UNIVERSIDADE ESTADUAL PAULISTA – UNESP
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Sistema reprodutor masculino e espermiotaxonomia em Dromiidae: existe um só padrão de produção de fluido seminal, empacotamento do espermatozoide e transferência espermática em caranguejos?

Maria Alice Garcia Bento

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Orientador: Dr. Fernando José Zara
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“Nunca deixe que lhe digam que não vale a pena
acreditar no sonho que se tem,
ou que seus planos nunca vão dar certo
ou que você nunca vai ser alguém”
Flávio Venturini / Renato Russo

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SUMÁRIO

RESUMO

Os caranguejos da família Dromiidae estão incluídos na seção Podotremata Guinot, 1977 e são pouco conhecidos do ponto de vista histo-morfológico e ultraestrutural. Na presente dissertação, apresentamos a ultraestrutura dos espermatozoides e os padrões anátomo-histológicos do sistema reprodutor dos dromiídeos que ocorrem no litoral brasileiro. Adicionalmente, o comportamento de cópula e a morfologia da espermateca em *Hypoconcha parasitica* foram estudados, com o intuito de levantar pistas sobre os mecanismos de fertilização em Dromiidae. As amostras foram processadas segundo as rotinas para histologia e histoquímica, microscopia, eletrônica de transmissão e varredura. As análises ultraestruturais do espermatozoide indicam que não existe um padrão distinto típico para os gametas entre todos os Dromioidea. Adicionalmente, apenas caracteres específicos foram observados, como a presença do flange esférico na zona eletronlúcida anterolateral e a zona acrossomal externa granular em *H. parasitica*, a ausência da zona acrossomal raiada e a presença de resquícios de flange em *Moreiradromia antillensis* e diferentes morfologias das câmaras perforatoriais entre todas as espécies estudadas. Apenas quando os caracteres da ultraestrutura dos espermatozoides dos Dromioidea foram comparados com Homolidae, Latreillidae e Raninidae, notaram-se características claras entre estas famílias. A produção do fluido seminal e o empacotamento dos espermatozoides no vaso deferente dos Dromiidae são totalmente distintos dos Eubrachyura. *Hypoconcha parasitica*, *Hypoconcha arcuata*, *M. antillensis* e *Dromia erythropus* não possuem espermatóforos, mas sim a presença de um cordão espermático interno envolto por secreções, os quais são mais similares aos Astacidae. O comportamento de cópula em *H. parasitica* é simples, sem corte e as fêmeas copulam em muda e intermuda. O armazenamento do material seminal na espermateca é distinto de Eubrachyura. A organização morfo-histológica da espermateca indica que a liberação dos espermatozoides para a fertilização ocorre por meio de contrações musculares da parede cuticular, com a participação do pleópodo 1 na movimentação dos ovócitos e espermatozoides, auxiliando o contato dos gametas.

PALAVRAS-CHAVE: Decapoda, transferência espermática, ultraestrutura; sistema reprodutor masculino, armazenamento dos espermatozoides.

ABSTRACT

The Dromiidae crabs are included in the section Podotremata Guinot, 1977 and are poorly known from histo-morphological and ultrastructural point of view. This dissertation shows the sperm ultrastructure and the anatomo-histological patterns of the reproductive system to the Brazilian dromiid species. In addition, we studied the mating behavior and spermathecal morphology in *Hypoconcha parasitica* to elucidate some points of the fertilization in dromiid crabs. The samples were processed according to the routine for histology and histochemistry and also, to transmission and scanning electron microscopy. There are no distinct ultrastructural characters to sperm ultrastructure among Dromioidea. Additionally, only specific characters were observed for studied Dromiidae as the presence of the spherical flange in the anterolateral eletron-lucid zone and the granular acrosomal outer zone founded in *H. parasitica*, absence of the acrosome ray zone and presence of the flange remnants in *Moreiradromia antillensis.* The perforatorial chambers show specific morphology among all species studied. The Dromiidae spermatozoa ultrastructure were not consistent to separate Dromiidae from the other Dromioidea. The ultrastructural differences were only found when compared Dromiodea with other Podotremata as Homolidae, Latreillidae e Raninidae. The seminal fluid production and spermatozoa package in the vas deferens of Dromiidae are totally different from Eubrachyura. In *H. parasitica*, *Hypoconcha arcuata*, *M. antillensis* and *Dromia erythropus*, the spermatophores are absent, and they show only a long internal spermatic cord surrounded by secretions in vas deferens, which is similar to the species of Astacidae. The mating behavior is simple without courtship and the females copulated in both soft and hard during moulting condition. The storage of seminal material inside the spermatheca is distinct from Eubrachyura. The morpho-histological organization of the spermathecae indicates that the process of sperm release for fertilization occurs through muscular contractions of its wall, and the pleopod 1 may promote the oocytes and spermatozoa movement to ensure the fertilization.

KEY-WORDS: Decapoda, sperm transfer; histology, ultrastructure, male reproductive system, sperm storage.

INTRODUÇÃO GERAL

A família Dromiidae (De Haan, 1883) pertence à infraordem Brachyura, incluída na seção Podotremata Guinot, 1977, na qual agrupa espécies em que o sistema reprodutor masculino abre-se na coxa do quinto pereópodo e as fêmeas mostram duas aberturas: uma do oviduto (gonóporo), por onde os ovócitos são liberados, localizada na coxa do terceiro pereópodo e a espermateca, cujo é um canal estendido que se abre em diferentes esternitos, dependendo das famílias e subfamílias (Guinot & Tavares, 2001; Guinot & Quenette, 2005; Guinot *et al*., 2013). Com aproximadamente 120 espécies taxonomicamente válidas, os representantes de Dromiidae encontram-se subdivididos nas subfamílias Dromiinae De Haan, 1833, Hypoconchinae Guinot & Tavares, 2003 e Sphaerodromiinae Guinot & Tavares 2003, as quais diferem-se principalmente pela organização do esterno torácico, abdômen, coxa do quinto pereiópodo e pênis nos machos, além das estruturas do esterno torácico 7/8 nas fêmeas (Guinot & Tavares, 2001; 2003; Ng *et al*., 2008). No Brasil são encontrados somente representantes das subfamílias Dromiinae e Hypoconchinae, representados pelas espécies *Dromia erythropus* (George Edwards, 1771), *Moreiradromia antillensis* (Stimpson, 1858), *Hypoconcha parasitica* (Linnaeus, 1763) e *H. arcuata* Stimpson, 1858 (Melo, 1996; Ng *et al*., 2008). Estes dromiídeos são comumente conhecidos pela associação com conchas de bivalves, esponjas ou ascídias, as quais são posicionadas dorsalmente ao animal, com auxílio dos dois últimos pares de patas, dobradas na região sub-dorsal da carapaça (Mclay, 1993; Melo, 1996; Guinot *et al*., 2013).

De uma maneira geral, o sistema reprodutor em Brachyura é um órgão bilateral em forma de letra “H”, constituído pelo par de testículos, localizado em ambas às margens superiores do cefalotórax, os quais são contínuos ao vaso deferente, estendendo-se longitudinalmente sobre o hepatopâncreas ou órgão perigástrico de acordo com Cervellione *et al*. (2017), abaixo do coração, terminando na região posterior do corpo (Krol *et al*., 1992). O testículo pode ser classificado como do tipo lobular ou tubular (para revisão ver Nagao & Munehara, 2003). O vaso deferente é anatomicamente dividido em três regiões: anterior (AVD), média (MVD) e posterior (PVD) (Krol *et al*., 1992). A AVD tem a função de produzir as secreções que levarão a formação dos espermatóforos

(Simeó *et al*., 2009; Nicolau *et al*., 2012; Zara *et al*., 2012). Por sua vez, a MVD e PVD são responsáveis por armazenar os espermatóforos e produzir a maior parte do fluido seminal (Krol *et al*., 1992; Simeó *et al*., 2009; Zara *et al.*, 2012). Em grande parte dos braquiúros, os espermatozoides são empacotados em espermatóforos do tipo coenospérmicos, nos quais vários espermatozoides encontram-se agrupados e delimitados por uma só parede do espermatóforo (El-Sherief, 1991; Jamieson, 1994; Guinot, 1997; Anilkumar *et al.*, 1999). Porém, em algumas espécies de caranguejos de água doce e, em ao menos uma espécie marinha, cada espermatóforo apresenta um só espermatozoide, sendo denominado como cleistoespérmico (Guinot, 1997; Klaus *et al.*, 2009; Tiseo *et al*., 2014; Tiseo *et al*., 2017). A ausência de espermatóforos é um evento raro em Brachyura e foi somente observada em alguns caranguejos de água doce (Klaus *et al*., 2009).

A descrição do sistema reprodutor masculino em espécies primitivas de Brachyura é bastante escassa, sendo que apenas o Raninidae *Ranina ranina* (Linnaeus, 1758) e o Dromiidae *Dromia personata* (Linnaeus, 1758) foram estudados sob este aspecto (Hartnoll, 1975; Subramoniam, 1993; Minagawa *et al*., 1994). O testículo de *R. ranina* foi classificado histologicamente como do tipo lobular e o vaso deferente mostrou três regiões distintas, i.e. AVD, MVD e PVD, sendo os espermatozoides, nesta estrutura, envolto por cápsula ou parede, positiva ao corante ácido periódico de Schiff (PAS), o qual evidencia a presença de polissacarídeos neutros, sendo denominada de espermatóforo (Minagawa, 1993; Minagawa *et al.*,1994). Adicionalmente, estes autores não discutem a evolução da massa espermática, o significado da cápsula ou se esta estrutura pode ser ou não considerada um espermatóforo verdadeiro. Similar ao padrão encontrado em *R. ranina*, o vaso deferente de *Dromia personata* (Linnaeus, 1758) também foi dividido em três diferentes regiões, porém ao invés de relacionada à anatomia, a divisão foi baseada nos diferentes tipos de secreções e constituição da parede do vaso deferente (Hartnoll,1975; Minagawa *et al*., 1994). A estruturação das secreções no vaso deste Dromiidae pareceu ser mais similar ao padrão encontrado para espécies de Astacidea, uma vez que não mostraram parede típica dos espermatóforos, mas sim diferentes tipos de secreções sendo aderidas em camadas (Hartnoll, 1975; Subramoniam 1993).

Desta forma, nota-se que o conhecimento do sistema reprodutor masculino em Podotremata carece de maiores aprofundamentos.

Os espermatozoides de Decapoda são imóveis e aflagelados, podendo ser classificados morfologicamente em duas categorias: uniestelado, encontrados em Dendrobranchiata e Caridea; ou multiestelado encontrados em Brachyura, Anomura e Pleocyemata (Jamieson & Tudge, 2000; Braga *et* al., 2013; Camargo *et al*., 2016; Tiseo *et al*., 2017). Em Brachyura, o espermatozoide é constituído em geral pelo núcleo, vesícula acrossomal, a qual porta o opérculo e uma estrutura perfuradora, a câmara perforatorial. A vesícula acrossomal encontra-se em um dos pólos do espermatozoide, o oposto ao núcleo. Esta estrutura é marcada por diferentes zonas ou camadas acrossomais (Jamieson, 1994; Jamieson & Tudge, 2000, Tudge, 2009). No ápice da vesícula acrossomal encontra-se o opérculo eletrondenso, a qual pode ser ou não perfurado. A câmara perforatorial (tubo perfurador) encontra-se na região central do acrossoma, a qual pode apresentar ampla variação de forma (Jamieson & Tudge, 2000; Tudge, 2009). O núcleo é preenchido por cromatina variando de granular a fibrosa e pode apresentar braços radiais com ou sem microtúbulos (Hinsch, 1986; Jamieson, 1994; Benetti *et al*., 2008; Klaus *et al*., 2009; Tudge, 2009). Em relação à ultraestrutura dos espermatozoides em Dromiidae, apenas os espermatozoides do Sphaerodromiinae *Sphaerodromia lamellata* Crosnier, 1994 e dos Dromiinae *Stimdromia lateralis* (Gray, 1831) e *Dromidiopsis edwardsi* Rathbun, 1919 foram estudados sob este ponto de vista (Jamieson *et al*., 1993 a,b,c; Jamieson, 1994; Guinot *et al.*, 1998; Jamieson & Tudge, 2000). De uma maneira geral, nestas espécies o espermatozoide apresenta acrossomo do tipo discóide com câmara perforatorial bilateralmente capitada e, a partir desta, ocorrem quatro zonas horizontais concêntricas: zona acrossomal interna, zona acrossomal raiada, zona acrossomal mediana ou eletronlúcida anterolateral e zona acrossomal externa (Jamieson *et al*., 1993 a,b,c; Jamieson, 1994; Guinot *et al.*, 1998; Jamieson & Tudge, 2000). O opérculo apical mostra-se perfurado e centralmente a perfuração existe uma protuberância apical (Jamieson, 1994). O núcleo é preenchido por cromatina fibrosa, podendo ou não apresentar braços radiais (Jamieson *et al*., 1993 a,b,c; Jamieson, 1994; Jamieson *et al*., 1995; Guinot *et al.*, 1998; Jamieson & Tudge, 2000). Apesar de existirem trabalhos com Dromiidae, as inclusões das espécies da fauna brasileira contribuirão para

uma maior elucidação filogenética das espécies que pode ser concatenada à molecular, existente na literatura. Adicionalmente, em Dromiidae uma das subfamílias, Hypoconchinae, tem o espermatozoide totalmente desconhecido do ponto de vista ultraestrutural. Desta maneira, conciliando os resultados deste estudo com a molecular, será possível aumentar o poder de resolução nas relações de parentesco.

A transferência espermática esta diretamente relacionada ao momento em que as fêmeas tornam-se receptivas aos machos, podendo realizar a atração destes por meio de, por exemplo, feromônios liberados na urina (mais frequente) ou estímulo tátil, visual ou auditivo (menos frequente) (Hartnoll, 1969, Kennedy & Cronin, 2007). Em relação à receptividade das fêmeas, aquelas capazes de copular durante a muda, (exoesqueleto mole) encontram-se receptivas aos machos apenas em curtos períodos, enquanto que as fêmeas capazes de copular em intermuda (exoesqueleto rígido) encontram-se receptivas continuamente (Diesel, 1991; Mclay & López Greco, 2011). Em Dromiidae, o acasalamento em *Dromia personata* Linnaeus, 1758 pode ocorrer pós muda (muda pré-ovígera), quando o exoesqueleto encontra-se mole e ao menos em um caso, a cópula ocorreu enquanto a fêmea estava na condição de intermuda, com o exoesqueleto rígido (Hartnoll, 1975). Apesar de mostrar estes resultados, o autor não discute no que implicaria o acasalamento ocorrer na condição de muda ou intermuda, tampouco descreve onde os gonópodos são inseridos para a transferência espermática. Adicionalmente, os repertórios comportamentais durante a transferência espermática ou sua relação com o ciclo de muda também são pouco abordados para *D. personata* e outras espécies de Podotremata (Hartnoll, 1975; Guinot *et al*.,2013). Desta maneira, nota-se uma lacuna a respeito da transferência espermática em Podotremata, visto que este tema nunca foi abordado na íntegra em espécies deste grupo, sendo este de grande importância para o entendimento de estruturas morfológicas, com ênfase na função da espermateca, nos modos reprodutivos e a evolução dos órgãos de armazenamento do fluido seminal e fêmeas dos caranguejos.

Nos Eubrachyura, as fêmeas possuem uma estrutura responsável em armazenar espermatozoides após a cópula, com origem ecto-mesoderme (Diesel, 1991; Guinot & Quenette, 2005; Guinot *et al*., 2013; Mclay & Becker, 2015). Esta estrutura já foi denominada como espermateca (*espermathecae*)

(Hartnoll 1969; Beninguer *et al*., 1988; López Greco *et al.*, 1999; Rotllant *et al.*, 2007; Sant´Anna *et al*., 2007). Porém, o termo mais apropriado para esta estrutura de origem mista é receptáculo seminal (Guinot & Ouenette, 2005; López Greco *et al*., 2009; Zara *et al*., 2014). No receptáculo seminal, o oviduto tem contato direto com a região de armazenamento dos espermatozoides e este contato pode ser dorsal ou ventral, influenciando decisivamente na fertilização dos ovócitos. Assim, este órgão pode favorecer o primeiro ou último macho que realizou a cópula, dependendo do local de abertura do oviduto (Diesel, 1991; Antunes *et al.*, 2016). O termo espermateca ficou restrito a estruturas de origem exclusivamente ectodérmica, observado em Podotremada (Tavares & Secretan, 1993; Guinot & Tavares, 2001; Guinot & Ouenette, 2005). Neste grupo, os machos possuem o par de pênis, gonóporos, gonópodos localizados na coxa do quinto par de pereópodos, e gonópodos, com diferentes morfologias e funções para cada família e subfamília (para revisão ver Guinot & Tavares, 2003; Guinot & Quenette, 2005; Guinot *et al*., 2013). Por sua vez, as fêmeas mostram duas aberturas: o par de gonóporos, localizados na coxa do terceiro pereópodo, cuja função é externalizar os ovócitos e o par de espermatecas. A espermateca é uma depressão no esterno, cuja abertura é alongada em forma de fenda, sem ligação interna com o oviduto (Guinot & Tavares, 2001; Guinot & Tavares, 2003; Guinot & Quenette, 2005; Guinot *et al*., 2013). Como a espermateca não contém conexão interna com o oviduto, acredita-se que os gametas masculinos precisam ser deslocados desta estrutura quitinosa até a região externa do corpo da fêmea para que a fertilização ocorra (Mclay & López Greco, 2011). Porém, o mecanismo funcional desta estrutura ainda continua sendo um mistério, sendo que em Homolidae foi sugerido que a ação de músculos na abertura espermatecal contribuem para a liberação dos espermatozoides (Becker & Scholtz, 2017).

Algumas espécies produzem o “plug espermático”, o qual é formado por secreções seminais dos machos. O plug pode se estender desde o receptáculo seminal até a região externa da vagina, ou ocupar somente a vagina (plug externo) ou formar uma massa semelhante à cera no interior do receptáculo seminal (plug interno) (Hartnol, 1968, 1969). Tais plugs parecem ser cruciais para o favorecimento de um único macho durante a cópula, pois a presença desta estrutura impossibilita ou dificulta o recebimento de material genético

adicional proveniente de outros machos (Hartnoll 1968, 1969; Zara *et al*., 2012; Nascimento & Zara, 2013). A presença de plug é frequentemente atribuída às espécies que acasalam com exoesqueleto ou carapaça mole, como por exemplo, Portunidae (Hartnoll, 1969; Johnson, 1980; Zara *et al.*, 2012; Nascimento & Zara, 2013; Guinot *et al.*, 2013; Zara *et al*., 2014). Além disso, estas espécies podem apresentar o comportamento de guarda pré e pós copulatória, nos quais os machos permanecem em "abraço" com a fêmea antes, durante e após a muda, a fim de impedir que outros machos tentem copular com a fêmea (Hartnoll, 1969; Zara *et al.,* 2012). Em Podotremata, estudos com o comportamento e transferência espermática são escassos, sendo que em *R. ranina*, observou-se a presença de pequenos espermatóforos depositados junto a espermateca após a cópula (Minagawa, 1993). Este mesmo autor propôs que parte do espermatóforo estavam internalizados na espermateca, porém não houve a devida comprovação por dissecção. No tocante a Dromiidae, o conhecimento é restrito ao que foi descrito para *Dromia personata* (Hartnoll, 1975*)*. Segundo o autor, no final da transferência espermática, nota-se material enrijecido ou "plug espermático" sobre a abertura da espermateca (Hartnoll, 1975). Apesar de acreditarem que Hypoconchinae não apresenta "plug espermático", a presença deste "plug" foi observada para também nos Dromiinae *Lauridromia intermedia* (Laurie, 1906), *Austrodromidia octodentata* (Haswell, 1882) e *Pseudodromia latens* Stimpson, 1858 (Guinot & Tavares, 2003; Guinot & Quenette, 2005; Guinot *et al.*, 2013). Entretanto, este assunto permanece ainda a ser aprofundado.

Assim, nesta dissertação são apresentados três capítulos em formato de artigos científicos. O primeiro trata- se da descrição ultraestrutural dos espermatozoides de *Dromia erythropus*, *Moreiradromia antillensis*, *Hypoconcha arcuata* e *H. parasitica*, com objetivo de relacionar os caracteres espermáticos entre os dromiídeos e outras famílias de Podotremata, buscando uma melhor compreensão sobre as relações filogenéticas deste grupo. Desta maneira, este capítulo produz um embasamento sobre o parentesco entre os Dromiidae para a elucidação do anatomo-histologia do sistema reprodutor desta família. O segundo descreve a morfologia do sistema reprodutor masculino dos Dromiidae encontradas no Brasil, com o intuito de verificar se este grupo de caranguejos apresenta o mesmo padrão usualmente descrito para o Eubrachyura, com a

presença de espermatóforos e características químicas do fluido seminal. O último capítulo utiliza a espécie *H. parasitica* para elucidar aspectos do comportamento de cópula e da morfologia da espermateca em Dromiidae, com o intuito de levantar pistas sobre os mecanismos de fertilização neste grupo de caranguejos primitivos.

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Capítulo I

Sperm ultrastructure of four crab species of the family Dromiidae (Crustacea: Brachyura): addition data to Hypoconchinae and Dromiinae subfamilies

Maria Alice Garcia Bento, Ivana Trettin, Fernando L. Mantelatto & Fernando José Zara

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Sperm ultrastructure of four crab species of the family Dromiidae (Crustacea: Brachyura): addition data to Hypoconchinae and Dromiinae subfamilies

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Condensed title: Ultrastructure of Dromiidae spermatozoa

ABSTRACT

We described the spermatozoa ultrastructure of Dromiidae *Hypoconcha parasitica*, *H. arcuata*, *Moreiradromia antillensis* and *Dromia erythropus* and compared the morphologies among species of Dromiidae, Dromioidea and Podotremata, to elucidate the relationship between different species of this brachyuran group. Specimens were collected from the Northern coast of São Paulo, Brazil and were fixed and processed following transmission electron microscopy routine. The Dromiidae spermatozoa studied are characterized by discoid acrosome, with three or four concentric zones, which are centrally separated by bilateral capitate perforatorial chamber, with the apex similar to the "mushroom" shape in the Hypoconchinae and letter “T” in Dromiinae. Above of of the perforatorial chamber there is an apical protuberance, continuous with the subopercular region and the operculum, which forms a low dome, centrally perforated. Under differential interference contrast phase, the spermatozoa show 3 to 4 radial arms. The spermatozoa characters in Hypoconchinae and Dromiinae were not consistent to separate these subfamilies from the Dromiidae and Dromioidea. The ultrastructural differentiation was only found between representatives Dromiidae and Raninidae. Thus, the spermiotaxonomy corroborated the previous morphological and molecular studies, supporting the monophyly of Dromiidae and Dynomenidae in relation to Homolidae and Latreilliidae.

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INTRODUCTION

Members of the family Dromiidae De Haan, 1833 are recognized by having intriguing morphological differences when compared with the basic true crab design, which is considered as primitive group within the species infraorder Brachyura, with approximately 50 recognized genera distributed worldwide (Melo, 1996; Ng *et al*., 2008). These species are commonly known as sponge crab or shellback crab by their association with shells of bivalves, sponges or ascidians (Mclay, 1993; Melo, 1996; Melo and Campos, 1999; Guinot *et al*., 2013). This family comprises the subfamilies Dromiinae, Hypoconchinae and Sphaerodromiinae, which are diagnosticated by the peculiar organization of the thoracic sternal, abdomen, coxa of the fifth pereopod and penis in males, and by the structure of the thoracic sternal 7/8 in females (Guinot and Tavares, 2003; Guinot *et al*., 2013). In Brazil only representatives of the Hypoconchinae and Dromiinae are found, represented by the two species of each subfamilies, *Hypoconcha parasitica* (Linnaeus, 1763), *H. arcuata* Stimpson, 1858, *Dromia erythropus* (George Edwards, 1771), *D. gouveai* Melo and Campos, 1999 and *Moreiradromia antillensis* (Stimpson, 1858) (Melo, 1996; Ng *et al*., 2008).

Regarding phylogeny, molecular works performed by Spears and Abele (1988) and Spears *et al*. (1992) included the family Dromiidae as part of Anomura. Further, evidences indicated that the larval morphology and the patterns of reproductive openings are more similar to Anomura species. However, subsequent morphological revisions by McLay (1993), Guinot and Tavares (2001; 2003), Guinot and Quenette (2005) and Guinot *et al*. (2013) and additional molecular studies (Martin & Davis, 2001; Ahyong *et al*., 2007; Tsang *et al*., 2014) have refuted the hypothesis of Dromiidae part of Anomura.

The study of the ultrastructure of spermatozoa has become an excellent tool in resolving taxonomic issues that include relationship among species in several groups of decapod crustaceans (Jamieson, 1994; Jamieson *et al*., 1995; Tudge, 1991; Tudge, 2009; Tudge *et al*., 2014). Additionally, studies based on the comparison of the ultrastructure of the spermatozoa of different

species, allied with molecular phylogeny studies has given support to new proposals of phylogeny to the group (Camargo *et al*., 2015, 2017; Assugeni *et al*., 2017). In Dromiidae, comparative studies with spermatozoa structures under transmission electron microscopy were carried out only with the species *Dromidiopsis edwardsi* Rathbun, 1919 and *Stimdromia lateraris* (=*Petalomera)* Gray, 1831 from the subfamily Dromiinae and *Sphaerodromia lamellata* Crosnier, 1994 from the subfamily Sphaerodromiinae (Jamieson *et al*., 1993a; Jamieson, 1994; Guinot *et al*., 1998). However, there are no studies with representatives of Hypoconchinae subfamily and other Dromiidae.

Therefore, considering the high diversity and the importance of this family as primitive group and the scanty knowledge on spermiotaxonomy, we described the sperm ultrastructure of the Hypoconchinae *Hypoconcha parasitica, H. arcuata* and of the Dromiinae *Moreiradromia antillensis* and *Dromia erythropus* and compared their morphology with Dromiidae, Dromioidea and Podotremata using literature data, in order to clarify the phylogenetic reproductive relationship between the different species of Podotremata.

MATERIALS AND METHODS

Animal samples

Mature males of *Hypoconcha parasitica*, *Hypoconcha arcuata*, *Moreiradromia antillensis* and *Dromia erythropus* were collected in the municipality of Ubatuba, São Paulo, Brazil (25°07´385´´S/47°52´508´´W), from September 2014 to August 2016, using a double-rig shrimp trawling. Subsequently, the animals were transported alive in Styrofoam boxes to the Invertebrate Morphology Laboratory (IML) at the Department of Applied Biology for Agriculture, of the Faculty of Agricultural and Veterinary Sciences at the Campus Jaboticabal at the São Paulo State University (UNESP). The animals were identified according to Melo (1996).

Sperm ultrastructure

The animals were anesthetized by thermal shock (−20 °C) for 15 minutes (López-Greco *et al*., 1999) and dissected. We used at least five animals *per* species, except *D. erythropus* of which we collected only one specimen. Fragments of the posterior vas deferens (1mm³) were fixed in Karnovsky fixative solution (2.5 % glutaraldehyde and 2 % paraformaldehyde) in a 0.1 M sodium cacodylate buffer (pH 7.4), containing 5% sucrose for 4 hours (Ro *et al*., 1990). After fixation, the samples were washed three times with the same buffer and post-fixed in 1% cacodylate-buffered osmium tetroxide for 2 hours. The samples were "en bloc" stained with 1 % aqueous uranyl acetate (overnight), dehydrated in an ascending acetone series (50 to 95 %), embedded and included in Epon-Araldite. Thin and ultrathin section (50-60nm) were obtained with a Leica UC7 ultramicrotome, and contrasted with 2% uranyl acetate in water for 45 minutes and 0.4% lead citrate in 0.1 N NaOH for 10 minutes. The samples were analyzed in JEOL-JEM 1010 transmission electron microscope operated with an 80 kV electron beam, and the digital images were obtained with Gatan camera in the Laboratory of Electron Microscopy, FCAV, UNESP – Jaboticabal Campus, São Paulo, Brazil. For the measures of acrosome lenght/width (C:L), the spermatozoa were arranged horizontally and the largest length and width of the acrosomal vesicle was used for the proportion.

For analyses of the spermatozoa under differential interference contrast phase microscope (DIC), the vas deferens fragments (1mm³) were maintained in sea water (pH 7.4) for the preparation of temporary slides. For this, portions of the vas deferens were macerated on slides containing ± 0.5 ml of sea water and covered with coverslips. These slides were observed in DIC Zeiss Axio Imager Z2.

RESULTS

General diagram

The studied Dromiidae spermatozoa are typically podotreme in overall shape ultrastructure (Fig. 1A). A large portion of the spermatozoon consists of the anteroposteriorly depressed

acrosome (Fig. 1A). The acrosome is discoid and the perforatorial chamber bilaterally capitate (Fig. 1A). Usually, four concentric horizontal zones are discernible occurring from the of the perforatorial chamber in the acrosome vesicle, each one with their own peculiar features. The zones are inner acrosome zone, ray acrosome zone, anterolateral electron-lucid zone and outer acrosome zone (Fig. 1A). The anterior surface of the acrosome is domed over the opercuwlum (Fig. 1A). This structure is perforated and centrally to this there is an apical protuberance with subopercular material (Fig. 1A). The nucleus is electron-pale, filled with fibrous chromatin and, under DIC, are observed two or three radial arms and the operculum perforation (Fig; 1A-D).

Species spermatozoa ultrastructure

Hypoconcha parasitica ─ In the vas deferens lumen, the spermatozoa are immersed in moderately electron-dense secretion, surrounded by granular electron-dense secretion, without typical spermatophore wall (Fig. 2A). Longitudinal section of entire spermatozoon in the wide axis of the perforatorial chamber shows a bilateral capitate perforatorial chamber in the inner acrosome, which has a slightly rounded apex resembling the form of a mushroom (Fig. 2B). The nucleus is filled with fibrous chromatin, surrounding almost all the whole extent of the acrosomal discoid vesicle (Fig. 2B). In longitudinal section the perforatorial chamber apex is rounded (Fig. 2C) and, above the bilateral capitate perforatorial chamber, there is an apical protuberance composed of subopercular material (Fig. 2D). The operculum is perforated centrally and is discontinuous with the acrosomal capsule (Fig. 2D). The acrosome has the form of a disc with C:L of 0.35 ± 0.05, and the horizontal zonation from the center to the margin being divided into: (1) electron-dense innermost zone, surrounded the stalk of the bilateral capitate perforatorial chamber*;* (2) outer acrosome zone, showing electron-dense granular lamellae, surrounded near the apical apex of the perforatorial chamber and extending laterally almost to the periphery of the acrosome; (3) acrosome ray zone, seated above the last zone and (4) the anterolateral electron-lucid zone, that is an extension of the flange of the inner zone and lies posteriorly to the acrosome ray zone (Fig. 2E). In the electron-lucid zone there is a sphere,

which is a discontinuous flange extension of the inner zone (Fig. 2F). The thin cytoplasm is vestigial with reduced lamellar complex and mitochondria with little crest (Fig. 2G). The nucleus is delimited by the nuclear envelope discontinuous with the acrosomal capsule (Fig. 2G). The cross section of the spermatozoon, near the anterior region of the perforatorial chamber, confirms the bilaterally capitate morphology of this structure as well as the presence of an inner acrosome zone, an acrosome ray zone and an anterolateral electron-lucid zone (Fig. 2H). The outer acrosome zone surrounds all the capitate edge of the perforatorial chamber and the ray zone, in which it forms a circumference below the operculum (Fig. 2H). The base of the perforatorial chamber is irregular, presenting three-pointed star-shape in a cross section near this region (Fig. 2I). This cross section also exhibits the extensions of the inner layer of the acrosome, the anterolateral electron-lucid zone and the nucleus (Fig. 2I).

Hypoconcha arcuata ─ the spermatozoa are immersed in moderately electron-dense type I secretion, surrounded by more electron-dense secretion, without spermatophore wall (Fig. 3A). The longitudinal section of entire spermatozoon in the wide axis of the perforatorial chamber shows bilaterally capitate perforatorial chamber with rounded apex, resembling the form of a mushroom with lamellae on the extremities (Fig. 3B). The perforatorial chamber showed lamellae at the ends and the nucleus is filled with fibrous chromatin, which involves almost all extension of the acrosomal vesicle (Fig. 3B). In longitudinal section in the narrow axis of the perforatorial chamber, rounded apex is observed (Fig. 3C). Furthermore, cytoplasmic membrane and mitochondria are present (Fig. 3C). Above the apical region of the perforatorial chamber there is an apical protuberance, which is continuous with the subopercular region and the operculum electron-dense centrally perforated (Fig. 3D). The acrosome is in shape of a disc, with C:L of 0.3 ± 0.05, with horizontal zonation from the center to the periphery of the acrosome vesicle, where four zones may be recognized. These are the innermost zone, which is homogeneous, electron-dense and filled the lower region of the acrosome; the outer zone that is less electron-dense adjacent to the perforatorial chamber; ray acrosome zone that is embedded on the outer zone, and the anterolateral zone that is electron-lucid (Fig. 3E). The almost cross

section over the perforatorial chamber apex confirms the bilateral slightly rounded bilateral-shaped of its extremities and shows the medial region between the operculum, the perforatorial chamber, the outer acrosome zone and the ray acrosome zone (Fig. 3F).

Moreiradromia antillensis ─ The vas deferens lumen showed the spermatozoa immersed in electron-dense secretion I, which is surrounded by electron-lucid secretion II with the presence of electron-dense granules, without typical spermatophore wall (Fig. 4A). The longitudinal section of the spermatozoon in the wide axis of the perforatorial chamber shows that it is bilaterally capitate, with flattened apex, in the form of the letter "T" (Fig. 4B). The acrosome is discoid and the nucleus is filled with fibrous chromatin (Fig. 4B). The longitudinal cross section of the spermatozoon in the narrow axis of the axis confirms that it has a flat apex (Fig. 4C). The operculum is centrally perforated and discontinuous with the acrosomal capsule (Fig. 4C - D). The perforatorial chamber apex contains horizontal lamellae and above this there are the operculum perforated and an apical protuberance filled with subopercular material (Fig. 4D). The acrosome has the form of a disc, with C:L of 0.4 ± 0.05, showing horizontal zonation from the center to the periphery, presenting an inner acrosome zone, composed by horizontal striation; a homogeneous outer acrosome zone; an anterolateral electron-lucid zone, which contains small portions of the inner zone (Fig. 4E). In cross section, it is possible to notice the narrow stalk of the perforatorial chamber, the inner acrosome zone, the anterolateral electron-lucid zone with small portions of inner zone and the nucleus (Fig. 4F).

Dromia erythropus ─ The spermatozoa are immersed in homogeneous, electron-dense type I secretion (Fig. 5A). The spermatozoa and type I secretion are surrounded by type II secretion, that it is moderately granular and electron-dense (Fig. 5A). The type II secretion is surrounded by type III secretion, which is electron-dense (Fig. 5A). This latter secretion type is surrounded by the type IV secretion that is heterogeneous and composed by two elements (Fig. 5A). One element more abundant on the face in contact with type III secretion, which is less electron-dense (Fig. 5A). The other element is next to the epithelium and is more electron-dense (Fig. 5A). The nucleus is filled with thin fibrous chromatin and the radial arms are not observed

under transmission electron microscopy (Fig. 5A-B). The acrosome has the form of a disc, with C:L of 0.38 ± 0.02 (Fig. 5B-C). The longitudinal section above the wide axis of the perforatorial chamber showed flat apex and, at the same time, confirms the bilaterally capitate morphology forming a "T" shaped perforatorial chamber apex (Fig. 5B). The nucleus is filled with fibrous chromatin (Fig. 5B). In the longitudinal section in the narrow axis, the perforatorial chamber is thinly bilaterally capitate and rounded in its edges (Fig. 5C). The operculum form a dome-shaped, with the thicker periphery and thinner centrally perforated inner edge and also the discoid acrosome and mitochondria. (Fig. 5C). The perforatorial chamber presents lamellae, which are perforatorium tubules (Fig. 5D). The operculum appears dome-shaped in longitudinal section, with thicker external periphery and thinner centrally perforated inner margin (Fig. 5C-D). The perforated subopercular region has an apical protuberance, which is subopercular material spillage from the opercular perforation (Fig. 5D). The acrosome shows horizontal zonation from the center to the periphery that is divided in four zones: the inner acrosome zone that is moderately striated and adjacent to the base of the perforatorial chamber, the outer homogeneous, electron-dense acrosome zone, located near the apical acrosome region, the ray acrosome zone and the electron-lucid acrosome zone, both being close to the periphery (Fig. 5E). The anterolateral electron-lucid zone can be clearly observed at the edge of the acrosome in longitudinal and transverse sections (Fig. 5E-F). The bilaterally capitate morphology becomes clear in cross sections near the apex that forms a tetrahedral structure of the head of the perforatorial chamber (Fig. 5F). We also observed that the anterolateral electron-lucid zone and the acrosome ray zone surround the acrosome completely (Fig. 5F).

Dromiidae spermatozoa have discoid acrosome, with billaterally capitate perforatorial chamber, perforated operculum, subopercular protuberance and two or three radial arms, as observed in other species of Dromiidae described in the literature (Table 1). A comparison of the fine ultrastructural characters of the spermatozoon among the Dromiidae species here described and from the previous literature is provided in table 1.

DISCUSSION

Overall, the spermatozoa of *Hypoconcha parasitica*, *H. arcuata*, *Moreiradromia antillensis* and *Dromia erythropus* followed the patterns found for both Dromiidae and Dromioidea species, with the following synapomorphies: depression of the acrosome making it discoid, acrosome zonation predominantly horizontal and perforated operculum. The capitate bilateral perforatorial chamber is consider the unique apomorphy of Dromioidea (Jamieson, 1994).

The ultrastructural characters found in the spermatozoa of Hypoconchinae *H. parasitica* and *H. arcuata* are more similar to each other than when compared to the Dromiinae species *M. antillensis* and *D. erythropus*. The only differences found among the Hypoconchinae were in relation to the outer acrosomal zone and to the presence of lamellae in the apex of the perforatorial chamber. The outer acrosome zone of *H. parasitica* has lamellar aspect, while in *H. arcuata*, this zone is homogeneous. In addition, the lamellae are present only at the apex of the perforatorial chamber of *H. arcuata*. Also, the studied species of Dromiinae share more sperm characteristics with species of this last subfamily, differing in the thickness of the operculum, presence of ray zone, flange and capsular projections.

The spermatozoa of Hypoconchinae, when compared to other dromiids, showed more similarity with the unique member described for the subfamily Sphaerodromiinae, *Sphaerodromia lamellata* Crosnier, 1994. The only differences of *H. parasitica* were the operculum thickness, presence of flange from the discontinuous inner acrosome zone on the anterolateral electron-lucid zone and the wide outer zone that have lamellae resembling fingerprints, below the ray acrosome zone, which does not occur in *S. lamellata* (Guinot *et al*., 1998). Moreover, the morphology of the perforatorial chamber of the mushroom type not is identical, once in the median portion of *H. parasitica* shows a narrowing, which does not occur in Sphaerodromiinae. When comparing spermatozoa characters between *H. arcuata* and the Sphaerodromiinae subfamily, we noticed differences in the operculum thickness and flange presence on the anterolateral electron-lucid zone.

The comparison of the spermatic characters among the Dromiinae shows that Dromiinae present greater similarity with *Dromidiopsis edwardsi* (Jamieson *et al*., 1993a). The only differences between *M. antillensis* and *D. edwardsi* were the C:L, the inner acrosome zone constitution, the flange in the anterolateral electron-lucid zone, the radial arms and the capsular projections. On the other hand, the differences between *Dromia erythropus* and *Dromidiopsis. edwardsi* (Jamieson *et al*., 1993a) were only in relation to the thickness of the operculum, the acrosome ray zone presence and in the constitution of the internal acrosomal zone.

The comparison between the spermatozoa of the Hypoconchinae and Dromiinae species studied and the other species of Dromiinae, revealed that the main difference was related to the acrosome ray zone, which is circular in species of Hypoconchinae. In *M. antillensis* and *Stimdromia lateralis* (Gray, 1831) the acrosome ray zone is absent, while in *Dromidiopsis edwardsi* an inconsistency in the descriptions were observed (Jamieson, 1994). Jamieson *et al*. (1993c) described the acrosome ray zone, which is elongated in *Dromidiopsis edwardsi*, being this zone later named intermediate zone (Jamieson, 1994). The varying thickness of the operculum was found only in *D. erythropus.* Due to the morphological similarity in spermatic characters, our results are in agreement with morphological based analysis of Hypoconchinae and Sphaerodromiinae that suggest that these subfamilies are the most basal within Dromiidae (Jamieson, 1994; Guinot & Tavares, 2003; Guinot *et al*., 2013).

When the Hypoconchinae species were compared with other representatives of Dromioidea, we noted a great similarity of the spermatozoa ultrastructure between Dynomenidae and Homolodromiidae (Jamieson *et al*., 1993a; Jamieson and Tudge, 2000). The only differences between *H. parasitica* and *Metadynomene tanensis* (Yokoya, 1933) were the absence of anterolateral electron-lucid zone, whereas *Paradynomene tuberculata* Sakai, 1963 compared with *H. parasitica* shows the thinner inner zone (Jamieson *et al*., 1993a). The differences found between *H. arcuata* and *Met. tanensis* are regarding C:L, thickness of the operculum, presence of the flange, lamellae at the apex of the perforatorial chamber and radial arms, whereas the differences between

this Hypoconchinae and *P. tuberculata* are thickness of the operculum and presence of lamellae at the apex of the of the perforatorial chamber (Jamieson *et al*., 1993a).

The comparison among the studied Dromiinae and the Dromioidea from the literature shows that *D. erythropus* shares more sperm characters with the species of Dynomenidae than with those of Homolodromiidae. The differences found between *D. erythropus* and *Met. tanensis* were the acrosome C:L ratio, the constitution of the internal acrosomal zone, the perforatorial chamber apex and the bilaterally capitate perforatorium chamber figure. While *D. erythropus* and *P. tuberculata* differ in the constitution of the internal acrosomal zone, presence of the flange, the perforatorial chamber apex and bilaterally capitate perforatorium chamber. The spermatozoon of *M. antillensis* is more distinct from species of Dynomenidae and Homolodromiidae and all characteristics of *M. antillensis* were observed in the spermatozoa of other Dromiidae species from the literature (Jamieson *et al*., 1993 a, b, c; Jamieson, 1994, Guinot *et al*., 1998, Jamieson and Tudge, 2000).

According to Jamieson and Tudge (2000), the spermatic characteristics found in Homolodromiidae represent a mix between the characters of Dromiidae and Dynomenidae. We also observed this mix of characters and therefore could not yet find any robust character to separate the Dromiidae from this study from species of Homolodromiidae. Thus, there is no distinct pattern of the ultrastructure of the spermatozoa for Dromioidea, and the representatives of this superfamily only differ from others Podotremata, which agrees with the proposal from Guinot *et al*. (1998). Moreover, the absence of distinct characters between Dromioidea families and subfamilies is in agreement with the monophyly of the group, previously proposed by means of spermiotaxonomy (Jamieson, 1994, Jamieson *et al*., 1995, Guinot *et al*., 1998) and molecular analyzes (Ahyong *et al*., 2007; Tsang *et al*., 2014). Although Ahyong *et al*. (2007) suggests that *Hypoconcha* is the most divergent branch within the topology for Dromiidae, no spermatic characteristic was observed to corroborate this finding. Furthermore, in this last studied the only representative of Dromiinae is closer to Dynomenidae in the phylogenetic tree. Regardless, the studied species of Hypoconchinae and Dromiinae have typically dromioid spermatozoa and any

attempt to include them in Anomura (Spears *et al*., 1992), must be abandoned and has no spermiotaxonomy support.

Tsang *et al*. (2014) demonstrated that Dromiidae and Dynomenidae are a monophyletic group, as well as Homolidae and Latreilliidae are also monophyletic. Additionally, due to the addition of Latreilliidae representatives these two groups are shown to be paraphyletic (Tsang *et al*., 2014). When we compared *H. parasitica*, *H. arcuata*, *M. antillensis* and *D. erythropus* as well as the Dromiidae and Dynomenidae described in the literature, we noticed a clear separation of sperm characteristics between the two clades. Homolidae and Latreilliidae, differ in spermiotaxonomy from each other and between Dromiidae and Dynomenidae, in the following characters: 1) the acrosome with less discoid-shape, 2) the apical protuberance undeveloped (Homolidae) or absent (Latreilliidae), 3) the capitate perforatorium chamber, 4) absence of the acrosome ray zone and 5) opercular projections (Homolidae). Although molecular data for Homolodromiidae were not included in the most current phylogeny of Brachyura (Tsang *et al*., 2014), based on the ultrastructure of spermatozoa, it is clear to us that Homolodromiidae is more related to Dromiidae and Dynomenidae than Homolidae and Latreilliidae.

When the spermatozoa of the studied Dromiidae was compared to the Podotremata from the Raninidae family, it was noticed a pronounced difference of structural organization (Jamieson, 1989; Jamieson and Tudge, 2000). In Raninidae, the spermatozoa shows another type of acrosomal zonation, which is more concentric than horizontal, presence of multiple capsular projections, posterior capsule chambers, tapered and reduced perforatorium chamber and specially, posterior median process (Jamieson, 1989), typical for Majoidea (for revision see Tudge *et al*., 2012). In a general analysis, the spermatozoon of *R. ranina* shared more characteristics with representatives of Cyclodorippoidea, Homolidae and Eubrachyura than with Dromiidae or other Dromioidea, which cohoborates what is observed in molecular phylogeny (Jamieson and Tudge, 2000; Tsang *et al*., 2014). Thus, the proposed grouping using of Dromiacea, Raninoidea and Cyclodorippoidea, supported by molecular phylogeny (Ahyong *et al*., 2007, 2011), appear to better represent the morphological variations of the spermatozoa ultrastructure than the use of Podotremata. Therefore,

the spermatozoa ultrastructural characteristics are a robust tool that could be essential for the resolution of taxonomic problems, in association with molecular tools.

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Figure 1. General diagram of Dromiidae spermatozoa under transmission electron microscope and differential interference contrast microscopy (DIC). (A) The spermatozoa have discoid acrosome, with bilaterally capitate perforatorial chamber. Note four concentric horizontal zones occurring from the perforatorial chamber, such as: inner acrosome zone, acrosome ray zone, anterolateral electron-lucid zone and outer acrosome zone. The operculum showed perforation and centrally to this there is an apical protuberance. The nuclei are filled with fibers of chromatin, with the presence or absence of radial arms. **(B)** Spermatozoa of *H. parasitica* and *H. arcuata* on DIC showing the operculum centrally perforated and the presence of two or three radial arms. **(C)** On DIC, the spermatozoa of *M. antillensis* and **(D)** *D. erythropus* show an operculum and three radial arms. RA = radial arms; O = operculum.

Figure 2. Spermatozoa ultrastructure of *Hypoconcha parasitica*. (A) Lumen of the posterior vas deferens showing the spermatozoa immersed in secretion moderately electron-dense, surrounded by the more electron-dense secretion, without the typical spermatophore wall. **(B)** Longitudinal section of entire spermatozoon in the wide axis of the perforatorial chamber, showing their bilateral capitate morphology and apex with a mushroom-shape. Notice the thin nucleus filled with fibrous chromatin, surrounding almost all the whole extent of the acrosomal discoid vesicle. **(C)** In longitudinal section, the apex of the perforatorial chamber is rounded. Observe the acrosome and the nucleus filled with fibrous chromatin. **(D)** Above the perforatorial chamber, there is the continuous apical protuberance with the subopercular region and the operculum is perforated (arrow) and discontinuous with the acrosomal capsule. **(E)** The acrosome zones are electron-dense innermost zone, surrounded the stalk of the perforatorial chamber; outer acrosome zone, showing electron-dense granular lamellae and surrounded near the apical apex of the perforatorial chamber; acrosome ray zone, seated above the last zone and the anterolateral electron-lucid zone, lies posterior to the acrosome ray zone. **(F)** The sphere on the anterolateral electron-lucid zone (arrow) is an extension of the flange of the inner zone. Note the acrosome ray zone and a little portion of the anterolateral electron-lucid zone. **(G)** Detail of the thin cytoplasm near the edge of the acrosome, showing a mitochondria with

little crest and the nucleus delimited by the nuclear envelope (arrow) discontinuous with the acrosomal capsule. **(H)** Cross section of the spermatozoon, near the anterior region of the perforatorial chamber, showing the bilaterally capitate morphology of this structure (arrows), inner acrosome zone, acrosome ray zone and anterolateral electron-lucid zone. Note that the outer acrosome zone surrounds all the capitate edge of the perforatorial chamber region and the ray zone, in which it forms a circumference below the operculum. **(I)** The irregular base of the perforatorial chamber, showing three-pointed star-shape in the cross section near this region. The base of the perfuratorium becames wider and forms an irregular figure (arrow). This cross section also shows the extensions of the inner layer of the acrosome, the anterolateral electron-lucid and the nucleus. AC = acrosome; AP = apical protuberance; ARY = ray acrosome zone; EA = anterolateral electron-lucid zone; IA = inner acrosome zone; M = mitochondria; N = nucleus; O = operculum; OA = outer acrosome zone; P = perforatorial chamber; PA = perforatorial chamber apex; PB = perforatorial chamber base; SI = type I secretion; SII = type II secretion; SZ = spermatozoa.

Figure 3. Spermatozoa ultrastructure of *Hypoconcha arcuata*. (A) The spermatozoa are immersed in less electron-dense type I secretion, which are surrounded by more electron-dense type II secretion. Note that typical spermatophore wall is absent. **(B)** Overview of spermatozoa in longitudinal section on the wide axis of the bilaterally capitate perforatorial chamber, showing the mushroom-shaped morphology of the apex. Observe the presence of lamellae at the ends of the perforatorial chamber and the nucleus filled with fibrous chromatin, which involves almost all extension of the acrosomal vesicle. **(C)** In longitudinal section of the narrow axis of the perforatorial chamber, their apex is rounded. Note the cytoplasmic membrane and mitochondria. **(D)** Above the perforatorial chamber is the apical protuberance and the operculum electron-dense centrally perforated. **(E)** The discoid acrosome composed of four concentric zones, such as: homogeneous and electron-dense inner acrosome zone, filled the lower region of the acrosome; the less electron-dense outer acrosome zone, adjacent to the perforatorial chamber apex; acrosome ray zone, embedded on the outer zone and the anterolateral electron-lucid zone. **(F)** The almost transverse cross over the apex of the perforatorial chamber (white arrows) confirms the slightly rounded bilateral-shape of its extremities and we can observe the anterolateral electron-lucid region (black arrows) between the perforatorial chamber and the outer

acrosome zone, and the ray acrosome zone. AC = acrossome; AP = apical protuberance; ARY = ray acrosome zone; CY = cytoplasm; EA = anterolateral electron-lucid zone; IA = inner acrosome zone; M = mitochondria; N = nucleus; L = lamellae; O = operculum; OA= outer acrosome zone; P = perforatorial chamber; PA = perforatorial chamber apex; SI = type I secretion; SII = type II secretion; SZ = spermatozoa.

Figure 4. Spermatozoa ultrastructure of *Moreiradromia antillensis.* (**A)** General appearance of the vas deferens lumen, showing spermatozoa in electron-dense secretion I, which is involved by the less electron-lucid secretion II with the presence of electron-dense granules, without the typical spermatophore wall. **(B)** The longitudinal cross in the wide axis of the perforatorial chamber shows their bilateral capitate morphology, with flattened apex, shaped similarly to the letter "T". Note the discoid acrosome and the nucleus filled with fibrous chromatin. **(C)** Longitudinal section in the narrow axis of the perforatorial chamber showing the flat apex and the centrally perforated operculum, which is discontinuous with the acrosomal capsule (arrow). **(D)** Perforatorial chamber apex with horizontal lamellae. Note the operculum (arrowhead) and the apical protuberance filled with subopercular material. **(E)** The discoid acrosome showing the horizontal zonation is formed by four zones: the inner acrosome zone, composed by horizontal striation (write arrow), the homogeneous outer acrosome zone, the anterolateral electron-lucid zone, which seems to contain electron-dense remnants of the inner acrosome zone (black arrow). **(F)** In cross section, note the narrow stalk of the perforatorial chamber, the inner acrosome zone, anterolateral electron-lucid zone with small portions of inner zone and the nucleus. AC = acrosome; AP = apical protuberance; EA = anterolateral electron-lucid zone;; IA = inner acrosome zone; N = nucleus; L = lamellae; O = operculum; OA = outer acrosome zone; P = perforatorial chamber; PA = perforatorial chamber apex; SI = type I secretion; SII = type II secretion; SZ = spermatozoa.

Figure 5. Spermatozoa ultrastructure of *Dromia erythropus*. (A) The spermatozoa are immersed in electron-dense, homogeneous type I secretion, which is surrounded by the type II secretion, that is moderately electron-dense with granules. The II secretion is involved by electron-dense type III secretion. Note that this latter secretion is surrounded by heterogeneous type IV secretion, composed of two elements: one more abundant on the

face in contact with type III secretion, which is less electron-dense and another, next to the epithelium, which is less electron-dense (arrows). **(B)** Discoid acrosome in longitudinal section to the wide axis of the bilaterally capitate perforatorial chamber, showing the morphology with the shape of the letter "T", with flat apex. Note the nucleus filled with fibrous chromatin. **(C)** Longitudinal section in the narrow axis of the perforatorial chamber, showing that it is thinly bilaterally capitate and rounded in the edges. In this section, also note the presence of the operculum forming a dome-shaped, with the thicker periphery (white arrowhead) and thinner centrally perforated inner edge (black arrowhead), and also the discoid acrosome and mitochondria. **(D)** Opercular region perforated (arrowhead) marked with the apical protuberance and the perforatorial chamber apex showing horizontal lamellae. **(E)** Acrosome with horizontal zonation, being divided in inner acrosome zone slightly striated, adjacent to the base of the perforatorial chamber, electron-dense, homogeneous outer acrosome zone, near the apical region of the acrosome, ray acrosome zone and anterolateral electron-lucid zone. **(F)** Morphology of the bilaterally capitate perforatorial chamber in cross section near the apex, forming a tetrahedral structure. Note the anterolateral electron-lucid and ray acrosome zones surrounded completely the acrosome. AC = acrosome; AP = apical protuberance; ARY = ray acrosome zone; EA = anterolateral electron-lucid zone; IA = inner acrosome zone; M = mitochondria; N = nucleus; L = lamellae; O = operculum; Ao = outer acrosome zone; P = perforatorial chamber; PA = perforatorium chamber apex; SI = type I secretion; SII = Type II secretion; SIII = type III secretion; SIV = type IV secretion; SZ = spermatozoa.

List of figures and table

Fig. 1

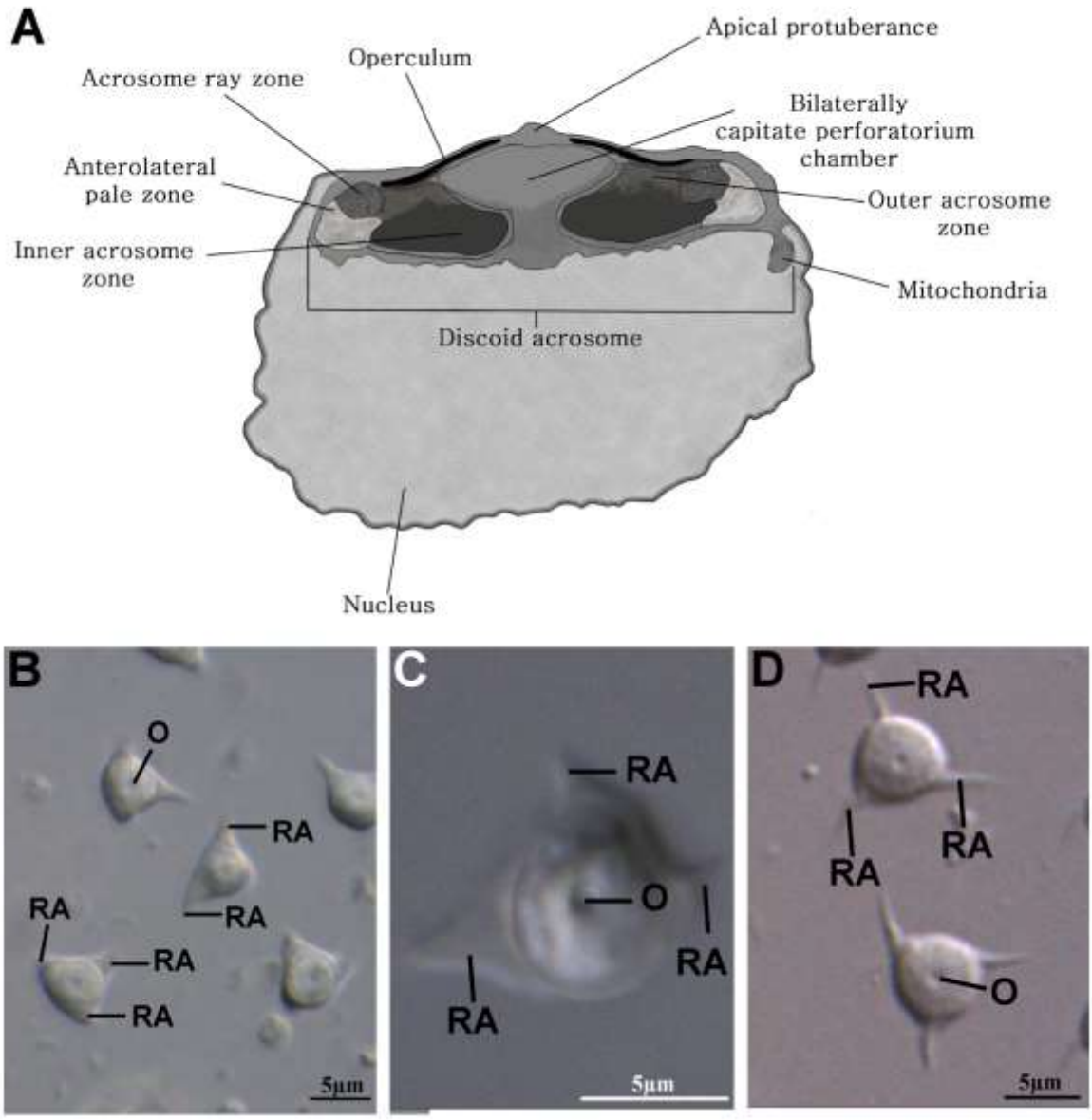
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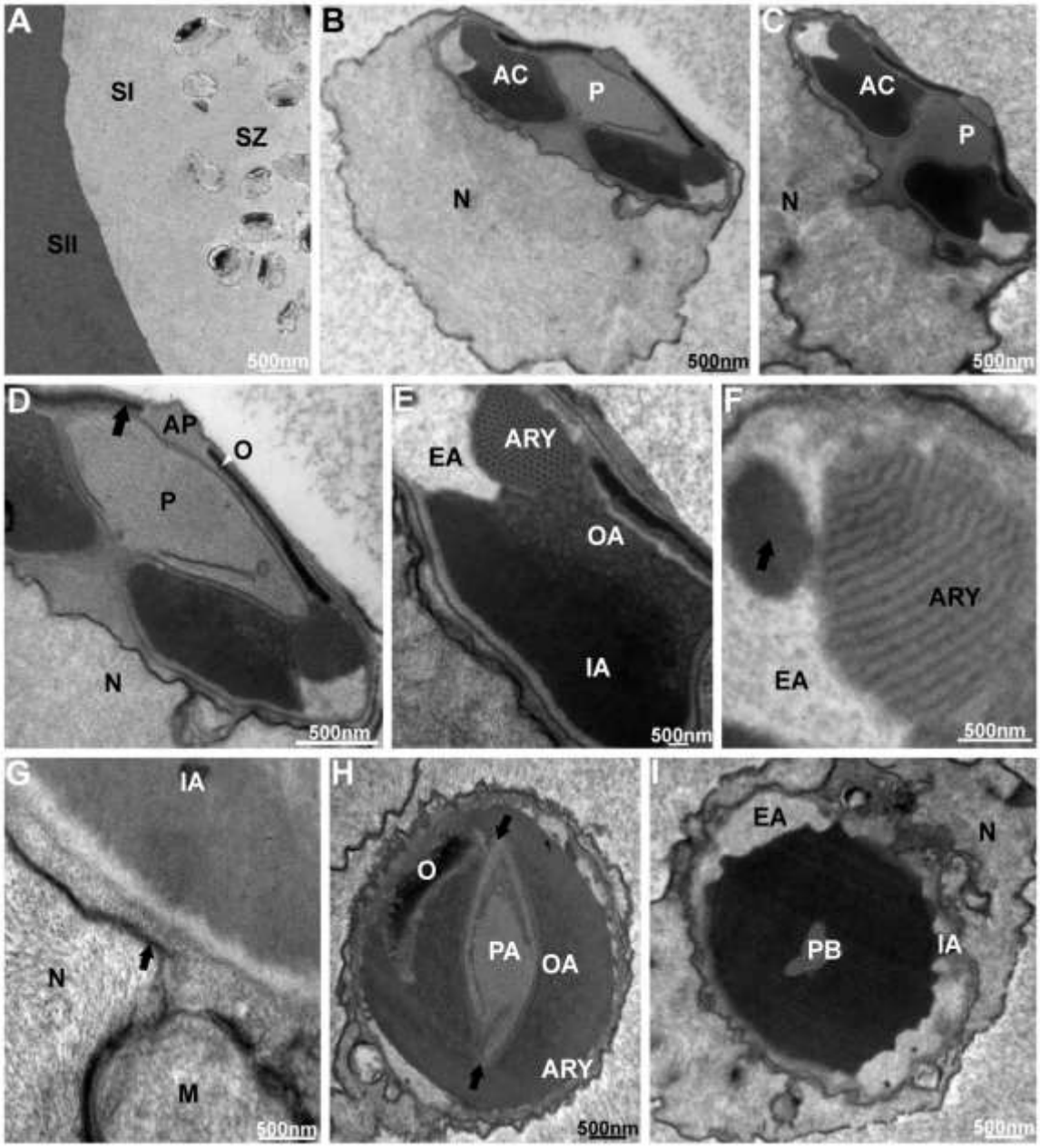
Fig. 2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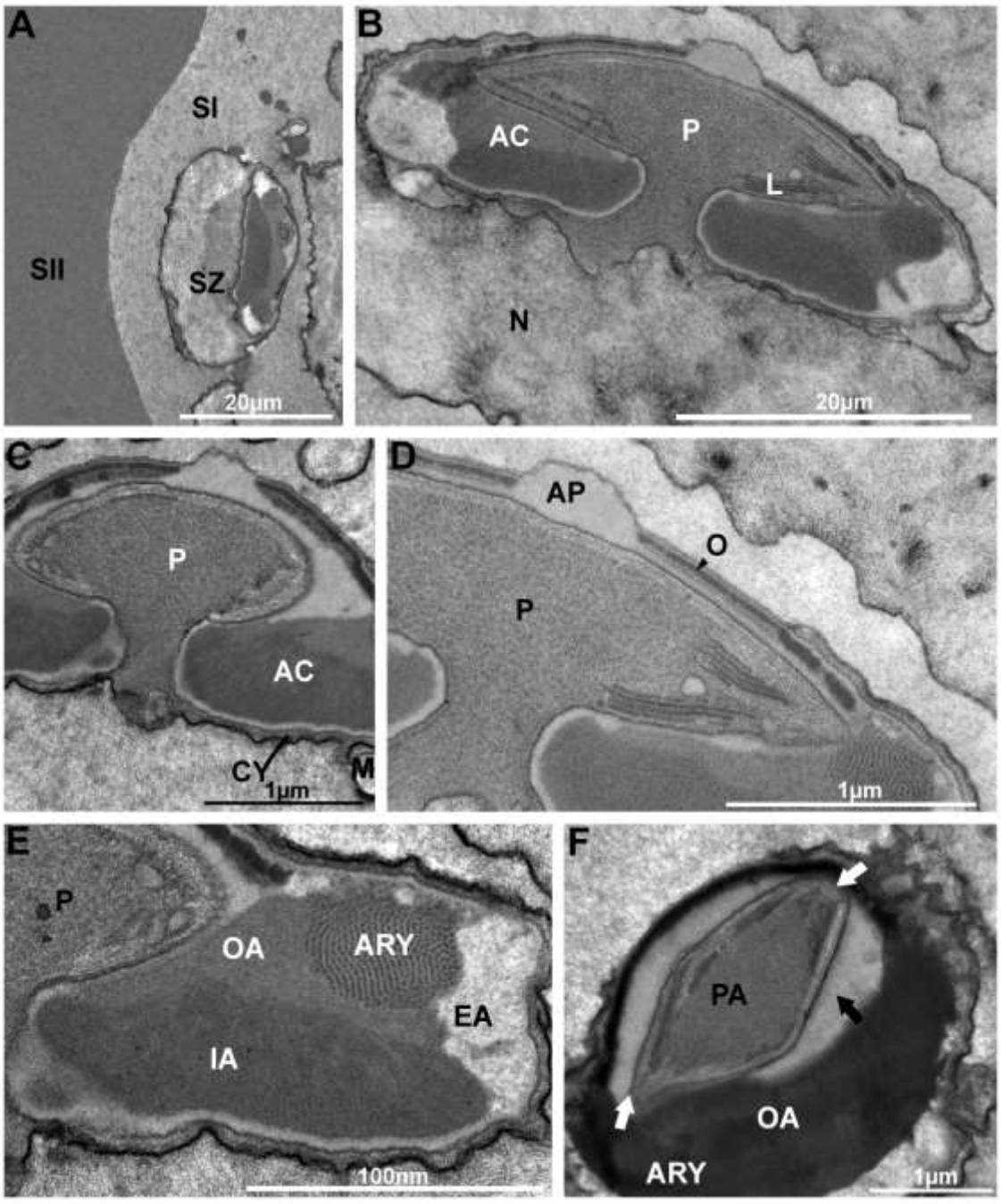
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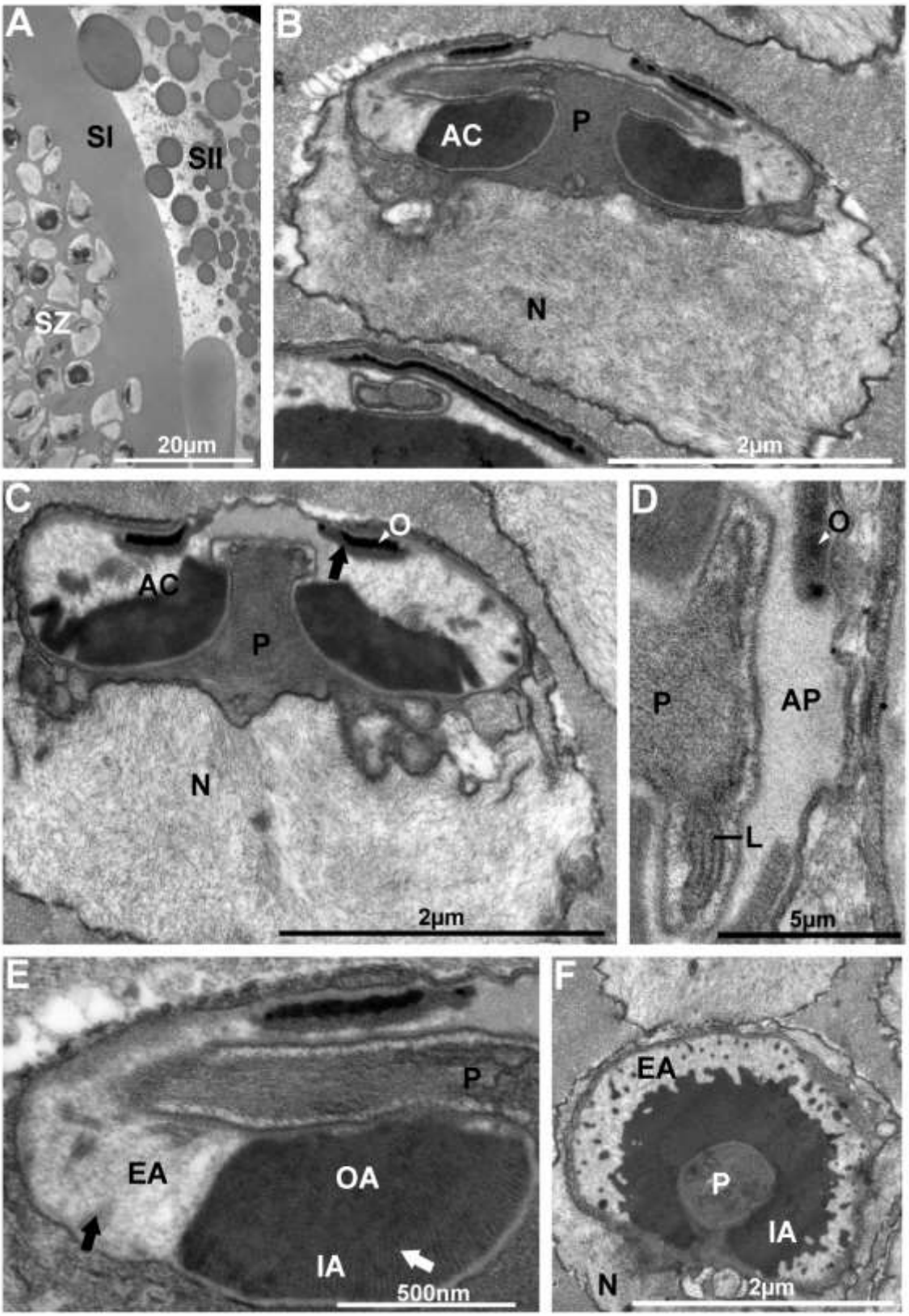
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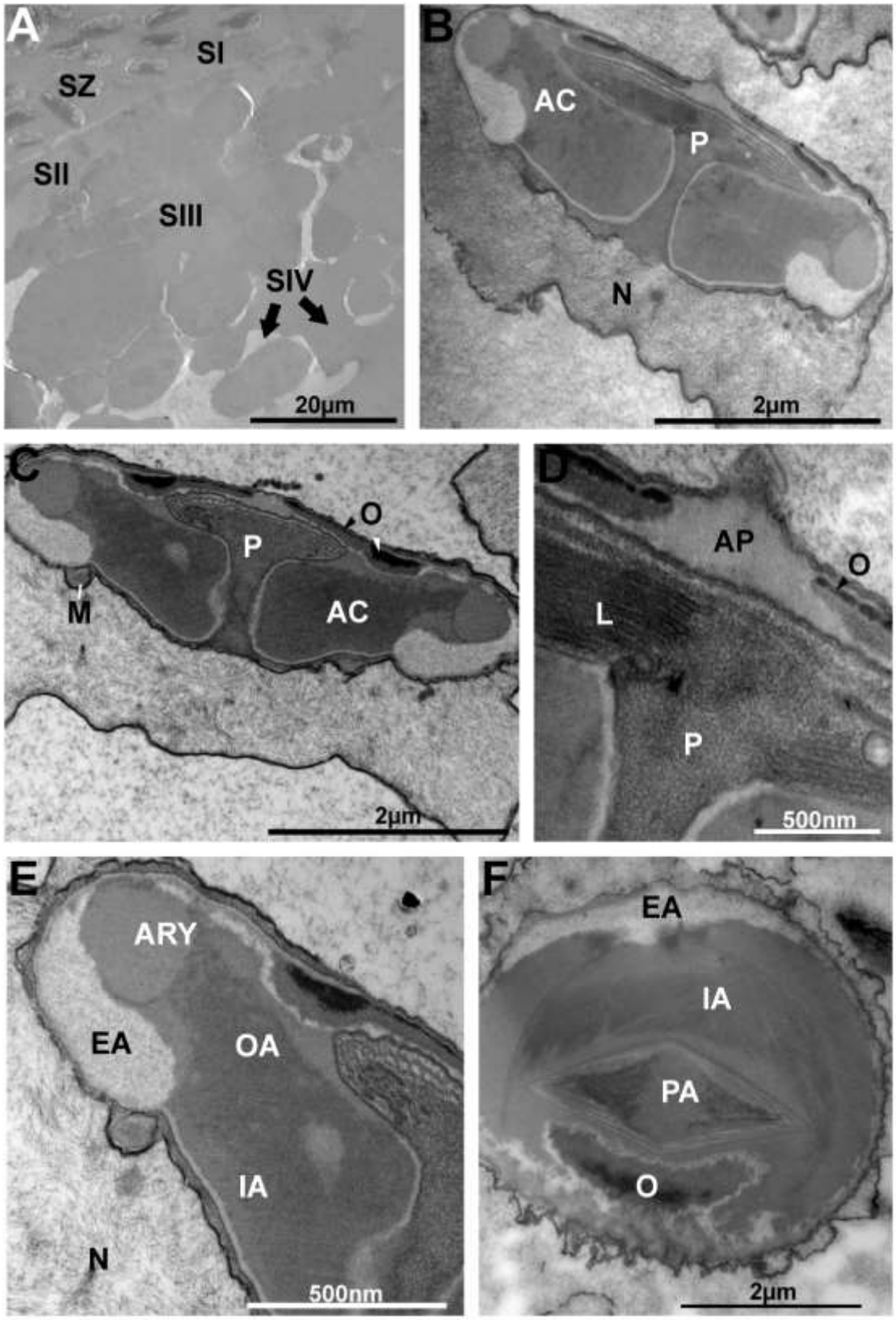
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Table 1. Comparison of the ultrastructural characters of the spermatozoid among species of the Dromiidae subfamilies, described in the literature and from this study.

		Hypoconchinae			Dromiinae			Sphaerodromiinae
Characteristics		*Hypoconcha parasitica*[1]	*Hypoconcha arcuata*[1]	*Dromia erythropus*[1]	*Dromidiopsis Edwardsi*[4,5]	*Moreiradromia antillensis*[1]	*Stimdromia Lateralis*[2,3,5]	*Sphaerodromia lamellata*[2,5]
	Length/ width	0.3				0.4		0.5
Acrosome	Anterolateral electron-lucid zone	Present						
	Ray acrosome zone – rounded on the outer acrosome zone	Present				Absent		Present
	Outer acrosome zone	Lamellar	Homogeneous					
	Inner acrosome zone	Homogeneous		Striated	Homogeneous	Striated	Homogeneous	
	Flange of the inner acrosomal layer discontinuous in the electron-lucid zone	Present	Absent			Present	Absent	
Aspect of chromatin		Granular						
Capsular projections		Absent				Present		Absent
Centriole		Present			Absent	Present	Absent	
Lamellar structure		Present						
Mitochondria		Present						
Operculum	Type	Perforate						
	Opercular projections	Absent						
	Continuity of the capsule and operculum	Discontinuous						
	Operculum thickness	Thin		Thick and thin	Thin			Thick
	Subopercular protuberance	Present						
Perforatorial chamber	Perforatorial chamber apex	Rounded		Flat			Rounded	
	Bilateral capitate perforatorial chamber (figure of)	Mushroom		Letter “T”			Mushroom	
	Lamellae at perforatorial chamber apex	Absent	Present				Absent	Present
Radial arms		Present						

[1]This study, [2]Jamieson (1994); [3]Jamieson et al (1993); [4] Guinot et al (1998); [5]Jamieson et al., (1995)

Capítulo II

SEMINAL FLUID PRODUCTION AND SPERM TRANSFER IN DROMIIDS: NEW INSIGHTS INTO THE EVOLUTION OF CRAB REPRODUCTION

Maria Alice Garcia Bento, Laura López Greco and Fernando José Zara

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SEMINAL FLUID PRODUCTION AND SPERM TRANSFER IN DROMIIDS: NEW INSIGHTS INTO THE EVOLUTION OF CRAB REPRODUCTION

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Short running title: SPERMATOPHORE PRODUCTION AND SPERM TRANSFER IN DROMIIDAE CRABS

ABSTRACT

Reproductive anatomy, including sperm storage structures and sperm transfer, is an important feature used to analyze phylogenetic relationships among taxa. We describe male reproductive anatomy, the seminal fluid production and the new spermatozoa packaging in the vas deferens of primitive crabs, also emphasizing spermatozoa transfer compared to true crabs. In all species of Dromiidae, the testes were tubular type and the vas deferens is a tubule with a simple epithelium. The spermatozoa are in a central mass immersed in type I secretion, forming a large spermatic cord. In Hypoconchinae species and *Moreiradromia antillensis*, the spermatic cord is surrounded by type II secretions, while in *D. erythropus* we also found secretions of types III and IV. These patterns produce a unique elongated kind of coenospermic "spermatophore," or continuous cord totally different from the spermatophores of all true crabs. In all Dromiidae species, the penial tube is a long conical-round paired organ. The apex of penial tubes has a mobile operculum, avoiding the reflux from gonopod G2 during the sperm transfer. A true sperm plug was not found in all studied species. Our results show a different pattern to sperm package and new insights about how the sperm transfer occurs in Podotremata.

ADDITIONAL KEYWORDS: anatomy – ultrastructure – male reproductive system – *Hypoconcha* – Podotremata – spermatheca.

INTRODUCTION

Detailed comparative morphological analyses of the reproductive systems and associated structures of Decapoda allow the use of these characters for phylogenetic and evolutionary inferences (McLay & López Greco, 2011; Guinot, Tavares & Castro, 2013; López Greco, 2013; McLay & Becker, 2015). Primitive crabs are included in Podotremata (Guinot, 1977). In the species in this group, the paired gonopores are sternal located on the coxae of both fifth pereopods of males and on the coxae of each third pereopod in females (Guinot & Tavares, 2003, Guinot & Quenette, 2005; Guinot *et al*., 2013). In addition, the females exhibit paired spermathecae, which are a enlarged intersegmental phragmata formed by adjacent boundaries of sternites 7 and 8, with the opening in different sternites, depending on the family or subfamily (Tavares & Secretan, 1993; Guinot & Tavares, 2003; Guinot & Quenette, 2005). The spermatheca is a structure of ectodermal origin in which the spermatozoa are stored (Hartnoll, 1969; Guinot & Quenette, 2005; Guinot *et al*., 2013). This structure has no connection to the ovaries; therefore, the fertilization process occurs outside the body (Hartnoll, 1969; Guinot & Quenette, 2005; Guinot *et al*., 2013). In contrast, in the true crabs (eubrachyuran), the gonopores are sternal, and the spermatozoa are stored in the pair of seminal receptacles located within the sixth thoracic sternite, with direct connection to the ovaries by the oviducts (for revision McLay & López Greco, 2011; McLay & Becker, 2015). Thus, the fertilization in these latter crabs takes place inside the seminal receptacles (Hartnoll, 1969; McLay & López Greco, 2011).

Generally, the male reproductive system of crabs is a bilateral organ forming an “H” shape that is composed of a pair of testis continuous with a pair of vasa deferentia ending in the posterior region of the pair of fifth pereopods (Krol, Hawkins & Overstreet, 1992). Spermatogenesis and spermiogenesis occur in the testes, and from a histological point of view, this organ can be classified as lobular or tubular (Nagao & Munehara, 2003). Mature spermatozoa are released in the vas deferens, which can be divided into three regions based on morphology and function: anterior (AVD), where the spermatophores are formed; a middle region (MVD); and a posterior region (PVD), where seminal fluid production occurs, surrounding the spermatozoa until copulation (Krol *et al*., 1992; Zara *et al*., 2012; Tiseo, Mantelatto & Zara, 2014). Additionally, the MVD and PVD of some brachyuran species present accessory glands, also called outpockets, diverticula or caeca (Johnson, 1980; Simeó, Ribes & Rotllant, 2009; Tiseo *et al*., 2014). These structures produce different types of secretions, which are added to the seminal fluid (Simeó *et al*., 2009, Tiseo *et al*., 2014).

In Decapoda, spermatozoa are packaged in different ways inside the vas deferens. In lobsters (Astacidea), there are different secretions sequentially produced along the vas deferens constituting the spermatophore wall (for revision Kooda-Cisco & Talbot, 1982; Dudenhausaennd & Talbot; 1983; Hobbs, Harvey & Hobbs, 2007; López Greco, Vazquez & Rodríguez, 2007; López Greco, 2013). In hermit crabs (Anomura) the spermatozoa are packed into complex spermatophores composed of an ampulla, peduncle, stalk and pedestal (Hinsch, 1991; Tudge, 1991; Mantelatto, Scelzo & Tudge, 2009; Fantucci & Mantelatto, 2011). In Brachyura, the spermatozoa are packaged into elliptical coenospermic spermatophores, in which several spermatozoa are grouped and delimited by a single wall (El-Sherief, 1991; Guinot, Jamieson & Tudge, 1997; Anilkumar, Sudha & Subramoniam, 1999; Tiseo *et al*., 2014; Tiseo, Mantelatto & Zara, 2017). In contrast, in some freshwater crabs and one marine species, each spermatophore has only one spermatozoon, known as a cleistoespermic spermatophore (Guinot *et al*., 1997; Klaus, Schubart & Brandis, 2009; Tiseo *et al*., 2014, 2017). The absence of spermatophores is a rare condition in Brachyura, but it has been observed in some freshwater crabs from Potamidae and Gecarcinucidae (Guinot *et al*., 1997; Klaus *et al*., 2009; Klaus & Brandis, 2011). In Podotremata, few articles have described the pattern of sperm packaging within the vas deferens. In Dromiidae and Raninidae, the male reproductive system is divided into three anatomical regions (Hartnoll, 1975; Minagawa *et al*., 1994), while in Homolidae, no different regions are described, despite them being obvious in the drawings of Hartnoll (1975). The histological differences schematized for *Dromia personata* (Linnaeus, 1758) need to be reviewed in Dromiidae since the anterior and median regions seem to be very similar in the drawings. In studies of Podotremata, the authors do not discuss the sperm packaging pattern or the presence of true spermatophores (Hartnoll, 1975). Thus, studies are required to determine if the sperm packaging patterns found in primitive crabs follows the same model of spermatophore morphology found in Eubrachyura species. It is expected that the spermatophores of the Dromiidae species studied here are of the coenospermic or cleistospermic type, following the commonly described models for crabs (El-Sherief, 1991; Guinot *et al*., 1997; Anilkumar *et al*., 1999; Klaus *et al*., 2009; Zara *et al*., 2012; Tiseo *et al*., 2014, 2017).

In Brachyura, seminal fluid is transferred to females through two pairs of structures known as gonopod one (G1) and gonopod two (G2), respectively (Hartnoll, 1969; Guinot *et al*., 2013; Mclay & Becker, 2015). In Podotremata, exclusive to the Hypoconchinae and Dromiinae subfamilies, there are also present penial tubes, which are mobile structures

formed by vas deferens protusions from gonopores on the coxa of the last thoracic pereopod (P5) (Guinot & Tavares, 2003; Guinot *et al*., 2013). This sclerotized tube receives the vas deferens contents, and it is inserted in the very long needle-like apex of the G2, which is inserted at the base of the G1, assisting in sperm transfer (Guinot *et al*., 2013). Despite being well-studied from a morphological point of view, the function of the mobile penial tubes and the gonopods in Dromiidae during the sperm transfer is poorly understood (Hartnoll, 1969; Guinot & Tavares 2003; Guinot *et al*., 2013), especially in species with a long G2, such as Hypoconchinae and Dromiinae (McLay & Becker, 2015). In some species of Brachyura, the end of the sperm transfer is marked by the formation of a spermatic plug, which blocks the entrance of the vagina or vulva, preventing successive copulations (Hartnoll, 1969; Guinot *et al*., 2013; McLay & Becker, 2015). Although the absence of a sperm plug has been confirmed for Hypoconchinae, the presence of hardened material deposited on the female sternum was found in some Dromiidae (Guinot & Tavares, 2003; Guinot *et al.*, 2013). However, different from the pattern found in Eubrachyura sperm plug, presence of traces of some spermatozoa was noticed in Dromiidae. Therefore, no studies proving the function of this secretion has carried out to Dromiidae crabs. Due to the importance of this group for understanding the evolution of Brachyura and the gap in knowledge of the male reproductive system of primitive crabs, this study describes the male reproductive system of four species of Dromiidae under light and electron microscopy. In addition, we describe the anatomy of the spermatozoa storage structures of ovigerous females of Hypoconchinae and Dromiinae, as seen by stereomicroscope analysis. We aim to elucidate the pattern of seminal fluid production and chemical composition and to describe the spermatozoa package in the vas deferens and the transfer of the seminal contents through the mobile penial tube and G2 of the male. Moreover, we investigated the presence of the sperm plug on the spermathecal aperture of females.

MATERIALS AND METHODS

Adult males of *Hypoconcha parasitica* (Linnaeus, 1763), *Hypoconcha arcuata* Stimpson, 1858, *Moreiradromia antillensis* (Stimpson, 1858) and *Dromia erythropus* (George Edwards, 1771) were collected by trawling (20 min) using a shrimp fishing boat in the municipality of Ubatuba, São Paulo, Brazil (25°07´385´´S/47°52´508´´W), from October of 2014 to August of 2016. The animals were transported alive in Styrofoam boxes with proper aeration to the laboratory, where they were maintained alive in tanks.

We used at least five animals per species for all techniques, except *D. erythropus,* because we collected only one specimen in two years. The animals were anesthetized by thermal shock (-20°C/10 min), and the cephalothorax was removed. After being anesthetized, the whole animal was fixed in 4% paraformaldehyde prepared with seawater and buffered 0.2 M sodium phosphate (pH 7.2) to view the gross anatomy of the male reproductive system under a Leica® stereomicroscope. For histological and histochemical description, the mature male reproductive system was removed and kept in the same fixative for 24 h (4°C). The samples were washed twice (30 min) in the same buffer, dehydrated in an ethanol series (70 a 95%), and embedded and enclosed in methacrylate historesin Leica®. Serial 4-7 μm thick sections were cut on a Leica® rotary microtome and stained with hematoxylin and eosin (Junqueira & Junqueira, 1983). For histochemistry, slides were stained with ponceau xylidine for proteins (Mello & Vidal, 1980) and periodic acid-Schiff (PAS) and Alcian Blue (pH 2.5) for neutral and acidic polysaccharides, respectively (Junqueira & Junqueira, 1983). PAS combined with hematoxylin (Junqueira & Junqueira, 1983) was also used to detect the different stages of spermiogenesis. In addition, ovigerous females of *H. parasitica* (N=6) and *M. antillensis* (N=2) from our collection preserved in 80% ethanol were also checked under a stereomicroscope to verify the structure of pleopod one (PL1) and two (PL2) and the presence of a sperm plug on the spermathecal aperture.

For the ultrastructure analysis, fragments of AMV, MVD and PVD ($1mm^3$) were fixed in 2.5% glutaraldehyde in marPHEM (PHEM 1.5X+ 9% sucrose) (Montanaro, Gruber & Leisch, 2016) for 4 h at 4°C, washed three times in PHEM buffer (Montanaro *et al*., 2016) and post-fixed with 1% osmium tetroxide buffered for 2 h. The samples were "en bloc" stained using 1% aqueous uranyl acetate (overnight), dehydrated in acetone series, embedded and included in Epon-Araldite resin. Thin and ultrathin sections were obtained with a Leica UC7 ultramicrotome and contrasted with 2% uranyl acetate in water for 45 min and 0.4% lead citrate in 0.1 N NaOH for 10 min (Reynolds, 1963). The images were obtained with a Jeol J1010 transmission electron microscope operated with an 80 kV electron beam.

The mobile penial tubes of *H. parasitica, H. arcuata, M. antillensis* and *D. erythropus* were removed with the last thoracic pereopod (P5) The samples were fixed in 3% glutaraldehyde in sodium phosphate buffer (pH 7.2) for 24 h. All structures were dehydrated in an ascending series of ethanol (30-100%) and completely dried in EMS 850 critical-point using liquid CO_2. The materials were placed on SEM stubs and sputter-coated with gold (5 nm) with Dayton vacuum sputtering. The samples were observed and

photographed in a scanning electron microscope (JEOL JSM5410) using a 15 kV electron beam.

To describe the mechanism by which seminal fluid packs the spermatozoa, 2 cm-long fragments of vas deferens of *H. parasitica* and *M. antillensis* were squeezed for luminal content extrusion using forceps to release the seminal fluid onto slides containing seawater. In addition, some material found on the female spermathecal aperture of both species were removed onto slides and stained with neutral red to check for the presence of spermatozoa. As the female *M. antillensis* was fixed in 100% alcohol, we used a spermatozoon fixed in 4% paraformaldehyde prepared with seawater and 0.2 M sodium phosphate buffered to compare the two morphologies. All samples were analyzed under a differential interference phase contrast microscope (Zeiss Axio Scope Z2). To verify the presence of a sperm plug and to describe the morphology of the paired female pleopods in Hypoconchinae and Dromiinae, females of *H. parasitica* (n=5) and *M. antillensis* (n=5) were obtained from the collection of the Invertebrate Morphology Laboratory (UNESP - Jaboticabal). The dorsal external structures were examined under a Leica® stereomicroscope and photographed using the Leica IM50 program.

RESULTS

GROSS ANATOMY

In *H. parasitica*, *H. arcuata*, *M. antillensis* and *D. erythropus*, the male reproductive system is a bilateral "H"- shaped organ composed of a pair of testes, located in both the superior cephalothorax sides, without reaching the lateral margins (Fig. 1A - C). Each pair of testes is a whitish and convoluted tubular organ. Connected to the testes is a pair of vasa deferentia, which extend longitudinally over the hepatopancreas and below the heart, toward the ventral posterior region of the body (Fig. 1A - C). Each vas deferens ends in the ejaculatory duct that opens in the mobile penial tube. The vas deferens has no different anatomical regions or accessory glands. The vasa deferentia are convoluted; however, in Hypoconchinae they are left-right folded (Fig 1A), while in the Dromiinae, *M. antillensis* and *D. erythropus* (Fig. 1B, C) are back and forth (posterior-anterior) convoluted (Fig.1B, C). In *D. erythropus*, the posterior region of the vas deferens is quite convoluted (Fig. 1C).

HISTOLOGY AND HISTOCHEMISTRY

TESTIS

The testes of *H. parasitica*, *H. arcuata*, *M. antillensis* and *D. erythropus* showed similar features both macroscopically and histologically. The testes were classified as tubular with a histological analysis, and they are internally filled by germ cells in different stages of spermatogenesis and spermiogenesis (Fig. 2A - D). Spermatogenesis starts in the germinal zone, which is composed of many spermatogonia localized at the periphery of a seminiferous tubule (Fig. 2E). The spermatogonia are differentiated by a large central nucleus, several nucleoli and an acidophilic cytoplasm (Fig. 2E). In all Dromiidae sections analyzed, the maturation zone beneath the germinal center only showed spermatocytes or spermatids in different maturation stages (Fig. 2F - J). The maturation zone was not found to contain different cell strata since spermatocytes until late spermatids in the same cross-section. The chromosomes in different stages of the meiotic prophase characterize the primary spermatocytes (Fig. 2F). The secondary spermatocytes have smaller nuclei, which are rounded and filled with homogeneously stained chromatin (Fig. 2G).

The spermiogenesis showed a similar pattern of cell maturation in all studied species by light microscopy. Early spermatids are identified by the appearance of the proacrosomal vesicle, which is reactive with homogeneous staining to PAS (Fig. 2H). The nucleus is rounded, homogeneously basophilic and occupies a large part of the cell volume (Fig. 2H). The intermediary spermatids are marked by the beginning of the nuclear modification becoming slightly irregular due to an increase in the round acrosome vesicle (Fig. 2I). The main characteristic of this cell is the presence of the PAS-positive acrosome exhibiting a more intense reaction at the perforatorial chamber and the acrosomal operculum, both during formation (Fig. 2I). Late spermatids contain thin “half-moon” nuclei that became discoid, positioned in the opposite pole of the acrosome vesicle (Fig. 2J). The nucleus has a cup appearance, almost surrounding the acrosome (Fig. 2J). Mature spermatozoa are similar to the late spermatids; however, they are found in the seminiferous tubule lumen (evacuation zone) (Fig. 2K). This cell shows a slightly thinner nucleus, which almost completely surrounds the heterogeneous acrosomal vesicle (Fig. 2K). The evacuation zone, or seminiferous tubule, of *H. parasitica, H. arcuata* and *M. antillensis* is filled with only one basophilic secretion, called a type I secretion (Fig. 2K). However, in *D. erythropus*, the type 1 secretion contains a lucid secretion that resembles bobbles near the periphery of the

evacuation zone, which spreads on a basophilic secretion where the spermatozoa are immersed (Fig. 2L).

VAS DEFERENS

Despite the absence of different anatomical and histological regions along the vas deferens or the presence of accessory glands, the species studied here differed in their seminal fluid secretions and chemical compositions as follows:

Hypoconcha parasitica. - The mature spermatozoa are conducted into the vas deferens immersed in type I secretion, the same type found in the testes (Fig. 3A). The vas deferens lumen exhibits, in the anterior region, spermatozoa in a type I secretion surrounded by type II secretion (Fig. 3A - C). The type II secretion is electron-dense and homogeneous, while the type I secretion is less electron-dense and finely granular (Fig. 3B). The volume of the type II secretion is smaller in regions near the testis (Fig. 3C), becoming larger along the vas deferens (Fig. 3D - G). The vas deferens did not show histological variation or different cell types in the anterior, middle or posterior regions. It has a simple epithelium, lying on a thick musculature surrounded by a thin layer of connective tissue (Fig. 3A). The epithelium along the vas deferens ranged from cubic to flattened due to the presence of a greater amount of type II secretions (Fig. 3A, C). The spermatozoa are compacted into large central masses in the type I secretion, forming a large spermatic cord (Fig. 3A - G). This cord is convoluted within the type II secretion in some places (Fig. 3G). A cross-section confirms the single, continuous tubular morphology of the vas deferens along its whole extension (Fig. 3C). The type I secretion is acidophilic (Fig. 3D) and reactive to proteins (Fig. 3E), being positive for neutral (Fig. 3F) and acidic polysaccharides (Fig. 3G). The type II secretion acts as a wrap around the spermatic cord, producing a unique extremely elongated kind of coenospermic "spermatophore". This type II secretion is basophilic (Fig. 3D), intensely stained to proteins (Fig. 3E) and to neutral polysaccharides (Fig. 3F), being weakly positive for acidic polysaccharides (Fig. 3G). Inside of the type II secretion, fibrous and acidophilic glycoprotein materials are present, along with strongly marked neutral polysaccharides (Fig. 3D - F). The basophilic spermatozoa are strongly reactive for proteins and positive for neutral and acid polysaccharides (Fig 3D - G).

Hypoconcha arcuata. – The mature spermatozoa in the vas deferens are also immersed in type I secretions (Fig. 4A). From the anterior to the posterior region of the vas deferens, the large spermatic mass is immersed in type I secretion, forming a cord surrounded by type II secretion (Fig. 4A - C). In a cross-section, the spermatozoa form a central mass

covered by the electron-dense and granular type I secretions, and the mass is surrounded by type II secretion, which is homogeneously electron-dense and basophilic (Fig. 4B, C). The internal sperm mass remains as a long spermatic cord surrounded by the type II secretion (Fig 4A, C). The vasa deferentia in all regions are formed by a simple epithelium, surrounded by musculature and a thin layer of connective tissue. In this species, the musculature is thicker than in the previous one, showing several layers (Fig. 4D). From a histochemical point of view, the type I secretion is weakly basophilic (Fig. 4D), slightly reactive for proteins (Fig. 4E) and neutral polysaccharides (Fig. 4F), but no acidic polysaccharides were detected (Fig. 4G). The type II secretion is acidophilic (Fig. 4D), weakly reactive for proteins (Fig. 4E) and neutral polysaccharides (Fig. 4F) and not reactive to acidic polysaccharides (Fig. 4G). The basophilic spermatozoa are strongly proteinaceous and also reactive to neutral and acidic polysaccharides (Fig. 4A, C - G).

Moreiradromia antillensis. – Mature spermatozoa in the vas deferens are immersed in the type I secretion, adjoin this from the anterior to the posterior region, and have an outer layer of type II secretion. The type II secretion contains many spheres or granules of different sizes immersed in a finely granular matrix (Fig. 5A - C). The type I secretion is electron-dense and homogeneous, while the type II is a finely granular matrix and is less electron-dense than the type I (Fig. 5B). The granules are electron-dense (Fig. 5B). The vas deferens is formed by a simple cubic epithelium on a thin layer of connective tissue, surrounded by strong musculature (Fig. 5A, D). Spermatozoa in the type I secretion form a long spermatic cord within the type II secretion (Fig. 5A). In cross-section, the vas deferens is a spermatic cord arranged centrally, surrounded by type II secretion (Fig. 5C). The type I secretion is basophilic (Fig. 5A, C, D), weakly reactive to proteins (Fig. 5E), and neutral (Fig. 5F) and acidic polysaccharides (Fig. 5G). Type II secretions have granules strongly reactive to proteins (Fig. 5E) and negative to polysaccharides (Fig. 5F, G). The type II secretion matrix is finely granular and positive for proteins (Fig. 5E) and neutral polysaccharides (Fig. 5F) but is weakly responsive to acidic ones (Fig. 5G). The basophilic spermatozoa are strongly reactive to all compounds studied (Fig. 5A, C - G).

Dromia erythropus. – This species showed a higher degree of complexity in the chemical composition of secretions and sperm packaging. Mature spermatozoa are conducted through the vas deferens immersed in type I secretion, which is electron-dense and homogeneous, forming the spermatic cord (Fig. 6A, B). The vas deferens lumen has a spermatic cord beginning from the anterior region of the vas deferens, surrounded by type II, III and IV secretions (Fig. 6A - C). The epithelium along the vas deferens varies from

cubic to squamous, probably due to the presence of varying amounts of type II secretions in the vas deferens, independent of the region (Fig. 6A, C, D). The spermatic cord is compacted by type II secretion, which contains lucid spherical granules under the light microscope; these granules are electron-dense under TEM (Fig. 6A, B). The matrix between the granules shows the same electron density as is found in the type I secretion of the spermatic cord (Fig. 6B). The type I secretion is weakly basophilic and glycoproteinaceous with the presence of only neutral polysaccharides (Fig. 6D - F). The type II secretion is granular and strongly proteinaceous (Fig. 6E) while the type III is electron-dense with discrete electron-lucid granules (Fig. 6B). This secretion seems more fluid, weakly fibrillar and basophilic and is negative for proteins and weakly reactive to neutral and acidic polysaccharides (Fig. 6D - G). The type IV secretion is heterogeneous and composed of two elements. One is more abundant on the face in contact with the type III secretion, which is less electron-dense (Fig. 6B), basophilic, and weakly reactive to proteins but strongly reactive to neutral polysaccharides and slightly reactive to acidic polysaccharides (Fig. 6D - G). The other element is next to the epithelium and is electron-dense, acidophilic, glycoproteinaceous, and positive for neutral polysaccharides (Fig. 6B – F). However, for acidic polysaccharides it is less intense and negative (Fig. 6G). The type IV secretion is thinner in regions near the testis (Fig. 6C), becoming thicker along the vas deferens (Fig. 6A, C - G). The basophilic spermatozoa are strongly reactive to proteins and neutral polysaccharides and reactive to acidic polysaccharides (Fig. 6D – G). The summary of the results from the histochemical reactions of the spermatozoa and the different types of secretions in the vas deferens for the four species of Dromiidae are shown in Table 1.

ULTRASTRUCTURE OF THE VAS DEFERENS

As the four species of Dromiidae vasa deferentia have the same cell ultrastructure, we will use *H. parasitica* as a model for the description. The vas deferens wall is composed of an external connective layer, a middle muscular layer and an internal epithelium (Fig. 7A). The lumen is filled by a less electron-dense secretion where the spermatozoa are distributed and other secretions that show different electron densities depending on the species. No spermatophore wall is observed around the spermatic cord (Fig. 7A). The thin connective tissue layer contains fibroblast-like cells with oval or flat nuclei (Fig. 7A, B). The cytoplasm contains polyribosomes, rough endoplasmic reticulum (RER) and some mitochondria (Fig. 7B). More than one layer makes up the striated muscular fibers, showing at least one longitudinal fiber and other oblique fibers (Fig. 7A,

B). Epithelial cells have a thick basal lamina, which is filled by different electron densities (Fig. 7B, C). The basal region of the epithelial cell depicts many deep basal plasma membrane folds and many mitochondria and polyribosomes are noticeable among them (Fig. 7C). The cytoplasm is filled with a large amount of RER, and the nucleus is positioned in the middle of the cell (Fig. 7D). The nucleus is usually flattened and contains more condensed chromatin near the nuclear envelope (Fig. 7A, D). The RER shows a traditional pattern composed of many parallel cisternae (Fig. 7E), and among them, many mitochondria and well-developed Golgi complexes are noticeable (Fig. 7E, F). The Golgi complex produces at least two types of vesicles, one electron lucent and another filled with granular and electron-dense material; both are released into the lumen by exocytose at the apical region (Fig. 7G, H). The epithelium contains small microvilli regularly distributed on the surface (Fig. 7G, H).

MOBILE PENIAL TUBE AND GONOPOD

The penial tube is a long conical-round paired organ, forming a mobile tube independent of the coxal structure, emerging from male P5 in all studied species (Fig. 8A - D). The scanning electron microscope shows that this structure in the Hypoconchinae *H. parasitica* and *H. arcuata* is composed of setae, mainly on the basal portion (Fig. 8A, B). One of the faces does not contain setae, forming a glabra margin (Fig. 8A, B). The setae are long and show many ramifications classified as plumose (Fig. 8A, B). In the Dromiinae *M. antillensis* and *D. erythropus*, the penial tube is long, flat dorso-ventrally, and slightly curved at the apex (Fig 8C, D). The basal setae surround the penial tube, and a line of setae occurs along the central margin 2/3 (Fig 8C, D). The setae are long in both the latter species and were classified as pappose types (Fig 8C, D). In all species, the penial tube apex has a very thin cuticle, noticeable by the wrinkles caused by dehydration, which forms a mobile operculum (Fig 8E - H). In the Dromiidae species studied here, the penial tube is inserted into the gonopod G2, which shows a needle-like apex and so easily fractures (Fig. 8I). The fractured G2 in *M. antillensis* contains the secretion that forms a smooth surface around the spermatozoa. However, the secretion is thinner under MEV, also due to the dehydration process (Fig 8I, J). The same pattern was observed in the other Dromiidae species.

DIFFERENTIAL INTERFERENCE CONTRAST PHASE MICROSCOPE (DIC)

The seminal fluid, when mechanically squeezed out of the vas deferens, maintains its structure, forming a central spermatic cord surrounded by secretions differing according to species. In the Hypoconchinae *H. parasitica*, the secretion around the spermatic cord is thin and homogeneous under DIC (Fig. 9A). A similar result was found in Dromiinae (Fig. 9B). In *M. antillensis*, the mechanically squeezed seminal fluid also formed an elongated cord composed of an outer rounded type II secretion and inner spermatozoa contained in type I secretion (Fig. 9B). This pattern of an elongated and unique coenospermic "spermatophore" is found in all Dromiidae species studied.

"SPERM PLUG" AND FIRST FEMALE PLEOPOD

The pair of uniramous first pleopods (PL1) is held in the medial portion of the spermathecae in *H. parasitica*, almost perpendicular to the body axis but not reaching the spermathecal apertures (Fig. 10A). Their lateral edge apices are fringed with setae (Fig. 10A, B). The pair of spermathecae is formed by the split of the intersegmental phragma seven and eight sternites, and the width brings them almost near the third coxae, where the gonopores are located (Fig. 10A). In ovigerous females of *H. parasitica*, material similar to a sperm plug on the spermathecal aperture was found and this material included traces of spermatozoa (Fig. 10A, C, D). In *M. antillensis*, the pair of uniramous PL1 is present in the 1/3 portion of the spermathecae, not reaching the spermathecal aperture (Fig. 10E). Their edge apices are also fringed with setae (Fig. 10E, F). The PL1 of ovigerous females of both subfamilies did not seem to be involved in carrying eggs once the PL1 is free, while the other pleopods held eggs. In the ovigerous females of *M. antillensis*, material similar to a sperm plug was also found on the aperture of spermathecae with traces of spermatozoa (Fig. 10G, H, I). The spermatozoa found in the sperm plug of *M. antillensis* were fixed in 100% alcohol and show a similar morphology compared to the sperm fixed in 4% paraformaldehyde prepared with seawater and 0.2 M sodium phosphate buffer (Fig. 10I, J).

DISCUSSION

The male reproductive system of Dromiidae showed significant differences from previously described Podotremata and especially with Eubrachyura. Based on observations of the male reproductive anatomy, no different vas deferens regions or accessory glands, caeca or lateral out pockets were found, which is distinct from the pattern described for the Podotremata *Ranina ranina* (Linnaeus, 1758) by Minagawa *et al.* (1994), *Dromia*

personata by Hartnoll (1975) and all eubrachyurans studied (Hartnoll, 1964; Beninger *et al.*, 1988; Krol *et al*., 1992; Garcia & Silva 2006; Castilho *et al.* 2008; Simeó *et al.*, 2009; Stewart *et al*., 2010; Nicolau *et al.*, 2012; López Greco, 2013; Ravi, Manisseri & Sanil, 2014; Tiseo *et al*., 2014; McLay & Becker, 2015). Small differences were noticed between the reproductive system subfamilies since the reproductive systems in the Dromiinae (*M. antillensis* and *D. erythropus)* are longer and convoluted, mainly in the distal portion of the vas deferens, compared to Hypoconchinae. In *R. ranina*, there are three anatomically recognized regions (anterior, middle and posterior) without any lateral expansions, caeca or accessory glands. However, the anterior portion is more modified, thin and convoluted (Minagawa *et al*., 1994). Despite the anatomy of *D. personata* not yet being described, there seems to be some differences in histology that may imply anatomical differences, though without any glands (Hartnoll, 1975). Thus, the male reproductive system in dromiids is extremely simplified and different from the patterns described for the other primitive crabs.

The Dromiidae testis was classified as tubular based on Nagao & Munehara (2003). The testes show different areas with different kinds of spermatogenesis cells, but all stages were never observed in the same transversal section, as observed in *Maja brachydactyla*, Balss, 1922 by Simeó *et al*. (2009). As described in this latter species and also in *Pachygrapsus*, Randall, 1840, the mature sperm cells are released to the evacuation zone (lumen of seminiferous tubule). Spermatogenesis in Dromiidae also shows the same histological characteristics found in other species, which seems to be the general model in Brachyura crabs (Ryan 1967; Garcia & Silva 2006; Castilho *et al*., 2008; Santos *et al*., 2009; Zara *et al*., 2012; Tiseo *et al*., 2014). Additionally, the classification of early, middle and late spermatids, based on the increase of the acrosomal vesicle, also follows the pattern found in Eubrachyuran crabs under light microscopy (Zara *et al.*, 2012; Nascimento & Zara, 2013; Tiseo *et al*., 2014). However, different from these authors, in the present study, the cell maturation states were easily identified by the use of PAS with hematoxylin techniques instead of toluidine blue stain. Therefore, we propose that the use of this technique is more appropriate for the description of spermiogenesis in Brachyura.

The vas deferens of Dromiidae is a single tubule, simple and continuous, and any significant morphological variation under light and electron microscopy is in agreement with anatomy. This pattern of vas deferens, without clear regions or glands, is unique among Brachyuran crabs. In Podotremata, the early descriptions of *R. ranina* report differentiated regions, including the presence of epithelium and secretions that were absent

in all species in the present work. However, despite being described as different regions by the characterization of the epithelium and the nature of the secretions, *D. personata* and *Homola barbata* (Fabricius, 1793) are discussed as having no gross morphology specialization (Hartnoll 1975). Thus, based on our studied species, we corroborate the results of *D. personata,* and this morphology can be considered the simplest model in Brachyura. Moreover, the simplified pattern of the vas deferens of Dromiidae is different from that described for shrimps in Dendrobranchiata and Caridea, where the vas deferens is differentiated into proximal, middle and distal regions, and also contains terminal ampoule (Bauer, 1986; Bauer & Martin, 1990; Chow *et al*., 1991; Bauer & Min, 1993; Bauer, 2004). However, the absence of distinct regions in the vas deferens is a commonly observed pattern in Anomura (Buranelli, Zara & Mantelatto, 2014) and some Astacidea (Kooda-Cisco & Talbot, 1982; Dudenhausaennd & Talbot, 1983; López Greco *et al*., 2007; López Greco, 2013). In these infraorders, although it was possible to divide these tubules into different regions, what was normally observed was the increase of the diameter of the posterior or distal region of the vas deferens.

The vas deferens wall in Dromiidae was divided into three layers: an outer thin connective layer, a middle muscular layer and an inner epithelium; this may be considered a pattern found in all decapods studied under TEM (Hinsch & Walker, 1974; Kooda-Cisco & Talbot, 1986; Ro *et al*., 1990; Benhalima & Moriyasu, 2000; Simeó *et al.,* 2009). The Dromiidae muscular layer is thick, and the fibers are longitudinal and oblique along the vas deferens, indicating that they have the same function throughout the tube and during sperm transfer. Usually, the muscular layers in Decapoda show different fiber orientations and may be more or less developed according to the vas deferens region associated with the role of each portion (Kooda-Cisco & Talbot, 1986; Ro *et al*., 1990; Benhalima & Moriyasu, 2000; Simeó *et al.,* 2009). For example, in the AVD and MVD, the musculature is related to spermatophore formation and movement to the PVD, where the fibers have a role in mixing the spermatophores with the accessory gland fluid, as well as in aiding sperm transfer (Ryan, 1967; Simeó *et al*., 2009; Tiseo *et al*., 2014). The cells from the epithelial layer along the vas deferens in Dromiidae show the same pattern as merocrine glycoprotein secretory cells. These cells are characterized by their basal membrane which is folded with many mitochondria among them, and this is likely related to the high ionic exchange in the secretory regions of crab vas deferens (Hinsch & Walker, 1974; Simeó *et al*., 2009), indicating an active uptake of compounds from the hemolymph, as has been suggested for insect salivary glands (Zara & Caetano, 2002). In addition, the cytoplasm is

filled with a large amount of RER and large, well-developed Golgi complexes, producing small secretory vesicles that are released by exocytosis, corroborating the histochemical results. This cell ultrastructure, including the small number of secretory vesicles, is similar to what is found in spider crabs and others Decapoda (Hinsch & Walker, 1974; Kooda-Cisco & Talbot, 1986; Ro *et al*., 1990; Simeó *et al*., 2009).

The spermatozoa packaging in the four dromiid species is different from what has been typically reported for crabs in general. These species exhibit a long spermatic cord, surrounded by glycoproteins, forming a kind of very elongated coenospermic "spermatophore", without wall or capsule. This pattern is different from all others described for Eubrachyura. Spherical or elliptical spermatophores of the coenospermic type is the most common structure observed for Heterotremata and Thoracotremata (for review, see Erkan *et al*., 2009; Zara *et al*., 2012; Tiseo *et al*., 2014, 2017). In the same groups of crabs, cleistospermic spermatophores are uncommon; however, they are found in some freshwater crabs, such as Potamidae, Gecarcinucidae and a single marine crab *Pachygrapsus gracilis* (Saussure, 1858) (Guinot *et al*., 1997, Klaus *et al*., 2009; Klaus & Brandis, 2011; Tiseo *et al*., 2014, 2017). In Podotremata, the only spermatophores described appear to be similar to those of the Dromiidae studied here, despite the phylogenetic distance between the Dromioidea and Raninoidea clades (Tsang *et al*., 2014). The spermatophore in *R. ranina* is composed of a central mass of cylindrical spermatozoa surrounded by two secretions that make up the wall, named the "capsule" (Minagawa *et al*., 1994). However, these authors do not discuss the role of the capsule or whether this structure can be considered a true spermatophore or not. Minagawa *et al*. (1994) did not show whether this spermatophore is a single structure with a spermatic cord inside, as was found in the species studied here. The only description of *R. ranina* reported the spermatophore as small, deposited near the exit of the spermathecae, and other authors have proposed that larger part of the spermatophore is internalized in the spermathecal, without due verification (Minagawa, 1993; Minagawa *et al*., 1994). In *D. personata*, the vas deferens was divided into three regions according to the epithelial cells and luminal content. The spermatozoa are maintained centrally, agglutinated in a secretion along the vas deferens, with a second layer of vacuolar secretion added at the MVD and a third secretion added at the PVD, where no spermatozoa are present (Hartnoll, 1975; Subramoniam, 1993). These luminal secretions are very similar to those described in *D. erythropus*; however, in this species the luminal secretion is the same from the AVD to the PVD, which also contain spermatozoa. The male reproductive system anatomy and

histology of Hypoconchinae and the Homolidae *Ho. barbata* are quite similar, including the presence of only S1 and S2 in the seminal fluid. However, no epithelial cell differences were found in Hypoconchinae as they occur in *Ho. barbata* (Hartnoll, 1975).

Thus, the Dromiidae secretion types in the vas deferens and the spermatic cord, forming the long coenospermic spermatophores, are more similar in structure to *D. personata* and *Ho. barbata* than *R. ranina*. These similarities are in agreement with molecular phylogenetic studies by Ahyong *et al*. (2007) and Tsang *et al*. (2014) using nuclear protein-coding and mitochondrial rRNA genes, which showed that Dromiidae and Homolidae are more closely related than Raninidae. Tsang *et al*. (2014) recognized Podotremata in separate sections: Dromiacea, Raninoidea and Cyclodorippoidea. Dromiidae and Homolidae are inside Dromiacea, which is in accordance with the male reproductive system morphology reported in this study and previous reports (Hartnoll, 1975; Guinot, 1977; Minagawa, 1993; Minagawa *et al.*, 1994).

The Dromiidae have already been included in the Anomura infraorder (Spears, Abele & Kim 1992). However, the sperm packaging in Dromiidae is clearly different from all Anomura spermatophores described, although the anatomy of the vas deferens is similar. In Anomura, the spermatozoa are included in pedunculate coenospermic spermatophores, which are composed of an ampulla, a peduncle and base or foot (Tudge, 1991; Scelzo, Mantelatto & Tudge, 2004; Amadio & Mantelatto, 2009; Buranelli & Mantelatto, 2012; Buranelli *et al*., 2014). In Astacidea, sperm masses form an elongated cord and are surrounded by different types of secretions in the vas deferens lumen, which seems to be a convergence with the species of this study. These secretions in lobsters are added from the anterior region of the vas deferens, with new layers added to the wall in the middle and posterior regions thus making them thicker (Hobbs *et al*., 2007, López Greco, 2013). This multilayer wall structure is called a tubular spermatophore, which is elongated and occurs in Parastacidae, Enoplometopidae, Nephropidae, Homaridae, Astacidae and Cambaridae (Kooda-Cisco & Talbot, 1982; Dudenhausen & Talbot; 1983; Hobbs *et al*., 2007; López Greco *et al*., 2007; López Greco, 2013). However, the spermatophores in *H. parasitica*, *H. arcuata*, *M. antillensis* and *D. erythropus* do not show these layers on the wall. Therefore, they are similar but not identical to the spermatophores found in Astacidea (Kooda-Cisco & Talbot, 1982; Dudenhausen & Talbot; 1983; Hobbs *et al.*, 2007; López Greco *et al*., 2007; López Greco, 2013). Thus, the present results strongly agree with the early proposition of Hartnoll (1975) that the long coenospermic spermatophores of *D. personata*

and the four species of Dromiidae in this analysis are more similar to Astacidae than Eubrachyura.

To insert the elongated spermatophores in the narrow spermathecae, the dromiid species use a very long, thin gonopore G2 attached to the gonopore G1. At the base of G2, the long mobile penial tube of the studied Dromiidae species emerges from P5 coxa, corroborating previous anatomical studies of Dromiinae and Hypoconchinae subfamilies (Guinot & Tavares, 2003; Guinot & Quenette, 2005; Guinot *et al*., 2013). According to Guinot & Tavares (2003), the penial tube is the vas deferens prolongation to the external environment, forming a sclerotized tube with a soft tip, and is only characteristic of subfamilies Hypoconchinae and Dromiinae. Our results confirm the soft tip, and we propose that the soft tip acts as an operculum, allowing the seminal fluid and the spermatic cord to pass in one direction, avoiding backflow during the copular pumping. The penial tube and the G2, including the operculum described her, enters into a single foramen in male gonopod G1. Additionally, the G2 with a needle-like apex may submit the high pressure the elongated spermatophores, during sperm transfer. Thus, this penial tube (and the operculum), as already stated, probably plays an important role in the reproductive strategy of these species (Guinot & Quenette, 2005). Moreover, we observe that during the spermatic transfer of Hypoconchinae, the male abdomen performs a movement of extension and distension, which seems to collaborate with the propulsion force to transfer the elongated spermatophore (Bento-Garcia MA & Zara FJ., unpublished data). In Eubrachyura, some species of Dorippidae have a long penial tube, similar to the mobile tube and independent from the coxa found in Dromiidae. However, the dorippid penial tube is coxo-sternal and is not completely free from the coxa (Guinot & Tavares, 2003; Guinot *et al*., 2013; Hayer *et al*., 2016; Becker & Scholtz, 2017), probably functioning in a different manner than dromiids. In Dorippidae, the seminal receptacle is also completely or almost completely cuticle-lined, very similar to the Dromiidae spermathecae (Hayer *et al*., 2016; Becker & Scholtz, 2017; Vehof, Scholtz & Becker, 2017). However, in doripids, the ovary and oviduct are in contact with the vagina or vulva where internal fertilization takes place (Hayer *et al*., 2016; Vehof *et al*., 2017). In Podotremata, including Homolidae and Dromiidae, the spermathecae has no internal connection to the oviduct, leading to an external fertilization (Guinot & Quenette, 2005; Guinot *et al*., 2013; Becker & Scholtz, 2017). In this case, the seminal receptacle of dorippids and the spermathecae of dromiids seem analogous instead of homologous since the positions on the body are also distinct

(i.e., sternite 6 in Dorippidae and sternite 7 and 8 in Dromiidae) (Guinot & Quenette, 2005; Guinot *et al*., 2013; Becker & Scholtz, 2017).

The spermatozoa or spermatophores stored in the spermathecae are poorly studied in Podotremata, especially in dromiids. Although the absence of "sperm plugs" in Hypoconchinae was earlier stated (Guinot & Tavares, 2003) and we also confirmed this absence in *H. parasitica* and *H arcuata*. The presence of sperm plug secretion was observed in the Dromiinae *D. personata* (Hartnoll, 1975), *Austrodromidia octodentata* (Haswell, 1882) and *Pseudodromia latens* Stimpson, 1858 and is described as a hardened material deposited on the female sternum (Guinot & Tavares, 2003; Guinot *et al.*, 2013). It was also observed in *Lauridromia intermedia* (Laurie, 1906), with traces of spermatozoa on this plug material (Guinot & Quenette, 2005). In our study, we found this material deposited on the aperture of spermathecae in *M. antillensis* ovigerous females, also including traces of spermatozoa, following the descriptions of Dromiinae (Guinot & Tavares, 2003; Guinot & Quenette, 2005; Guinot *et al.*, 2013). According to Guinot & Tavares (2003) and Guinot *et al.* (2013), this secretion is a sperm plug, acting as a rigid adhesive secretion on the spermathecal aperture in Podotremata. The sperm plug (internal or external) in brachyurans is considered a barrier that prevents successive copulations, among other functions (for review, see Hartnoll, 1969; Zara *et al.*, 2012; Zara, Raggi Pereira & Sant'Anna, 2014). If this secretion founded in Dromiinae act as a sperm plug it shows the same function observed in Eubrachyura, however, the main composition is sliglithly different since eubrachyurans sperm plug lacks the presence of spermatozoa. In addition, we also founded a presence of secretion in *H. parasitica* apertures and it was only observed in ovigerous females. Thus, further studies need to be carried out to demonstrate if this secretion is a sperm plug in Hypoconchinae or if this secretion is an evidence of material sternalized from spermatheca during the external fertilization in Dromiidae crabs.

McLay & Becker (2015) proposed the use of "sperm plaque" in Podotremata because this secretion is not necessarily injected in the spermathecae. This structure is more similar to a "barrier" that can prevent the loss of spermatozoa and the influx of water and also avoids subsequent copulations in the spermatic competition (McLay & Becker, 2015; Becker & Scholtz, 2017). However, based on the patterns of seminal fluid production and sperm packaging in all species studied here, and based on the presence of spermatozoa in the secretions on the spermathecal aperture of ovigerous *H. parasitica* and *M. antillensis* females, the sperm plug function seems questionable to us. Although mating has been described once for Dromiidae and Podotremata (Hartnoll, 1975; Guinot *et al*., 2013), we

propose two hypotheses: 1) The long coenospermic spermatophores formed by seminal fluid and the inner spermatic cord are inserted inside the spermathecae and some of the seminal fluid and seminal cord are released from the spermathecae on the aperture of spermathecae as a result of the copulation; 2) As the fertilization in Podotremata occurs outside the body, the spermatozoa and seminal fluid are released from the storage chamber (spermathecae) to meet the extruded eggs during the ovulation process. Thus, the material found outside the spermathecal aperture can be remnants of spermatozoa and seminal fluid extruded by the female during ovulation. The production of the glycoprotein secretion S1 acts as an energetic resource to maintain viable spermatozoa as proposed in many Eubrachyura (Zara *et al*., 2012, 2014), while the other external layers with acidic and neutral polysaccharides may act as an adhesive secretion, attaching the material in the spermathecae and also avoiding loss of spermatozoa. This hypothesis was proposed when the vas deferens was squeezed and observed under DIC microscopy, where the spermatozoa remained enveloped by a secretion, maintaining its long constitution. This last observation consolidated the proposal that the secretion continues to surround the spermatozoa after ejaculation and until the fertilization. Hartnoll (1975) also described the spermatozoa surrounded by several types of secretions inside the spermathecae of *D. personata*, strengthening our hypothesis. On the other hand, in species of Homoloidea, free spermatozoa were found mixed with seminal fluid inside the spermathecae, without the presence of a sperm plug (Hartnoll 1975, Becker & Scholtz, 2017). Hartnoll (1975) reported that the "plug secretion" appears after the copulation, and the acid polysaccharides found in the external layers can also promote the extravasations of material from the inseminated spermathecae. The acidic polysaccharides can attract sodium and water from the seawater, leading to the hydration of the S1 and S2 secretions in *M. antillensis*, as well the S3 secretion in *D. erythropus*, producing the expansion of these secretions inside the spermathecae, leading to the formation of the plug. Since *Hypoconcha* secretions are weakly responsive to acidic polysaccharides, the hydration process probably would be not so effective to release material on the spermathecal aperture.

The spermathecae in Homolidae are very similar to the Dromiidae and also show muscles attached to the inner wall (Hartnoll, 1975; Becker & Scholtz, 2017). The females of *Hypoconcha* and *Moreiradromia* have a similar structure of the PL1 as observed in the Homolidae *H. barbata* and *H. orientalis*, Henderson, 1888, by Becker & Scholtz (2017). Furthermore, the PL1 in the observed Dromiidae ovigerous females did not seem to be

destined to carry eggs once the PL1 was free, while the other pleopods held eggs. In Homolidae, the PL1 seems to draw extruded eggs into the fertilization chamber (Becker & Scholtz, 2017). When the eggs are draws the PL1 are held erect so that the eggs remain in this chamber until the sperm are released for fertilization; afterwards, the PL1 return to their initial position to fertilize eggs attained by PL2- PL5 in the brood chamber (Becker & Scholtz, 2017). This mechanism has been described in American Lobster *Homarus americanus* Milne Edwards, 1837(Aiken, Waddy & Mercer, 2004), and although the PL1 do not cover the spermathecal apertures, they probably play a similar role in the Dromiid species studied here.

In conclusion, this research provides descriptions of a different pattern of male reproductive system that can be considered characteristic of the primitive Brachyura. The vas deferens showed no anatomical, histological and cellular variations from the anterior to the posterior region. Additionally, the spermatozoa package on the spermatic cord, forming an elongated spermatophore, can be considered unique and is probably the simplest and most basal coenospermic spermatophore described for Eubrachyura, similar to the external fertilization of species of Astacidae. This pattern seems to be exclusive to Podotremata (at least Dromiidae and Homolidae), and no direct evidence of intermediary steps can link Podotremata and Eubrachyura, in the same way to the presence of function sperm plug in Dromiidae.

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Figure 1. Diagram of the male reproductive system of Dromiidae of Brazil. **A,** testis and vas deferens of *Hypoconcha parasitica* and *H. arcuata*. The pair of testes is located on both superior sides of the cephalothorax and is continuous with the pair of vasa deferentia, which extend longitudinally over the hepatopancreas and below the heart, towards the ventral posterior region of the body (gonopod). Note that the vas deferens does not contain different anatomical regions, accessory glands or external pouches. **B,** in *M. antillensis*, the pair of testes is also located on both superior sides of the cephalothorax, is continuous with the pair of vasa deferentia and ends in the posterior region of the body. Observe that the vas deferens does not exhibit different regions or glands and the posterior part of the vas deferens is more folded. **C,** the pair of testes of *Dromia erythropus* is located on both superior sides of the cephalothorax and is continuous with the vasa deferentia, ending in the gonopod. The vas deferens did not show different regions, accessory glands or pouches. Only the posterior region of the tubule is quite convoluted. T, testes; VD, vas deferens.

Figure 2. Testis, spermatogenesis and spermiogenesis. A, tubular testis of *Hypoconcha parasitica* stained with HE. The seminiferous tubule exhibits germinal, maturation and evacuation zones with mature spermatozoa. **B,** the tubular testis of *H. arcuata* visualized by toluidine blue. Note the germinal, maturation and evacuation zones. **C and D,** tubular testes of *Moreiradromia antillensis* and *Dromia* stained with HE. **E,** germinal center filled with spermatogonia representing all species. These cells have a large central nucleus (arrow), several little nucleoli and an acidophilic cytoplasm. **F,** details of primary spermatocytes in different stages of the meiotic prophase (arrowheads) of Dromiidae. **G,** secondary spermatocytes, with a smaller rounded nucleus and homogeneous chromatin (arrow) in all species. **H,** initial spermatids starting spermiogenesis. Observe that these cells show pro-acrosomal vesicles reactive to PAS (black arrow), producing small vesicles with homogeneous staining. The nucleus occupies a large part of the volume of these cells and is basophilic, homogeneous and rounded (white arrow). **I,** the intermediate spermatids are marked by the nucleus at the beginning of the formation of the nuclear cup (arrowheads). Noted in the PAS-positive (white arrow) acrosomal vesicle, a more intensely marked region (black arrow) indicates the beginning of the formation of the perforatorium and operculum. **J,** the final spermatids with a flat nucleus (black arrow) associated with the acrosomal vesicle (white arrow), both discoid. **K,** mature spermatozoa with a thin nucleus

(black arrow), involving almost the entire extent of the heterogeneous acrosomal vesicle (white arrow) of *H. parasitica*, *H. arcuata* and *Moreiradromia antillensis*. Note that the evacuation zone is filled by basophilic secretion, classified as a type I secretion. **L,** in *Dromia erythropus*, the evacuation zone is filled by a type I secretion, which contains heterogeneous granules near their periphery. These granules diffuse over the basophilic secretion where the spermatozoa are immersed. EST, early spermatid; EZ, evacuation zone; GZ, germinal zone; LST, late spermatid; MST, middle spermatid; MT, maturation zone; SI, type I secretion; SPG, spermatogonia; SPC1, spermatocytes I; SPC2, spermatocytes II; SZ, spermatozoa.

Figure 3. ***Hypoconcha parasitica*** **vas deferens . A,** in a longitudinal section, the vas deferens with HE is a single tubule, continuous and without differentiation. The spermatozoa are immersed in type I secretion and compacted by type II secretion with the presence of a fibrous material (**), forming a large spermatic cord, producing a unique extremely elongated kind of coenosoermic "spermatophore". Note the simple epithelium (arrowheads), supported on a thin layer of connective tissue, surrounded by marked musculature. **B,** general aspect of the vas deferens ultrastructure, showing spermatozoa in a homogeneous type 1 secretion, which is surrounded by a finely granular type II secretion, both with different electron densities. **C,** the cross-section stained with HE confirms the tubular morphology of the vas deferens. Note that the spermatozoa in the lumen of the tubule are surrounded by type II secretion, which is thin in the regions near the testis and thicker along the vas deferens. The rectangular area is shown in panels D, E, F and G. **D,** vas deferens submitted to HE. The basophilic spermatozoa are immersed in acidophilic type I secretion. The type II secretion is basophilic, and the fibrous material is acidophilic (**). **E,** Vas deferens to ponceau xylidine, with positive reactions in type I secretion. The type II secretion is stained strongly for proteins. Note that the fibrous material strongly protein (**). **F,** type I secretion and connective tissue (arrow) reactive to neutral polysaccharides. Note that type II secretion is strongly reactive to PAS, such as the fibrous material (**). **G,** when stained with Alcian Blue, the type I and II secretions were positive and weakly reactive to acidic polysaccharides, respectively. Note the convolute vas deferens within the type II secretion. EP, epithelium; M, muscular layer; SI, type I secretion; SII, type II secretion; SZ, spermatozoa.

Figure 4. *Hypoconcha arcuata* vas deferens. A, longitudinal section of the vas deferens by HE, showing that the spermatozoa are immersed in type I secretion and are compacted by type II secretion, forming a large spermatic cord, producing a unique extremely elongated coenosoermic spermatophore. **B,** Ultrastructure of the vas deferens. Note that the spermatozoa are immersed in an electron-dense and strongly granular type I secretion, where they are compacted by a homogeneous type II secretion. **C,** The cross-section shows that the vas deferens has no histological variations or different cell types from the anterior, middle and posterior regions stained with HE. Note the simple epithelium on a thin layer of connective tissue surrounded by strong musculature, displaying multiple layers. The rectangular area is shown in panels D, E, F and G. **D,** the longitudinal section of the vas deferens by HE shows that the type I secretion is weakly basophilic, and the type II secretion is acidophilic. **E,** the type I and II secretions are slightly reactive to ponceau xylidine and to **F,** PAS. **G,** type I and II secretions are negative for acidic polysaccharides stained with Alcian Blue. Note that the spermatozoa are only reactive when stained. EP, epithelium; M, muscular layer; SI, type I secretion; SII, type II secretion; SZ, spermatozoa.

Figure 5. *Moreiradromia antillensis* vas deferens. A, longitudinal section of the vas deferens, showing the extremely elongated kind of coenosoermic spermatophore stained with HE. The spermatozoa are immersed in a type I secretion, which is surrounded by the type II secretion, composed of heterogeneous granules in a finely granular matrix (arrowhead). Note that the vas deferens is formed by a simple epithelium, on a thin connective layer, covered by musculature. **B,** the ultrastructure of the vas deferens shows the spermatozoa in an electron-dense and homogeneous SI secretion, compacted by a type II secretion that is less electron-dense than a type I secretion, with the presence of homogeneously electron-dense granules. **C,** cross-section of the vas deferens, confirming the continuous and tubular morphology of the elongated spermatophore. The rectangular area is shown in panels D, E, F and G. **D,** spermatozoa immersed in basophilic type I secretion, which is compacted by a granular and heterogeneous type II secretion. Observe that the type II secretion matrix is basophilic (arrowhead). **E,** vas deferens submitted to the ponceau xylidine. The type I secretion and type II secretion matrix are slightly reactive to proteins (arrowhead). Note the heterogeneous granules of the type II secretion is strongly reactive to proteins. **F,** the type I secretion and the secretion matrix II (arrowhead) are weakly reactive to neutral polysaccharides with PAS. **G,** when submitted to Alcian Blue,

the type I secretion is slightly reactive, and the type II secretion and its matrix (arrowhead) are negative for the stain. EP, epithelium; M, muscular layer; SI, type I secretion; SII, type II secretion; SZ, spermatozoa.

Figure 6. *Dromia erythropus* vas deferens. A, the lumen of the vas deferens with HE shows that the anterior region spermatozoa are immersed in type I secretion and surrounded by a type II secretion, which is covered by types III and IV secretions. This arrangement of secretions forms an elongated coenosoermic spermatophore and is the most complex type of structuring among all the species in this study. **B,** under transmission electron microscopy, the spermatic cord is formed by a type I secretion (electron-dense and homogeneous), and the spermatozoa are compacted by a type II secretion, which shows electron-dense granules. The type III secretion is electron-dense, with discrete electron-lucid granules surrounding the type II secretion, and is covered by the type IV secretion, which exhibits a portion that is electron-dense and another that is less electron-dense. **C,** the cross-section of the vas deferens in HE shows the unique and continuous structure of the elongated spermatophore. Note that the spermatozoa in the vas deferens lumen are immersed in type I secretion. The other type II, III and IV secretions are added externally to the lumen of the vas and are thicker near the posterior region. The rectangular area is shown in panels D, E, F and G. **D, v**as deferens by HE, showing that the spermatozoa are immersed in the weakly basophilic type I secretion. Note the granular type II secretion, being the limit of these granules marked by acidophilic secretion. Observe that the type III secretion is slightly basophilic, and the type IV secretion is composed of two layers: one external to the basophilic type III secretion (white arrow) and another acidophilic next to the epithelium (black arrow). **E,** submitted to ponceau xylidine the type I, II, III secretions are weakly, strongly and negatively reactive to proteins, respectively. The outer layer of the type III secretion is slightly reactive to proteins (white arrow), and the other layer next to the epithelium is positive for proteins (black arrow). **F,** vas deferens by PAS. Note that the type I secretion is weakly reactive to the stain, and the type II shows a negative reaction. The type III secretion is slightly positive, and the first layer of type IV secretion (the one external to the type III secretion) is strongly positive (white arrow). The second layer (next the epithelium) is positive to the stain (black arrow). **G,** type I and type II secretions are negative for acidic polysaccharides when stained with Alcian Blue. Note that the type III secretions are weakly reactive to acidic polysaccharides. The first and second

layers of secretion IV are slightly reactive (white arrow) and negative (black arrow), respectively, when stained by this technique. EP, epithelium; SI, type I secretion; SII, type II secretion; SIII, type III secretion; SIV, type IV secretion; SZ, spermatozoa.

Figure 7. Ultrastructure of Dromiidae vas deferens. A, the Dromiidae vasa deferentia studied show the same cell ultrastructure along the tube; thus, we use the cells of *H. parasitica* as a model for the description. The vas deferens wall was composed of an external connective layer, a middle muscular layer and an internal epithelium, and the lumen is filled by a less electron-dense secretion where the spermatozoa are distributed and other secretions, which show different electron-densities depending on the species. Note that the thin connective tissue layer contained fibroblast-like cells with oval or flat nuclei. The rectangular areas (1 and 2) are shown in panel B and C, respectively. The rectangular (3) shows the panels D, E and F, and the rectangular (4) is shown in panels G and H. **B,** the cytoplasm contains polyribosomes, rough endoplasmic reticulum (RER) and some mitochondria. Observe that one layer is composed of striated muscular fibers, showing at least one longitudinal fiber and other oblique fibers. **C,** epithelial cells show a thick basal lamina, which is filled with components of different electron-densities, and the base of the epithelial cell depicts many deep basal plasma membrane folds and many mitochondria and polyribosomes. **D,** the cytoplasm is filled with a large amount of RER, and the nucleus is positioned at the middle of the cell. Note that the nucleus is flattened, showing more condensed chromatin associated with the nuclear envelope (arrows). **E,** RER composed of many parallel cisternae, and among them, there are a lot of mitochondria and well-developed Golgi complexes. **F,** the well-developed Golgi complexes are present in the epithelium cytoplasm. Note that this organelle produces vesicles that are released to the lumen by the apical region of the cell (arrows). **G,** the Golgi complexes produce at least two types of vesicles, one electron lucent (white arrow) and another filled with granular and electron-dense material (black arrow). **H,** the epithelium shows small microvilli, which are regularly distributed on the surface. Note the vesicles produced by the Golgi complexes being released to the lumen by exocytosis at the apical region. BL, basal lamina; CG, Golgi complex; CY, cytoplasm; EP, epithelium; L, lumen; M, musculature; N, nuclei; MT, mitochondria; MV, microvilli; RER, rough endoplasmic reticulum; S, secretion; SZ, spermatozoa; TC, connective tissue.

Figure 8. Scanning electron microscopy of the penial tube and two gonopod (G2) in Dromiidae. Mobile penial tube of **A,** *H. parasitica* and **B,** *H. arcuata*, showing the long plumose setae mainly located in the basal portion (black arrows) and on one of the side margins (white arrow). The penial tube is longer, flat dorso-ventrally, and slightly curved at the apex in **C,** *M. antillensis* and **D,** *D. erythropus.* In this latter species, the basal setae surround the penial tube, and a line of setae occur along the central 2/3 of the margin and exhibit pappose setae only on the lateral margin until the apex (arrow). **E,** the penial tube apices of *H. parasitica* show a very thin cuticle, noticeable by the wrinkles caused by dehydration, which form a mobile operculum. **F,** the penial tube apex of *H. parasitica* showing the operculum. **G,** *Moreiradromia antillensis* penial tube apex forming the operculum. **H,** the penial tube apex of *D. erythropus* showing the operculum. **I,** the fractured G2 of *M. antillensis* representing all species studied here, showing the secretion that forms a smooth surface around the spermatozoa. **J,** details of G2 fractured apex of *M. antillensis*. Note the thinner secretion due to the dehydration process (white arrow) and spermatic cord portion (black arrow). G2, two gonopod; PT, penial tube.

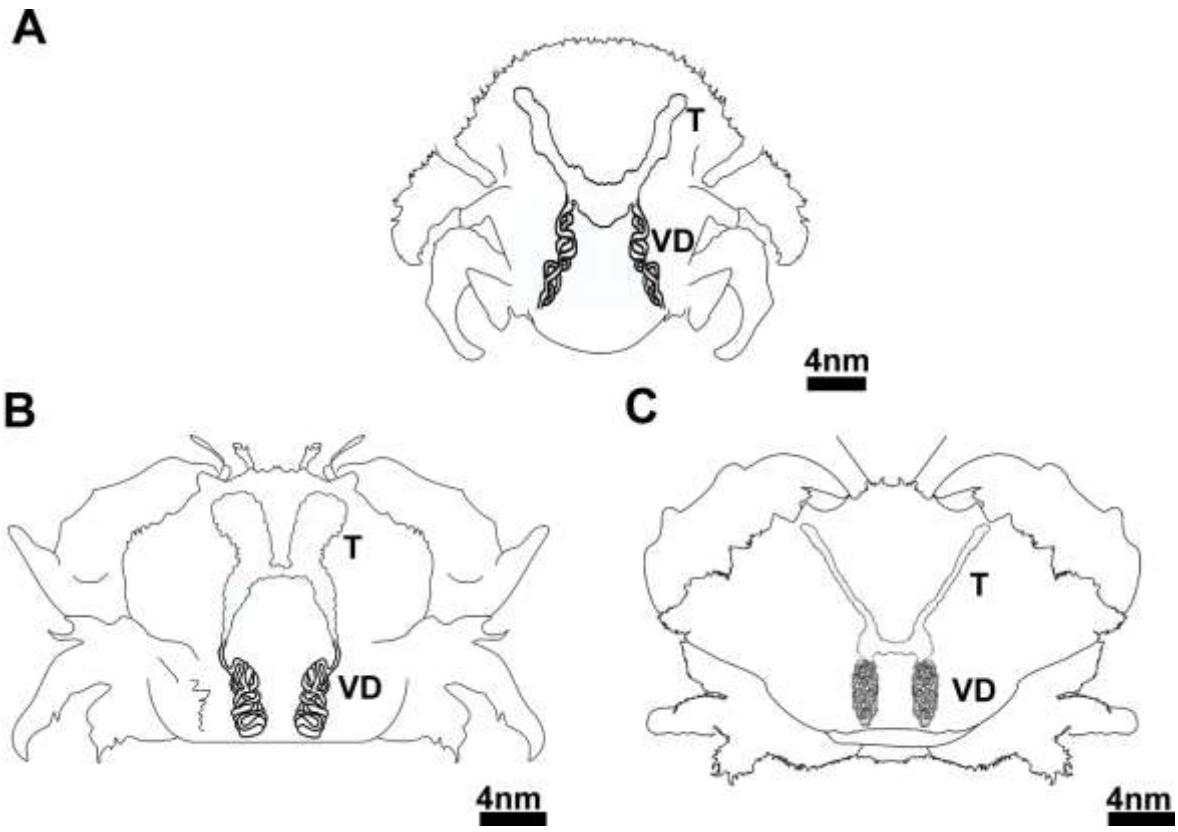
Figure 9. *Hypoconcha parasitica* and *Moreiradromia antillensis* seminal fluid under DIC microscope. The seminal fluid squeezed from the vas deferens maintains the structure, forming a sperm cord showing spermatozoa immersed in the type I secretion surrounded by the type II secretion in both **A,** Hypoconchinae and **B**, Dromiinae. SI, type I secretion; SII, type II secretion; SZ, spermatozoa; VD, vas deferens.

Figure 10. "Sperm plug" and female first pleopod of *H. parasitica* and *M. antillensis*. A, overview of *H. parasitica* sternum showing the location of the pair of first pleopod, the spermathecal aperture and the first, second and third pereopods. Note a material similar to a sperm plug (**) on the spermathecae aperture, with traces of spermatozoa and the gonopores on the third coxae. **B,** details of the first pleopods of *H. parasitica*, in which they are almost perpendicular to the body axis but not reaching the spermathecal apertures, and their lateral edge apices are fringed with setae. **C,** the material, similar to a "sperm plug" (**), included traces of spermatozoa in *H. parasitica*. **D,** details of the spermatozoa

found in the material on the spermathecal aperture of *H. parasitica*. Note the spermatozoon with a centrally perforated operculum. **E,** the first pleopod in *M. antillensis* is present in the 1/3 portion of the spermathecae, not reaching the spermathecal aperture. Note the first, second and third pereopod in the female sternum. **F,** the first pleopod of *M. antillensis*, showing the edge apex also fringed with setae. **G,** ovigerous female *M. antillensis* with material similar to a sperm plug (**) on the spermathecal aperture. **H,** the material found in the aperture of spermathecae of *M. antillensis* showing traces of spermatozoa (**). **I,** details of the spermatozoa found in the material on the spermathecal aperture of *M. antillensis*, fixed in 100% alcohol. **I,** spermatozoa fixed in 4% paraformaldehyde prepared with seawater and 0.2 M sodium phosphate buffer. AP, spermathecal aperture; GO, gonopore; N, nucleus; O, operculum; Pl1, first pleopod; P1, first pereopod; P2, second pereopod; P3, third pereopod; P4, fourth pereopod; P5, fifth pereopod; SZ, spermatozoa.

List of figures and table

Fig. 1



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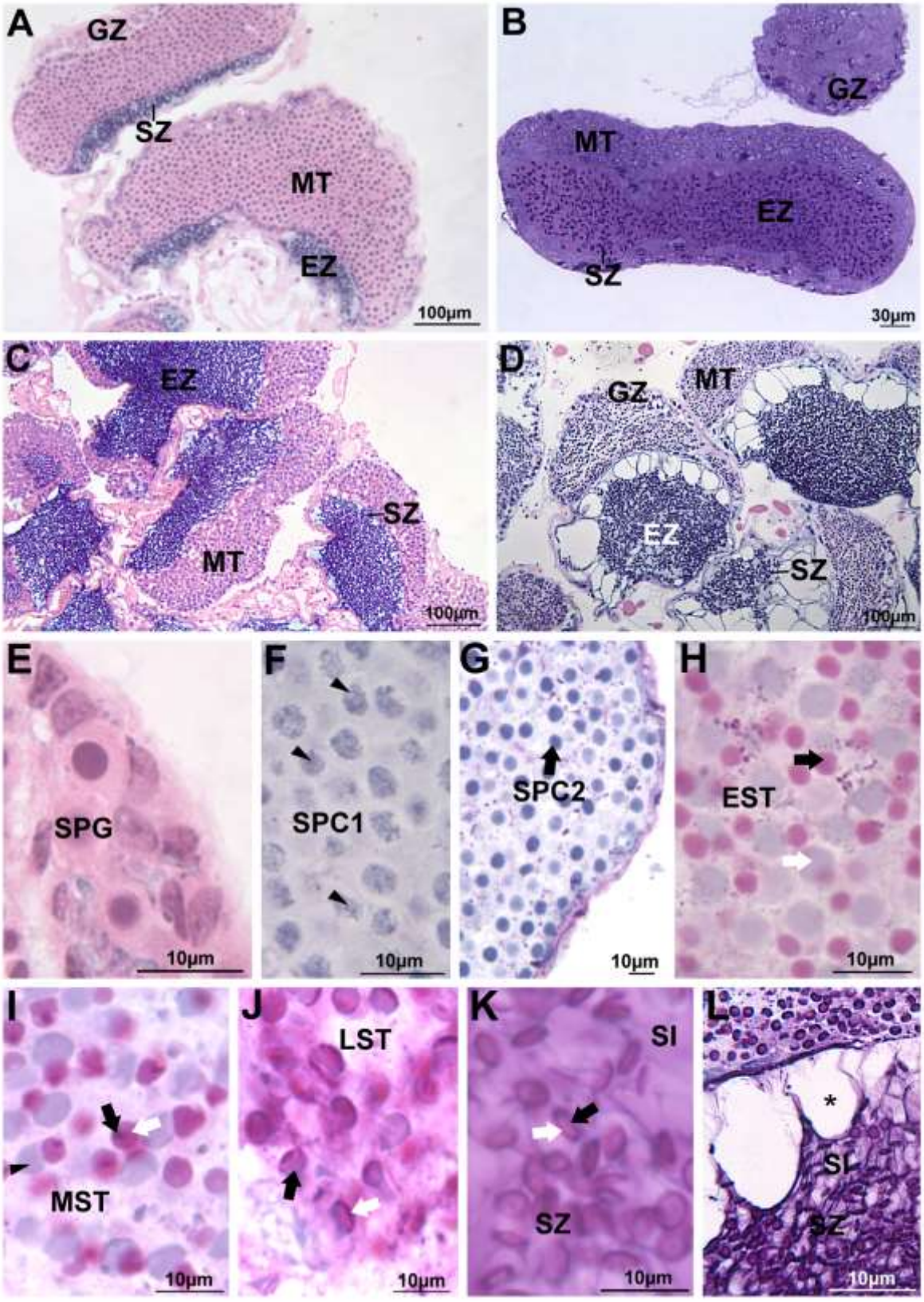
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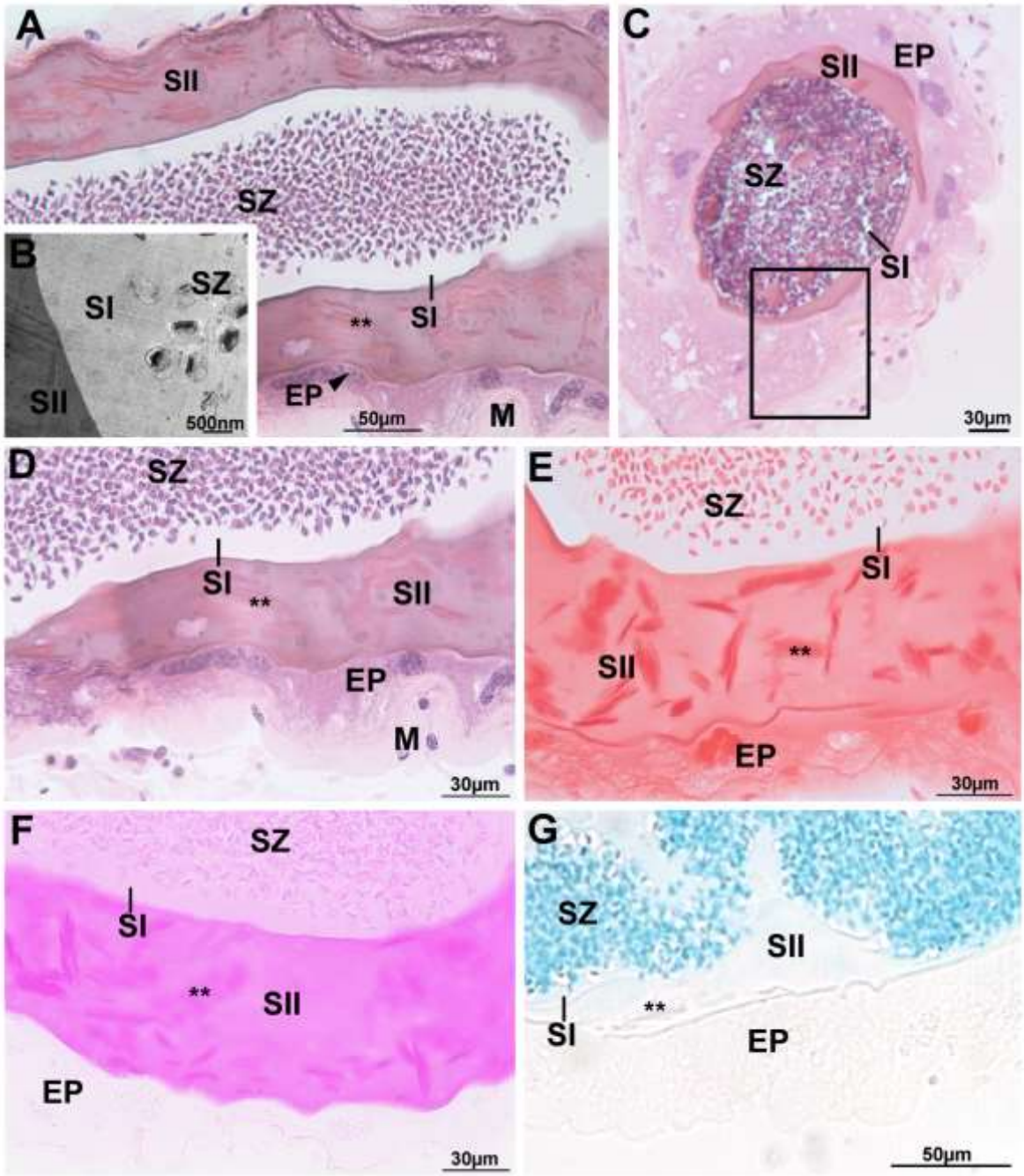
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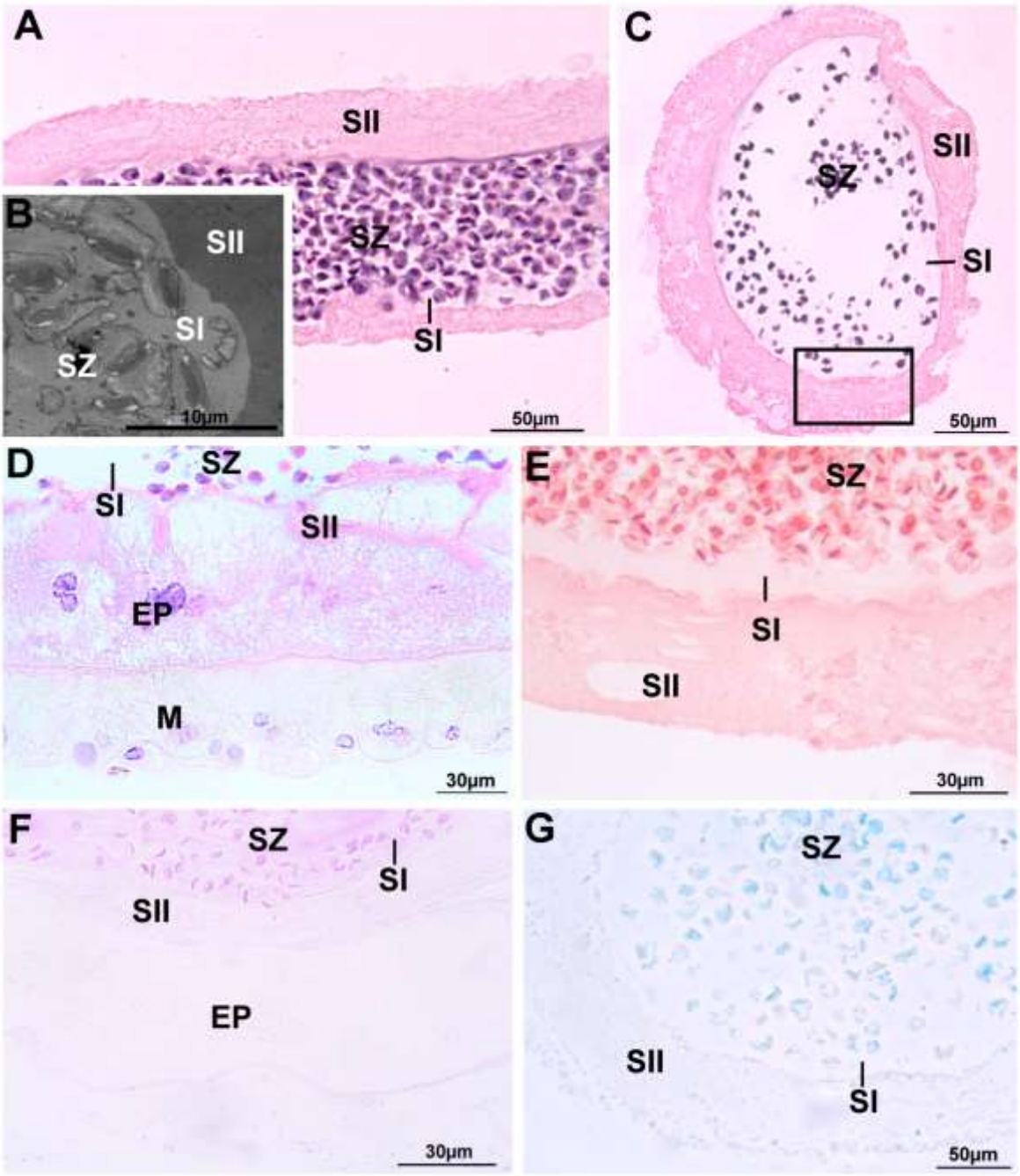
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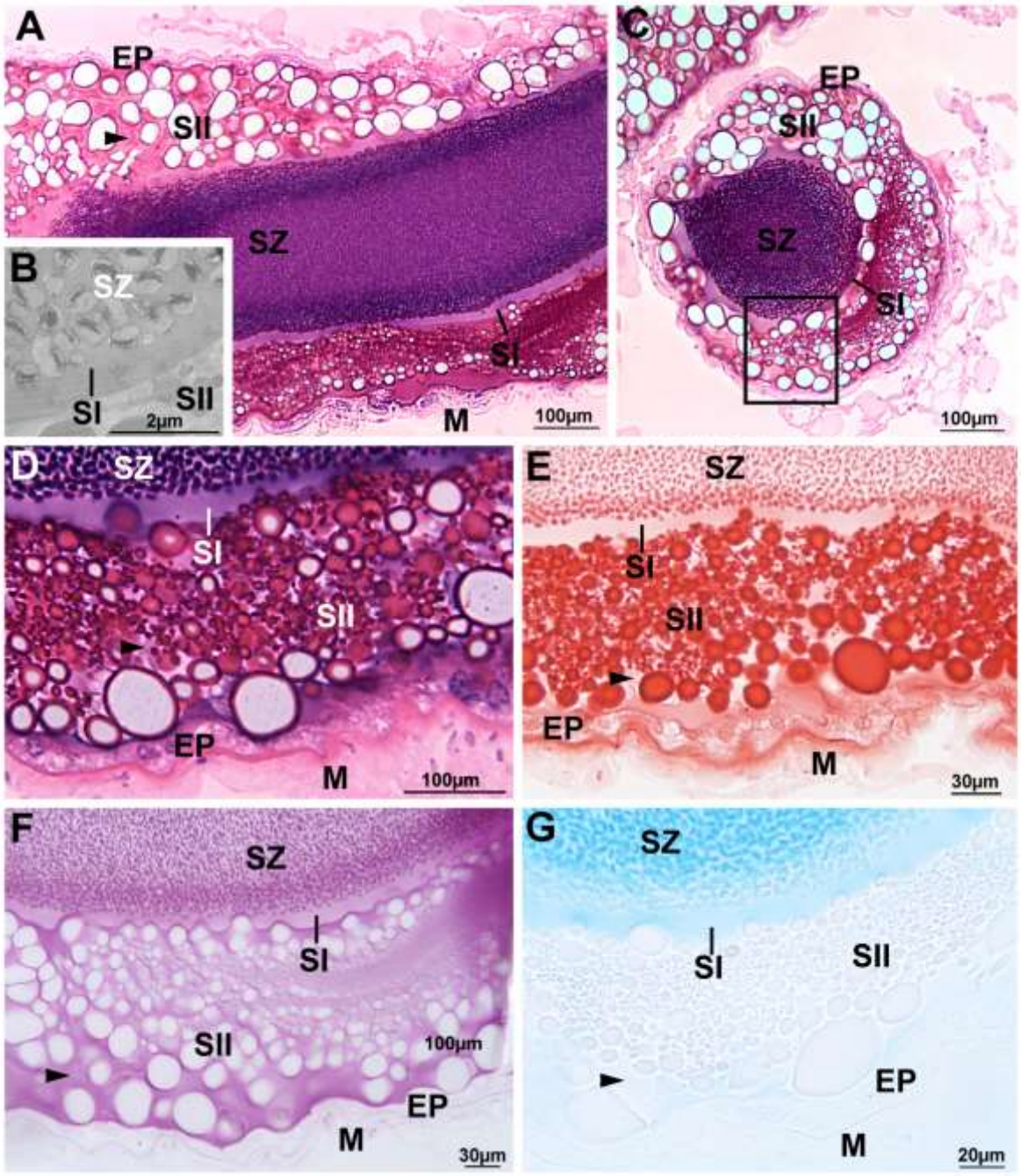
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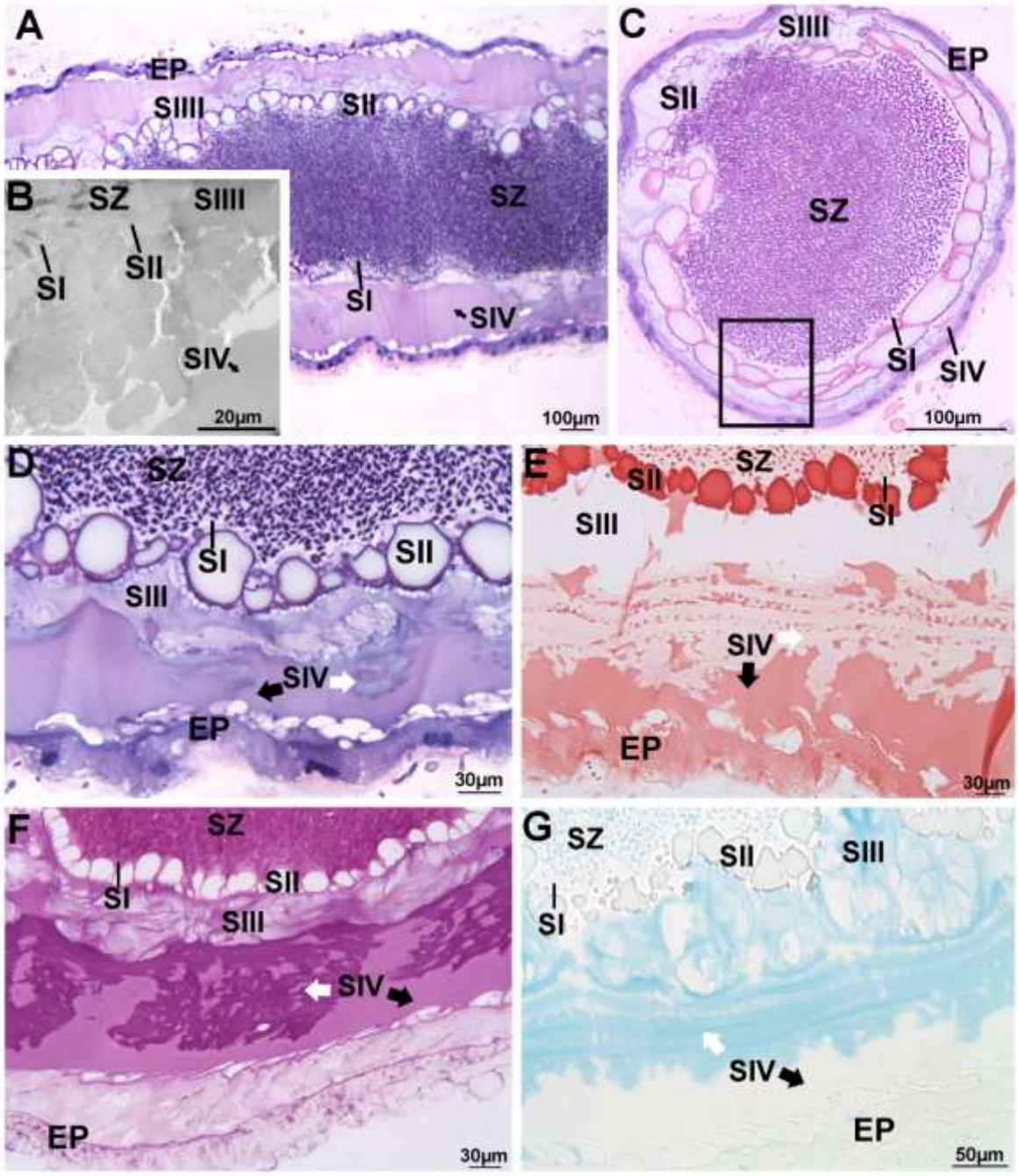
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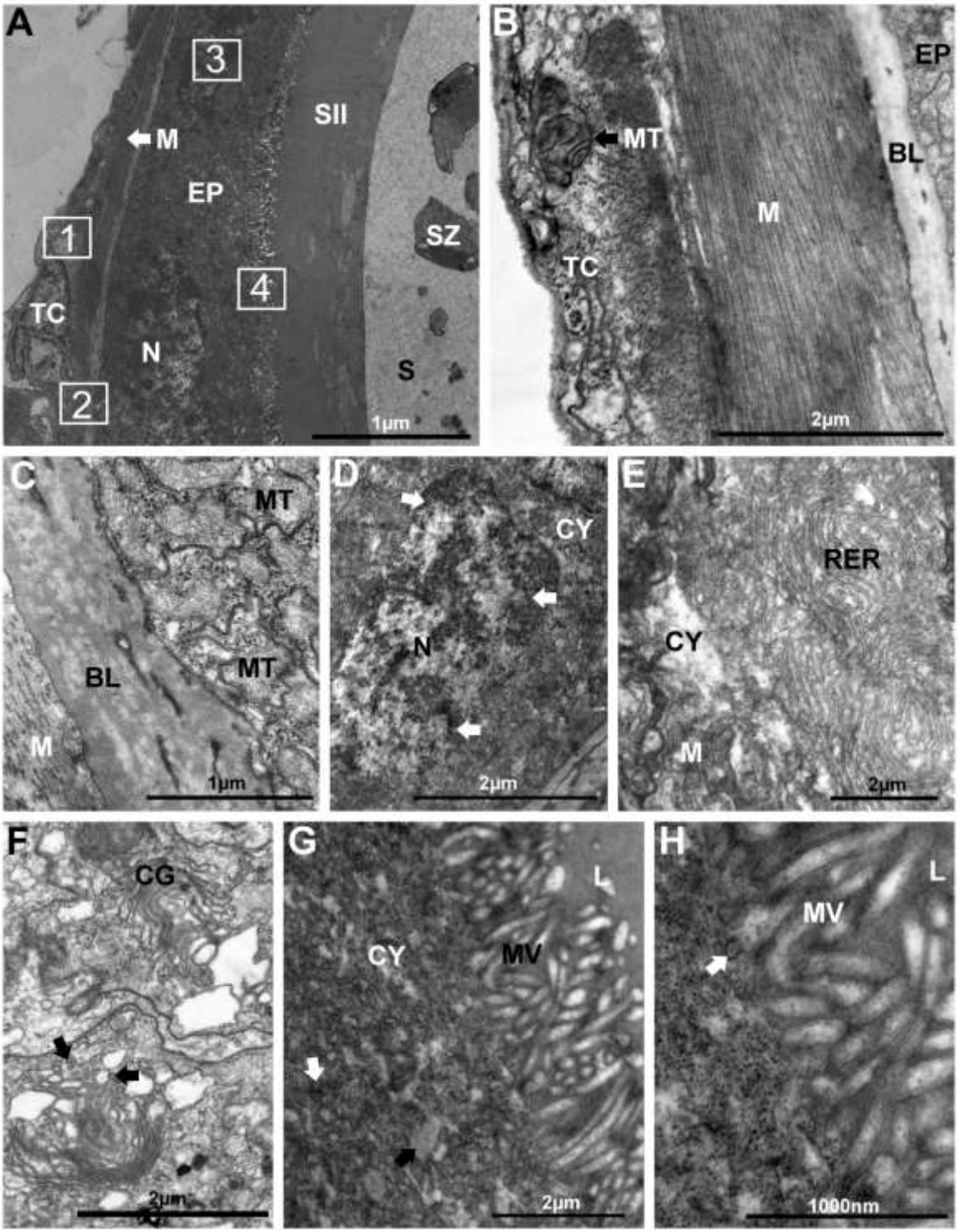
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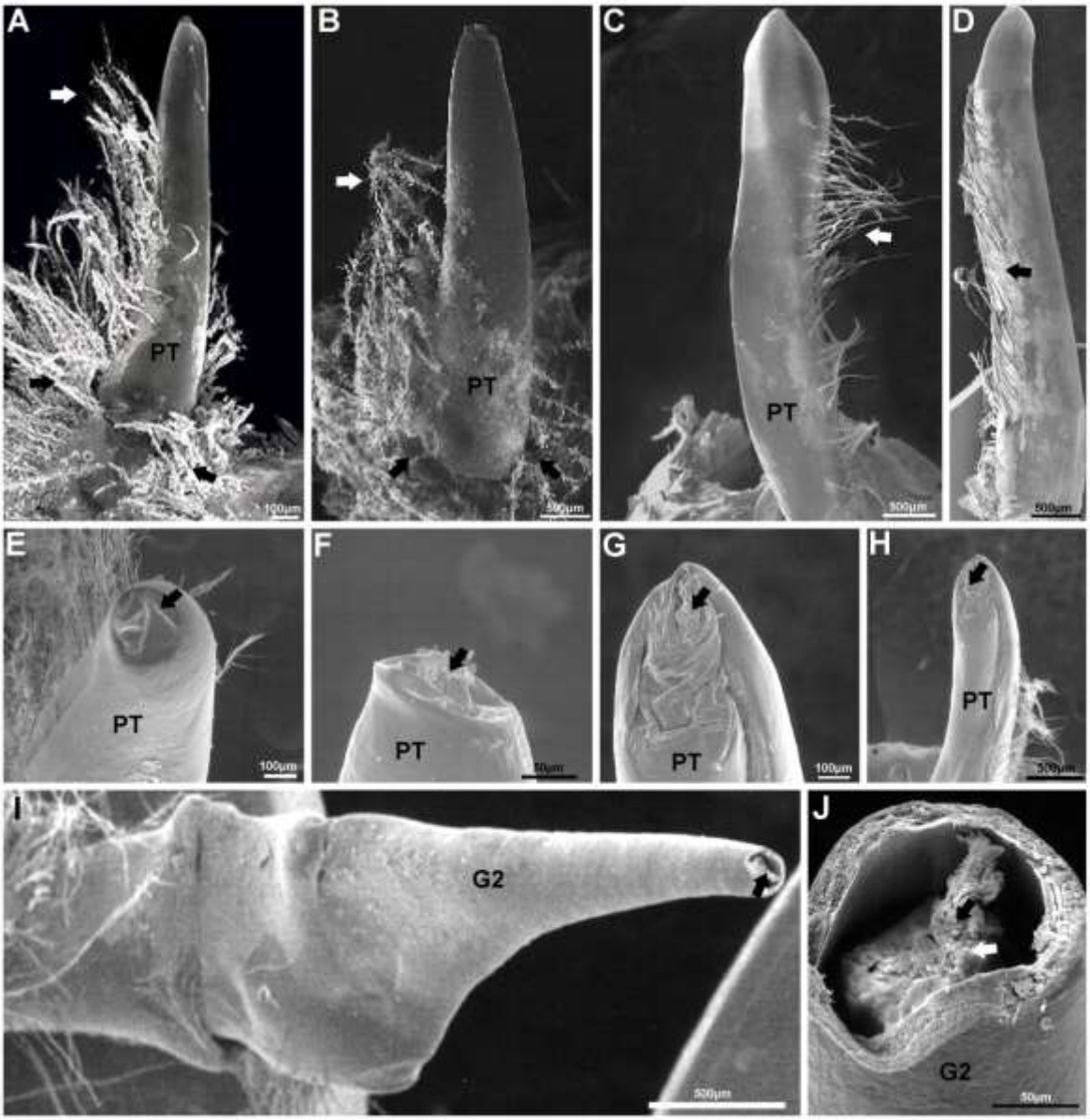

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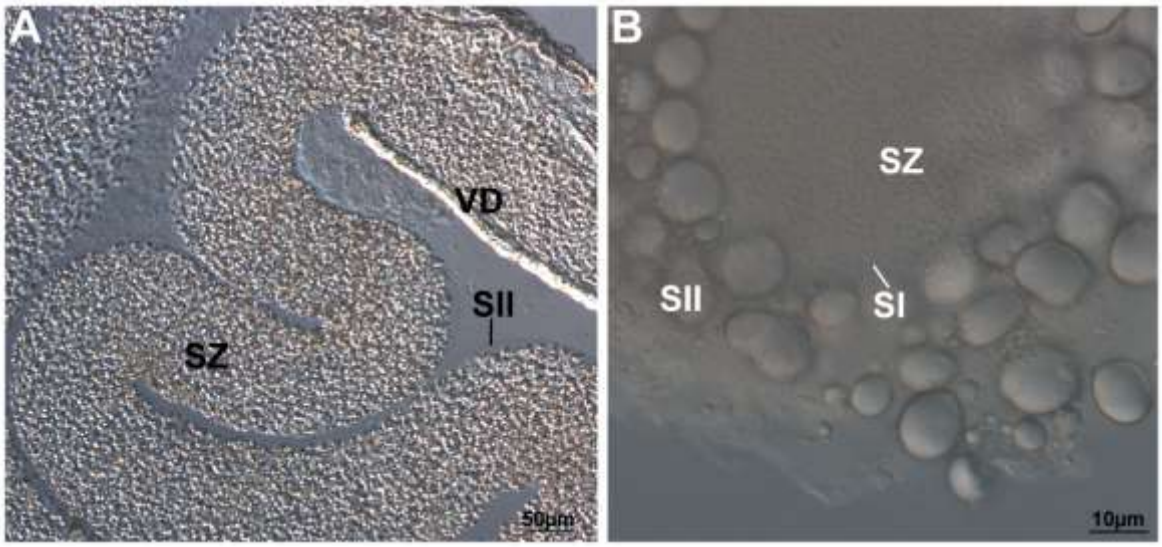

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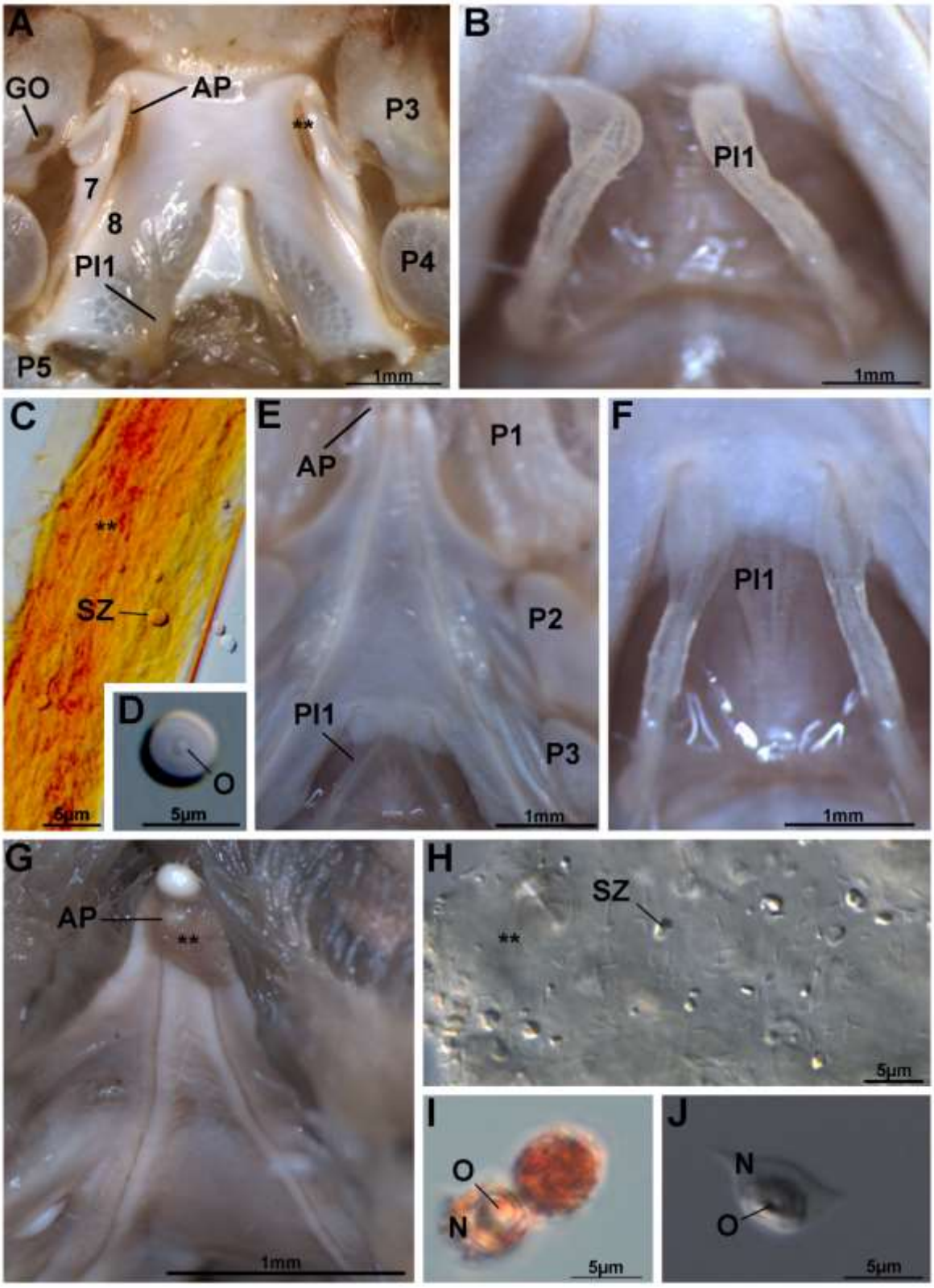
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Table 1: Histochemistry of spermatozoa and secretions from the vas deferens in *Hypoconcha parasitica*, *H. arcuata*, *Moreiradromia antillensis* and *Dromia erythropus*. +++ = strongly positive; ++ = positive; + = weakly positive; - = negative.

		Ponceau xylidine (total proteins)	PAS (neutral polysaccharides)	Alcian blue (acidic polysaccharides)
Hypoconcha parasitica	Spermatozoa	++	++	+++
	Type I secretion	+	++	++
	Type II secretion	+++	+++	+
	Fibrous material	+++	+++	-
Hypoconcha arcuata	Spermatozoa	+++	+++	++
	Type I secretion	++	++	+
	Type II secretion	+++	+	-
Moreiradromia antillensis	Spermatozoa	+++	+++	+++
	Type I secretion	++	++	++
	Type II secretion (granular)	+++	-	-
	SII matrix	++	++	+
Dromia erythropus	Spermatozoa	+++	+++	+
	Type I secretion	+	++	-
	Type II secretion (granular)	+++	-	-
	Type III secretion	-	++	++
	Type IV secretion (external to SIII)	+	+++	+++
	Type IV secretion (next to epithelium)	++	++	-

Capítulo III

Comportamento de cópula e armazenamento espermático no caranguejo *Hypoconcha parasitica* (Podotremata: Dromiidae)

Maria Alice Garcia Bento & Fernando José Zara

Capítulo redigido de acordo com as normas do periódico *Acta Zoologica*

Comportamento de cópula e armazenamento espermático no caranguejo *Hypoconcha parasitica* (Podotremata: Dromiidae)

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Condensed title: Comportamento de copula e armazenamento espermático em Dromiidae

RESUMO

Neste trabalho descrevemos o comportamento de cópula de *Hypoconcha parasitica*, em condições laboratoriais, e adicionalmente, analisamos a morfologia da espermateca e o padrão de armazenamento dos espermatozoides e fluido seminal. Os casais foram mantidos em aquários e os comportamentos de acasalamento foram filmados e quantificados. As espermatecas foram processadas seguindo as rotinas para microscopia eletrônica de varredura, histologia e histoquímica, com prévio tratamento em EDTA. O comportamento de corte e guarda copulatória estão ausentes em *H. parasitica*. A transferência espermática ocorreu enquanto os casais encontravam-se em "postura bivalve". Três cópulas foram registradas, com machos sempre em intermuda, enquanto uma fêmea copulou em muda e duas em intermuda. A espermateca é uma invaginação dos seguimentos torácicos esternos 7/8, recoberta exclusivamente por cutícula, seguindo o padrão de Podotremata. Externamente a parede da espermateca notam-se fibras musculares associadas à cutícula, principalmente nas regiões mais distais, oposta a abertura. A organização da espermateca indica que o processo de liberação dos espermatozoides para a fertilização ocorre por meio de ação muscular exercida na parede da câmara. Assim, a distribuição da musculatura em Hypoconchinae é diferente do descrito para o Homolidae *Paromola cuvieri*, a qual é concentrada na abertura da espermateca. Como em Homolidae, o pleopodo 1 parece estar envolvido na movimentação de espermatozoides e ovócitos no momento da fertilização em *H. parasitica*. Assim, a morfologia da espermateca e estruturas associadas trazem novas informações sobre os mecanismos envolvidos na reprodução de caranguejos primitivos e como ocorreu a modificação desta estrutura armazenadora entre os Podotremata.

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INTRODUÇÃO

O comportamento de acasalamento tem sido estudado em diversos crustáceos Decapoda (Hartnoll, 1969; Waddy & Aiken, 1990; Diesel, 1991; Bauer, 1996, Pinheiro & Fransozo, 1999). Entretanto, este assunto é pouco conhecido para os caranguejos primitivos pertencentes à seção Podotremata Guinot, 1977. Esta seção agrupa aproximadamente 89 gêneros em que sistema reprodutor feminino e masculino abrem-se por meio de orifícios coxais (gonóporos), pertencentes aos terceiros e quintos pereópodos, respectivamente (Guinot & Tavares, 2001; Guinot & Quenette, 2005; Ng *et al*., 2008; Guinot *et al*., 2013). Adicionalmente, as fêmeas portam um par de estruturas que são responsáveis pelo armazenamento dos espermatozoides até a fertilização, denominadas espermatecas (Tavares & Secretan, 1993; Guinot & Quenette, 2005; Guinot *et al*., 2013).

A cópula foi somente estudada no Dromiidae *Dromia personata* (Linnaeus, 1758) e no Raninidae *Ranina ranina* (Linnaeus, 1758) dentre os Podotremata (Hartnoll, 1975; Skinner & Hill, 1987; Guinot *et al*., 2013). Em *D. personata* o acasalamento pode ocorrer com fêmeas em muda e em intermuda, enquanto que em *R. ranina*, a cópula ocorre com fêmeas em intermuda, sendo que ambas espécies não exibem comportamentos de corte e guarda (Hartnoll, 1975; Skinner & Hill, 1987). Para as espécies de Eubrachyura, dois principais padrões de acasalamento foram estabelecidos. O primeiro padrão é aquele em que o acasalamento ocorre rapidamente após a muda da fêmea, quando estas encontram-se sob a condição de exoesqueleto mole (Hartnoll, 1969; Christy, 1987). Neste caso, os comportamentos de corte e guarda prolongada são bastante comuns (Hartnoll, 1969; Christy, 1987; Jivoff & Hines, 1998; Mclay & López Greco, 2011). No segundo padrão, o acasalamento ocorre quando as fêmeas encontram-se em intermuda (exoesqueleto rígido) e o comportamento de corte (quando presente) é breve e a guarda prolongada normalmente está ausente (Hartnoll, 1969; Christy, 1987).

A espermateca tem origem exclusivamente ectodérmica, sendo constituída por invaginações dos seguimentos torácicos esternos adjacentes 7/8, nos quais formam uma depressão no esterno, cuja abertura é alongada em forma de fenda, sem apresentar ligação interna com o oviduto (Guinot & Tavares, 2001; Guinot & Tavares, 2003; Guinot & Quenette, 2005; Guinot *et al*., 2013). Desta maneira, os ovócitos dos Podotremata são ovulados via gonóporos coxais e os espermatozoides são liberados da espermateca para que a fertilização externa ocorra (Hartnoll, 1968, 1969; Guinot & Quenette, 2005; Guinot *et al*., 2013; Mclay & Becker, 2015). Este padrão encontrado em Podotremata é diferente ao observado nos caranguejos Eubrachyura, nos quais as fêmeas portam gonóporos nos esternos do sexto segmento torácico e são ligados internamente aos receptáculos seminais de origem ecto- mesodérmica. O receptáculo seminal recebe o oviduto, por meio de uma conexão dorsal ou ventral, tendo papel importante na competição espermática (Diesel, 1989, 1991, McLay & López-Greco, 2011; Antunes *et al*., 2016). Assim, a fertilização em Eubrachyura ocorre internamente ao corpo destes, podendo ocorrer nos receptáculos seminais ou em outras estruturas internas associadas (Hartnoll, 1968, 1969; Guinot *et al*., 2013; Mclay & Becker, 2015; Hayer *et al*., 2016).

Apenas dois trabalhos descrevem a histologia da espermateca em Podotremata, sendo um deles apresentado de maneira esquemática (Hartnoll, 1975; Becker & Sholtz, 2017). O processo de liberação dos espermatozoides da espermateca para a fertilização externa parece ocorrer meio de ação muscular, e foi proposto somente para a Homolidae (Becker & Sholtz, 2017). Assim, o mecanismo de liberação dos espermatozoides da espermateca em Dromiidae ainda é desconhecido e, com a lacuna de conhecimento da morfologia funcional desta estrutura, não é possível traçar hipóteses sobre como ocorre à fertilização em Podotremata (Becker & Sholtz, 2017). Desta maneira, no presente trabalho realizamos a descrição detalhada do comportamento de cópula e da morfologia da espermateca de

Hypoconcha parasitica (Linnaeus, 1763), a fim de elucidar os mecanismos de fertilização nesta espécie de Dromiidae e verificar se os comportamentos exibidos durante a transferência espermática e a maneira como os espermatozoides são armazenados no interior da espermateca são semelhantes ao padrão comumente encontrado para caranguejos Brachyura.

MATERIAL E MÉTODOS

Animais

Machos e fêmeas maduras de *Hypoconcha parasitica* de 19,0 a 22,0 mm de largura da carapaça (LC) foram coletados no município de Ubatuba, São Paulo, Brasil (25°07´385´´S/47°52´508´´W) no período de agosto de 2016 a novembro de 2017, por meio de arrasto (20 min.), utilizando-se barco de pesca camaroneira, com redes do tipo "double rig", a uma profundidade de 10 a 18 metros. Posteriormente, os animais foram transportados vivos em caixas de isopor com a devida aeração para o Laboratório de Morfologia de Invertebrados do Departamento de Biologia Aplicada à Agropecuária, FCAV, UNESP- Jaboticabal, onde foram mantidos em aquários para devida manutenção.

Comportamento reprodutivo

As análises do comportamento reprodutivo de *H. parasitica* foram realizadas em condições laboratoriais. Para tal, os casais foram mantidos em aquários (45x30x20cm) com um casal cada, em água do mar aerada (salinidade de 33 a 35 Psu e temperatura média de 25ºC). Para a separação dos casais nos aquários, foram utilizadas telas plásticas removíveis para que o contato químico e visual fossem mantidos. Para identificar o período de preferência de cópula, a tela plástica foi removida cinco vezes no período entre 09:00h e

18:00h e cinco vezes entre 19:00h e 05:00h da manhã. Para observações em período noturno, os aquários foram iluminados por luz infravermelha (Pinheiro e Fransozo, 1999). Além disso, os períodos de contato entre os animais após a remoção da tela removível, foram adicionalmente registrados por meio de câmera digital (GoPro Hero 5 e Sony DCR HC40). Sedimentos arenosos e conchas de bivalves foram utilizados como substrato, para mimetizar o ambiente natural. Todas os animais utilizados encontravam-se em intermuda, exceto em um evento em que a fêmea sofreu muda durante a experimentação, estando assim, com o exoesqueleto não calcificado (carapaça mole). As filmagens foram armazenadas em cartão de memória e posteriormente analisadas em computador para averiguação do repertório comportamental reprodutivo de *H. parasitica*. Ao término da cópula, foi realizada a checagem da região ventral das fêmeas para verificação do aspecto da espermateca quanto a presença de plug espermático. As fêmeas que copularam nos aquários foram checadas a cada duas horas nas primeiras 24h e após este período, a checagem foi realizada a cada dia, para verificar o momento do evento de ovulação.

Análise da espermateca e ovários

Para as análises histológicas e ultraestruturais, seis fêmeas tiveram uma de suas espermatecas fixadas em solução Karnovsky (glutaraldeído 2,5% e parafolmaldeído 2% em tampão cacodilato de sódio 0.1M, pH 7.4 (Karnovski, 1965), com a adição de sacarose 5% (Ro *et al*., 1990) durante três a quatro dias a 4ºC (pH 7.4). As outras espermatecas, bem como os ovários foram fixadas em paraformaldeído 4% em tampão fosfato de sódio 0.1M (pH7.4) pelo mesmo período. Após a fixação em Karnovsky, as amostras foram lavadas três vezes em tampão cacodilato de sódio 0.1M (pH 7.4), com duração de 10 minutos cada e pós - fixadas em tetróxido de ósmio 1% no mesmo tampão por 30 minutos. Posteriormente, os materiais foram desidratados em séries crescentes de álcool (70 a

100%) por 15 minutos cada. Quando em álcool 100%, as espermatecas foram envolvidas em Parafilm (Bemis) e congeladas rapidamente em nitrogênio líquido. Após o congelamento, com uma lâmina previamente congelada, o material foi fraturado, sendo posteriormente descongelado no álcool 100% (Hayat, 1978). Em seguida as amostras foram submetidas a completa secagem em ponto crítico CPD 030 (Balzer Union), com CO2 líquido. Posteriormente, foram fixadas em "stubs" de alumínio e levadas ao metalizador (SC 070 - Balzer Union) para serem vaporizadas com ouro (camada de 10 milímetros). Ao final, todas as amostras foram analisadas e fotografadas em microscópio eletrônico de varredura Zeeis EVO 10, com intensidade do feixe de elétrons variando de 10 - 20KV.

Para as análises histológicas e histoquímicas, as espermatecas das seis fêmeas fixadas em paraformaldeído 4%, sendo uma após a cópula e outras duas após a ovulação foram descalcificadas em ácido etilenodiaminotetracético (EDTA) 10% por 48 a 72 horas e desidratadas em séries crescentes de etanol de 70 a 90%. Após a desidratação, as amostras foram embebidas e incluídas em historesina glicol-metacrilato Leica®. Os cortes seriados foram obtidos em micrótomo rotativo (5 a 7µm). Para descrição histológica, o material foi corado com Hematoxilina & Eosina (Junqueira & Junqueira, 1983) e para a histoquímica, as lâminas foram submetidas às técnicas de Xylidine Ponceau (Mello & Vidal 1980) para evidenciar proteínas totais e PAS e Azul de Alcian 2.5% (Junqueira & Junqueira, 1983) para polissacarídeos neutros e ácidos, respectivamente.

RESULTADOS

Comportamento reprodutivo

Os casais submetidos às análises de cópula apresentavam LC de 19,8 a 25 mm para os machos, enquanto as fêmeas apresentaram LC variando 19 a 21, 5 mm. Das cinco

seções de tentativa de pareamento, somente em três a cópula foi efetivada, sendo este evento sempre durante o dia, duas no período da manhã e uma no final da tarde. Nenhuma cópula foi observada nos experimentos durante o período da noite. Das três cópulas realizadas em laboratório, duas aconteceram com fêmeas em estágio de intermuda e uma em estágio de muda. O LC dos casais foram selecionados de maneira aleatória, sendo que os casais que efetivamente copularam apresentaram a seguinte proporção macho e fêmea de LC: 20mm: 21,10 mm; 21, 4 mm: 19,8mm; 25,0 mm: 21, 5mm.

Logo após a remoção da tela removível que separava os casais, os animais continuaram caminhando normalmente sobre o substrato (Fig.1A). Nenhum tipo de corte elaborada foi observado até o momento da cópula. Em todas as seções de tentativa de pareamento, os machos ao encontrar a fêmea, tocavam o seu corpo e iniciaram o comportamento de escalada sobre a concha da fêmea. Para os três casais que efetivamente copularam, o macho encontra a fêmea e toca a sua concha com um dos quelípodos e posteriormente, dirige o outro quelípodo até a concha. Imediatamente, o macho inicia a escalada sobre a concha da fêmea, a qual se mantém estática ou com movimentos limitados (Fig. 1B, C). Os pereópodos dois e três do macho estão sempre em contato com a concha e/ou substrato. Posteriormente, os machos passam a se posicionar em cima da concha da fêmea. O macho começa a tocar a região ventral da concha onde encontra-se a fêmea. Posteriormente, o macho apoia a sua concha em posição lateral ao subtrato e com ação dos quelípodos e pereópodos, ao mesmo tempo que começa a levantar a fêmea, empurrando o corpo e a concha da mesma (Fig. 1D). Ao levantar completamente a fêmea, o casal atinge a "postura bivalve", momento no qual o macho com o os pereópodos dois e três, abre o abdômen da fêmea, posicionando o seu abdômen sobre o da mesma (Fig. 1E). O tempo entre os primeiros comportamentos e a postura bivalve foi de 4 a 5 minutos. Antes de alcançar a postura bivalve, algumas fêmeas não permitiram sua imobilização pelos machos

e iniciaram um comportamento de fuga, ou seja, comportamento de evitação da cópula, o que ocorreu em duas seções comportamentais. Quando o macho atinge a posição bivalve, este insere os segundos gonópodos na abertura da espermateca da fêmea, iniciando a transferência espermática (Fig. 1E, F). A transferência espermática é marcada pelo movimento contínuo do abdômen do macho, em vai e vem. Dois tipos de postura bivalve foram observados, um no qual o macho e fêmea com a região anterior voltada para o substrato, enquanto que a região posterior do corpo permanece elevada (Fig. 1E), o qual foi observada uma única vez. A segunda postura bivalve foi observada duas vezes e nesta, o macho posiciona-se sobre o corpo da fêmea, a qual permanece em decúbito dorsal, com a concha apoiada sobre o substrato (Fig. 1F). A duração média de cópula foi de 173,3 ± 70 minutos. O término da transferência espermática é marcado pelo movimento dos quelípodos dos machos em direção à parte anterior do seu corpo, a fim de abandonar a postura bivalve, ao mesmo tempo que a fêmea move seus pereópodos em direção ao substrato (Fig. 1G). Ao alcançarem o substrato, os casais se separam e voltam a se mover livremente no aquário (Fig. 1H). Os machos, sempre em intermuda, copularam com duas fêmeas em intermuda, as quais tiveram a duração de cópula em 100 e 180 minutos. Em um só evento de cópula, com a fêmea em muda, a cópula ocorreu durante o período de 240 minutos.

Espermateca e estruturas associadas

A espermateca é um órgão par, localizada na superfície ventral do cefalotórax, mais especificamente presente na região inclinada da parte posterior do esterno e é constituída por suturas torácicas derivadas de invaginações dos integumentos externos 7/8 (Fig. 2A, B). As porções mais posteriores das suturas têm início no mesmo nível da coxa dos pereópodos quatro (P4), e estendem-se próximo ao nível do par de gonóporos, os quais são estruturas independentes da espermateca, presentes nas coxas dos terceiros pereópodos

(P3) (Fig. 2A, B). Acima da extremidade posterior da espermateca existe uma região curta e estreita, denominada por tubo espermatecal ou câmara de armazenamento, onde, internamente, o fluido seminal permanece armazenado até o processo de fertilização (Fig. 2A, B). O tubo espermatecal é contínuo e termina na abertura da espermateca, a qual está localizada na região anterior do esterno torácico, próximo ao P3 (Fig. 2A, B). Em fêmeas não ovígeras, a abertura da espermateca é oval e pequena (Fig. 2A). Enquanto que em fêmeas logo após a ovulação (ovígeras), esta região apresenta preenchida por material seminal (Fig. 2B). Ao microscópio eletrônico de varredura nota-se que a abertura da espermateca encontra-se no mesmo nível do gonóporo operculado na altura da coxa do terceiro pereópodo (Fig. 3A). O gonóporo tem forma arredondada e é recoberto por opérculo membranoso (Fig. 3B). Ao redor do gonóporo nota-se inúmeras cerdas coxais do tipo plumosas (Fig. 3A, B). A abertura da espermateca em fêmeas logo após a ovulação apresenta ejaculado no seu interior, onde observam-se claramente os com espermatozoides (Fig. 3C, D). Em cortes longitudinais ao eixo ântero-posterior da espermateca, nas fêmeas que acasalaram sob condições laboratoriais, observa-se a presença de fluido seminal com espermatozoides no seu interior (Fig. 3E, F). Uma das faces da espermateca apresenta cutícula mais delgada em relação a outra e, nota-se a inserção das fibras musculares principalmente nas regiões mais distais, ou seja, no lado oposto da abertura da espermateca, sobre a face cuticular mais espessa (Fig. 3E, 4A, B).

Por meio da histologia, a abertura da espermateca mostra-se estreita e curta, com curvatura, sem a presença de botões ou estruturas membranosas cuticulares que a separam da região onde os espermatozoides encontram-se armazenados (Fig. 4A, B). A espermateca da fêmea dissecada logo após a cópula, bem como fêmeas sacrificadas logo após a ovulação, mostraram a presença de secreção seminal com espermatozoides junto da abertura da espermateca. A espermateca mostra espaço interno amplo, a qual se torna cada

vez mais estreito na direção posterior (Fig. 4A, B). A musculatura corpórea está diretamente ancorada na face de cutícula mais espessa da espermateca e as células epidérmicas mostram aspecto pavimentoso e em certos pontos descontínua, o que devido ao poder de resolução do microscópio de luz assemelha-se a um contato direto da musculatura na cutícula (Fig. 4C). Por sua vez, a epiderme associada a porção cuticular mais delgada da espermateca apresenta epitélio colunar simples, com núcleos basais e citoplasma acidófilo (Fig. 4D). No lúmen da espermateca notam-se dois tipos de secreção, a secreção do tipo um (SI), onde estão imersos os espermatozoides e a secreção do tipo dois (SII), sem a presença de espermatozoides. A SI não sofreu alteração histoquímica em relação às fêmeas recém-copuladas (Fig. 5A - D) e as fêmeas sacrificadas após a ovulação (Fig. 5E - H), sendo esta uma secreção glicoproteica, com pequena reatividade para polissacarídeos ácidos (Fig. 5E- H). A secreção tipo II na fêmea recém-copulada é acidófila, intensamente reativa para proteínas e positiva para polissacarídeos neutros, sendo negativa para polissacarídeos ácidos (Fig. 5A - D). Porém, nas fêmeas após a ovulação, a SII apresentou modificação histoquímica, onde esta passou a ser basófila, menos intensamente corada para proteínas e com intensa reação para polissacarídeos neutros (Fig. 5E - H). Não houve alteração da reação para polissacarídeos ácidos (Fig. 5H).

As fêmeas que copularam no estado de intermuda possuem ovócitos em estágio avançado de desenvolvimento, com a presença de grande quantidade de grânulos de vitelo em seu interior (6A, B). A única fêmea que copulou no estágio de muda apresentou ovócitos rudimentares, caracterizados por citoplasma fortemente basófilo, com várias dilatações menos basófilas no seu interior (Fig. 6C). A análise dos pleópodos das fêmeas mostrou que o primeiro par (PL1) não carrega ovos em fêmeas ovígeras, permanecendo livres na câmara de incubação (Fig. 6D). Os PL1 são curtos, com a região distal cônica e circundados por inúmeras cerdas plumosas, particularmente abundantes na região apical

(Fig. 6E). Estes pleópodos são unirremes e com o abdômen fechado se aproximam da abertura da espermateca (Fig. 6F, G).

DISCUSSÃO

Em Podotremata, o comportamento de cópula foi pouco estudado, sendo as descrições restritas aos representantes de Dromiidae *D. personata* e ao Raninidae *R. ranina* (Skinner & Hill, 1987; Guinot *et al*., 2013). Em *Hypoconcha parasitica* não foi verificado nenhum tipo de comportamento de corte e todos os machos ao encontrar a fêmea iniciaram a tentativa de cópula, não havendo evidência de sinais visuais, como observado em outros Eubrachyura (Ryan, 1967; Hartnoll, 1969, Dunham, 1978; Kennedy & Cronin, 2007). Assim, *H. parasitica* seguiu o padrão conhecido para os outros Podotremata (Hartnoll, 1975; Skinner & Hill, 1987). Em *H. parasitica* uma característica interessante da cópula foi o posicionamento do macho segurando a fêmea levando a junção das conchas sobre o corpo ou postura bivalve, que em dois casos, o término da cópula ocorreu com o macho sobre a fêmea. Nos outros Podotremata descritos na literatura, o macho posiciona a fêmea sobre o seu corpo, estando este em decúbito dorsal (Hartnoll, 1975; Skinner & Hill, 1987). A adoção desta postura bivalve por parte de *H. parasitica* parece ser devido ao fato destes animais recobrirem o seu corpo com uma concha rígida, tendo em vista as poucas observações de cópula. Em *D. personata*, o período de cópula foi ligeiramente menor ao observado para *H. parasitica*. Além disso, neste trabalho observamos que o abdômen do macho deste Hypoconchinae permaneceu em constante movimento de abertura e fechamento durante o período de transferência espermática. Assim, o longo período de cópula e a movimentação abdominal podem estar associados à pequena musculatura observada no vaso deferente de Dromiidae (Bento-Garcia *et al*., capítulo II; Hartnoll, 1975). Adicionalmente, em Eubrachyura existem várias espécies que possuem musculatura

delgada ao longo do vaso deferente e o período de cópula é geralmente prolongado, sendo que neste caso o material seminal é transferido para o receptáculo seminal, o qual é um órgão de origem ecto-mesodérmica bastante distinta da espermateca (Hartnoll, 1975; Mclay & López- Greco, 2011; Mclay & Becker, 2015).

Outro aspecto a ser levado em consideração, é a condição de muda. No caso de *H. parasitica*, em condições laboratoriais pode-se constatar que existe a transferência espermática tanto com fêmeas em condição de muda, como em intermuda, similar ao observado para *D. personata* (Hartnoll, 1975). Assim, a posição da fêmea sob o macho se mantém a mesma, independente do estágio de muda. Hartnoll (1975) observou a presença de material seminal depositado sobre a abertura da espermateca logo após a separação forçada dos casais durante a cópula. A presença de material seminal na abertura da espermateca também foi descrita para outros Podotremata, como os Dromiinae *Lauridromia intermedia* (Laurie, 1906), *Austrodromidia octodentata* (Haswell, 1882), *Pseudodromia latens* Stimpson, 1858 e Raninidae *Symethis variolosa* (Fabricius, 1973) e *R. ranina*, sendo este material considerado como plug espermático (Guinot & Tavares, 2003; Guinot & Quenette, 2005; Guinot *et al*., 2013). A presença de plug espermático ou qualquer outro material rígido na espermateca estão ausentes em Homolidae (Becker & Sholtz, 2017). Evidências de plug espermático não foram encontradas para Hypoconchinae. Com base em nossos resultados, confirmamos a ausência de plug espermático em *H. parasitica.* Na verdade, a presença de secreção na abertura da espermateca foi evidenciada nesta espécie, porém, este material foi exclusivamente encontrado em fêmeas ovígeras, tanto provenientes dos experimentos laboratoriais, como provenientes do campo preservadas em álcool. Logo após a finalização natural do processo de cópula, nenhum material foi encontrado preenchendo a abertura da espermateca. Este material observado nas fêmeas ovígeras tratava-se de secreção seminal contendo

espermatozoides, o que não evidencia o plug espermático, sendo que este mesmo material também foi observado na abertura da espermateca de *Moreiradromia antillensis* (Stimpson, 1858) (Garcia- Bento *et al*.; capítulo II). O material observado na abertura da espermateca parece permanecer nesta estrutura como um resquício do último processo de liberação do fluido seminal para a fertilização dos ovócitos, uma vez que nos Podotremata a fertilização é externa. Durante este evento, a câmara de fertilização formada pelo abdômen, na qual recobre o esterno, permite que os ovócitos liberados pelos gonóporos da coxa dos P3 entrem em contato com a secreção liberada da espermateca (Becker & Scholtz, 2017).

Em *H. parasitica*, assim como nos outros Podotremata, o órgão de armazenamento de espermatozoides é exclusivamente revestido por cutícula, portanto com origem ectodérmica. A espermateca de *H. parasitica* possui um arranjo de fibras musculares inseridas particularmente na região mais distante da abertura da espermateca, onde a cutícula é mais espessa. Uma descrição bastante similar a esta também foi encontrada para outro Podotremata, o Homolidae *Homolochunia valdiviae* (Gordon, 1950). Esta inserção muscular na parede da espermateca pode atuar no momento da ovulação para promover a movimentação do esternito 7 em relação ao esternito 8, fazendo com que o ejaculado seja liberado da espermateca por meio da ação muscular, para que ocorra a fertilização. Este mecanismo foi proposto para os Homolidae *Paromola cuvieri* (Risso, 1816), *Homola barbata* (Fabricius, 1793), *Homologenus malayensis* Ihle, 1912, contudo as estruturas responsáveis pelo processo de contração muscular estão concentradas na abertura da espermateca, sem a presença de músculos nas demais regiões (Becker & Scholtz, 2017). Desta maneira, o mecanismo de liberação do material para a fertilização parece estar diretamente envolvido com a ação muscular, porém, estudos a respeito da organização da espermateca e principalmente dos pontos de inserção muscular e regiões de cutícula mais

membranosas, devem ser aprofundados, pois organizações muito similares são encontradas tanto em Dromiidae como em Homolidae (Gordon, 1950; Becker & Scholtz, 2017). Becker & Scholtz (2017) propõem para Homolidae, que o Pl1 atua de maneira similar ao observado para *Homarus americanus* H. Milne Edwards, 1837 durante o processo de ovulação (Aiken *et al*., 2004). As análises da morfologia do Pl1 em *H. parasitica* apontam para uma grande similaridade com os homolídeos e *Homarus americanus* H. Milne-Edwards, 1837. Na espécie aqui estudada, o Pl1 não participa como uma estrutura na qual ocorre a adesão dos ovos nas fêmeas ovígeras, apresentando uma organização unirreme e uma ampla ornamentação de cerdas plumosas. Estes podem atuar na movimentação do material contido na câmara de fertilização, misturando os espermatozoides liberados da espermateca aos ovócitos provenientes do gonóporo da coxa dos P3, sendo este um mecanismo observado em Dromiinae e Homolidae (Becker & Scholtz, 2017).

O material seminal armazenado no lúmen da espermateca é composto por espermatozoides livres, circundados por massas disformes de secreção. A distribuição deste material luminal teve uma organização bastante variável, não obedecendo nenhum padrão, os quais relembram aquelas apresentadas nos esquemas propostos para *D. personata* e *Ho. barbata* (Hartnoll, 1975). Hartnoll (1975) observou secreção acumulada próximo à região da abertura da espermateca. Em *H. parasitica* tal secreção também foi observada, mas não se trata de um plug espermático, pois tanto em fêmeas recém-copuladas como naquelas próximas a ovulação, notou-se a presença deste material constituído pela SII, que junto a esta ocorre a SI contendo os espermatozoides. Desta maneira, o lúmen da espermateca não apresenta plug espermático ou pacotes espermáticos que relembrem aqueles descritos para os Eubrachyura (Hartnoll, 1969; Diesel, 1989; Johnson, 1980; Zara *et al*., 2014; Antunes *et al*., 2016). Estes dois tipos de secreção são semelhantes àquelas observadas no vaso deferente para esta mesma espécie, inclusive em

termos histoquímicos (Bento-Garcia *et al*., capítulo II). O ejaculado no interior da espermateca sofreu modificação histoquímica em relação à fêmea recém-copulada e aquelas após a ovulação, em condições laboratoriais. A SII reduz a intensidade para proteínas totais, ao mesmo tempo que a reação para polissacarídeos neutros torna-se mais intensa nas fêmeas após a ovulação. Desta maneira algum tipo de maturação deve ocorrer desde a cópula até a postura dos ovos, porém, o mecanismo que leva a esta alteração permanece um mistério, uma vez que o epitélio cuticular que reveste a espermateca não apresenta canais internos que possam indicar a liberação de algum tipo de secreção na luz da espermateca. Nos Eubrachyura, os quais apresentam receptáculos seminais, a presença de secreção glicoproteica é amplamente descrita (Sainte-Marie & Sainte-Marie, 1998; Sant'Anna *et al*., 2007; Zara *et al*., 2014; Antunes *et al*., 2016). São propostas a estas secreções diferentes funções, como evitar a perda de espermatozoides e garantir a proteção e nutrição (Jonhson, 1980; Sant'Anna *et al*., 2007; Zara *et al*., 2014; Antunes *et al*., 2016). Adicionalmente no caso da espermateca, a presença desta secreção além de proteger os espermatozoides também tem função de evitar o processo de reação do acrossoma, uma vez que em experimentos laboratoriais somente espermatozoides removidos da SII apresentaram as etapas iniciais de reação do acrossoma (Bento-Garcia *et al*., dados não publicados).

A fêmea que copulou em muda apresentava os ovários rudimentares e assim parece haver a necessidade da fêmea enrijecer seu esqueleto para subsequentemente desenvolver os ovócitos, indicando que o processo de fertilização deverá ocorrer no estágio de intermuda. Este padrão não deve ocorrer em fêmeas que copularam em intermuda, uma vez que estas encontram-se com os ovários desenvolvidos e o exoesqueleto rígido, o que permite que a ação muscular para a liberação dos espermatozoides contidos na espermateca ocorra a qualquer momento, ao término da maturação dos ovócitos. Assim, como as

fêmeas em intermuda apresentam material seminal preservados no interior da espermateca, parece factível que a fêmea realize múltiplas posturas de ovos, sem a necessidade de uma nova muda, o que é semelhante a diversas espécies de Eubrachyura, inclusive espécies que realizam muda terminal como *Maja brachydactyla* Balss, 1922 e *Callinectes danae* Smith, 1869 (Simeó *et al*., 2010; Zara *et al*., 2014; Mollenberg *et al*., 2017).

Em conclusão, após a transferência espermática em *H. parasitica*, não foi encontrada a presença de plug espermático na abertura da espermateca, sendo este material observado apenas após a fertilização e consequente ovulação. Assim, confirmamos a ausência do plug espermático no Hypoconchinae estudado e o material encontrado nas fêmeas ovígeras é resquício do processo de fertilização. Adicionalmente, a organização da espermateca observada em *H. parasitica* indica que o processo de liberação dos espermatozoides ocorre por meio de ação muscular exercida contra a parede da câmara. A distribuição desta musculatura neste Dromiidae segue o padrão descrito ao menos para um Homolidae, sendo distinto de outras espécies desta família recentemente descritas, indicando que um maior número de Podotremata precisam ser estudados histologicamente para se entender a evolução dos mecanismos de liberação dos espermatozoides desta estrutura ectodérmica. Adicionalmente, através das análises do comportamento de cópula e da organização interna geral da espermateca podemos observar que existe grande similaridade com as descrições existentes na literatura para Podotremata. Entretanto, cada espécie apresenta ao menos uma característica única, as quais podem estar relacionadas com as estratégias reprodutivas de cada família. A espermateca exclusivamente ectodérmica parece ser uma estrutura homóloga entre os Podotremata estudados, porém devido ao grande número de espécies deste grupo, novos estudos precisam ser realizados para que esta proposta seja confirmada.

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Lista de legendas

Figura 1. Comportamentos do acasalamento em *Hypoconcha parasitica*. (A) Após a retirada da tela plástica removível, os casais se movimentam normalmente pelo aquário. **(B)** Os animais se encontram, posteriormente o macho inicia uma escalada sobre a concha da fêmea com o auxílio dos quelípodos e dos primeiros e segundos pereópodos. Note que após a escalada, o macho se posiciona em cima da concha da fêmea. **(C)** Macho apoiando a sua concha em posição lateral ao substrato. Observe que o macho começa a movimentar os quelípodos para alcançar a região ventral da fêmea. **(D)** Macho levantando a fêmea (setas). Note que este empurra o corpo e a concha da fêmea sobre o substrato. **(E) "**Postura bivalve", na qual o macho posiciona a sua superfície ventral em frente da superfície ventral da fêmea, posicionando seu abdômen sobre o dela. Observe que nesta posição o macho e fêmea estão com sua região anterior voltada para o substrato, enquanto que a região posterior permanece elevada. **(F)** Em "postura bivalve", o macho insere os gonópodos dois na abertura da espermateca da fêmea, iniciando a transferência espermática (seta). Nesta posição, o macho posiciona-se sobre o corpo da fêmea, permanecendo em decúbito dorsal, com a concha apoiada sobre o substrato. **(G)** Nota – se o término do acasalamento quando o macho movimenta seus quelípodos em direção à parte anterior do seu corpo, abandonando a postura bivalve. Além disso, a fêmea move os seus pereópodos em direção ao substrato. **(H)** Macho e fêmea alcançam o substrato, separam-se e voltam a se mover livremente no aquário. ♂= macho; ♀= fêmea.

Figura 2. Anatomia da espermateca e estruturas associadas de *H. parasitica*. (A) A espermateca de fêmeas não ovígeras é uma estrutura par, localizada na região inclinada da parte posterior do esterno e é constituída por suturas torácicas derivadas de invaginações dos integumentos esternos 7/8. Note que a porção mais posterior da sutura que constitui a espermateca, tem início no mesmo nível da coxa dos pereópodos quatro (seta), no qual terminam quase no mesmo nível do par de gonóporos, localizados na coxa dos terceiros pereópodos. Adicionalmente, observa-se a abertura oval e pequena da espermateca. **(B)** Espermateca das fêmeas após a ovulação, mostrando o tubo espermatecal formado pela invaginação dos seguimentos esternos 7/8 e pela abertura da espermateca. Note que a abertura da espermateca é preenchida por material seminal (seta). Ap= abertura da espermateca; Cx3= coxa do terceiro pereópodo; Cx4= coxa do quarto pereópodo; Cx5= coxa do quinto pereópodo; Go= gonóporo; Spt= tubo espermatecal; St= esterno torácico. 7, 8= esternitos sete e oito.

Figura 3. Espermateca e estruturas associadas de *H. parasítica* vistos sob microscopia eletrônica de varredura. (A) Abertura da espermateca situada próxima ao nível do gonóporo operculado, na qual se situa na coxa dos terceiros pereópodos. **(B)** Detalhe do Gonóporo operculado com forma arredondada, mostrando-se coberto por um opérculo membranoso. Observe a presença das cerdas coxais plumosas situadas ao redor do gonóporo (setas). **(C)** Abertura da espermateca de fêmeas logo após a ovulação. Note a presença do ejaculado em seu interior (**). A área retangular é mostrada na figura D. **(D)** Detalhe dos espermatozoides encontrados na abertura da espermateca das fêmeas após a ovulação (setas). **(E)** Fratura ligeiramente obliqua da espermateca mostrando o conteúdo seminal e duas camadas cuticulares, sendo uma mais espessa (seta preta) e outra mais

delgada (setas brancas), onde está inserida a musculatura. A área retangular é mostrada na figura F. **(F)** Detalhe dos espermatozoides (setas) e secreção encontrados no interior da espermateca das fêmeas que acasalaram. Ap= abertura da espermateca; Cu= cutícula; Cx3= coxa do terceiro pereópodo; Go= gonóporo; Mu= musculatura; Op= opérculo; S= secreção; 7, 8= esternitos sete e oito.

Figura 4. Histologia da espermateca das fêmeas de *H. parasitica* após a cópula. (A) Vista geral da espermateca da fêmea antes da postura dos ovos, corada com Hematoxilina e Eosina (HE). Observe que uma das faces da espermateca apresenta cutícula mais delgada (seta branca) em relação à outra (seta preta) e que ambas apresentam a inserção de fibras musculares. Além disso, a espermateca mostra espaço amplo com o interior preenchido por espermatozoides imersos em secreção até a região próxima da abertura da espermateca. A abertura é estreita e curta. **(B)** Vista geral da espermateca da fêmea após a postura dos ovos (ovulação), mostrando-se envolvida por cutícula ao longo de toda sua extremidade e a inserção da musculatura. Note a presença dos espermatozoides imersos em secreção encontrados no interior da espermateca. **(C)** Detalhe da musculatura ancorada à face mais espessa da espermateca, mostrando também as células epidérmicas com aspecto pavimentoso (seta). **(D)** A epiderme associada à porção cuticular mais delgada da espermateca apresenta epitélio colunar simples, com núcleos basais e citoplasma acidófilo. Cu= cutícula; Ep= epitélio colunar simples da epiderme; Mu= musculatura; S= secreção; Sz= espermatozoides; 7,8= esternitos sete e oito.

Figura 5. Histologia e histoquímica das secreções encontradas no interior da espermateca das fêmeas de *H. parasitica*. (A) Lúmen da espermateca da fêmea antes da postura dos ovos corada com Hematoxilina e Eosina (HE), mostrando a secreção do tipo I (SI), levemente basófila e a secreção do tipo II (SII) acidófila. **(B)** Quando a espermateca da fêmea antes da ovulação foi corada com Xylidine Ponceau, a SI mostrou-se positiva para proteínas, enquanto a SII foi fortemente reativa para proteínas. **(C)** Em PAS, a SI e SII da fêmea antes da postura dos ovos foram positiva para polissacarídeos neutros. **(D)** Quando as lâminas foram submetidas ao corante azul de Alcian, a SI e a SII da fêmea antes da ovulação mostraram-se negativas a este corante. **(E)** A espermateca das fêmeas após a ovulação mostraram-se preenchidas pela SI basófila e pela SII fortemente basófila, quando submetidas a HE. **(F)** A SI manteve a quantidade de proteínas e a SII passou a ser menos proteica após a ovulação. **(G)** Quando coradas com PAS, a SI encontrada nas espermatecas das fêmeas após a ovulação mostrou-se positiva e a SII foi fortemente reativa para polissacarídeos neutros. **(H)** A SI e a SII das fêmeas após a ovulação continuaram negativas para polissacarídeos ácidos quando submetidas ao corante Azul de Alcian. Cu= cutícula; Mu= musculatura; SI= secreção do tipo um; SII= secreção do tipo dois; Sz= espermatozoides.

Figura 6. Histologia dos ovócitos e pleópodos das fêmeas ovígeras de *H. parasitica*. (A, B) Nas fêmeas que copularam em intermuda, os ovócitos apresentaram-se em estágio avançado de desenvolvimento, mostrando a presença de grande quantidade de grânulos de

vitelo em seu interior (seta). **(C)** Ovócitos rudimentares da fêmea que copulou durante a muda, os quais são caracterizados por citoplasma fortemente basófilo, com várias dilatações menos basófilas no seu interior (seta). **(D)** Vista geral da região ventral das fêmeas ovígeras sob estereomicroscópio, mostrando que os primeiros pleópodos (Pl1) não são destinados ao carregamento dos ovos (setas), vistos que estes permanecem livres na câmara de incubação. **(E)** Pl1 curtos, com região distal cônica e circundados por inúmeras cerdas plumosas (setas), abundantes principalmente na região apical. **(F)** Pl1 unirremes (setas). **(G)** Quando os abdômens das fêmeas ovígeras estão fechados (seta), os Pl1 se aproximam da abertura da espermateca. Eg= ovos; N= núcleo; Ov= ovócitos; Pl1= pleópodo um; St= esterno; V= grânulos de vitelo**.**

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Fig. 1

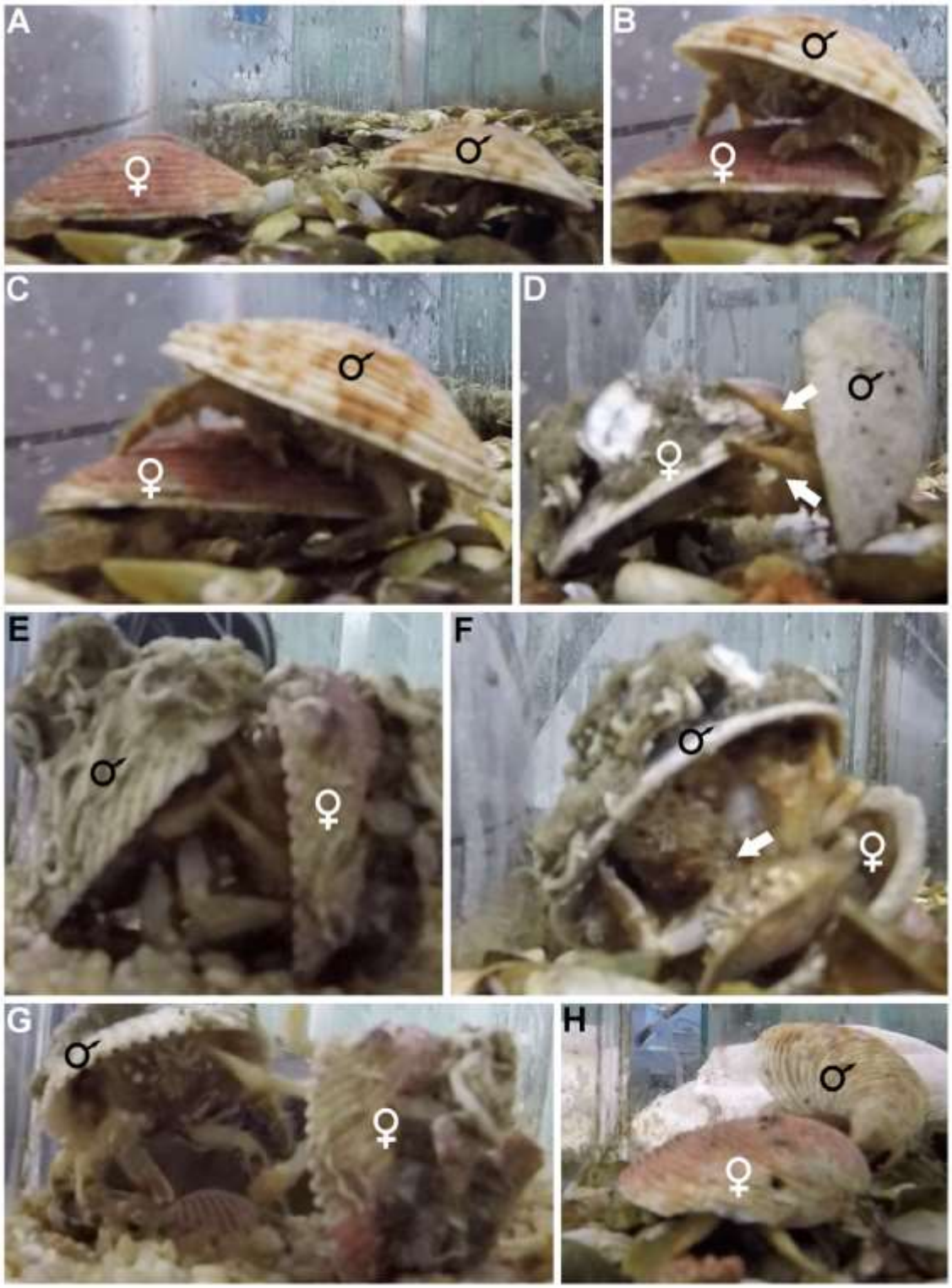


Fig. 2

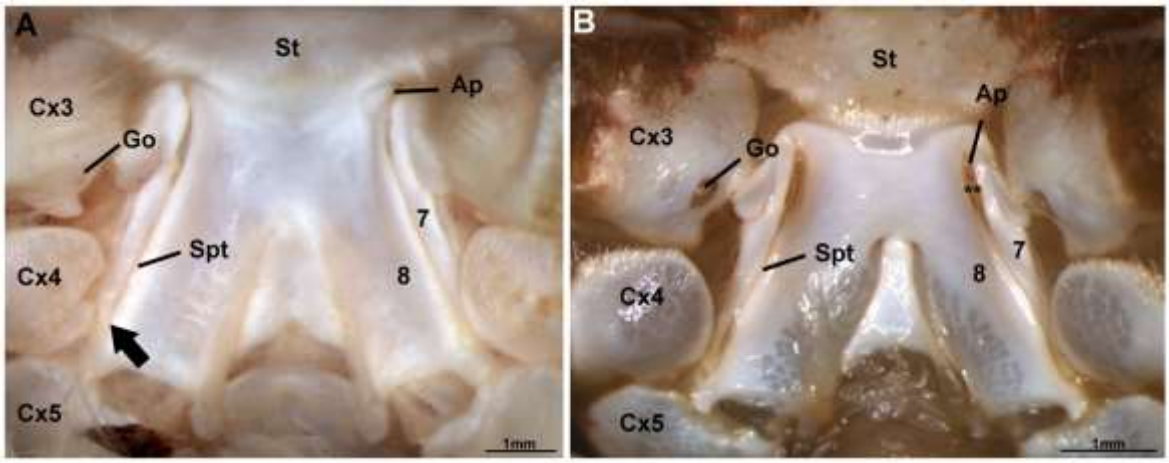


Fig. 3

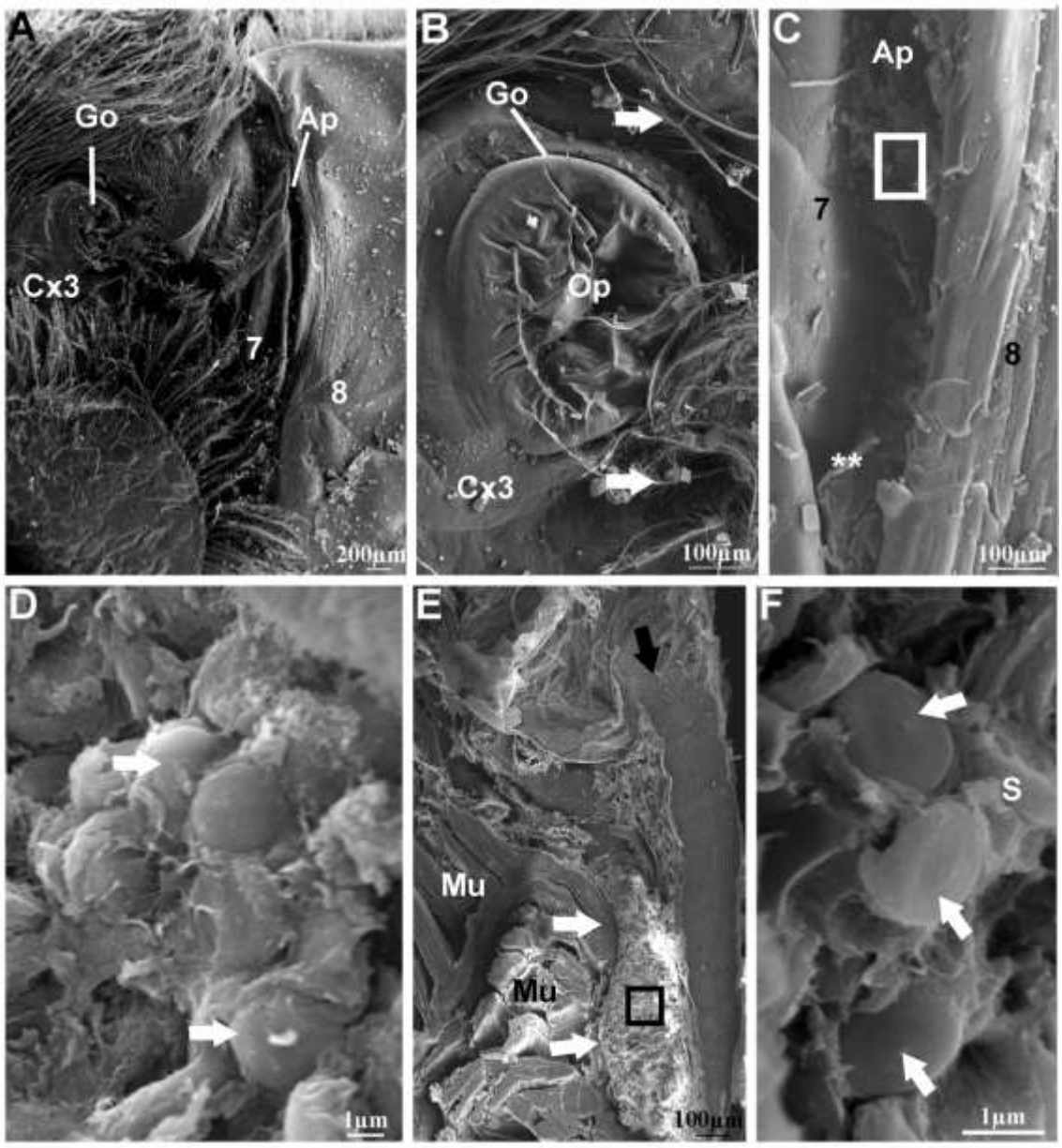

A
Go
Ap
Cx3
7
8
200µm
B
Go
Op
Cx3
100µm
C
Ap
7
8
**
100µm
D
1µm
E
Mu
Mu
100µm
F
S
1µm

Fig. 4

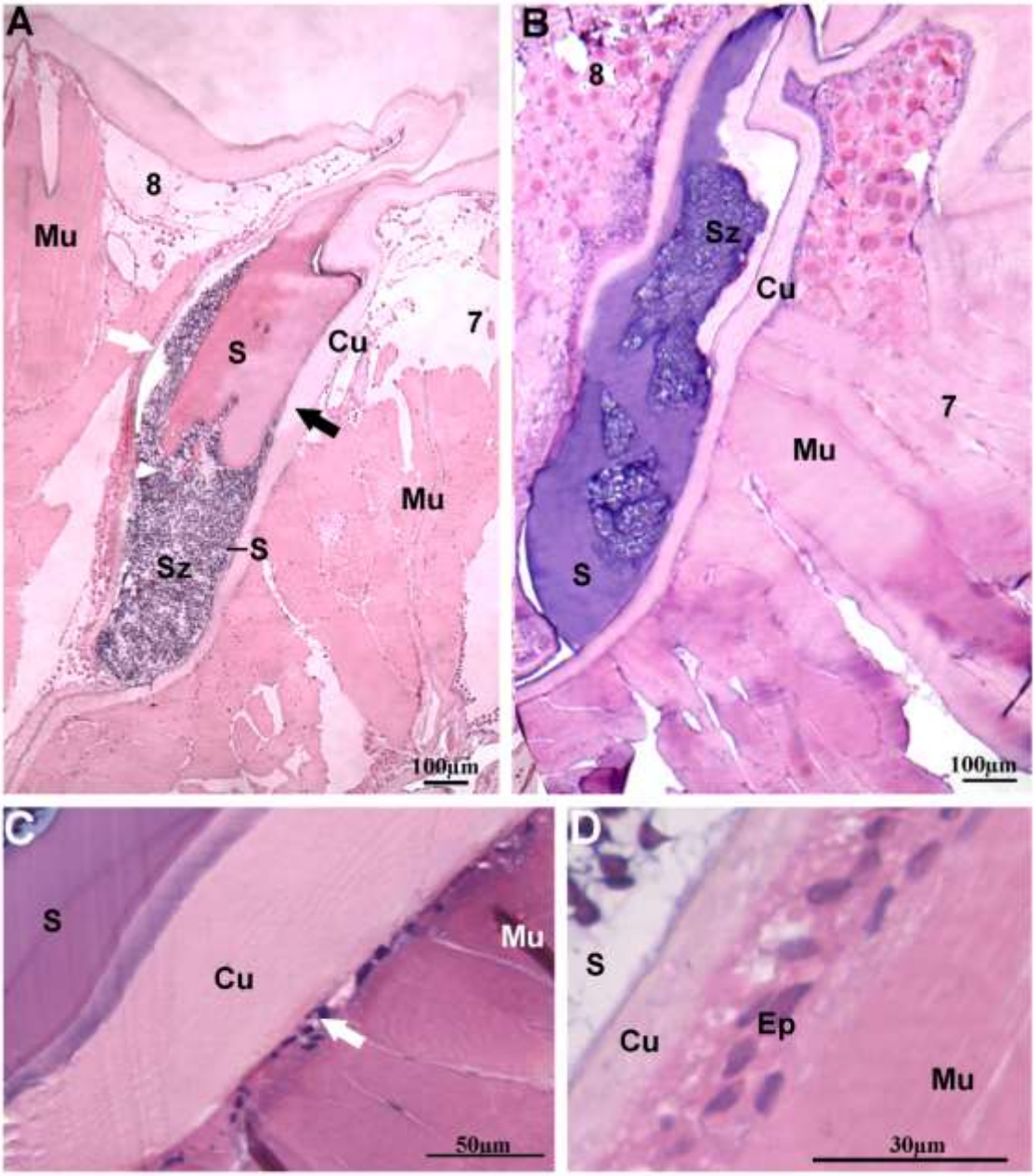

A
8
Mu
S
Cu
7
Mu
Sz
S
100µm
B
8
Sz
Cu
7
Mu
S
100µm
C
S
Cu
Mu
50µm
D
S
Cu
Ep
Mu
30µm

Fig. 5

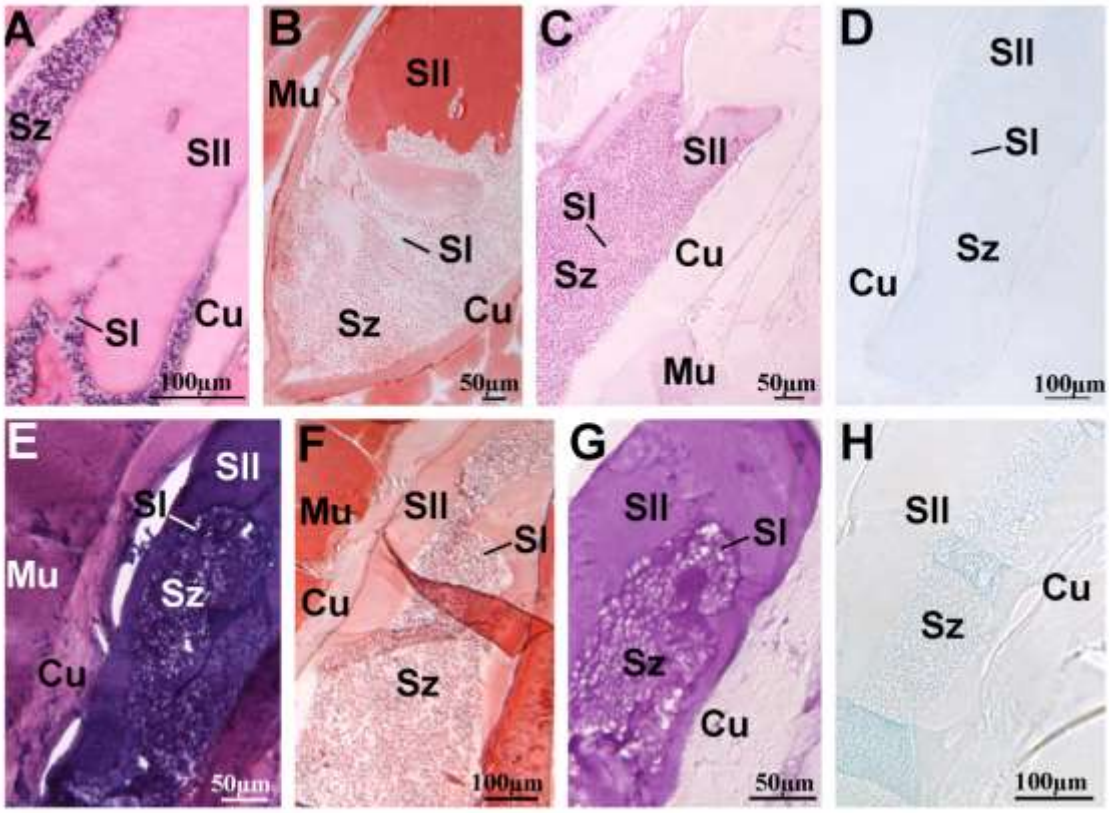
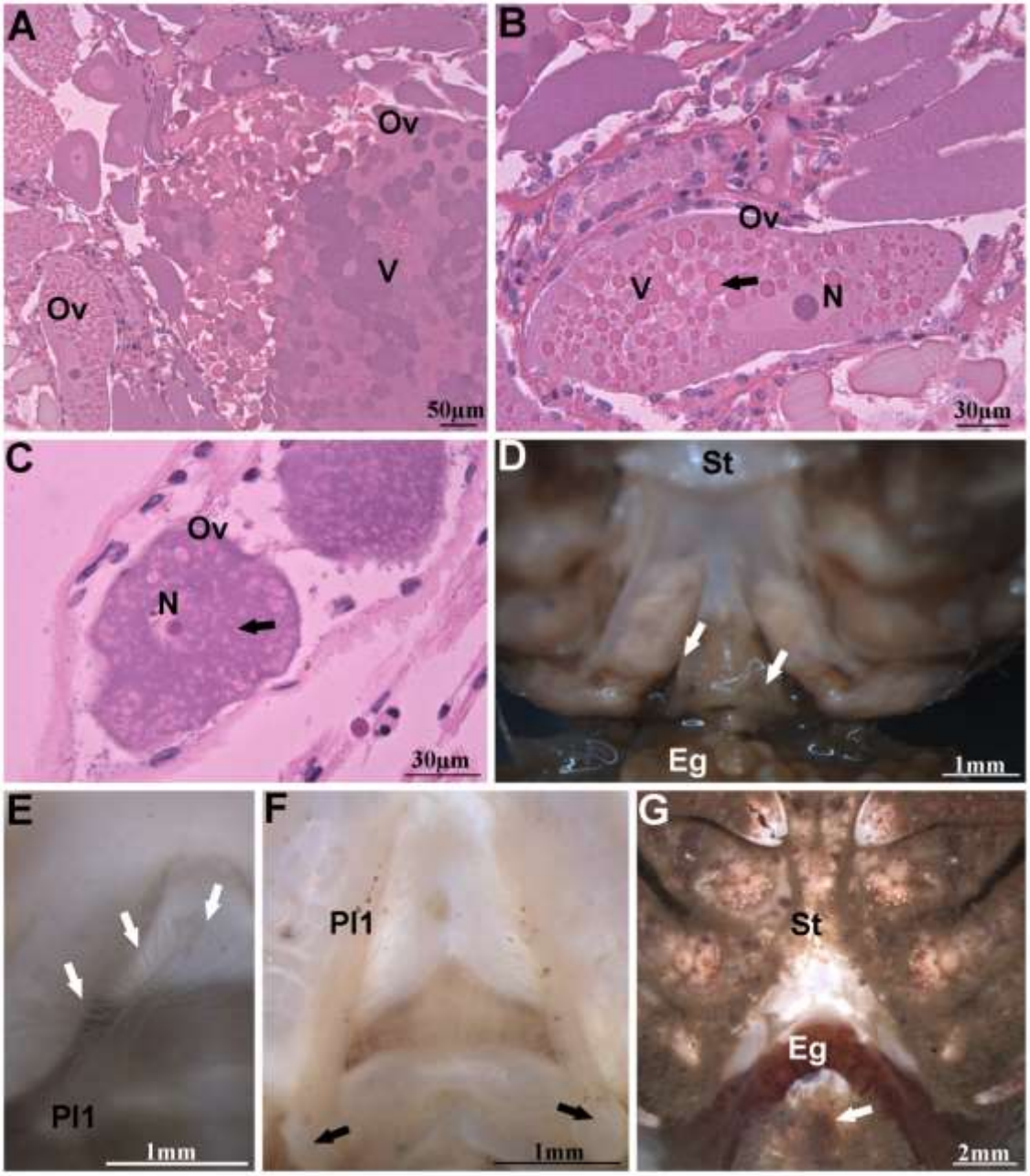

A
Sz
SII
SI
Cu
100µm
B
Mu
SII
SI
Sz
Cu
50µm
C
SII
SI
Sz
Cu
Mu
50µm
D
SII
SI
Sz
Cu
100µm
E
SII
SI
Mu
Sz
Cu
50µm
F
Mu
SII
SI
Cu
Sz
100µm
G
SII
SI
Sz
Cu
50µm
H
SII
Cu
Sz
100µm

Fig. 6


A
Ov
V
Ov
50µm
B
Ov
V
N
30µm
C
Ov
N
30µm
D
St
Eg
1mm
E
Pl1
1mm
F
Pl1
1mm
G
St
Eg
2mm

CONCLUSÃO GERAL

A análise da ultraestrutura dos espermatozoides dos Hypoconchinae *H. parasitica* e *H. arcuata* e dos Dromiinae *M. antillensis* e *D. erythropus* mostraram que não existe um padrão distinto da ultraestrutura dos espermatozóides entre os representantes de Dromioidea. Todos os caracteres espermáticos são comuns aos representantes desta superfamília, sem apresentar um caractere exclusivo para separação entre gêneros, subfamílias e entre as famílias Dromioidea. Somente caracteres específicos foram observados, sendo as seguintes estruturas: a presença do flange esférico na zona eletronlúcida anterolateral e a zona acrossomal externa granular de *H. parasitica*, a ausência da zona acrossomal raiada e a presença de resquícios de flange em *M. antillensis* e quanto às morfologias das câmaras perforatoriais, típicas para as espécies Brasileiras, aqui estudadas. Porém, quando as características da ultraestrutura dos espermatozoides dos Dromioidea é comparada com outros Podotremata, das famílias Homolidae, Latreilliidae e Raninidae, notam-se características claras que os diferem destas famílias, como vesícula acrossomal pouco discoide, protuberância apical pouco desenvolvida (Homolidae) ou ausente (Latreillidae), câmara perforatorial capitada, ausência de zona da zona acrossomal raiada, a presença de projeções operculares (Homolidae).

O padrão de produção do fluido seminal e empacotamento dos espermatozoides no vaso deferente dos Dromiidae são totalmente distintos dos Eubrachyura. Em Dromiidae não existe um espermatóforo, mas sim cordão espermático interno envolto por secreções, com maior similaridade às espécies de Astacidae.

Assim, o armazenamento do material seminal no interior da espermateca torna-se distinto do observado aos Eubrachyura, porém características intermediárias entre a espermatéca e o receptáculo seminal não foram encontradas, sendo a evolução entre os órgãos entre Podotremata e Eubrachyura ainda um mistério. A organização morfo-histológica da espermateca sugere fortemente que o processo de liberação dos espermatozoides para a fertilização ocorre por meio de ação mecânica de sua parede, por meio da musculatura, com

participação do pleópodo 1 na movimentação dos ovócitos e espermatozoides, para garantir o contato dos gametas.