · Reproduction · Light microscopy

## Introduction

Mosquitoes (Culicidae Meigen, 1818 family) belong to the order Diptera and are widely recognized as vectors of arboviruses, protozoa, and nematodes, which cause diseases in humans (Consoli and Lourenço-de-Oliveira 1994; Foster and Walker 2002). For this reason, scientific literature emphasizes research on mosquito vectors (Andereck et al. 2010; Barbosa Da Silva et al. 2019; Fiaz et al. 2019; Pascini et al. 2020; Godoy et al. 2021; Miranda et al. 2021; Rodrigues et al. 2021). However, understanding the biological aspects of non-vector mosquitoes is very important. This knowledge contributes to the taxonomic data on mosquitoes and aids in comprehending the evolutionary history of the Culicidae, a field still marked by gaps (Reidenbach et al. 2009).

There are 3,719 known species of mosquitoes, distributed within two subfamilies (Anophelinae Grassi, 1900 and Culicinae Meigen, 1818). The Anophelinae subfamily is composed of the genera *Anopheles* Meigen, 1818, *Bironella* Theobald, 1905, and *Chagasia* Cruz, 1906. On the other hand, the Culicinae subfamily comprises 110 genera and 11 tribes, which includes the monophyletic tribe Culicini Meigen, 1818. It is made up of the genera *Culex* Linnaeus, 1758, *Deinocerites* Theobald 1901, *Galindomyia* Stone & Barreto, 1969, and *Lutzia* Theobald 1901 (Reidenbach et al. 2009; Harbach et al. 2012; Harbach 2023).

Over time, the taxonomic classification of many mosquitoes within the Culicini tribe has undergone revisions, leading to controversies in the nomenclature of many species. This occurred with the genus *Lutzia*, previously considered a subgenus of *Culex* (Harbach et al. 2012). However, a reevaluation based on morphological and molecular data supported the *Lutzia* as a distinct genus (Tanaka 2003; Harbach et al. 2012; Sun et al. 2019). This update classification

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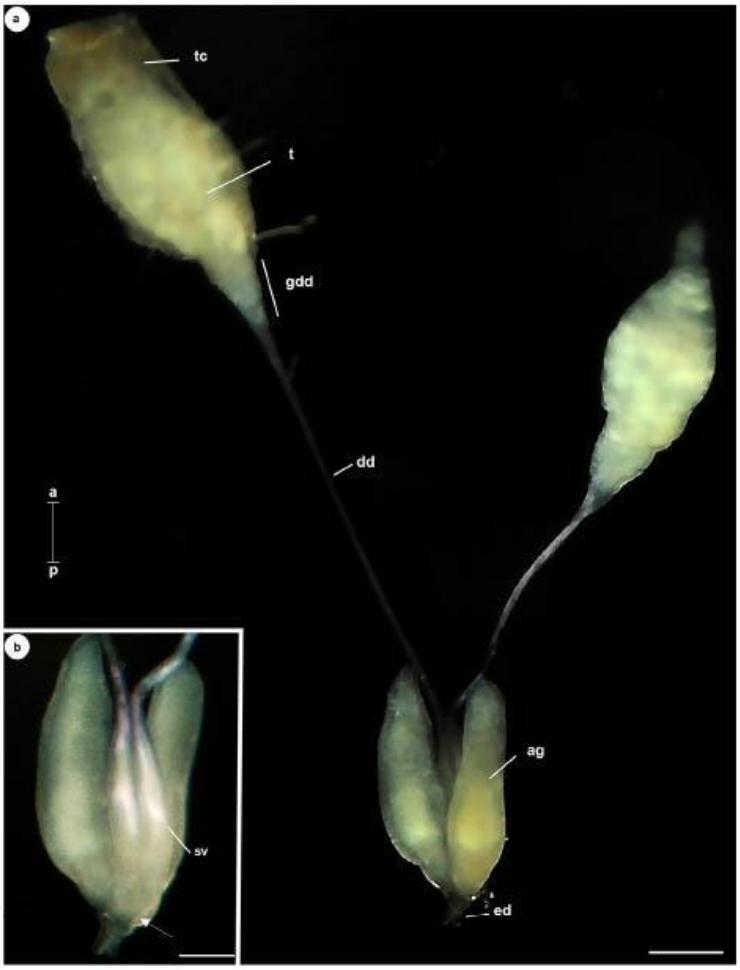



**Fig. 1** Morphology of the male reproductive system (MRS) of *L. bigoti*. **a** In the ventral view, it is observed that the MRS is formed by a pair of testes (t) covered by a yellowish testicular capsule (tc), a pair of deferent ducts (dd), whose apical portion has a goblet shape (gdd), a pair of accessory glands (ag) and an ejaculatory duct (ed). **b** In the dorsal view, the pair of seminal vesicles (sv) is most visible a: anterior region (p): posterior region. Scale: 50 µm

is currently in the Catalog of Life (https://www.catalogueoflife.org/).

Information regarding the biology of *Lutzia* remains limited, although it is known that the larvae exhibit voracious predators behavior (Harbach 2023). The potential utilization of mosquito larvae with such predation tendencies for the biological control of other culicids has been proposed (Lopes 1999). In contrast, adults possibly feed on sugary plant substances, and there is no evidence supporting hematophagy in this species (Harbach 2023). Morphological studies of the genus are scarce, with literature primarily focusing on external character descriptions. Such as a recent master's thesis that analyzed the antennal sensilla of adults of *Lutzia bigoti* Bellardi, 1862 (Albergaria 2022). That way, there is still much uncovered regarding the biology of this genus of mosquitoes.

In insects, spermatozoa exhibit great morphological diversity, even among related taxa, making them a promising subject for taxonomic studies (Dallai et al. 2014; Dallai et al. 2016; Fitzpatrick et al. 2022). These cells are produced by the male reproductive system (MRS) through the intricate process of spermatogenesis, involving several steps. For example, in *Drosophila melanogaster* Meigen, 1830, spermatogenesis initiates in the apical region of the testes, where germline stem cells differentiate into gonialblasts. These cells undergo mitotic and meiotic divisions along

the testes, forming spermatogonia that develop into spermatocytes, spermatids, and finally, spermatozoa (Demarco et al. 2014). Typically, the MRS in most insects consists of a pair of testes, deferent ducts, seminal vesicles, an ejaculatory duct, and accessory glands (Snodgrass 1959; Chapman 1998; Klowden 2013). Numerous descriptions have documented the morphology of spermatozoa and the MRS in different insects (e.g., Spiegel et al. 2013; Dias et al. 2017, 2022; Dallai et al. 2018; Munhoz et al. 2021; Rezende et al. 2021; Salazar et al. 2022). However, data on the morphology of mosquito spermatozoa and MRS are still scarce.

This entire scenario highlights the problems related to the taxonomic classification of the Culicini tribe and the necessity to enhance our comprehension of the morphology of the genus *Lutzia*. In this study, we employed *L. bigoti* as a model to elucidate the characteristics of spermatozoa and MRS. We discussed and compared our results to existing data from other insects.

## Materials and methods

### Collection, dissection of mosquitoes, and whole-mount of the MRS

Immatures *L. bigoti* were collected in dark buckets containing fluvial water installed in the Mata do Paraíso Forest Reserve (20°45′ 14″ S, 42°52′ 55″ W) (Viçosa, Minas Gerais, Brazil), under License N° 569,172 ICMBIO/SISBIO. The specimens were them transported to the insectarium of the Department of General Biology at the Federal University of Viçosa (DBG-UFV). The larvae were kept individually in transparent pots with dechlorinated water. Due to their predatory habit, *L. bigoti* larvae were fed with *Aedes aegypti* Linnaeus, 1762 larvae obtained from the DBG-UFV insectarium. Pupae, both collected and obtained from the insectarium were transferred to breeding cages. Upon reaching adulthood, mosquitoes were fed with a 10% sugar water solution ad libitum. The individuals were identified under a Zeiss Stemi 2000-C® stereomicroscope, using the taxonomic key of Tanaka (2003), the description of the species proposed by Theobald (1901), and a description available on the Mosquito Taxonomic Inventory website (https://mosquito-taxonomic-inventory.myspecies.info/) (Harbach 2023).

Adult males at least 7 days old were anesthetized with $CO_2$, and their MRS was dissected in 0.1 M sodium phosphate buffer (PBS), pH 7.2. After dissection, the MRS organs were photographed using a Zeiss 2000-C® stereomicroscope and an Olympus CX31® light microscope with an Electro-Optical System (EOS) Rebel Canon T7$^{+}$® camera for the whole-mount analysis. For histology, the MRS from five individuals was fixed in a 2.5% glutaraldehyde solution for at least 2 h.

### Measures of the spermatozoa

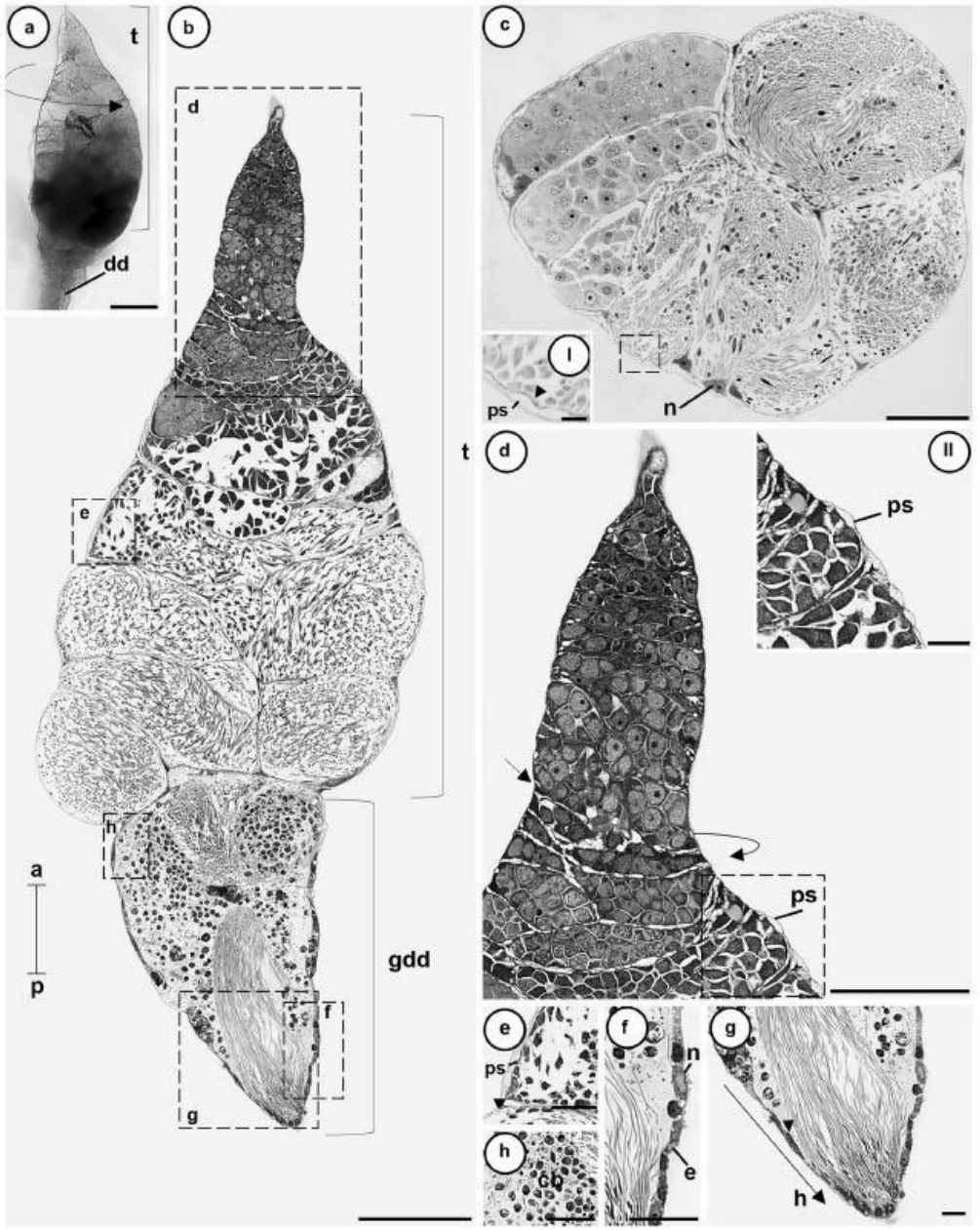
To obtain spermatozoa, the contents of the seminal vesicles of three mosquitoes were individually spread on histological slides under drops of PBS. Subsequently, the slides were stained with Giemsa (Merck KGaA, Darmstadt, Germany®) (1:15 in distilled water) for 15 min, followed by washing in running water and air-drying at room temperature. The material was analyzed, and 20 spermatozoa from each individual were photographed using an Olympus BX60® photomicroscope equipped with an Olympus Qcolor® digital camera. For precise measurements, encompassing total spermatozoa measurements (head + flagellum), head components (acrosome + nucleus), and a distal thin portion of the flagellum were performed with the free ImageJ software (https://imagej.nih.gov/ij/) (Schneider et al. 2012).

### Histology

The fixed samples were washed in distilled water and then post-fixed in 1% osmium tetroxide solution (Sigma Aldrich®) for 1 h. Following another round of distilled water, the samples were subjected to dehydration in an ascending alcohol series (30%, 50%, 70%, 90%, and three times in 100%) for 10 min in each bath. After, samples were pre-infiltrated in three solutions of historesin (Leica, Historesin, Heidelberg, Germany®) and alcohol (1:2; 1:1; 2:1) for 1 h in each mixture. Complete infiltration was achieve with pure historesin, and after an overnight incubation, samples were transferred to silicone with historesin added with the catalyst at a ratio 15:1. The molds were placed in an oven at 60 °C until complete polymerization. Sections, 1.0 µm thick, were obtained using a Leica RM 2255® microtome and stained with Giemsa solution (diluted at 1:15 in distilled water) or 1% toluidine blue (Synth®) in 0.5% sodium borate. Analysis and photography were performed using an Olympus CX31® microscope with an EOS Rebel Canon T7® digital camera attached. Several frames were captured using a 40× or 100× objective, and the images were then assembled into panoramic images using the Photomerge tool in Adobe Photoshop 2022 Software®.

## Results

The MRS of *L. bigoti* consists of two isolated testes, each featuring a single spiral follicle connected to its corresponding deferent duct. Notably, each testis exhibits a well-compacted follicle encased within a yellow-pigmented testicular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◂**Fig. 2** Testes and goblet of the deferent duct of *L. bigoti*. **a** Whole-mount of the testis (t) and the initial portion of the deferent duct (dd). The testis has a spiral appearance (arrow). **b** Longitudinal section of the t and the goblet of the deferent duct (gdd). Note that the t has only one follicle which are germ cells at different stages of the spermatogenesis. Cells in the young stage of the process are located in the anterior regions, while the more mature ones are located in the posterior regions. Rectangles d–h demarcate details in images **d–h**, respectively. **c** Cross-section of the testis in which it is possible to observe that the testicular follicle is single and spiraled. Note that in the same section, there is the presence of germline cells at different stages of formation. See the nuclei (n) of cystic cells with decondensed chromatin and evident nucleolus. In inset **I**, it is possible to observe the peritoneal sheath (ps) that covers the entire testis and the limit of the testicular cyst (arrowhead). **d** The anterior portion of the t in which the spiral aspect is evident (arrows), also note the ps which is seen in inset **II**. **e** Details of the t in which the ps and the limit of the testicular cyst are observed (arrowhead). This region is thinner than the gdd epithelium **e** seen in **f**. See that the nucleus (n) has decondensed chromatin. **g** The posterior portion of the gdd. In this region, the spermatozoa are positioned with their heads (h) facing the posterior region of the gdd, according to the orientation of the arrow. Arrowhead: flat n of epithelial cell. **h** Still in the gdd, it is possible to notice many cytoplasmic bodies (cb). Staining: toluidine blue (a–g) and Giemsa **h**. a: anterior region (p): posterior region. Scales: a, b, c, h: 100 µm; d, e, f, g: 25 µm; inset I: 10 µm; inset II: 25 µm

capsule (Fig. 1a). The first portion of the deferent duct, located near the testis, displays a morphology reminiscent of a goblet shape. Subsequently, the second portion of the ducts undergo noticeable thinning, and a little after the half, they expand, forming the seminal vesicles responsible for the storage of spermatozoa. Following the seminal vesicles, the deferent ducts join the two accessory glands, converging to form the ejaculatory duct (Fig. 1a, b).

The freshly dissected testes exhibit alternating and dark regions (Fig. 2a), indicating that these organs are spirals. Histological sections confirm the spiral arrangement, revealing each testis has only one follicle (Fig. 2b). The follicle is filled with cysts at different stages of spermatogenesis, initiating in the anterior region and ending in the posterior region (Fig. 2b). Within each cyst, germ cells from the same spermatogonia are surrounded by somatic cystic cells (Fig. 2c). In addition, histological analysis reveals the peritoneal sheath covering each testicular follicle (Fig. 2b–e).

The goblet of the deferent duct features a simple epithelium with flattened cell nuclei predominantly displaying decondensed chromatin (Fig. 2f). Its lumen contains cytoplasmic bodies and spermatozoa with nuclei oriented toward the posterior region of the goblet (Fig. 2g, h).

Observing histological sections of the testes, cysts with germ cells at different stages of formation are evident. Those with younger are concentrated in the anterior testicular region, while more mature ones are located in the posterior region, close to the goblet of the deferent ducts (Fig. 2b).

The two seminal vesicles are juxtaposed (Fig. 1b). Histological sections show the epithelia separating the two lumens, both filled with spermatozoa (Fig. 3a, b). The simple epithelium consists of cubic cells with decondensed chromatin nuclei (Fig. 3c). Each vesicle is externally covered by a thin muscular layer (Fig. 3a, c).

The accessory glands, located close to the seminal vesicles, exhibit a fusiform shape, with thinner apical and basal regions compared to the median (Figs. 1 and 3e). They are connected only in the basal region (Fig. 3d). The lumen of each gland contains numerous secretion granules produced by epithelial cells. The anterior glandular region, secrete a material that presents a slight color (Fig. 3a, f). In the posterior region of the glands, the cells secrete a material that stains more intensely (Fig. 3g). Both secretions display decondensed chromatin and an evident nucleolus, indicative of an apocrine secretion type (Fig. 3f, g). In addition, the accessory glands are covered by a muscular layer (Fig. 3a, f, g).

The spermatozoa of *L. bigoti* are filiform and lengthy, measuring 219.51 (±21.12) µm in length (Fig. 4a). The head region, measuring 13.53 (± 1.23) µm in length, with 2.36 (±0.76) µm occupied by the acrosome and 11.17 (±0.77) µm by the elongated nucleus (Fig. 4b). The flagellum, with a diameter close to that of the head, spans approximately 200 µm in length. Its distal portion is thinner, measuring approximately 30 µm (Fig. 4c).

## Discussion

Our study represents the first morphological characterization of MRS and spermatozoa from a mosquito belonging to the genus *Lutzia*. We observed that *L. bigoti* has the basic organizational plan of the MRS commonly found in insects, as documented in previous studies (e.g., Snodgrass 1959; Chapman 1998; Klowden 2013). According to the classification proposed by Hiroyoshi and Reddy (2022), we classify the MRS arrangement in *L. bigoti* as type B. In this case, spermatozoa traverse the deferent ducts to reach the seminal vesicles, which connect to the ejaculatory duct alongside the ducts of the accessory glands. While this organization has been observed in Culicidae (Hodapp and Jones 1961; Mahmood and Reisen 1982, 1994; Huho et al. 2006), it is not commonly observed in all Diptera. For example, families such as Tephritidae Newmman, 1834 and Glossinidae Theobald, 1903, exhibit a type C configuration (similar to type B but without seminal vesicles), whereas type H (characterized by the absence of accessory glands) occurs in Psychodidae Newmman, 1834 (Drew 1969; Odhiambo et al. 1983; Valdez 2001; Spiegel et al. 2013; Hiroyoshi and Reddy 2022).

The occurrence of testes with just only one follicle is not unique to *L. bigoti* and is observed in other Diptera (Snodgrass 1959; Chapman 1998; Valdez 2001; Spiegel

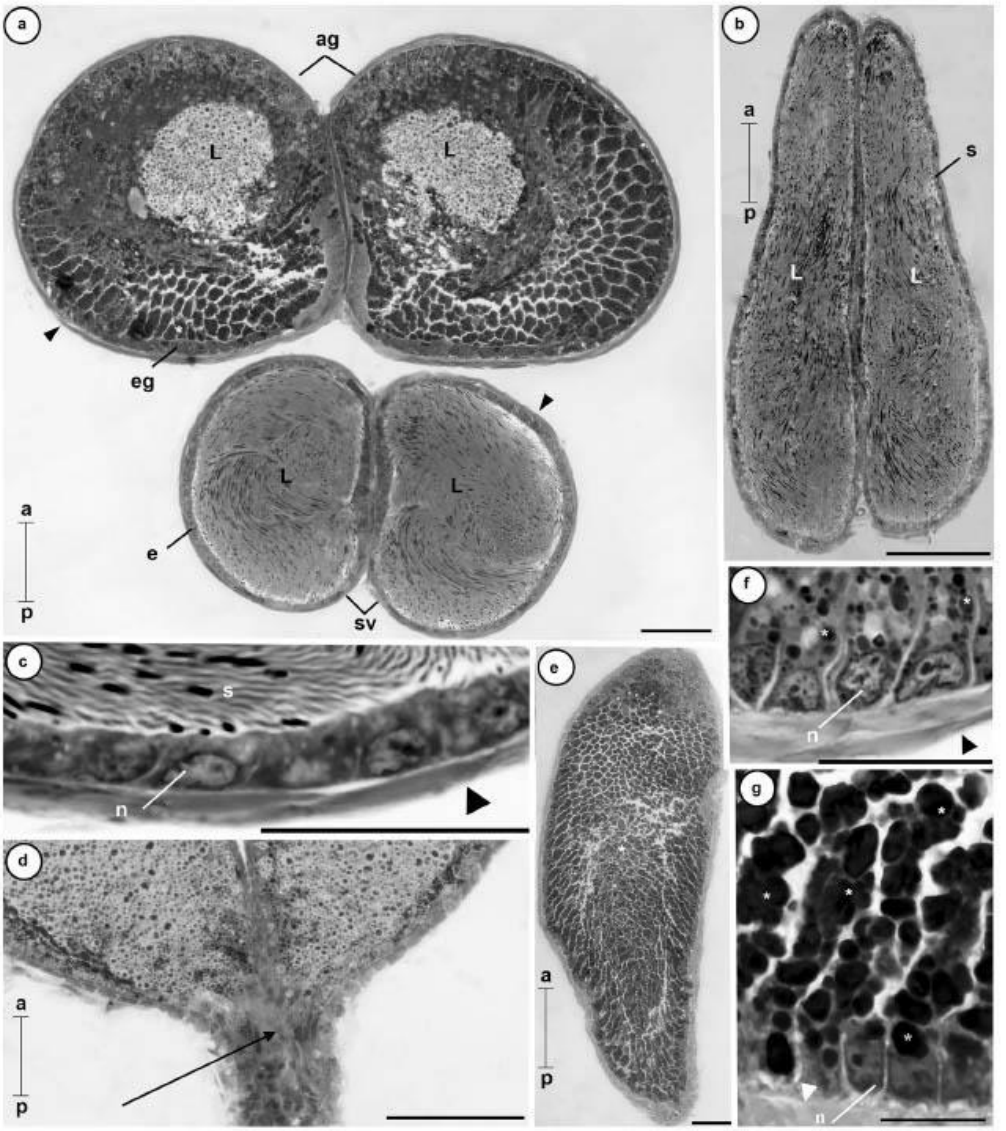


**Fig. 3** Accessory glands and seminal vesicles of *L. bigoti*. **a** Cross-section of accessory glands (ag) and seminal vesicles (sv). Note that the glandular epithelium (eg) releases numerous packets (*) containing secretions into the lumen (L) of the ag. See that the vesicular epithelium **e** is juxtaposed and separates the lumen (L) of the sv, which is full of spermatozoa. The organs are covered by a muscular layer (arrowhead). **b** Longitudinal section of the seminal vesicles, whose lumen (L) is filled with spermatozoa (s). **c** Details of the simple cubic epithelium of sv. The nuclei (n) have a cubic shape and decondensed chromatin. Note the presence of spermatozoa (s) and the muscular layer (arrowhead). **d, e** Longitudinal section of the ag. In **d**, it is possible to observe the point at which they come together (arrow). (*) packets of secretions in the lumen of the ag. **f, g** Details of cells secreting an ag. In **f**, it is possible to observe cells in the anterior region and g in the posterior region of ag. Note that the secretion (*) in g is more stained than in f. See the nuclei (n), and the muscular layer (arrowhead) that covers the ga. (a) anterior region; (p) posterior region. Giemsa stain. Scale: 50 µm

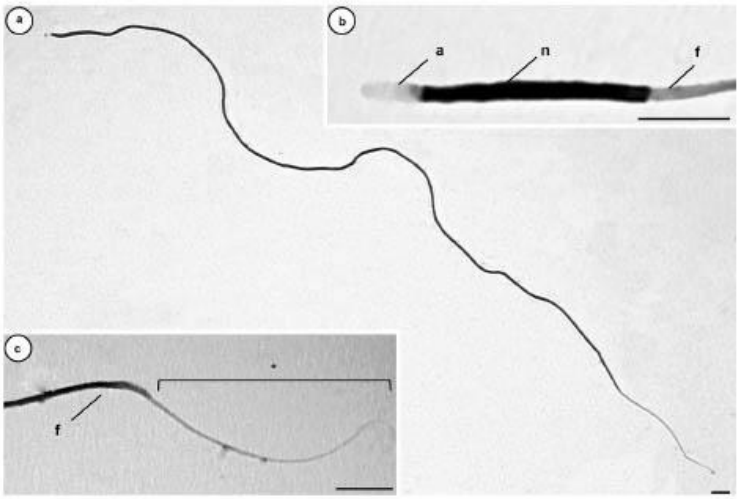


**Fig. 4** Spermatozoa of *L. bigoti* **a** general appearance. **b** Spermatozoa head. See the acrosome **a** and elongated nucleus (*n*). Furthermore, it is possible to see that the flagellum **f** has a similar diameter to the head region. **c** Note that the final portion of the flagellum (*) is thinner than the rest of it. Scale: 5 µm

et al. 2013; Drew 1969; Valdez 2001; Hassan 2017) and other insects (Chapman 1998; Glowacka et al. 1995; Will et al. 2005; Klowden 2013). Although the testes of some mosquitoes have already been studied (Hodapp and Jones 1961; Mahmood and Reisen 1982; Mahmood and Reisenand 1994; Huho et al. 2006), this is the first time their compactly spiral organization has been highlighted through histology. This spiral organization results in larger and elongated testes, occupying less space and reducing the contact surface, as observed in Gelastocoridae Kirkaldy, 1897 and Notonectidae Latreille, 1802 (Pereira et al. 2015). While the copulatory behavior of *L. bigoti* remains unknown, male mosquitoes from other species copulate many times throughout their adult lives (Alfonso-Parra 2022; Meuti and Shorth 2019). This must require a high quantity of spermatozoa available for transfer to females. We hypothesize that this organization of the testes in *L. bigoti* may confer advantages in meeting the substantial spermatozoa requirements associated with multiple copulations. This structural adaptation likely facilitates continuous spermatozoa production, ensuring an adequate supply for transfer to females.

In addition to spermatozoa production, testes can secrete nutrients and hormones and play a role in spermatozoa storage in some Diptera (Hiroyoshi and Reddy 2022). In mosquitoes of the genus *Anopheles* (*A. gambiae* Giles, 1902; *A. stephensis* Liston, 1901; and *A. culicifacies* Giles, 1901), despite the presence of seminal vesicles, whole-mount examinations of the reproductive tract revealed that the posterior portion of the testes functions as a storage site for spermatozoa. For these species, the posterior portion of the testes exhibited thread-like striations, believed to correspond to stored spermatozoa (Mahmood and Reisen 1982, 1994; Huho et al. 2006). Similarly, we observed a striated feature in the posterior portion of the *L. bigoti* testes. However, histological analyses showed the presence of an epithelium in this region, which is continuous with the deferent duct. Therefore, we assert that this region does not correspond to the testicular region but is, in fact, the first portion of the deferent ducts adjacent to the testes. Given its distinctive shape, we refer to as "the goblet of the deferent ducts". Due to the presence of many cytoplasmic bodies and free spermatozoa, we hypothesize that these bodies may contain secretions potentially involved in the degradation of the cystic envelope, facilitating the release of spermatozoa. This phenomenon bears similarities to observations in the fly *Anastrepha ludens* Loew, 1873 (Diptera: Tephritidae), as reported by Valdez (2001), a topic not yet explored in the context of mosquitoes.

We observed that the testes of *L. bigoti* are covered solely by the peritoneal sheath, distinguishing them from testicular structure in the fly *A. ludens* and the mosquito *Culex pipiens* Linnaeus, 1758, which feature a testicular wall formed by both the peritoneal sheath and a thin muscular layer (Valdez 2001; Hassan 2017). This disparity in structural composition underscores the morphological diversity among dipterans. Some studies have suggested that the presence of a muscular layer is related with a single follicle (Valdez 2001). However, our histological findings for *L. bigoti*'s unifollicular testes did not reveal the presence of such a muscular layer.

In most insects, spermatozoa, following their production in the testes, traverse the deferent ducts to reach the seminal vesicles, where they are stored until copulation occurs (Chapman 1998; Klowden 2013). Seminal vesicles are enlargements located in the posterior portions of

the deferent ducts, typically one in each duct. In *L. bigoti* and other mosquitoes such as *A. aegypti*; *C. pipiens*; *Culex quinquefasciatus* Say, 1823; *Culex tritaeniorhynchus* Giles, 1901; *Haemagogus equinus* Theobald, 1903; and *Psorophora howardii* Coquillett, 1901, the walls of the seminal vesicles are juxtaposed (Hodapp and Jones 1961). However, this characteristic is not present in the seminal vesicles of *Anopheles* mosquitoes (Hodapp and Jones 1961; Mahmood and Reisen 1982, 1994; Huho et al. 2006). These data may indicate that juxtaposed vesicles are a character within Culicidae family and possibly only present in the Culicinae subfamily. Nonetheless, a comprehensive analysis of other genera and species of mosquitoes is essential to confirm this hypothesis.

Accessory glands in insects play a role in producing and secreting substances that constitute seminal fluid (Chapman 1998; Klowden 2013). The fusion of the two accessory glands at their basal portion appears to be a shared characteristic among all Culicidae, as observed in different mosquito genera, including *Aedes* Meigen, 1818, *Anopheles*, *Culex*, *Haemagogus* Williston, 1896, and *Psorophora* Robineau-Desvoidy, 1827 (Hodapp and Jones 1961; Mahmood and Reisen 1982, 1994; Huho et al. 2006). Notably, the accessory glands of *L. bigoti* showed regions with distinct affinities to the dye, similar to observations in *A. aegypti* (Dapples et al. 1974), suggesting that each glandular region specializes in the production of a specific set of compounds, though characterization remains challenging (Meuti and Short 2019). It is known that many secretions from the male MRS are transferred to females during copulation, influencing their physiology and reproductive behavior (Leopold 1976; Gillott 2003). However, the role of accessory gland secretions in *L. bigoti* is yet to be studied.

In the specie under investigation, the epithelium of each seminal vesicle and each accessory gland is covered by a thin muscular layer, likely playing a role in expelling the secretions their produced. An examination conducted on *Bactrocera tryoni* Froggatt, 1897 (Diptera: Tephritidae) showed a high degree of innervation in the accessory glands, suggesting that males may exert some control over the production and transfer of their fluid (Radhakrishnan et al. 2009). However, this observation is yet to be confirmed in mosquitoes, warranting further studies to substantiate this hypothesis.

The morphological diversity exhibited by insect spermatozoa holds significant value for taxonomy, likely are result of the high selective pressure these cells undergo (Dallai 2014). Although some information on spermatozoa morphology and ultrastructure for certain mosquito species (Justine and Mattei 1988; Ndiaye et al. 1996; Klowden and Chambers 2004), these data remain largely unexplored for taxonomy purposes. Surprisingly, the SpermTree platform (https://spermtree.org/database/), which includes spermatozoa morphometric data from over 5,500 animal species, lacks information on Culicidae family species (Fitzpatrick et al. 2022). Yet, there is enormous potential to leverage the morphology of these cells for species discriminate, a strategy successfully employed in the study of other insects (Pereira et al. 2008; Barcellos et al. 2015; 2018).

Although there is no data for culicids on the SpermTree platform, there is information for other dipterans, showing a varied size between species of the same genus, as occurs in the genus *Drosophila* Fallén, 1823 (Fitzpatrick et al 2022). Therefore, we suggest the analysis of spermatozoa from other species of mosquitoes, as this information can be useful in describing species and even in the construction of taxonomic keys, since structures of the male genitalia are cited for the discrimination of many species (Tanaka 2003; Sallum 2020a; Sallum 2020b; Harbach 2023).

In summary, this study provided a basis for understanding the morphological pattern of the MRS in *L. bigoti*, suggesting that the basic pattern of organization of the system is common in Culicidae. In our results, we observed the fusion of the two accessory glands in their basal portion, as occurs in other Culicidae. We also highlight the presence of juxtaposed vesicles, which in mosquitoes must be present only in Culicinae. Furthermore, we describe the spiral pattern of the testicular follicle. The morphology and measurements of *L. bigoti* spermatozoa and the male reproductive system added information to the Culicidae, which could be useful for future taxonomic investigations. Finally, this work opens perspectives for future analyses of the reproductive biology of *Lutzia* and other mosquitoes, including the ultrastructure of the MRS and spermatozoa, in addition to taxonomic investigations within the family.

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**Data availability** No datasets were generated or analysed during the current study.

## Declarations

**Conflict of interest** The authors declare no conflict of interest.

**Ethical approval** Not applicable.

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## 5. CAPÍTULO 2: New findings on the male reproductive system and spermatozoa of *Aedes aegypti* (Diptera: Culicidae)

Neste capítulo foi investigado a organização morfológica do SRM ao longo do desenvolvimento de *Ae. aegypti*. Buscou-se compreender a relação entre o desenvolvimento dos testículos e a espermatogênese nesses mosquitos. Nesta análise o SRM foi revisto, sanando inconsistências existentes na literatura. Medimos os espermatozoides e destacamos sua ultraestrutura.

Artigo em preparação para submissão à revista “Parasites and Vectors”.

**Title**

New findings on the male reproductive system and spermatozoa of *Aedes aegypti* (Diptera: Culicidae)

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**Abstract**

**Background:** *Aedes aegypti* is one of the most important arbovirus vectors, characterized by its widespread distribution and exceptional reproductive capacity. This study reexamines this species's male reproductive system (MRS), focusing on its morphology throughout post-embryonic development and the structure of its spermatozoa.

**Methods:** We analyzed the MRS of *A. aegypti* in the larval L4, pupal and adult stages using bright-field light microscopy, fluorescent microscopy, and transmission electron microscopy techniques. Spermatozoa measurements were made using the ImageJ software.

**Results:** In L4 larvae, the MRS was composed by the two testes, a thin deferent duct and a pair of seminal vesicles. The MRS is fully developed in pupae and adults, with two testes, deferent ducts, seminal vesicles, accessory glands, and ejaculatory duct. Histological sections revealed that each testis is formed by a single follicle, which appeared to spiral at all stages. In pupae and adults, the testes showed germ cells at different stages of development, while the goblet portion of the deferent duct contained cytoplasmic bodies and spermatozoa. In adults, the seminal vesicles were filled with spermatozoa soon after emergence. Secretions from accessory glands are apocrine type. The spermatozoa were thin and long, measuring around 335 µm in length. Ultrastructural analysis revealed a very short acrosome covering the apical nucleus, in the flagellar region an axoneme with an unusual 9 + 9 + '1' microtubule pattern and two mitochondrial derivatives along the flagellum, narrowing at the terminal portion.
**Conclusions:** Our analysis revealed a clear link between testicular development and spermatogenesis. Additionally, we identified seminal vesicles present at all life stages and accessory glands visible only in pupae and adults. The characterization of sperm structure and ultrastructure indicated similarities with other mosquito species. Finally, our study provided valuable information that may support research in comparative biology and vector control strategies.

**Keywords:** Spiraled testicular follicle, Sexual development, Mosquito reproduction, Microscopy.

**Background**

The mosquito *Aedes aegypti* Linnaeus, 1762, is a significant vector of arboviruses responsible for diseases such as chikungunya, dengue fever, yellow fever, and Zika [1]. These diseases are of great concern because the mosquito is anthropophilic, well-adapted to urban environments, and influenced by urbanization and climate change [1-4]. Half of the world's population is at risk of dengue, with a substantial increase in cases in the last five years [5]. Although vaccines exist for some arboviruses, such as yellow fever and dengue, reducing the mosquito population remains the primary control strategy [1-2,6-7].

Despite not feeding on blood, male mosquitoes are essential for maintaining the mosquito population, making research into their biology crucial. The male reproductive system (MRS) produces, stores, and releases spermatozoa. In insects, this system consists of a pair of testes, deferent ducts, seminal vesicles, an ejaculatory duct, and accessory glands [8-9]. The testes produce spermatozoa, and these cells exhibit great diversity, aiding in identifying taxonomic groups [10].

Data on the MRS organization in *A. aegypti* are limited and outdated, with the descriptions dating back 60 years ago [11-13]. These works generally provide anatomical descriptions of the MRS and data on *A. aegypti* spermatozoa morphology and ultrastructure are scarce. While some researchers have measured their sperm size, these measurements are inconsistent [12,14]. Additionally, the ultrastructure of these cells is presented, but it needs to be emphasized [15].

Although information on the MRS of *A. aegypti* is limited, the MRS of the species *Lutzia bigoti* (Diptera: Culicidae) Bellardi, 1862 was recently analyzed by us, revealing that it is formed by a pair of testicles, deferent ducts, seminal vesicles, a pair of accessory glands and an ejaculatory duct. Histological analysis showed that each testis is composed of a single spiral follicle [16]. Illustrations of the testicles of *A. aegypti* showed that it has a spiral appearance [12]. So, we hypothesize that this organization is a distinctive and functional characteristic of the testis, since the relationship between testicular development and sexual maturation is already known [17-18]. Our assumption is that the spiraling of the testicular follicle is already present in late larvae (L4), since spermatogenesis begins at this stage in *Aedes* mosquitoes [19-20]. In this work, we also describe MRS throughout development, measure spermatozoa and analyze their ultrastructure.

## Methods

### 1. Collection, dissection of mosquitoes, and whole-mount of the MRS

Larvae of the last instars (L4), pupae, and adults of *A. aegypti* (strain PPCampos, Campos dos Goytacazes) were obtained from the colony maintained at the insectary of the Department of General Biology at the Federal University of Viçosa (DBG- UFV). Mosquitoes in the insectary are kept under controlled conditions: 26 ± 3°C, 60 ± 5% relative humidity, and a 12-hour light-dark cycle. Larvae were fed turtle food (Reptolife®), and adults are provided with a 10% sucrose solution ad libitum.

The *A. aegypti* larvae do not have external characteristics for determining sex, but males develop more quickly [21]. Considering this data, we collected the first larvae to reach the L4 stage of an oviposition and dissected them. We cut the abdomen of these larvae with micro-scissors and analyzed the anatomy of the gonad. To describe the testicular anatomy, numerous larvae (L4) were dissected, and the testes were examined in whole-mount under a light microscope. For subsequent steps, ten male mosquitoes were fixed in 2.5% glutaraldehyde solution (Electron Microscopy Sciences®) in 0.1M sodium cacodylate buffer.

An initial screening was conducted to collect male pupae, as smaller pupae in *A. aegypti* are usually males [22-23]. Ten of these pupae (at least 24 hours old) were dissected, and the reproductive system was examined under a stereomicroscope to confirm it as the MRS. Some samples were prepared for whole-mount photography, and then all were fixed in 2.5% glutaraldehyde solution in 0.1M sodium cacodylate buffer.

For the collection of adult males, pupae identified as males were transferred to individual cages. Upon emergence, the sex was confirmed from the antennae, which are plumose in males [12]. These were kept in female-free cages, ensuring that only virgin individuals were used. Five newly emerged males and 30 sexually mature males (from two or five days of age, with five males at 21 days) were selected at this stage. Some of these MRS were mounted for photography.

Immatures were anesthetized on ice, and adults were anesthetized with $CO_2$ before their reproductive systems were dissected in 0.1M sodium phosphate buffer (PBS), pH 7.2 solution (0.1 M NaCl, 20 mM $KH_2PO_4$ and 20 mM $Na_2HP_4$). For whole-mounts, the MRS was placed on a histological slide with a drop of PBS and covered with coverslips. Photographs were taken using an Olympus CX31® optical microscope

and Zeis Primo Star® stereomicroscope equipped with a Rebel Canon T7+Electro-Optical System (EOS)® camera.

**2. Measures of the spermatozoa**

To obtain the spermatozoa, the content of the seminal vesicles from 17 adult *A. aegypti* was individually spread on histological slides under drops of PBS. Ten slides were stained with Giemsa (Merck®) (1:15 in distilled water) for 20 minutes, rinsed with running water, and air dried at room temperature. 370 spermatozoa were analyzed, with measurements of total length (head + tail) and the thin distal portion of the tail, using an Olympus BX60® photomicroscope equipped with an Olympus Qcolor® digital camera. To highlight the nuclei, four slides were incubated in 0.2g/LM DAPI solution (4.6-diamino-2-phenylindole) (Sigma-Aldrich®), washed with running water, and 60 nuclei were photographed using the same microscope, equipped with a BP360-370 nm excitation filter. Some spermatozoa had their nuclei photographed using both fluorescent microscopy and a bright field for image overlay. Additionally, three slides were stained with 1% eosin (Dinâmica®) for one minute, washed, stained with crystal violet (Dinâmica®), and washed again. The nucleus-flagellum transition region of 45 spermatozoa was photographed using the same setup. All measurements were performed using the free software ImageJ® (https://imagej.nih.gov/ij/) [24].

The seminal vesicles of five newly emerged adult mosquitoes were dissected, and individual slides of their contents were prepared as previously described. At this stage, we examined whether the newly emerged mosquito already possessed spermatozoa in its seminal vesicles. Analysis and photography were performed using an Olympus CX31® microscope with a Canon EOS Rebel T7+® digital camera.

**3. Light microscopy**

The larvae, pupae, and adult *A. aegypti* samples underwent a procedure where they were first rinsed in distilled water and then treated with 1% osmium tetroxide solution (Sigma Aldrich®) for an hour. Following this, they were dehydrated using a series of increasing concentrations of alcohol (30%, 50%, 70%, 90%, and three times at 100%) for 10 minutes each. Subsequently, the samples were pre-treated with three different solutions of Historesin (Leica®) and alcohol (at 1:2, 1:1, and 2:1) for an hour in each mixture. Complete infiltration was achieved by incubating the samples in pure

Historesin overnight. Afterward, the samples were transferred to silicone molds containing Historesin with a catalyst added at a ratio of 15:1 and polymerized in an oven at 60°C.

Sections with a thickness of 1.0 µm were obtained using a Leica RM 2255® microtome. These sections were stained with 1% toluidine blue (Synth®) in 0.5% sodium borate. Analysis and photography were performed using an Olympus CX3® microscope with a Canon EOS Rebel T7+® digital camera. Multiple frames were captured using either a 40× or 100× objective, and these images were later combined into panoramic views using the Photomerge tool in Adobe Photoshop 2022 Software®.

**4. Transmission Electron Microscopy**

Testes and seminal vesicles from adult mosquitoes previously fixed in a glutaraldehyde solution were used in this stage. The material was washed in distilled water and then, shielded from light and at low temperature, post-fixed for 2 hours in 1% osmium tetroxide. Subsequently, the material was washed again in distilled water and dehydrated in ethanol of increasing concentrations (30%, 50%, 70%, 90%, and three times in 100%). Dehydration continued using 100% ethanol and pure acetone in increasing concentrations (2:1, 1:1, and 1:2) and pure acetone. The material was infiltrated with Epon resin (Electron Microscopy Sciences®) overnight. The samples were then transferred to silicone molds and placed in an oven at 60ºC until complete polymerization.

Ultrathin sections were obtained using the RMC Boeckeler® ultramicrotome with a diamond knife (Diatome®). The material was then collected on grids (Electron Microscopy Sciences®) and contrasted. At this stage, the grids were placed for 40 minutes in a 2% aqueous uranyl solution, shielded from light. The grids were washed in distilled water and placed in lead citrate (Electron Microscopy Sciences®) for 20 minutes. The material was washed again in distilled water. The grids were analyzed, and the sections were photographed using the Zeiss EM 109® transmission electron microscope.

**Results**

**1. Reproductive system in whole-mount.**

During the dissection of the L4 stage larvae, we observed the pair of testes, and each testes already displayed a slight degree of spiralization. At this stage, there is an accumulation of fat bodies surrounding them. In addition, a thin deferent duct emerges from each testis. However, no other structures were observed in whole-mount MRS (Fig. 1A).

In pupae and adults the MRS was fully formed. It's consisted of a pair of testes, with more evident spiralization than in L4. At this stages, a thin deferent duct that emerge from each testis has a dilation in its lower portion, forming the seminal vesicles. As observed in L4 larvae, many fat bodies were still associated with the testicular walls and the deferent ducts. Adjacent to the seminal vesicles, a pair of accessory glands can be distinguished. Glands and seminal vesicles merge at their bases, converging into the ejaculatory duct (Figs. 1B and 2A-B). In adults, it was evident that the initial portion of the deferente duct had a goblet-like shape, known as the goblet of a deferent duct. (Fig. 2C).

## 2. Histology of MRS

### 2.1. Testes

Histological sections showed that the testes from L4 larvae, pupae, and adults possess a single spiraled follicle (Figs. 3 and 4A). The histological sections confirmed the slight spiralization in the L4 testes as observed in the whole-mount. Globular germ cells, probably in the early stages of spermatogenesis, were identified in larval testes (Fig. 3A).

The sections showed that the pupal testes have a more pronounced spiraling than larvae. We observed germ cells at all stages of development. Cell development occurs in an anterior-posterior direction, with globular cells occupying the upper region of the testicles and elongated cells in the lower region (Fig. 3B). At the base of each testis, the goblet of the deferent duct can be identified, where cytoplasmic bodies and spermatozoa were observed in the lumen. In this region, a simple epithelium is present (Fig. 3C).

Adult testes exhibit an organization like the pupae, including in 21-day-old mosquitoes (Figs. 4A-E). At all stages of life, each testicular cyst is surrounded by cystic cells (Figs. 3, 4A and B). Through transmission electron microscopy, we

observed the goblet region of the deferent duct is lined by a simple epithelium (Figs. 4F). The lumen of this portion contains spermatozoa and cytoplasmic bodies that correspond to cellular debris with varying shapes (Figs. 4F and G).

**2.2. Seminal Vesicles and Accessory Glands**

Seminal vesicles were observed side by side at all life stages (Figs. 5A-C and 6A). Histological sections revealed that in L4 larvae, the seminal vesicles had a very thin epithelium (Fig. 5A) and contained some secretion in the lumen but lacked spermatozoa. In pupae, the seminal vesicles exhibited a simple epithelium and an empty lumen, covered by a thin muscle layer extending between the two vesicles. At the basal region, this muscle layer encircled both vesicles, juxtaposing their epithelia with no muscle layer separating them in this portion (Figs. 5B-C). In adult mosquitoes, the seminal vesicles were filled with spermatozoa (Fig. 6A), present since their emergence (Fig. 7G). These vesicles had a thick epithelium composed of cuboidal cells and were enveloped by a muscle layer (Fig. 6B).

The accessory glands of pupae and adult mosquitoes featured a high epithelium producing apocrine secretions. They are lined by a muscular layer (Figs. 5D-F and 6C). The dye differentially stained the secretions of the anterior and posterior regions, with the anterior region appearing lighter than the posterior (Figs. 6D-F). The accessory glands merged at their basal portion, forming a continuous connection with the ejaculatory duct (Fig. 6G), which was internally lined by a cuticle (Fig. 6H). In L4 larvae, the accessory glands could not be identified or distinguished.

**3. Spermatozoa**

Spermatozoa are characterized by a filiform shape, with an average length of 335.14 ± 17.58 µm (CV= 5.24%) (Fig. 7A). Observations using overlays of DAPI- and Giemsa-stained photographs of spermatozoa showed that the head of these cells is nearly entirely occupied by the nucleus, which has an average size of 38.5 ± 1.6 µm (Figs. 7 B-D). The terminal portion of the flagellum is narrower than the rest, with an average length of 30.4 ± 1.8 µm (Fig. 7E). The double staining with crystal violet and Giemsa revealed the centriole adjunct, whose average length was 19.6 ± 1.4 µm. (Fig. 7F).

The ultrastructure of the spermatozoa features a short acrosome covering the apical portion of the nucleus, which measures 0.04 µm in diameter at this region (Fig.

8A). The nucleus has condensed chromatin, and is circular in cross-section. The axoneme, arranged in a 9 + 9 + '1' pattern (nine accessory microtubules, nine peripheral microtubule doublets, and a central element), inserts at the nuclear base. Below this insertion, the centriole adjunct consists of granular material surrounding two mitochondrial derivatives and the axoneme (Figs. 8B-C). Further along, only the mitochondrial derivatives and axoneme remain, and the flagellar terminal portion has only the axoneme (Fig. 8D). Illustration 1 shows relevant aspects of *A. aegypti* spermatozoa.

**Discussion**

Our analysis revealed a clear link between testicular development and spermatogenesis, as the spiralization of the testes became more pronounced in pupae and adults compared to L4 larvae. This relationship is supported by the fact that spermatogenesis begins in L4 larvae and continues throughout the mosquito's lifespan [19-20; 25]. Therefore, we demonstrate a strong correlation between the organ's structural development and its primary function - sperm cells production.

Compact spiraled testes were also found in *L. bigoti*, another culicid specie [16]. It is possible to assume that this spiral organization results in larger and elongated testes, occupying less space. This seems directly linked to the mosquito's reproductive efficiency, maximizing internal space for many sperm production. This idea is supported by the observation of 5,000 sperm in the seminal vesicles of sexually mature mosquitoes at least three days old [26]. Additionally, we observed that older mosquitoes continue to produce spermatozoa, allowing *A. aegypti* to be classified as sinespermatogenic [27].

Each testis is continuous with the deferent duct, which, in pupae and adults, has a funnel-shaped initial portion, so we refer to it as the goblet of the deferent duct [16]. Previous studies have suggested that in the testes of *A. aegypti* pupae, a mass prevents spermatozoa from leaving these organs [12]. We propose that the cytoplasmic bodies described in this study may correspond to the mass observed in the previous study but do not block sperm passage, as they are already observed in the seminal vesicles of newly emerged mosquitoes. Furthermore, these cytoplasmic bodies appear to be cellular debris, possibly originating from the process of spermiogenesis [28].

In many insects, spermatozoa are stored in seminal vesicles [8-9]. In *A. aegypti*, these structures are observable in histological sections as early as L4, becoming more prominent in later life stages. Although seminal vesicles have been reported in various mosquito species, including the genus *Aedes* [11, 13, 16], they have been misidentified as the ejaculatory duct in *A. aegypti* [12]. Seminal vesicles are an enlargement of the deferent ducts and store spermatozoa. In contrast, the ejaculatory duct is a shared channel that forms the aedeagus, the male intromittent organ that releases sperm cells and secretions from the accessory glands [8, 9]. Our study observed both structures, indicating that the previous classification needed to be rectified.

Accessory glands were only observed in pupae and adults. The non-observation of accessory glands in larvae could be, in fact, because of their absence or due to their small size at this stage. Our findings align with a previous study that did not describe these glands in larvae [12]. In adults, the accessory glands are relatively larger than in pupae, likely due to the accumulation of secretory material beginning in the pupal stage and continuing through post-emergence maturation. This idea is supported by previous findings showing that the glands become empty and shrink after successive copulations, regaining their size following a period without mating [29-30]. In pupae and adults, it was observed that the accessory glands fuse at their basal portion, a characteristic shared with other Culicinae adults [13, 16], suggesting a common trait among different genera. The ejaculatory duct receives secretions from the accessory glands and sperm from the seminal vesicles [8-9].

The ejaculatory duct is ectodermal in origin, as evidenced by the presence of a cuticle. Our histological sections demonstrated the continuity of the accessory glands to the ejaculatory duct, indicating their ectodermal origin. Therefore, we can classify them as ectadenia [8].

In *A. aegypti*, there were conflicting reports on spermatozoa length. For instance, one study reported that spermatozoa heads measure 40-45 µm and the flagellum 200 µm [12], while another only mentioned that total length ranges from 250 to 300 µm [14]. The earliest study, conducted in the 1960s, lacks specific methodological details [12], while the second used a flat tool on a computer monitor to take measurements [14]. In our work, we used ImageJ software, which is widely used in research involving measurements and microscopy and allows for more accurate measurements [24].

Compared to the *L. bigoti* data [16], our spermatozoa measurements suggest significant differences in sperm cell length across mosquitões, showing remarkable variation within Culicidae. Another relevant finding is that the acrosome in *L. bigoti* is easily distinguishable in the sperm head [16], whereas in *A. aegypti*, the acrosome was visible only under transmission electron microscopy. We propose that a small acrosome may be a characteristic of the genus *Aedes*, as *Aedes mariae* Sergent & Sergent, 1903 (Diptera: Culicidae) acrosome measures only 0.12 µm in length [31].

The method combining eosin and crystal violet, previously demonstrated in *Apis mellifera* Linnaeus, 1758 (Hymenoptera: Apidae), can identify the acrosome, nucleus, and flagellum in spermatozoa [32-33]. Interestingly, the same method revealed the centriole adjunct in *A. aegypti* sperm, allowing us to measure it. This structure, rich in RNA and ribonucleoproteins, is deposited beneath the nucleus during spermiogenesis and appears as an electron-dense material under transmission electron microscopy [34]. We believe this staining was possible because crystal violet, as a basic dye, has a strong affinity for acidic components in this region.

We observed a low coefficient of variation in sperm size in *A. aegypti*. In species subject to sperm competition, it is common for more uniform sperm to be produced, with a tendency toward an optimal phenotype [35-41]. Polyandry, characterized by the successive mating of a female with more than one male [42], has been observed in *Aedes* females [25, 43-44]. Thus, the low variability in gamete length may be explained by female polyandry.

In Diptera, there is a wide variety of spermatozoa, and many differences lie in the axoneme pattern [10]. The ultrastructure of *A. aegypti* spermatozoa revealed that the axoneme is inserted at the base of the nucleus and exhibits a 9 + 9+ ‘1’ arrangement, meaning there are nine accessory microtubules, nine pairs of microtubules, and a central element that does not correspond to a true microtubule. This element is not a true microtubule, as it appears more solid than tubular [45-46]. Although this axoneme pattern is unusual, the same arrangement has been found in other mosquito species, such as *Culex* sp., *Aedes canadensis* Theobald, 1901, and *Toxorhynchites brevipalpis* Theobald, 1901 [45-47]. Other aspects, such as the presence of the centriole adjunct and mitochondrial derivatives, were similar [45-47]. Furthermore, at the posterior end of the sperm cells, we did not observe the mitochondrial derivatives and noticed a successive loss of the axoneme elements. For

this reason, under light microscopy, the posterior portion of the flagella appeared narrower.

**Conclusions**

Our findings contribute to a better understanding of mosquito anatomy, particularly the morphology and post-embryonic development of the MRS in Aedes aegypti. By highlighting the importance of the spiral structure of the testes, the presence of seminal vesicles at all life stages, and the observation of accessory glands only in pupae and adults, we provide valuable insights into the mosquito's reproductive biology. Furthermore, our focus on the structure and ultrastructure of sperm cells revealed sperm characteristics common to other mosquito species. These discoveries open new perspectives for researchers working in comparative biology or exploring male reproductive potential as a tool for vector control.

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**Availability of data and materials**

All data and materials are available upon request from the corresponding author.

**Authors' contributions**

HBS: planning, experimental execution, data analysis, and scientific article writing. RCB, PHR, DCA: experimental execution and article revision. JLN: planning, supervision, and scientific article writing.

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

All authors read and approved the final manuscript.

**Competing interests**

The authors declare no competing interests.

## Figures

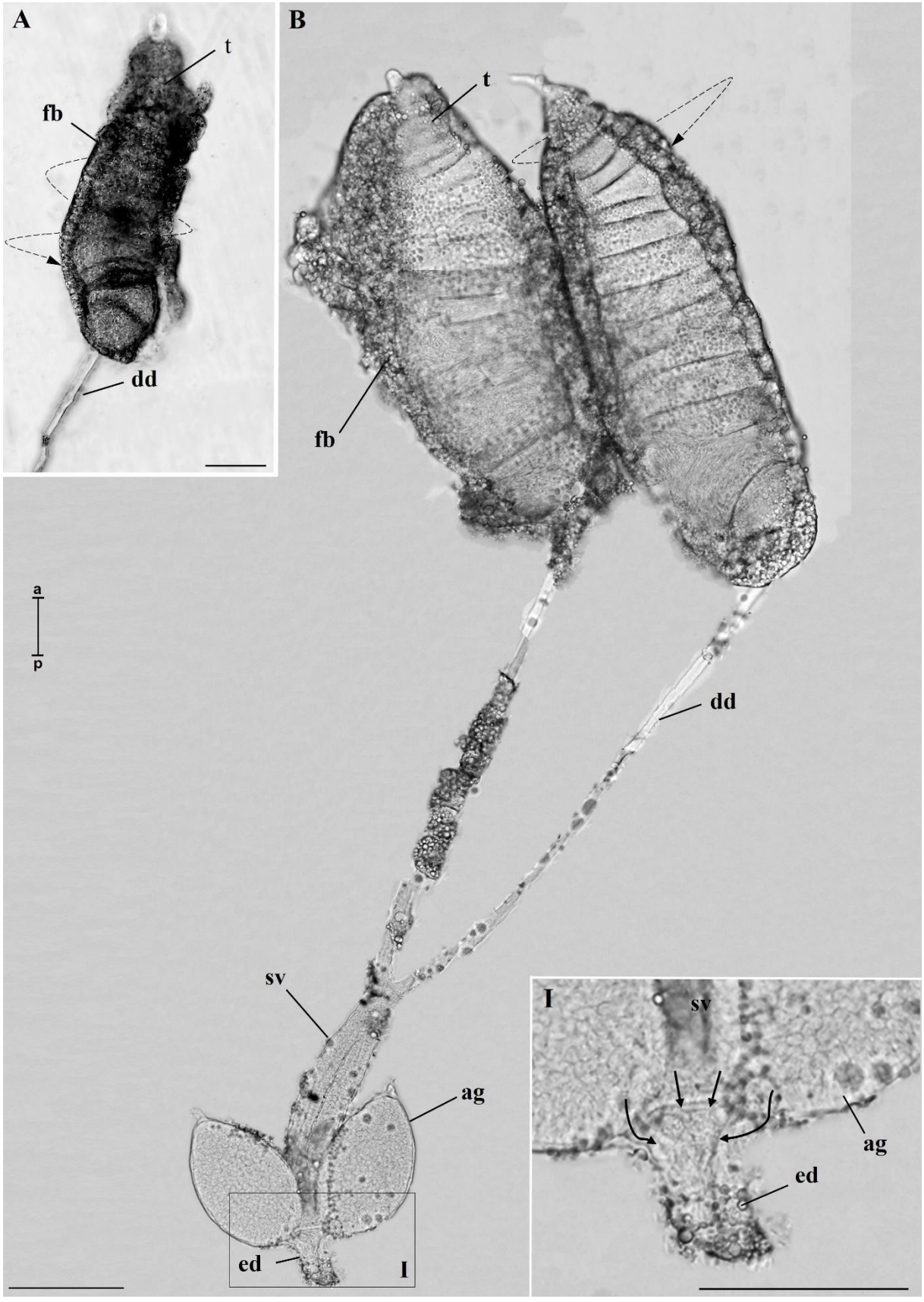

**Figure 1.** Whole-mount of MRS of immature *A. aegypti*. A: Testis (t) of L4 larva with a spiral shape (dotted arrow) continuous with the deferent duct (dd). B: MRS of pupa. The spiral shape (dotted arrow) of the testis (t) is more evident than in the L4. Each deferent duct (dd) has a dilated portion forming the seminal vesicles (sv). Note the presence of a pair of accessory glands (ag) and the ejaculatory duct (ed). Inset I shows that the ag and sv converge into the ed (arrows). In both phases, fatty bodies (fb) are noted. Scale bars: 100 µm. a: anterior region; p: posterior region.

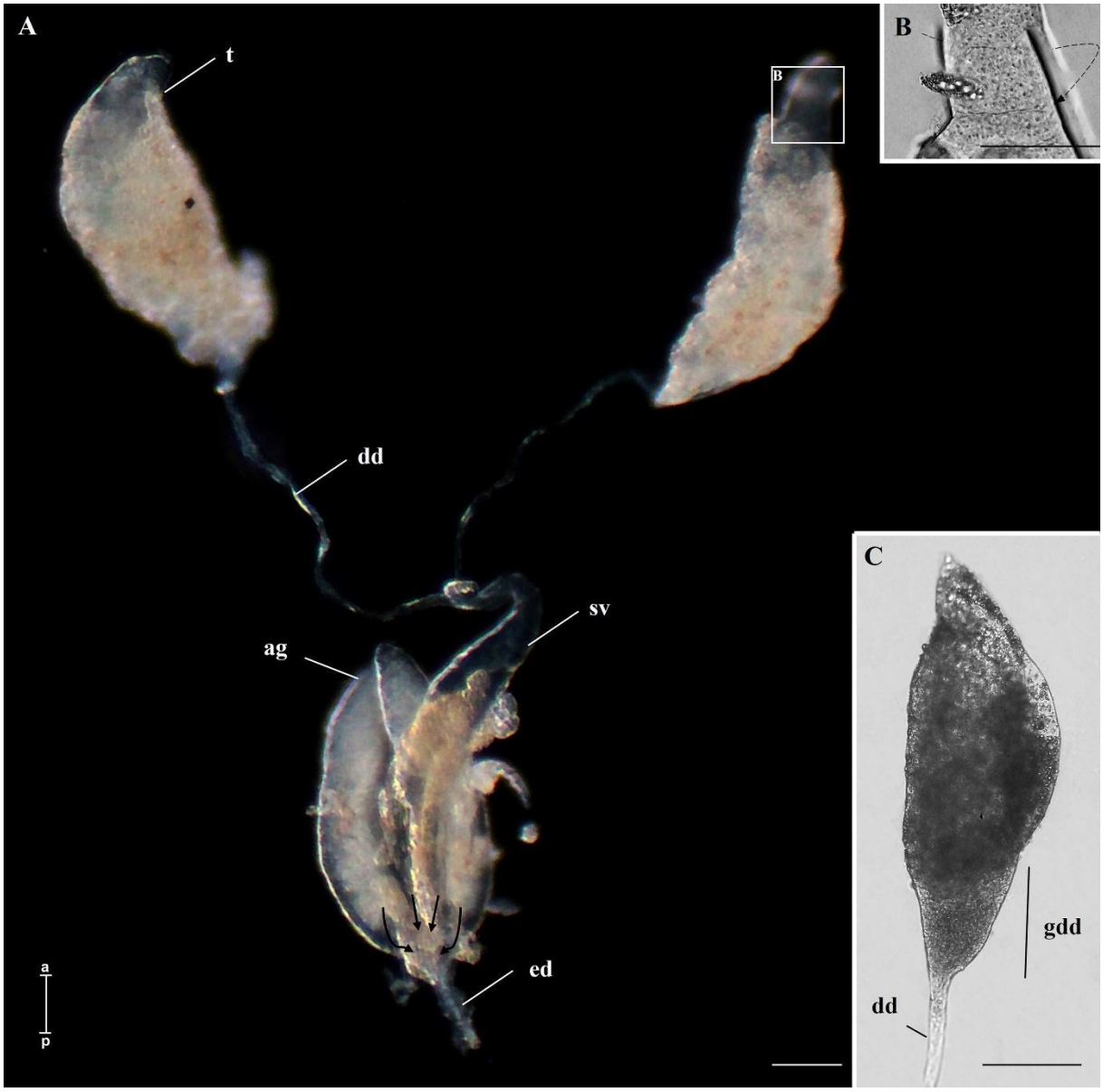


**Figure 2.** Whole-mount of MRS of *A. aegypti* adults. A: Testis (t), deferent duct (dd), seminal vesicles (sv), accessory glands (ag) and ejaculatory duct (ed). The arrows indicate that the accessory glands and the seminal vesicles empty into the ejaculatory duct. (B) The spiral shape of the testis (dotted arrow).C: Detail of the testis showing

the goblet of the deferent duct (gdd). Scall bars: 100 µm . a: anterior region; p: posterior region.

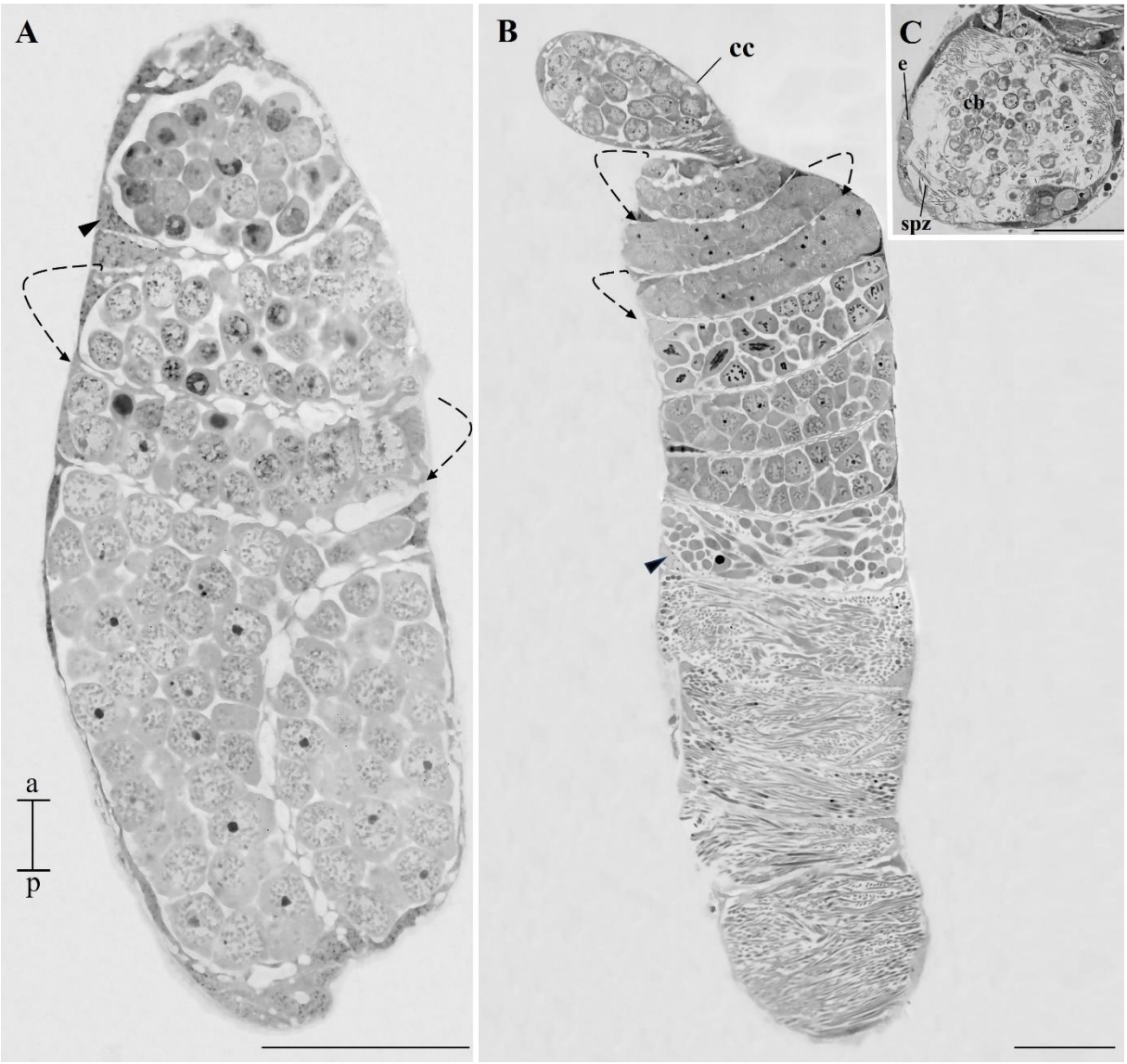


**Figure 3.** Histology of the testis of immature *A. aegypti*. A: Testis of larva with evident spiraling of the testicular follicle (dotted arrow). Note the cytoplasm of cystic cells (cc) and their nucleus (arrowhead). The cysts present young cells of the spermatogenic lineage. Note the presence of globular cells throughout the organ. B: Testis of pupa. The spiraling of the testicular follicle (dotted arrow) is more pronounced. The cytoplasm of cystic cells (cc) and their nucleus (arrowhead) are also visible. Globular cells (newer in the spermatogenic lineage) are seen in the upper region of the testis while more elongated cells (older in the spermatogenic lineage) are seen in the lower regions of the testis. C: Goblet of deferent duct in the testis of pupa. See the presence of an

epithelium (e) in this region. Spermatozoa (spz) and cytoplasmic bodies can be seen in the lumen of the goblet. Staining: toluidine blue. Scale bar: 50 µm. a: anterior region; p: posterior region.

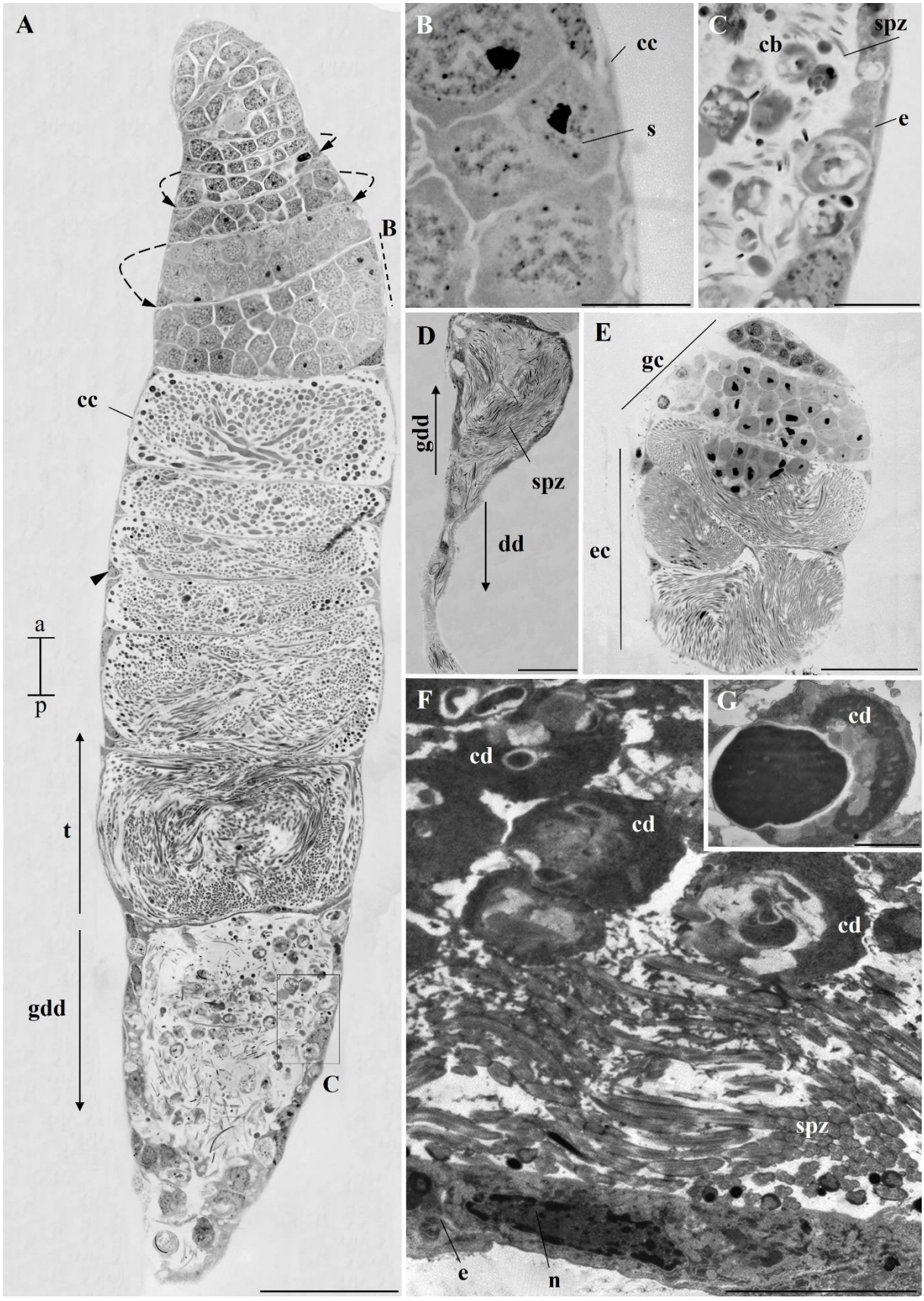


**Figure 4.** Histology and ultrastructure of the testis of adult *A. aegypti*. A: Longitudinal histological section of the testis (t) and goblet of the deferent duct (gdd), revealing the

spiral aspect of the testis (dotted arrow) and the cytoplasm of cystic cells (cc) covering it, detailed in B. Cyst cell nuclei (arrowhead) are present throughout the testis. The gdd is located posterior to the t and is further detailed in C. B: Cytoplasm of cystic cells (cc), developing sperm cell (s). C: Detail of the gdd showing epithelium (e), numerous cytoplasmic bodies (cb), and spermatozoa (spz). D: Histological section of the goblet of the deferent duct (gdd) and the deferent duct (dd). Note the presence of numerous spermatozoa (spz) in the lumen. E: Cross section of the testis of *A. aegypti* at 21 days of age. The dotted arrows indicate the spiral shape of the organ. Note the presence of globular cells (gc) (newer in the spermatogenic lineage) and elongated cells (ec) (older in the spermatogenic lineage), indicating that spermatogenesis is still occurring at this age. F: Ultrastructure of the goblet of the deferent duct, identified by the presence of the epithelium (e) and a lumen filled with spermatozoa (spz). n: nucleus. Note the presence of cellular debris (cd) of various shapes in F and G. Staining A-e: toluidine blue. Scale bar: A, D-E: 50 µm; B-C: 10 µm; F: 5 µm; G: 1 µm.

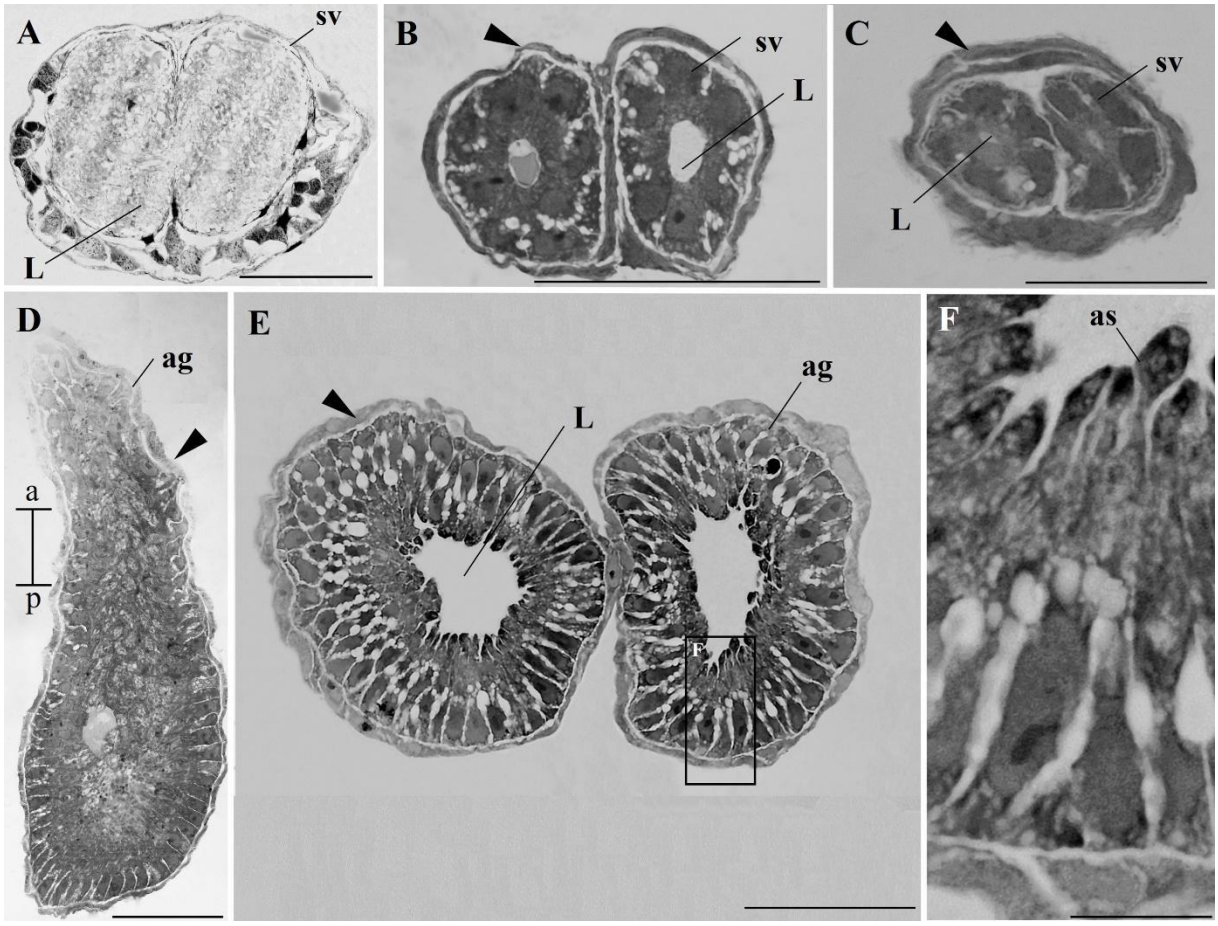

**Figure 5.** Histology of seminal vesicles and accessory glands of immature *A. aegypti*. A: Seminal vesicle (sv) of a larva, showing a wide lumen (L). B: Upper portion of the pupal seminal vesicles (sv), covered by a musculature (arrowhead) surrounding each vesicle completely. C: Lower portion of the same sv as in B, with a narrower lumen (L) and no individualized muscular covering for each gland. D: Longitudinal section of a pupal accessory gland (ag). E: Cross-section of two pupal accessory glands, showing the lumen (L) and the surrounding musculature (arrowhead). F: Detail of secretory cells in the ag with apocrine secretions (as). Staining: toluidine blue. Scale bars: A-B and D-E: 50 µm; C: 25µm; F: 10 µm. a: anterior region; p: posterior region.

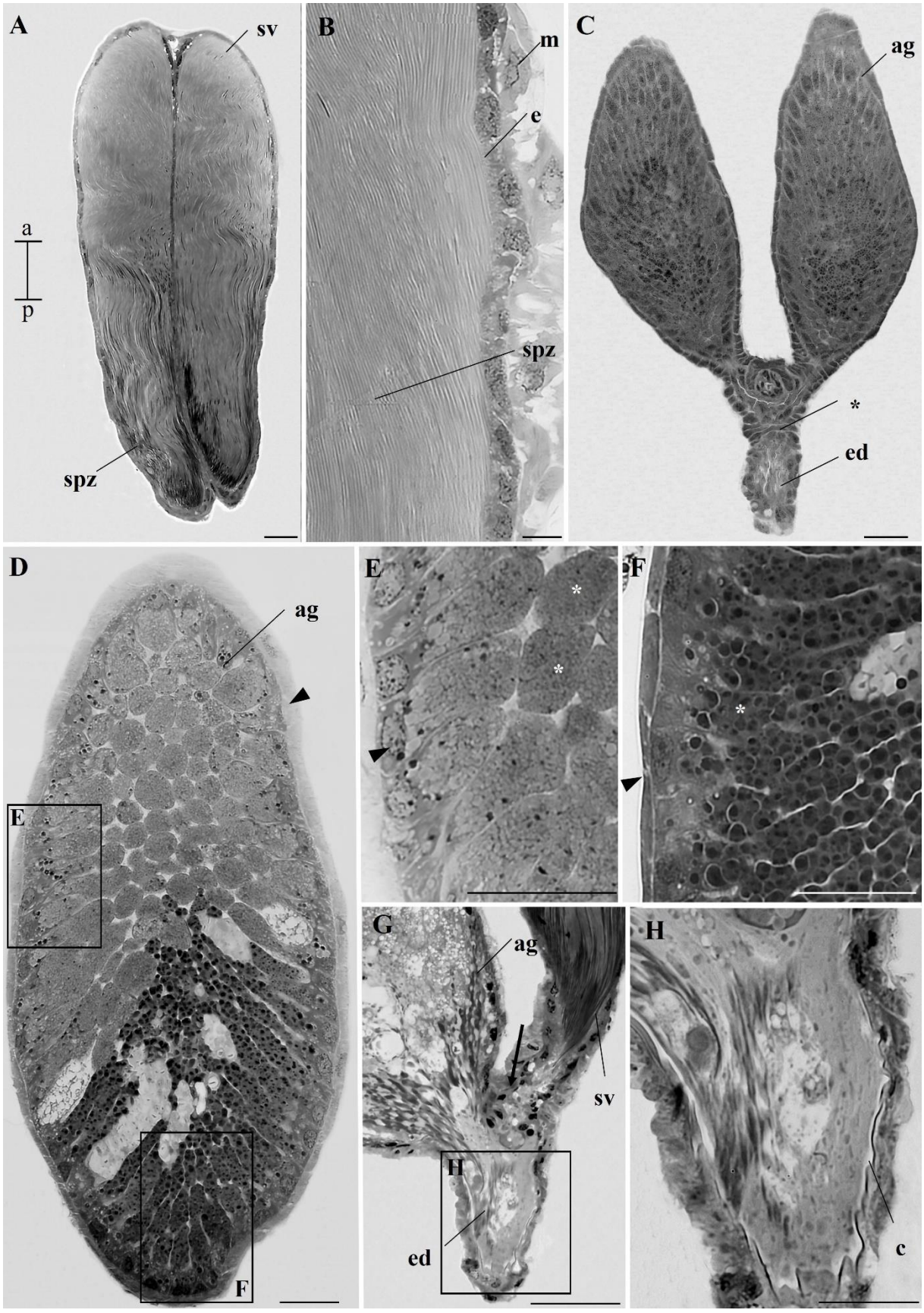


**Figure 6.** Histological sections of the seminal vesicle and accessory gland of adult *A. aegypti*. A: Longitudinal section of two seminal vesicles (sv) filled with spermatozoa

(spz). B: Detail of a seminal vesicle, showing a simple epithelium (e) and a muscular layer (m) surrounding the vesicle. Spermatozoa (spz) are visible in the lumen. C: Junction point of two accessory glands (ag) and the ejaculatory duct (ed). D: Longitudinal section of an accessory gland (ag) filled with apocrine secretions produced by the glandular epithelium. The ag has two distinct regions; the anterior region is stained more lightly than the posterior region. Musculature (arrowhead). E-F: Longitudinal sections showing details of the secretions (*) in the anterior (E) and posterior (F) regions of the gland. G: Junction point (arrow) of an ag with a seminal vesicle (sv), also highlighting the ejaculatory duct (ed). H: Detail of the cuticle (c) in the ejaculatory duct. Staining: toluidine blue. Scale bars: A-G: 25 µm; H: 10 µm. a: anterior region; p: posterior region.

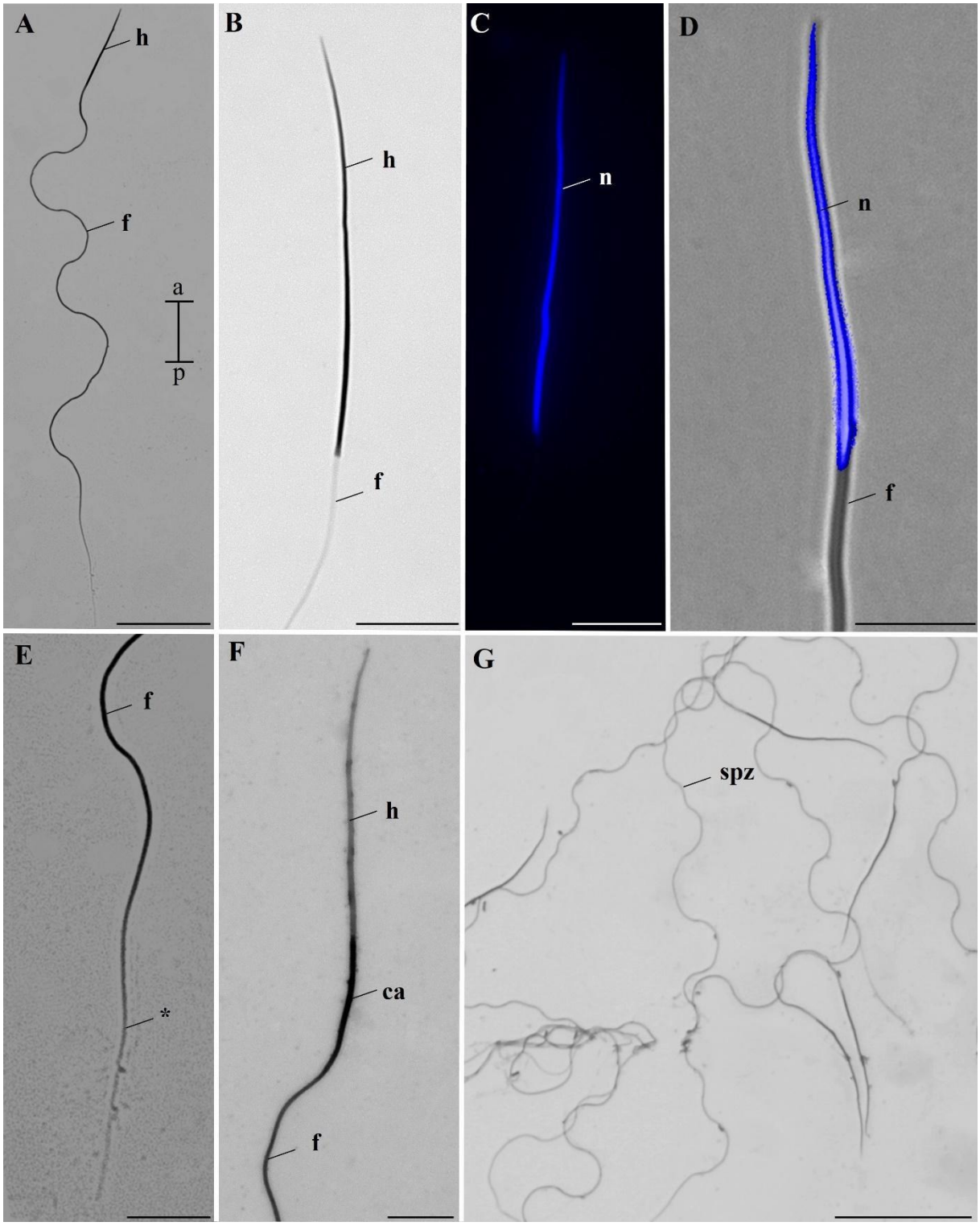


**Figure 7.** *A. aegypti* spermatozoa. A: Overview of the spermatozoon showing the head (h) and flagellum (f). B: Detail of the head (h) of a spermatozoon. C: Nucleus (n) of a spermatozoon stained with DAPI. D: DAPI and light microscopy overlay, demonstrating that the nucleus (n) occupies nearly the entire head region; f: flagellum. E: Terminal portion (*) of the flagellum (f). F: Spermatozoon with the centriolar adjunct (ca) highlighted. G: Spermatozoa from a newly emerged adult. Staining: A, B, E, G:

Giemsa. F: crystal violet and eosin. Scale bar: A and G: 50 µm; B-F: 10 µm. a: anterior region; p: posterior region.

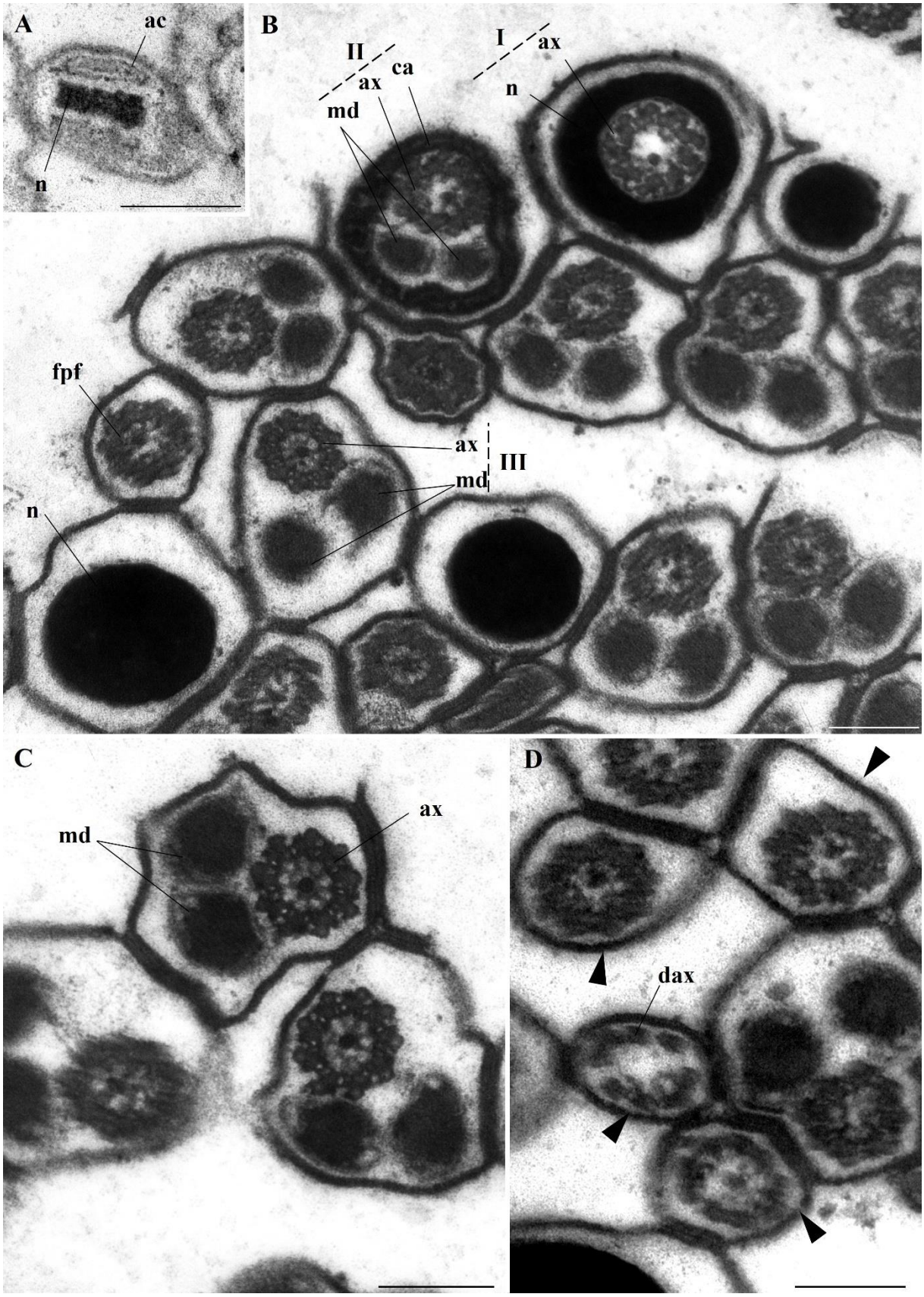

**Figure 8.** Electron micrograph of *A. aegypti* spermatozoa. A: Anterior region of the head, showing the nucleus (n) surrounded by the acrosome (ac). B: Several spermatozoa where the nucleus (n) is visible; in I: the insertion of the axoneme (ax) into the nucleus (n); II: the centriole adjunct (ca) region, containing two mitochondrial derivatives (md) and the axoneme (ax); III: the median portion of the flagellum, also with two mitochondrial derivatives (md) and the axoneme (ax); and the final portion of the flagellum (fpf). C: Median portion of the flagellum, highlighting the 9+9+'1' arrangement of the axoneme (ax) and the two mitochondrial derivatives (m). D: Final portions of the sperm flagella (arrowhead), in one of them the disintegrating axoneme (dax) can be observed. Scale bar: 200 nm.

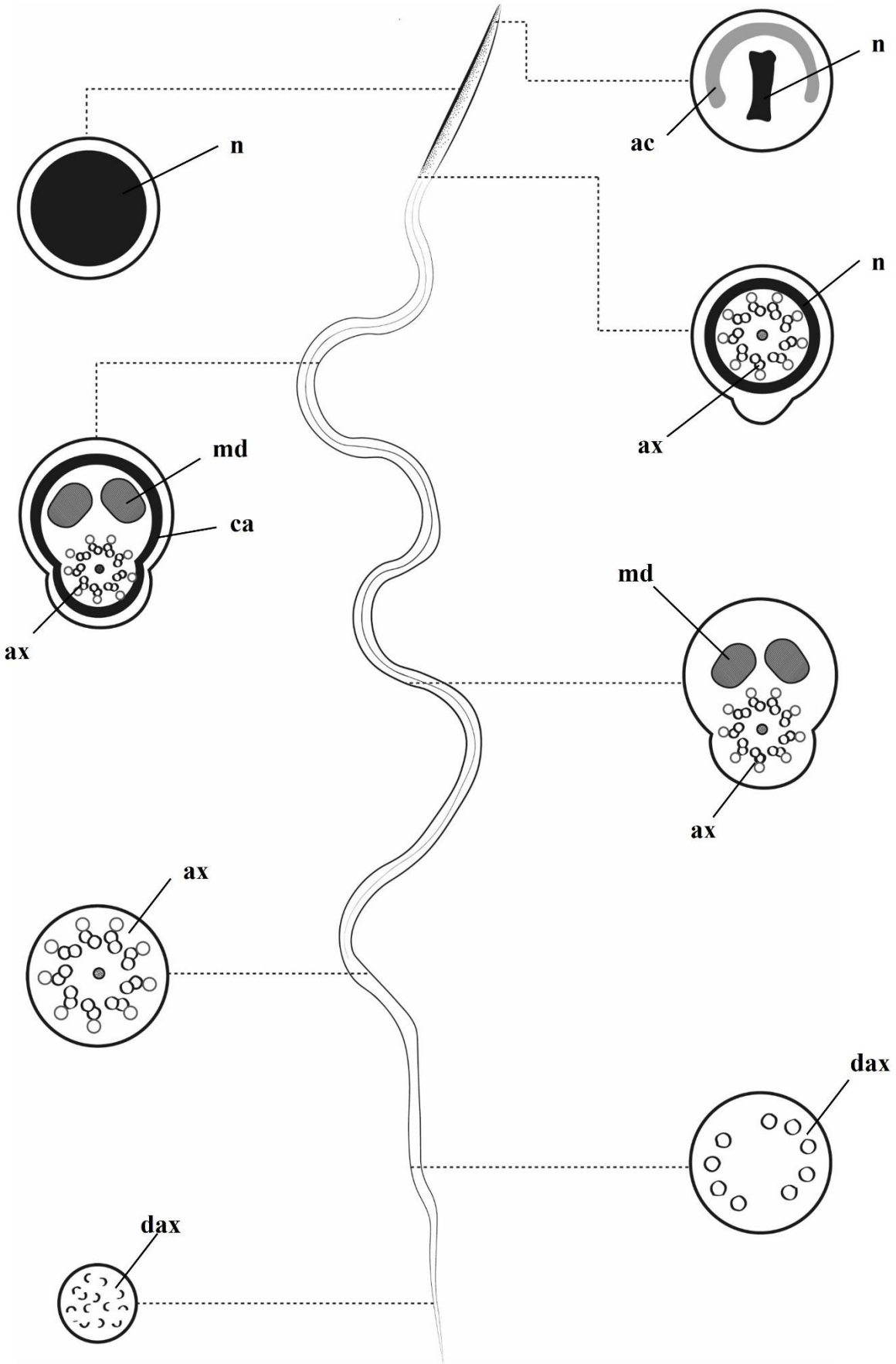
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**Illustration 1.** Diagram of the spermatozoa of *A. aegypti*, indicating its structures and the locations where they are found: nucleus (n), acrosome (ac), flagellum (f), axoneme (ax), centriolar adjunct (ca), mitochondrial derivatives (m), and disintegrated axoneme (dax).

## 6. CAPÍTULO 3: Morphology of the male reproductive system and spermatozoa variation in *Anopheles darlingi* (Diptera: Culicidae).

Nesse trabalho, analisamos se, de fato, há polimorfismo espermático em *An. darlingi*. Além disso, utilizamos essa espécie para comparar o SRM de Anophelinae com o de mosquitos Culicinae.

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# Morphology of the male reproductive system and spermatozoa variation in *Anopheles darlingi* (Diptera: Culicidae).

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**Abstract**

In this study, we present an analysis of the male reproductive system and spermatozoa of *Anopheles darlingi* Root, 1926, the primary malaria vector in Brazil. The reproductive system consists of a pair of unifollicular testes, deferent ducts, a muscular ejaculatory duct, and a pair of accessory glands. The average spermatozoa length was 188 µm, with a continuous variation from 92 to 246 µm. This significant variation may be associated with the mosquito's copulatory behaviour, in which females are monandrous. This scenario may reduce the selective pressure for uniformity of male gametes in this species.

**Keywords:** spermatozoa; testes; sperm variation coefficient; mosquitoes; copulation

**Introduction**

Sperm polymorphism refers to different morphological or functional types of spermatozoa within the same individual or species (Ward 1998, Simmons 2001, Swallow and Wilkinson 2002, Pitnick et al. 2009). A classic example is found in Lepidoptera, which produce two sperm morphotypes: eupyrene (nucleated) and apyrene (anucleated) (Friedländer et al. 2005). In Diptera, dimorphism has been reported in 13 species of the *Drosophila obscura* complex (Drosophilidae), characterised by producing of long and short sperm (Joly and Lachaise 1994). At the same time, polymorphism has been reported in *Anopheles* Meigen, 1818 mosquitoes (Culicidae) (Klowden and Chambers 2004).

Many mosquito species are vectors of arboviruses, protozoa, and nematodes (Consoli and Oliveira 1994, Foster and Walker 2019). Currently, 3,726 species have been described and grouped into three subfamilies: Anophelinae Grassi, 1900, Burmaculicinae Borkent & Grimaldi, 2016, and Culicinae Meigen, 1818. The Anophelinae subfamily comprises three genera, including *Anopheles* (Harbach 2007, Reidenbach et al. 2009, Harbach, 2024). Mosquitoes of this genus are well known as vectors of the protozoan *Plasmodium* Marchiafava & Celli, 1885, the causative agent of malaria (Consoli and Oliveira 1994, Fikadu and Ashenafi 2023). This disease is prevalent in tropical and subtropical regions, with 249 million cases and 608,000 deaths reported in 2022 (Fikadu and Ashenafi 2023, WHO 2024). In Brazil, malaria is primarily concentrated in the Amazon region, where the most critical vector is *Anopheles darlingi* Root, 1926 (Deane 1948, Consoli and Oliveira 1994, Magris et al. 2007).

The mating behavior of *Anopheles* has been studied in African species, such as *Anopheles gambiae* Giles, 1902. Males form swarms to attract females for copulation. At the end of mating, it has been observed that in some species males transfer a gelatinous mating plug, making females refractory to subsequent mating (Giglioli 1966, Meuti and Short 2019, Cator et al. 2021, Alfonso-Parra et al. 2022). Polyandry, a situation in which females mate sequentially with two or more males, is considered rare, and therefore, the probability of sperm competition in must be very low (Tripet et al. 2003).

The male reproductive system (MRS) of mosquitoes consists of a pair of testes, deferent ducts, seminal vesicles, a pair of accessory glands, and an ejaculatory duct

(Snodgrass 1959, Christophers 1960, Hodapp and Jones 1961, da Silva et al. 2024). Histological descriptions of this system have revealed intriguing aspects of the unifollicular and spiral organization of the testes in Culicinae (da Silva et al. 2024).

In this work, we describe the morphology of the MRS of *An. darlingi* and we compare our findings with those of other dipterans in literature. Additionally, we performed a morphometric analysis of its spermatozoa, discussed the concept of sperm polymorphism applied to this mosquito, and related these data to their mating behaviour.

**Materials and Methods**

Twenty adult males of *An. darlingi* were obtained from rearing cages maintained at the Medical Entomology Laboratory of FIOCRUZ, Belo Horizonte, Minas Gerais, Brazil. The MRS of nine mosquitoes were dissected and prepared for histological analysis following the procedures described by Silva et al. (2024). In this study, we use Giemsa stain (Merck®). The remaining eleven individuals had their deferent ducts carefully pierced to release sperm onto microscope slides. One slide was prepared from each individual, also following the protocol described by Silva et al. (2024). Previously, there was difficulty obtaining individualized sperm in *An. gambiae* was highlighted (Voordouw et al. 2008). We minimized this challenge by using needles to spread the gametes carefully. 213 individualized and complete spermatozoa were photographed (20x objective with 0.40 numerical aperture or 40x objective with 0.65 numerical aperture) and measured using ImageJ® software (Schneider et al. 2012). To do this, the software was calibrated according to the scale of the microscopic image. Using the segmented line tool, we carefully measured the entire length of each spermatozoon. The data obtained were tabulated in the Jamovi® statistical analysis software. We also calculated the sperm coefficient of variation (CV) using the mathematical formula: CV= (standard deviation/ mean) x100.

**Results**

The MRS of *An. darlingi* comprises a pair of testes, deferent ducts, accessory glands, and an ejaculatory duct (Fig. 1A). The testes are entirely separated and oval-shaped. Histological sections revealed that they consist of a single follicle. In cross-sections, we observed globular cells in the testicular central region of the organ, likely

spermatogonia and spermatocytes. We also noted elongated cells, probably spermatids and spermatozoa, and cytoplasmic bodies along the entire testicular periphery (Fig. 1B).

Each testis connects directly to its respective deferent duct (Fig. 1C), which is a thin, relatively long tube where spermatozoa were visible throughout the lumen (Fig. 1D). The accessory glands are kidney-shaped and located on either side of the transition region of the deferent ducts with the ejaculatory duct (Figs. 1A, E). The glandular epithelium produces two distinct types of apocrine secretions, and each gland is encased in a circular layer of muscle tissue (Fig. 1F). Short ducts from the accessory glands emerge centrally and connect to the anterior region of the ejaculatory duct near the openings of the deferent ducts (Fig. 1A,G). The ejaculatory duct is a tube covered by a thick muscular layer (Fig. 1H).

Spermatozoa are filiform, with lengths ranging from 92.6 µm to 246.5 µm (Fig. 2), averaging 188.79 ± 30.4 µm and showing a high coefficient of variation (16.1%). These data were also calculated for each mosquito and are shown in Table 1. A scatter plot revealed continuous variation in sperm length, with no distinct size classes evident (Graph 1).

**Discussion**

The MRS of *An. darlingi* differs from mosquitoes of the subfamily Culicinae in three key aspects: the linearity of the testes, the absence of dilated regions in the deferent ducts which represent seminal vesicles, and the presence of a muscular ejaculatory duct. The testes of *An. darlingi* are unifollicular, as observed in other dipterans (Snodgrass 1959, Christophers 1960, Hodapp and Jones 1961, Valdez 2001, Spiegel et al. 2013, Hassan et al. 2017, da Silva et al. 2024). However, unlike Culicinae mosquitoes, the follicle is not spiralled, as seen in *Lutzia bigoti* Bellardi, 1862 (da Silva et al. 2024), *Aedes aegypti* Linnaeus, 1762, *Aedes albopictus* Skuse, 1894, and *Toxorhynchites theobaldi* Dyar & Knab, 1906 (personal observations by da Silva). We classified spermatogenesis as radial based on the organization of the germ cells. This characteristic differs from what is observed in *An. gambiae*, where spermatogenesis occurs from the anterior to the posterior region (Vitale et al. 2023).

In many male insects, the seminal vesicles are dilated deferent duct regions that store sperm (Chapman 1998, Klowden 2013). In Culicinae, they are evident and

located in the posterior region of these ducts (da Silva et al. 2024). However, this structure was not observed in the anopheline studied here. The presence of spermatozoa along the deferent ducts suggests that these structures, despite lacking dilations, function as storage sites for mature spermatozoa. The absence of differentiated seminal vesicles is common among males that engage in multiple matings (Hiroyoshi and Reddy 2021), a behavioral trait typically observed in *Anopheles* mosquitos (Tripet et al. 2003, Meuti and Short 2019, Cator et al. 2021, Alfonso-Parra et al. 2022,). The muscular ejaculatory duct appears to be a characteristic shared among anophelines, differing from the short ejaculatory duct found in Culicinae (Snodgrass 1959, Hodapp and Jones 1961, Mahmood and Reisen 1982, 1994, Huho et al. 2006, da Silva et al., 2024). We suggest that these highlighted morphological particularities mark the Anophelinae subfamily.

The spermatozoa of *An. darlingi* exhibited uniform morphological characteristics but showed a pronounced but continuous variation in length. As a result, it was only possible to classify these cells into a single group. Variation in sperm length in *An. darlingi* had already been observed (Klowden and Chambers 2004), with 55% of gametes measuring between 200-250 µm, but the authors considered this variation a sperm polymorphism. However, in cases of polymorphism, male reproductive cells are typically categorized into at least two distinct types, usually according to total length (or that of its regions) and/or morphology (Joly and Lachaise 1994, Chawanji et al. 2005, Sasakawa 2009, Araújo et al. 2011, Baffa et al. 2017). For instance, the 13 analyzed species of the *D. obscura* complex exhibited a bimodal distribution in sperm size (Joly and Lachaise 1994). This distribution highlighted the presence of two distinct groups of gametes, providing a clear example of dimorphism. In *Drosophila azteca* Sturtevant & Dobzhansky, 1936, long sperm measured an average of 925 µm, while short sperm measured 143 µm, representing almost a tenfold difference (Joly and Lachaise 1994). Similarly, in *Triatoma brasiliensis brasiliensis* (Hemiptera: Reduviidae) Neiva, 1911, long sperm are nearly three times the size of short sperm (Baffa et al. 2017). In contrast, the variation in sperm length observed in *Anopheles* does not demonstrate a clear separation into distinct size classes (Klowden and Chambers, 2004). Therefore, we suggest that sperm polymorphism is absent in *An. darlingi*.

The reproductive behavior of *An. darlingi* has not been elucidated. However, it is known that males in rearing cages form pseudo-swarms to attract females, with

evidence of competition among multiple males for the same female (Villarreal-Treviño et al. 2015). This same behavior is known in other *Anopheles* species, such as *An. gambiae* (Alfonso-Parra et al. 2022). In these anophelines, the hormone 20-hydroxyecdysone (20E) present in the ejaculate renders females refractory to subsequent mating (Mitchell et al. 2015). Moreover, various studies have reported that most *Anopheles* females are monandrous (Tripet et al. 2003, Mitchell et al. 2015; Meuti and Short 2019; Cator et al. 2021, Alfonso-Parra et al. 2022). We propose that the significant variation in sperm length in *An. darlingi* is linked to this female trait.

In this context, several studies have shown that gametes tend to be less uniform when the risk of sperm competition is minimal or nonexistent, or more uniform when the risk is high (Hunter and Birkhead 2002, Calhim et al. 2007, Kleven et al. 2008, Fitzpatrick and Baer 2011, Parker et al. 2013, Van der Horst and Maree 2014, Varea-Sánchez et al. 2014, Rowley et al. 2019, de Souza et al. 2023). Furthermore, it has been suggested that when sperm competition is low or absent, less energy is invested in producing highly uniform or optimized sperm (Van der Horst and Maree, 2014). This may result in sperm production by the same individual with lengths varying within a wide range, as observed in *An. darlingi*. The confirmation of the copulatory behavior of *An. darlingi* and/or the identification of 20E in the male ejaculate could further strengthen our hypothesis.

Sperm of varying lengths have been observed in the spermatheca of *An. gambiae*. However, these were significantly larger than those found in the testes, suggesting that selection may favor longer sperm within the female reproductive tract (Klowden and Chambers 2004). This phenomenon could represent intra-ejaculate selection (Sutter and Immler 2020). Conversely, in the same species, it has been reported that males with a higher proportion of short sperm exhibit greater reproductive success as their sperm fill the spermatheca, signaling to the female that mating has occurred (Voordouw et al. 2008). However, further studies are needed to determine whether *An. darlingi* exhibits the intra-ejaculate selection pattern observed in *An. gambiae*.

In summary, our study highlights features of the male reproductive system of *An. darlingi* that differentiate this Anophelinae from the studied Culicinae, namely: non-spiralized testes, absence of differentiated seminal vesicles, and a muscular ejaculatory duct. Additionally, we observed that the male gametes of *An. darlingi*

exhibit variation in length. However, this variation is continuous and does not constitute sperm polymorphism. It is important to note that these findings were derived from colonized mosquitoes, thus not subjected to the same environmental stressors found in natural habitats. Finally, we propose that sperm length variation can be explained by low (or absent) selective pressure for sperm homogeneity, probably resulting from monandrous mating behavior.

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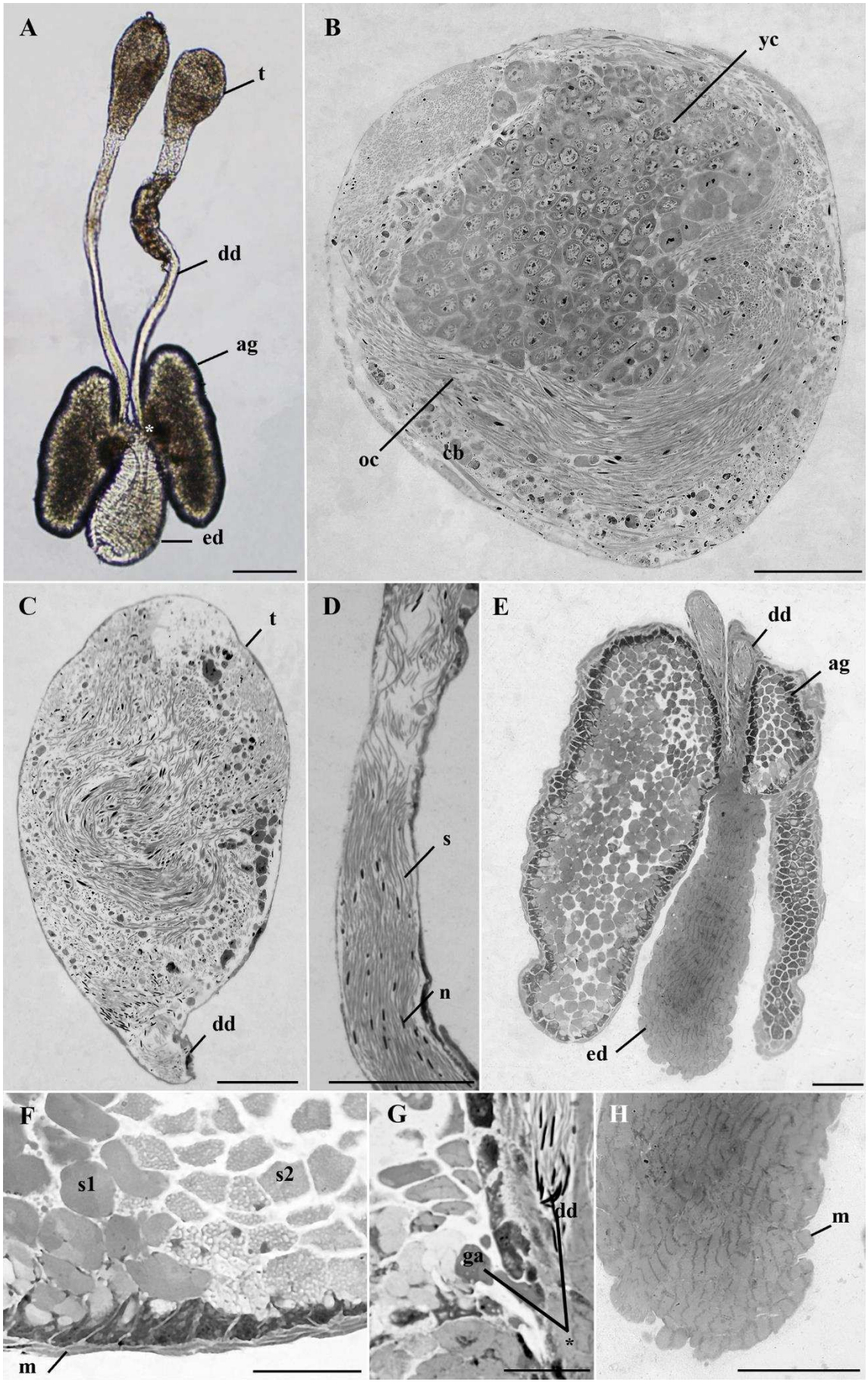
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**Figure 1.** Male reproductive system of *An. darlingi*. (A) Whole-mount of the system, consisting of a pair of oval-shaped testes (t), each giving rise to a deferent duct (dd). A pair of accessory glands (ag) with their central glandular ducts (*) can also be observed. The dd and ag converge at the anterior portion of the ejaculatory duct (ed). (B) Cross histological section of a testis, composed of a single follicle, where young cells (yc) and old cells (oc) of the germ cell lineage, as well as cytoplasmic bodies (cb), can be distinguished. (C) Longitudinal section showing the continuity between the testis (t) and the deferent duct (dd). (D) Lumen of a deferent duct (dd) filled with spermatozoa (s), also showing their nuclei (n). (E) Adjacent to the deferent ducts (dd), a pair of accessory glands (ag) was identified. The ejaculatory duct (ed) is also visible. (F) Detail of an accessory gland, showing two types of secretions: s1 and s2. The gland is surrounded by muscular tissue (m). (G) Detail showing that the accessory glands (ag) and the deferent ducts (dd) jointly empty (*) into the ejaculatory duct (ed). (H) Detail of the muscular tissue (m) of the ejaculatory duct. Staining: Giemsa. Scale bar: A 200 µm; B-E, H: 50 µm; F: 10 µm; G: 5 µm.

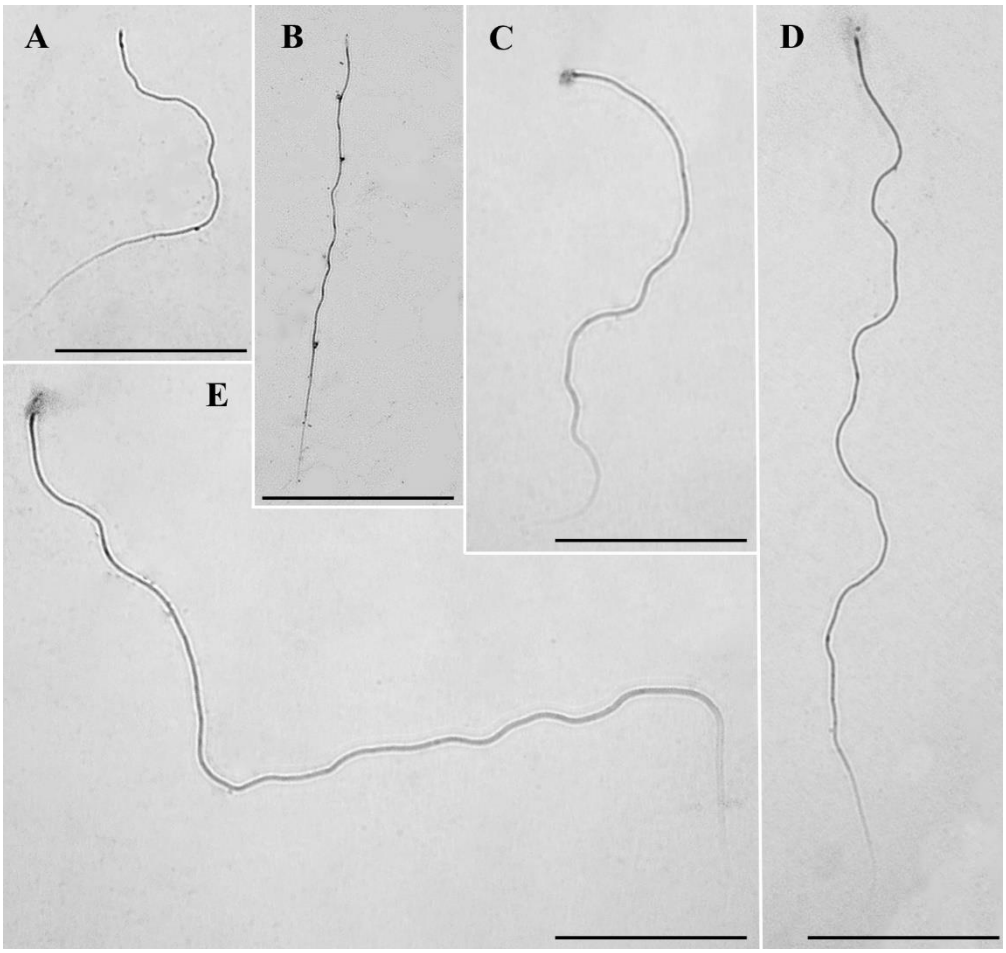
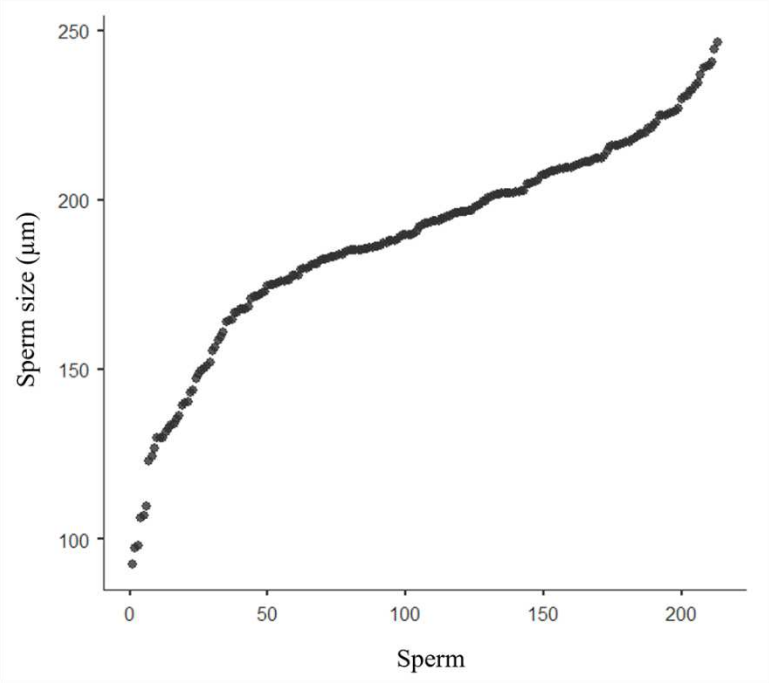


**Figure 2.** Spermatozoa of *An. darlingi*. They measure approximately (A) 100 µm, (B) 130 µm, (C) 150 µm, (D) 190 µm, and (E) 230 µm. Staining: Giemsa. Scale bar: 50 µm.




**Graph 1.** Continuous distribution curve relating spermatozoa to size. Each black dot represents a spermatozoon.

| Individual | Average ± standard deviation | Coefficient of variation |
|---|---|---|
| **1** | 162.58 ± 44.14 | 27.14% |
| **2** | 188 ± 16.6 | 8.82% |
| **3** | 202.45 ± 20.25 | 10% |
| **4** | 182.93 ± 35.88 | 19.61% |
| **5** | 181.55 ± 12.13 | 6.68% |
| **6** | 198.03 ± 20.9 | 10.55% |
| **7** | 207.71 ± 21.21 | 10.21% |
| **8** | 186.01 ± 40.37 | 21.7% |
| **9** | 170.79 ± 30.01 | 17.57% |
| **10** | 181.75 ± 35.35 | 19.44% |
| **11** | 196.24 ± 22.44 | 11.43% |

**Table 1.** Average sperm length and coefficient of variation (CV) for each individual mosquito analyzed

# 7. CONCLUSÃO

Os estudos apresentados nesta tese destacaram importantes avanços no entendimento da morfologia do SRM e dos espermatozoides de mosquitos. As observações realizadas contribuíram para a compreensão das adaptações morfofuncionais desses organismos, como a presença de testículos espiralados em *Ae. aegypti* e *L. bigoti* e a ausência de vesículas seminais em *An. darlingi*. Observamos que o SRM dos representantes da subfamília Culicinae é similar entre si, diferenciando-se de *An. darlingi*, representante da subfamília Anophelinae. Em relação aos espermatozoides, foi verificado que em *L. bigoti* há um acrossomo bem evidente na região da cabeça, enquanto em *Ae. aegypti* essa estrutura é bem pequena. Além disso, verificou-se que para as três espécies estudadas o tamanho dos espermatozoides é diferente. A variação em comprimento dos espermatozoides de *An. darlingi* foi explicada pelo comportamento monândrico das fêmeas que resulta em baixa pressão seletiva para homogeneidade nos espermatozoides. Esses achados preenchem lacunas que não apenas ampliam o conhecimento sobre o SRM e espermatozoides de mosquitos, mas também abrem caminhos para investigações futuras.