UNIVERSIDADE ESTADUAL DE CAMPINAS
INSTITUTO DE BIOLOGIA

ARIANE CAMPOS

ULTRASTRUCTURAL ANALYSIS OF THE SPERMATOZOA OF FIVE SPECIES OF THE ANOMALODESMATA AND IMPARIDENTIA CLADES (MOLLUSCA, BIVALVIA)

ANÁLISE ULTRAESTRUTURAL DE ESPERMATOZOIDES DE CINCO ESPÉCIES PERTENCENTES AOS CLADOS ANOMALODESMATA E IMPARIDENTIA (MOLLUSCA, BIVALVIA)

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Dissertation presented to the Institute of Biology of the University of Campinas in partial fulfillment of the requirements for the degree of Master in Animal Biology, in the area of Animal Biodiversity.

Dissertação apresentada ao Instituto de Biologia da Universidade Estadual de Campinas, como parte dos requisitos exigidos para a obtenção do Título de MESTRA em BIOLOGIA ANIMAL na área de Biodiversidade Animal.

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RESUMO

A análise ultraestrutural comparativa de espermatozoides dos bivalves tem sido utilizada para identificar (1) a morfologia dos gametas, (2) estruturas celulares, (3) constituição do espermatozoide, (4) estratégias reprodutivas e também contribui para (5) a elucidação das relações filogenéticas. Nesse contexto, a análise dos gametas das espécies pertencentes aos clados Anomalodesmata e Imparidentia poderá contribuir com informações para a compreensão da biologia reprodutiva do grupo e também para o esclarecimento de questões relacionadas a taxonomia. Enquanto Anomalodesmata representa um dos mais raros e especializados grupos de bivalves, por exemplo, as valvas dos cuspidariídeos possuem um rostro que é o local onde o sifão inalante se insere para capturar as presas. Imparidentia integra as espécies que são conhecidas pela grande importância ecológica e econômica, como os bivalves popularmente conhecidos como berbigões que são utilizados amplamente na culinária. No presente estudo foram analisados os gametas de cinco espécies, incluindo três anomalodesmados – *Cardiomya cleryana* (Cuspidariidae), *Pandora brevirostris* (Pandoridae), *Thracia similis* (Thraciidae) – e dois imparidênteos, *Laevicardium brasilianum* e *Trachycardium muricatum* (Cardiidae). A análise dos espermatozoides foi realizada por microscopia eletrônica de transmissão. Os espermatozoides apresentam uma ampla variedade morfológica principalmente do núcleo e acrossomo. Em geral, o acrossomo está localizado no ápice nuclear e as mitocôndrias são simétricas como ocorre em *C. cleryana*, *L. brasilianum* e *T. muricatum.* Curiosamente observou-se em *P. brevirostris* e *T. similis* a localização incomum do acrossomo na região posterior do núcleo, em contato com as mitocôndrias assimétricas. Em *T. similis* verificou-se durante a espermiogênese uma possível migração do acrossomo inicialmente localizado no ápice para a região da peça intermediária. Os espermatozoides das espécies analisadas apresentam características similares às espécies pertencentes aos clados “thraciid”, “lyonsiid” e “cuspidariid” de Anomalodesmata, como por exemplo, a forma do núcleo, localização do acrossomo e arranjo mitocondrial. Em relação às espécies de Cardiidae foi possível diferenciar os espermatozoides, uma vez que *L. brasilianum* apresenta o acrossomo curto e o núcleo em forma de barril, enquanto em *T. muricatum* o acrossomo é cônico e o núcleo possui formato de garrafa. Os resultados aqui apresentados são de grande valia, visto que na literatura há pouca informação sobre

a ultraestrutura dos espermatozoides dos anomalodesmados com a descrição de apenas cinco espécies. Com a análise ultraestrutural de espermatozoides foi possível encontrar diferenças morfológicas significativas, que contribui para a identificação dos representantes tanto do grupo Anomalodesmata quanto Imparidentia.

ABSTRACT

The comparative ultrastructural analysis of bivalve spermatozoa has been used to identify (1) gamete morphology, (2) cellular structures, (3) the composition of the sperm, and (4) reproductive strategies, as well as contributing to (5) the elucidation of phylogenetic patterns. In this context, the analysis of the gametes of species belonging to clades of the Anomalodesmata and Imparidentia may contribute to the understanding of the reproductive biology and the taxonomy of these groups. While the Anomalodesmata is one of the rarest and most specialized groups of bivalves, for example, valves of the cuspidariids have a rostrum where is inserted the inhaling siphon to capture the prey. Imparidentia includes a number of species of considerable ecological and economic importance, like bivalves popularly known as cockles that are widely used in a cooking. In the present study, the gametes of five species were analyzed, including three anomalodesmatans – *Cardiomya cleryana* (Cuspidariidae), *Pandora brevirostris* (Pandoridae) and *Thracia similis* (Thraciidae) – and two imparidentians, *Laevicardium brasilianum* and *Trachycardium muricatum* (Cardiidae). Using transmission electron microscopy, male gamete cells were described. The morphology of the spermatozoa varies considerably, especially in the nucleus and acrosome. In general, the acrosome is located at the apex of the nucleus, and the mitochondria are symmetrical, as observed in *C. cleryana*, *L. brasilianum* and *T. muricatum*. Unusually, the acrosome of *P. brevirostris* and *T. similis* was located posteriorly to the nucleus, in contact with the asymmetrical mitochondria. In *T. similis*, the acrosome, located initially at the apex of the nucleus, appeared to migrate to the midpiece during spermiogenesis. The spermatozoa of the anomalodesmatan study species were consistent with those observed previously in the "thraciid", "lyonsiid" and "cuspidariid" clades, including the shape of the nucleus, the position of the acrosome, and the arrangement of the mitochondria. In the two cardiids, the species can be differentiated by the morphology of the spermatozoa, with a short acrosome and barrel-shaped nucleus in *L. brasilianum*, in contrast with a conical acrosome and bottle-shaped nucleus in *T. muricatum.* The results of this study are of great value, since on the ultrastructure of the spermatozoa of anomalodesmatans are virtually nonexistent in the current literature, with only five described species. The ultrastructural description of spermatozoa allowed to find

significant morphological differences, which contributed to the identification of the representatives of both the Anomalodesmata and Imparidentia species.

SUMÁRIO

I. INTRODUÇÃO

1. A classe Bivalvia

Os bivalves integram o filo Mollusca e representam a segunda maior classe em relação a diversidade de espécies, sendo superado apenas pelos gastrópodes (Ponder & Lindberg, 2008). A estimativa do número de bivalves no mundo é de 7500 espécies, sendo que aproximadamente 400 são registradas para a costa brasileira (Rios, 1994; Simone, 1999; Gosling, 2008).

São reconhecidos principalmente pela concha que é constituída por duas valvas, unidas por um ligamento (elástico parcialmente calcificado) responsável por sua abertura quando os músculos adutores relaxam e pela charneira que atua antagonicamente, mantendo a oclusão das valvas (Giribet & Wheeler, 2002; Ponder & Lindberg, 2008). O corpo é comprimido lateralmente, a cabeça está ausente e na ampla cavidade do manto estão as brânquias (Gosling, 2008). Esses organismos ocorrem em ambientes marinhos e de água doce, podem ocupar diferentes substratos e desempenham um papel essencial na condução de energia e nutrientes na coluna de água (Doering & Oviatt, 1986).

A classe Bivalvia representa um grupo estudado há séculos pela humanidade, mas sua ampla diversidade ainda proporciona inúmeras possibilidades para a pesquisa, considerando que pouco se conhece sobre a biologia das espécies (Amaral & Nallin, 2011). Nos últimos anos houve um aumento de informações sobre os bivalves, principalmente devido ao desenvolvimento de novos métodos de análises filogenéticas, bem como pela aplicação cada vez maior de ferramentas moleculares, anatômicas e ultraestruturais que contribuem com informações sobre os aspectos morfológicos e funcionais das espécies, fornecendo caracteres que auxiliam para melhor compreensão da filogenia (Ponder & Lindberg, 2008; Amaral & Nallin, 2011).

2. Estudo dos espermatozoides

Os gametas masculinos de moluscos são morfologicamente os mais diversos entre os Metazoa (Maxwell, 1983). A análise das características ultraestruturais dos espermatozoides tem sido utilizada para relacionar os diferentes modos de fertilização e contribuir para a compreensão da taxonomia, visto que provavelmente não há duas espécies distintas de bivalves que produzam

espermatozoides idênticos (Popham, 1979; Retzius, 1904 *apud* Franzén, 1983; Healy, 1996b; Gwo *et al*., 2002; Introíni *et al*., 2013; Bieler *et al*., 2014). A aplicação da morfologia comparativa de espermatozoides em Bivalvia pode ser utilizada para reconhecer os diferentes táxons estabelecidos para espécie e gênero, e até mesmo no nível de família e superfamília, com base em alguns traços das células gaméticas masculinas (Healy, 1996a).

Segundo Popham (1979) as estruturas menos variáveis dos espermatozoides de bivalves são: cauda, centríolos e mitocôndrias. Enquanto a forma do núcleo é amplamente diversificada, por exemplo, *Brachidontes darwinianus* (Mytilidae) apresenta o núcleo esférico (Introíni *et al*., 2004), *Anomalocardia brasiliana* (Veneridae) possui o núcleo ligeiramente curvo (Introíni *et al*., 2009), em *Lunulicardia hemicardium* (Cardiidae) é curto e tem forma de barril (Healy *et al*., 2008), em *Vasticardium vertebratum* (Cardiidae) é curvo e em forma de bastão (Healy *et al*., 2008), é alongado e curvo em *Codakia orbicularis* (Lucinidae) (Moueza & Frenkiel, 1995) e na espécie *Cerastoderma edule* (Cardiidae) o núcleo apresenta a forma helicoidal (Souza & Azevedo, 1988).

O acrossomo geralmente consiste em uma vesícula envolvida em material subacrossomal. Ele apresenta uma extensa variedade na morfologia, constituição e localização no espermatozoide e assim pode ser considerado como uma estrutura eficaz para inferências taxonômicas (Popham, 1979; Franzén, 1983; Healy, 1989; Eckelbarger *et al*., 1990; Reunov, 2005). Na superfamília Galeommatoidea, o acrossomo está deslocado, inclinando-se lateralmente em relação ao eixo longitudinal do núcleo (Foighil, 1985; Eckelbarger *et al*., 1990). As espécies *Ruditapes decussatus, Eurhomalea rufa* (Veneridae) apresentam no ápice nuclear um *perforatorium* (Pochon-Masson & Gharagozlou 1970; Guerra *et al*. 2003). Algumas espécies do grupo Paleoheterodonta apresentam espermatozoides com múltiplas vesículas pré-acrossomais não fundidas (Healy, 1989). E enfim, os anomalodesmados *Laternula limicola* (Laternulidae), *Lyonsia ventricosa* (Lyonsiidae), *Myadora brevis* e *Myochama anomioides* (Myochamidae) apresentam o acrossomo localizado na peça intermediária, próximo as mitocôndrias (Kubo, 1977; Kubo & Ishikawa, 1978; Popham, 1979).

3. Classificação dos espermatozoides

De acordo com a classificação proposta por Retzius em 1904 (*apud* Franzén, 1983), os espermatozoides podem ser classificados de acordo com a sua morfologia em:

"Tipo primitivo", possui a cabeça esférica ou cônica, a peça intermediária com mitocôndrias esféricas agrupadas em anel ao redor dos centríolos proximal e distal e o flagelo possui arranjo microtubular simples.

"Tipo modificado", apresenta o acrossomo e núcleo alongados em forma de haste, com aparato centriolar, as mitocôndrias são alongadas e na região do flagelo pode conter estruturas adicionais.

"Tipo aberrante", não exibe um plano básico comum.

Rouse & Jamieson (1987) propuseram uma reclassificação e sugeriram termos que categorizam os aspectos funcionais, levando em consideração o método de fertilização do espermatozoide.

"Ect-aquaspermatozoa" proposto para os organismos que liberam os espermatozoides no ambiente aquático e a fertilização é externa.

"Introspermatozoa" se refere aos organismos que depositam os gametas diretamente nas fêmeas e a fertilização é interna.

"Ent-aquaspermatozoa" os gametas masculinos são liberados na água e capturados pelas fêmeas (por exemplo pelo sifão inalante), onde ocorre a fertilização interna (na maioria dos bivalves, na cavidade palial do manto).

4. Anomalodesmata

Anomalodesmata inicialmente foi descrito por Dall (1889), que caracterizou o grupo baseando-se na ausência dos dentes articulados na charneira, o que ele interpretou como arcaico (Harper *et al*., 2006). Os anomalodesmados representam aproximadamente 16% das famílias de bivalves existentes e ao longo da sua história evolutiva há uma proporção semelhante de diversidade de espécies (disponíveis pelo amplo registro fóssil), contudo, esse incrível grupo ainda permanece como sendo um dos menos conhecidos e compreendidos (Harper *et al*., 2006). Atualmente a ordem compreende 15 famílias (Clavagellidae, Cleidothaeridae, Cuspidariidae, Euciroidae, Laternulidae, Lyonsiellidae, Lyonsiidae, Myochamidae,

Pandoridae, Parilimyidae, Pholadomyidae, Periplomatidae, Poromyidae, Thraciidae e Verticordiidae) (Harper *et al*., 2006).

Todos os anomalodesmados são marinhos e ocupam uma vasta gama de *habitats* da zona entre-marés até as regiões mais profundas do oceano. As espécies viventes exploram uma ampla variedade de hábitos de vida. A ordem, em sua maioria, é composta por organismos escavadores infaunais, mas existem também grupos que vivem aderidos a rochas, a outros bivalves ou constroem tubos revestidos com pequenos fragmentos de concha (Clavagellidae). Exclusivamente, alguns bivalves desenvolveram adaptações notáveis, como por exemplo, 54 espécies da família Cuspidariidae, 27 espécies de Verticordiidae e 6 espécies pertencente a Poromyidae são carnívoras (Harper *et al*., 2000, 2006; Allen, 2008). Acredita-se que essa pluralidade de modos de vida seja consequência de uma grande radiação adaptativa que se iniciou no final da Era Mesozóica, e parece ter ocorrido simultaneamente a diminuição do número de espécies que viviam em águas rasas (Harper *et al*., 2006).

Atualmente, a maioria das espécies viventes dos anomalodesmados são geograficamente restritas (ocorrendo principalmente em regiões do infralitoral) e consideradas muito raras. Representantes das famílias Cuspidariidae, Pandoridae, Periplomatidae e Thraciidae já foram encontradas no litoral norte de São Paulo (Migotto *et al*., 1993; Salvador *et al.*, 1998; Soares-Gomes & Pires-Vanin, 2003; Amaral & Nallin, 2011; Tallarico *et al.*, 2014). Os anomalodesmados possuem uma morfologia muito diversificada, o que torna difícil a diagnose. Apesar de uma série de caracteres elencados frequentemente, por exemplo, a presença de *lithodesma* (reforço no ligamento interno da concha) e glândulas secretoras arenofílicas, nenhum pode ser considerado exclusivo para os anomalodesmados (Yonge *et al*., 1980; Morton, 1985).

Contudo, análises morfológicas e moleculares indicam que os anomalodesmados formam um grupo monofilético (Scheneider, 2001; Giribet & Wheeler, 2002; Dreyer *et al.,* 2003; Harper *et al.,* 2006.; Taylor *et al*., 2007; Plazzi *et al*., 2011; Sharma *et al*., 2012), considerado como grupo irmão de Imparidentia (Bieler *et al*., 2014; González *et al*., 2015). Sugere-se uma distribuição em três grupos principais: (a) "cuspidariid" (Cuspidariidae); (b) “thraciid” (Thraciidae, Cleidothaeridae e Myochamidae); (c) “lyonsiid” (Lyonsiidae, Clavagellidae,

Laternulidae e Pandoridae) (Dreyer *et al*., 2003; Harper *et al*., 2006; Healy *et al*., 2008).

Uma curiosa questão na evolução dos anomalodesmados é o modo de vida dos bivalves carnívoros septibrânquios, membros das famílias Cuspidariidae, Poromyidae e Verticordiidae que possuem as brânquias modificadas em septos musculares utilizados para capturar a presa (Harper *et al*., 2000, 2006).Considerando a problemática existente quanto às relações de proximidade e principalmente a alocação das famílias, Runnegar (1974), a partir de caracteres morfológicos, e Healy (1996a), com a análise ultraestrutural dos espermatozoides, sugerem a exclusão da família Cuspidariidae do clado Anomalodesmata.

A escassez de dados na literatura sobre a ultraestrutura dos espermatozoides dos anomalodesmados é um convite a investigar mais espécies (Healy *et al*., 2008). Com os dados disponíveis até o momento, não é possível confirmar a hipótese dos septibrânquios carnívoros como grupo irmão dos outros anomalodesmados, logo, são necessárias mais informações sobre a morfologia dos espermatozoides das famílias Verticordiidae e Poromyidae (Healy, 1996a).

5. Imparidentia

Bieler *et al*. (2014) baseando-se em dados morfológicos e moleculares propôs um novo clado definido como Imparidentia, que corresponde ao grupo Euheterodonta, excluindo Anomalodesmata. O nome Imparidentia se refere aos dentes desiguais que predominam no ligamento das valvas desse grupo. Dentre as famílias mais numerosas pertencentes aos Imparidentia, destacam-se Cardiidae, Tellinidae, Veneridae e Lucinidae (Taylor *et al*., 2007). Mas, apesar da grande variedade de espécies de importância ecológica e econômica, os aspectos reprodutivos são pouco estudados, especialmente para espécies localizadas na América do Sul (Penchaszadeh & Salaya, 1989).

A superfamília Cardioidea, composta pelas famílias Cardiidae e Hemidonacidae, são estudadas do ponto de vista conquiliológico, anatômico e mais recentemente por análises moleculares, indicam uma posição incerta desses grupos e sugerem que Hemidonacidae esteja relacionada à ordem Veneroida (Taylor *et al*., 2007; Healy *et al*., 2008) e a família Cardiidae mostra-se como um grupo irmão de Tellinoidea (Taylor *et al.*, 2007). Na América do Sul, o único trabalho realizado sobre

o aspecto reprodutivo de espécies da família Cardiidae foi com a espécie *Laevicardium laevigatum*, proveniente do Golfo Triste (Venezuela) (Penchaszadeh & Salaya, 1989). Já para espécies encontradas no Brasil, nenhum registro em literatura foi encontrado quanto aos membros dessa família.

Há continuamente uma expansão de dados sobre a ultraestrutura dos gametas masculinos que demonstra ser um valioso recurso para contribuir com dados para as relações taxonômicas e filogenéticas (Healy, 1995; Gwo *et al*., 2002). Entretanto, até o momento, nenhum traço morfológico pode ser definido como evidente peculiaridade da família, já que a forma espermática de Cardiidae varia amplamente entre os táxons (Healy *et al*., 2008), o que demonstra a necessidade de estudos mais detalhados com número maior de espécies.

II. JUSTIFICATIVA

Dados sobre a ultraestrutura de espermatozoides dos anomalodesmados e cardiídeos são inexistentes para as espécies que habitam o litoral brasileiro. Na literatura há pouca informação sobre os anomalodesmados, havendo descrição da ultraestrutura de espermatozoides de apenas cinco espécies. Esses estudos têm demonstrado características atípicas em Myochamidae (Popham, 1979; Healy *et al*. 2008), Laternulidae (Hosokawa & Noda, 1994) e Lyonsiidae (Kubo & Ishikawa, 1978), curiosamente o acrossomo está localizado na região posterior do núcleo, diferindo do usual, que se encontra no ápice nuclear, como ocorre na maioria das espécies de bivalves do clado Imparidentia (aqui representado pelas espécies da família Cardiidae). Portanto, a análise comparativa dos espermatozoides das espécies pertencentes às famílias Cuspidariidae, Pandoridae, Thraciidae são necessários, para verificar a existência da localização atípica do acrossomo e a morfologia dos espermatozoides, em geral, que poderá contribuir significativamente para a compreensão da biologia reprodutiva e com dados para a taxonomia do grupo.

III. OBJETIVOS

Ampliar o conhecimento da morfologia dos espermatozoides, com o intuito de encontrar diferenças que contribuam para identificar representantes do clado Anomalodesmata, *Cardiomya cleryana* (Cuspidariidae), *Pandora brevirostris*

(Pandoriidae) e *Thracia similis* (Thraciidae) e da família Cardiidae (Imparidentia), *Laevicardium brasilianum* e *Trachycardium muricatum*.

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V. ARTIGO I

Ultrastructural analysis of the spermatozoa of three species of the Anomalodesmata (Mollusca, Bivalvia) from Brazilian waters

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Running title: Spermatozoan of three anomalodesmatan species
Keywords: ultrastructure, spermatozoa, Anomalodesmata

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Abstract

Anomalodesmata is the rarest and most specialized group of the class Bivalvia. Few data are available on the spermatozoa of anomalodesmatans, considering that in previous studies only five species were analyzed. The spermatozoa of cuspidariid species were analyzed by transmission electron microscopy, one thraciid species was studied using light microscopy and no published data were found on any pandorid. To contribute to the understanding of the morphology of the spermatozoa in these families, the present study describes the male gametes of three species – *Cardiomya cleryana* (Orbigny, 1842) (Cuspidariidae), *Pandora brevirostris* Güller & Zelaya, 2016 (Pandoridae) and *Thracia similis* Couthouy, 1839 (Thraciidae) – using Transmission Electron Microscopy (TEM). In *Cardiomya cleryana* the acrosome is located in an anterior position, and the mitochondria are arranged symmetrically. By contrast, the spermatozoa of *P. brevirostris* and *T. similis* presented a number of unique characteristics, in particular the association of the acrosome with the midpiece, and asymmetrical mitochondria. This position of the acrosome appears to be unique in Anomalodesmata, and is unknown for non-anomalodesmatan bivalves. In *T. similis*, the later stages of spermiogenesis appear to involve the probable migration of the acrosomal vesicle, resulting in a final position in the posterior region of the nucleus. The present ultrastructural description of spermatozoa contributes to differentiate the studied species.

Introduction

The Anomalodesmata is composed exclusively of marine bivalves, which occupy a wide range of habitats from the intertidal zone to the deep ocean, where they adopt an enormous variety of lifestyles. Most species are now restricted to relatively limited geographic ranges and have specialized niches, and the group is considered to be extremely rare (Harper *et al*., 2000, 2006). Species of the families Cuspidariidae, Pandoridae, Periplomatidae and Thraciidae have been recorded on the northern coast of São Paulo state, in southeastern Brazil (Migotto *et al*., 1993; Salvador *et al*., 1998; Soares-Gomes & Pires-Vanin, 2003; Amaral & Nallin, 2011; Tallarico *et al*., 2014).

The molecular data indicate that the Anomalodesmata is monophyletic and forms a basal clade within the Heterodonta (Steiner & Hammer, 2000; Giribet & Wheeler, 2002; Bieler & Mikkelsen, 2006). Most families of this group appear to be hermaphroditic, with the exception of some species of the families Cuspidariidae, Laternulidae and Clistoconchidae (Boss, 1982; Morton, 1973; Morton, 2012; Machado *et al*., 2016).

In general, molluscan spermatozoa are among the most morphologically diversified in the Metazoa (Maxwell, 1983). In most animals, the sperm cell is composed of three basic parts, the head, midpiece and tail. Considerable variation has been described in the shape of the nucleus and the acrosome, which appears to be correlated with the evolution of the different species and specific features of the reproductive biology of this group (Popham, 1979; Franzén, 1983; Rouse & Jamieson, 1987; Healy, 1989, 1995; Healy *et al*., 2008, 2014; Eckelbarger *et al*., 1990; Souza & Oliveira, 1994, Komaru & Konishi, 1996; Gwo *et al*., 2002; Bieler *et al*. 2014).

The variation found in composition, morphology, size, and position of the acrosome of bivalve spermatozoa is remarkable. The principal functions of the acrosome are to dissolve the outer layer of the oocyte and contribute to the fusion of the plasma membrane of the spermatozoon to the oocyte. Comparative spermiocladistics, focusing in particular on the features of the acrosome and nucleus, has considerable potential for phylogenetic analyses in bivalves through the correlation of sperm structure with evolutionary processes (Popham, 1979; Franzén, 1983; Bernard & Hogdson 1985; Eckelbarger *et al.*, 1990).

In most cases, the nucleus occupies most of the sperm head and the acrosome lies either very close to, or in direct contact with the apex of the nucleus (Popham, 1979; Franzén, 1983; Hodgson *et al*., 1990; Healy, 1989, 1996; Healy *et al.*, 2000, 2001; Hosokawa & Noda, 1994). In most bivalve spermatozoa, the acrosome is located in an anterior position, as observed in the Cuspidariidae (septibranch). In the sperm of *Cuspidaria latesulcata*, the acrosome is located apically at the mitochondria are arranged symmetrically in relation to the centrioles and flagellum (Healy *et al*., 2008). The sperm cells of *Cuspidaria cuspidata* and *Cardiomya costellata* have been analyzed by light microscopy (by Retzius, 1905 *apud* Healy *et al*., 2008, and Morton, 2016, respectively) although no data were provided on the location of the acrosome, only that *C. costellata* has an elongated head and is biflagellate.

The first ultrastructural study of anomalodesmatan spermatozoa focused on the Japanese species *Laternula limicola* (Laternulidae). This study found that the spermatozoon has an elongated vesicle lying posterior to the midpiece, and that this structure briefly occupies a position at the apex of the nucleus, being referred to as a “temporary acrosome” (Kubo, 1977; Kubo *et al*., 1979). This same acrosome configuration was observed previously in *Lyonsia norwegica* (Lyonsiidae) by Franzén (1955) using light microscopy, and was classified initially as a “dictyosome” (Golgi complex). Healy *et al*. (2008) analyzed the spermatozoa of *Myochama anomioides* (Myochamidae) and described the acrosome in a posterior region of the nucleus, as also found to *Myadora brevis* (Popham,1979). In the Thraciidae, only one species – *Thracia papyracea* – has been studied by light microscopy (Franzén, 1955) and the acrosome was not detected.

Studies of fertilization in *Laternula limicola* (Laternulidae) have demonstrated a unique mode of interaction between the spermatozoa and oocytes (Kubo *et al*., 1979, Hosokawa & Noda 1994, Healy *et al*. 2014). In the first of these studies, Kubo *et al*. (1979) reported that the acrosome and mitochondria are excluded during sperm penetration, and were thus referred to as a “temporary acrosome”. However, Hosokava & Noda (1994) subsequently described an unusual process of fertilization in *L. limicola*, with the sperm being incorporated laterally, beginning with the acrosome, which is located on the midpiece. Following this reaction, the inner membrane of the acrosome is exposed in contact with a

microvillus of the oocyte. Fertilization occurs rapidly at the site of contact, indicating that the acrosome is a functional structure, rather than a temporary one.

The present study focused on the morphology and ultrastructure of the male gametes of three anomalodesmatan species – *Cardiomya cleryana* (Orbigny, 1842) (Cuspidariidae), *Pandora brevirostris* Güller & Zelaya, 2016 (Pandoridae) and *Thracia similis* Couthouy, 1839 (Thraciidae) – found on the coast of Brazil. The study also presents a comparison of the findings with congeners and other species of the respective families.

Material and Methods

Specimens of anomalodesmatan bivalves were obtained from the subtidal zone of the São Sebastião Channel (23º49.278' S, 45º24.107' W), on the northern coast of São Paulo state, in southeastern Brazil. Samples of sediment were collected by boat, using a van Veen grab (sampling area of 0.25 m^2) and trawl dredger with a base of 40 cm x 80 cm (conical net with a 5 mm mesh). The live specimens were retrieved using a sieve with a mesh of 0.3 to 0.5 mm. Each specimen was identified prior to dissection at the Center for Marine Biology of the University of São Paulo (CEBIMar - USP). Specimens of three species of Anomalodesmata were collected: (i) *Cardiomya cleryana* (Cuspidariidae), (ii) *Pandora brevirostris* (Pandoridae) and (iii) *Thracia similis* (Thraciidae). After dissection, the shells were separated and deposited in the "Prof. Dr. Adão José Cardoso" Museum of Zoology (ZUEC) at the State University of Campinas (UNICAMP). For ultrastructural analysis, small pieces (1–3 mm^3) of male gonadal tissue were removed and fixed in 2.5% glutaraldehyde (prepared in 0.1 M sodium cacodylate buffer pH 7.2, containing 7% sucrose and 5 mM calcium chloride) for 24 hours at 4ºC, and then rinsed in the same buffer. The samples were post-fixed in 1% osmium tetroxide for 2h at 4ºC, and then dehydrated in a graded acetone series followed by gradual infiltration with EPON resin. The ultrathin sections were stained with 2% uranyl acetate, lead citrate and examined and photographed in a Zeiss Leo 906 Transmission Electron Microscope (TEM), operating at an accelerating voltage of 60 kV. The size of the mature sexual cells was estimated using UTHSCSA Image Tool Version 3.0 (http://compdent.uthscsa.edu/dig/itdesc.html).

Results

The ultrastructural morphology of spermatozoa is described below for each species. In relation to the absolute measurements of sperm structures, *Pandora brevirostris* has the greater acrosome and *Cardiomya clearyana* has the largest length of the nucleus (Table 1).

Cardiomya cleryana (d'Orbigny, 1842) (Cuspidariidae)

The spermatozoon presents a short, slightly curved nucleus with the acrosome in an apical position, a midpiece and a single flagellum (Fig. 1A). The contents of the nucleus are highly electron-dense, with the exception of some electron-lucent lacunae (Figs. 1A-C). The acrosome is short, with a vesicle in the form of a basal ring located at the apex of the nucleus, occupying a deposit of subacrosomal material (Figs. 1A-B). The longitudinal section of the midpiece consists of mitochondria with a rounded profile, with proximal and distal centrioles arranged perpendicularly to one another (Fig. 1C). The transversal section of the five mitochondria revealed an approximately triangular formation (Fig. 1D). The simple flagellum has the standard arrangement of 9 + 2, i.e., nine peripheral, microtubular doublets surrounding a central pair of single microtubules (Fig. 1E). There is an acrosome at the apex of the nucleus in both the late spermatids and mature spermatozoa (Figs. 2A-C). The scheme used for the measurement of the different compartments, and the longitudinal and transversal sections of the spermatozoa is summarized in Figure 7A, and the measurements are provided in Table 1.

Pandora brevirostris Güller & Zelaya, 2016 (Pandoridae)

The nucleus is short and tapered, with an attenuated apex. The contents of the nucleus are highly electron-dense, and no acrosomal structure is present in the apex (Fig. 3A). The acrosome can be observed as a large vesicle located at the posterior extremity of the nucleus. The acrosome is short and dish-shaped, with a loose, granulo-fibrous deposit of material in the subacrosomal area (Figs. 3A-D), and is probably anchored by the centrioles, as observed in the oblique section (Fig. 3C). Electron-dense granules, probably glycogen deposits, can be observed in a perimitochondrial position (Figs. 3A, 3C). The midpiece consists of five mitochondria, a pair of centrioles and the acrosome. The proximal and distal centrioles are

perpendicular to each other (Fig. 3D). The transversal section of the mitochondria shows the flattening of the adjoining mitochondria, with rounded outer-facing surfaces. The flagellum has the 9+2 configuration and cytoplasmic residues can be observed in the transversal sections (Fig. 3F). We observed the position of the acrosome at different stages of spermatogenesis, represented by the loose chromatin found in the spermatids and the more condensed chromatin in the mature spermatozoa. In the sperm cell of *P. brevirostris* the acrosome is found in the posterior region of the nucleus in all stages (Figs. 4A-C). The ultrastructural features of the spermatozoon and the scheme used for the measurement of the different compartments are summarized in Figure 7B, and the measurements are provided in Table 1.

***Thracia similis* Couthouy, 1839 (Thraciidae)**

The nucleus is short and bullet-shaped with a markedly convex apex and flattened base. The contents of the nucleus are highly electron-dense, with the exception of some electron-lucent lacunae (Figs. 5A, 5C). The acrosome consists of a membrane-bound, ring-like electron-dense and granular material within an enclosed lumen (Figs. 5A-C). The midpiece contains five mitochondria, together with the acrosome and a pair of centrioles (Figs. 5A, 5C). In the longitudinal section, the profile of the mitochondria is circular, although it is more triangular in the transversal section. The mitochondria surround a pair of centrioles, which are oriented perpendicularly to each other (Figs. 5C, 5D). The flagellum has the classical 9+2 structure (Fig. 5E). During spermatogenesis, highly electron-dense granules were observed in the nucleus. The acrosome was initially located at the apex of the nucleus, although, towards the end of the process, it was observed apparently migrating down one side of the nucleus to a position in the posterior region of the midpiece (Figs. 6A-D). The scheme used for the measurement of the different compartments, and the longitudinal and transversal sections of the spermatozoa are summarized in Figure 7C, and the measurements are provided in Table 1.

Discussion

The ultrastructural morphology of male gametes has been investigated in only five anomalodesmatan species, *Laternula limicola* (Laternulidae) (Kubo, 1977; Hosokawa & Noda, 1994), *Lyonsia ventricosa* (Lyonsiidae) (Kubo & Ishikawa, 1978), *Myadora brevis* (Myochamidae) (Popham, 1979) and *Myochama anomioides* (Myochamidae) (Healy *et al*. 2008) and *Cuspidaria latesulcata* (Cuspidariidae) (Healy *et al*. 2008). Our analysis of *Cardiomya cleryana* provides ultrastructural data on the sperm of this cuspidariid obtained from the gonads of live specimens fixed specifically for transmission electron microscopy, which contrasts with the study of *C. latesulcata*, which was based on museum material. Our description of the ultrastructure of the spermatozoa of *Thracia similis* and *Pandora brevirostris* represents the first data for Thraciidae and Pandoridae families, respectively. The sperm ultrastructure of these three species varies primarily in the shape of the nucleus and acrosome, the position of the acrosome, the symmetry of the mitochondria, and the apparent migration in *T. similis*.

The comparative ultrastructure of the spermatozoa may help to clarify the taxonomy among the anomalodesmatans (Healy *et al*., 2008). The atypical position of the acrosome (close to or in contact with the midpiece) and the asymmetrical arrangement of the mitochondria in relation to the centrioles and flagellum have been observed in *Myadora brevis* (Popham, 1979), *Myochama anomioides* (Healy *et al*. 2008), *Laternula limicola* (Kubo, 1977; Hosokawa & Noda, 1994), *Lyonsia ventricosa* (Kubo & Ishikawa, 1978), *Pandora brevirostris* (present study) and *Thracia similis* (present study).

In the cuspidariids, by contrast, the acrosome is located at the apex of the nucleus. Healy (1996) has argued that the Cuspidariidae is unrelated to the Anomalodesmata and should be removed from this group. The hypothesis that the carnivorous septibranchs are a sister group of the other anomalodesmatans has been raised by a number of authors (see Runnegar, 1974; Healy, 1996; Dreyer *et al*., 2003) and the cuspidariids *Cuspidaria latesulcata* and *Cardiomya cleryana* share a number of characteristics, including the location of the acrosome at the apex of the nucleus, a slightly curved nucleus and symmetrical mitochondria. If these traits are shared by the other septibranch anomalodesmatans (Cuspidariidae, Poromyidae and Verticordiidae), further research will be necessary.

Based on the available molecular data, the Anomalodesmata is monophyletic and forms a basal clade within the Heterodonta (Steiner & Hammer, 2000; Giribet & Wheeler, 2002; Dreyer *et al*., 2003; Bieler & Mikkelsen, 2006). The study of Dreyer *et al*. (2003), based on the analysis of the 18S ribosomal RNA gene, suggested the formation of three main groups (see Fig. 8): (i) the “cuspidariid” clade (members of the family Cuspidariidae), (ii) the “thraciid” clade (Cleidothaeridae, Myochamidae and Thraciidae), and (iii) the “lyonsiid” clade (Clavagellidae, Laternulidae, Lyonsiidae and Pandoridae).

These clades are also characterized by some common aspects of sperm morphology, with the “thraciids” *Thracia similis* (Thraciidae) and *Myadora brevis* and *Myochama anomioides* (Myochamidae) sharing a rounded nuclear apex. In the "lyonsiid" clade, the genera *Lyonsia* and *Laternula* and the species *Pandora brevirostris* have a tapered nucleus and an attenuated apex. In the “cuspidariid” clade (*Cuspidaria cuspidata* and *Cardiomya cleryana*), the acrosome is located at the apex of the nucleus of the spermatozoon, which is slightly curved, and has symmetrical mitochondria.

The representatives of the "thraciid" and "lyonsiid" clades analyzed in the study presented an atypical trait observed only in these anomalodesmatans, in which the acrosome is located in the region of the midpiece, laterally to the mitochondria, in contrast with the configuration in most bivalve species, in which is located at the apex of the nucleus. The ultrastructural analysis of the species belonging to "thraciid", "lyonsiid" and “cuspidariid” clades shows that this atypical location of the acrosome can be considered to be a shared characteristic of species from "thraciid" and "lyonsiid" clades, as observed in the adapted phylogeny (Fig. 8).

In most anomalodesmatan spermatids, the acrosome temporarily occupies a position at or near the apex of the nucleus, but subsequently moves to a posterior position, close to the mitochondria (Kubo, 1977; Kubo & Ishiwaka, 1978; Popham, 1979; Healy *et al*., 2008). Franzén (1955) observed the acrosome of *Lyonsia norvegica* at the apex of the spermatid nucleus for a short period, but at a later stage in the development of the spermatid, this structure moves to the posterior region and forms a large vesicle behind the mitochondria.

In *Laternula limicola* and *Lyonsia ventricosa*, Kubo (1977), Kubo & Ishikawa (1978) and Hosokawa & Noda (1994) observed no acrosomal structure at

the apex of the nucleus, nor any evidence of nuclear folding. However, the images of the interaction between the spermatozoa and the oocyte indicate that the nuclear folds just before the acrosome reacts with the microvilli of the oocyte.

In the spermatids of *Myochama anomioides* (Myochamidae), Healy *et al*. (2014) observed an acrosome at the apex of the nucleus, but subsequently, during cell maturation, the acrosome moves to the region of the midpiece. In this species, the nucleus folds in half during chromatin condensation, and the acrosome appears to be "guided" to its final position in contact with the midpiece. In the last stage of chromatin condensation, the nucleus is curved and bends into a U-shape, which the acrosome located near or in contact with the midpiece. In the most advanced stage, the acrosome is located in a posterior position, the nucleus apparently loses its U-shape and continued to condense the chromatin up until maturity. During the nuclear condensation and development, the folding of the nucleus and the migration of the acrosome may take place without involving the cytoplasmic microtubules or microfilaments.

In the present study, the condensation of the chromatin during spermiogenesis was described in three species, *Cardyomia cleryana*, *Pandora brevirostris* and *Thracia similis*. Our analysis of *T. similis* revealed a number of differences in comparison with *M. anomioides*, including a lack of nuclear folding, although the probable migration of the pre-acrosome from the apical region of the nucleus towards the posterior region of the spermatozoon was observed. As the chromatin is remodeled during condensation, cytoskeleton elements may support the migration of the pre-acrosome, although this hypothesis requires confirmation.

Conclusions

The present study provides the first analysis of the morphological features of spermatozoa of three Brazilian anomalodesmatan species. The ultrastructure of the spermatozoa of the studied species shares a number of characteristics with that of other species of the three clades proposed by Dreyer *et al*. (2003). The shape of the nucleus of *T. similis* is similar to that of the myochamid species, which supports their inclusion in the “thraciid” clade. In addition, the acrosome of *T. similis* migrated during spermiogenesis, and this species does not have nuclear folding, as described for *Myochama anomioides*. The shape of de nucleus of *Pandora brevirostris* is similar

to that of the lyonsiid species of “lyonsiid” clade. The sperm morphology of *Cardiomya cleryana* is similar to that of *Cuspidaria latesulcata*, of the “cuspidariid” clade, with an anterior acrosome, a slightly curved nucleus, and a radial arrangement of the mitochondria. The spermatozoa of *P. brevirostris* and *T. similis* share a posterior acrosome and asymmetrical mitochondria, which suggests that they are most closely related to the non-septibranch anomalodesmatans. By contrast, the acrosome of *C. cleryana* is located at the apex of the nucleus, and its mitochondria are symmetrical. The investigation of the acrosome and other sperm structures contributes to the understanding of the phylogenetic relationships among these groups, although more data from a broader range of taxa will be required for a more conclusive analysis of the systematics of the Bivalvia.

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Tables

TABLE 1. Dimensions (µm) of spermatozoan structures in the anomalodesmatan species analyzed in the present study. Mean ± Standard Deviation (µm).

Species	**Number of spermatozoa analyzed (*n*)**	**Acrosome**		**Nucleus**		
		Length	Width	Length	Width of apex	Width of base
Cardiomya cleryana	20	0.27 ± 0.04	0.38 ± 0.03	2.71 ± 0.17	0.37 ± 0.04	0.87 ± 0.09
Pandora brevirostris	20	0.34 ± 0.04	0.96 ± 0.12	2.24 ± 0.13	0.31 ± 0.05	1.03 ± 0.10
Thracia similis	20	0.16 ± 0.02	0.47 ± 0.05	1.88 ± 0.18	0.47 ± 0.08	1.04 ± 0.07

TABLE 2. Ultrastructural sperm features of anomalodesmatan species described in the present study.

Species	**Nuclear shape**	**Acrosome position**	**Acrosomal shape**	**Mitochondria**
Cardiomya cleryana	Short, slightly curved nucleus	Apex of the nucleus	Short, with a vesicle in the form of a basal ring	Symmetrical
Pandora brevirostris	Short and tapered, with an attenuated apex	Posterior region of the nucleus	Short, widely dish-shaped	Asymmetrical
Thracia similis	Short and bullet-shaped	Initially located at the apex of the nucleus, and then in a posterior region	Short, dish-shaped	Asymmetrical

Figure Captions

Figure 1. Spermatozoon of *Cardiomya cleryana.* **(A)** Longitudinal section (LS) of the acrosomal complex, nucleus and midpiece. **(B)** LS of the acrosome at the nuclear apex. **(C)** Midpiece: LS of mitochondrion with proximal and distal centrioles arranged orthogonally to one another. **(D)** Transversal Section (TS) of the five mitochondria. **(E)** TS of the flagellum. **Abbreviations**: *a,* acrosome*; dc,* distal centriole; *f,* flagellum; *m,* mitochondria; *n,* nucleus; *pc,* proximal centriole; *sa*, subacrosomal area.

Figure 2. Condensation of the nuclear chromatin in *Cardiomya cleryana*. **(A)** Poorly condensed chromatin. **(B)** Loosely condensed chromatin. **(C)** Strongly condensed chromatin. **Arrows**: acrosome located at nuclear apex in all stages. **Abbreviations**: *a,* acrosome; *m,* mitochondria; *n,* nucleus.

Figure 3. Spermatozoon of *Pandora brevirostris*. **(A)** Longitudinal section (LS) of nucleus, acrosome associated with the midpiece. **(B)** Transversal Section (TS) showing details of the acrosome and subacrosomal area. **(C)** Oblique Section of the acrosome, centriole and electron dense granules. **(D)** LS of the midpiece with mitochondria, centrioles and acrosomal complex. **(E)** TS of the five mitochondria and acrosome. **(F)** TS of flagellum and cytoplasmic residues. **Abbreviations**: *a,* acrosome*; dc,* distal centriole; *f,* flagellum; *m,* mitochondria; *n,* nucleus; *pc,* proximal centriole; *sa*, subacrosomal area.

Figure 4. Condensation of the nuclear chromatin in *Pandora brevirostris*. **(A)** Poorly condensed chromatin. **(B)** Loosely condensed chromatin. **(C)** Strongly condensed chromatin. **Arrows:** acrosome located in the posterior region of the nucleus, near the midpiece, in all three stages. **Abbreviations**: *a,* acrosome; *m,* mitochondria; *n,* nucleus.

Figure 5. Spermatozoon of *Thracia similis*. **(A)** Longitudinal Section (LS) of the nucleus and the acrosome, in contact with the midpiece. **(B)** Transversal Section (TS) of the acrosome and subacrosomal area. **(C)** LS of the acrosome, lying posterior to the nucleus. **(D)** TS of the five mitochondria, centrioles and acrosome. **(E)** TS of

the flagellum. **Abbreviations**: *a,* acrosome*; dc,* distal centriole; *f,* flagellum; *m,* mitochondria; *n,* nucleus; *pc,* proximal centriole; *sa*, subacrosomal area.

Figure 6. Condensation of the nuclear chromatin in *Thracia similis*. **(A)** Poorly condensed chromatin. **(B)** Loosely condensed chromatin. **(C)** Strongly condensed chromatin. **Arrows**: in the first stage, the acrosome is located at the apex of the nucleus, but subsequently migrates to the midpiece. **Abbreviations**: *a,* acrosome; *m,* mitochondria; *n,* nucleus.

Figure 7. The compartments of the sperm measured in the present study, drawn in longitudinal and transversal views **(A)** *Cardiomya cleryana*. **(B)** *Pandora brevirostris*. **(C)** *Thracia similis*. **Abbreviations**: *a,* acrosome*; dc,* distal centriole; *f,* flagellum; *m,* mitochondria; *n,* nucleus; *pc,* proximal centriole; *sa*, subacrosomal area.

Figure 8. Redrawn of summary cladogram of the analysis of the 18S ribosomal RNA gene in Dreyer *et al*. (2003: Figure 5) with the data from the present study superimposed. *****probable appearance of (i) acrosome at the apex of the nucleus, and (ii) asymmetric mitochondria.

Figure 1

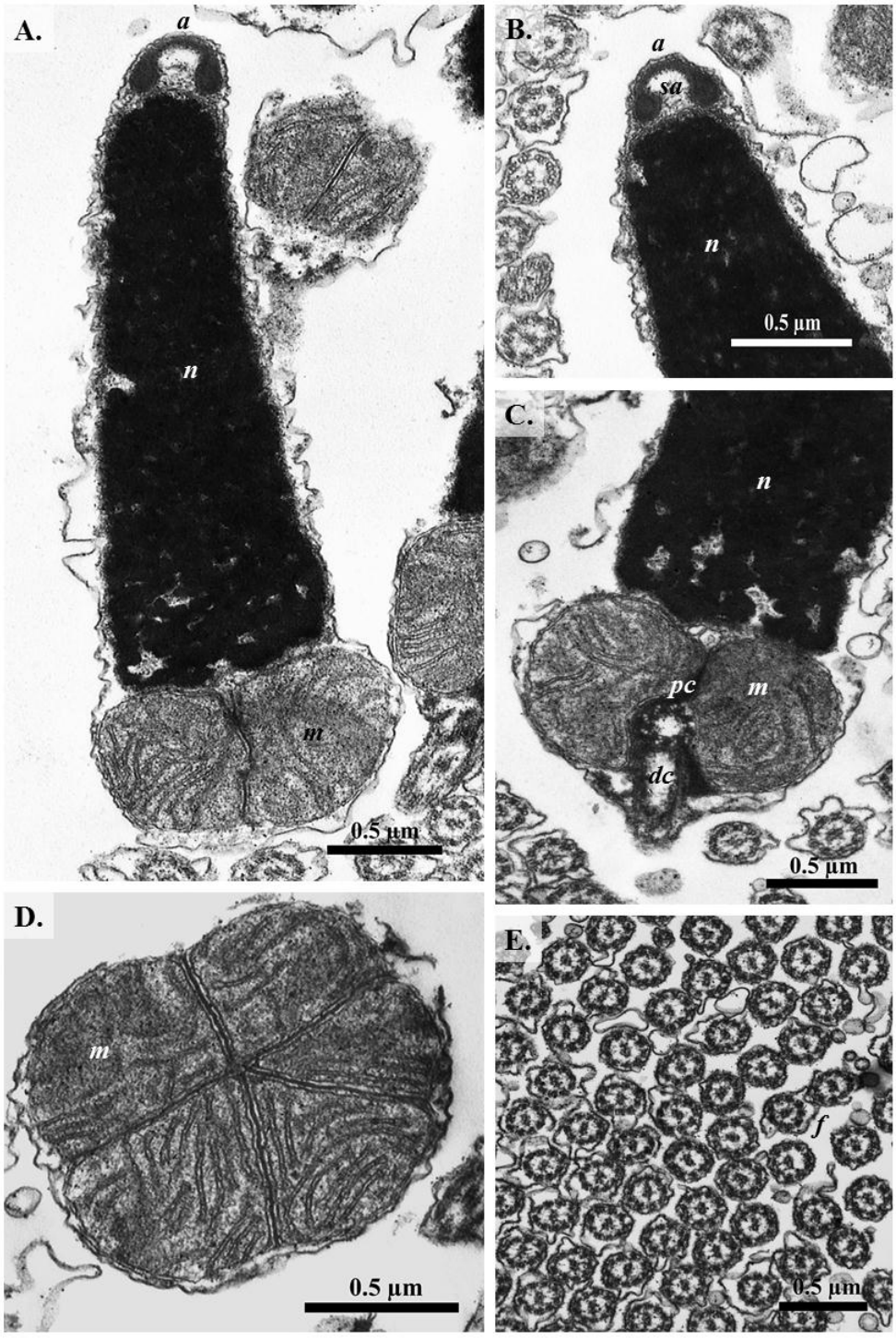

Figure 2

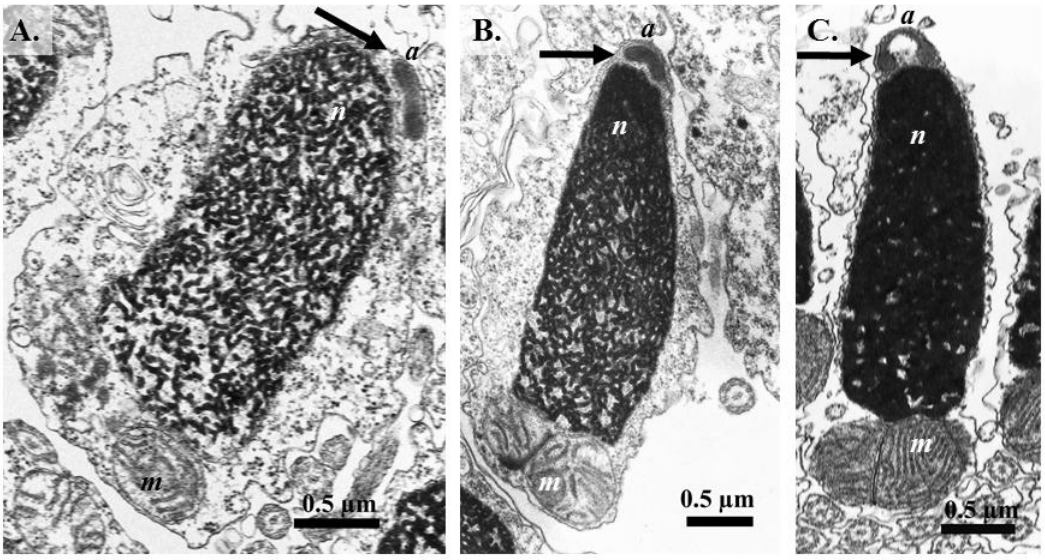

Figure 3

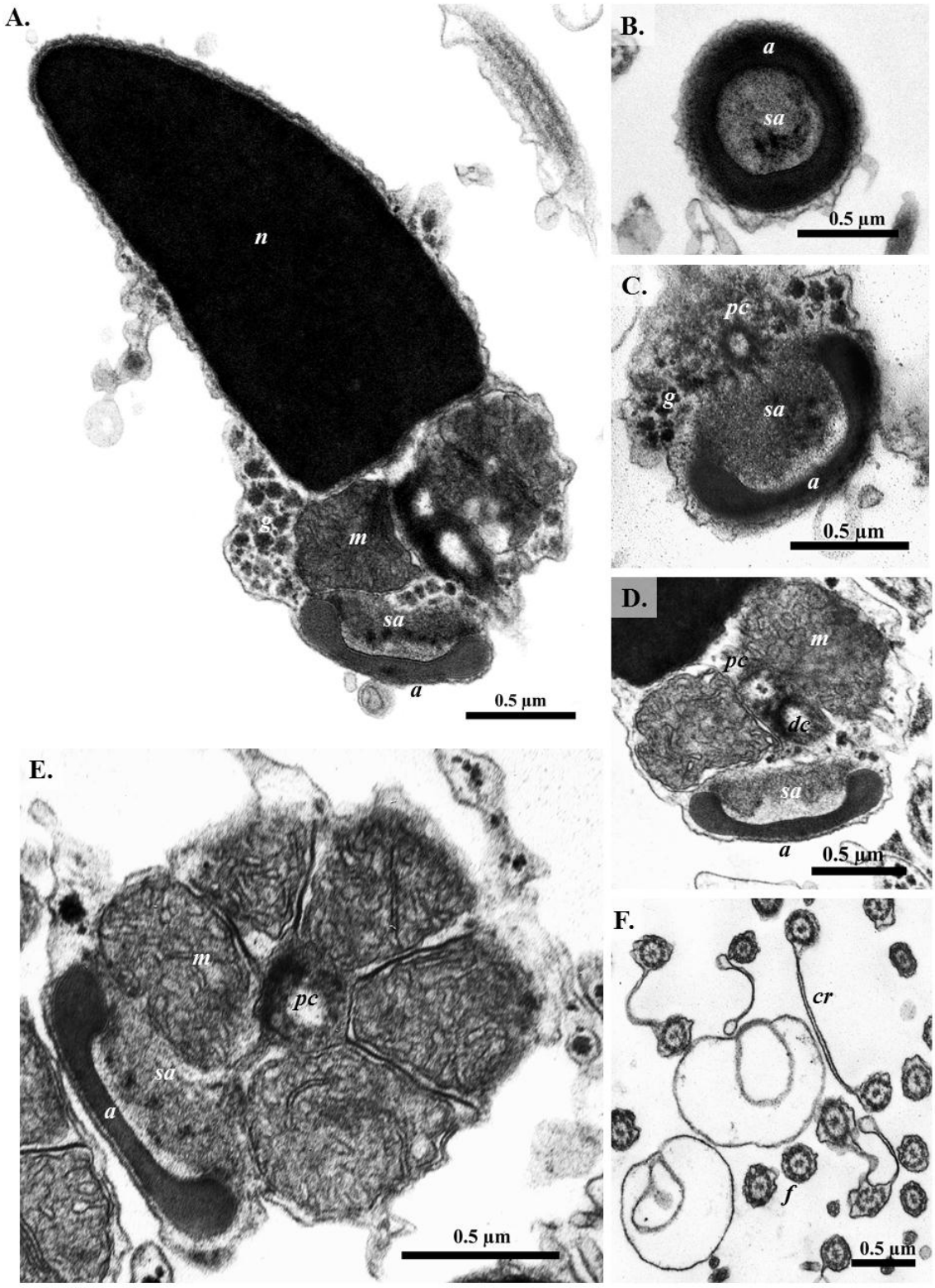
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Figure 4

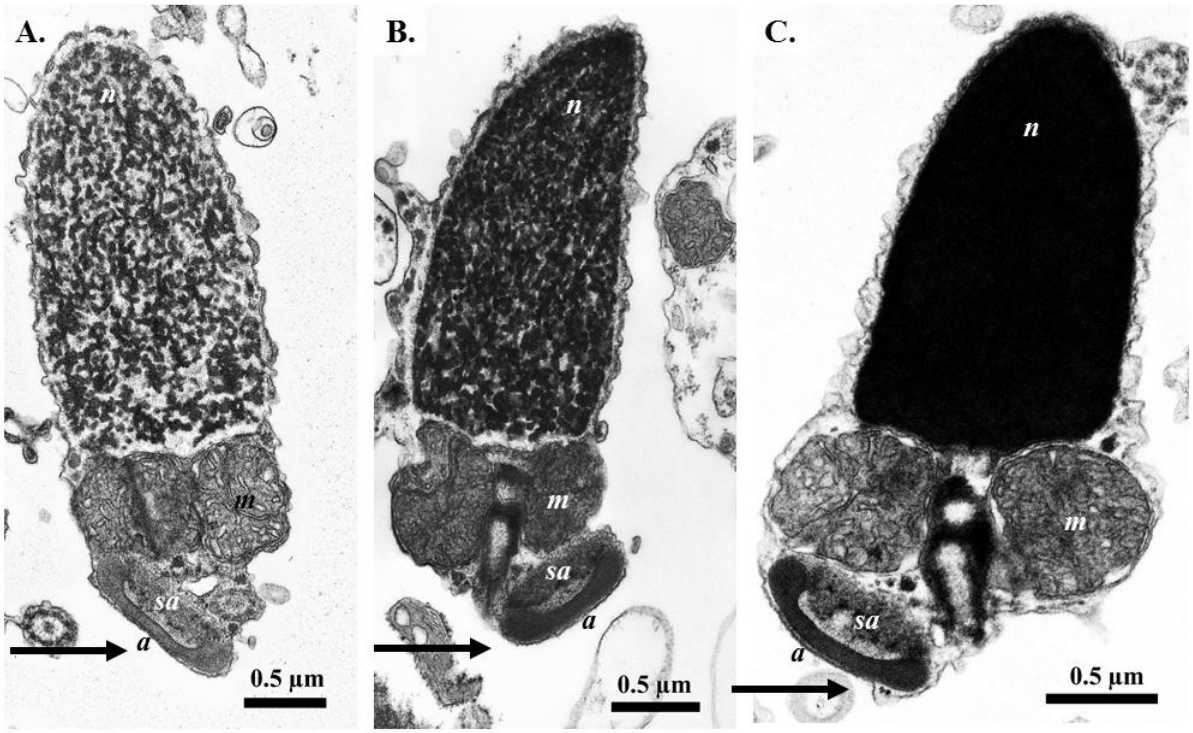

Figure 5

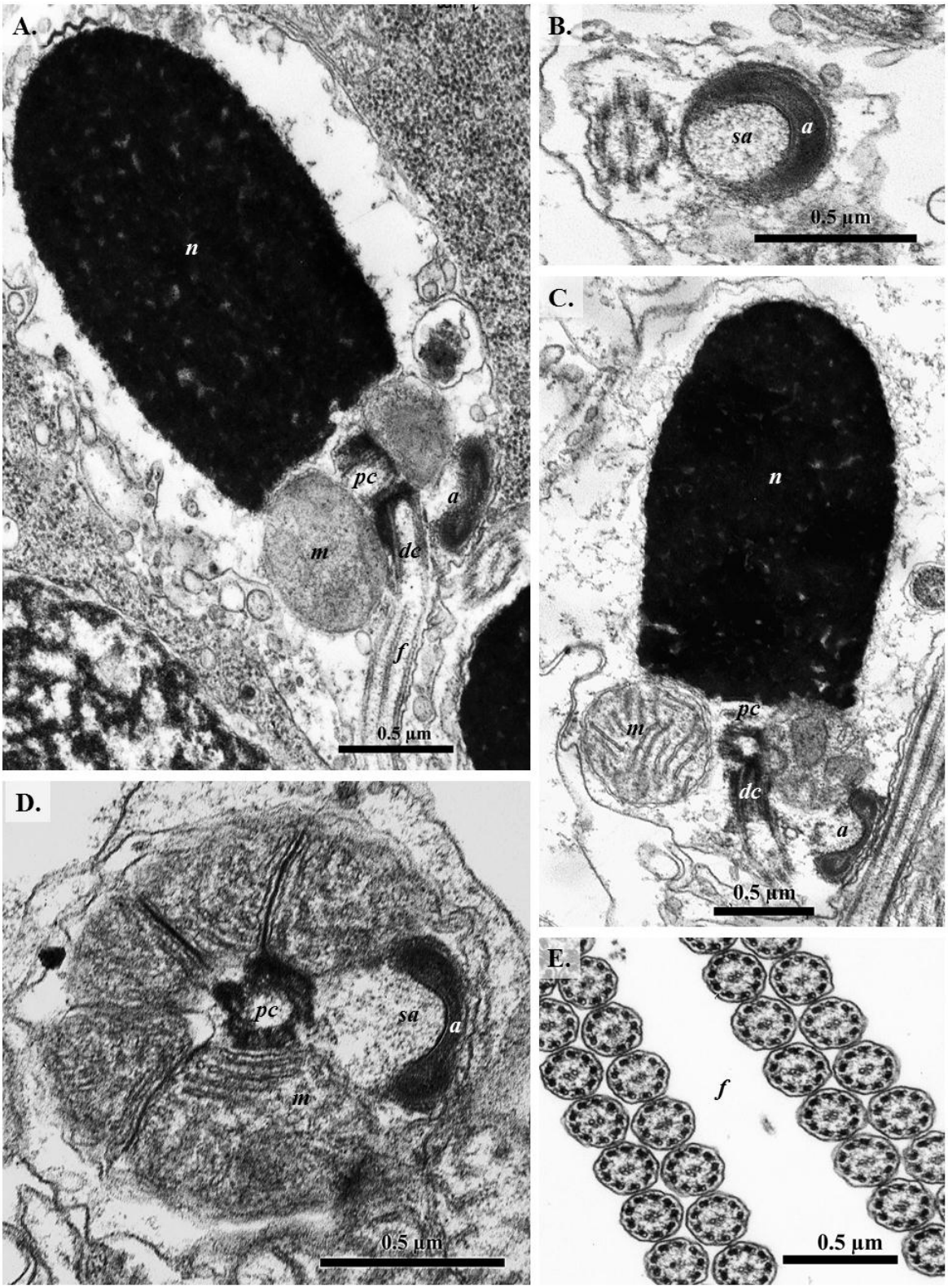
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Figure 6

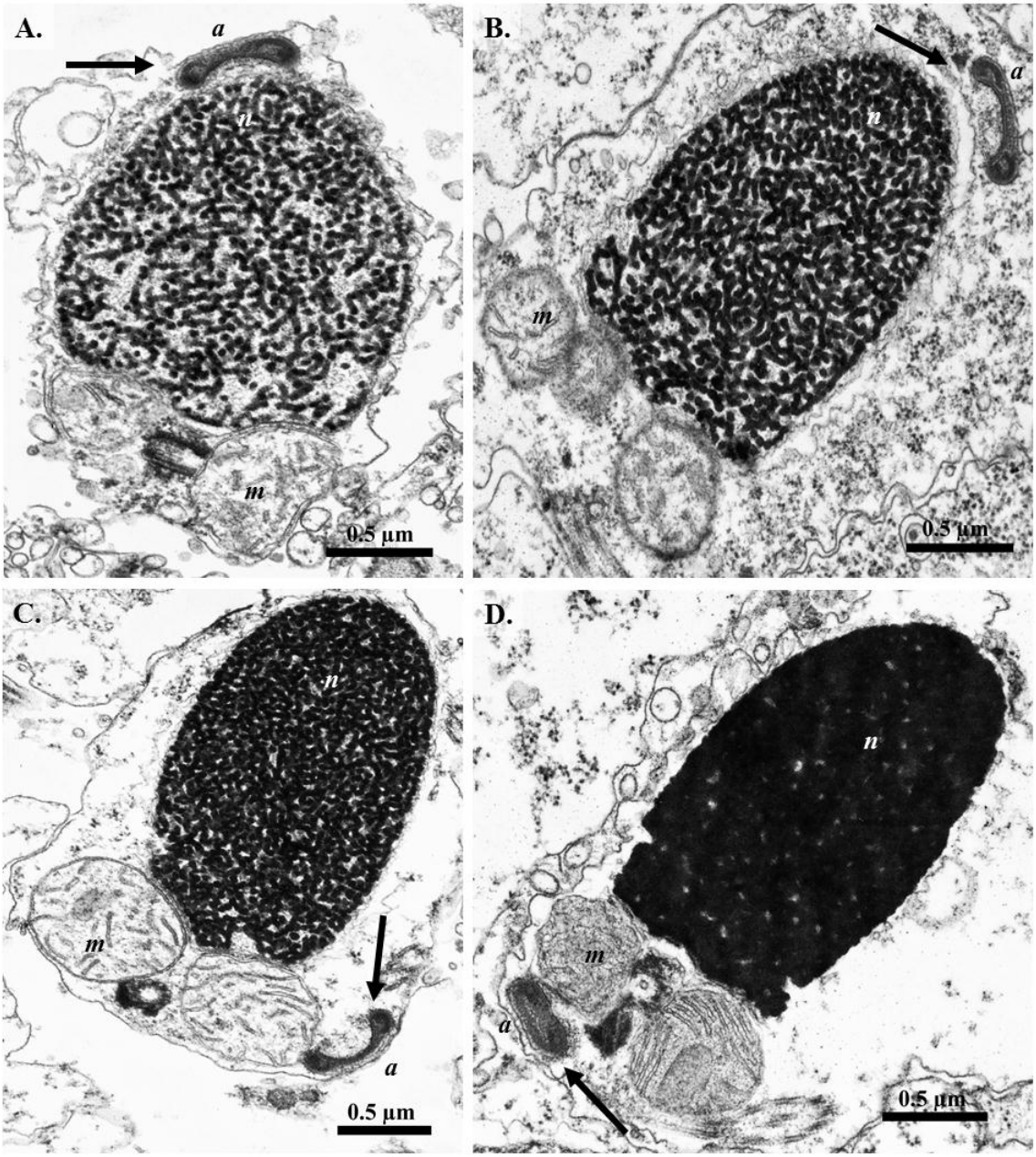

Figure 7

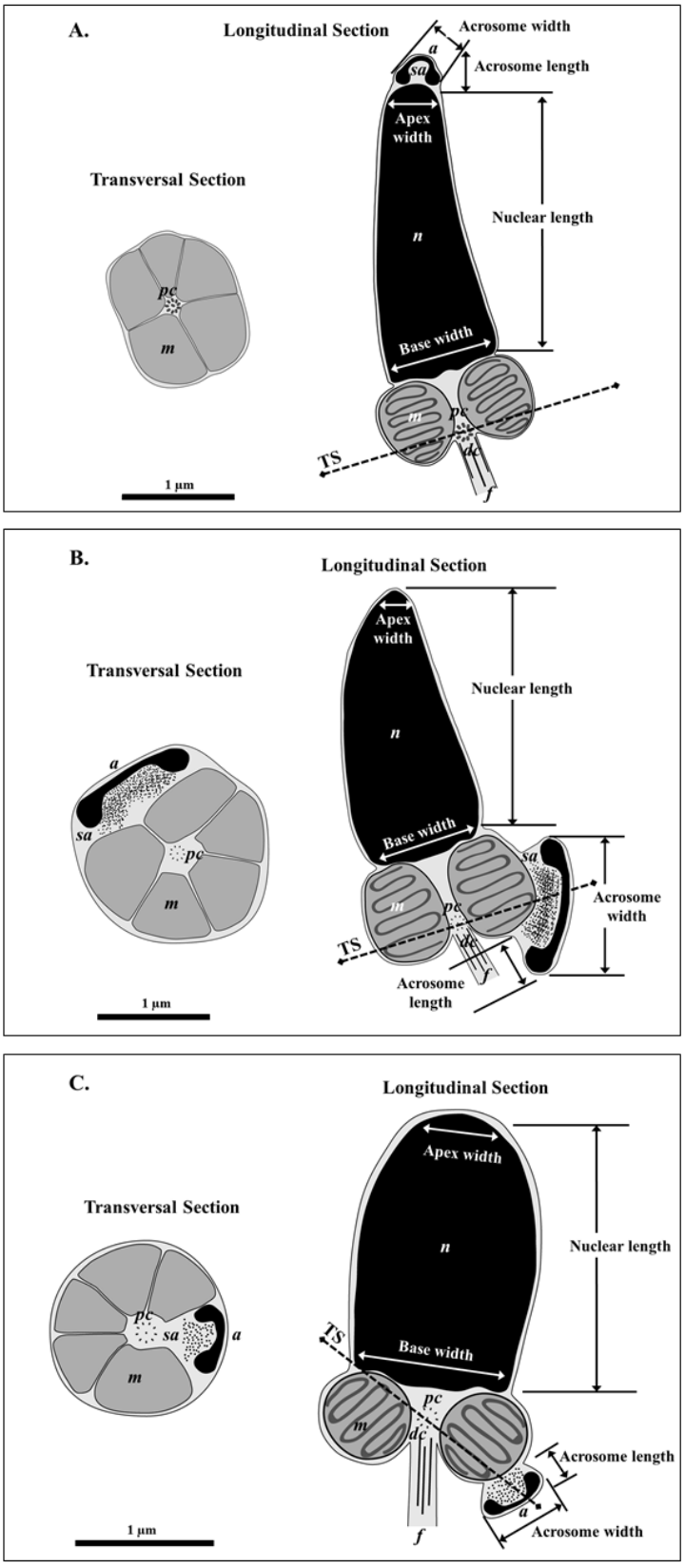
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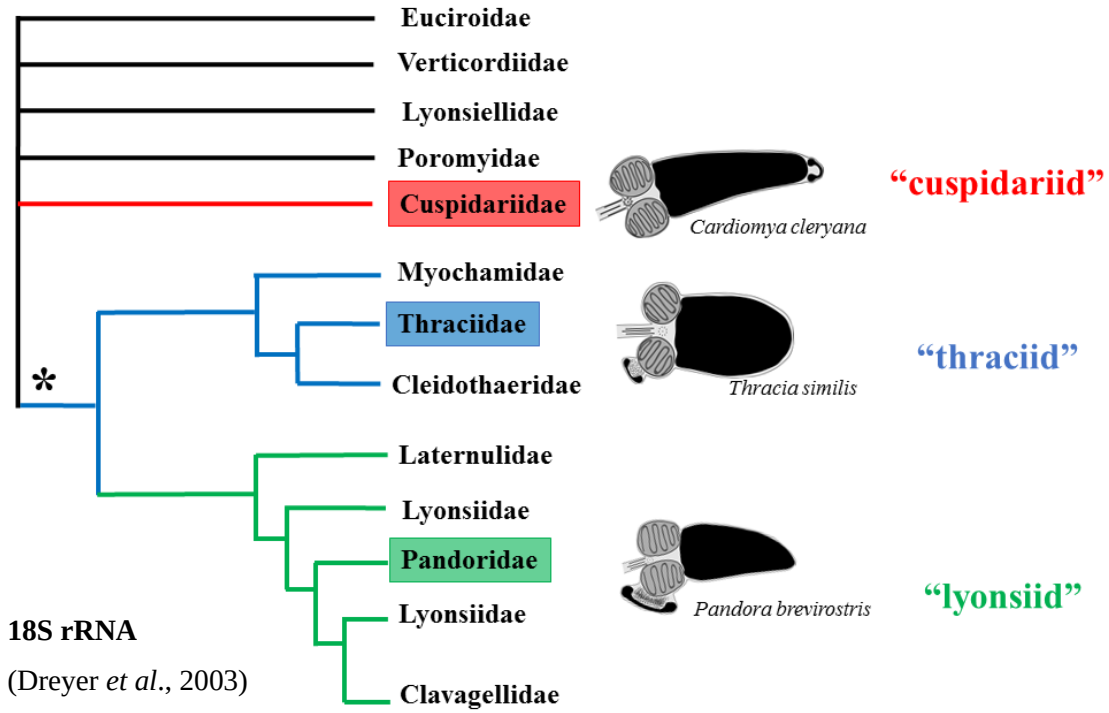
Figure 8



18S rRNA

(Dreyer *et al*., 2003)

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VI. ARTIGO II

Ultrastructure of the spermatozoa of *Trachycardium muricatum* (Linnaeus, 1758) and *Laevicardium brasilianum* (Lamarck, 1819) (Bivalvia: Cardiidae: Imparidentia)

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Abstract

The ultrastructure of the mature spermatozoa of two cardiid species, *Trachycardium muricatum* and *Laevicardium brasilianum*, was described using transmission electron microscopy. The spermatozoa of both species present the following characteristics in common: the base of the nucleus is broader than the apex, the chromatin is well condensed, the midpiece consists of four spherical mitochondria, a pair of centrioles is arranged perpendicularly to each other, and the flagellum has a simple arrangement. The two species could nevertheless be differentiated by the short acrosome and barrel-shaped nucleus observed in *L. brasilianum*, which contrast with the conical acrosome and bottle-shaped nucleus of *T. muricatum*.

Introduction

The bivalve family Cardiidae includes a number of marine species of economic and ecological importance, such as cockles and giant clams (Healy *et al*., 2008), with a total of approximately 265 extant species (Poorten, 2014). Most cardiids are found in tropical and temperate seas, although a few species occur in arctic waters. In general, these animals are suspension feeders, but there are some exceptions such as zooxanthellate cardiids (Maruyama *et al*., 1998; Schneider, 1998; Kirkendale, 2009).

An increasing number of data are available on the ultrastructure of the male gametes, which have become an essential resource for the interpretation of taxonomic and phylogenetic relationships, contributing to the differentiation of some species with highly similar shell morphology (Healy 1989; Souza & Oliveira, 1995; Healy, 1995; Kafanov & Drozdov, 1998; Gwo *et al*., 2002). Up to now, however, no morphological trait has been identified as a diagnostic characteristic of the family Cardiidae, given that the morphology of the sperm varies widely among taxa (Healy *et al*., 2008), reinforcing the need for more detailed studies of a wider range of species.

Up to now, the systematics of the family Cardiidae has been based mainly on the morphological features of the shell, soft anatomy (Schneider, 1992, 2002), and shell microstructure (Carter & Schneider, 1997; Schneider & Carter, 2001), or phylogenetic analyses combining these characters (Schneider, 1995, 1998; Nevesskaja *et al*., 2001; Schneider, 2002). The available data are inconclusive on the relationships among the different group, and indicate that the Cardioidea and Tellinoidea form a clade within the Imparidentia, forming the sister group of the Neoheterodontei (Taylor *et al*., 2007; Bieler *et al*., 2014; Combosh *et al*., 2016). In this context, comparative studies of sperm morphology may provide valuable insights into the evolutionary history of these forms.

Studies of the reproductive biology of cardiids are of considerable importance, and the comparative analysis of the fine structure of molluscan sperm has been used increasingly as a tool for the assessment of taxonomic and phylogenetic relationships (Popham, 1979; Franzén, 1983; Healy, 1995; Erkan & Souza, 2002; Introíni *et al*., 2013; Bieler *et al*., 2014). Despite the diversity of the

cardiids, little is known of their reproductive features, in particular in the South America species (Penchaszadeh & Salaya, 1989).

In the present study, we analyze and describe the ultrastructure of the spermatozoa of *Trachycardium muricatum* and *Laevicardium brasilianum* and compare the results with the data available on the cardiid and tellinid species.

Material and Methods

Specimens were collected from the intertidal zone of the São Sebastião Channel, northern coast of São Paulo state, in southeastern Brazil. Specimens were obtained from samples of the sediment from Velho Barreiro Beach (23°45′ S, 45°20′ W), which were sieved with a mesh size of 0.3 to 0.5 mm. Each specimen was identified and then dissected at the Center for Marine Biology of the University of São Paulo (CEBIMar - USP). Specimens of two species of Cardiidae were collected – (i) *Laevicardium brasilianum* and (ii) *Trachycardium muricatum*. The shells were deposited in the "Prof. Dr. Adão José Cardoso" Museum of Zoology (ZUEC) at the State University of Campinas (UNICAMP). For the ultrastructural analysis, small pieces (1–3 mm^3) of male gonadal tissue were removed and fixed for 24 hours at 4ºC in 2.5% glutaraldehyde (prepared in 0.1 M sodium cacodylate buffer pH 7.2, containing 7% sucrose and 5 mM calcium chloride), and then rinsed in the same buffer. The samples were post-fixed in 1% osmium tetroxide for 2h at 4ºC, and then dehydrated in a graded acetone series before being embedded gradually in EPON resin. The ultrathin sections were stained with 2% uranyl acetate, lead citrate, and examined and photographed in a Zeiss Leo 906 Transmission Electron Microscope (TEM), operating at an accelerating voltage of 60 kV. The size of the mature sexual cells was estimated using UTHSCSA Image Tool Version 3.0 (http://compdent.uthscsa.edu/dig/itdesc.html).

Results

Laevicardium brasilianum (Lamarck, 1819)

The acrosome is short and dome-shaped, resting on a slight protrusion at the apex of the nucleus. The acrosome presents two distinct regions of electron density, a basal ring with a high density of electrons, and the anterior half of the vesicle, which is less electron-dense. The electron-dense basal ring can be seen in the transversal section of the acrosomal vesicle (Fig. 1B). The nucleus is short and barrel-shaped, and the diameter of its anterior region is smaller than that of the base. The chromatin is highly condensed, with the exception of some electron-lucid lacunae (Figs. 1A, 1C). Apically, the nucleus projects slightly into the acrosomal vesicle, a structure known as a *nuclear peg* (Fig. 1A). A fossa at the base of the nucleus comes into contact with the midpiece, apparently forming an anchorage point for the centriolar apparatus (Fig. 1C)**.** The midpiece consists of four spherical mitochondria and a pair of centrioles arranged perpendicularly to each other, anchoring fibers (microtubules) that support the attachment of the centrioles to the midpiece, and the tail. There is evidence of the presence of glycogen deposits near the anchoring fibers (Figs. 1C-F). The flagellum presents the 9 + 2 pattern (Fig. 1F). The scheme used for the measurement of the different compartments, and the longitudinal and transversal sections of the spermatozoa are summarized in Fig. 3A, and the measurements are provided in Table 1.

Trachycardium muricatum (Linnaeus, 1758)

Transversal section of the acrosome shows that the region of the base is electron-dense (Fig. 2B). The acrosome is short and conical. The vesicle contains a highly electron-dense basal ring (Figs. 2A, 2B, 2C). The nucleus is conical and bottle-shaped, with the base broader than the apex and well-condensed chromatin, with electron-lucid lacunae. The surface of the nucleus is flat towards the apex (Figs. 2A, 2C). The midpiece consists of four spherical mitochondria, a pair of centrioles arranged perpendicularly to each other, while the microtubules of the flagellum have a 9 + 2 arrangement (Figs. 2D, 2F). Electron-dense glycogen granules were observed in the midpiece, dispersed randomly or near the centriolar apparatus (Fig. 2E). The longitudinal and transversal sections of spermatozoa and different sperm

compartments measured during the study are detailed in Fig. 3B. The measurements of the sperm cells are presented in Table 1.

Discussion

Laevicardium brasilianum have a short acrosome and barrel-shaped nucleus in contrast with the conical acrosome and bottle-shaped nucleus of *Trachycardium muricatum*.

About the width and length of the acrosome and the nucleus, *T. muricatum* is the largest sperm compared to *L. brasilianum* (Table 1).

The ultrastructure of the spermatozoa of *Laevicardium brasilianum* revealed a slight projection of the nuclear apex that invaginates the subacrossomal area, which is known as a nuclear peg. This structure is also present in all the *Tridacna* (Cardiidae) analyzed to date, although the size of this structure varies among the different species. A pronounced nuclear peg is found in *Tridacna* and *Cerastoderma edule* (Sousa & Azevedo, 1988; Sousa *et al*., 1995). Interestingly, a short but broad nuclear peg is also present in *Fulvia tenuicostata* and *Lunulicardia hemicardium,* although it is less well developed in the latter species (Popham, 1979; Keys & Healy, 1999, 2000). A minor nuclear peg has also been detected in *Parvilucina pectinella* (Lucinidae) (I.P. Corbo – personal information). Therefore, the presence of this structure in the two groups does not necessarily reflect their phylogenetic proximity, but rather probably an adaptive convergence in gametes.

Some gastropod mollusks of the genus *Patella* also present a narrowing of the nucleus that extends towards the subacrossomal area (Keys & Healy, 2000). The authors did not refer to this narrowing specifically as a nuclear peg, although it does appear to be the same type of structure.

A structure similar to a nuclear peg has also been observed in the spermatozoa of lampreys, and its function is presumably to support the acrosomal vesicle or, in the case of *Tridacna maxima* and *Tridacna crocea*, to anchor the acrosome (Keys & Healy, 2000). However, further comparative studies on the fertilization process and more detailed data on the ultrastructure of the nuclear peg will be necessary to elucidate its function in the cardiids.

In the midpiece of *L. brasilianum* stores of glycogen are deposited near the anchoring fibers. The anchoring fibers ensure the maintenance of the structural integrity of the gametic cell even after vigorous movements of the flagellum for propulsion through turbulent water. In *T. muricatum*, the presence of indistinct,

electron-dense glycogen granules can be observed close to the mitochondria and the centriolar apparatus.

Sperm released into the surrounding water for external fertilization are known ect-aquasperm (Rouse & Jamieson, 1987). When released into the aquatic environment, these spermatozoa are able to survive on low oxygen levels, and are able to persist for weeks or even months, depending on the intracellular reserves of glycogen for the production of energy, and is essential to the metabolism and motility of the species (Anderson & Personne, 1970).

In this case, the storage of glycogen in the midpiece may represent an important source of endogenous energy for the metabolism of the spermatozoa. As mentioned above, the presence of glycogen deposits in the midpiece can prolong the longevity of the male gamete. An increase in the life expectancy of the sperm implies an increase in the probability of reaching an oocyte, which maximizes the chances of fertilization occurring, and may be especially advantageous in turbulent water (Anderson, 1970; Introíni *et al*., 2009).

The available molecular data indicate a close relationship between the Cardioidea and Tellinoidea clades (Campbell, 2000; Dreyer *et al*., 2003; Taylor *et al*., 2007; Bieler *et al*., 2014). The findings of the present study are entirely consistent with the results of Healy *et al*. (2008), who were unable to define an exclusive, diagnostic characteristic for the spermatozoa of cardiid species. However, four common characteristics are observed in the male gametes of the cardiids: (1) the acrosomal vesicle generally exhibits a rounded or truncated apex, (2) the acrosomal complex does not depend on a depression at the apex of the nucleus, (3) the subacrosomal material does not organize itself as an "axial rod" or perforatorium, although polymerization may occur, which would contribute to the acrosomal reaction, and (4) the midpiece usually has four mitochondria.

The male gametes produced by cardiid species, such as *Fulvia tenuicostata*, have a barrel-shaped nucleus (Popham, 1979) similar to that observed in *L. brasilianum* in the present study and is different in relation to the nucleus of *T. muricatum. Cerastoderma edule*, by contrast, has an elongated and helical nucleus (Souza & Azevedo, 1988), and *Cerastoderma glaucum* is similar to *C. edule* with the exception of the midpiece, where the mitochondria extend laterally beyond the base of the nucleus. The spermatozoa of *Turtonia minuta* (Veneracea) (Ockelmann, 1964)

have elongated and helical nuclei, which reflects the phylogenetic proximity between the Tellinidae and *Cerastoderma* sp. Studies in light microscopy have shown that spermatozoa with helical nuclei are produced by a number of cardiid species, and at least one tellinid species (Karpevich, 1961). The spermatozoa of *Tellina lineata, Strigilla carnaria, Strigilla pisiformis* (Tellinidae) and *Nuttalia japonica* (Psamobiidae) also have an elongated and helical nucleus (G.O. Introíni – personal communication).

At this stage, however, any conclusion on the diversity of the morphology of the male gametes in the Tellinoidea and Cardioidea would be highly speculative given that the similarities in their ultrastructure may be the result of an adaptive convergence. The morphological diversity of spermatozoa can be interpreted as evidence of polyphyly or it may simply reflect the successful radiation of species into new habitats, where alternative strategies of fertilization have been acquired.

Tables

TABLE 1. Dimensions (µm) of spermatozoa structures in the cardiid species analyzed in the present study. Mean ± Standard deviation (µm).

Species	Number of spermatozoa analyzed (*n*)	Acrosome		Nucleus		
		Length	Width	Length	Width of apex	Width of base
Laevicardium brasilianum	20	0.31 ± 0.03	0.58 ± 0.06	1.51 ± 0.09	0.60 ± 0.07	1.15 ± 0.12
Trachycardium muricatum	20	0.40 ± 0.04	0.48 ± 0.04	2.37 ± 0.12	0.51 ± 0.06	1.13 ± 0.09

TABLE 2. Ultrastructural sperm features of anomalodesmatan species described in the present study.

Species	Nuclear shape	Acrosomal shape	Mitochondria
Laevicardium brasilianum	Short and barrel-shaped	Short and dome-shaped	Symmetrical
Trachycardium muricatum	Conical and bottle-shaped	Short and conical	Symmetrical

Figure Captions

Figure 1. Spermatozoon of *Laevicardium brasilianum* (Lamarck, 1819). **(A)** Longitudinal section (LS) of the barrel-shaped nucleus. Note that the apex of the nucleus projects into the acrosomal lumen, forming a nuclear peg. **(B)** Transversal section (TS) of the ring-like acrosome. **(C)** LS of the acrosome, nucleus with well condensed chromatin, basally a nuclear fossa and midpiece. (**D)** Midpiece with stock of glycogen deposited near the anchoring fibers. **(E)** Midpiece. Observe the anchoring fibers. **(F)** TS of four spherical mitochondria, surrounding a pair of centrioles arranged perpendicularly to each other. **Abbreviations**: *a*, acrosome; *sa*, subacrosomal area; *dc*, distal centriole; *pc*, proximal centriole; *f*, flagellum; *af*, anchoring fibers; *pnf*, post nuclear fossa; *g*, glycogen; *m*, mitochondria; *n*, nucleus. **Arrow**, nuclear peg.

Figure 2. Spermatozoon of *Trachycardium muricatum* (Linnaeus, 1758). **(A)** Transversal section (TS) of the acrosome. **(B)** Longitudinal section (LS) of the nucleus with well-condensed chromatin. **(C)** LS of the conical acrosome (**D)** Midpiece: mitochondria, a pair of centrioles oriented perpendicularly to each other. **(E)** Midpiece with granules of glycogen. **(F)** TS of spherical mitochondria surrounding by glycogen. **Abbreviations**: *a*, acrosome; *as*, subacrosomal area; *cp*, proximal centriole; *f*, flagellum; *n*, nucleus; *m*, mitochondria. **Arrowhead,** glycogen granules.

Figure 3. Schematic drawing showing the measurement of the different compartments of the spermatozoa of cardiids, with longitudinal and transversal views. **(A)** *Laevicardium brasilianum*. **(B)** *Trachycardium muricatum*. **Abbreviations**: *a,* acrosome*; dc,* distal centriole; *f,* flagellum; *m,* mitochondria; *n,* nucleus; *pc,* proximal centriole; *sa*, subacrosomal area.

Figure 1

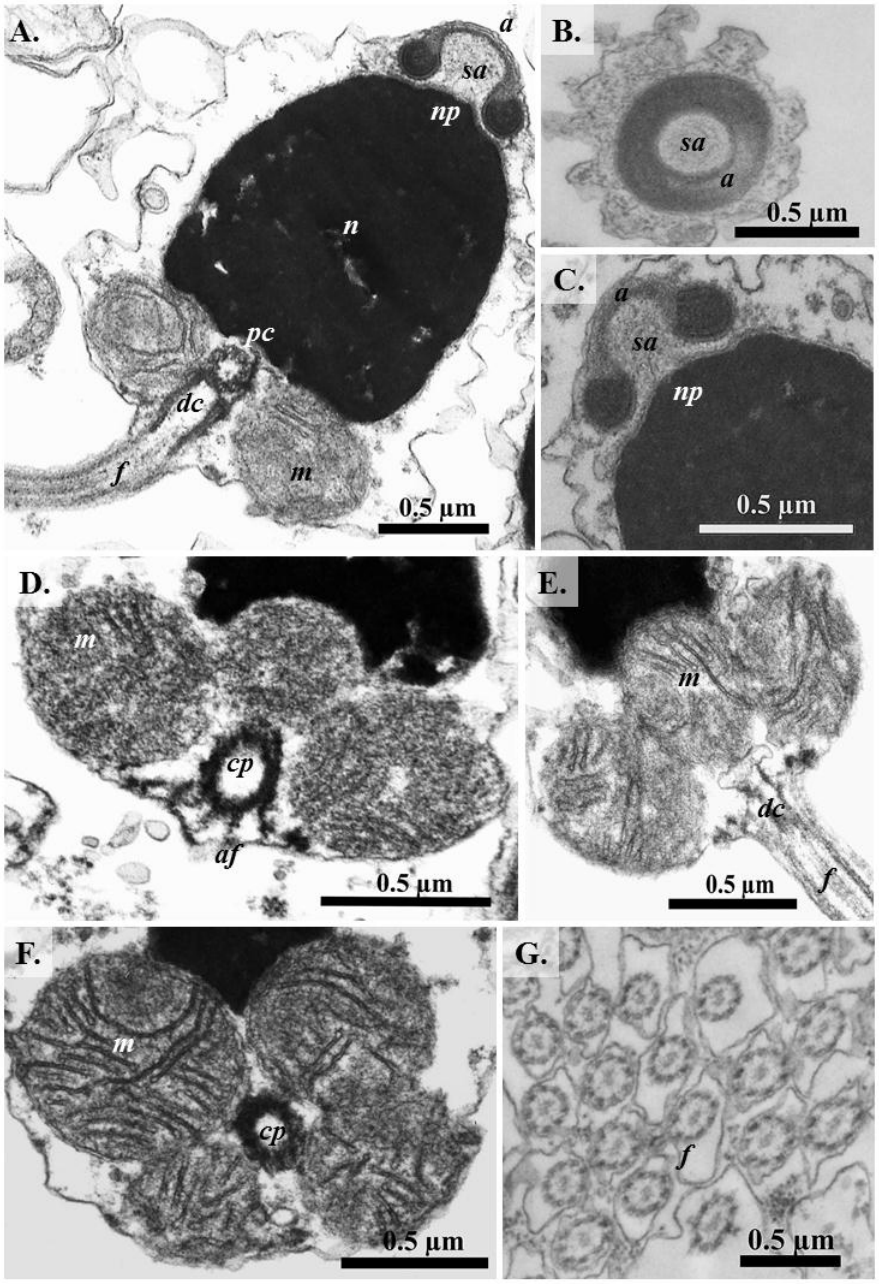
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Figure 2

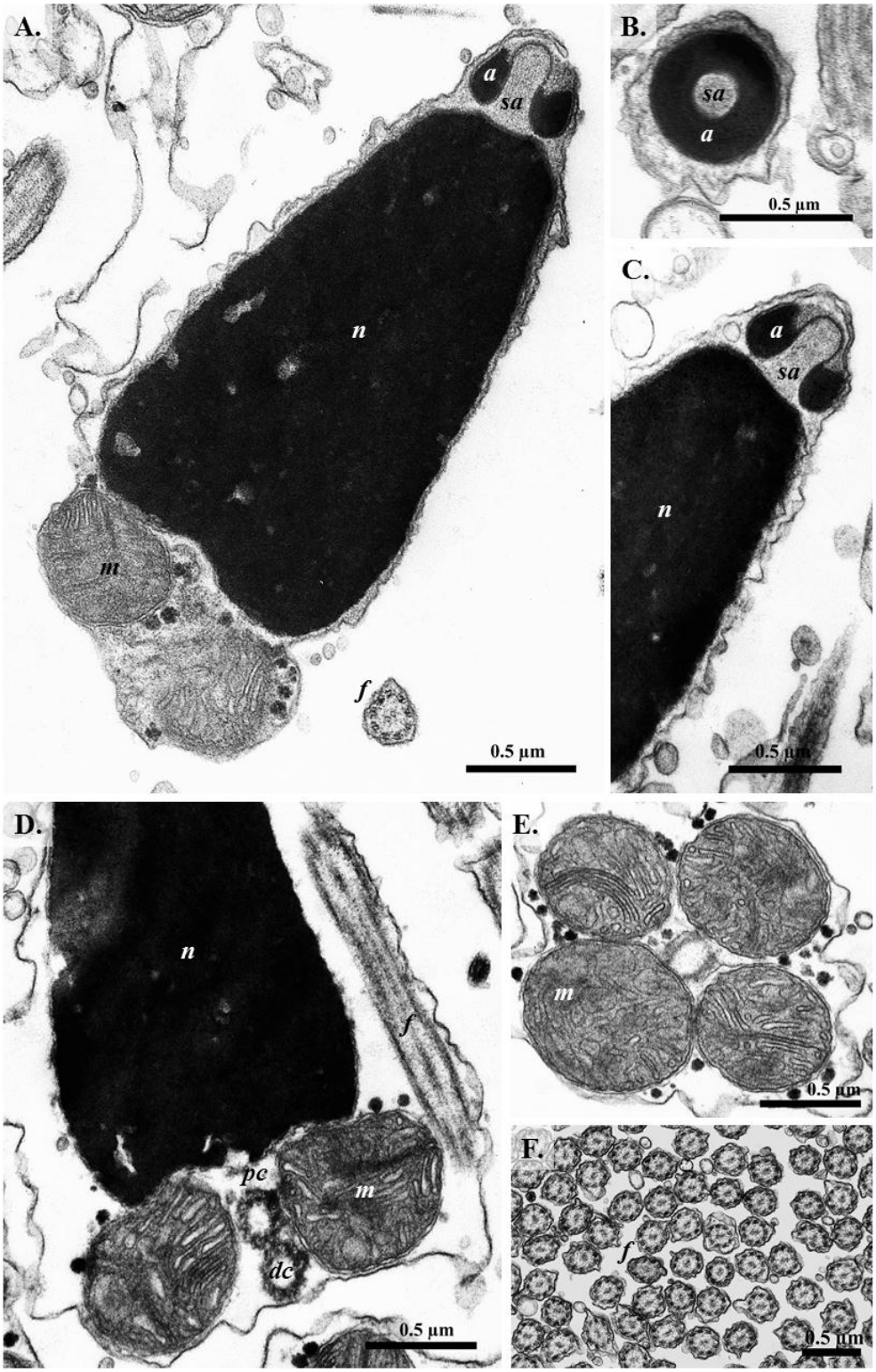
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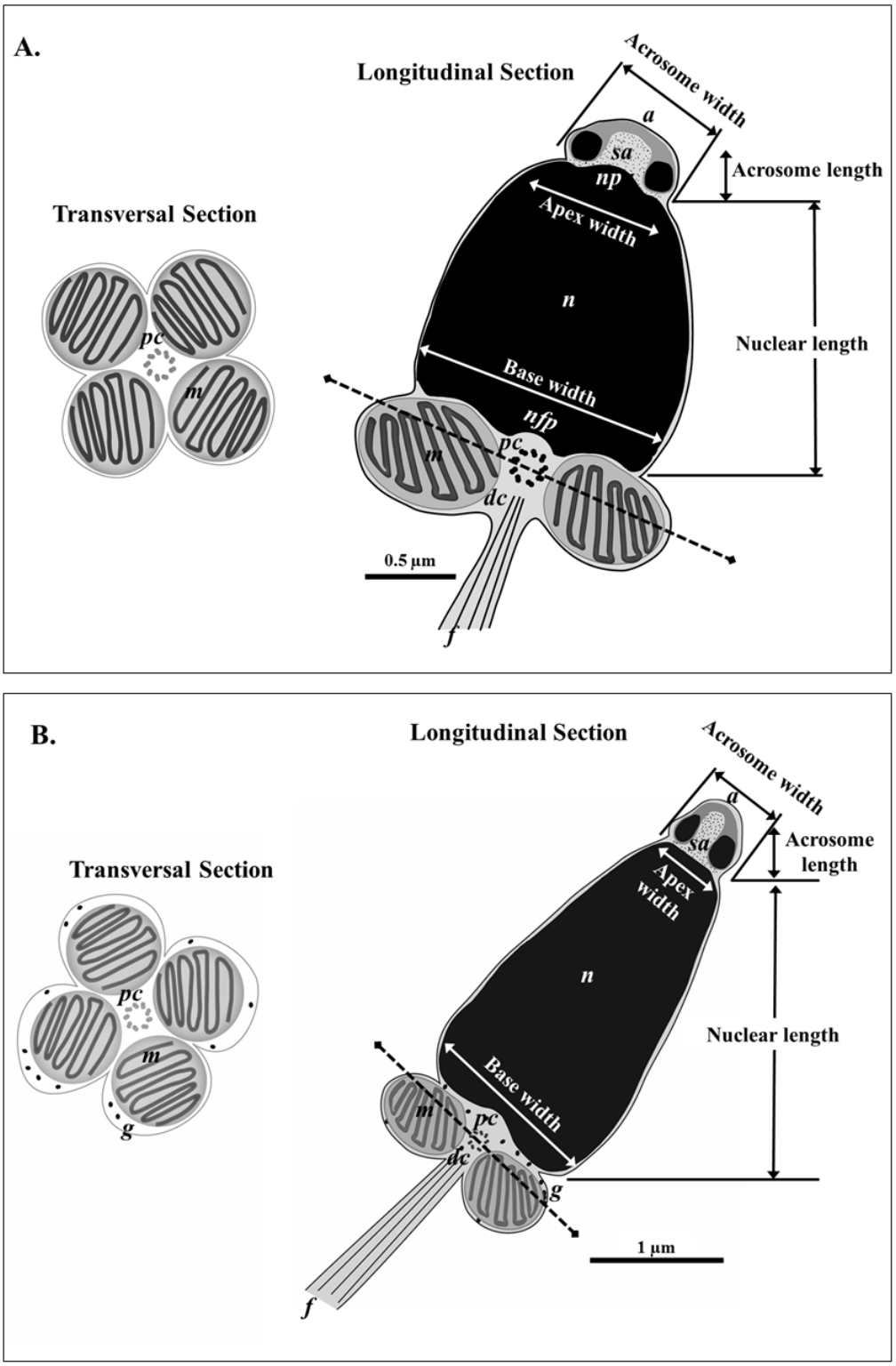
Figure 3


A.
Longitudinal Section
Acrosome width
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sa
np
Acrosome length
Apex width
n
Nuclear length
Base width
nfp
pc
m
dc
0.5 µm
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Transversal Section
pc
m
B.
Longitudinal Section
Acrosome width
a
sa
Acrosome length
Apex width
n
Nuclear length
Base width
m
pc
dc
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1 µm
f
Transversal Section
pc
m
g

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VII. APÊNDICE

CONSIDERAÇÕES FINAIS

Os resultados apresentados possibilitaram a descrição da ultraestrutura do espermatozoide e a distinção de três espécies pertencentes ao clado Anomalodesmata e duas de Imparidentia. Esses dados podem contribuir com a taxonomia e, futuramente, com a filogenia de bivalves.

O estudo dos espermatozoides revelou uma característica atípica dos espermatozoides das espécies *Pandora brevirostris* e *Thracia similis*, em que o acrossomo se localiza na região posterior do núcleo, diferindo do que ocorre na maioria das espécies.

Os espermatozoides de *P. brevirostris* e *T. similis* compartilham as características, quanto à posição posterior do acrossomo e mitocôndrias assimétricas, sugerindo que são proximamente relacionadas aos anomalodesmados não-septibrânquios.

Em relação as espécies de Imparidentia, a presença do nuclear peg em famílias distintas de bivalves pode representar uma proximidade filogenética, entretanto, a semelhança ultraestrutural pode ser fruto de uma convergência adaptativa.

VIII. ANEXOS

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DECLARAÇÃO

Em observância ao **§5º do Artigo 1º da Informação CCPG-UNICAMP/001/15**, referente a Bioética e Biossegurança, declaro que o conteúdo de minha Dissertação de Mestrado, intitulada "***Análise ultraestrutural de espermatozoides de cinco espécies pertencentes aos clados Anomalodesmata e Imparidentia (Mollusca, Bivalvia)***", desenvolvida no Programa de Pós-Graduação em Biologia Animal do Instituto de Biologia da Unicamp, não versa sobre pesquisa envolvendo seres humanos, animais ou temas afetos a Biossegurança.

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