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ESPERMIOGÊNESE E MORFOLOGIA DOS ESPERMATOZÓIDES DE *Iporangaia pustulosa* (ARACHNIDA: OPILIONES: LANIATORES)

Este exemplar corresponde à redação final da tese defendida pelo(a) candidato (a) Juliana Siqueira Moya

e aprovada pela Comissão Julgadora.

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SUMÁRIO

RESUMO

Iporangaia pustulosa (Arachnida: Opiliones) é uma espécie de opilião pertencente à sub-ordem Laniatores. Machos adultos desta espécie tiveram seus aparelhos reprodutores dissecados, e seus testículos e vesículas seminais processados para microscopias de luz e eletrônicas de transmissão (convencional e citoquímica - E-PTA) e varredura. O aparelho reprodutor de *I. pustulosa* é composto por um testículo tubular, dois ductos deferentes que se unem em uma grande vesícula seminal. O testículo apresenta cistos com células germinativas em diferentes fases de desenvolvimento. A espermiogênese é centrípeta e caracterizada por: (1) ausência de formação de flagelo; (2) centro cinético reduzido a dois centríolos do tipo 9+0; (3) formação de manchete de microtúbulos, que auxiliam na compactação nuclear e na determinação da forma celular; (4) gradual compactação cromatínica nas diferentes fases das espermátides até a total compactação nos espermatozóides; (5) desenvolvimento de uma invaginação citoplasmática em direção ao núcleo, desprovida de organelas; (6) acrossomo formado a partir de vesículas do Golgi, composto por uma vesícula acrossomal, um material denso sub-acrossomal, mitocôndrias e pequenas vesículas associadas; (7) espermatozóides ovais, aflagelados, com membrana plasmática ondulada. Pelo método de E-PTA foram detectadas proteínas básicas em diferentes estruturas ao longo do desenvolvimento da espermiogênese. Os espermatozóides formados são liberados dos cistos no centro do testículo e são armazenados na vesícula seminal. Dispersos pela luz da vesícula, os espermatozóides apresentam os mesmos componentes observados no testículo, porém adquirem projeções extracelulares aderidas às ondulações da membrana recobrindo a superfície celular, exceto na região do acrossomo. Essas projeções são adquiridas gradualmente ao longo da extensão da vesícula seminal e são recobertas por um material amorfo na extremidade semelhante a um glicocálice. A espermiogênese de *I. pustulosa* e a morfologia dos espermatozóides testiculares e vesiculares são semelhantes ao descrito para a espécie *Vonones sayi*, também pertencente à sub-ordem Laniatores. Por não ocorrer formação de axonema durante a espermiogênese, os espermatozóides de *I. pustulosa* são aflagelados e tais projeções podem estar envolvidas no transporte do espermatozóide, sua ancoragem no interior da fêmea ou ainda no reconhecimento do óvulo.

ABSTRACT

Iporangaia pustulosa (Arachnida: Opiliones) is a harvestman species belonging to the Laniatores suborder. Adult males of this species had their reproductive tracts dissected and their testis and seminal vesicles were processed for light, transmission (routine preparations and cytochemistry: E-PTA) and scanning electron microscopy. The reproductive tract of *I. pustulosa* is composed of a tubular testis and two deferent ducts that connect to a large seminal vesicle. The testis presents cysts of germ cells in different developmental stages. Spermiogenesis in *I. pustulosa* is centripetal and characterized by (1) the lack of flagellum formation; (2) the kinetic center reduced to two centrioles of the 9+0 type; (3) the microtubule manchette formation; (4) the gradual chromatin condensation from spermatids to spermatozoa; (5) the cytoplasmic invagination development, without organelles, that extends into the nucleus; (6) the acrosome formed by Golgi vesicles, composed of acrossomal vesicles, a subacrosomic dense material, associated mitochondria and small vesicles; (7) the oval aflagellated spermatozoa, with a wavy plasma membrane. With the E-PTA method, basic proteins were detected in different structures throughout spermiogenesis. The spermatozoa are released from the cysts in the center of the testis and are kept in the seminal vesicle lumen. There, the spermatozoa present the same components observed in the testis, however they acquire extracellular projections, which adhere to the wavy membrane covering the cell surface, except in the acrosome area. These projections are acquired gradually along the seminal vesicle length and are covered by an amorphous material on their tips, resembling a glycocalix. The spermiogenesis of *I. pustulosa* as well as the morphology of testicular and seminal vesicle spermatozoa are similar to that described for *Vonones sayi*, also belonging to the Laniatores suborder. As axoneme formation does not occur during the spermiogenesis, *I. pustulosa* spermatozoa are aflagellated and their projections could help to transport sperm along the male and female reproductive tracts, anchor the spermatozoa inside the female and/or play a role in oocyte recognition.

INTRODUÇÃO

A ordem Opiliones

Opiliões são artrópodes pertencentes à classe Arachnida (sub-filo Chelicerata), juntamente com aranhas (Araneae), escorpiões (Scorpiones), ácaros, carrapatos (Acari) entre outras ordens. Possuem o corpo ovóide e compacto, medindo de 5 a 20 milímetros, sem constrição entre o prossoma (cefalotórax) e o opistossoma (abdômen) e, em geral, apresentam pernas extremamente longas, que podem chegar a 15 cm (Berland, 1949; Storer *et al*., 2000).

São comumente confundidos com aranhas, mas diferem pela ausência de glândulas produtoras de seda ou veneno, ausência de um pecíolo ou pedicelo, conferindo a não-segmentação do corpo, e presença de um único par de ocelos e quelíceras queladas (Berland, 1949). No Brasil, os opiliões são conhecidos por diversos nomes populares atribuídos a sua semelhança com as aranhas e ao forte odor que emitem: aranha bode, bodum, aranha fedida, frade fedorento, aranha de chão. No século XVI, na Epístola do padre jesuíta José de Anchieta, existe uma citação sobre a presença de opiliões no Brasil: *"Há aqui umas aranhas de gênero diverso, tendo também um nome diferente do destas, e que exalam muito mau cheiro: são frias por natureza, não saem das casas senão quando o sol está muito ardente"* (Mello-Leitão, 1936).

Constituem um grupo com grande diversidade morfológica e relativa diversidade de hábitos alimentares. Em geral são carnívoros, mas podem ser encontradas espécies sapróforas ou onívoras. Adicionalmente, a maioria das espécies é fotofóbica,

consequentemente se alimenta e se reproduz à noite (Savory, 1938), sendo que logo ao entardecer eles iniciam sua atividade e voltam aos seus refúgios no alvorecer (Gnaspini, 1996).

Como mecanismo de defesa, os opiliões usam a secreção de glândulas exócrinas bilaterais na porção dorsal do prossoma (Eisner *et al.*, 1971; Clawson, 1988), que possui propriedades anti-bacterianas (Gnaspini, 1995). Essa secreção possui um forte odor e coloração variada, podendo ser branca, amarela ou ainda laranja. Além da função de defesa, essa secreção parece estar relacionada com a eliminação de excretas, proteção contra microrganismos, reconhecimento sexual, alarme e comportamento de agregação (Holamberg, 1986).

Além da emissão de substâncias repelentes, outras estratégias de defesa estão presentes em opiliões. A autotomia, ou perda, de apêndices locomotores é utilizada para desviar a atenção do predador enquanto o animal realiza a fuga (Guffey, 1998). Algumas espécies sacodem o corpo rapidamente a fim de dificultar a identificação e ataque ao corpo do animal por parte do predador. Outras espécies utilizam a tanatose, na qual o opilião se mantém rígido e imóvel por certo tempo.

Diferente dos demais aracnídeos, os opiliões apresentam comportamento gregário, no qual se formam grupos de pelo menos três indivíduos, cujas pernas se sobrepõem (Machado *et al.*, 2000). As explicações para tal comportamento são variadas e relacionam-se com a escolha de ambientes que possam diminuir o risco de desidratação e evaporação, além de aumentar as habilidades defensivas pela ação coletiva da descarga das substâncias repugnatórias.

A diversidade dos opiliões está mais concentrada nos trópicos úmidos, onde sua biomassa pode superar a das aranhas, e decresce consideravelmente nas regiões mais frias e secas (Hillyard & Sankey, 1989). Em todo mundo, o Brasil é o país com maior riqueza de opiliões, com cerca de 950 espécies (Pinto-da-Rocha, 1999) das 6000 descritas na ordem (Cokendolpher & Lee, 1993). A Floresta Atlântica, das regiões sul e sudeste do Brasil, apresenta a maior diversidade de opiliões, possuindo em algumas Reservas Federais e Estaduais, de 30 a 50 espécies em cada área (Pinto-da-Rocha, 1999). O sudeste brasileiro, em especial os Estados de São Paulo e Rio de Janeiro, apresenta a fauna mais rica e estudada no país, com 232 e 212 espécies descritas, respectivamente. Nas demais regiões brasileiras, os levantamentos são incompletos ou, muitas vezes, inexistentes.

Algumas espécies vivem no folhiço e na vegetação mais baixa de áreas inundadas. Na época da cheia, essas espécies sobem às árvores e passam a viver no dossel, onde se reproduzem, descendo novamente no período da vazante seguinte. A fauna de formações mais secas, como cerrado e caatinga, possui diversidade muito menor, podendo ocorrer menos de 10 espécies por área; entretanto, ao contrário, espécies de áreas úmidas, possuem uma distribuição geográfica muito mais ampla. No Brasil, por exemplo, a fauna de caverna compreende pelo menos 26 espécies de 6 famílias (Adis, 1997).

Inicialmente a ordem Opiliones era dividida em três sub-ordens: Cyphophthalmi, Palpatores e Laniatores. Recentemente, os Palpatores foram divididos em Eupnoi e Dyspnoi, daí o fato de muitos trabalhos antigos referirem-se à sub-ordem Palpatores. Assim, atualmente, a ordem é dividida em quatro sub-ordens: Cyphophthalmi, Eupnoi, Dyspnoi e Laniatores (Giribet *et al.*, 2002). Os Cyphophthalmi são um grupo pequeno, com cerca de 130 espécies distribuídas pelo mundo. Seus representantes são semelhantes a

ácaros, pois são muito pequenos, apresentando corpo arredondado e pernas curtas. As sub-ordens Eupnoi e Dyspnoi apresentam um pouco mais de 2000 espécies concentradas, principalmente, nas regiões temperadas e seus representantes possuem corpo pequeno e arredondado e pernas longas. Os Laniatores possuem aproximadamente 3750 espécies concentradas nas regiões tropicais, mais especificamente, na América do Sul e seus representantes apresentam uma grande variação morfológica, mas, em geral, possuem corpo robusto, pernas longas, e pedipalpos armados com espinhos (Shultz, 1998). No Brasil, a sub-ordem Laniatores é a mais abundante e não existem registros de ocorrência de espécies da sub-ordem Cyphophthalmi.

Iporangaia pustulosa (Mello-Leitão, 1935), espécie estudada no presente trabalho, pertence à família Gonyleptidae (sub-ordem Laniatores), encontrada na América do Sul, com exceção do Chile e Argentina (Pinto-da-Rocha, 1999). A família Gonyleptidae é a mais bem representada no Brasil e concentra a maior parte dos estudos ecológicos e comportamentais com opiliões da região neotropical (Gnaspini, 1996; Machado & Oliveira, 1998; Machado *et al.*, 2000).

Aspectos reprodutivos em opiliões

O dimorfismo sexual entre os opiliões é muito acentuado, principalmente na família Gonyleptidae. Os machos, em geral, são maiores, possuindo pernas mais longas e quelíceras mais dilatadas (Berland, 1949). Nas espécies de Laniatores, Dyspnoi e Eupnoi, os indivíduos se reproduzem durante todo o ano, podendo apresentar variações sazonais (Juberthie, 1965; Gnaspini, 1996).

O aparelho reprodutor masculino dos opiliões, nas poucas espécies estudadas, é constituído por um testículo tubular único, de onde partem dois ductos deferentes que desembocam em uma vesícula seminal. Apresentam um órgão propulsor para ejaculação e, finalmente, um pênis retrátil (Berland, 1949; Juberthie, 1965; Cokendolpher & Jones, 1991). O aparelho reprodutor feminino, por sua vez é composto por ovários unidos em forma de U, de onde partem dois delgados ovidutos que desembocam em uma bolsa. Desta bolsa segue um ducto estreito que se dilata em um útero riniforme. Do útero parte um longo ducto que apresenta continuidade com o ovipositor, um cilindro rígido revestido por quitina (Berland, 1949). Segundo Shear (2004), somente em algumas espécies de opilião foi encontrado um reservatório seminal nas fêmeas. Tanto o ovipositor quanto o pênis são estruturas projetadas para fora do orifício genital durante os processos de oviposição e cópula, respectivamente (Machado *et al.*, 2000).

Os opiliões apresentam, em geral, fecundação interna com transferência direta de espermatozóides (Shear, 1975), e a competição espermática é aparentemente muito alta (Thomas & Zeh, 1984). O processo de cópula ainda é pouco conhecido, tendo sido observado em poucas espécies; entretanto sabe-se que geralmente não é precedida por corte. As fêmeas colocam os ovos horas ou semanas após a inseminação (Juberthie, 1965) e o número de ovos varia de 20 a 30 em algumas espécies de Laniatores, até centenas em Eupnoi e Dyspnoi (Machado & Raimundo, 2001). Os ovos são depositados no substrato (solo, madeira, cavidades de troncos) e o cuidado à prole, parental ou maternal, pode ser observado em algumas espécies, conferindo proteção contra predadores e, em alguns casos, contra a disseminação de fungos (Mora, 1990). O canibalismo de ovos é muito comum

entre os opiliões (Canals, 1936; Capocasale & Bruno-Trezza, 1964; Edgar, 1971; Mora, 1990) favorecendo, portanto, a evolução do cuidado à prole.

Diversidade de espermatozóides na classe Arachnida

O filo Chelicerata possui mais de 100.000 espécies descritas e é considerado, entre os artrópodes, o grupo de maior diversidade morfológica de espermatozóides (Alberti, 1995).

Na classe Arachnida são encontradas desde espécies que apresentam espermatozóides de forma alongada flagelada, como na ordem Scorpiones, passando por forma espiralada, como nas ordens Pseudoscorpiones, Amblypygi, Araneae e Ricinulei, até formas esféricas aflageladas, como nas ordens Palpigradi, Solifugae, Acari e Opiliones. Em paralelo, podem variar em comprimento, apresentando espermatozóides de 1mm, como em carrapatos (Acari), até inferiores a 2μm, como em opiliões (Alberti, 1995). Segundo Baccetti (1979, 1985), a presença de espermatozóides espiralados e aflagelados são dois traços evolutivos importantes dentro da classe Arachnida.

O acrossomo é encontrado em todas as ordens de Arachnida, com exceção de algumas espécies em Acari e Opiliones (Alberti, 1979). É formado, em geral, por uma vesícula acrossomal e um filamento, também denominado *perforatorium*. Em algumas espécies são ainda observados materiais pré-, peri- e sub-acrossomais. O *perforatorium* é, em geral, considerado um material sub-acrossomal, sendo composto por filamentos de actina altamente ordenados (Alberti, 1995).

O núcleo apresenta cromatina densamente compactada, e pode ser filiforme, nos espermatozóides flagelados, ou ovóide, nos espermatozóides aflagelados. Adicionalmente, os núcleos filiformes podem ser espiralados, como em Pseudoscorpiones e Araneae, e ainda podem apresentar um enrolamento, como observado em Pseudoscorpiones, Amblypygi, Uropygi e Araneae (Alberti, 1983, Alberti & Weinmann, 1985; Boissin, 1974; Jespersen, 1978; Tripepi & Saita, 1985; Werner & Bawa, 1988). Nos espermatozóides aflagelados, o núcleo também apresenta cromatina densamente compactada, e sua morfologia pode variar entre oval, globular e em forma de U (Alberti, 1995).

O axonema, presente somente nos espermatozóides flagelados, pode apresentar padrão microtubular do tipo 9+3 (9 duplas periféricas + 3 centrais), como ocorre em Araneae, Uropygi e Amblypygi (Osaki, 1969; Phillips, 1976; Tripepi & Saita, 1985) ou 9+2 (9 duplas periféricas + 2 centrais), como ocorre em algumas espécies de Araneae (Alberti, 1990). Em Acari, Solifugae e Opiliones, os espermatozóides são aflagelados e, portanto, não existe formação de axonema nos espermatozóides. O único exemplo de ocorrência de axonema em espermatozóides aflagelados foi relatado no gênero *Siro* (Opiliones: Cyphophtalmi), mas neste caso o axonema é temporário, estando presente durante a espermiogênese e sendo perdido no espermatozóide (Juberthie *et al.,* 1976).

Mitocôndrias estão presentes tanto em espermatozóides flagelados quanto nos aflagelados. Nos flagelados, estas estão localizadas na peça intermediária, enquanto nos aflagelados, elas são encontradas em regiões variadas, como por exemplo, embebidas no núcleo (Alberti, 1980b, Alberti *et al.*, 1991; Juberthie *et al.,* 1976; Witalinski, 1982).

Materiais extracelulares ou secreções são responsáveis pela agregação dos espermatozóides em Scorpiones, Solifugae, Acari (Alberti, 1980b, 1984, 1988; Alberti &

Storch, 1976). Em opiliões Cyphophytalmi os agregados incluem espermatozóides dimórficos (Juberthie *et al.,*1976).

Em função da diversidade morfológica dos espermatozóides em inúmeros grupos de vertebrados e invertebrados, características espermáticas têm sido utilizadas para a inserção de novos caracteres em análises filogenéticas (Baccetti, 1970; Jamieson *et al.*, 1995). Nos artrópodes, em especial, esses caracteres também têm sido empregados de maneira comparativa na filogenia de diversos grupos (Baccetti, 1970; Alberti, 1995; Jamieson, 1987; Jamieson *et al.*, 1999). Dentro de Arachnida, entretanto, apenas as ordens Opiliones, Acari e Araneae apresentam trabalhos com considerações sistemáticas suficientemente amplas. Além da aplicação em problemas filogenéticos, a morfologia espermática em Arachnida também é utilizada em questões relacionadas à biologia da fertilização (Alberti, 1995).

Ocorrência de espermatozóides aflagelados

Considera-se um espermatozóide típico, uma célula germinativa masculina composta por uma cabeça, que apresenta o material genético; uma peça intermediária, que contém mitocôndrias responsáveis pela motilidade, e um flagelo, com o axonema (Baccetti & Afzelius, 1976). Essa descrição é também considerada a forma ancestral ou mais primitiva (Franzén, 1956). Por trás dessa forma típica, existe uma vasta diversidade morfológica de espermatozóides, que abrange desde variações de comprimento até arranjos entre seus componentes celulares (Morrow, 2004).

Um exemplo dessa diversidade ocorre em relação à estrutura flagelar, em que espermatozóides multi-flagelados são encontrados em muitos grupos de invertebrados, e uma única célula pode possuir até 100 flagelos, como na espécie de cupim *Mastotermes darwiniensis* (Baccetti & Dallai, 1978). Por outro lado, em outros grupos, assim como em Opiliones, ocorre a produção de espermatozóides desprovidos de qualquer estrutura flagelar (Juberthie & Manier, 1978).

Segundo Morrow (2004), espermatozóides aflagelados evoluíram independentemente em pelo menos 36 taxa, desde algas vermelhas até vertebrados. Em 20 destes taxa, os espermatozóides não apresentam qualquer motilidade coordenada e em 11, os espermatozóides apresentam uma leve vibração, como forma alternativa de movimento.

A competição espermática é identificada como uma das mais importantes forças evolutivas relacionadas às mudanças morfológicas observadas nos espermatozóides de um determinado grupo (Parker, 1970). Nas espécies que possuem competição espermática, a seleção natural tende a ser favorável à evolução da produção de pequenos espermatozóides em grande quantidade (Parker, 1982, 1998) ou ainda, de longos espermatozóides com alta motilidade (Katz & Drobnis, 1990). Em ambos os casos, a competição espermática seleciona a motilidade, uma vez que a produção de espermatozóides imóveis, ou com baixa motilidade, seria uma estratégia ineficiente. Assim nas espécies onde não ocorre competição espermática, parece existir uma tendência na seleção para produção de espermatozóides imóveis. Observações em alguns taxa indicam que existe uma gradual perda do flagelo, ocorrendo um estágio intermediário, onde o flagelo é imóvel nos grupos mais basais, até sua regressão total nos grupos mais derivados. Exemplos dessa ocorrência

podem ser observados em Ophryotrocha (Annelida) e Cecidomyiidae (Insecta) (Baccetti & Afzelius, 1976; Dallai, 1979), assim como em Opiliones.

Espermiogênese em opiliões

Os trabalhos existentes na literatura descrevem os aspectos gerais da espermatogênese em Opiliones e, muito poucos, a respeito da morfologia de seus espermatozóides. A grande maioria desses trabalhos data das décadas de 1970-80 (Reger, 1969; Juberthie *et al.*, 1976; Juberthie & Manier, 1977a, b, c, 1978; Tripepi, 1983; Jones & Cokendolpher, 1985) e em geral, o registro fotográfico é insatisfatório e as informações incompletas.

De maneira geral, a espermatogênese é um processo no qual células germinativas são transformadas de células indiferenciadas diplóides (espermatogônias) em espermatozóides haplóides altamente especializados (Sharpe, 1994). Este complexo processo envolve uma série de divisões celulares até a formação de espermátides, que por um processo de diferenciação celular, denominado espermiogênese, originarão os espermatozóides (Recco-Pimentel & Aguiar Junior, 2007).

Em todas as espécies de opiliões estudadas até o momento, os espermatozóides são desprovidos de flagelo e possuem formato oval. Entretanto, baseando-se nas variações morfológicas que ocorrem durante a espermiogênese em Opiliones, pode-se dividir esse grupo em dois processos: aquele no qual se formam espermátides transitoriamente flageladas, encontrado nas espécies de Cyphophtalmi, a sub-ordem mais basal, e aquele nas

quais espermátides e espermatozóides são completamente aflagelados, descrito nas demais sub-ordens. (Juberthie & Manier, 1978).

A espermiogênese nos Cyphophthalmi, estudada unicamente na espécie *Siro rubens* (Latreille, 1804) (Juberthie *et al.*, 1976; Juberthie & Manier, 1978) é caracterizada pelo desenvolvimento de invaginação citoplasmática, acrossomo com *perforatorium*, presença de um par de centríolos e formação transitória de espermátides flageladas. Durante o processo, forma-se um axonema que sofre gradual retração, restando somente um par de centríolos no espermatozóide. Além disso, os espermatozóides apresentam inúmeras microvilosidades em sua superfície. A presença de dimorfismo de espermatozóides é outra característica dos Cyphophthalmi, ou seja, ocorre a produção de dois tipos de espermatozóides por um mesmo indivíduo: um típico e outro aberrante (desprovido de acrossomo e cromatina).

A espermiogênese em Eupnoi, Dyspnoi e Laniatores é bastante semelhante (Juberthie & Manier, 1977a, b, c; Reger, 1969; Tripepi, 1983), não existindo formação de espermátides transitoriamente flageladas. O processo de formação de espermatozóides é caracterizado pelo desenvolvimento de invaginação citoplasmática, acrossomo, com mitocôndrias e membranas associadas, e presença de um par de centríolos. A formação do *perforatorium* e o nível de compactação cromatínica são variáveis.

Em Laniatores, sub-ordem a qual pertence *Iporangaia pustulosa,* somente 3 espécies tiveram sua espermiogênese descritas em nível ultra-estrutural (Juberthie & Manier, 1977c; Jones & Cokendolpher, 1985), entretanto, sem o devido detalhamento da morfologia dos espermatozóides formados.

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OBJETIVOS

Levando-se em consideração a escassez de informações a respeito da biologia reprodutiva em Opiliones, bem como o processo de espermiogênese e a morfologia dos espermatozóides formados, o presente estudo investigou a espécie *Iporangaia pustulosa* com os objetivos de:

- Descrever a ultra-estrutura da espermiogênese visando compreender melhor o processo de formação dos espermatozóides aflagelados;
- Analisar a ultra-estrutura dos espermatozóides tanto no testículo como na vesícula seminal;
- Evidenciar a presença de proteínas básicas em diferentes estruturas das células germinativas masculinas, a fim de se obter uma marcação morfológica das estruturas durante sua formação;
- Comparar os resultados aqui obtidos com aqueles existentes na literatura para outras espécies de opilião, identificando possíveis diferenças morfológicas, contribuindo para uma melhor compreensão da espermiogênese e da biologia reprodutiva desta espécie e dos demais opiliões.

RESULTADOS

A partir dos resultados obtidos com *Iporangaia pustulosa* foram elaborados 2 artigos científicos.

O primeiro trabalho é um manuscrito completo, redigido em inglês, que trata da descrição estrutural, ultra-estrutural e citoquímica da espermiogênese e dos espermatozóides testiculares.

O segundo trabalho refere-se a um artigo publicado no periódico *Arthropod Structure and Development*, que descreve a morfologia dos espermatozóides vesiculares, bem como a estrutura do aparelho reprodutor da espécie mencionada.

ARTIGO 01

SPERMIOGENESIS OF *Iporangaia pustulosa* (ARACHNIDA: OPILIONES: LANIATORES)

SPERMIOGENESIS OF *Iporangaia pustulosa* (ARACHNIDA: OPILIONES: LANIATORES)

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ABSTRACT

The present study described the spermiogenesis of the harvestman *Iporangaia pustulosa* (Arachnida: Opiliones: Laniatores). Adult males were dissected and their testes were processed for light microscopy and by conventional and E-PTA methods for transmission electron microscopy. In general, the testis presents uniform populations of germ cells arranged in cysts, which give a compartmentalized aspect. Early spermatids present chromatin poorly condensed at the nucleus periphery, large cytoplasm, acrosomal vesicles and the beginning of a cytoplasmic invagination. During the spermiogenesis, the cytoplasm is gradually cast off by vesicles. The chromatin appears as thin filaments, thickening into strands, until a homogeneous compacted mass can be found in the spermatozoa. The acrosome is formed by many vesicles from the Golgi complex. It is located, initially, near the nucleus and then attached to it, being encircled by vesicles and mitochondria. The cytoplasmic invagination, opposite the acrosome region, presents a granular aspect, different from the cytoplasm and has a pair of centrioles in its opening. The spermatozoon is oval and devoid of a flagellum. It presents a compact nucleus, a dense acrosome, with a subacrosomal compartment and mitochondria associated, and a periform cytoplasmic invagination with a centriole. The E-PTA technique presented positive staining in the poorly condensed chromatin and in the centriolar proteins, while the dense chromatin, the invagination opening and the acrosome stained negatively. These data provide the most complete description of a Laniatores spermiogenesis and their sperm morphology.

INTRODUCTION

Harvestmen (Arachnida: Opiliones) are distributed all over the world and are grouped in 4 suborders: Cyphophthalmi, Eupnoi, Dyspnoi and Laniatores (Giribet *et al.*, 2002). The Cyphophthalmi is the basal and smallest group, with species distributed around the world. The Eupnoi and Dyspnoi are mostly found in the temperate regions, whereas the Laniatores, that comprise about 3,500 species, are concentrated in the tropical region, especially in South America (Shultz, 1998).

Brazil has the richest harvestman fauna in the world, with about 950 species (Pinto-da-Rocha, 1999) of the 6,000 described in the order (Cokendolpher & Lee, 1993). The Brazilian states of São Paulo and Rio de Janeiro have the most complete studies of harvestmen fauna of this country, with 232 and 212 described species, respectively. In the other states the data is still incomplete.

The male reproductive tract of *Iporangaia pustulosa* (Mello-Leitão, 1935), the species studied here, as well as in other harvestman species, is composed of a tubular U-shaped testis connected to two deferent ducts that transfer spermatozoa into a large seminal vesicle. Following the seminal vesicle, there are a propulsive organ and an eversible penis (Berland, 1949; Juberthie, 1965; Cokendolpher and Jones, 1991; Moya *et al.*, 2007). Spermatogenesis occurs in the testis, resulting in a population of germ cells in different stages of differentiation. In the seminal vesicle, however, the spermatozoa are concentrated and stored until fertilization (Juberthie and Manier, 1978; Moya *et al.*, 2007).

The spermatozoa of *I. pustulosa* present a U-shaped nucleus that surrounds a cytoplasmic invagination, as well as a complex acrosome (Moya *et al.*, 2007). In the

seminal vesicle, projections are observed covering its surface. As a representative species of the suborder Laniatores, *I. pustulosa* has aflagellated sperm. This sperm type is apparently produced by all Opiliones species, except for members of the suborder Cyphophtalmi, which retain a non-motile axoneme in the sperm (Alberti, 1995, 2000, 2005).

Ultrastructural description of the Laniatores spermatozoa is known for only 8 species, including *I. pustulosa* (Juberthie and Manier, 1976, 1977c; Jones and Cokendolpher, 1985; Moya *et al.*, 2007). These studies describe general aspects of spermiogenesis and present little information about the spermatozoan morphology.

Studies of sperm morphology, furnishing a basis for understanding the spermatogenetic process, are often used in phylogenic and taxonomic studies (Alberti and Peretti, 2002; Alberti, 2005).

The present study described the spermiogenesis in the harvestman *I. pustulosa*, with an ultrastructural view, to improve the knowledge concerning the reproductive biology and to provide new information about the spermiogenesis and spermatozoa in the order Opiliones.

MATERIAL AND METHODS

Adult males of *Iporangaia pustulosa* were collected at the Parque Estadual Intervales (24°14'S; 48°04'W; 800 m alt.), close to the municipality of Ribeirão Grande, southern of São Paulo state, Brazil (Leonel, 1994). The males were dissected in 0.1M sodium cacodylate buffer and their testes were processed for light and transmission electron microscopies.

Testes were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde with 3% sucrose in 0.1M sodium cacodylate buffer, for 12-72 hours at 4ºC. They were post fixed in 1% osmium tetroxide in the same buffer for 3-5 hours, dehydrated in acetone and embedded in epoxy resin. Other testes were also fixed in 2.5% glutaraldehyde and 1% tannic acid in 0.1M sodium cacodylate buffer, with 1.5% sucrose and 5mM calcium cloride for three days at 4ºC. They were washed in the same buffer and stained with 1.5% uranyl acetate for 2 hours at room temperature before dehydration in acetone and epon embedding (Dallai & Afzelius, 1990). Thin sections were cut at 1-2µm, mounted on microscope slides, stained with toluidine blue, pH 4.0 and photographed with a light microscope. Ultra-thin sections, stained with uranyl acetate and lead citrate, were observed in the electron microscope.

For basic proteins detection, testes were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer with 1.5% sucrose and 5mM calcium chloride for 4 hours at 4ºC. After washing in the same buffer, they were dehydrated in ethanol and then stained with 2% phosphotungstic acid in absolute ethanol (E-PTA). The fragments were infiltrated for 3 days and embedded in epoxy resin.

RESULTS

The testis of *Iporangaia pustulosa* is a single U-shaped tubule organ that presents populations of germ cells in various stages of differentiation, arranged in cysts along the testis length, which gives a compartmentalized testis organization (figs. 1A-F). There is a tendency of cysts containing the earliest stages of differentiation to be located in the testis periphery while cysts with the latest stages concentrate preferably in the testis center (figs. 1A and B). Sequential cross sections showed that this centripetal orientation occurs along all the testis´ extension.

Spermatogonias and spermatocytes are dense cells with large amount of cytoplasm and a spherical nucleus, weakly stained. The cysts containing these cells present cell junctions and intercellular bridges. Spermatids present a reduced cytoplasmic volume and a cytoplasmic invagination invades the nucleus, which can be seen by the weak central stain (figs. 1C-E). The spermatozoa are aflagellated cells, oval-shaped and tapering to fine points. They can be observed in cysts or freely dispersed in the testis center immersed in a secretion, depending on the developmental stage and testicular maturation (figs. 1A, B, D and F).

The entire process of spermatogenetic development is carried out in cysts bordered by somatic cells (or cystic cells) (figs. 2A and B, 3D, 7A and B). In the first differentiation steps, each cyst contains numerous germ cells in the same developmental stage, while the spermatid cysts may contain cells in different stages (figs. 2A, 8A and B). Therefore, cells in all developmental stages can be seen in sections for electron microscopy, which cover a small area (figs. 2A and B, 3D, 7A and B).

In the first steps of spermiogenesis, the early spermatids have an irregular shape and their chromatin is irregularly aggregated at the nucleus periphery (figs. 2A and B). The cytoplasm is rich in endoplasmic reticulum and mitochondria (figs. 2A and D). In this phase, the centriole can be identified in a small indentation of the nuclear membrane (fig. 2A). This indentation marks the opening of the future cytoplasmic invagination, which will invade the nucleus. A reorganization of the nuclear membrane occurs, as chromatin condensation progresses, and portions of cast off membrane with aggregated nuclear pores can be found in the cytoplasm (fig. 2C). In the cytoplasm, near the nucleus, vesicles produced by the Golgi complexes begin to contribute to the acrosome formation (fig. 2D). Simultaneously, at the opposite pole of the cell, an invagination of cytoplasm begins to penetrate the nucleus (fig. 2E). As a result, these opposing features determine a polarized cell where the invagination region is opposed to the acrosome region.

In the next steps, the cytoplasmic invagination expands into the nucleus, and membranes enveloping cytoplasmic elements occupy the opening of this invagination. As a result, the entrance is occupied by these membranes, isolating the invaginated portion of the cytoplasm from that surrounding the nucleus (fig. 2F). The E-PTA technique showed that these membranes are stained negatively (fig. 2G). The chromatin condensation occurs in a centripetal pattern (fig. 2F). In this stage of chromatin aggregation, it is still E-PTA positive, while the clear central region is negative (fig. 2H). Mitochondria, specifically their matrix, also appear strongly E-PTA negative (fig. 2H).

The innumerable pre-acrosomic vesicles originating from the Golgi complexes, in the following stage, fuse into a single large acrosomic vesicle, which is located in the cytoplasm, near the nucleus (fig. 3A). The latter contains chromatin still loosely compacted

and encased in a nuclear envelope with pores. During this phase, the acrosomic vesicle is affixed to a specific region of the nuclear membrane, lined by dense material, and positioned opposite to the cytoplasmic invagination that has begun to invade the nuclear center (fig. 3B). On the cytoplasmic side of the invagination opening, a pair of centrioles, arranged orthogonally so that one is observed longitudinally while the cross-sectioned centriole shows the typical pattern of nine triplets of microtubules (fig. 3C).

In intermediary spermatids, the irregular cell shape is lost and it becomes increasingly rounded, with its center dominated by the growing cytoplasmic invagination, which is distinct from the extra nuclear cytoplasm (figs. 3D and E). Also in this phase, the cytoplasm of the posterior portion of the cell, surrounding the invagination aperture, is filled with a compact layer, or manchette, of microtubules that is identified in smaller magnifications as a denser cytoplasmic layer covering the nucleus (figs. 3D and E). This manchette extends along the length of the cell on the invagination side of the nucleus (figs. 3D and E). With gradual elimination of the cytoplasm, the few remaining organelles congregate near the microtubule manchette (fig. 3D). In these intermediate spermatids, the acrosome is accommodated in a large nuclear depression and the chromatin now undergoes reorganization forming entwined filaments with a regular diameter (figs. 3D and E). They appear electron dense in routine preparations for electron microscopy and electron transparent, or negative, when using the E-PTA method (fig. 3F). These filaments that are initially fine, grow thicker and with varied thickness as spermiogenesis progresses (fig. 3G). Fine bridges interconnect the microtubules without, however, making a very precise microtubule arrangement (fig. 3G).

The progressive chromatin condensation leads to thicker, but also denser fibers (figs. 4A and C). This greater density is confirmed by the E-PTA method, in which the condensed chromatin fibers are negative, staining only on the surface of each fiber, which makes them stand out clearly (figs. 4B and E). The acrosome, that is very dense in this stage, is stained by routine methods for ultrastructural observation (fig. 4A) but is negative with the E-PTA treatment (fig. 4B). A subacrosomic compartment appears in this phase, and is strongly E-PTA positive. The presence of a centriole is still detected at the opening of the invagination (fig. 4C), but can be detected with difficulty when using the cytochemical method which only stains the centriolar proteins, and not the microtubules that are not, therefore, well defined (fig. 4E). Microtubules are aligned along the nucleus, lining the invagination side (figs. 4C and D).

In the final stages of spermiogenesis, the late spermatids have greatly reduced cytoplasm, densely compacted chromatin, a cytoplasmic invagination with areas of different densities and an acrosome immersed in a deep nuclear cavity (fig. 5A). Also in this final differentiation stage, it is possible to find a centriole at the opening of the invagination (fig. 5B) and the manchette of microtubules in the remaining cytoplasm (fig. 5C).

The testicular spermatozoa are shaped as an elongated oval and characteristically have the surrounding cytoplasm reduced to a fine layer, which no longer contains microtubules, a densely compacted chromatin, an invagination with few areas of lesser density and an acrosome located in a deep depression (fig. 5D). The previously irregular plasma membrane has now attained a discrete and regularly scalloped arrangement covering a fine layer of dense cytoplasm (figs. 5D and E). With the E-PTA technique, the

compacted chromatin and the cytoplasmic invagination appear E-PTA negative, while the central and peripheral proteins of a centriole, located at the opening of the invagination, appear lightly positive (fig. 5F).

The acrosome has a variable morphology during the different phases of spermiogenesis, and even in fully formed spermatozoa. Acrosomal development during spermiogenesis begins with the formation of an acrosomic vesicle near the nucleus of early spermatids (fig. 3A). Later, this vesicle is attached to the nucleus by a dense layer and fits into a nuclear depression (figs. 3B and E), as previously mentioned. Beginning with an early globular shape (fig. 3A), the acrosome gradually acquires a rectangular/columnar format (fig. 6A). Subsequently, vacuolization occurs around the acrosome (fig. 6B). Part of the vesicles remains in the spermatozoon, near the top of the acrosome (figs. 6C and D). Vesicle remodeling is suggested by the opening of some vesicles to the extra cellular medium (fig. 6D). A subacrosomic compartment, in contact with the nucleus, is observed even from the first stages of the acrosomic insertion (figs. 4B and 6A-D); first, with a granular aspect (fig. 6A) and then, with a dense amorphous appearance (figs. 6B-D). Some mitochondria are also found near the acrosome (fig. 6A, C and D).

In relation to the testicular cyst organization, late spermatids, with condensed nuclei, irregular cytoplasm and the microtubule manchette are still maintained inside cysts (fig. 7A), while spermatozoa can be found inside cysts (fig. 7B) or, later, dispersed in the lumen, located in the testis center (fig. 7C).

The great condensation and gradual elimination of almost all the cytoplasm in spermatids, during the process of spermiogenesis, leads to a strong reduction of the germ cell size, resulting in greatly reduced sperm dimensions (figs. 8A and B).

FIGURE LEGENDS

Fig. 1A-F: Light microscopy. Testis of *Iporangaia pustulosa*. **(A)** and **(B)** Testis organization, showing the centripetal orientation, with cysts of early cells (asterisks) in the periphery, surrounding spermatozoa (z). Note cysts of spermatids (t) and spermatozoa dispersed in the testis lumen. **(C)** to **(E)** Early germ cells (asterisks) can be seen in the testis periphery. Note the volume cell reduction and the nuclear condensation of the spermatids (t). **(F)** Spermatozoa located, preferably, in the testis center. Note that the spermatozoa can be seen in cysts or dispersed in the lumen.

Scale bars: 25μm

Abbreviations: spermatid (t); spermatozoa (z).

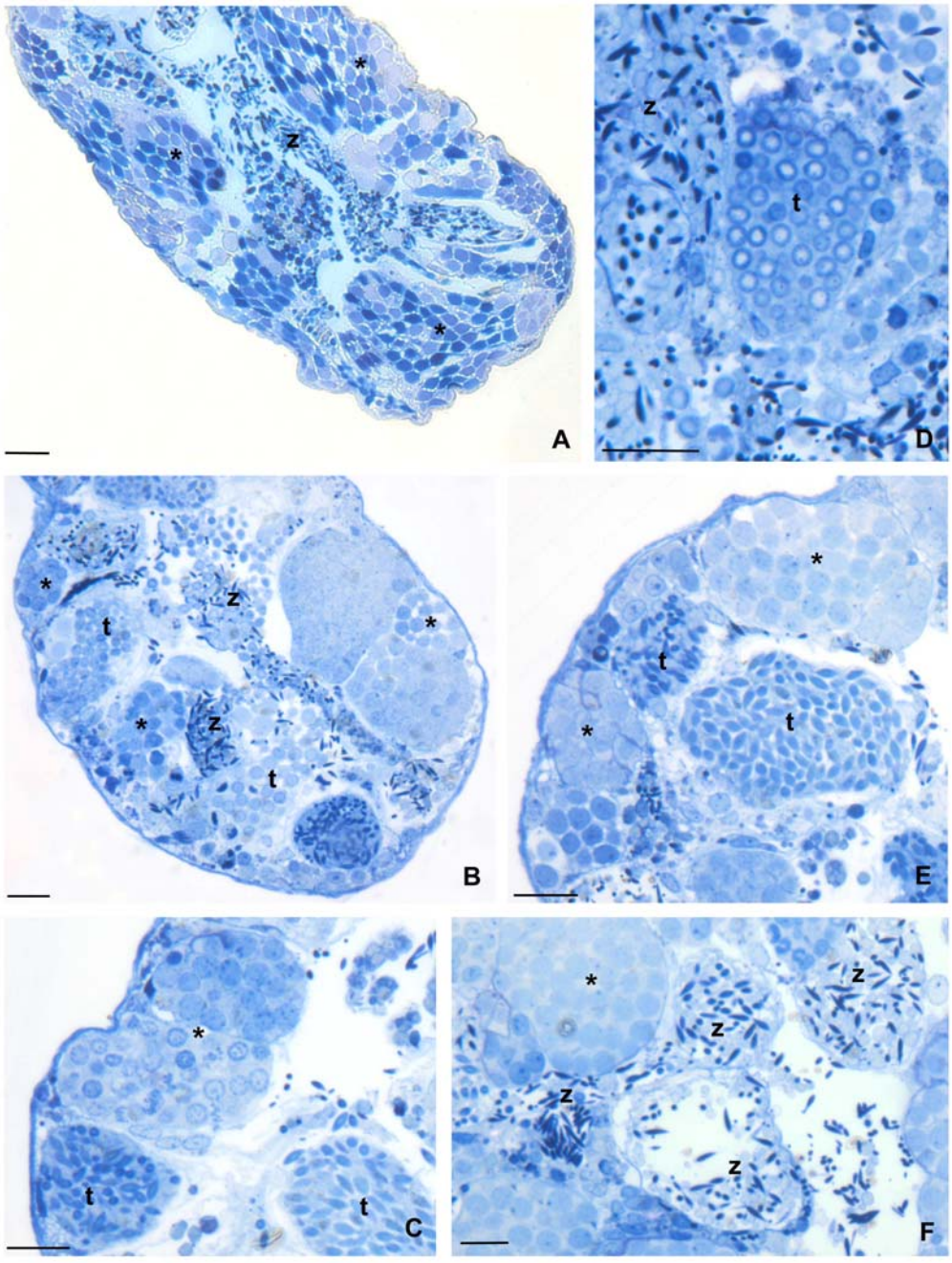
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Fig. 2A-H: Early spermatids. **(A)** Initial chromatin condensation (asterisks). Note the large cytoplasmic volume with several organelles and the presence of a centriole (black arrow) near the nucleus. **(B)** The chromatin condensation (asterisk) starts at the nuclear periphery. **(C)** As result of the chromatin condensation and nuclear reduction, pore complexes are eliminated into the cytoplasm. **(D)** Beginning of acrosome development. The Golgi complex produces many vesicles to constitute the acrosome. **(E)** Opposite to the nucleus, the cytoplasmic invagination initiates with a small opening (double arrowhead). **(F)** As the cytoplasmic invagination develops, membranes (white arrows) can be seen at the opening of the invagination. Note the chromatin condensation (asterisk). **(G)** and **(H)** The E-PTA method shows that the cytoplasmic invagination opening and mitochondria are E-PTA negative, while the loosely condensed chromatin (asterisks) is E-PTA positive.

Scale bars: 1µm

Abbreviations: cystic cell (cc); cytoplasm (c); cytoplasmic invagination (ci); endoplasmic reticulum (er); Golgi complex (gc); mitochondria (m); nucleus (n); pore complexes (p); spermatozoon (z); vesicles (v).

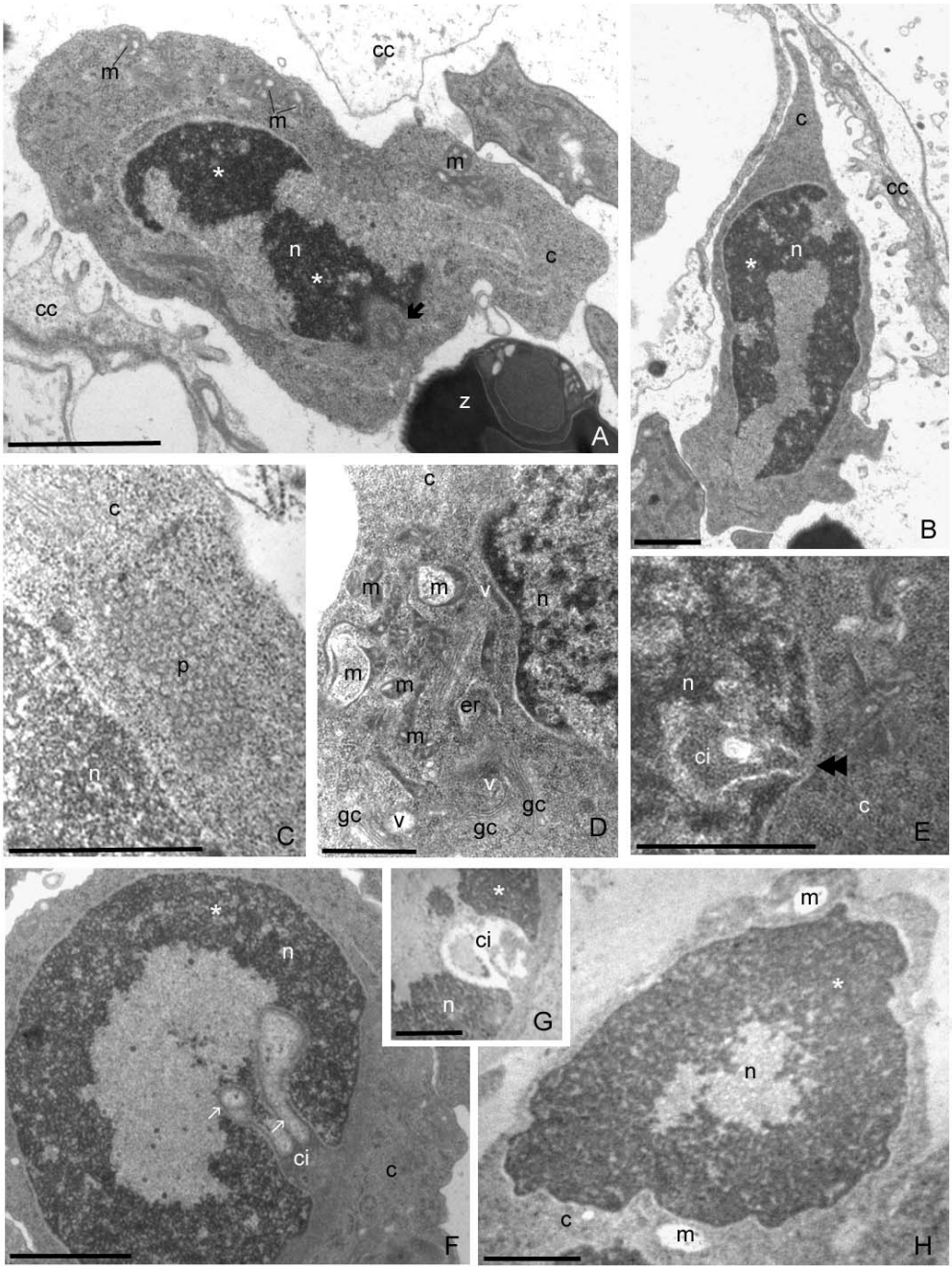
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Fig. 3A-G: Spermatids in higher levels of condensation. **(A)** A single acrosomal vesicle is formed near to the nucleus. Note the presence of mitochondria near the vesicle and pore complexes. **(B)** Acrosome attachment (open arrow) on the nucleus. On the opposite side, the cytoplasmic invagination presents several membranes (white arrows). Note the progress of chromatin condensation. **(C)** Two orthogonal centrioles at the opening of the cytoplasmic invagination. **(D)** and **(E)** Acrosome attachment in a nuclear cavity (open arrows). Note the progress of chromatin condensation, with short, thin filaments, presence of membranes (white arrows) in the cytoplasmic invagination and microtubules in the opposite side of the acrosome. **(F)** The E-PTA method shows that the cytoplasmic invagination is E-PTA negative, while the condensed chromatin presents E-PTA negative filaments surrounded by positive granules. **(G)** Increased chromatin condensation, showing dispersed thick dense fibers. Note the microtubular bridges interconnecting the microtubules (black arrows).

Scale bars: 1µm; Fig. C: 0,5µm

Abbreviations: acrosome (a); cystic cell (cc); cytoplasmic invagination (ci); microtubules (mt); mitochondria (m); nucleus (n); pore complexes (p).

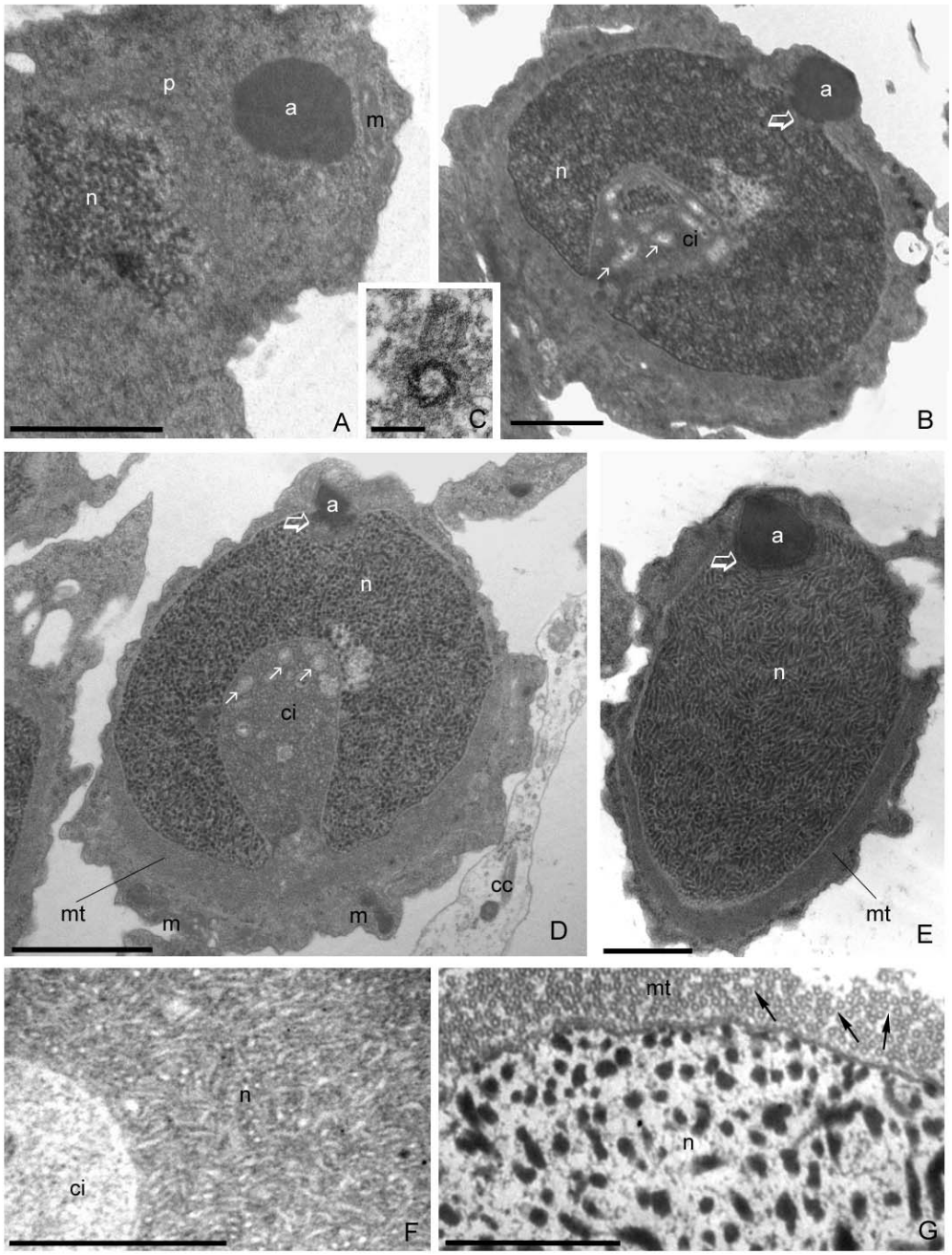
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Fig. 4A-E: Intermediary spermatids. **(A)** The spermatid assumes almost its final shape. The chromatin presents thick compacted strands. The acrosome is totally inserted in the nuclear cavity and some mitochondria are located near to the acrosome. Note the granular aspect of the cytoplasmic invagination. **(B)** The E-PTA method shows that the acrosome is strongly E-PTA negative, while the subacrosomal material is E-PTA positive. Also, the thickening chromatin fibers are E-PTA negative but react positively on their surface. Notice the E-PTA positive cytoplasmic invagination. **(C)** Progressive chromatin condensation, showing very thick compacted strands. Note the granular aspect of the cytoplasmic invagination, the centriole (arrow) and the microtubule manchette in longitudinal section. **(D)** Detail of the microtubule manchette in cross section. **(E)** The E-PTA method shows that the very thick compacted chromatin strands are E-PTA negative, but coated with a positive layer. Notice the E-PTA positive cytoplasmic invagination and the lightly stained centriole in the opening (arrow).

Scale bars: 1µm

Abbreviations: acrosome (a); cytoplasmic invagination (ci); microtubules (mt); nucleus (n).

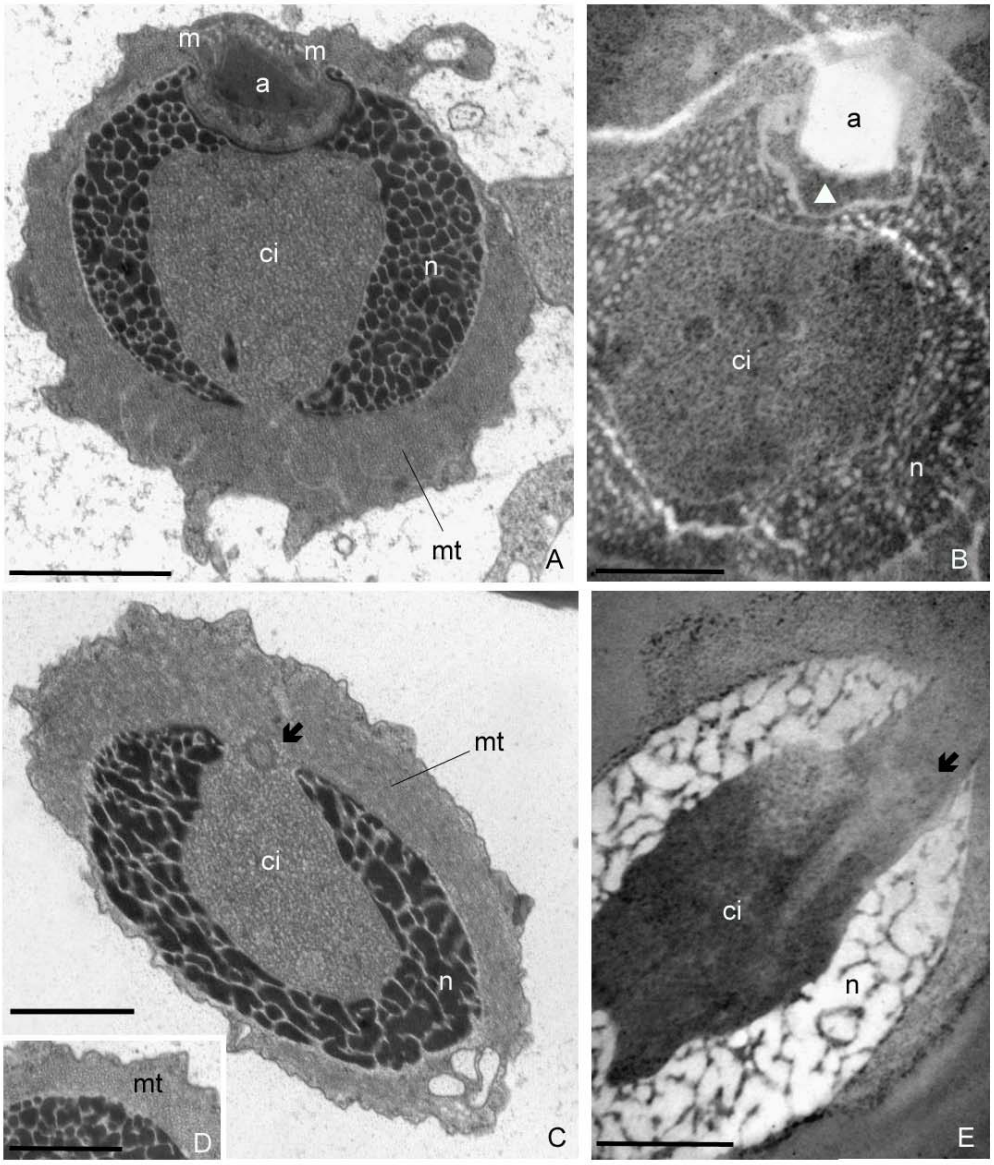
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Fig. 5A-F: Late spermatids and spermatozoa. **(A)** Late spermatid with highly condensed, homogenous chromatin and reduced cytoplasm. The cytoplasmic invagination presents some electron lucid points. **(B)** A centriole at the opening of the invagination. **(C)** In this stage, nuclear condensation is complete but microtubules are still observed. **(D)** Spermatozoa showing the absence of cytoplasm and microtubules. The sperm cells are oval-shaped and present a compacted nucleus, cytoplasmic invagination and acrosome with some associated mitochondria. The cell surface presents regular scallops filled with dense material (double arrows). **(E)** Detail of the cell surface, where regular scallops are filled with dense cytoplasm material. **(F)** The E-PTA method shows that the homogenously condensed chromatin and the cytoplasmic invagination are E-PTA negative, with the latter very strongly negative. Note the presence of a centriole (black arrow), in which the associated proteins are E-PTA positive.

Scale bars: 1µm; figs. B and F: 0,25µm; fig. E: 0,1µm.

Abbreviations: acrosome (a); cytoplasm (c); cytoplasmic invagination (ci); microtubules (mt); mitochondria (m); nucleus (n).

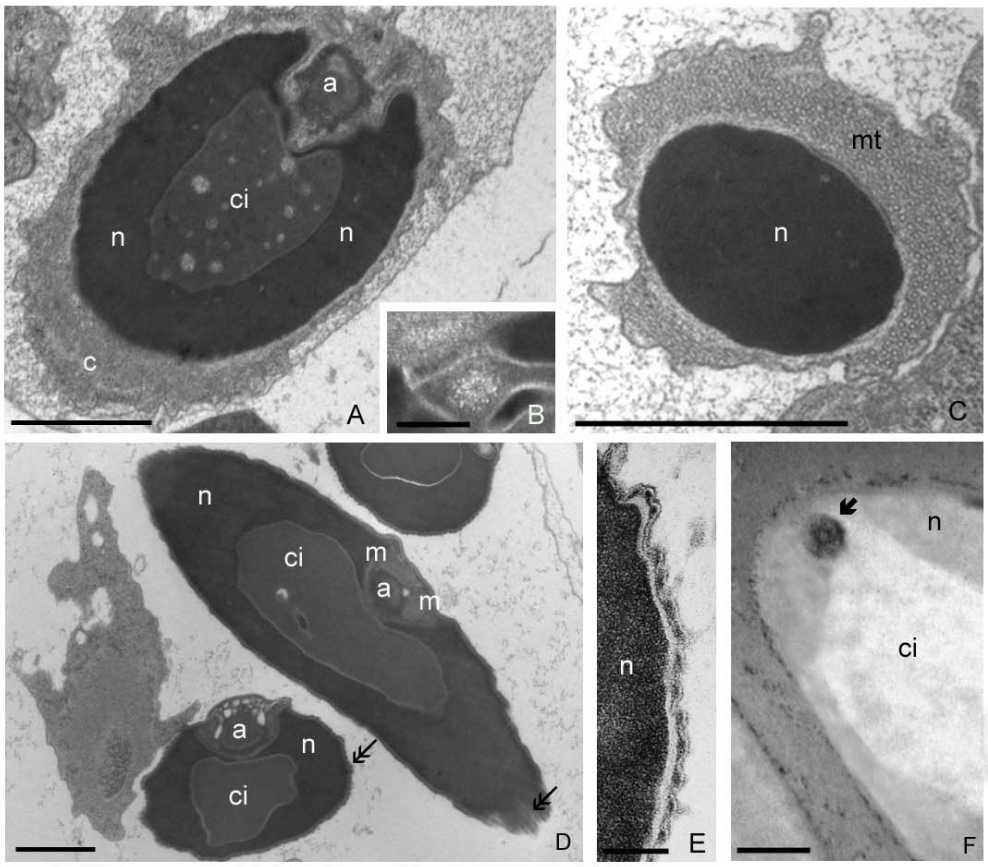
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Fig. 6A-D: Acrosome morphology. **(A)** Intermediate spermatid showing mitochondria associated with the acrosome. The subacrosomal material (arrowhead) has a granular aspect. **(B)** Late spermatid showing the abundance of vesicles near the top of the acrosome. The subacrosomal material (arrowhead) presents an amorphous, dense aspect. **(C)** and **(D)** Spermatozoa showing the acrosome almost in its final shape, with associated mitochondria and some vesicles. The subacrosomal material (arrowhead) presents a dense aspect. The arrows show the elimination vesicles.

Scale bars: 1µm

Abbreviations: acrosomal vesicles (av); acrosome (a); cytoplasmic invagination (ci); mitochondria (m); nucleus (n).

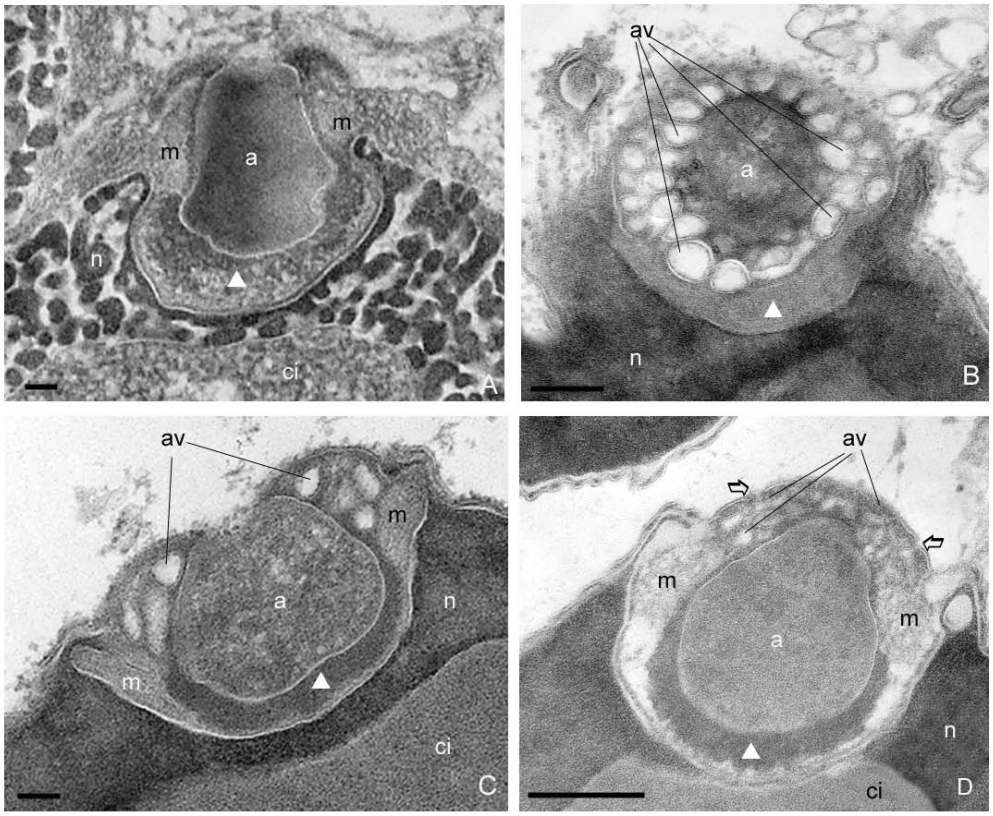
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Fig. 7A-C: Organization of late spermatids and spermatozoa in the testis. **(A)** Late spermatids immersed in a cyst with cystic cells. **(B)** Spermatozoa immersed in a cyst with cystic cells. **(C)** Spermatozoa dispersed in the testis lumen.

Scale bars: 1µm

Abbreviations: cystic cells (cc); lumen (lu).

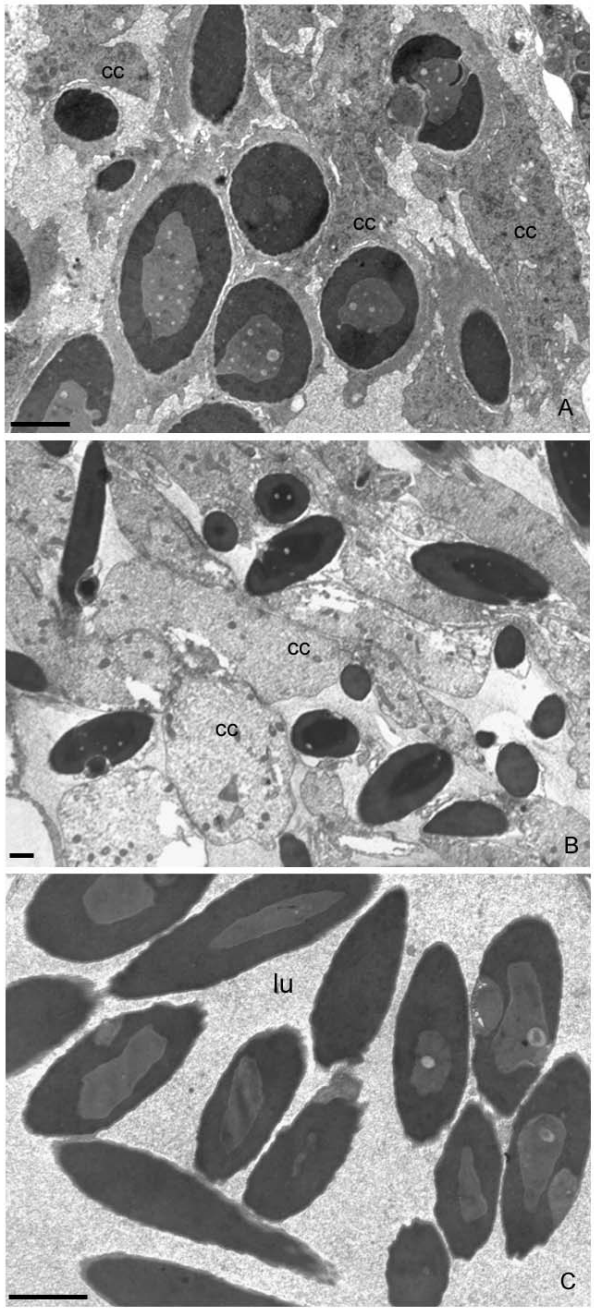
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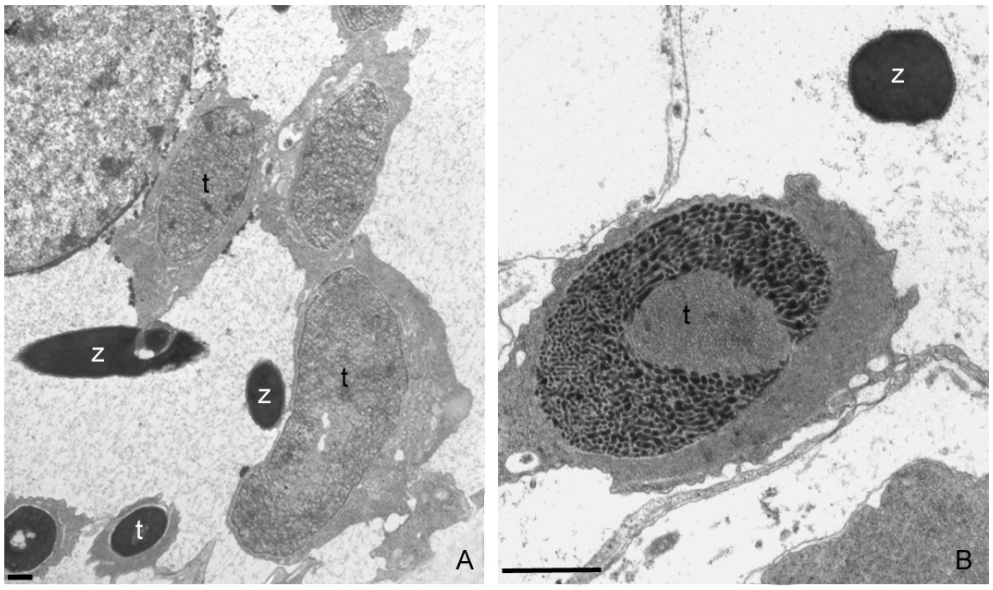
Fig. 8A-B: Reduction of the cell size during the spermiogenesis.

Scale bars: 1µm

Abbreviations: spermatids (t); spermatozoa (z)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DISCUSSION

Besides this study of *Iporangaia pustulosa* (Gonyleptidae), only three other Laniatores species have had their spermiogenesis described ultrastructurally: *Cynorta cubana* (Banks, 1909) (Cosmetidae), *Strisilvea cavicola (*Roewer, 1927) (Phalangodidae) (Juberthie & Manier, 1977c) and *Vonones sayi* (Simon, 1879) (Cosmetidae) (Jones & Cokendolpher, 1985). Of these descriptions, the species *V. sayi* is the most detailed and has the closest resemblance to *I. pustulosa.*

The reproductive tract of *I. pustulosa* has the same components as previously described for other harvestman species (Berland, 1949; Juberthie, 1965; Cokendolpher & Jones, 1991), containing a tubular testis where the spermatozoa are produced to be transferred to the large seminal vesicle, suggesting that males are able to produce large amounts of sperm (Birkhead & Moeller, 1998).

The testis organization of *I. pustulosa*, with all developmental stages arranged in cysts that are present along the entire testis length was also reported for the Eupnoi, *Phalangium opilio* (Linneaus, 1758) (Tripepi, 1983) and to the Laniatores, *V. sayi* (Jones & Cokendolpher, 1985). However, in the latter the occurrence of a centripetal spermiogenesis, as occurs in *I. pustulosa* and *P. opilio*, was not reported. In this Laniatores, early spermatid cysts are often bordered by late spermatid cysts, and no linear or belted arrangement of developing sperm are observed. In *I. pustulosa*, as in the Eupnoi, *P. opilio* (Tripepi, 1983), cysts containing early stages are located in the testis periphery; while spermatozoa are seen in the testis center. This organization, suggests that this final differentiation stage is ready

to be transferred to the seminal vesicle by the deferent ducts, which connect with the testis lumen.

The cystic spermatogenesis, as observed in *I. pustulosa*, is a common characteristic in the Opiliones order. The testis compartmental arrangement indicates that the spermatozoa develop in cysts, liberating the sperm into a central duct, or lumen, and finally, in the seminal vesicle, where a large amount of sperm is stored.

In *I. pustulosa*, in the early stages of spermatogenesis, spermatogonia and spermatocyte cysts present cell junctions and intercellular bridges. Thus, the contact between the cells is specific, and only one population type of cells is found in the cysts. On the other hand, in the spermatid and spermatozoa cysts, cell junctions or intercellular brigdes were no longer observed, permitting a loss of coordinated development and leading to mixed populations of younger and more mature cells, together in the same cyst. However, intercellular bridges in very young spermatids were frequently observed in the Cyphophtalmi *Siro rubens* (Latreille, 1804) (Juberthie *et al.*, 1976), the Eupnoi *P. opilio* (Tripepi, 1983) and the Laniatores *V. sayi* (Jones & Cokendolpher, 1985).

The spermiogenesis of *I. pustulosa* is similar to that described of other Laniatores species. It is characterized by the absence of flagellum formation, the kinetic center is reduced to two centrioles of the 9+0 type, the formation of a microtubular manchette under the nucleus, an acrosome composed of a dense material and some associated mitochondria.

Centripetal chromatin compacting observed in early *I. pustulosa* spermatids also occurs in the Laniatores, *V. sayi* (Jones & Cokendolpher, 1985), where the nuclear center is filled with chromatin that is not yet compacted, while chromatin condensation begins at the nuclear periphery. In the Eypnoi, *P. opilio* (Tripepi, 1983), the chromatin of spermatids is

rearranged into small dispersed granules that migrate to the nuclear periphery, accumulating in dense masses. On the other hand, in the Eupnoi, *Leiobunum* sp., the nuclear membrane becomes totally fused in the more advanced spermatids and the chromatin is transformed into filaments that are dispersed in the nucleus (Reger, 1969). The phases of chromatin condensation during spermiogenesis of Opiliones are very similar for the different species studied, but the final form found in the spermatozoa may vary considerably. In *I. pustulosa*, as also occurs in the Laniator, *V. sayi* (Jones & Cokendolpher, 1985), chromatin condensation begins at the nuclear periphery, obtaining a homogeneous condensation into fine dispersed filaments, then thick fibers that become totally compacted in the spermatozoa. In the Dyspnoi, *Ischyropsalis luteipes* (Simon, 1872) (Juberthie & Manier, 1976), the spermatozoan nuclei are very poorly compacted, consisting in scattered dense fibers. In the other known species the chromatin is irregularly compacted (Reger, 1969; Juberthie *et al.*, 1976; Juberthie & Manier, 1976, 1977a, b; c; Tripepi, 1983).

The earliest spermatids with loose chromatin have irregular nuclear membranes with abundant nuclear pores. As the chromatin condenses, it can no longer transcribe RNA and becomes inactive. As a consequence, the membrane near the condensed chromatin does not need pores and these are removed from the membrane as it is diminished to accompany the shrinking nucleus. Nuclear remodeling implies in the elimination of part of the nuclear membrane and pores that existed in the round spermatids. Membranes with nuclear pores can be found in the cytoplasm of spermatids of many different animals during nuclear condensation, and will be eliminated together with the excess cytoplasm. These structures can form complex arrangement, called *annulate lamellae*, in cells such as oocytes and cancerous cells (Kessel, 1992). Without pores, the two nuclear membranes can be

distinguished by the regular, clear space between them. Reger (1969) and Jones & Cokendolpher (1985) claimed that the two nuclear membranes fused in advanced spermatids of the studied species but this does not seem to be a general characteristic.

Like the other Laniatores species studied, *I. pustulosa* has a microtubular apparatus during spermiogenesis, which is used for shaping during the cellular transformations. According to Phillips (1974) the function of the microtubular manchette is related to cytoplasm redistribution during spermiogenesis. In fact, in *I. pustulosa*, the microtubules are found in great quantity near the opening of the cytoplasmic invagination. Besides this, microtubules are observed around the nucleus, probably contributing to chromatin condensation and to the final cell format.

Bridges interconnecting microtubules in durable arrangements are important to give the tubules a greater stability and a stronger interaction. They are seen in trypanosomes and cytostomes of protozoans, for example, where the associations of microtubules have a skeletal function. This is probably the function of the microtubule sheath on the invagination side of the spermatids of *I. pustulosa*.

Since the germ cells are aflagellate, the centrioles are the only microtubular structures present in the large majority of the Opiliones studied to date. They can be found throughout spermiogenesis, remaining as the only microtubular structure in the spermatozoa. In Cyphophtalmi, besides the presence of a pair of centrioles in spermatids, microtubule doublets occur dispersed in the cytoplasm. However, in spermatozoa, the pair of centrioles are no longer encountered, and only the doublets are retained. On the other hand, in Eupnoy, Dyspnoi and Laniatores, including *I. pustulosa*, microtubule doublets were never observed in any stage of spermiogenesis, while the centriole pair is present

during spermiogenesis and maintained in the spermatozoa of some species. The exceptions occur among the Laniatores, *V. sayi* (Jones & Cokendolpher, 1985), in which no centriole was identified during the entire spermiogenesis process, and in the Eupnoi, *Leiobunum* sp. (Reger, 1969), in which centrioles were not identified in the spermatozoa. The presence of microtubule doublets in Cyphophtalmi can be attributed to the fact that in this first suborder, a temporary flagellum is developed. Thus, these doublets are the result of the disorganization/regression of this axoneme.

During all of the spermiogenesis of *I. pustulosa*, organelles were never found inside the cytoplasmic invagination, only amorphous membranous bodies that appear to be part of the invagination process. On the other hand, in *V. sayi* (Jones & Cokendolpher, 1985), the organelles migrated to the invagination region, conferring a polarization to the cells. However, in later stages Jones & Cokendolpher (1985) affirmed that only the cytoplasmic matrix of uniform density is incorporated into the invagination. In *I. pustulosa*, organelles, mostly mitochondria, are identified in the cytoplasm near the invagination. In early spermatids, the cytoplasm in the invagination was homogeneously granular, with a few membranes that resulted from the process of invagination and do not belong to organelles. In late spermatids, this cytoplasm is dense with a few electron transparent points, without any membrane boundaries, which was not encountered in other Opiliones.

The only structure found near the invagination is a pair of centrioles. These were also observed in spermatids and spermatozoa of other Opiliones species, with the exception of *V. sayi* (Jones & Cokendolpher, 1985). A difference in morphology and constitution between the extra nuclear cytoplasm and that inside the invagination, as observed in *I. pustulosa*, was also reported in the Eupnoi, *Leiobunum* sp. (Reger, 1969). In *I. pustulosa*,

this difference is subtle, contrasting with the situation of the Eupnoi mentioned, in which a large quantity of granular material of different densities was described. Cytochemical differences confirm this separation of the two cytoplasmic regions (discussed below).

The acrosome of *V. sayi* is an electron-translucent structure protruding from the side of the spermatozoon. It is a spherical structure with a homogenous content. This translucent structure is associated with a more dense structure embedded in the spermatozoon wall, but separated from it by peri-nuclear material.

The acrosome complex in Opiliones consists in an acrosomic vesicle, located near the nucleus. Exceptions occur in the aberrant spermiogenesis of Cyphophtalmi, *S. rubens* (Juberthie *et al.*, 1976), where there is no acrosome and the Eupnoi, *Leiobunum* sp. (Reger, 1969), in which the acrosome loses its identity, being incorporated into the nuclear content. In the normal spermiogenesis of Cyphophtalmi (Juberthie *et al.*, 1976) and in two other species of Dyspnoi (Juberthie & Manier, 1977b, 1978), besides the vesicle, there is the presence of an acrosomal rod (*perforatorium*), located in the subacrosomal region and extending to and penetrating the nucleus. In the other known species, including Laniatores (Juberthie & Manier, 1977c, Jones & Cokendolpher, 1985), Eupnoi (Reger, 1969; Tripepi, 1983) and the two Dyspnoi species (Juberthie & Manier, 1977a) this rod was never observed.

In *I. pustulosa*, the acrosome presents a dense material in the subacrosomal compartment, as well as mitochondria and clear vesicles near the plasma membrane. The electron transparent vesicles that are found encircling the acrosome vary greatly in number, and this plasticity may be due to the expulsion of some vesicles from the cells near

maturity, as it is shown in this study. Mitochondria associated with the acrosome occur in other species, such as the Laniatores, *C. cubana* e *S. cavicola* (Juberthie & Manier, 1977c).

The E-PTA technique used in this study, and for the first time in the study of Opiliones spermiogenesis, showed the presence of basic proteins in different structures of the spermatids and of the spermatozoa of *I. pustulosa*. The staining of basic proteins in the nuclei of early spermatids and their later loss of stainability in late spermatids is due to the chromatin condensation. Due to initial, lighter chromatin condensation in early spermatids, the stain penetrates the chromatin, showing the basic proteins such as the histones associated to chromatin in the nucleus. As the condensation progresses in late spermatids, the basic proteins gradually become inaccessible to the large phosphotungstate molecule, so that there are now portions that are E-PTA negative and positive. In the spermatozoa, the condensation is such that the stain can no longer penetrate and the nucleus is completely negative. As mentioned above, in *I. pustulosa*, the cytoplasm of the invagination has a different morphology and probably a different constitution from the surrounding cytoplasm. This is confirmed by the E-PTA reaction which was distinct for the two types of cytoplasm during spermiogenesis. Also, the content of the invagination shows different reactions as the cells develop, which we believe reflects the changes to which the invagination is submitted.

The acrosome, in spite of containing a large number of proteins, is E-PTA negative, due to its very high condensation in late spermatids and spermatozoa. On the other hand, the subacrosomal compartment is positive, indicating basic proteins and an intermediate level of condensation, as seen also with routine preparation methods. This substance has

probably a role in adhesion of the acrosome and may be separated from the vesicle so that it comes in contact with the ovum after the acrosome, in order to have a sequential effect.

Although microtubules do not contain basic proteins, parts of the centriole reacted positively to E-PTA because the microtubular organization of the organelle consists in nine triplets of microtubules, maintained by many proteins that make up an irradiating internal structure, as well as external surrounding proteins, which must include basic proteins.

The mitochondria have a very dense matrix with clear, cylindrical cristae, in routine preparations. E-PTA staining shows this area to be highly negative, possibly due to the density of this matrix, and/or because the internal membrane does not permit the passage of the stain molecule. Since the external mitochondrial membrane is not distinguished, being as strongly stained, as are also the cristae, this suggests that it is permeable to the stain and that this compartment contains basic proteins.

The morphology of the intratesticular spermatozoa of *I. pustulosa* is quite similar to that described for *V. sayi* (Jones & Cokendolpher, 1985), with a compact, homogeneous nucleus, a cytoplasmic invagination devoid of organelles and a small, regular undulation of the plasma membrane (called “bud” in *V. sayi*). However, the morphology of the acrosome differentiates the two species. In *V. sayi*, there are lamella near the nucleus and no mention of associated vesicles or mitochondria. Another difference between the two species is the highly condensed, or “encysted” spermatozoa in *V. sayi*, that represent groups of non-viable spermatozoa to be eliminated, which was not found in *I. pustulosa*.

Morrow (2004) revealed that at least 36 taxonomic groups, from red algae to fish, evolved independently to produce this type of aflagellated sperm. In the Opiliones, aflagellated spermatozoa are produced by all members of the order (Reger, 1969; Juberthie

& Manier, 1977a, b, c, 1978; Juberthie *et al.*, 1976; Tripepi, 1983; Jones & Cokendolpher, 1985; Alberti, 1995; Moya *et al.*, 2007).

The spermatozoa organization affects reproductive biology, therefore it is accepted for species with immobile sperm, that fertilization should be different from the classic spermatic model (Afzelius, 1979). The concept that the evolution process begins with mobile, flagellated spermatozoa, passing to spermatozoa that remain flagellated but lose their motility, and finally become aflagellated (Dallai *et al.*, 1990), is widely accepted. Thus, the lack of a flagellum is considered a characteristic of a high specialization level (Baccetti & Afzelius, 1976; Baccetti, 1985; Jamieson, 1987; Dallai *et al.*, 1990).

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ARTIGO 02

SPERM MORPHOLOGY OF THE NEOTROPICAL HARVESTMAN *Iporangaia pustulosa* (ARACHNIDA: OPILIONES): COMPARATIVE MORPHOLOGY AND FUNCTIONAL ASPECTS

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Sperm morphology of the neotropical harvestman *Iporangaia pustulosa* (Arachnida: Opiliones): Comparative morphology and functional aspects

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Abstract

We describe herein the sperm morphology of the harvestman *Iporangaia pustulosa*. Adult males were dissected, the reproductive tract was schematized and the seminal vesicle was processed by light, transmission and scanning electron microscopy. The male reproductive tract is composed of a tubular testis, two deferent ducts, a seminal vesicle, a propulsive organ and a penis, similar to that observed in other Opiliones. The spermatozoa from the seminal vesicle are oval, aflagellate and immotile, presenting a nucleus surrounding an invagination of the cytoplasm, as well as a complex acrosome and projections on the cell surface. In the testis, spermatozoa are devoid of projections. In the seminal vesicle, they gradually acquire the projections with tufts adhering to it. Consequently, spermatozoa in various distinct stages of projection development can be found in the seminal vesicle. We believe that these projections (1) could help transport sperm along the male and perhaps female reproductive tracts; (2) are used to anchor the spermatozoa inside the female spermatheca in order to avoid mechanical displacement by the genitalia of other males and (3) may play a role in oocyte recognition. We propose that the evolution of aflagellarity in Opiliones is related to the unique morphology of the female reproductive tract. Since eggs are fertilized on the tip of the ovipositor just prior to being laid, there is no advantage favoring sperm mobility. Additionally, female sperm receptacles are small and males that produced small spermatozoa would have a higher chance of fertilizing more eggs.

Keywords: Aflagellate sperm; Harvestman; Sexual selection; Sperm competition; Surface projections

1. Introduction

The phylum Chelicerata comprises more than 100,000 described species and is the most diversified arthropod group considering sperm morphology (reviewed in Alberti, 1995, 2000). Especially within the class Arachnida, the spermatozoa present great morphological variation, including filiform flagellate forms as observed in the order Scorpiones, spiraled forms in the orders Pseudoscorpiones, Amblypygi, Araneae, and Ricinulei, and even spherical and aflagellate forms in the orders Opiliones, Palpigradi, Solifugae, and Acari. Arachnid spermatozoa may also vary in length, ranging from less than 2 μm in some harvestmen to nearly 1 mm in ticks (Alberti, 1995, 2000).

Most studies describing sperm morphology in Opiliones were published in the 1970s and 1980s (Reger, 1969; Juberthie and Manier, 1976, 1977a,b,c, 1978; Juberthie et al., 1976; Tripepi, 1983; Jones and Cokendolpher, 1985). These studies focused on species from the northern hemisphere belonging to the families Sironidae (suborder Cyphophthalmi), Phalangiidae (suborder Eupnoi), Nemastomatidae (suborder Dyspnoi), and Cosmetidae (suborder Laniatores). In all cases, the aflagellate spermatozoa are solely comprised of a nucleus and an acrosome, which has not always been properly identified. The spermatozoa are densely concentrated in the seminal vesicle, rendering recognition of their organelles very difficult (review in Juberthie and Manier, 1978).

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Although a general framework of sperm morphology exists for the Opiliones, there are still many uncertainties on ultrastructural characteristics, the motility process, and the fusion of gametes in these species in which the acrosome is absent. Since the early papers on the subject, the methodology has changed considerably with improvements in instrumentation. Ultrastructural analysis using the current techniques and equipment could provide new information regarding the composition and location of the organelles, including acrosome, nucleus, centrioles, and mitochondria, and also increase our understanding of their structure and function. Moreover, detailed information on sperm morphology in arachnids may also provide useful characters for both phylogenetic analysis (e.g., Alberti and Peretti, 2002; Alberti, 2005) and studies of sexual selection (e.g., Morrow, 2004).

In this study we describe the male reproductive tract and spermatozoa morphology of the harvestman *Iporangaia pustulosa* Mello-Leitão 1935 (Laniatores: Gonyleptidae). Females of *Iporangaia pustulosa* lay eggs on the undersurface of shrub leaves growing at the margin of streams and the eggs are covered by an abundant transparent mucus coat. The offspring is guarded by the males, which may be found resting at the leaf base or on the upper surface of the leaf containing the egg-batch. Males copulate with several females and the batches are generally composed of eggs in several stages of embryonic development. Females are iteroparous and copulate with several males throughout their lives (Machado et al., 2004). This is the first study to investigate a representative of the neotropical Gonyleptidae, which is one of the largest family in the order and has been the focus of many recent behavioral studies (see references in Machado and Raimundo, 2001; Machado, 2002; Hara et al., 2004). Since the reproductive biology of *I. pustulosa* has been recently reported (Machado et al., 2004), we also integrate our morphological data with behavioral information in this harvestman species.

2. Methods

Twenty adult males of *Iporangaia pustulosa* were collected at the Parque Estadual Intervales (24°14′S; 48°04′W; 800 m alt.), close to the municipality of Ribeirão Grande, southern São Paulo State, Brazil (for details on this site see Leonel, 1994). Voucher specimens were deposited in the Museu de Zoologia da Universidade de São Paulo (MZSP) and Museu de História Natural da Universidade Estadual de Campinas (ZUEC).

2.1. Male reproductive tract anatomy

Live males were ventrally dissected in 0.1 M sodium phosphate buffer using a stereoscopic microscope. The reproductive tract was removed and a schematic drawing was made.

2.2. Light microscopy

2.2.1. Spermatozoa suspension

Drops of sperm obtained from the seminal vesicle, suspended in 0.1 M sodium phosphate buffer, were spread on glass slides, fixed in 4% paraformaldehyde for 30 min at room temperature and quickly washed in the same buffer. To study general characteristics, the suspensions were stained with toluidine blue, washed, mounted and observed with a photomicroscope. To study the nuclear structure, sperm suspensions were stained with 0.2 μg/ml 4,6-diamino-2-phenylindole (DAPI) in PBS for 30 min, washed in running water and immersed in 0.1 M McIlvane buffer for 5 min in the dark, at room temperature. They were mounted and photographed with a microscope equipped with a BP 360-370 filter (Olympus BX60).

2.2.2. Histology

Seminal vesicles were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 12 h at 4 °C, rinsed in 0.1 M sodium phosphate buffer, dehydrated in acetone and embedded in epoxy resin. The tissues were sectioned at 1–2 μm, mounted on microscope slides, stained with toluidine blue, pH 4.0 and photographed with a microscope (Olympus BX60).

2.3. Transmission electron microscopy

Testis and seminal vesicles were fixed in 2.5–3% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium cacodylate or sodium phosphate buffers for intervals of 12–72 h, at 4 °C. They were postfixed in 1% osmium tetroxide in the same buffer for 3–5 h, dehydrated in acetone and embedded in epoxy resin. For better protein and microtubule preservation, other testis and seminal vesicles were fixed in 2.5% glutaraldehyde and 1% tannic acid in 0.1 M sodium phosphate buffer, with 1.5% sucrose and 5 mM calcium chloride for 3 days at 4 °C. They were washed in phosphate buffer and stained with 1.5% uranyl acetate for 2–5 h at room temperature before dehydration in acetone and epon embedding (Dallai and Afzelius, 1990). Sections, stained with uranyl acetate and lead citrate, were observed in a LEO 906 Zeiss electron microscope.

2.4. Scanning electron microscopy

Sperm suspensions were spread on round cover slips and fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer with 1.5% sucrose and 5 mM calcium chloride for 15 min, rinsed in the buffer for 15 min and post fixed in 1% osmium tetroxide. After rinsing in buffer and dehydrating in ethanol or acetone, they were critical point dried, gold sputtered and observed in a JEOL 5800LV scanning electron microscope.

3. Results

The reproductive tract of *Iporangaia pustulosa* is comprised of a tubular U-shaped testis connected to two deferent ducts that transfer spermatozoa into a large seminal vesicle. Following the seminal vesicle, there is a propulsive organ and an eversive penis (Fig. 1).

The spermatozoa of *I. pustulosa* obtained from the seminal vesicles are dispersed in the lumen (Figs. 2 and 3) and present no apparent motility. They are aflagellate, oval and average 12 μm in length by 2 μm in width, tapering to fine points (Figs. 3 and 4). Spermatozoa from the seminal vesicle consist of a nucleus surrounding an invagination of the cytoplasm, as well as an acrosome and many projections on the cell surface (Figs. 5–8).

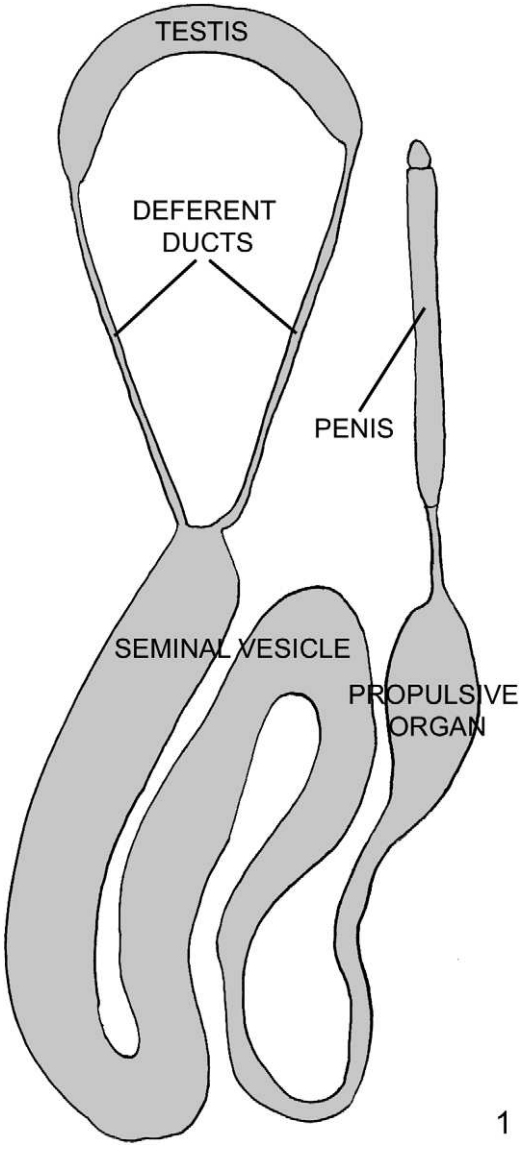


Fig. 1. Schematic drawing of the male reproductive tract of *Iporangaia pustulosa*.

The nucleus is uniformly condensed, occupying approximately 90% of the spermatozoon volume, resembling the shape of this cell (Figs. 4–6).

The invagination, a cytoplasmic portion that is engulfed by the nucleus during the spermiogenesis, conforms to the general oval pattern, appearing pear-shaped in cross sections, remaining open on the surface opposite to the acrosome (Figs. 7 and 8). This cytoplasm is less electron-dense than the nucleus and is devoid of organelles. No microtubular structures, such as an axoneme or centriole, were found. The acrosome is partially immersed in the nucleus, opposite to the nuclear opening, and is comprised of a globular acrosomal vesicle in a cup-shaped layer of remaining cytoplasm, which underlies the vesicle and contains associated folded membranes and mitochondria-like structures (Figs. 8–10). Externally, a thin, smooth extracellular layer covers the acrosome.

Cell suspensions from the seminal vesicle, viewed in scanning electron microscopy, showed different types of spermatozoa: a thinner cell with a smooth surface, measuring about 11.42 μm by 1.7 μm, and a wider one with a rough surface, measuring about 14.12 μm × 3.2 μm. In all the suspensions observed, the number of wider spermatozoa was considerably higher than the thinner ones (Fig. 11). Transmission electron microscopy revealed the presence of a great number of projections covering the cell surface in the seminal vesicle, corresponding to the rough surface observed with the scanning electron microscope (Fig. 12).

All of the observed sperm types presented the same intracellular structures, with the same arrangement. In the testis, the late spermatids and early spermatozoa presented a simple, irregular waving plasma membrane without external coats (Fig. 13).

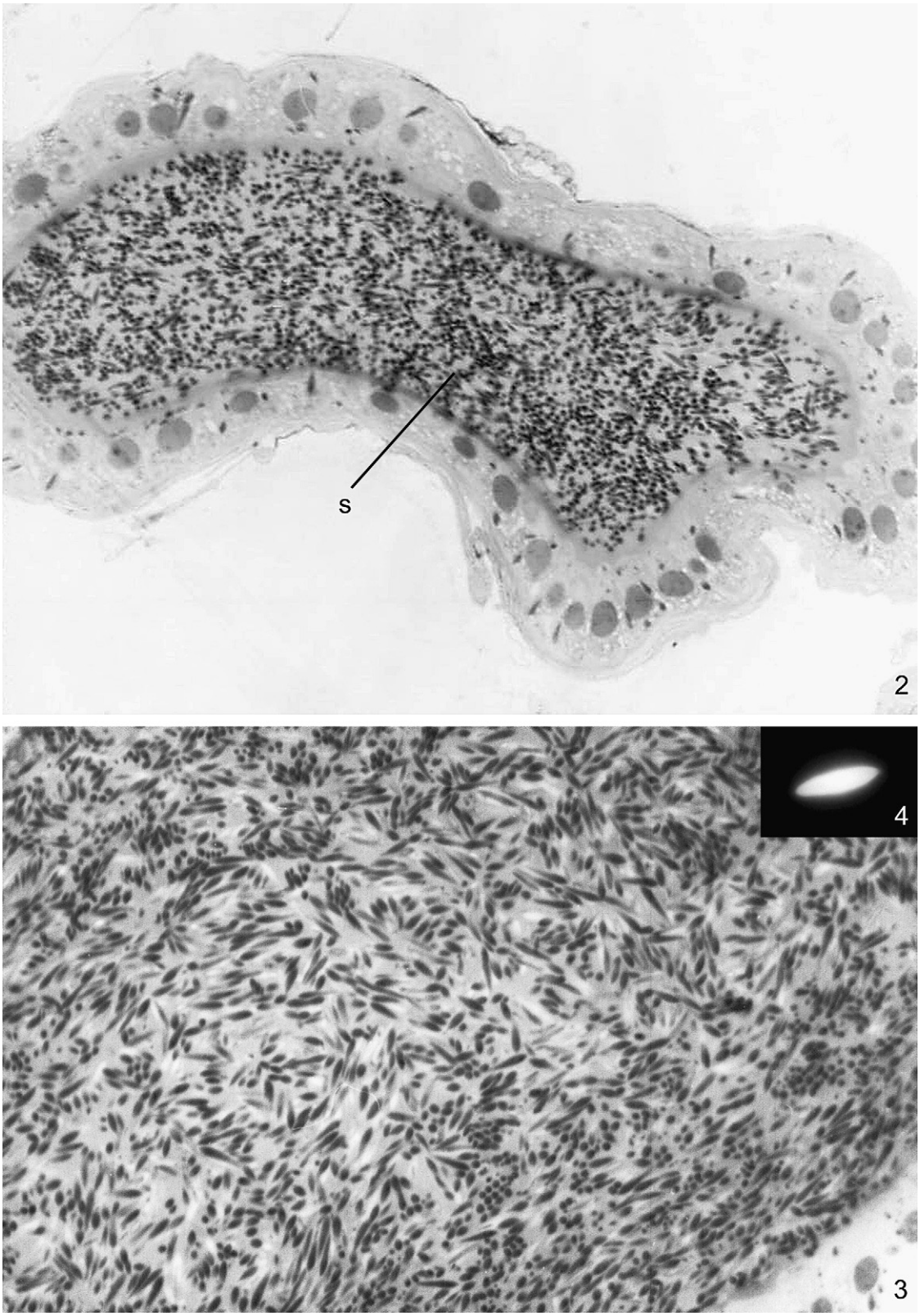
After leaving the testis, the sperm membrane forms regular scallops filled with a dense material and small irregular external tufts adhering to each scallop (Figs. 14 and 15). Then, the spermatozoa gradually acquire projections adhering to the scallops of plasma membrane (Figs. 16 and 17) except above the acrosome (Fig. 5). These projections are covered by an amorphous material on their tips, resembling a glycocalyx and a thin electron-lucent layer (Figs. 16 and 18). As a result, spermatozoa in various distinct stages of projection development can be found in the seminal vesicle.

In the proximal region of the seminal vesicle, in relation to the testis, more spermatozoa are found with short projections with a delicate linear covering. In the medial region, they occur in approximately equal numbers, while in the terminal region the majority of the spermatozoa have long projections.

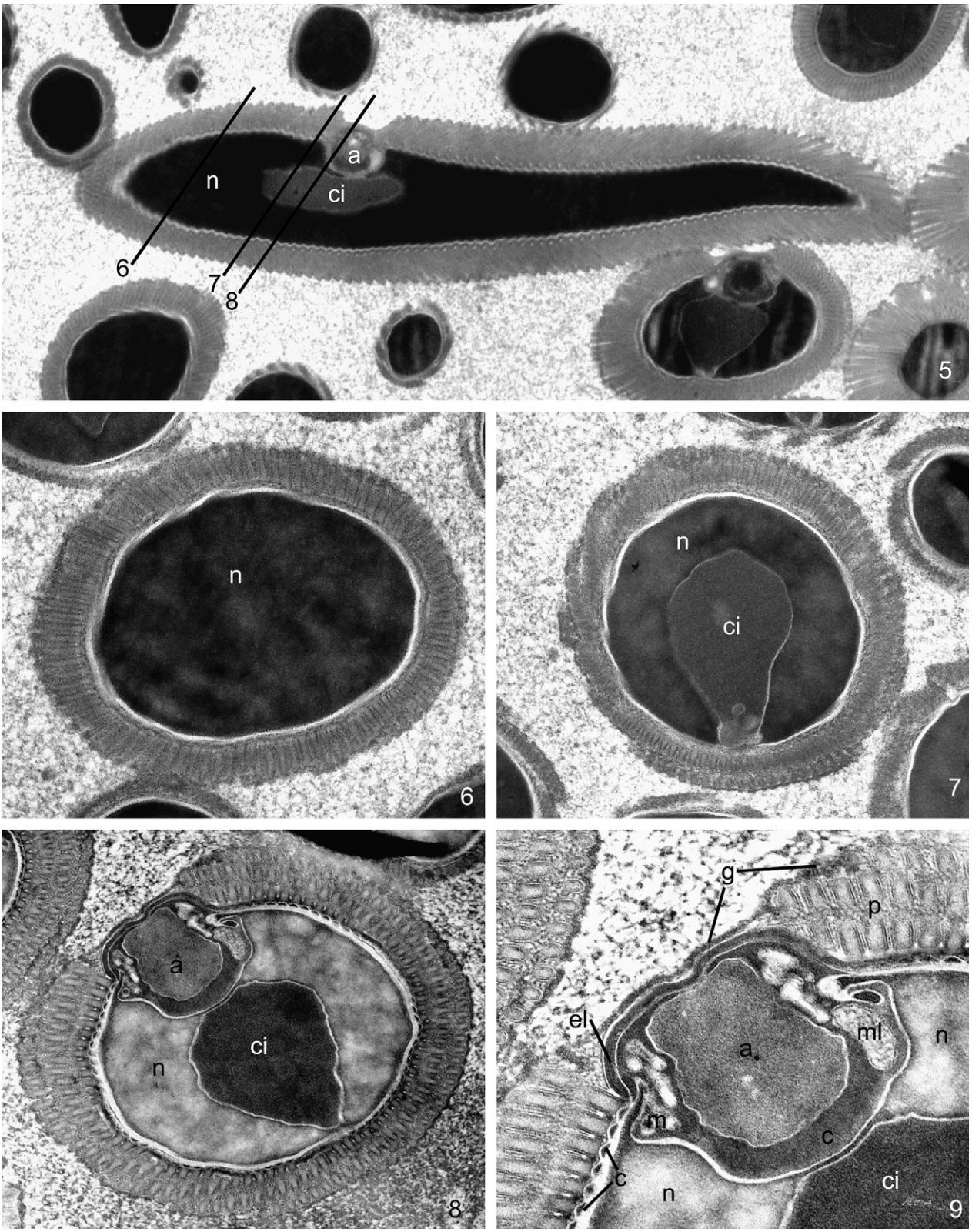
4. Discussion

4.1. Comparative morphology and functional aspects

The reproductive tract of *Iporangaia pustulosa* has the same components as previously described in the literature for other harvestman species (Berland, 1949; Juberthie, 1965; Cokendolpher and Jones, 1991). There is a tubular testis



Figs. 2–4. Light microscopy. (2) Spermatozoa (s) dispersed in the lumen of the seminal vesicle (×67). (3) Large magnification of spermatozoa dispersed in the lumen of the seminal vesicle (×120). (4) Nucleus stained with DAPI technique (×300).



Figs. 5–9. Transmission electron microscopy (TEM). (5) Longitudinal section of the spermatozoon showing the nucleus (n); the acrosome (a) and the cytoplasmic invagination (ci). The lines represent the cross sections of Figs. 6–8 (×12,900). (6–8) Cross sections corresponding to different regions of the spermatozoon shown in Fig. 5 (×27,500), (×24,300), (×29,400). a, acrosome; ci, cytoplasmic invagination; n, nucleus. (9) Detail of the acrosomal region. (×79,800). a, acrosome; c, cytoplasm; ci, cytoplasmic invagination; el, extracellular layer; g, glycocalyx; m, folded membranes; ml, mitochondria like structure; n, nucleus; p, projections.

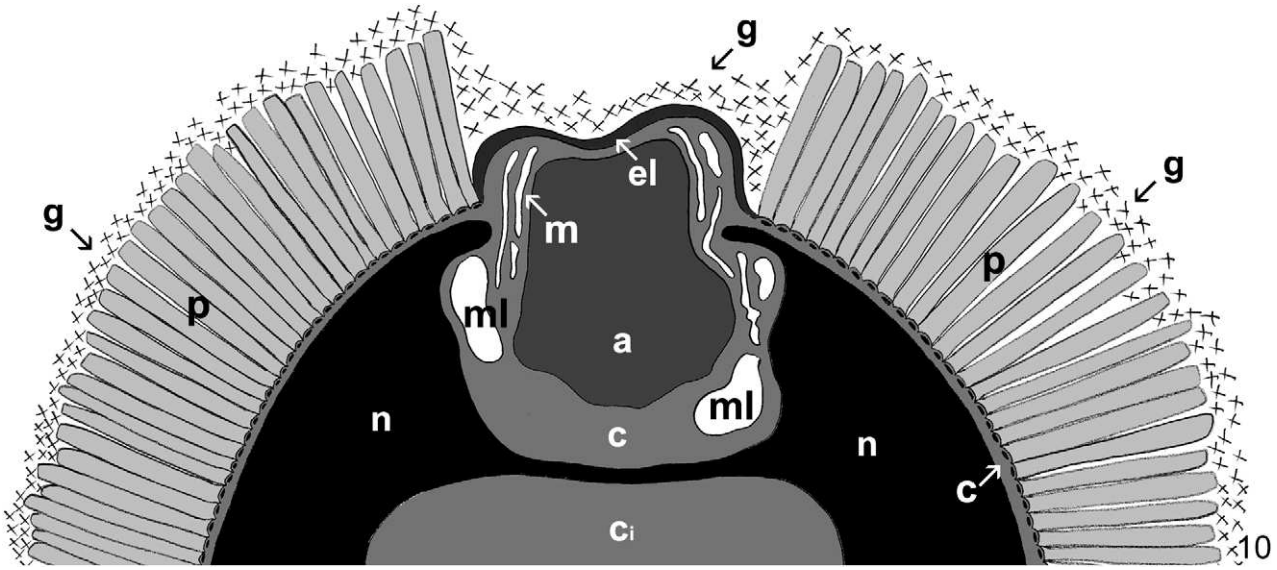


Fig. 10. Schematic drawing of the acrosomal region. a, acrosome; c, cytoplasm; ci, cytoplasmic invagination; el, extracellular layer; g, glycocalyx; m, folded membranes; ml, mitochondria like structure; n, nucleus; p, projections.

and a large seminal vesicle, suggesting that males are able to produce large amounts of sperm (Birkhead and Moeller, 1998). Two deferent tubules connect the testis to the seminal vesicle, where the sperm is stored. A propulsive organ is located immediately in front of the penis, which is probably related to the eversion of the male genitalia through hydraulic pressure of the hemolymph (J.W. Shultz, personal communication).

An extensive review of the literature revealed that aflagellate sperm independently evolved in at least 36 taxonomic groups, including representatives of many arthropod orders (Morrow, 2004). Aflagellate sperm are apparently produced by all species of the order Opiliones, except for members of the suborder Cyphophthalmi, which retain a non-motile axoneme in the sperm (Alberti, 1995, 2000, 2005).

As a representative of the suborder Laniatores, the spermatozoa of *I. pustulosa* lack a flagellum. However, contrary to prior studies of most harvestman species (e.g., Juberthie and Manier, 1976, 1977a,b,c; Tripepi, 1983) no evidence of centrioles was encountered in spermatozoa of *I. pustulosa*. Even after applying a specific fixation for microtubules using tannic acid (Dallai and Afzelius, 1990), we did not identify these structures.

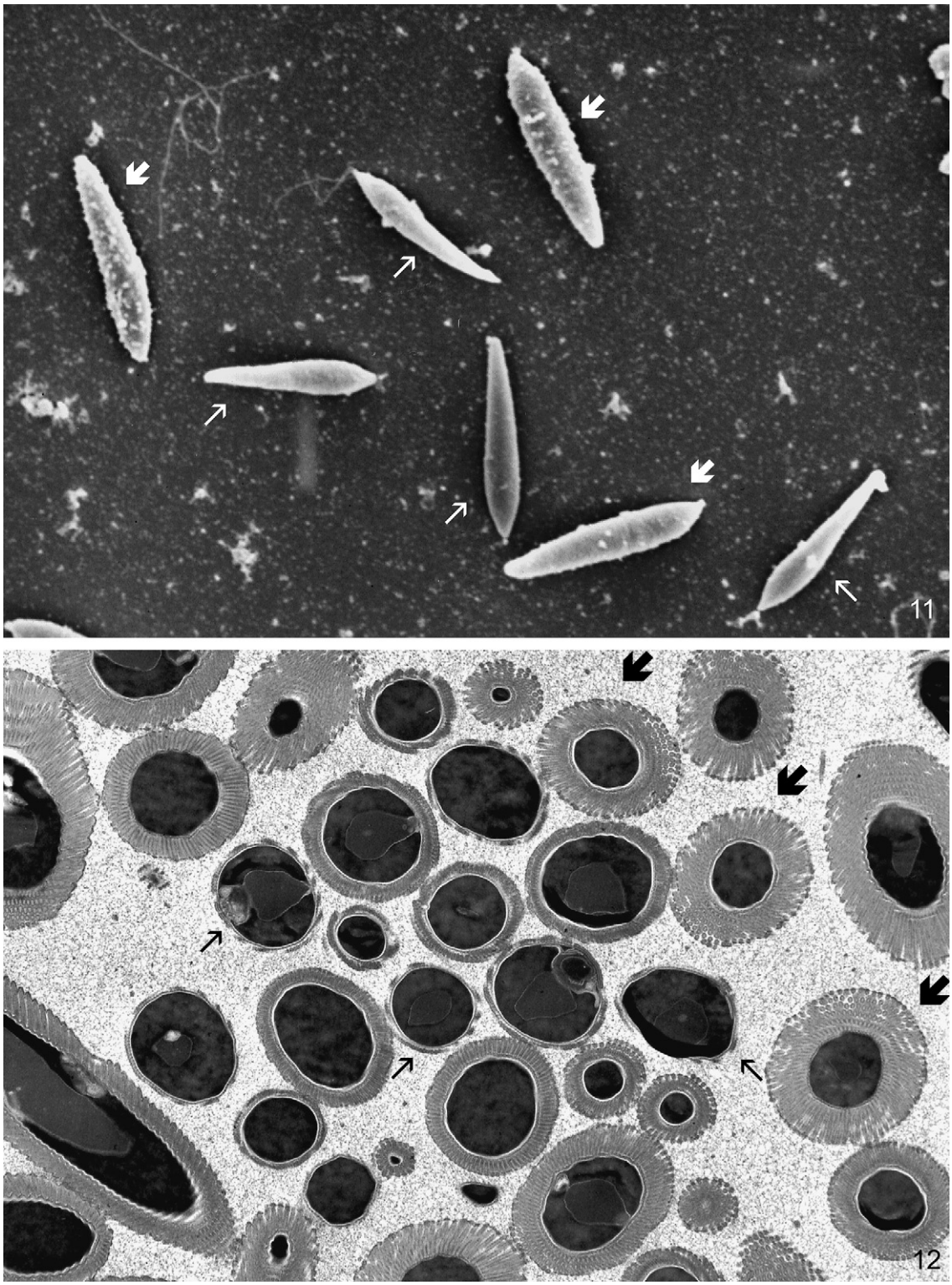
The ultrastructure of the acrosome of *I. pustulosa* has never been described in other harvestmen species. A globular acrosomal vesicle, as occurs in *I. pustulosa*, was observed only for the Laniatores *Epedanellus tuberculatus*, *Cynortoides cubanus* (Juberthie and Manier, 1977c) and *Vonones sayi* (Jones and Cokendolpher, 1985), but for these three species, the descriptions are not detailed. In *V. sayi*, the acrosome represented ''an electron translucent structure protruding from the side of the spermatozoa connected to a dense structure embedded in the spermatozoa wall'', as described by the authors. We consider that the ''dense structure'' described for *V. sayi* is the same as the acrosome of *I. pustulosa*, while their ''electron translucent structure'' is really a large secretion deposit similar to what we also found covering different regions of the spermatozoa (not shown in this study).

In general, the harvestman acrosome is a flat dense structure as observed in the Dyspnoi *Mitostoma pyrenaeum* and *Nemastoma bimaculatum* (Juberthie and Manier, 1977a) and in the Eupnoi *Phalangium opilio* (Tripepi, 1983). In the Cyphophthalmi *Siro rubens* (Juberthie et al., 1976) and in the Dyspnoi *Trogulus nepaeformis* (Juberthie and Manier, 1977b) and *Ischyropsalis luteipes* (Juberthie and Manier, 1976), the flat dense acrosome possesses a rod inserted into the nucleus.

The cytoplasmic invagination penetrating the nucleus is not a unique characteristic of *I. pustulosa* and has been previously found in representatives of the suborders Laniatores, such as the cosmetids *Vonones sayi* (Jones and Cokendolpher, 1985), *Cynortoides cubanus* and *Epedanellus tuberculatus* (Juberthie and Manier, 1977c), as well as in the Eupnoi, such as the sclerosomatid *Leiobunum* sp. (Reger, 1969); and Dyspnoi, such as the nemastomatids *Mitostoma pyrenaeum* and *Nemastoma bimaculatum* (Juberthie and Manier, 1977a).

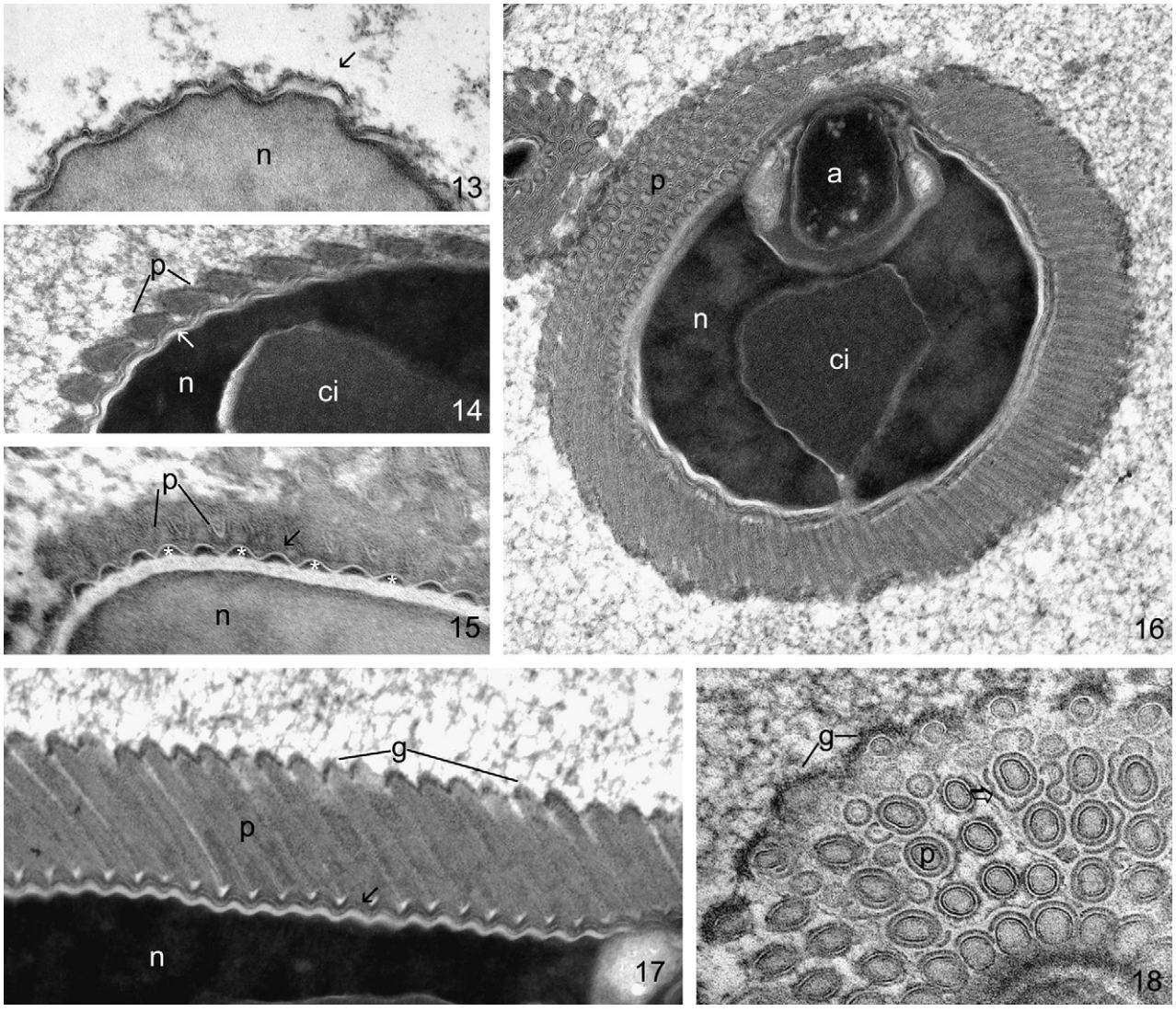
The ultrastructure of spermatozoa obtained from the seminal vesicle of *I. pustulosa* is particularly similar to that of the cosmetid *V. sayi*, in which the cytoplasmic invagination, the homogeneously condensed nucleus, the acrosome and surface projections were also reported (Jones and Cokendolpher, 1985).

Unfortunately, most prior studies do not report the morphology of sperm obtained from the seminal vesicle. The projections of *I. pustulosa* and *V. sayi* occur only in spermatozoa from the seminal vesicle. Although the contents of the seminal vesicle have already been investigated in the



Figs. 11 and 12. Two sperm morphologies: observed in Scanning (Fig. 12) and Transmission (Fig. 11) Electron Microscopes. The thinner one with smooth surface (thin arrow) and the wider one with rough surface (large arrows). In Fig. 12. notice the presence (large arrows) and absence (thin arrows) of projections on the surface of the spermatozoa representing both surfaces observed in Fig. 11 (×5,100) (×10,200).

phalangiid *Phalangium opilio* these structures were not found (Tripepi, 1983). It is possible that this morphological feature is restricted to the suborder Laniatores, or at least to the superfamily Gonyleptoidea. Projections of the surface are also found in the aflagellate sperm of the Solifugae *Oltacola gomezi* and *Procleobis patagonicus* (Alberti and Peretti, 2002) and in the aflagellate sperm of some Acari (Reger, 1961, 1963, 1971; Witalinski and Dallai, 1994; Alberti,



Figs. 13–18. TEM. (13) Spermatozoon in the testis with simple and irregularly waving plasma membrane (arrow). (×36,000) n, nucleus. (14) Plasma membrane (arrow) with irregular small tufts and early projection. (×55,700). ci, cytoplasmic invagination; n, nucleus; p, projections. (15) Plasma membrane (arrow) with regular scallops filled with dense material (*). (×98,900). n, nucleus; p, projections. (16) Spermatozoon showing developed projections around the cell (×26,900). a, acrosome; ci, cytoplasmic invagination; n, nucleus; p, projections. (17) Longitudinal section showing the detail of the projections with glycocalyx. (×41,900). g, glycocalyx; n, nucleus; p, projections; arrow, plasma membrane. (18) Cross section of the projections (p) with linear covering (large arrow) and glycocalyx (g) (×80,900).

2000). These projections found in different groups do not necessarily represent the same function.

We believe that the projections on the cell surface of *I. pustulosa* sperm are extracellular structures, and do not represent microvilli as observed in *Vonones sayi*. First, the mature germ cells in the testis do not present projections and, in this late spermiogenesis stage, these cells no longer possess the organelles necessary for developing the projections. Second, we believe that the scalloped membrane represents a modified plasma membrane; therefore, these projections are externally located. The thin electron-lucent layer and the amorphous material located on the projections, as well as the dense layer over the acrosome, probably present carbohydrate constituents, similar to a glycocalyx.

The morphologically different spermatozoa of *I. pustulosa* do not represent a true dimorphism. They can be considered different developmental stages that occur during their maturation process. The thin spermatozoa, with a smooth surface, are cells that recently exited the testis, while the wide ones with a rough surface are mature cells. We believe that the projections are developed during the final, extratesticular spermatic maturation; therefore, their presence identifies mature cells. The origin of these projections is unknown, but we believe that the epithelial cells from extratesticular ducts (deferent ducts and seminal vesicle) produce the material that will be organized into these complex coats since the complete projections were found only posterior to the testis.

Projections of the cell surface are also found in the aflagellate sperm of some Acari (Reger, 1961, 1963, 1971; Witalinski and Dallai, 1994; Alberti, 2000); however, in this group, the projections are not extracellular structures. According to Reger (1961, 1963), Feldman-Muhsam (1986) and Witalinski

and Dallai (1994), the sperm motility is due to these foldings. Since we observed no sperm motility *in vitro* and we consider these projections extracellular structures, we cannot affirm this activity. Although, the role of the projections in Opiliones sperm remains unclear, we propose here three non-mutually exclusive hypotheses to explain their function. First, the rough projections may facilitate sperm transport through the male and perhaps female reproductive tracts. A second possibility is that the projections are used to anchor the spermatozoa inside the female spermatheca to prevent mechanical displacement by the genitalia of other males. Third, the presence of carbohydrate tufts on the projection tips suggests that they may play a role in oocyte recognition, as occurs in other animals.

4.2. Sexual selection and sperm morphology

One of the most important selective forces in the evolution of sperm morphology is sperm competition (Birkhead and Moeller, 1998). Since aflagellate sperm is probably less costly to produce, both in terms of energy and time, selection could favor the loss of the sperm flagellum and any other motile mechanisms in monandrous species (Morrow, 2004). Conversely, species showing paternal care are expected to present numerous male strategies to insure paternity. In these species, male–male competition is likely to be intense either by means of sperm displacement of previous males or through sperm–sperm interaction (Birkhead and Moeller, 1998). Recently, Morrow (2004) found no evidence suggesting that the evolution of aflagellate sperm could be linked to the removal of selective pressures generated by sperm competition.

In this paper we provide information on sperm morphology of the neotropical harvestman *I. pustulosa*, whose males take care of eggs and early hatched nymphs (Machado et al., 2004). Since females are polyandrous, sperm competition is typically severe, since low confidence of paternity reduces the benefits and increases the costs of male caring behavior (Kokko and Jennions, 2003). In this scenario, sperm should be highly motile, but our results show that the sperm of *I. pustulosa* are aflagellate, and direct observation indicates that they are incapable of independent movement.

Observations of some taxonomic groups indicate that the loss of the flagellum is to some extent gradual (Morrow, 2004). In these cases, an intermediate stage occurs where the flagellum first becomes immotile in phylogenetically basal groups before the axoneme degenerates entirely in derived groups. Opiliones provide an example of this sequence of events since species of the basal suborder Cyphophthalmi retain a non-motile axoneme within the sperm (Alberti, 1995). The complete loss of the axoneme in the suborders Eupnoi, Dyspnoi, and Laniatores, however, is probably not correlated with relaxation of the selection pressure from sperm competition on sperm morphology.

Recent studies on species of Cyphophthalmi suggest that the first harvestmen transferred the sperm via spermatophores (Karaman, 2005). Females probably stored the spermatozoa in the sperm receptacles, placed near the tip of the ovipositor, as they do presently (Juberthie and Manier, 1978). Since eggs are fertilized at the tip of the ovipositor just prior to being laid (Blanc, 1880; de Graaf, 1882), sperm do not need to travel; thus, there is no pressure favoring sperm mobility. This hypothesis may also apply to the remaining suborders of Opiliones because the position of the sperm receptacles is the same.

Another hypothesis for the evolution of aflagellarity in Opiliones is related to the size of the sperm receptacles, which is very small when compared to other arachnid groups, such as spiders (Foelix, 1996) and scorpions (Sissom, 1990). Due to their small size, it is possible that these organs have a limited capacity to store sperm. In this context, the loss of the flagellum is expected because this structure would needlessly occupy space inside the sperm receptacles and because the spermatozoa are not engaged in a race to reach the eggs. It is noteworthy that the two hypotheses raised here are not mutually exclusive, and both rely on the unique morphology of the female ovipositor to explain the evolution of aflagellarity in the order Opiliones.

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CONSIDERAÇÕES FINAIS

Tendo em vista a escassez de informações a respeito da biologia reprodutiva em opiliões, este estudo teve por objetivo contribuir para um maior conhecimento nesta área, através da Biologia Celular e suas ferramentas. Os resultados apresentados são referentes à espécie *Iporangaia pustulosa,* um representante da família Gonyleptidae (sub-ordem Laniatores), a mais bem representada no Brasil e que concentra a maior parte dos estudos ecológicos e comportamentais envolvendo opiliões na região Neotropical. Estes resultados geraram dois manuscritos que se complementam. O primeiro deles (Artigo 2) retrata a morfologia dos espermatozóides na vesícula seminal bem como a estrutura do aparelho reprodutor masculino da espécie mencionada. Com objetivo de compreender o processo de formação destes espermatozóides, o segundo manuscrito (Artigo 1) descreve detalhadamente a ultra-estrutura da espermiogênese e dos espermatozóides intratesticulares.

Com as informações obtidas em *I. pustulosa* podemos considerar que:

- O aparelho reprodutor de *I. pustulosa* é semelhante àquele encontrado nas poucas espécies de Opiliones estudadas;
- A espermiogênese ocorre de maneira centrípeta (da periferia para o centro testicular) e em cistos que contêm células germinativas em diferentes estágios de desenvolvimento, assim como observado para a maioria das espécies estudadas.
- O processo de espermiogênese aqui relatado é o mais detalhado entre os trabalhos existentes e se assemelha com o observado na espécie *Vonones sayi* (Laniatores), exceto pela ausência de centríolo nesta espécie;

- Assim como ocorre na maioria das espécies de Opiliones estudadas, os espermatozóides são aflagelados.
- A morfologia dos espermatozóides testiculares também é semelhante àquela descrita para *V. sayi*, exceto pela presença de lamelas associadas ao acrossomo e ausência de mitocôndrias associadas ao mesmo nesta espécie;
- Assim como ocorre no testículo, os espermatozóides de *I. pustulosa* apresentam morfologia semelhante à descrita para *V. sayi*, com presença de projeções nas membranas celulares. Entretanto, nesta espécie, tais projeções são consideradas microvilosidades.
- A estrutura identificada como compartimento sub-acrossomal nas espermátides e nos espermatozóides testiculares, corresponde ao citoplasma mencionado nos espermatozóides vesiculares de *I. pustulosa*. Da mesma forma, as estruturas identificadas como "mitochondria-like" nos espermatozóides vesiculares, são efetivamente mitocôndrias, conforme verificado durante a espermiogênese.
- Os espermatozóides aflagelados de *I. pustulosa*, aparentemente não possuem motilidade, apesar de apresentarem alta competição espermática, o que sugere que os aparelhos reprodutores masculinos e femininos apresentem estruturas, como vilosidades e músculos, que auxiliem no deslocamento destas células.

Vale ressaltar que, em muitos taxa, a determinação da motilidade espermática é difícil, uma vez que os espermatozóides podem se tornar móveis somente no trato reprodutor feminino. Assim, a análise de espermatozóides no trato reprodutor masculino pode levar a conclusões equivocadas com relação a sua motilidade. Em adição, a observação de espermatozóides no trato reprodutor feminino é bastante complicada, uma

vez que em muitos taxa não foi ainda encontrada ou descrita uma espermateca ou qualquer órgão de armazenamento de espermatozóides nas fêmeas. Este é o caso de *I. pustulosa*, uma vez que após inúmeras observações, feitas durante este trabalho, não foi encontrado nenhum órgão de armazenamento de espermatozóides próximo ao ovipositor em fêmeas.

Finalmente, os objetivos propostos foram cumpridos e as informações aqui obtidas poderão ser usadas para uma melhor compreensão da biologia reprodutiva, filogenia e desenvolvimento dos espermatozóides aflagelados em Opiliones.