Luiza de Oliveira Saad

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Orientador

A minha querida vozinha,
que sempre estará comigo,
Ruth.

“The important thing is not to stop questioning. Curiosity has its own reason for existence. One cannot help but be in awe when he contemplates the mysteries of eternity, of life, of the marvelous structure of reality. It is enough if one tries merely to comprehend a little of this mystery every day. Never lose a holy curiosity”

Albert Einstein

(Old Man's Advice to Youth: ‘Never Lose a Holy Curiosity’)

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Introdução Geral

Seleção Sexual

A teoria da seleção sexual pressupõe que características que tragam vantagens à reprodução de determinados indivíduos podem ser selecionadas durante o seu processo evolutivo (DARWIN, 1871). Tais características seriam capazes de maximizar a possibilidade de um determinado indivíduo encontrar um parceiro, copular e gerar descendentes férteis. A seleção sexual entre os indivíduos pode ocorrer de forma intrassexual ou intersexual. A primeira atua entre membros do mesmo sexo, como nas disputas entre machos pelo acesso às fêmeas; já a segunda atua entre membros de sexos diferentes, como na escolha de parceiros pelas fêmeas (HOSKEN & HOUSE, 2011; MORENO-GOMEZ et al., 2015).

A seleção sexual é composta por processos pré- e pós-copulatórios (BIRKHEAD & MØLLER, 1998). Dentre os processos pré-copulatórios, há a escolha do parceiro sexual devido à existência de ornamentos atrativos, comportamento de corte, disputa entre machos pelo acesso à fêmea, dominância de um harém, entre outros (DANIELSSON, 2001; ANDERSSON & SIMMONS, 2006; BRENNAN, 2012). Já os processos pós-copulatórios podem ser representados pela remoção física ou diluição dos espermatozoides de cópulas prévias por outros machos, armazenamento de espermatozoides de diferentes machos, depósito de espermatozoides mais próximos do sítio de fertilização, entre outros (BIRKHEAD & MØLLER, 1998). A importância dos processos pós-copulatórios é inegável, pois o sucesso na aquisição de um parceiro pode não resultar necessariamente no sucesso da fertilização dos ovos. Neste caso, os artifícios pós-copulatórios passam a atuar diretamente na seleção sexual dos indivíduos (PARKER, 1970; BIRKHEAD & MØLLER, 1998).

Dentre os processos pós-copulatórios, há a competição espermática, que consiste na competição entre os espermatozoides de dois ou mais machos pela fertilização de um dado conjunto de ovócitos (PARKER, 1970). Vários mecanismos relacionados à competição espermática são conhecidos: machos podem impossibilitar fisicamente a fêmea de estocar espermatozoides de outros machos (e.g., com inserções de plugues; FROMHAGE & SCHNEIDER, 2006); espermatozoides de um macho podem ser mais velozes e formar agregações, elevando seu sucesso reprodutivo (FISHER & HOEKSTRA, 2010); se a fêmea apresenta locais de armazenamento de espermatozoides (i.e., receptáculos seminais ou

espermatecas), a forma como os gametas masculinos são dispostos nesses órgãos pode garantir vantagem a um determinado macho em detrimento de outro (IWATA & SAKURAI, 2007); machos podem ainda apresentar mecanismos de remoção de espermatozoides estocados pela fêmea (WAAGE, 1979; ONO et al., 1989; BIRKHEAD & MØLLER, 1998; PRICE et al., 1999; BIRKHEAD, 2010).

Embora os machos apresentem uma série de estratégias associadas à competição pela geração de descendentes, a "arena" na qual ocorre a competição espermática, geralmente, é no trato reprodutivo feminino ou em outro local de fertilização no corpo da fêmea, permitindo que ela atue na escolha desses espermatozoides (WIGBY & CHAPMAN, 2004). Entretanto, apenas após a década de sessenta as fêmeas foram consideradas como influenciadoras ativas na seleção sexual (THORNHILL, 1983; EBERHARD, 1996; BIRKHEAD & MØLLER, 1998; BIRKHEAD, 2010). A "escolha críptica da fêmea" refere-se à influência da fêmea nos processos pós-copulatórios, que favorecem a utilização dos gametas de um ou mais machos em detrimento de outros, por meio de, por exemplo, interrupção prematura da cópula, dificuldade ao acesso à genitália, transporte de esperma para locais de armazenamento e/ou fertilização, incapacitação/digestão de esperma e rejeição ou remoção de plugues de acasalamento, entre outros (EBERHARD, 1994; PRICE et al., 1999; MILLER & PITNICK, 2003; WIGBY & CHAPMAN, 2004).

Com base no exposto, considera-se que a seleção sexual seja resultado da co-evolução entre os indivíduos de ambos os sexos, i.e., a seleção favoreceria atributos masculinos que aumentassem a probabilidade de gerar descendentes, as fêmeas podendo responder a esses atributos e recuperar o controle sobre a fertilização (BIRKHEAD, 2010). Entretanto, compreender esse complexo processo evolutivo depende de informações dais quais não detemos completo conhecimento, por exemplo, os detalhes morfológicos de como a genitália masculina e feminina funcionam durante a transferência de esperma, como os espermatozoides são manipulados dentro da fêmea antes da fertilização, e qual o controle do animal sobre a ovulação, oviposição e armazenamento de espermatozoides (EBERHARD, 2004).

Seleção Sexual em Cefalópodes

Moluscos da classe Cephalopoda apresentam aspectos reprodutivos peculiares e variados dentre seus representantes (ROCHA et al., 2001). Sua reprodução envolve

comportamentos de combate entre machos, corte, poligamia e até cuidado parental (HANLON & MESSENGER, 1996; HANLON, 1998). A transferência de espermatozoides é indireta, por meio de espermatóforos, os quais são transferidos pelo macho geralmente com auxílio de um braço modificado, o hectocótilo (HANLON & MESSENGER, 1996). Durante a transferência à fêmea, os espermatóforos passam por mudanças estruturais, durante o fenômeno conhecido por "reação espermatofórica", no qual suas túnicas evaginam e formam o espermatângio (i.e., espermatóforo evertido contendo a massa espermática), que se fixa ao corpo da fêmea (MARIAN, 2012a, 2012b, 2014).

Algumas espécies de lulas da família Loliginidae apresentam dois tipos de cópula: a paralela, na qual o macho (geralmente de maior porte, denominado *consort*) deposita espermatóforos dentro da cavidade do manto da fêmea, próximo à abertura do oviduto; e cópula frontal, na qual os espermatóforos são depositados próximos a um receptáculo seminal localizado na membrana peribucal (cópula efetuada geralmente por machos de menor porte denominados *sneakers*; HANLON & MESSENGER, 1996). Em ambos os casos, após a reação espermatofórica, há liberação de espermatozoides a partir dos espermatângios, que podem ser utilizados imediatamente para fertilização dos ovócitos se a fêmea estiver em fase de postura de ovos (MARIAN, 2014). Espermatozoides oriundos de espermatângios depositados na membrana peribucal podem ainda ser armazenados no receptáculo seminal (MARIAN, 2014). Em Decapodiformes (i.e., lulas e sépias), o receptáculo seminal é uma invaginação da membrana peribucal, geralmente ventral à boca, circundada por musculatura circular e secretora de uma substância que mantém os espermatozoides em estado inativo (DREW, 1911; OORDT, 1938).

Dessa forma, no caso de loliginídeos, a fertilização dos ovócitos pode ocorrer por espermatozoides provenientes de três sítios diferentes: próximo à abertura do oviduto, onde espermatozoides de machos *consort* estão presentes; na membrana peribucal, pelos espermatozoides liberados dos espermatângios de *sneakers*; e pelos espermatozoides de *sneakers* armazenados no receptáculo seminal (HANLON & MESSENGER, 1996). Como as fêmeas copulam com vários parceiros durante o período reprodutivo, a competição espermática parece exercer uma pressão importante nessas espécies. Dentre os mecanismos possivelmente associados à competição espermática em lulas, destacam-se aqueles documentados para a espécie *Doryteuthis bleekeri*, na qual foram observadas diferenças morfológicas, de comportamento e de longevidade entre espermatóforos e espermatozoides de *sneakers* e *consorts* (IWATA, et al., 2011; IWATA et al., 2014; HIROHASHI et al.,

2016).

O número e a localização dos receptáculos seminais variam dentre as famílias de Decapodiformes. Especificamente, Loliginidae, Sepiidae, Spirulidae, Idiosepiidae e Bathyteuthidae possuem apenas um receptáculo localizado na região ventral da membrana peribucal (MARIAN, 2014) 2014), ao passo que Ommastrephidae e Thysanoteuthidae apresentam vários receptáculos distribuídos por toda a margem dessa membrana (IKEDA et al., 1993; NIGMATULLIN et al., 1995). A hipótese mais recente sobre o surgimento dos receptáculo seminais, baseada em otimizações de caracteres reprodutivos sobre topologias resultantes de recentes análises filogenéticas, sugere que o órgão surgiu ao menos duas vezes independentemente na evolução de Decapodiformes. Entretanto, o grande número de topologias disponíveis na literatura não permitiu uma resposta única e a grande quantidade de dados faltantes na matriz de caracteres morfológicos utilizada resultou em otimizações ambíguas para uma série de caracteres (MARIAN, 2014).

Existem apenas alguns estudos que examinaram a morfologia e histologia do receptáculo seminal de espécies de Decapodiformes, como *Bathyteuthis berryi* (BUSH et al., 2012), *Idiosepius paradoxus* (SATO et al., 2010), *Sepia officinalis* (HANLON et al., 1999), *Sepia apama* (NAUD et al., 2005), *Todarodes pacificus* (IKEDA et al. 1993), *Doryteuthis pealeii* (WILLIAMS, 1909; DREW, 1911), *Loligo forbesi* (LUM-KONG, 1992) e *Loligo vulgaris* (OORDT, 1938). Entretanto nenhum deles descreveu detalhadamente a estrutura desse órgão (e.g., constituição das células glandulares, organização espacial da musculatura). Consequentemente, a anatomia funcional do receptáculo seminal e possíveis mecanismos de captação de espermatozoides pelo órgão são pouco compreendidos. Sabe-se que, após a reação espermatofórica, os espermatozoides são liberados para o meio externo, porém a forma pela qual esses gametas adentram o receptáculo seminal é desconhecida. Com base em *Bathyteuthis berryi* (Bathyteuthidae), BUSH et al. (2012) sugeriram que a massa bucal da fêmea teria alguma participação na manipulação do espermatângio e, consequentemente, na transferência de esperma para dentro do receptáculo, embora não tenham apresentado evidências sólidas para esse cenário. HANLON et al. (1999), investigando a cópula de *Sepia officinalis* (Sepiidae), sugeriram que a ação do hectocótilo poderia auxiliar a entrada dos espermatozoides no receptáculo, mas concluíram que mais estudos seriam necessários para compreender esse processo. SATO et al. (2010) sugeriram que os espermatozoides poderiam nadar ativamente para dentro do receptáculo seminal em *Idiosepius paradoxos* (Idiosepiidae). Por meio de experimentação *in vitro*, OORDT (1938) demonstrou que não há atração química

oriunda do receptáculo de *Loligo vulgaris* (Loliginidae) e levantou a hipótese de que a ação da musculatura associada ao receptáculo poderia prover sucção dos espermatozoides liberados próximos ao órgão.

Apesar de seu papel central em processos pós-copulatórios de seleção sexual, o funcionamento do trato reprodutor feminino ainda é pouco compreendido em diversas espécies (MILLER & PITNICK, 2003). No caso de cefalópodes, os processos de captação, armazenamento e liberação de espermatozoides são desconhecidos. Consequentemente, mecanismos de escolha críptica da fêmea não foram documentados nesse grupo, embora haja evidências indiretas (SHASHAR & HANLON, 2013; NAUD et al., 2016). A ausência de elementos básicos sobre a morfologia funcional dos receptáculos seminais dificulta a proposição de hipóteses sobre mecanismos de seleção sexual que possam ser efetivamente testadas experimentalmente. Dessa forma, se faz necessário reunir elementos da morfologia que possam elucidar o funcionamento desses órgãos.

Objetivos

Esta dissertação teve como objetivo geral investigar a estrutura e função do receptáculo seminal de *Doryteuthis plei*, espécie de lula adotada como modelo, como base para compreensão de mecanismos pós-copulatórios de seleção sexual em cefalópodes. Mais especificamente, o trabalho procurou atingir as seguintes metas:

• Analisar a morfologia do receptáculo seminal com base em microscopia integrativa (microscopia óptica com emprego de técnicas histoquímicas, microscopia eletrônica de varredura e de transmissão, microscopia confocal e microCT), visando a uma caracterização detalhada da estrutura do órgão, incluindo suas células secretoras e os sistemas muscular e nervoso associados;

• Analisar a morfologia e histologia/histoquímica do receptáculo seminal em três situações experimentais distintas: (1) antes de cópula recente, (2) após cópula recente (e antes da desova) e (3) após a desova, para investigar alterações morfológicas no órgão (i.e., conformação interna, modificações na musculatura e aspecto das células glandulares) e volume de espermatozoides, como base para compreensão dos mecanismos de captação, armazenamento e liberação de gametas masculinos pela fêmea.

Organização da Tese

Além desta "Introdução Geral" e das "Considerações Finais", a Dissertação está organizada em dois capítulos estruturados como artigos científicos e redigidos em inglês. As respectivas referências, tabelas e figuras estão apresentadas independentemente ao final de cada capítulo.

O primeiro capitulo, intitulado “Functional morphology of the seminal receptacle indicates cryptic female choice in the squid *Doryteuthis plei* (Blainville, 1823) (Cephalopoda: Loliginidae)”, reúne as informações obtidas a partir de microscopia integrativa, apresentando uma descrição detalhada da estrutura do receptáculo seminal. Além disso, o manuscrito documenta os resultados das manipulações experimentais e as respectivas análises histológicas.

O segundo capítulo, intitulado “A mating plug in a squid? Sneaker spermatophores blocking the female seminal receptacle in *Doryteuthis plei* (Blainville, 1823)”, apresenta evidências do primeiro registro de plugues copulatórios em lulas, que foram descobertos durante a investigação morfológica realizada no capítulo anterior.

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Capítulo 1

Functional morphology of the seminal receptacle indicates cryptic female choice in the squid *Doryteuthis plei* (Blainville, 1823) (Cephalopoda: Loliginidae)

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Abstract

Sexual selection exerts a significant pressure on the evolution of reproductive attributes. Males show a diverse array of strategies to gain advantage in mating and fertilization success, but females also play a crucial role in pre- and post-copulatory sexual selection processes. Within this context, cephalopod mollusks show peculiar reproductive strategies, including sperm transfer via spermatophores, and the presence of female sperm-storage organs (seminal receptacles). However, the knowledge of the functioning of the cephalopod seminal receptacles is scarce, the mechanisms involved with sperm uptake, storage and release being unknown. To shed light on post-copulatory mechanisms in cephalopods, the present study aimed at investigating the structure and function of the seminal receptacle of the squid *Doryteuthis plei*. The morphology of the seminal receptacle was thoroughly analyzed applying integrative microscopy (light microscopy including histochemical techniques, scanning & transmission electron microscopy, confocal microscpy and microCT). Moreover, to investigate morphological and sperm volume changes associated with possible mechanisms of sperm uptake, storage and release by the organ, the morphology, histology and histochemistry of the seminal receptacle was also analyzed under three distinct experimental manipulations: (1) before recent mating (2) after recent mating and (3) after egg release. The results show a complex and striking diversity of secretory cells and associated muscle fibers in the seminal receptacle. There were changes in the composition and

predominance of secretory cells between major reproductive events, suggesting a secretory activity associated with mating and spawning. The possible roles of these secretions in sperm uptake, storage and release are discussed in light of our data. Moreover, the structure of the nervous and muscular systems associated with the receptacle suggests that females have control over precise deformations of the organ, possibly related to sperm uptake and release. Putative post-copulatory sexual selection mechanisms in squids are discussed in light of these data.
Key Words - sperm storage, sexual conflict, sperm transfer mechanisms, decapodiforms.

Introduction

Sexual selection is composed of pre-copulatory (e.g., courtship behavior and fighting among males to gain access to females) and post-copulatory processes (e.g., physical removal or dilution of sperm from previous mating by rival males) (Parker, 1970; Birkhead and Møller, 1998; Birkhead 2010; Hosken and House, 2011). Sperm competition is a type of post-copulatory process occurring whenever the sperm of two or more males compete for fertilization of a given set of eggs (Parker, 1970). Although males present a series of strategies associated with sperm competition, the site where this competition occurs (i.e., the female reproductive tract or elsewhere in or on the female's body) allows the female the possibility of actively selecting these sperm (Wigby and Chapman, 2004). Therefore, another type of post-copulatory process is cryptic female choice, which occurs whenever the female actively biases the fertilization success of males, favoring the selection or differential use of sperm of a particular male (Eberhard, 1994). Cryptic female choice might occur through, for example, premature mating termination, impeding male access to female genitalia, selective transport of sperm to storage or fertilization sites, sperm digestion, and removal of mating plugs (Eberhard, 1994; Prince et al., 1999; Pizzari and Birkhead, 2000; Miller and Pitnick, 2003; Wigby and Chapman, 2004; Kamimura, 2013). The morphology of the female body (specially the reproductive tract) is also regarded as intimately associated with the evolution of male sperm competition strategies (Keller and Reeve, 1995; Eberhard, 1996; Birkhead and Møller, 1998; Miller and Pitnick, 2003; Briceño and Eberhard, 2009; Birkhead, 2010). Therefore, the understanding of post-copulatory sexual selection ultimately requires details of the functional morphology of both male and female genitalia during and after sperm transfer (Eberhard, 2004).

Cephalopod mollusks have unique and quite variable reproductive strategies (Rocha et al., 2001). They are usually polygamous, and complex behaviors, such courtship, male agonistic contests, and parental care, have been widely documented in many of its representatives (Hanlon and Messenger, 1996; Hanlon, 1998; Bush et al., 2012; Shashar and Hanlon, 2013). Sperm transfer is indirect via spermatophores, which are transferred by the male generally with the aid of a modified arm called hectocotylus (Hanlon and Messenger, 1996). During copulation, spermatophores produced by male cephalopods undergo the spermatophoric reaction, a complex process of evagination that culminates in the autonomous attachment of the spermatangium (everted spermatophore containing the sperm mass) on the female's body (Marian, 2012a; 2012b; 2014).

Some squids of the family Loliginidae have two possible mating positions: "male-parallel", in which large (consort) males transfer spermatophores into the female's mantle cavity close to the opening of the oviduct; and "head-to-head", in which small (sneaker) males deposit spermatophores near a seminal receptacle located in the buccal membrane (Hanlon and Messenger 1996; Hanlon, 1998). After the spermatophoric reaction, spermatozoa are released from the spermatangium, and sperm can immediately be used for fertilization if the oocytes are released by the female (Marian, 2014). However, sperm coming from spermatangia deposited on the buccal membrane might also be stored within the seminal receptacle (Marian, 2014).

The fact is, spermatophores are deposited by the male on the female buccal membrane, but outside the seminal receptacle (Hanlon and Messenger, 1996; Marian, 2014). Consequently, after the spermatophoric reaction, sperm are released to the external environment, and how exactly the sperm get into the seminal receptacle is unknown (Hanlon et al., 1999; Sato et al., 2010; Bush et al., 2012). The squid seminal receptacle has been described as an invagination of the ventral buccal membrane, surrounded by circular muscles and provided with secretory cells that release a content that keeps the sperm immotile (Drew, 1911; Oordt, 1938). Only a few decapodiform cephalopods (i.e., squids and cuttlefishes) have been examined concerning the morphology of the seminal receptacle (e.g., *Bathyteuthis berryi*, Bush et al., 2012; *Doryteuthis pealeii*, Williams, 1909, Drew, 1911; *Idiosepius paradoxus*; Sato et al., 2010; *Loligo forbesi*, Lum-Kong, 1992; *Loligo vulgaris*, Oordt, 1938; *Sepia officinalis*, Hanlon et al., 1999; *Sepia apama*, Naud et al., 2005; *Todarodes pacificus*, Ikeda et al. 1993). However, most of these studies briefly describe the organ using basic histological techniques, and no detailed information on its structure is available (e.g., nature

and formation of glandular cells). Several key questions about squid sperm transfer dynamics remains, therefore, unknown.

Despite its central role in post-copulatory sexual selection processes, the functioning of the female reproductive tract is poorly understood in several species (Miller and Pitnick, 2003), and squids are no exception. Therefore, this study aimed at thoroughly investigating the morphology of the seminal receptacle of the squid *Doryteuthis plei*, applying several microscopy techniques combined with experimental manipulations, to shed some light on its putative roles in uptake, storage and release of sperm.

Material and methods

Animals

During the summers months of 2014-2016, mature specimens of *Doryteuthis plei* were captured using hand-jigging off the northern coast of São Sebastião Island (Ilhabela municipality, São Paulo state, Brazil), between 23º44'00'' S and 45º17'50'' W. Captured specimens were maintained alive using the boat's live bait tank (ca. 80 l) and transported to the Marine Biology Center of the University of São Paulo (CEBIMar-USP, São Paulo, Brazil), where animals were transferred to 500 l tanks supplied with a continuous flow of fresh seawater. Squids were fed *ad libitum* with dead shrimps once a day until the moment they were used for the experiments. We followed the methods of collection, transportation and temporary maintenance of living specimens recommended elsewhere (Moltschaniwskyj et al., 2007) Specimens were collected under SISBIO/ICMBio permit #44738. Before dissection, female specimens were over-anesthetized using an isotonic solution of $MgCl_2$ (Messenger et al., 1985).

Light microscopy

The seminal receptacles of 10 females were dissected and fixed either in 4% paraformaldehyde (in PBS 0.1 M, pH 7.2) for 8 h at 4°C, or in a modified Karnovsky's fixative (2% paraformaldehyde, 2.5% glutaraldehyde in cacodylate buffer 0.1 M, pH 7.4, $CaCl_2$ 2.5 mM, adjusted to 1000 mOsm with sucrose) for 4 h at 4°C (Marian, 2012a). The material was then dehydrated and embedded in glycol methacrylate resin (Leica Historesin Embedding Kit, Leica Microsystems Nussloch GmbH, Germany), following the manufacturer's protocol. Serial transverse and sagittal 3-5 µm sections were subjected to the

following staining procedures (according to Humanson, 1962; Bancroft and Stevens, 1982; Pearse 1985): Hematoxylin-Eosin (H&E – general stain), Toluidine Blue (TB – general stain; sometimes counterstained with basic fuchsin), Periodic Acid–Schiff (PAS – identification of polysaccharides), Alcian Blue at pH 2.5 (AB – identification of acidic polysaccharides), Mercury-Bromophenol Blue (BB – identification of basic proteins); a combination of the AB and PAS techniques (AB-PAS – differentiation of acidic and neutral polysaccharides); and Gömöri Trichrome (GT – to evidence muscle fibers). To obtain control sections for the Periodic Acid–Schiff test, the Schiff's reagent was also applied without pretreatment with periodic acid. The reverse combination of PAS and AB was also tested (first and second stains, respectively; Yamabayashi, 1987). Images were obtained using a Carl Zeiss Axio Imager M2 microscope (Carl Zeiss, Oberkochen, Germany).

Scanning and transmission electron microscopy

For scanning electron microscopy (SEM), 5 samples were fixed in Karnovsky's fixative (see above). Post-fixation was performed for 30 min in 1% OsO_4, followed by 15 min in 1% tannic acid in buffer solution and additional 15 min in fresh solution of 1% OsO_4 at 4 °C. After dehydration in a graded ethanol series, the samples were critical point dried, mounted on stubs, coated with gold and examined in a Zeiss DSM 940 (Carl Zeiss, Oberkochen, Germany).

For transmission electron microscopy (TEM), 15 seminal receptacles were fixed in Karnovsky's fixative (see above), washed in buffer solutions, post-fixed for 1h at room temperature in 1% OsO_4 in buffer solution, dehydrated in a graded ethanol series and embedded in Epoxy resin (Leica Historesin Kit, Germany). Ultrathin sections (50–70 nm) were obtained using a Leica Ultracut UCT microtome (Leica, Illinois, USA). Ultrathin sections were mounted on copper slot-grids, and contrasted with uranyl acetate and lead citrate. The slot-grids were examined using a Zeiss EM 900 transmission electron microscope (Carl Zeiss, Oberkochen, Germany). Images were captured using the F-View Soft Imaging System (Soft Imaging System GmbH, Munster, Germany).

Additionally, 3 seminal receptacles were fixed in 4 % paraformaldehyde in 0.1M phosphate buffer (PB) for 1 h, followed by four rinses in the buffer solution and were stored in 0.1 M PB containing 0.1 % NaN_3 at 4 °C. Then, for post-fixation, the samples were treated with 1% OsO_4 in PB for 1 hour at room temperature. Dehydration was performed via acidified dimethoxypropane, and embedding in Agar Low Viscosity Resin (Agar Scientific,

Stansted, Essex, UK). Ultrathin (60 nm) sections were generated using a Leica UC6 ultramicrotome (Leica Microsystems, Wetzlar, Germany). Ultrathin sections were collected on mesh copper grids and contrasted with uranyl acetate and lead citrate. The slot-grids were examined in a Zeiss Libra 120 transmission electron microscope (Carl Zeiss AG, Oberkochen, Germany). Images were captured using the F-View Soft Imaging System (Soft Imaging System GmbH, Munster, Germany).

Microscopic X-ray computed tomography (microCT)

Five seminal receptacles were fixed in 4% paraformaldehyde in 0.1M PB at room temperature for 2h and stored in PB with 0.1% NaN_3 at 4 ºC. Then, for post-fixation, the samples were treated with 1% OsO_4 in PB for 1 hour at room temperature. Dehydration was performed via acidified dimethoxypropane and embedding in Agar Low Viscosity Resin. Resin blocks were mounted vertically and scanned in air. MicroCT scans were acquired with an Xradia microXCT-400 (Carl Zeiss X-ray Microscopy, Pleasanton, CA) using the 4× lens. Samples were scanned at 40kV source voltage and 200µA intensity. Projections were recorded with 40s exposure time (camera binning = 1) and an angular increment of 0.16667°; reconstructed slices measured 2028x2028 pixel. Isotropic voxel resolution of reconstructed volumes was 1.75µm. Image stacks were exported in *.dcm (DICOM) format and imported into Amira 6 (FEI Visualization Sciences Group, Mérignac Cédex, France). The Amira 6 software allowed the combination of different standard visualization devices for viewing volume data, polygonal surfaces and individual slices, resulting in a 3D reconstruction of the seminal receptacle structure.

Confocal laser scanning microscopy

Samples of 14 seminal receptacles were fixed in 4% paraformaldehyde in 0.1M phosphate buffer (PB) at room temperature (RT) for 2h and stored in PB with 0.1% NaN_3 at 4 ºC. At the Department of Integrative Zoology (U. Vienna, Austria), these samples were partially dissected, preserving the tissue surrounding the organ, and embedded in a gelatine-ovalbumine solution and stored in PBS with 10% formaldehyde for 4h up to 15h at 4 ºC. Subsequently, samples were sectioned into 100-300µm thick sections using a vibratome (VT1000S, Leica Microsystems, Wetzlar, Germany). Sections of the seminal receptacle were washed six times in PBS with 2-10% Triton X-100, added to increase tissue permeability. Samples were blocked for 4h up to 15h in PBT with 5% normal swine serum (NSS; Jackson

ImmunoResearch, West Grove, PA, USA). For α-tubulin staining, sections were incubated in a 1:1000 dilution of a primary antibody against acetylated α-tubulin (Sigma) for 24h. For neuronal staining, sections were incubated in a 1:500 dilution of a primary antibody against FMRFamide (Immunoestar) or anti-Serotonin (Immunoestar) for 15h. Then the samples were rinsed six times in PB with 0.1% Triton X-100, and after this step secondary fluorochrome-coupled antibodies were diluted in a blocking solution with 1% NSS and applied at a 1:800 (for acetylated α-tubulin visualization: Alexa Fluor 633) or a 1:1200 (for FMRFamide or serotonin visualization: Alexa Fluor 568) dilution for 15h. For nucleic acid staining, 0.5% Hoechst (Sigma) was added and the samples were incubated for 12h at room temperature in the dark. For F-actin staining, the sections were incubated in a 4% dilution of Alexa Fluor 488 phalloidin (Invitrogen) in PBT for 12h at room temperature in the dark. Combinations of different labeling were performed in order to obtain diverse staining in the same sample. Subsequently, sections were washed six times for 20 min each in PBS, and then mounted in Fluoromount G (Southern Biotech, Birmingham, Alabama, USA) on glass slides. All preparations were examined with a Leica DM IRBE microscope equipped with a Leica TCS SP2 confocal unit (Leica Microsystems, Wetzlar, Germany). Optical sections with a Z-step size of 1 μm were generated and digitally merged to yield maximum projection images. The confocal laser scanning microscopy methodology was adapted from Wollesen et al. (2009).

Experiments

Females (n = 30; ML 100-180mm) were divided into three experimental groups. In Group 1 (“before mating”; n = 10; ML 140-180mm), females were isolated in a tank without males for at least 48 h after capture, and then processed for histological analysis (see below). In Group 2 (“after recent mating”; n = 10; ML 100-166mm), females were first isolated in a tank without males for at least 24 hours after capture. Then, they were each isolated in a tank with 2 to 4 males of different sizes (sneakers and consorts) to stimulate head-to-head mating by sneaker males (Shashar and Hanlon, 2013). They were then observed until head-to-head mating had occurred (from 16 min up to 5 hours). After 30 min of the end of head-to-head mating, the female was removed from the tank and processed for histological analysis. No male-parallel mating had occurred in this group. In Group 3 (“after spawning”; n = 10; ML 120-165mm), females were first isolated in a tank without males for at least 24 hours after capture. Then, each female was isolated in a tank until spawning. Spawning was stimulated by either placing consort males (up to two) or egg strings from other females inside the tank

(Buresch et al., 2009). The female was removed from the tank and processed for histological analysis from 30 min (when spawning was followed by an observer) to ca. 10 h after spawning (when spawning occurred at night, with no observer). Male-parallel mating occurred in 5 cases. All eggs strings were checked for the occurrence of fertilization.

After the end of the experiments, all females were over-anesthetized as described above, and their seminal receptacles were dissected and fixed in the modified Karnovsky's fixative (see above). The samples were then analyzed regarding number of spermatangia attached on the buccal membrane, and aspect of these spermatangia (empty, full, or fresh, i.e., with a large aggregated mass of sperm released from the spermatangia tips). Then, the seminal receptacles were processed for histology as described above, the resulting sagittal 3µm sections being stained with HE or AB-PAS. Digital images were captured using Carl Zeiss Axio Imager.M2 microscope (Carl Zeiss, Germany). To calculate the seminal receptacle volume, we first captured the images of the sagittal sections in every 45 µm; the area of sperm heads in each image was then calculated using ImageJ software, and the total volume of sperm was estimated by multiplying the total sperm area (sum of the area of the captured images) by the 45 µm thickness (adapted from Sato et al., 2010). The volume of sperm was compared between the experimental groups using one-way ANOVA followed by Tukey's test at a significance level of $p < 0.05$.

Results

Structure of the seminal receptacle

The seminal receptacle of *Doryteuthis plei* is located at the ventral region of the buccal membrane, between left and right ventral arms (Fig. 1A). It is a small (ca. 1.5 mm in height and 3 mm in length) kidney-shaped organ with a pronounced opening located anteriorly (Fig. 1B). The buccal membrane that surrounds the seminal receptacle forms a depression around the organ (Fig. 1B). Attached spermatangia were found near the seminal receptacle opening and over the entire ventral buccal membrane, but mainly concentrated at the depression around the organ (Fig. 1C,D). These spermatangia have a typical sneaker club-like morphology (Marian 2012a; Apostólico and Marian, submitted), i.e., with a wider base and gradually tapering to its tip, from where spermatozoa are released (Fig. 1C). Spermatangia with different aspects (i.e., empty, full and fresh) could be found attached on the same sample, possibly corresponding to distinct mating events. Full and empty

spermatangia are whitish and translucent, respectively (Fig. 1C). Fresh spermatangia are also whitish but present a large aggregated mass of sperm that obscures the seminal receptacle opening (Fig. 1D).

The seminal receptacle is an invagination of the buccal membrane epithelium, and is surrounded by the buccal membrane's connective tissue (Fig. 2A,B,D). The organ is divided into several bulbs (Fig. 2A-D), each bulb bearing several subdivisions (Fig. 2A-C). The bulbs are interconnected at some point, all of them converging to a common duct, which leads to the opening and connects the seminal receptacle to the exterior (Fig. 2A-D). Spermatozoa are stored mainly within the lumen of the bulbs, but also within the common duct (Fig. 2A,B); the seminal receptacle is usually not completely filled with spermatozoa. The internal volume of the seminal receptacle analyzed under microCT was 1.28 mm^3 (Fig. 2C). Muscle fibers and blood vessels are interspersed within the connective tissue surrounding the seminal receptacle (Fig 3C).

At the region of the seminal receptacle, the buccal membrane is covered by a thick cuticle (Fig. 2B,D). The cuticle extends over the whole buccal membrane depression (Fig. 3A), reaching the seminal receptacle opening, and entering a few micrometers into the common duct (Fig. 3B). The cuticle is divided into several parallel layers, indicating that it is possibly secreted intermittently (Fig. 3C). The bases of the spermatangia found on the buccal membrane are implanted into the cuticle layers, often causing ruptures (Fig. 3A,C). Spermatangia are sometimes attached to other portions of the buccal membrane devoid of a cuticle, resulting in injuries to the epithelium and underlying connective tissue; nevertheless, they are far more abundant at the cuticle (Fig. 2B; 3A). The cuticle is secreted by the buccal membrane's simple columnar epithelium, which has cells with oval, darkly stained and basal nuclei (Fig. 3C), being covered by microvilli (Fig. 3D). Small electron-lucent double-membrane vesicles are apparently involved in the secretion of the cuticle (Fig. 3D,E).

At the region devoid of a cuticle (i.e., outside the buccal membrane depression), the buccal membrane epithelium projects into folds and bear numerous secretory cells (Fig. 3A; 4A-D). The secretions from the majority of these cells showed a strong positive reaction for AB (blue; Fig. 4B), PAS (magenta; Fig. 4C) and for the combination of AB-PAS (purple; Fig. 4A,D), but they all showed negative reaction to BB and H&E. Cells with only AB-positive or only PAS-positive secretions were rare (Fig. 4A,D). When the reverse combination of PAS-AB was applied, there were no major differences in the general affinities for the stains.

Within the seminal receptacle bulbs, sperm are usually organized with their heads facing the bulb epithelium (Fig. 5A,B), although sometimes they do not present any clear pattern of organization (Fig. 5C). The bulbs are lined with columnar to cuboidal epithelial cells with round nuclei (Fig. 5A-C). Large secretory cells are rarely found interspersed within the bulb epithelium; their content is strongly PAS-positive (Fig. 5B) and AB-negative, with fine acidophilic granules (Fig. 5C). These cells probably secrete the PAS-positive matrix in which the sperm are immersed, given that strongly PAS-positive droplets are sometimes found within this matrix (Fig. 5A,B). The bulb epithelium may bear cilia and microvilli (5D,E) or just microvilli on its surface (Fig. 5F).

The epithelium lining the common duct and the region of the bulbs converging to the common duct is provided with a much larger amount of secretory cells (Fig. 6A,B). In general, the secretions from these cells showed strong positive reaction for AB (blue), PAS (magenta) or for the combination of both AB and PAS (purple) (Fig. 6B), but none for BB (Fig. 6C) or for H&E (Fig 6A). The secretions from some of these cells are apparently constantly released into the lumen of the common duct (Fig. 6B). Differently from the secretory cells of the outer epithelium, when the reverse combination of PAS-AB was applied to the inner epithelium, there was a predominance of AB-positive cells, indicating a very distinct chemical nature for these cells.

The secretory cells from the common duct differed in the affinity for the applied stains as well as in the texture of their content. For example, some cells had very fine and disperse granules located mainly at the apical region and showing strong reactivity to PAS (magenta) but none to AB (Fig. 6D,F); these cells are hereafter called “PAS-fine-secretory cells”. The remaining cells had medium to coarse round granules, and stained either with AB-only (blue), PAS-only (magenta) or AB-PAS (purple) (Fig. 6D-F). Interestingly, AB-PAS cells with medium granules might be homogeneously stained with a strong purple color (Fig. 6E), or the lower half might be stained with blue (AB) and the upper half with purple (AB-PAS) (Fig. 6F).

Ultrastructural analyses suggest at least three major groups of secretory cells: (I) cells with fine (<1 µm) electron-lucent granules located mainly at the apical region of the cell (Fig 7A), most probably corresponding to PAS-fine-secretory cells identified under light microscopy; (II) cells with medium (1 µm to 2µm; Fig 7B,D) to coarse (2µm to 3µm; Fig. 7C) electron-lucent granules, each granule containing very thin and elongated electron-dense marks (Fig. 7B-D), sometimes with round electron-dense elements (Fig. 7D); (III) cells with

medium (1 µm to 2µm; Fig 7E) to coarse (2µm to 3µm; Fig. 7F) electron-lucent granules, without the conspicuous marks of group II, but sometimes with varying electron density (Fig. 7F).

Associated musculature

Light and confocal microscopy indicates that the muscle system is divided into muscle fibers associated with the ventral buccal membrane, and muscle fibers directly associated with the seminal receptacle. The dense musculature of the buccal membrane is composed of numerous muscle fibers interspersed within the connective tissue (Fig. 8A). The musculature is denser at the region where the buccal membrane secretes the cuticle than at the region devoid of the cuticle (Fig. 8B). Three major types of fibers compose the bulk of the buccal membrane musculature: transverse, longitudinal and helical (or oblique). Transverse muscle fibers run perpendicular to the longitudinal axis, extending through the entire tissue (Fig. 8B,C) and being composed of fibers running vertically and horizontally across the buccal membrane (Fig. 8B-D,F). Transverse vertical muscle fibers are attached to the basement membrane of the epithelium and to the adjacent muscles layers (Fig. 8C,D). Longitudinal muscle fibers run parallel to the longitudinal axis (Fig. 8D,F), and helical fibers are arranged obliquely, with layers of opposite handedness (Fig. 8E,F).

At the region of the buccal membrane where the seminal receptacle is present, transverse, longitudinal and oblique fibers from the buccal membrane musculature are still present, mainly located adjacent to the seminal receptacle (Fig. 8A; 9A,B), but are sometimes dislocated by the organ (Fig. 9B). For example, vertical and horizontal transverse musculature fibers, which are normally straight, are curved when surrounding the organ (Fig. 9B).

The musculature directly associated with the seminal receptacle is composed of thinner fibers forming a diffuse meshwork that surrounds each internal bulb (Fig. 9C,D). These fibers run in different directions (longitudinal, transverse and diagonal), surrounding each bulb individually and also large groups of bulbs (Fig. 9C,D). Meshwork fibers are inserted in adjacent tissues (Fig. 9C).

The seminal receptacle opening is surrounded by the transverse musculature, which is attached to the basement membrane of the buccal membrane epithelium (Fig. 10A,B), as well as by the meshwork fibers that extend from the bulbs and common duct (Fig. 10C). In frontal section, the configuration of these fibers forms a circumferential array around the opening (Fig. 10D).

Ultrastructure analysis shows that the musculature of the buccal membrane and the seminal receptacle is apparently composed of smooth fibers (Fig. 11A,B).

Associated nervous system

Two large ventral nerves extend longitudinally and flank the ventral region of the seminal receptacle (Fig. 8A; 12A). From them, serotonergic fibers extend throughout the seminal receptacle and constitute an extensive submuscular nerve plexus (Fig. 12B,C).

Morphological changes associated with reproductive events

Most females in Group 1 ("before mating") had spermatangia attached on the buccal membrane (Table 1), but almost all of them were empty (Fig. 13A), except for a single female with half-full spermatangia. All seminal receptacles in Group 1 were largely filled with sperm, which occupied the bulbs and the common duct up to the opening of the receptacle (Fig. 13B). Within the internal bulbs, sperm were generally organized with their heads facing the bulb epithelium, but in the common duct there was no clear organization pattern (Fig. 13C). All types of secretory cells were found, but AB-PAS cells were slightly predominant (Fig. 13C,D). In the common duct, spermatozoa were mixed with secretions from AB-only and AB-PAS cells (Fig. 13D).

Females from Group 2 ("after recent mating") had a large amount of full spermatangia attached on the buccal membrane (Fig. 14A; Table 1). Most of the females (n=8) also presented a large aggregated mass of sperm released from the fresh spermatangia obscuring the seminal receptacle (Fig. 1D). Spermatangia with empty, full or fresh aspects could be found attached on the same sample, possibly corresponding to distinct mating events. Occasionally (n=3) the seminal receptacle was not completely filled with spermatozoa, the bulbs close to the opening and the common duct having much less sperm than the more peripheral bulbs (Fig. 14B). Even in these cases, spermatozoa were observed within the opening of the receptacle (Fig. 14B). Sperm heads organization was similar to Group 1. Spermatozoa were also mixed with secretions from AB-only and AB-PAS cells in the common duct (Fig. 14C,D). Although all types of secretory cells were found, AB-only cells were slightly predominant, and PAS-fine-secretory cells were rare (Fig. 14C,D).

Most females from Group 3 ("after spawning") presented spermatangia attached on the buccal membrane (Table 1). However, they were completely empty, except for three females with half-full spermatangia (Fig. 15A). All seminal receptacles were almost empty,

except for their most posterior bulbs, which still contained spermatozoa (Fig. 15A,B). Within the internal bulbs, sperm heads organization varied from facing the bulb epithelium to no clear organization pattern. Although all types of secretory cells were found, many cells were empty (Fig. 15C); AB-only cells were slightly predominant, and PAS-only cells were rare (Fig. 15D). A large amount of secretion was observed also at the seminal receptacle opening (Fig. 15D).

The volume of spermatozoa inside the organ was statistically different between the groups (ANOVA one-way test: gl = 2; F = 9.8685; p = 0.0009). After spawning, females showed a significant decrease in sperm volume compared to the females of the other two groups (Fig. 16). However, sperm volume in Groups 1 and 2 was not significantly different (Fig. 16). We found no evident correlation between the number of spermatangia attached on the buccal membrane and the volume of sperm inside the seminal receptacle (Table 1). For example, in Group 2, there was a large variation in sperm volume (Min: 0.063; Max: 0.264), despite all females presenting full to fresh spermatangia attached on the buccal membrane (Table 1). There was also apparently no correlation between male-parallel mating and sperm volume within the seminal receptacle in Group 3. In this experimental group, five females (#2, #4, #7, #8 and #10 in Table 1) copulated with consort males before spawning, but they showed large variation in sperm volume, similar to isolated females (Table 1).

Discussion

Secretory cycle associated with major reproductive events

The present study demonstrated that the female sperm storage organ of the squid *Doryteuthis plei* shows a striking diversity of secretory cells, which differ in texture and chemical composition. Although the receptacle is an invagination of the buccal membrane, the secretory cells of both outer and inner epithelia were shown to be quite distinct using regular and reverse combinations of AB and PAS techniques, suggesting they perform different functions.

Moreover, there were changes in the composition and predominance of cells between major reproductive events, suggesting a secretory activity associated with mating and spawning. For example, soon after head-to-head mating, when sneaker male deposits spermatangia on the buccal membrane of the female, the seminal receptacle apparently

releases the content of the PAS-fine-secretory cells. Also, after egg release, several types of cells (AB-only, PAS-only and AB-PAS) have their contents released together with the stored sperm. As will be discussed, these secretions could participate in distinct functions, such as sperm uptake, storage, nourishment and activation.

Muscle system suggesting high degree of control over precise deformations

The morphology and organization of the muscle fibers associated with the buccal membrane and the seminal receptacle have similarities to other cephalopod muscle systems (e.g., skin papillae; Allen et al., 2013, 2014; arms and tentacles; Kier, 2016). The three-dimensional organization of muscle, the presence of connective tissue fibers, and the lack of fluid-filled cavities or rigid skeletal elements characterize the whole buccal membrane as a muscular hydrostat (Kier, 1982; Kier and Smith, 1985; Kier and Stella, 2007; Kier, 2012). In general, muscular hydrostats provide support and precise and complex movements, having multiple oriented bundles of muscle fibers allowing for more control over three-dimensional deformation (Kier, 2012).

The musculature of the buccal membrane is diverse and probably responsible for moving and deforming the membrane. Several groups of fibers are present in this region. The buccal membrane connect the oral arms, and squids are capable of contract this entire region (Naef, 1972). Movements of the buccal membrane and oral arms are maybe correlated with feeding (e.g., Boucaud-Camou and Boucher-Rodoni, 1983), but they might possibly also affect the structure of the seminal receptacle. The transverse musculature is composed of fibers extending across the transversal plane of the buccal membrane, in two orientations that are perpendicular to each other. Contraction of the transverse vertical fibers might result in the flattening of the buccal membrane. Contraction of both horizontal and vertical transverse fibers might decrease the cross-sectional area and consequently result in the elongation of the buccal membrane. Contraction of the longitudinal fibers should shorten the buccal membrane, their differential contraction probably resulting in the arching of the buccal membrane. In addition, right- and left-handed helical muscle layers might generate some degree of torsion of the whole structure. Combination of the action of these fibers should confer precise and complex movements to the buccal membrane: elongation, shortening, bending and possibly torsion, directly affecting the seminal receptacle.

The contraction of the meshwork surrounding exclusively the seminal receptacle internal bulbs most probably cause the compression of the whole organ or of the individual bulbs, resulting in the decrease of their diameter and internal volume, most probably causing the release of stored sperm. The meshwork that surrounds the opening might function as a sphincter, controlling the passage of sperm.

Lines of evidence for female control over sperm uptake, storage and release

The two large nerves of the buccal membrane and the extensive submuscular nerve plexus are possibly involved in controlling the musculature and secretory cells of the buccal membrane and seminal receptacle. These large nerves originate from the brachial nerves, which are connected to the buccal lobe (Nixon and Young, 2003). In light of our findings, in the following sections we discuss the major processes that could be under control of the female by means of the seminal receptacle: sperm uptake, storage/nourishment and activation/release.

1. Sperm uptake

Previous hypotheses concerning sperm uptake into the seminal receptacle either involve (1.1) chemical attraction by secretions inside the receptacle followed by sperm active swimming (Sato et al., 2010); (1.2) sperm uptake by ciliary action from the receptacle (e.g., Hanlon et al., 1999); (1.3) muscular sperm uptake by the receptacle (i.e., by suction; Oordt, 1938); (1.4) some kind of manipulation not involving the seminal receptacle, such as the buccal mass actively picking up attached spermatangia (Sato et al., 2010, 2013, 2014a). Hypothesis #1.4 involves a behavior documented so far only for Idiosepiidae, and seems a specialized strategy to that particular group, so it is not discussed here.

1.1. Chemical attraction. The only experimental approach to test the hypothesis of chemical attraction in the seminal receptacle of squids was performed by Oordt (1938), who concluded that the secretion of the seminal receptacle does not attract spermatozoa in *Loligo vulgaris*. However, at that time intrasexual dimorphism was not known, and we are now not sure from which male morph (sneaker or consort) Oordt (1938) extracted sperm to perform his experiments. Several studies have now demonstrated that the behavior and physiology of sneaker and consort sperm are strikingly different (e.g., *D. bleekeri*, Hirohashi and Iwata, 2013; Hirohashi et al., 2013; *D. plei*, Apostólico and Marian, in prep.). Sneaker sperm was

demonstrated to form a cluster in response to their own respiratory CO_2 (Hirohashi and Iwata, 2013; Hirohashi et al., 2013). It is reasonable to suppose that a secretion from the seminal receptacle leading to CO_2 formation would presumably result in attraction of the sperm cluster into the receptacle. Considering the results of experimental group 2, almost all PAS-fine-secretory cells and part of the AB-PAS cells have their contents released soon after mating. If chemical attraction underlies sperm uptake in squids, and if this chemical attraction is provided by the secretory cells of the receptacle, then they should be involved in this process.

Sato et al. (2010), however, suggested that the vacuoles from the internal bulbs of the seminal receptacle of *Idiosepius paradoxus* would secrete a sperm attractant, because most sperm heads faced the bottom of the receptacle. This same organization was observed in the internal bulbs of *D. plei*, but not within the common duct. Secretory PAS-positive cells from the internal bulbs of *D. plei* apparently continuously secrete the polysaccharide matrix within which the sperm are immersed, and could presumably be involved in this process.

1.2. Ciliary action. The seminal receptacle of *D. plei* is lined with a ciliated epithelium, as already documented for other decapodiforms (e.g., Hanlon et al., 1999; Naud et al., 2005; Sato et al., 2010). Drew (1911), based on *Loligo pealei*, suggested an "outside force" that would pull the sperm into the organ, given that the sperm entering the seminal receptacle had their tails pointing in the same direction, suggesting not a swimming activity but an active force moving the spermatozoa. If a ciliary mechanism is present, the aggregating behavior of sneaker sperm (Hirohashi and Iwata, 2013) should facilitate sperm uptake. However, further investigation on ciliary currents within the seminal receptacle is required to test this hypothesis.

1.3. Muscular action. Muscle fibers around the seminal receptacle were also reported for other species (e.g., *Loligo vulgaris*, Oordt, 1938; *Sepia officinalis*, Hanlon et al, 1999; *Sepia apama*, Naud et al., 2005). Oordt (1938) hypothesized these muscle fibers would be responsible for sucking the sperm into the receptacle, basing his hypothesis on the results of his experiments of sperm attraction (see above) and on the fact that spermatangia were sometimes found within the receptacle (i.e., that they would have presumably been sucked into the organ). Our results based on histology and confocal microscopy suggest that muscular suctioning would be possible considering that the buccal membrane is a muscular hydrostat (Kier, 2012). This action would involve expansion of the internal volume of the organ and could be achieved by contraction of the fibers that cause compression of the organ,

followed by their relaxation. The thick connective tissue could provide storage of elastic energy (Kier, 2012) necessary for this suction action, but additional studies are needed to test this hypothesis. A controlled contraction wave would also be plausible within the muscular hydrostat context, and would have the same effect: moving anteriorly, contraction waves would release sperm; a reverse wave would bring sperm from the tip of the organ to the bottom of the bulbs.

2. Sperm storage and nourishment

Drew (1911) and Oordt (1938) suggested that the goblet cells present in the epithelium of the seminal receptacle of *Doryteuthis pealei* and *Loligo vulgaris*, respectively, would release a substance that inactivates sperm. Oordt (1938) revealed the seminal receptacle was slightly acidic (pH=6.2), and that sperm would become inactive *in vitro* if the pH of the seawater was brought to around 6. The goblet cells observed by Drew (1911) and Oordt (1938) probably correspond to the medium to coarse AB-only, PAS-only and AB-PAS secretory cells of *D. plei*, which are largely abundant in the epithelium of the common duct. The AB-positive cells might contain acidic polysaccharides (e.g., Bancroft and Stevens, 1982), and could presumably be involved with pH control within the organ and, hence, with sperm immobilization and storage. If so, this secretion would permit long-term maintenance of stored sperm by minimizing energy expenditure.

Nourishment during hibernation could presumably be provided by the secretory PAS-positive cells from the bulbs, that apparently produce the polysaccharide matrix within which the sperm are immersed. This matrix could serve as a source of energy for stored sperm, given that sneaker spermatozoa of *D. bleekeri* were demonstrated to be able to use extracellular glucose (Hirohashi et al., 2016).

3. Sperm activation and release

The histology of the seminal receptacle showed that after spawning there is active secretion by almost all types of cells, together with a drastic reduction of stored sperm volume. For example, AB-PAS cells, which were abundant before and after recent mating, have their contents released after spawning. The glandular cells of the seminal receptacles of the squid *Todarodes pacificus* also release their contents during spawning (Ikeda et al., 1993).

The role of these secretions could be involved with sperm activation. Sneaker spermatozoa of *D. bleekeri* have longer swimming lifetime because of their higher glycogen

content when compared to consort sperm (Hirohashi et al., 2016). This difference was hypothesized to be related to the more external deposition site of sneaker spermatangia (the buccal membrane), which would imply a higher risk of sperm dilution (Hirohashi et al., 2016) than the mantle cavity. Sneaker sperm are also able to use extracellular glucose to sustain motility (Hirohashi et al., 2016), so these secretions from the seminal receptacle could presumably supply nourishment for stored sperm during activation and release.

Our results on the receptacle muscle system strongly suggest that stored spermatozoa are released by female controlled muscular action, with participation of both the seminal receptacle meshwork and the buccal membrane musculature, allowing the female to control sperm release when the egg capsule is passed to the space between the arms during spawning.

Putative post-copulatory sexual selection mechanisms in squids

1. Sperm competition

The presence of a large number of spermatangia in different stages of freshness (empty, full, fresh) in our experimental groups indicates that females are promiscuous and copulate with several sneaker males, even if they are not close to spawn. Also, spermatangia might release sperm for at least 48 hours, given that one female in Group 1 had full spermatangia at the buccal membrane. Sperm release duration from attached spermatagia is quite variable, varying from one to several days (Drew 1919; Sato et al., 2014b). Previous *in vitro* experimentation with *D. plei* demonstrated that sperm release by sneaker spermatangia might last more than 5 hours, although they still have considerable sperm reserves even after one day (Apostólico and Marian, in prep.).

If spawning occurs when spermatangia from two or more males are releasing sperm at the same time, or if sperm from two or more males are present within the seminal receptacle, they will compete for fertilization, and we should expect diverse strategies associate with sperm competition (Birkhead and Møller, 1998; Hanlon et al., 1999). Cuttlefish males, for example, can flush strong jets of water directly at the female's buccal membrane, in order to expel spermatangia placed by other males, and hence reduce sperm competition (Hanlon et al., 1999). In our sample, some females had sperm stored within the receptacle but no spermatangia attached on the buccal membrane (Table 1), indicating either a very old copulation event or some unknown form of spermatangia removal strategy.

Intrasexual dimorphism is also associated with sperm competition, with consort males guarding the female after mating until the moment of spawning, and sneaker males employing alternative mating tactics and trying to ensure fertilization success by increasing the number of sexual partners (Hanlon, 1998; Buresch et al., 2009; Shashar and Hanlon, 2013; Naud et al., 2016; Apostólico and Marian, submitted).

Results from the experiments might also help cast some light into sperm competition conditions within the seminal receptacle. Apparently, during spawning, the female first uses the sperm stored in the anterior bulbs. Although some mixture of sperm inside the seminal receptacle is expected, it seems that the concept "first in/last out" applies to sperm in the squid seminal receptacle. If that is the case, the last male to copulate with the female would have an advantage over other males.

2. Cryptic female choice

During copulation with sneaker males, females are usually chosen by their partners, apparently not having a potential choice (e.g., Hanlon and Messenger 1996; Hanlon et al, 2002). However, after copulation spermatangia are deposited on the buccal membrane, and they might be depleted after some variable time. If the female does not spawn shortly after mating, and if the available sperm is not stored within the seminal receptacle, the last male to copulate will not sire the offspring of that female. The seminal receptacle allows the female to store sperm from several copulations, permitting a delay between mating and fertilization, and being potentially associated with more genetically variable offspring (Eberhard and Cordero, 1995; Wigby and Chapman, 2004). So, if the female possesses any control of the seminal receptacle, there is potential for cryptic female choice.

Changes in the chemical composition of sperm storage organs associated with major reproductive events, as documented herein for *D. plei*, were already documented in several taxa (e.g., Heifetz and Wolfner, 2004; Adams and Wolfner, 2007; Zara et al., 2014). If the female can control the microenvironment of the seminal receptacle, she can choose to secrete substances that will make the sperm viable or non-viable, biasing paternity in favor of some males over others, demonstrating cryptic female choice (Birkhead and Møller, 1998).

Also, as discussed above, the fact that some females of our sample had sperm stored within the receptacle but no spermatangia attached on the buccal membrane (Table 1) might indicate an old copulation event or some form of spermatangia removal, which could be performed by the female. For example, in *Idiosepius paradoxus*, females often remove

spermatangia using their mouth (buccal mass) after copulation (Sato et al., 2013; Sato et al., 2014b); in *Sepioteuthis sepioidea*, the female is apparently able to accept or reject spermatophores placed on her arms during mating (Hanlon and Messenger 1996); and in *D. pealeii* the female might eject spermatophores placed in the mantle cavity by the consort male, flushing them with her mantle (Buresch et al., 2009).

Although the volume of spermatozoa significantly decreased in the females of Group 3 when compared with Groups 1 and 2, even after spawning a considerable amount of sperm remained in the most posterior bulbs of the seminal receptacle (Table 1; Fig. 16). This result suggests that the female might control the quantity of sperm to be released during spawning, contrarily to previous hypotheses indicating that sperm release from the organ is a passive process for the female (e.g., that release would be by simple pressure of the egg capsule; Lum-Kong, 1992).

Although we expected an increase in sperm volume in the seminal receptacle of females after recent mating, there were no significant differences between Groups 1 and 2. Maybe the 30 min time interval after mating was not enough to permit sperm uptake by the organ. However, Group 2 females showed a large variation in sperm volume, despite all females evidently having received fresh spermatangia on their buccal membrane (Table 1). This could be indicative of some process of sperm removal from the seminal receptacle (e.g., by either the copulating male, to avoid sperm competition, or the female, as cryptic female choice), but additional studies are necessary to confirm this hypothesis.

Although no clear pattern was evident, some females that copulated with consort males apparently used part of the stored sneaker sperm for fertilization (Table 1), even having a fresh and abundant supply of consort spermatangia available within their mantle cavity (pers. obs.). This, too, could be indicative of cryptic female choice: the female choosing which source of sperm to use (seminal receptacle or mantle cavity). Recent data on the paternity and fertilization patterns of the squid *Loligo reynaudii* showed that females might change the source of sperm used to fertilize the same egg capsule, also suggesting cryptic female choice (Naud et al., 2016).

Concluding remarks

The present study shed new light on the potential of postcopulatory sexual selection mechanisms in the complex squid mating systems by focusing on an integrative and

experimental study of the sperm storage organ. Future research should focus on a deeper chemical characterization of the secretory products from the seminal receptacle (e.g., using immunocytochemistry) and also on testing sperm chemical attraction using both sneaker and consort spermatozoa.

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TABLE

Table 1 – Number of attached spermatangia and volume of stored sperm within the seminal receptacle of the females of *Doryteuthis plei* subjected to experimental manipulations (i.e., before mating, after recent mating and after spawning). In Group 3, five females (#2, #4, #7, #8 and #10) copulated with consort males before spawning. Abreviations: N. S. A., number of spermatangia attached on the buccal membrane; S. E., standard error.

	Before mating (Group 1)		**After recent mating (Group 2)**		**After egg release (Group 3)**	
	N. S. A.	**Volume (mm^3)**	**N. S. A.**	**Volume (mm^3)**	**N. S. A.**	**Volume (mm^3)**
Female 1	31	0.168	24	0.128	30	0.120
Female 2	11	0.169	37	0.106	0	0.057
Female 3	13	0.186	25	0.063	13	0.096
Female 4	5	0.157	11	0.084	43	0.141
Female 5	23	0.198	29	0.128	29	0.053
Female 6	27	0.150	40	0.236	49	0.048
Female 7	25	0.102	36	0.145	48	0.063
Female 8	0	0.173	23	0.119	68	0.111
Female 9	18	0.246	33	0.264	36	0.076
Female 10	0	0.200	29	0.223	8	0.043
Average ± S. E.	15.3 ± 3.5	0.175 ± 0.01	28.7 ± 2.6	0.150 ± 0.02	31.6 ± 6.9	0.081 ± 0.01

FIGURES

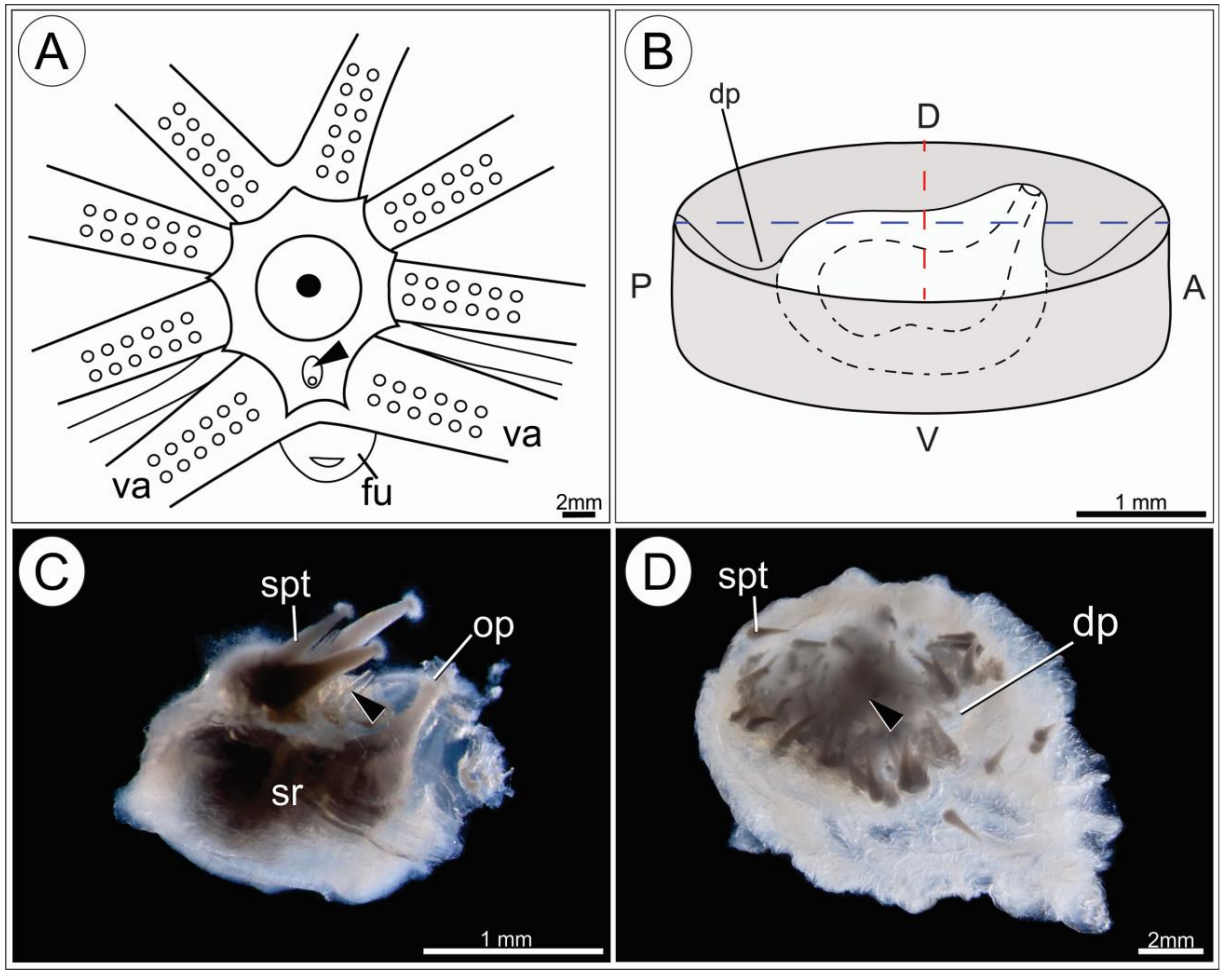

Figure 1 - General structure of the seminal receptacle of *Doryteuthis plei*. **A)** Schematic illustration of the arms and buccal membrane. Oral view. The seminal receptacle (arrowhead) is located between left and right ventral arms, on the ventral region of the buccal membrane. **B)** Schematic illustration of the buccal membrane, showing the position of the seminal receptacle. Lateral view. The seminal receptacle opening is dorsal and located anteriorly. The buccal membrane depression around the organ is where spermatangia are usually implanted. **C)** Lateral view of the dissected seminal receptacle, showing the spermatangia attached around the organ and close to the seminal receptacle opening. Spermatangia have the typical sneaker club-like morphology. Full spermatangia are whitish and empty ones are translucent (arrowhead). **D)** Dorsal view of the buccal membrane with numerous spermatangia attached around the seminal receptacle. A large aggregated mass of sperm obscures the organ (arrowhead). Attached spermatangia are mainly concentrated at the depression around the organ, but they can also be found over the whole buccal membrane. Abbreviations: A, anterior; D, dorsal; dp, buccal membrane depression; fu, funnel; op, opening; spt, spermatangia; P, posterior; sr, seminal receptacle; V, ventral; va, ventral arms.

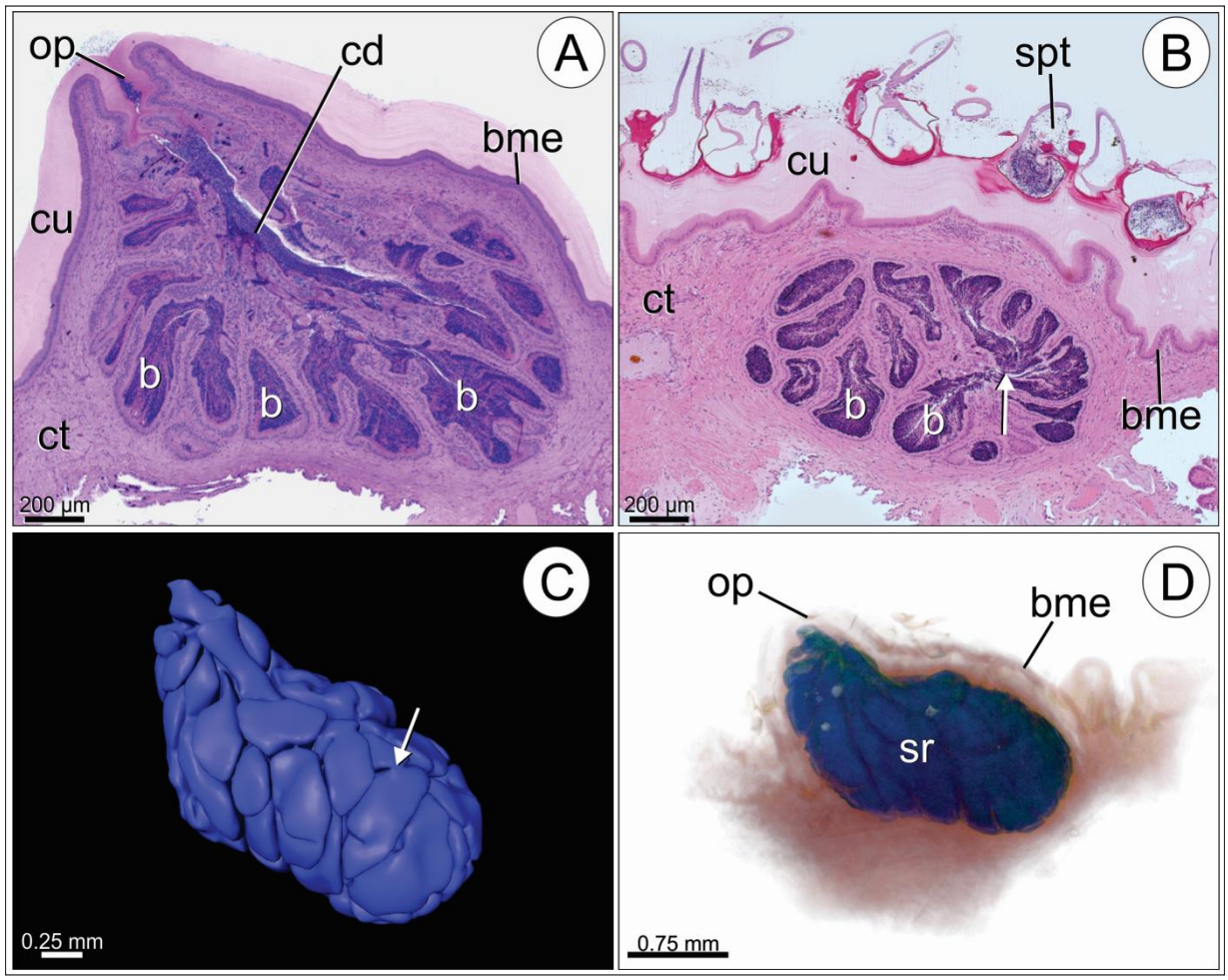

Figure 2 - Internal structure of the seminal receptacle of *Doryteuthis plei*. **A)** Sagittal section through the seminal receptacle showing its general organization. The organ is an invagination of the buccal membrane epithelium and is surrounded by the buccal membrane's connective tissue. Above the buccal membrane epithelium there is a thick cuticle layer. The seminal receptacle is divided into several interconnected bulbs converging to a common duct, which leads to the opening and connects the seminal receptacle to the exterior. AB-PAS-H staining. **B)** Sagittal section through the seminal receptacle showing that the internal bulbs are interconnected (arrow). Spermatangia are usually implanted into the cuticle layer. H&E staining. **C)** MicroCT data showing a 3D reconstruction of the internal bulbs of the seminal receptacle, showing the interconnection between the bulbs (arrow). **D)** MicroCT data showing a lateral view of the seminal receptacle within the ventral buccal membrane; transparency was intensified and the internal bulbs were colored in blue. Abbreviations: b, internal bulbs; bme, buccal membrane epithelium; cd, common duct; ct, connective tissue; cu, cuticle; op, opening; spt, spermatangia; sr, seminal receptacle.

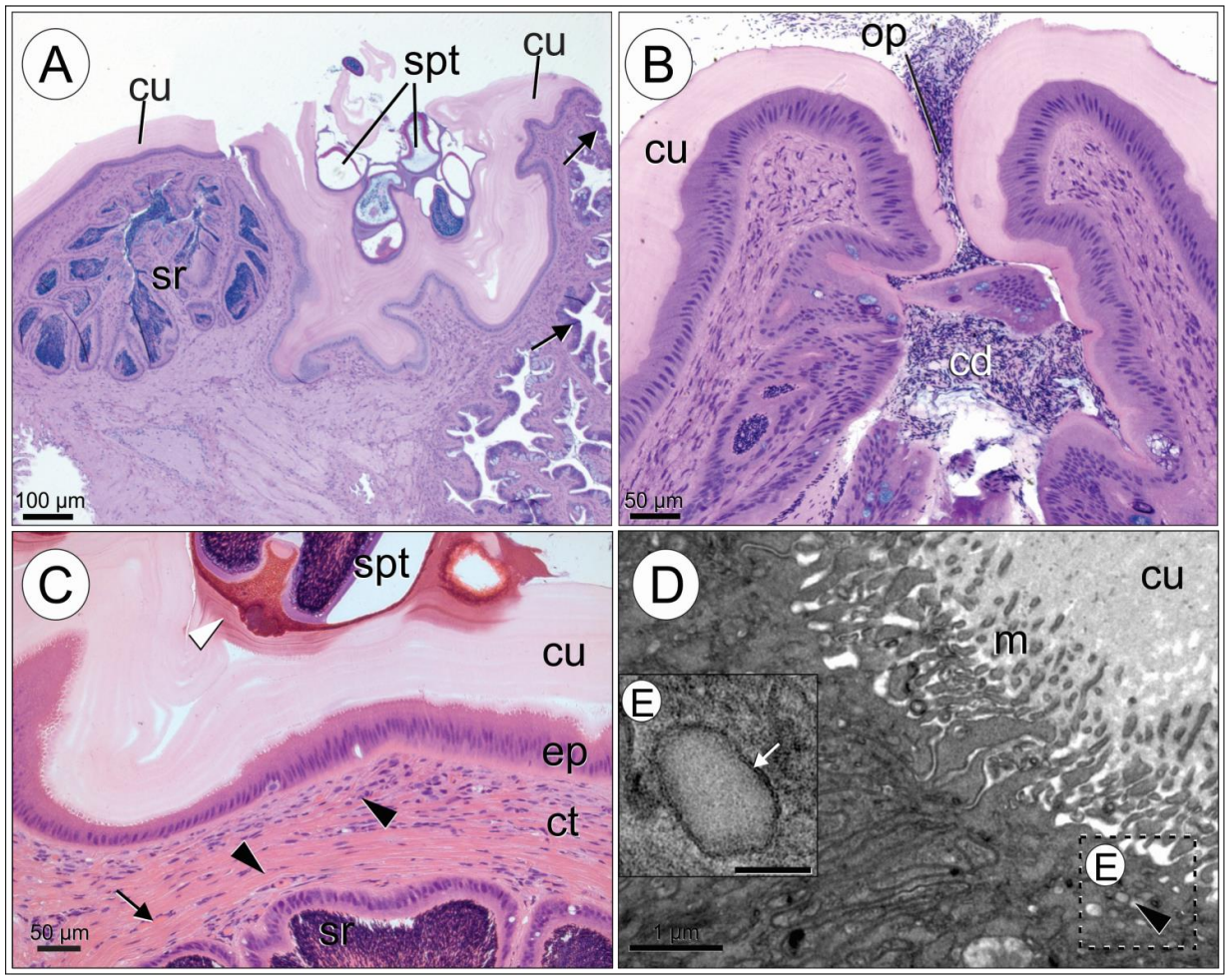

Figure 3 - Protective cuticle of the seminal receptacle of *Doryteuthis plei*. **A)** Sagittal section through the buccal membrane showing the cuticle layer that extends over the whole buccal membrane depression. Spermatangia are implanted into the cuticle layers. At the region devoid of a cuticle the buccal membrane epithelium projects into folds (arrows). AB-PAS-H staining. **B)** Sagittal section through the seminal receptacle opening. The cuticle reaches the seminal receptacle opening and enters a few micrometers into the common duct. AB-PAS-H staining. **C)** Cross-section through the cuticle region. The bases of the spermatangia are implanted into the cuticle layers, often causing ruptures (white arrowhead). Below the epithelium that secretes the cuticle, muscle fibers (black arrowheads) and blood vessels (arrow) are interspersed within the connective tissue surrounding the seminal receptacle. H&E staining. **D)** Transmission electron micrograph of the buccal membrane epithelium that secretes the cuticle, showing microvilli. Notice the small electron-lucent vesicles that are apparently involved in the secretion of the cuticle (arrowhead). **E)** Detail of the vesicle shown in D, showing its double-membrane. Scale bar: 0.1µm. Abbreviations: cd, common duct; ct, connective tissue; cu, cuticle; ep, epithelium; m, microvilli; op, opening; spt, spermatangia; sr, seminal receptacle.

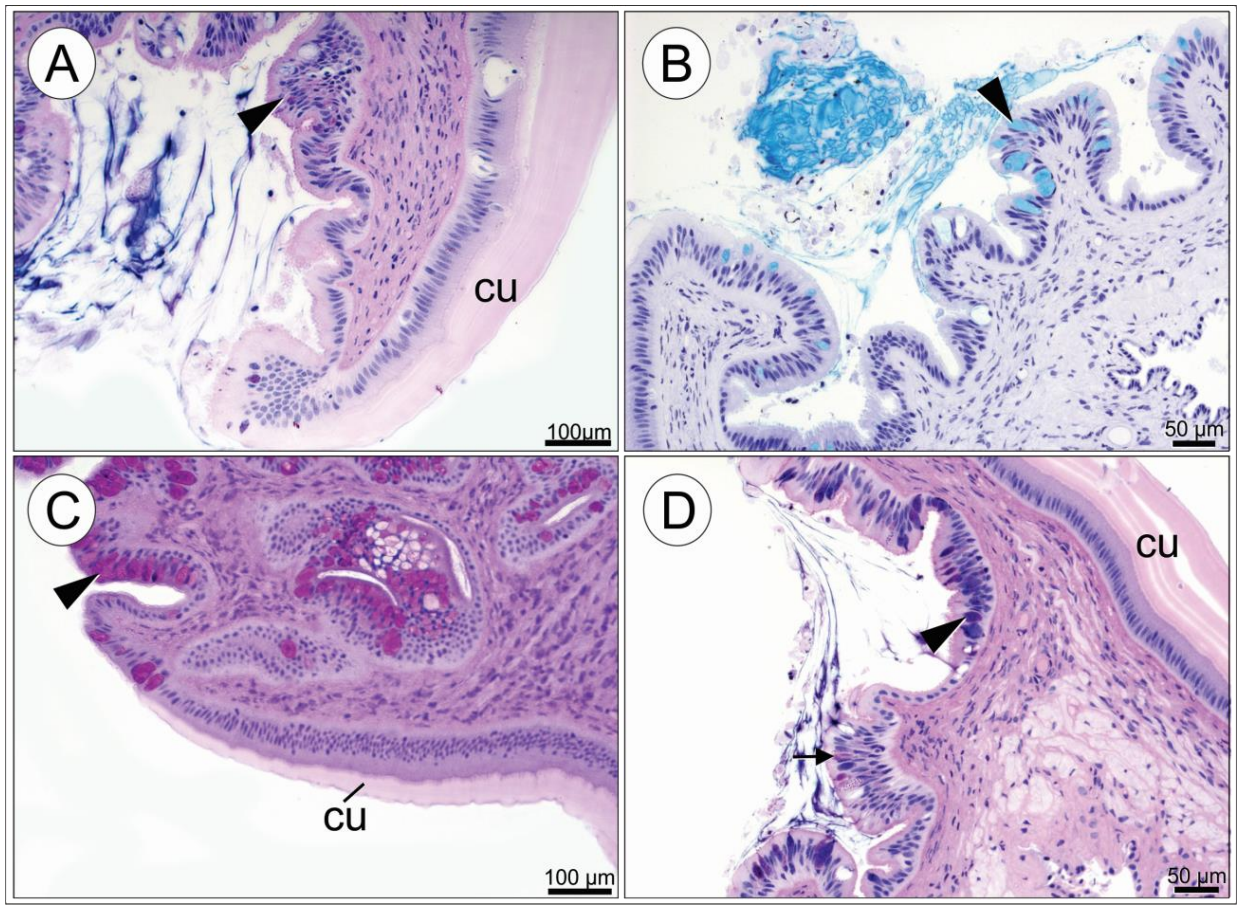

Figure 4 - Histological sections through the buccal membrane of *Doryteuthis plei* at the region devoid of a cuticle. **A)** Cross-section through at the interface between both regions (with and devoid of a cuticle). At the region devoid of a cuticle, the epithelium projects into folds (arrowhead) and bear numerous secretory cells that are releasing their content to the exterior. AB-PAS-H staining. B) Cross-section showing secretory cells with strong positive reaction for AB (arrowhead). AB-H staining. C) Sagittal section showing secretory cells with strong positive reaction for PAS (arrowhead). PAS-H staining. D) Cross-section showing the rare PAS-positive (arrow) and the more frequent AB-PAS secretory cells (arrowhead). AB-PAS-H staining. Abbreviation: cu, cuticle.

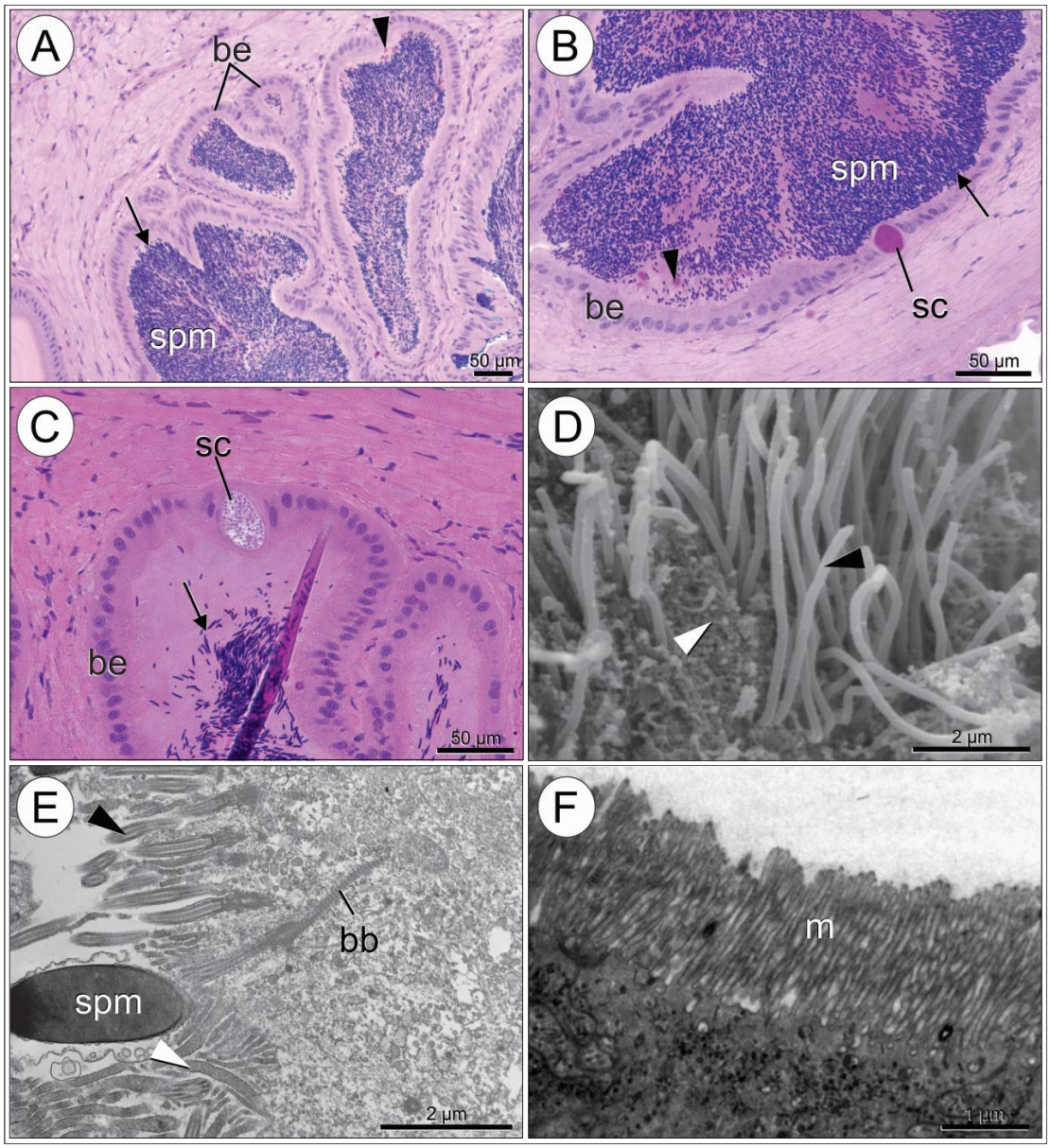

Figure 5 - Morphology of the internal bulbs of the seminal receptacle of *Doryteuthis plei*. **A)** Sagittal section showing the columnar to cuboidal epithelium, the PAS-positive matrix in which the sperm are immersed, and strongly PAS-positive droplets (arrowhead). Sperm heads are facing the bulb epithelium (arrow). AB-PAS-H staining. **B)** Sagittal section showing the rarely found bulb secretory cell with strong affinity for PAS. Sperm heads are facing the bulb epithelium (arrow). Spermatozoa are immersed within a PAS-positive matrix. Arrowhead points to the PAS-positive droplets. AB-PAS-H staining. **C)** Sagittal section showing the rarely found secretory cell with fine acidophilic granules. In this case sperm heads are not facing the bulb epithelium (arrow), and present no clear organization pattern. H&E staining. **D)** Scanning electron micrograph of the surface of the bulb epithelium, which bears cilia (black arrowhead) and microvilli (white arrowhead). **E)** Transmission electron micrograph of the bulb epithelium bearing cilia (black arrowhead) and microvilli (white arrowhead). It is possible to distinguish the cilia by the presence of the basal body. **F)** Transmission electron micrograph of the bulb epithelium bearing only microvilli. Abbreviations: bb, basal body; be, bulb epithelium; m, microvilli; sc, secretory cell; spm, spermatozoa;

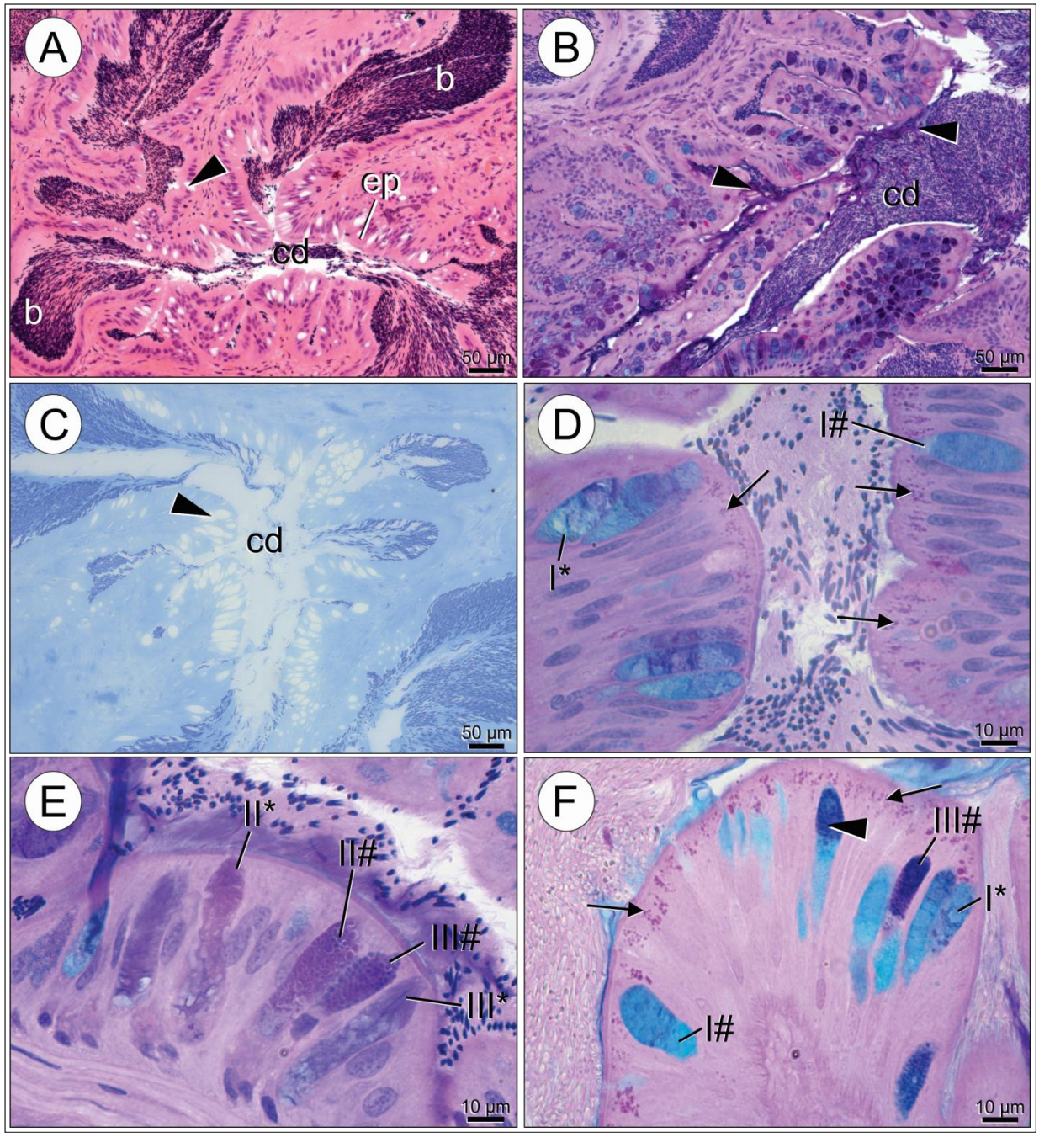
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Figure 6 - Histological sections through the seminal receptacle of *Doryteuthis plei* at the region of the common duct. **A)** Sagittal section through showing a large amount of secretory cells in this region. They presented negative reaction for H&E (arrowhead). H&E staining. **B)** Sagittal section showing numerous secretory cells (arrowheads) with strong positive reaction for AB (blue), PAS (magenta) or for the combination of both AB and PAS (purple). AB-PAS-H staining. **C)** Cross-section showing the secretory glands with negative reaction for Mercury-Bromophenol Blue (arrowhead). BB staining. **D-F)** Detail of the different types of secretory cells. PAS-fine-secretory cells have very fine and disperse granules located mainly at the apical region and show strong reactivity to PAS (arrows). Secretory cells with coarse round granules (marked with an asterisk *) may be stained with: I) AB-only (blue); II) PAS-only (magenta); or III) AB-PAS (purple). Secretory cells with medium granules (marked with a hash #) may be stained with: I) AB-only (blue); II) PAS-only (magenta); or III) AB-PAS (purple). **D)** AB-PAS-H staining. **E)** AB-PAS-H staining. **F)** Secretory AB-PAS cells with medium granules might also be stained the lower half with blue (AB) and the upper half with purple (AB-PAS) (arrowhead). AB-PAS staining. Abbreviations: b, internal bulbs; cd, common duct; ep, epithelium.

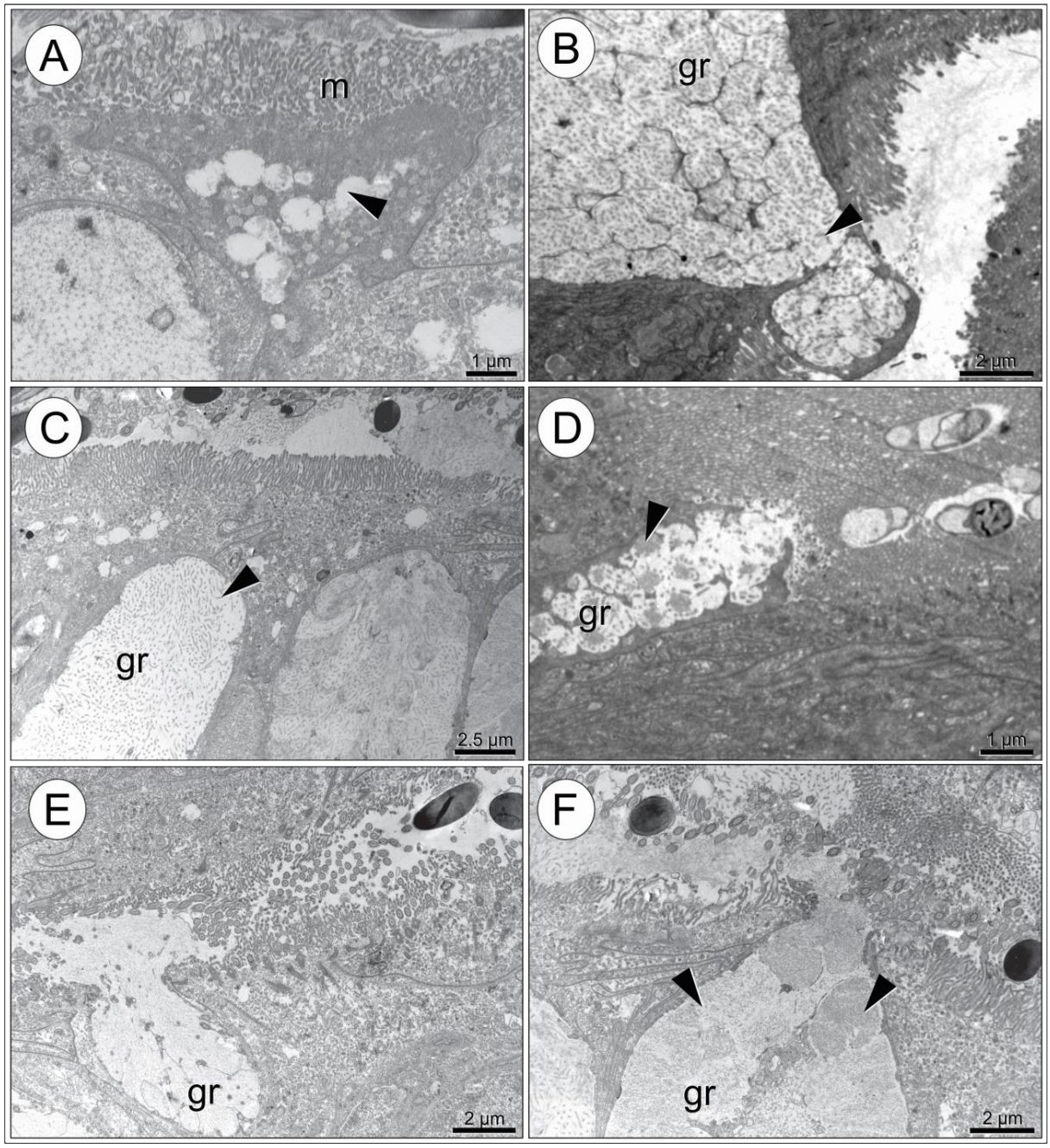

Figure 7 - Transmission electron micrograph of the major groups of secretory cells located at the seminal receptacle common duct epithelium of *Doryteuthis plei*. **A)** Detail of the apical region of the cell showing fine electron-lucent granules (arrowhead). **B)** Detail of a secretory cell with medium electron-lucent granules containing very thin and elongated electron-dense marks (arrowhead). **C)** Detail of a secretory cell with coarse electron-lucent granules containing very thin and elongated electron-dense marks (arrowhead). **D)** Detail of a secretory cell with medium electron-lucent granules containing round electron-dense elements (arrowhead). **E)** Detail of a secretory cell with medium electron-lucent granules without conspicuous marks. **F)** Detail of a secretory cell with coarse electron-lucent granules without conspicuous masks, and with variations of electron density between the granules (arrowheads). Abbreviations: gr, granule; m, microvilli.

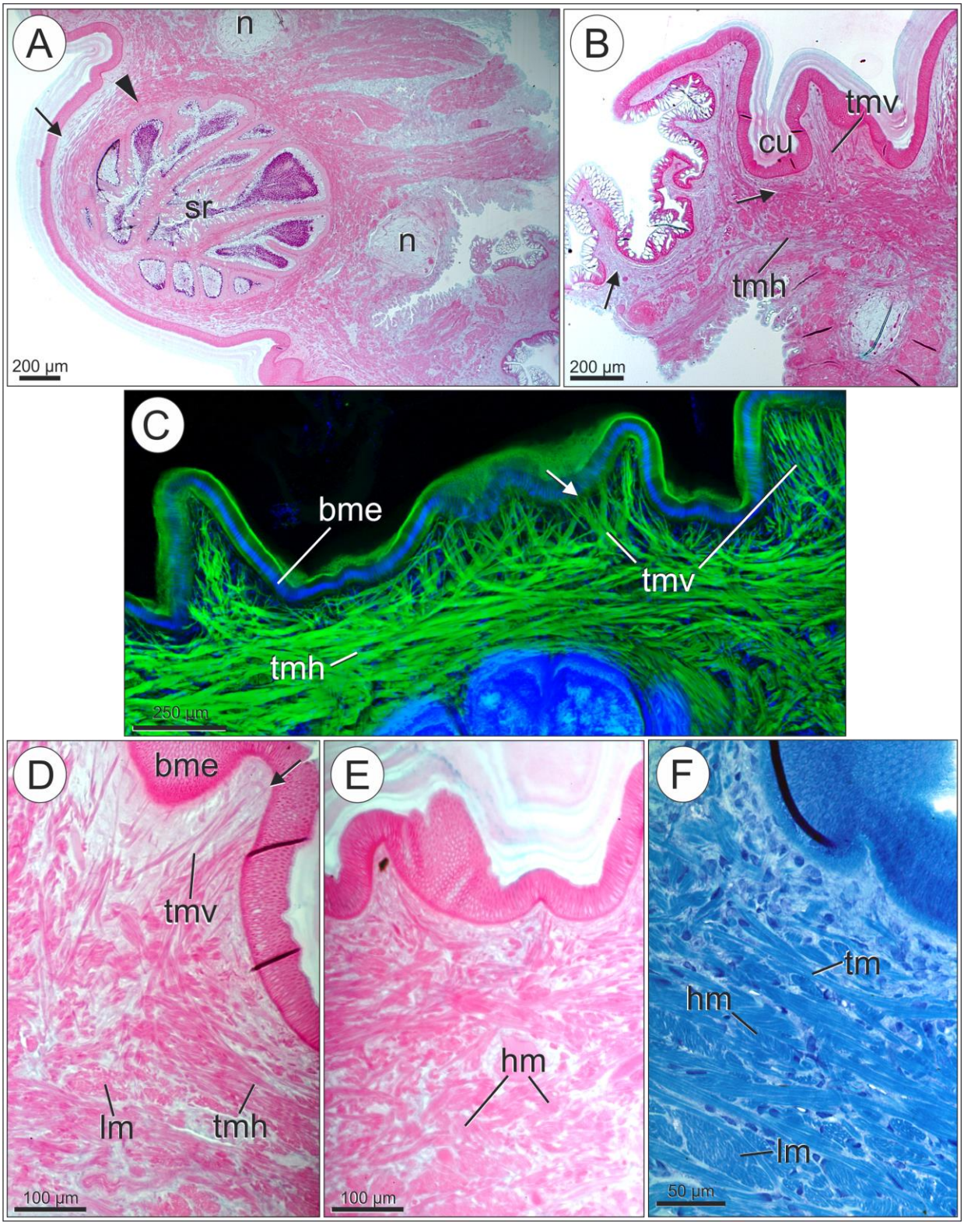
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Figure 8 - Musculature associated with the buccal membrane and seminal receptacle of *Doryteuthis plei*, showing transverse, longitudinal and helical fibers. **A)** Cross-section showing the muscle fibers stained in pink (arrowhead), interspersed within the connective tissue stained in light blue (arrow). At the region close to the seminal receptacle, fibers are concentrated around the organ. Notice the two large nerves, extending longitudinally longitudinally and flank the ventral region of the seminal receptacle. GT staining. **B)** Cross-section showing the end of the cuticle layer and the region devoid of a cuticle (arrow at left). The musculature is denser at the region where the buccal membrane secretes the cuticle (arrow at right). Transverse muscle fibers run perpendicular to the longitudinal axis, extending vertically or horizontally. GT staining. **C)** Confocal micrograph (cross-section) of the buccal membrane musculature showing the transverse musculature. Transverse vertical muscle fibers are attached to the basement membrane of the epithelium (arrow) and to the adjacent muscles layers. Nuclei are represented in blue and muscle fibers in green. **D-F)** Cross-sections of the buccal membrane musculature. **D)** Detail showing the transverse musculature composed of vertical and horizontal fibers. The vertical fibers are attached to the basement membrane of the epithelium (arrow). Longitudinal muscle fibers are running perpendicular to the transversal axis, and are seen in cross-section. GT staining. **E)** Detail showing helical fibers, with layers of opposite handedness. GT staining. **F)** Detail showing helical, transversal and longitudinal fibers. TB staining. Abbreviations: bme, buccal membrane epithelium; cu, cuticle; hm, helical musculature; lm, longitudinal musculature; n, nerves; sr, seminal receptacle; tmh, horizontal fibers of transverse musculature; tmv, vertical fibers of transverse musculature.

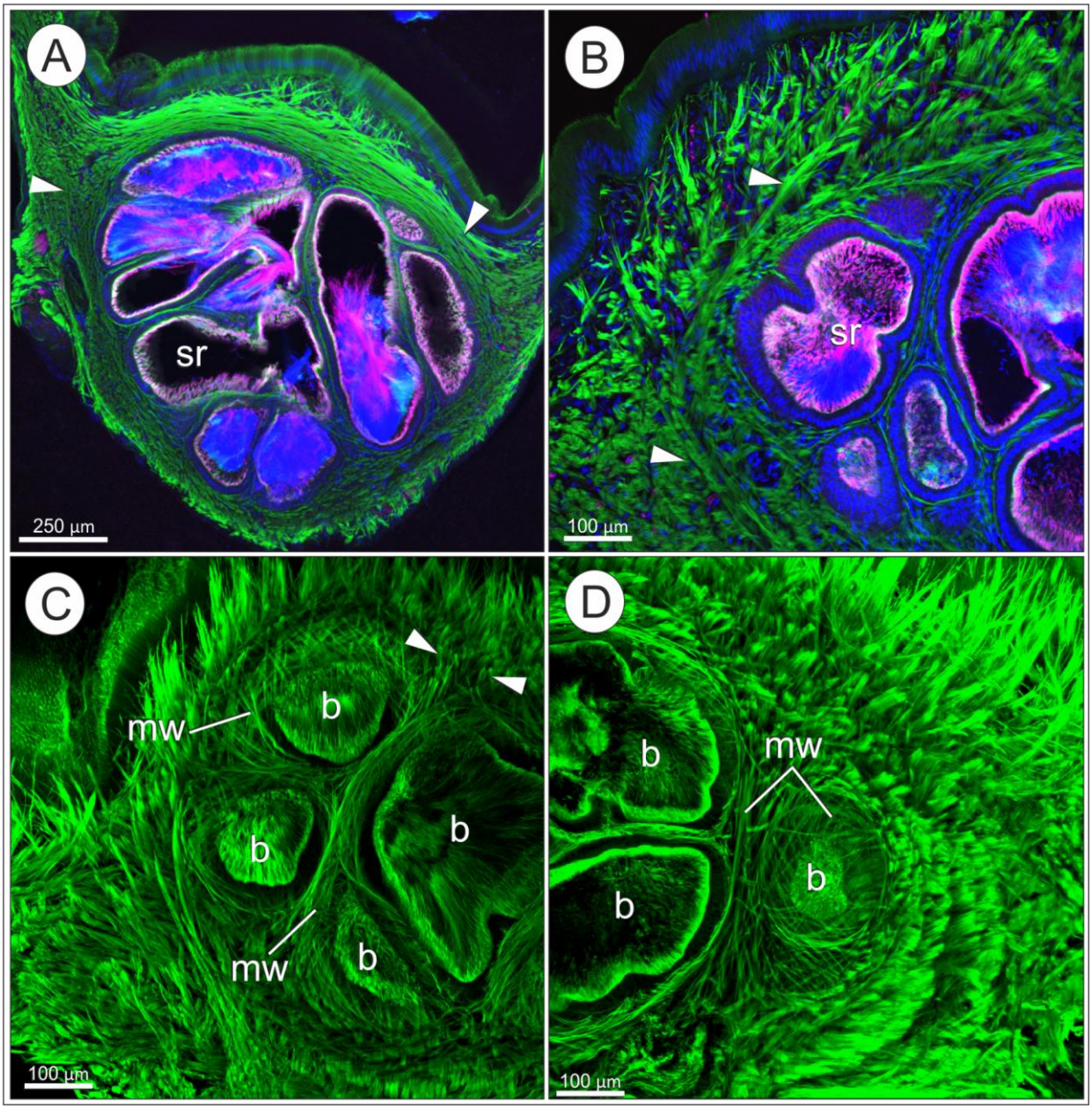

Figure 9 - Confocal images of the buccal membrane and seminal receptacle musculature of *Doryteuthis plei*. Muscle fibers are represented in green, nuclei in blue and anti-serotonin fibers in magenta. **A)** Cross-sections of the seminal receptacle, showing the buccal membrane musculature located adjacent to the organ (arrowheads). **B)** Transverse musculature dislocated by the presence of the seminal receptacle (arrowheads). **C)** Musculature directly associated with the seminal receptacle forming a diffuse meshwork that surrounds each internal bulb; fibers of the meshwork are inserted in adjacent tissues (arrowheads). D) Detail of the meshwork surrounding the internal bulbs. Abbreviations: b, internal bulbs; mw, diffuse meshwork, sr, seminal receptacle.

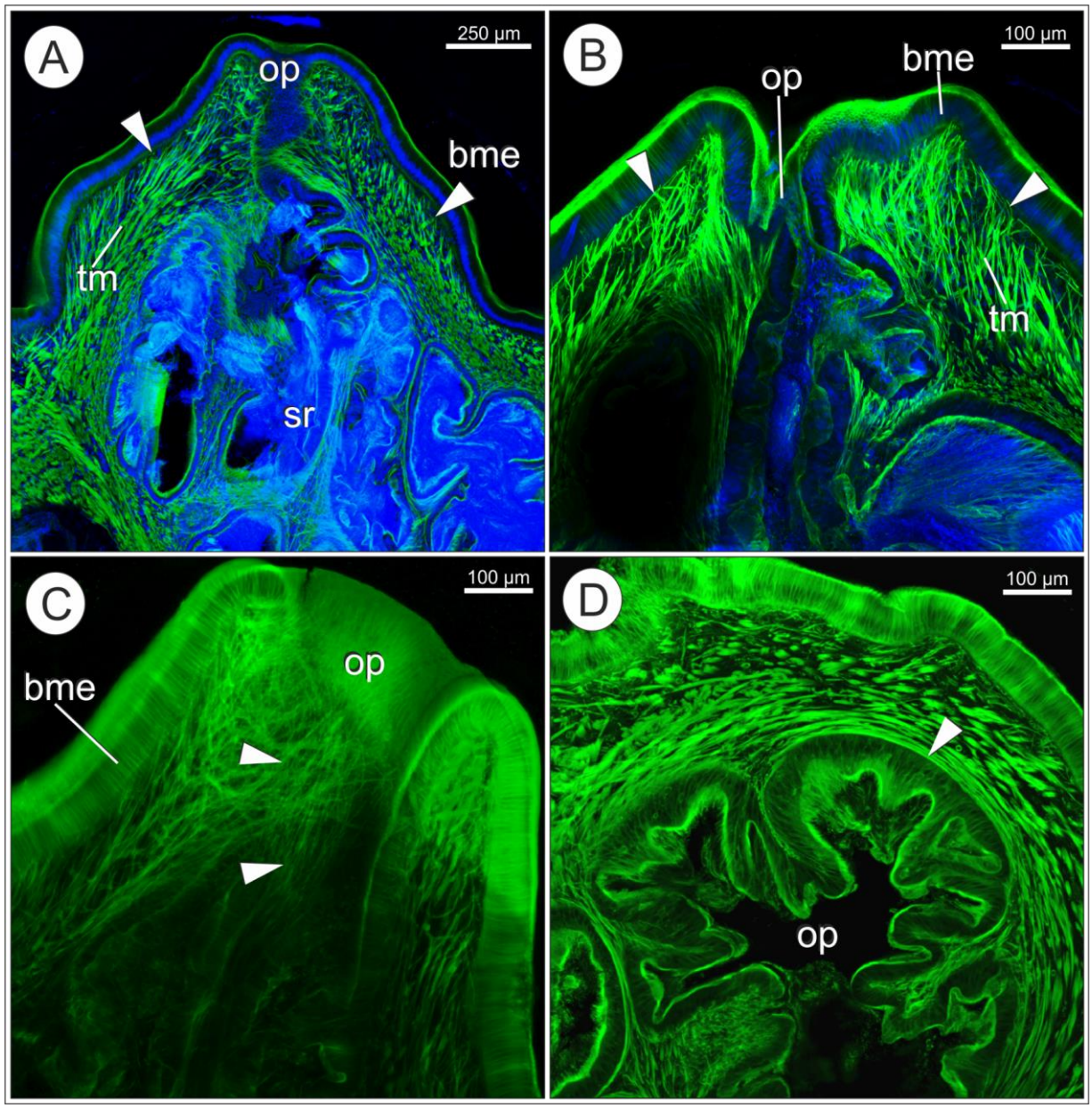

Figure 10 - Confocal images of the musculature that surrounds the seminal receptacle opening in *Doryteuthis plei*. Muscle fibers are represented in green and nuclei in blue. **A)** Sagittal section of the seminal receptacle, showing the opening surrounded by the transverse musculature, which is attached to the basement membrane of the buccal membrane epithelium (arrowheads). **B)** Cross-section of the seminal receptacle opening showing the transverse musculature attached to the basement membrane of the buccal membrane epithelium (arrowheads). **C)** Seminal receptacle opening musculature showing the meshwork fibers that extends from the bulbs and surrounds the opening (arrowheads). **D)** Frontal section of the seminal receptacle opening, showing circumferential muscle fibers (arrowhead). Abbreviations: bme, buccal membrane epithelium; op, opening; sr, seminal receptacle; tm, transverse musculature.

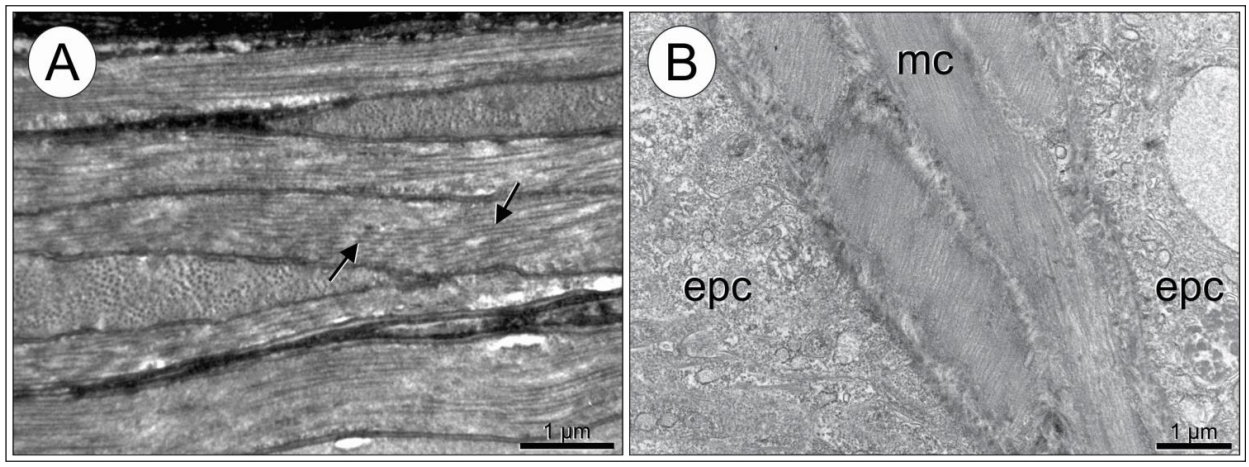

Figure 11 - Transmission electron micrograph of the musculature associated with the seminal receptacle of *Doryteuthis plei*. The apparent absence of striations suggests that the musculatures of the buccal membrane (A) and seminal receptacle (B) are composed of smooth fibers. **A)** Musculature of the buccal membrane located adjacent to the seminal receptacle. Arrows point to individual fibers. **B)** Seminal receptacle musculature found between opposed bulb epithelia. Fibers of the meshwork run in different directions. Abbreviations: epc, epithelium cells; mc, muscle fibers.

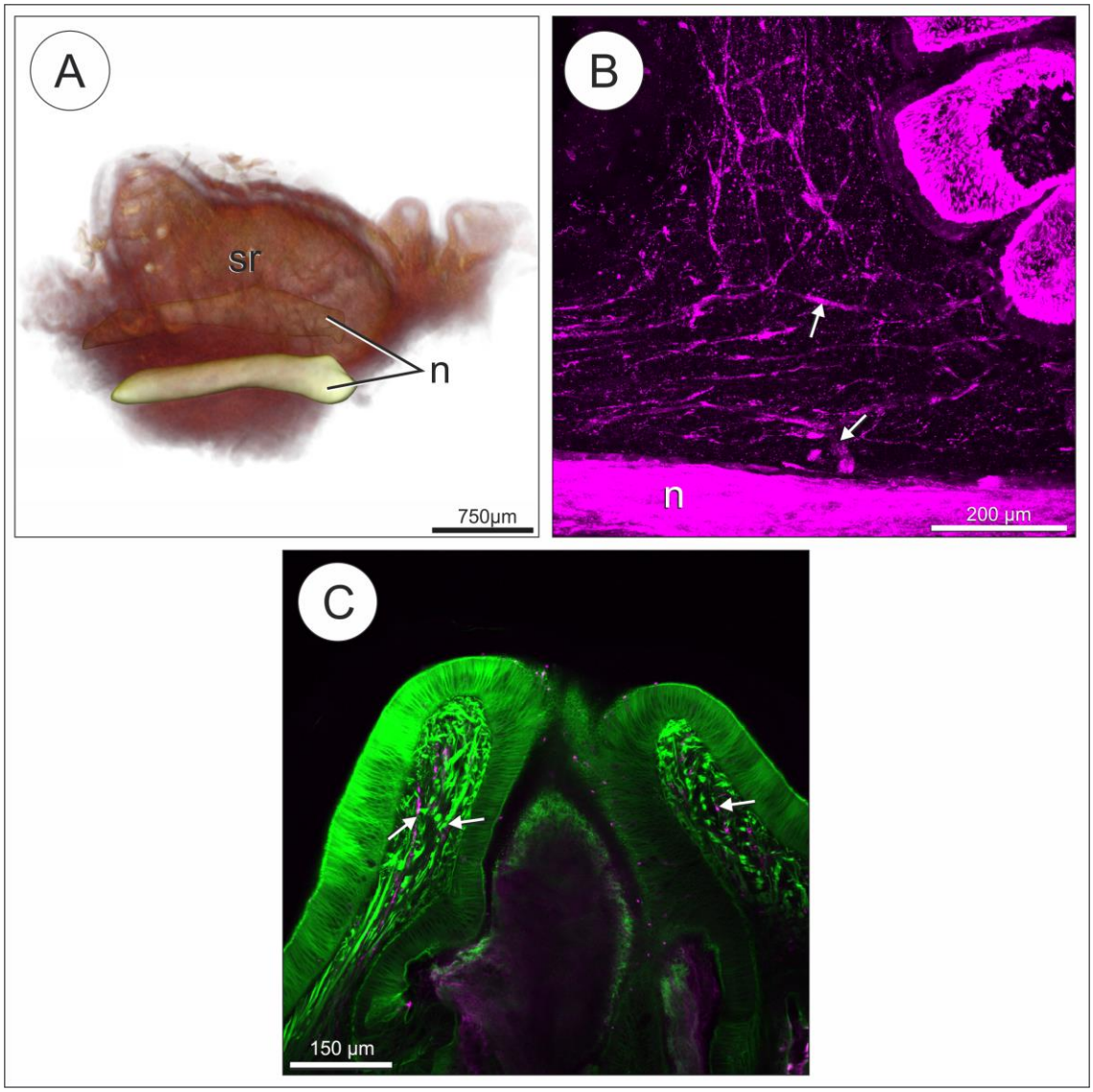

Figure 12 - Nerve system associated with the seminal receptacle and buccal membrane of *Doryteuthis plei*. **A)** MicroCT data with transparency intensified showing the two large ventral nerves (colored in yellow) extending longitudinally and flanking the ventral region of the seminal receptacle; lateral view. **B)** Confocal micrograph of serotonergic fibers (arrows) starting from the large nerve and extending throughout the seminal receptacle; sagittal section. **C)** Confocal micrograph of the musculature (in green) and associated nerve plexus (magenta, arrows) at the region of the opening of the seminal receptacle. Abbreviations: n, nerve; sr, seminal receptacle.

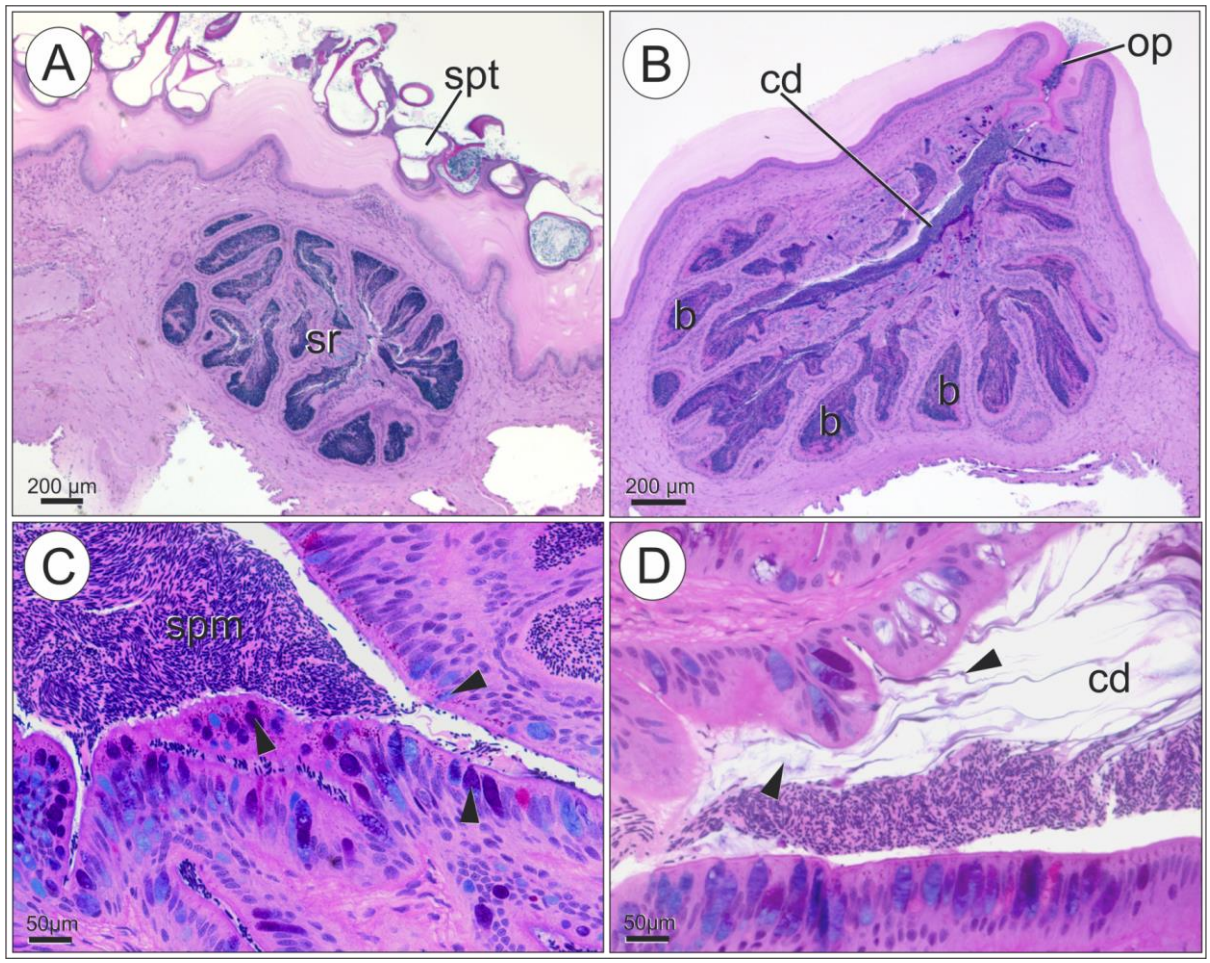

Figure 13 - Sagittal sections of the seminal receptacles from females of *Doryteuthis plei* from experimental Group 1 ("before mating"). AB-PAS-H staining. **A)** Large amount of empty spermatangia attached on the buccal membrane. **B)** Seminal receptacle with stored sperm occupying the internal bulbs and common duct up to the opening. **C)** Detail of the common duct showing spermatozoa heads with no clear organization pattern. Notice all types of secretory cells present in this region (arrowheads), with a slight predominance of AB-PAS cells (purple). **D)** Detail of the secretion being released in the common duct (arrowheads). The secretions were positive for AB and AB-PAS staining. Abbreviations: b, internal bulbs; cd, common duct; op, opening; spm, spermatozoa; spt, spermatangia; sr, seminal receptacle.

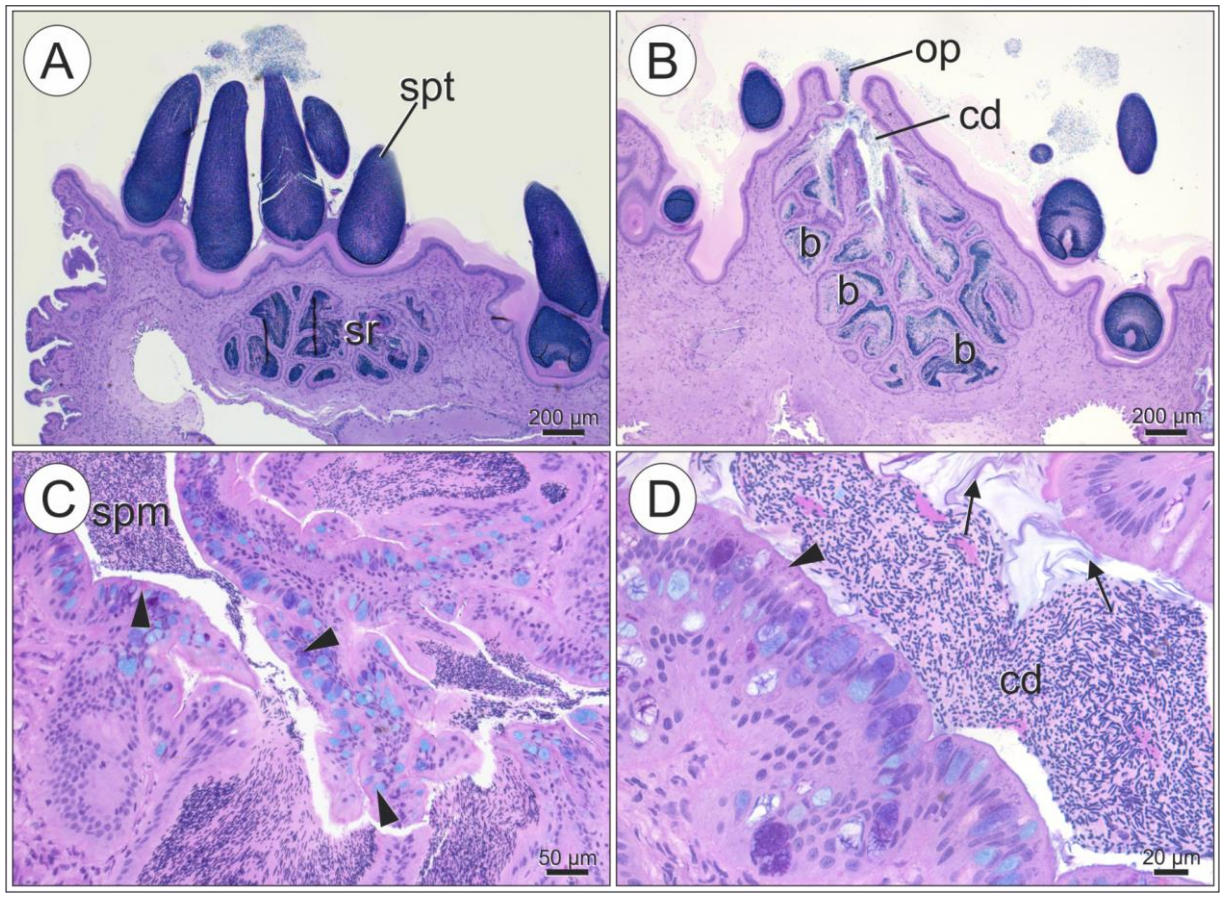

Figure 14 - Sagittal sections of the seminal receptacles from females of *Doryteuthis plei* from experimental Group 2 ("after recent mating"). AB-PAS-H staining. **A)** Large amount of full spermatangia attached on the buccal membrane. **B)** Occasional aspect from females in this group. The seminal receptacle is not completely filled with spermatozoa, with the bulbs close to the opening and the common duct having much less sperm than the more peripheral bulbs. Spermatozoa are also found within the the opening. **C)** Detail of the common duct showing spermatozoa heads with no clear organization pattern. All types of secretory cells are present in this region (arrowheads), with a slight predominance of AB-only cells (blue). **D)** Detail of the secretion being released in the common duct (arrows). The secretions were positive for AB and AB-PAS staining. Although all types of secretory cells are present, PAS-fine-secretory cells were rare (arrowhead). Abbreviations: b, internal bulbs; cd, common duct; op, opening; spm, spermatozoa; spt, spermatangia; sr, seminal receptacle.

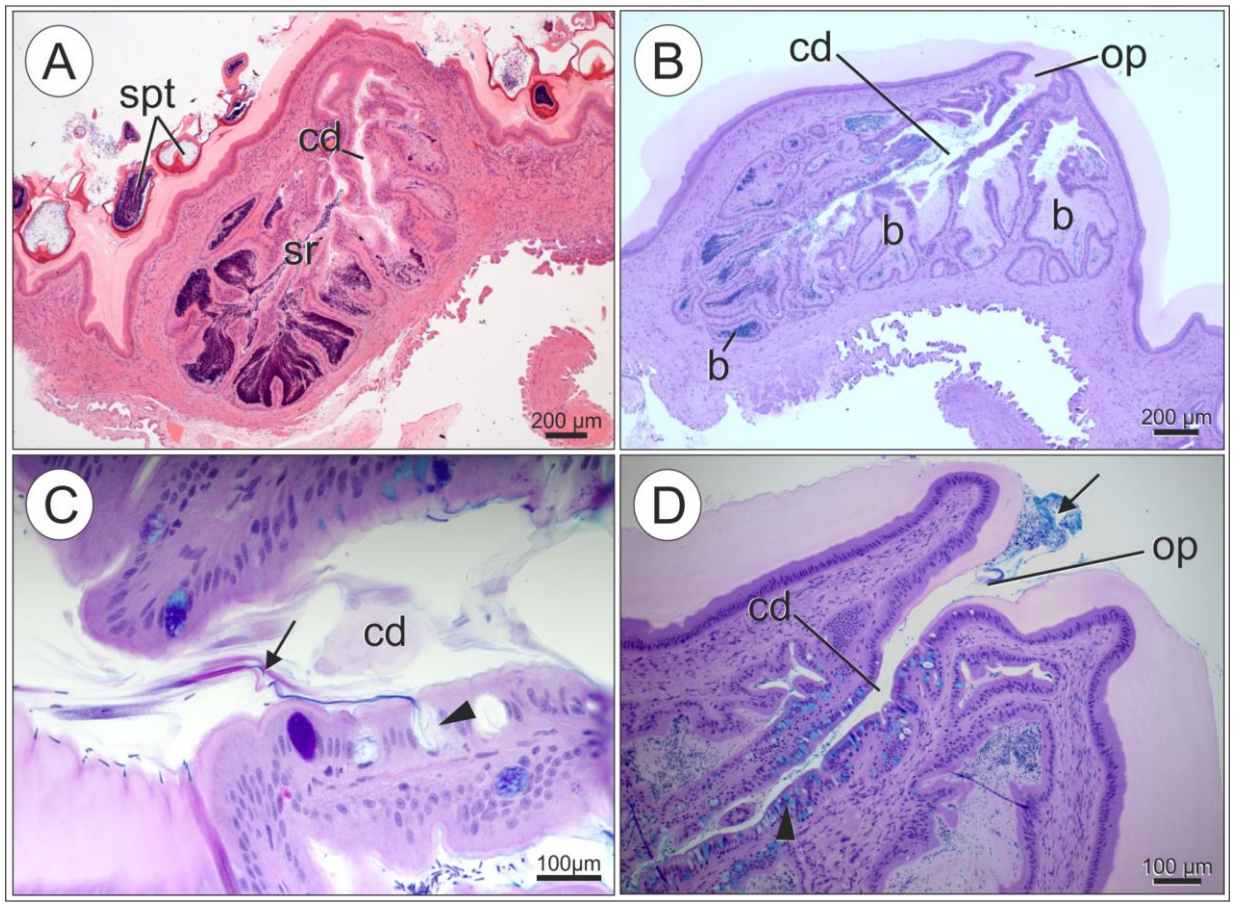

Figure 15 - Sagittal sections of the seminal receptacles from females of *Doryteuthis plei* from experimental Group 3 ("after spawning"). **A)** Large amount of empty or half-full spermatangia attached on the buccal membrane. The region of the commun duct is empty. H&E staining. **B)** General aspect from females in this group. The seminal receptacle is almost empty, except for the most posterior bulbs. AB-PAS-H staining. **C)** Detail of the common duct showing empty secretory cells (arrowhead) and the secretion from these cells (arrow). AB-PAS-H staining. **D)** Detail of the common duct and opening, showing the predominance of AB-only cells (arrowhead) (blue). Notice the large amount of secretions mixed with sperm at the seminal receptacle opening (arrow). AB-PAS-H staining. Abbreviations: b, internal bulbs; cd, common duct; op, opening; spt, spermatangia; sr, seminal receptacle

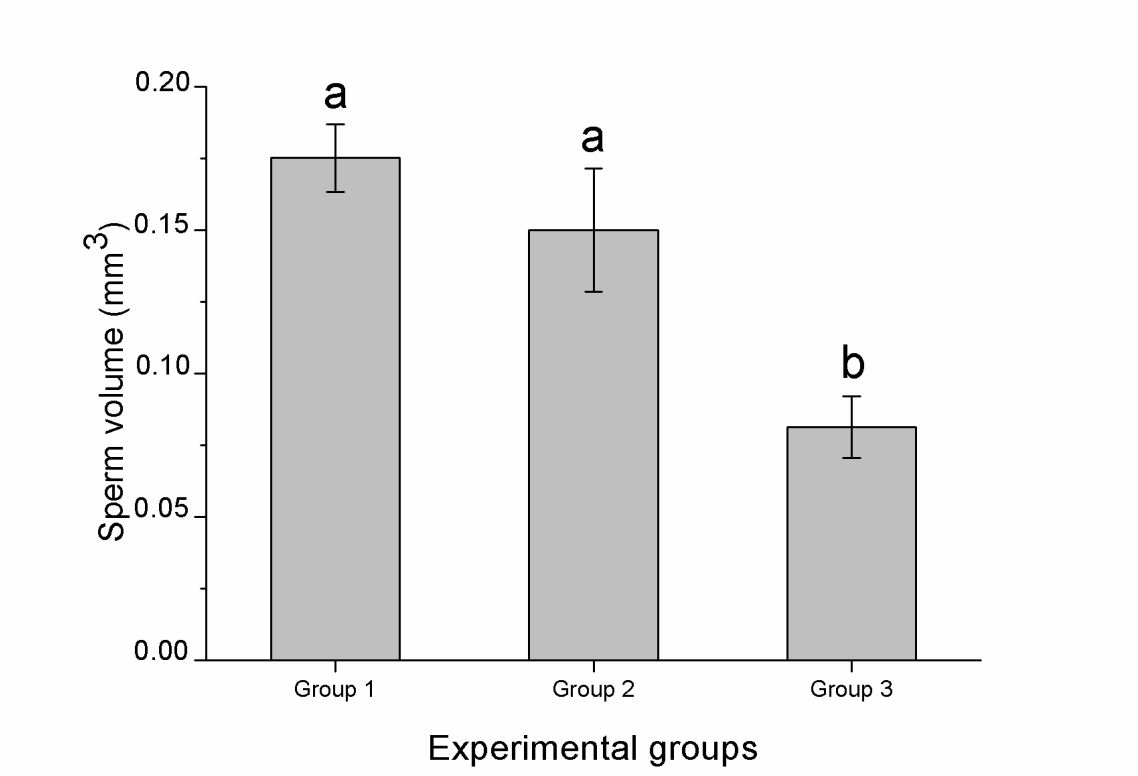

Figure 16 - Comparison between the volumes of stored sperm in the seminal receptacle of the females from the three experimental groups (Group 1: before mating; Group 2: after recent mating; Group 3: after spawning), showing mean and standard error. Different letters represent statistically significant differences ($p<0.05$).

Capítulo 2

A mating plug in a squid? Sneaker spermatophores blocking the female seminal receptacle in *Doryteuthis plei* (Blainville, 1823)

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Abstract

Males from numerous species have evolved a strategy of obstructing the female genitalia with copulatory plugs, reducing the risk of sperm competition and resulting in an advantage in sexual selection. Several lines of evidence suggest that sperm competition is a common feature in the complex squid mating systems, which include the evolution of alternative mating tactics (consort vs. sneaker). However, mating plugs have hitherto not been recorded for the group. During an investigation of the functional morphology of the seminal receptacle of the squid *Doryteuthis plei*, we found everted spermatophores (i.e., spermatangia) implanted into the storage organ and blocking its opening. Here, we describe this finding of "plugged spermatangia" based on microscopy analyses (histology and microCT) of seminal receptacles of females from three experimental manipulations (before and after recent mating and after egg release). We show that sneaker male spermatophores might block the opening of the seminal receptacle, possibly functioning as temporary copulatory plugs that physically obstruct the female storage organ. Plug efficiency should be higher after mating, when spermatangia are turgid and full of sperm, clogging the organ's opening, gradually decreasing its efficiency with time, when spermatangia lose their turgidity by releasing part of their sperm content. However, one experimental female still had a plugged spermatangium blocking a major portion of the opening even after 48 hours without mating. Within the context of squid mating systems and sexual selection, we hypothesize that

plugged spermatangia are a sneaker strategy associated with minimizing sperm competition between sneaker males.

Key Words – Cephalopoda, sexual conflict. copulatory plug, sperm transfer mechanisms

Introduction

Sperm competition – the competition between the sperm of different males for fertilization of a given set of eggs (Parker, 1970) – is considered a powerful selective force in postcopulatory sexual selection, and a major mechanism driving ejaculate and genital evolution in polyandrous taxa (e.g., Birkhead and Pizzari, 2002; Hosken and Stockley, 2004). Together with cryptic female choice – a postcopulatory mechanism involving the female actively biasing the fertilization success of males – sperm competition might result in the complexity and diversity of sperm and genital morphology seen in many animals (e.g., Birkhead and Pizzari, 2002; Hosken and Stockley, 2004; Higginson et al., 2012). Some adaptations resulting from sperm competition, however, are not directly involved with the competition between spermatozoa per se, but with reducing the risk of sperm competition (Wigby and Chapman, 2004; Parker and Pizzari, 2010). Behavioral adaptations (e.g., such as mate guarding and sperm displacement; Parker, 1970; Wigby and Chapman, 2004) are common examples, as well as the obstruction of the female genitalia with some sort of plug, which is a widespread phenomenon across animal taxa (Mann, 1984). The so called "mating" or "copulatory plugs" have already been recorded in several taxa (e.g., nematodes, Timmermeyer et al., 2010; acanthocephalan worms, Poulin and Morand, 2000; arthropods, Parker, 1970; Bauer and Min, 1993; Kuntner et al., 2012; vertebrates, Devine, 1975; Dixson and Anderson, 2002; Schneider et al., 2016), and may vary from coagulating glandular secretions to hard structures or even body parts (e.g., Mann, 1984; Kuntner et al., 2012; Schneider et al., 2016), which are used to seal the female genitalia. This physical barrier, which may be chemically enhanced by seminal fluid components (Mann, 1984; Perry et al., 2013), might temporarily prevent remating or reinsemination, thus reducing the risk of sperm competition.

Cephalopods have complex and diverse reproductive strategies (Rocha et al., 2001). Intricate courtship behavior (Hanlon et al., 1999), male agonistic contests (Hanlon et al., 2002), and even parental care (Bush et al., 2012) have been reported for the group. They are usually promiscuous (Hanlon and Messenger, 1996), and sperm is encased within

spermatophores, which are transferred to the female with the aid of a modified arm in the male (the hectocotylus; Nesis, 1987). Cephalopods spermatophores are generally complex structures capable of functioning autonomously and extracorporeally (Marian, 2012a,b). During transfer to the female, they undergo the so-called spermatophoric reaction, implanting themselves on the female tissue and forming the spermatangium (i.e., everted spermatophore containing the sperm mass; Marian, 2012a,b). From the spermatangium's distal tip (the extremity opposed to the implanted base), sperm is released and may be used immediately for fertilization or stored in the female seminal receptacle (Marian, 2012a,b).

Loliginid squids have two spermatophore deposition sites: within the mantle cavity close to the opening of the oviduct, or at the buccal membrane, where a seminal receptacle is located (Hanlon and Messenger 1996). Large consort males usually perform the "male-parallel" mating, inserting hook-like spermatangia near the oviduct opening, egg-laying usually occurring shortly after (Hanlon et al., 2002; Marian, 2012a; Apostólico and Marian, submitted). Most mature eggs are probably fertilized by sperm released by consort spermatangia as they leave the oviduct opening (Lum-Kong, 1992; Marian, 2012a). Small sneaker males perform the "head-to-head" mating, inserting club-like spermatangia on the female buccal membrane (Hanlon et al., 2002; Apostólico and Marian, submitted). Sneaker sperm might then fertilize the eggs when they pass through the female mouth region (i.e., between the arms) just before they are deposited on the substrate, or they might otherwise be stored within the female seminal receptacle (Hanlon and Messenger, 1996; Hanlon et al., 2002). The squid seminal receptacle is an invagination of the ventral buccal membrane, surrounded by circular muscles and provided with secretory cells that release a substance that keeps the stored sperm immotile (Drew, 1911; Oordt, 1938).

Several lines of evidence suggest that sperm competition is a common feature in the complex squid mating systems, such as polyandry, presence of different sperm storage sites, and complex mating behaviors, such agonistic contests and mate guarding (Hanlon, 1998; Shaw and Sauer, 2004; Iwata et al., 2011; Hirohashi and Iwata, 2013; Hirohashi et al., 2016; Naud et al., 2016). However, to our knowledge, mating plugs have hitherto not been recorded for the group.

During an investigation of the functional morphology of the squid *Doryteuthis plei* seminal receptacle (Saad et al., in prep.[1]), we found spermatangia implanted into the storage organ and blocking its opening. Here, we describe this striking finding based on mating

[1] Capítulo 1.

experiments and microscopy investigation. We suggest that sneaker spermatophores might act as copulatory plugs that temporarily obstruct the female seminal receptacle, while still being capable of releasing sperm to the exterior to fertilize the eggs. We then discuss our findings within the context of squid mating systems and sexual selection.

Material and methods

Animals

Specimens of *Doryteuthis plei* were caught by hand-jigging off the northern coast of São Sebastião Island (Ilhabela municipality, São Paulo state, Brazil), between 23º44'00'' S and 45º17'50'' W, during the summer months of 2014-2016. At the Marine Biology Center of the University of São Paulo (CEBIMar-USP), squids were kept alive in 500 l tanks supplied with a continuous flow of fresh seawater. Until the moment they were used for experiments, squids were fed *ad libitum* with dead shrimps once a day. Specimens were collected under SISBIO/ICMBio permit #44738. Before dissection, female specimens were over-anesthetized using an isotonic solution of $MgCl_2$, following guidelines for the welfare of cephalopods (Moltschaniwskyj et al., 2007).

Microscopic X-ray computed tomography (microCT)

A seminal receptacle from a female (ML 135 mm) with spermatangia obstruction was fixed in 4% paraformaldehyde in 0.1M phosphate buffer (PB) at room temperature (RT) for 2h and stored in PB with 0.1% NaN_3 at 4 ºC. The sample was then post-fixed in 1% osmium tetroxide in PB for 1 hour at room temperature, dehydrated using acidified dimethoxypropane, and embedded in Agar Low Viscosity Resin (Agar Scientific, Stansted, Essex, UK). The resin block containing the sample was mounted vertically and scanned in air. MicroCT scans were obtained with an Xradia microXCT-400 (Carl Zeiss X-ray Microscopy, Pleasanton, CA) using the 4× lens. The sample was scanned at 40kV source voltage and 200µA intensity. Projections were recorded with 40s exposure time (camera binning = 1) and an angular increment of 0.16667°; reconstructed slices measured 2028x2028 pixel. Isotropic voxel resolution of reconstructed volumes was 1.75µm. Image stacks were exported in *.dcm (DICOM) format and imported into Amira 6 (FEI Visualization Sciences Group, Mérignac Cédex, France). The Amira 6 software allowed the combination of different standard

visualization devices for viewing volume data, polygonal surfaces and individual slices, resulting in a 3D reconstruction of the spermatagia within the seminal receptacle.

Experiments

Females (n = 30; ML 100-180mm) were divided into three experimental groups. In Group 1 ("before mating"; n = 10; ML 140-180mm), females were isolated in a tank without males for at least 48 h after capture, and then processed for histological analysis (see below). In Group 2 ("after recent mating"; n = 10; ML 100-166mm), females were first isolated in a tank without males for at least 24 hours after capture. Then, they were each isolated in a tank with 2 to 4 males of different sizes (sneakers and consorts) to stimulate head-to-head mating by sneaker males (Shashar and Hanlon, 2013). They were then observed until head-to-head mating had occurred (from 16 min up to 5 hours). After 30 min of the end of head-to-head mating, the female was removed from the tank and processed for histological analysis. No male-parallel mating had occurred in this group. In Group 3 ("after spawning"; n = 10; ML 120-165mm), females were first isolated in a tank without males for at least 24 hours after capture. Then, each female was isolated in a tank until spawning. Spawning was stimulated by either placing consort males (up to two) or egg strings from other females inside the tank (Buresch et al., 2009). The female was removed from the tank and processed for histological analysis from 30 min (when spawning was followed by an observer) to ca. 10 h after spawning (when spawning occurred at night, with no observer). All eggs strings were checked for the occurrence of fertilization.

Histology

The dissected seminal receptacles were fixed in a modified Karnovsky's fixative (see Marian, 2012a) for 4 h at 4°C, dehydrated in a graded ethanol series and embedded in glycol-methacrylate resin (Leica Historesin Embedding Kit, Leica Microsystems Nussloch GmbH, Germany). Serial sagittal sections (3 µm) were obtained on a Leica RM2255 microtome (Leica, Wetzlar, Germany), and stained with hematoxylin and eosin (HE), toluidine blue (TB), or with a combination of Alcian blue, periodic acid-Schiff and hematoxylin (AB-PAS-H) (see Saad et al., in prep.). Digital images were captured using a Carl Zeiss Axio Imager M2 microscope (Carl Zeiss, Germany).

Results

The kidney-shaped seminal receptacle of *Doryteuthis plei* is located at the ventral region of the buccal membrane (Fig. 1A), has a single anterior opening (Fig. 1B-D), and is surrounded by a depression of the buccal membrane (Fig. 1B-D), where spermatangia are usually found attached (Fig. 1C,D; 2A). Internally, the receptacle is divided into several interconnected bulbs leading to a common duct, which connects the stored sperm to the exterior (Fig. 1D). Spermatangia found externally on the buccal membrane are generally club-like (i.e., with a broader base and tapering towards the distal tip; Fig. 1C, 2A,B), typical of sneaker male spermatangia (Marian 2012a; Apostólico and Marian, submitted). They are composed of the cement body, which is implanted into the seminal receptacle protective cuticle, and by the inner tunic, which envelops the sperm mass, sperm being released at the distal tip (Fig. 2B).

Spermatangia were also found inside the seminal receptacle (hereafter referred to as "plugged spermatangia"), in both wild (Fig. 2) and experimental samples (Fig. 3-5). Although some plugged spermatangia had the club-like morphology as those found externally (Fig. 2D; 3B), some were convoluted and intertwined with each other, although a broader base was still present (Fig. 2D,E; 3B).

In all experimental groups, we found spermatangia inside the seminal receptacle, but they differed in frequency and aspect. In Group 2 ("after recent mating") they were not only more frequent (60% of the females) than in Group 1 ("before mating"; 30%) and Group 3 ("after spawning"; 40%), but they were also generally very turgid and full of sperm, indicating they were freshly implanted (Fig. 3B; Fig. 4A; Table 1). In Groups 1 and 3 (Fig. 3A,C; Table 1), they were usually empty (Fig. 5C), with intermediate content (Fig. 5A,B) or present just as remnants, in some cases only recognizable by the presence of parts of the cement body or of the inner tunic (Fig. 3C; Fig. 5D). Full, intermediate, empty and remant spermatangia most probably correspond to different copulation events, from more recent to older matings, respectively. Up to three plugged spermatangia were found within a single seminal receptacle; in several cases of multiple plugs, they presented distinct aspect (Fig. 4A; Table 1).

In all cases of plugging, the cement body was found implanted inside the organ (e.g., Fig. 3B; 4A,C; 5D). The distal tip of the spermatangium, however, could either (1) pass through the opening of the organ and release sperm to the exterior (Fig. 2A,C,D,E; 3B; 4A;

Table 1); or (2) be located inside the organ, with the tip facing the aperture of the seminal receptacle and apparently releasing sperm to the exterior (Fig. 3B; Table 1); or (3) be located inside the organ, but with the tip pointing to the internal bulbs or common duct, sperm release occurring inside the organ (Fig. 4A,B; Table 1).

Only full spermatangia (present only in Group 2) appear to effectively block the opening of the seminal receptacle, because of their turgidity, which pressed their inner tunic against the walls of the opening of the seminal receptacle (Fig. 3B, 4A). Intermediate spermatangia (found in Groups 1 and 3) might partially obstruct the opening (Fig. 5A,B), while empty spermatangia and remnants apparently offer little resistance to sperm passage (Fig. 5C,D). The coiling of some plugged spermatangia, with some of them even intertwined (Fig. 2D,E; Fig. 3B), also appears to contribute to block the passage of stored sperm to the exterior.

Plugged spermatangia implantation was often associated with some kind of rupturing or tearing of the internal epithelium of the organ, in all experimental groups (Fig. 4C; 5C,D). Near the attachment of the plugged spermatangium, the epithelium was often absent or separated from the underlying connective tissue (Fig. 4C). Moreover, plugged spermatangia displaced stored sperm, occupying the more anterior space of the seminal receptacle (Fig. 3B; 4A). In Group 3, the seminal receptacle was evidently emptier than in Groups 1 and 2, with sperm present only in the posterior internal bulbs (Fig. 3C), evidencing the use of stored sperm for spawning.

Discussion

We have shown that sneaker male spermatophores might block the opening of the seminal receptacle in *Doryteuthis plei*, possibly functioning as copulatory plugs that physically obstruct (from partially to almost completely) the female storage organ. Experimental manipulations together with morphological data suggest that plug efficiency should be higher after mating, when spermatangia are full and turgid, clogging the organ's opening, gradually decreasing its efficiency with time, when spermatangia lose their turgidity by releasing part of their sperm content. However, one experimental female still had a plugged spermatangium blocking a major portion of the opening even after 48 hours without mating (Fig. 5A). Previous *in vitro* experimentation demonstrated that sperm release by sneaker spermatangia might last more than 5 hours, although they still have considerable

residual sperm even after 24 hours (Apostólico and Marian, in prep.). This suggests plug efficiency should be higher from immeadiately after mating until at least one day.

The autonomous evagination process that occurs during spermatophore transfer (i.e., the spermatophoric reaction) is responsible for anchoring, implanting and attaching the spermatangium on the female tissue (Marian, 2011; 2012a; 2012b; Marian et al. 2012). The everting ejaculatory apparatus of the spermatophore is responsible for anchoring on and tearing of the female tissue, opening a puncture for final chemical and physical adhesion by the cement body (Marian, 2011; 2012a; 2012b; Marian and Domaneschi, 2012; Marian et al., 2012). The aspect of the plugged spermatangia was similar to spermatangia implanted outside on the buccal membrane: the cement body is adhered to a region where there is damaged, scarified tissue (Marian, 2012a). Since all plugged spermatangia were found with their cement bodies attached inside the organ, it is reasonable to suppose that they were autonomously implanted within the seminal receptacle through the spermatophoric reaction, most probably not being forcibly inserted by the male. The fact that some plugged spermatangia were found with a convoluted morphology reinforces this assumption, because the spatial constraints within the seminal receptacle could affect the spermatophoric reaction and, hence, spermatangium formation.

During copulation, the male transfers several spermatophores to the female (Drew, 1919). Presumably, during this process, most spermatophores are implanted outside the organ, while some of them (up to three in our sample), possibly those positioned over the seminal receptacle's opening, might get plugged within the organ. It is not known if the male has any control of this process, since several females did not have plugged spermatangia, even after recent head-to-head mating (Table 1).

When spermatangia are implanted externally, the outer epihelium of the seminal receptacle remains generally intact because of the protection provided by its thick cuticle (Fig. 2B), but we have shown that plugged spermatangia result in injury to the seminal receptacle's inner epithelium (Fig. 5). Moreover, these plugs, if indeed efficient, might decrease the genetic variation of the female offspring, as present data on other loliginids suggest multiple paternity is a common feature of their mating systems (Shaw and Sauer, 2004; Buresch et al., 2009; Naud et al., 2016), and that seminal receptacles presumably store sperm from several males (Hanlon and Messenger, 1996; Naud et al., 2016). Therefore, sexual conflict might play a role in this process, and females might have evolved some mechanism to avoid plugs (e.g., Hosken and Stockely, 2004), which would also explain the

several females missing plugs after recent mating (Table 1). Females of other taxa usually respond to mating plugs by expelling them from their reproductive system (e.g., Devine, 1975), but expelling plugged spermatangia from the seminal receptacle might be delayed because of the strong adhesion provided by the cement body (e.g., Marian, 2012a). This could explain the presence of plugs of varied aspects even after 48 hours without mating, and even after spawning (Table 1). However, recent data on the structure of the seminal receptacle of *D. plei* suggest a high degree of female control over sperm uptake, storage and release (Saad et al., in prep.), so a female strategy to avoid or expel plugs in this case would not be surprising.

The dynamics of fertilization in squids are still obscure. Egg capsule formation probably starts as the eggs are released from the oviduct (Boletzky, 1986), so sperm from consort spermatangia, which are attached near the oviduct opening, probably have first access to the eggs (Naud et al., 2016). Sperm from sneaker spermatangia, which are placed on the buccal membrane, would presumably only contact the eggs as the capsule is transferred to the space between the arms of the female, just before the capsule is deposited on the substrate (e.g., Fields, 1965; Naud et al., 2016). Sperm stored within the seminal receptacle are probably from sneaker spermatangia, although the dynamics of sperm attraction into the seminal receptacle are still unknown (Hanlon et al., 1999; Sato, et al., 2010; Bush et al., 2012; Hirohashi and Iwata, 2013; Saad et al., in prep.). Several hypotheses have been proposed to explain this process, such as chemical attraction, sperm active swimming, or muscular sucking action by the seminal receptacle, but further investigations are needed to test them (see review in Saad et al., in prep.). The fact is, spermatangia blocking the opening of the female storage organ might prevent the release and subsequent use of stored sperm from previous matings, and also prevent or pose difficulties for the storage of sperm from subsequent matings.

The strategy of preventing both the use and storage of sperm from previous and subsequent matings, respectively, is advantageous as long as the male employing the plug also provide enough sperm for fertilization. In all cases of plugged spermatangia in Group 2, spermatangia were also found implanted externally. Given that both external and plugged spermatangia often showed the same aspect (full and turgid), we assume they are from the same male. Since sneaker spermatangia might retain considerable sperm content for at least 24 h (Apostólico and Marian, in prep.), depending on the time of female spawning, plugged spermatangia might successfully prevent use of previous sperm while both plugged and

external spermatangia would still provide sperm for fertilization. However, if the time interval between mating and spawning is too long, the male employing spermatangia as plugs would be under the risk of preventing its own sperm from being used for fertilization, given that his own spermatozoa would be prevented from being stored within seminal receptacle. However, several females in our sample had some plugged spermatangia with their tips releasing sperm also inside the organ (Table 1), which suggests that, in these cases, the male employing the plugs would not only prevent use of previous sperm, but also guarantee that some of his sperm are also stored within the organ for future use.

Fertilization success is often much higher for consort males. For the three loliginid species for which paternity rates are available (*D. bleekeri*, Iwata et al., 2005; *D. pealei*, Buresch et al., 2009; *L. reynaudii*; Naud et al., 2016), consort males have a minimum of 70% overall fertilization success. We suggest that plugged spermatangia are employed only by sneaker males (because of their typical club-like morphology), and that they would not affect fertilization success of consort males, being rather a strategy related to sperm competition between sneaker males. Recent data on *D. plei* male dimorphism revealed that sneaker males show higher gonadal investment, and produce spermatophores with discontinuously smaller sperm mass content than consort males (Apostólico and Marian, submitted). So sneakers apparently maximize their reproductive success by both investing more in gonad growth and partitioning their sperm mass into extra copulations, consort males investing more in somatic growth and, hence, mate guarding (Apostólico and Marian, submitted). In this context, we hypothesize that plugged spermatangia are an additional sneaker strategy, but associated with minimizing sperm competition between sneaker males.

Although several studies aimed at investigating the structure of the seminal receptacle in diverse cephalopods (e.g., *Loligo forbesi*, Lum-Kong, 1992; *Loligo vulgaris*, Oordt, 1938); *Doryteuthis pealeii*, Drew, 1911; *Bathyteuthis berryi*; Bush et al., 2012; *Idiosepius paradoxus*, Sato et al., 2010; *Sepia officinalis*, Hanlon et al., 1999; *Sepia apama*, Naud et al., 2005; *Todarodes pacificus*, Ikeda et al. 1993), plugs obstructing the female sperm storage organ have never been recorded for the group. Only Oordt (1938) mentioned that "sometimes, a whole sperm-reservoir may be found in the interior of the spermatheca" in *L. vulgaris*, but he speculated that spermatophores would be sucked into the seminal receptacle by muscular action, not mentioning any kind of obstruction. At least for *D. plei*, we reject the hypothesis of a sucking action to explain the presence of spermatangia within the organ, due to evident implanted aspect of these plugged spermatangia.

Mating plugs blocking the access to female genitalia have evolved many times independently in Metazoa (Birkhead and Moller, 1998; Colonello and Hartfelder, 2005; Uhl et al., 2010). While copulatory plugs in most taxa are composed of coagulating or solidifying secreted substances (e.g., insects, Parker, 1970; Ehrlich and Ehrlich, 1978; Polak et al., 1998; crustaceans, Bauer and Min, 1993; snakes, Devine, 1975; rodents, Baumgard et al., 1982; and primates, Dixson and Anderson, 2002), hard secreted plugs are also found (e.g., crustaceans; Spalding, 1942; arachinids, Peretti, 2003), including severed body parts (fragments of the copulatory organs in some insects, Colonello and Hartfelder, 2005; Fromhage and Schneider, 2006; and some arachnids; Contreras-Garduno et al., 2006). Squids are apparently another example of animals that employ their spermatophores not just for sperm transfer, but also for blocking the female genitalia, a strategy also observed in some insects (e.g., *Rhodnius*, *Melolontha melolontha*, *Locusta migratoria migratoides*, *Euthystira brachyptera* and *Chrysochraon dispar*; Davey, 1958; Davey, 1960; Mann, 1984).

The present finding of spermatangia blocking the female storage organ of *D. plei* should stimulate future studies regarding copulatory plugs in squids. For example, experimental manipulations associated with paternity analyses could confirm their role and efficiency as mating plugs. Also, investigations of the chemical properties of sneaker spermatangia might reveal other factors influencing their effectiveness, such as substances with sex-repellent properties or that might inhibit female receptivity (e.g., Mann, 1984; Baer et al., 2000; Shine et al., 2000; Bretman et al., 2010; Perry et al., 2013). Understanding the exact role of plugged spermatangia would help gaining insight into the pressure of sexual selection mechanisms (e.g., sperm competition, cryptic female and sexual conflict) within the complex mating systems of cephalopods.

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TABLE

Table 1 - Frequency, number, aspect and location of sperm release of the plugged spermatangia in the experimental groups (G 1 = before mating; G 2 = after recent mating; G 3 = after spawning). Full: fresh, turgid spermatangium from recent mating; intermediate: spermatangium with considerable sperm content, but not full and turgid; empty: spermatangium with almost all sperm content released; remnant: spermatangium identifiable by the presence of parts of the cement body or inner tunic. The position of the distal tip of the spermatangium determined if sperm release occurred inside or outside the organ; this information could not be confirmed for a collapsed spermatangium. Abbreviation: ML, mantle length.

Female	ML (mm)	Number	Aspect	Sperm release
G 1 (n=3/10)				
Female 1	160	1	1 empty	1 inside
Female 2	140	1	1 intermediate	1 outside
Female 3	170	1	1 remnant	1 collapsed
G 2 (n=6/10)				
Female 1	152	1	1 full	1 inside
Female 2	100	1	1 full	1 outside
Female 3	156	2	2 full	1 inside, 1 outside
Female 4	166	2	1 full, 1 remnant	1 inside, 1 collapsed
Female 5	120	3	3 full	2 outside, 1 inside
Female 6	120	3	2 full, 1 empty	1 outside, 1 inside, 1 collapsed
G 3 (n=4/10)				
Female 1	135	1	1 empty	1 collapsed
Female 2	120	1	1 empty	1 collapsed
Female 3	146	3	1 intermediate, 2 empty	1 outside, 2 collapsed
Female 4	160	1	1 remnant	1 collapsed

FIGURES

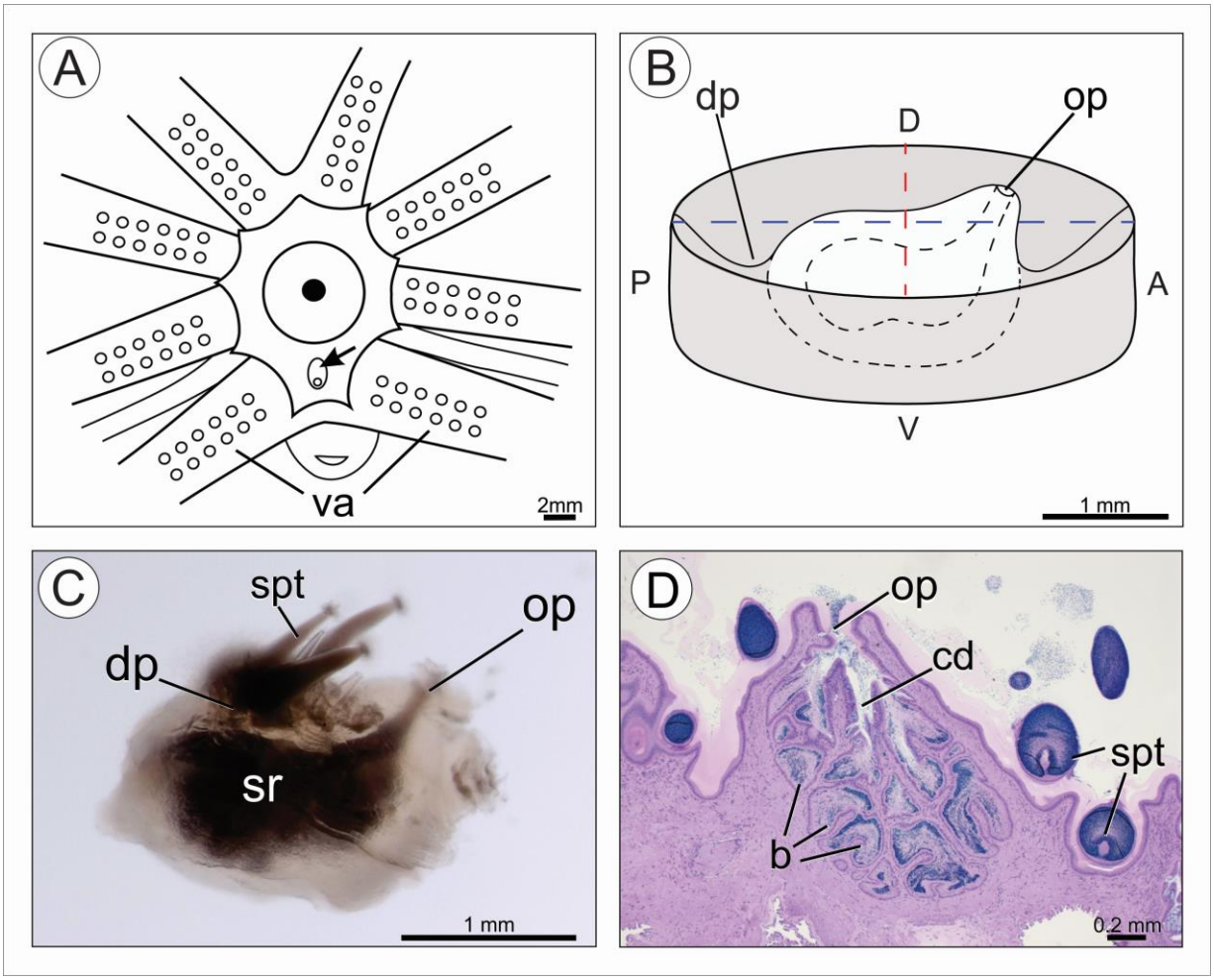


Figure 1 - General structure of the seminal receptacle of *Doryteuthis plei*. **A)** Schematic illustration of the oral view of the arms. The seminal receptacle (arrow) is located on the ventral region of the buccal membrane between left and right ventral arms. **B)** Schematic illustration of the lateral view of the seminal receptacle. The opening is dorsal and located anteriorly. Spermatangia are usually implanted at the buccal membrane depression around the organ. **C)** Lateral view of the dissected seminal receptacle, showing the spermatangia attached at the depression around the organ and close to the seminal receptacle opening. **D)** Sagittal section through the seminal receptacle showing its general organization. Several interconnected bulbs lead to a common duct and a single opening. Spermatangia are usually implanted externally to the seminal receptacle. AB-PAS-H staining. Abbreviations: A, anterior; b, internal bulbs; cd, common duct; D, dorsal; dp, buccal membrane depression; op, opening; spt, spermatangia; P, posterior; sr, seminal receptacle; V, ventral; va, ventral arms. Figure 1A and 1B originally published in Saad et al. (in prep.) and reproduced with permission

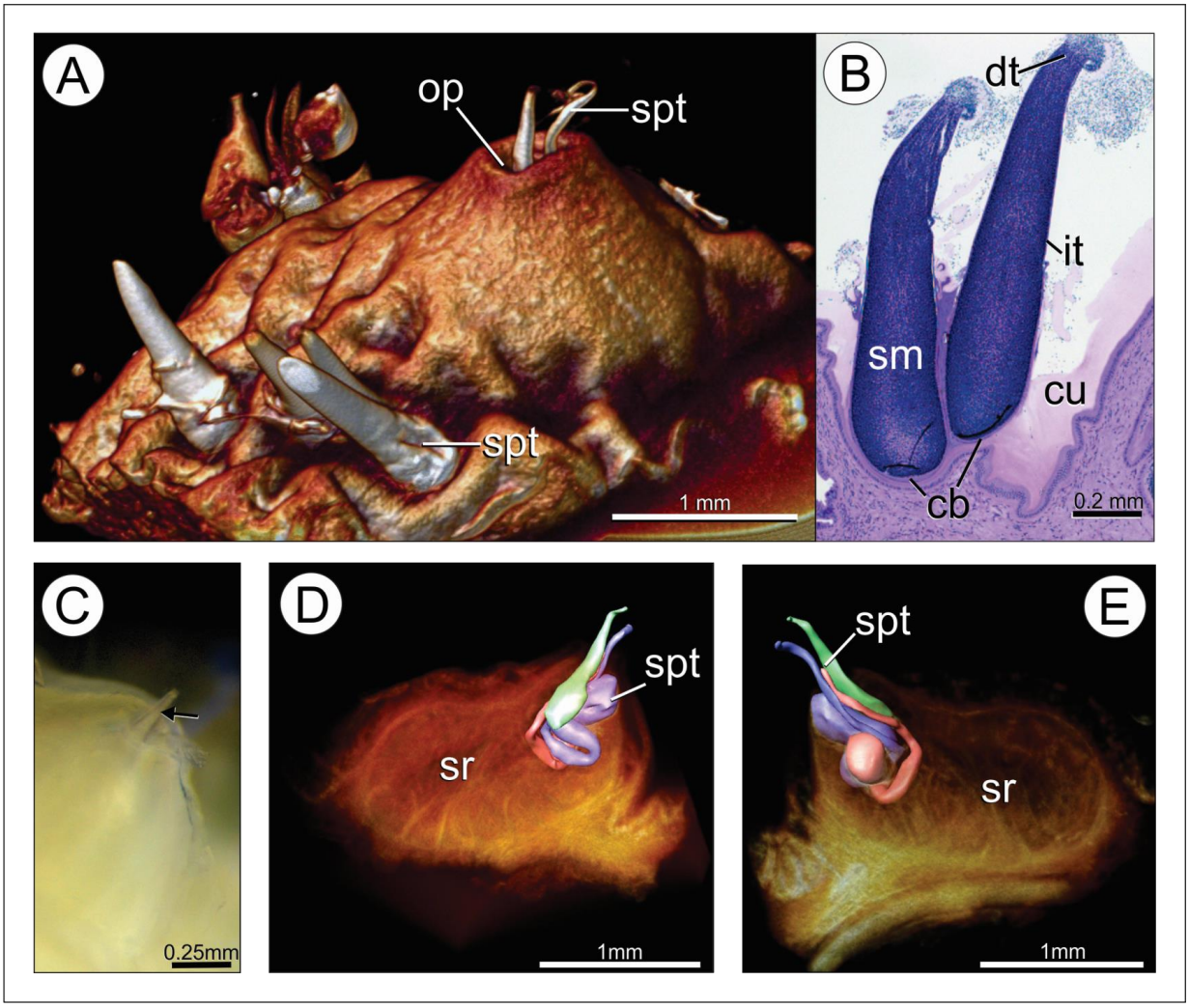
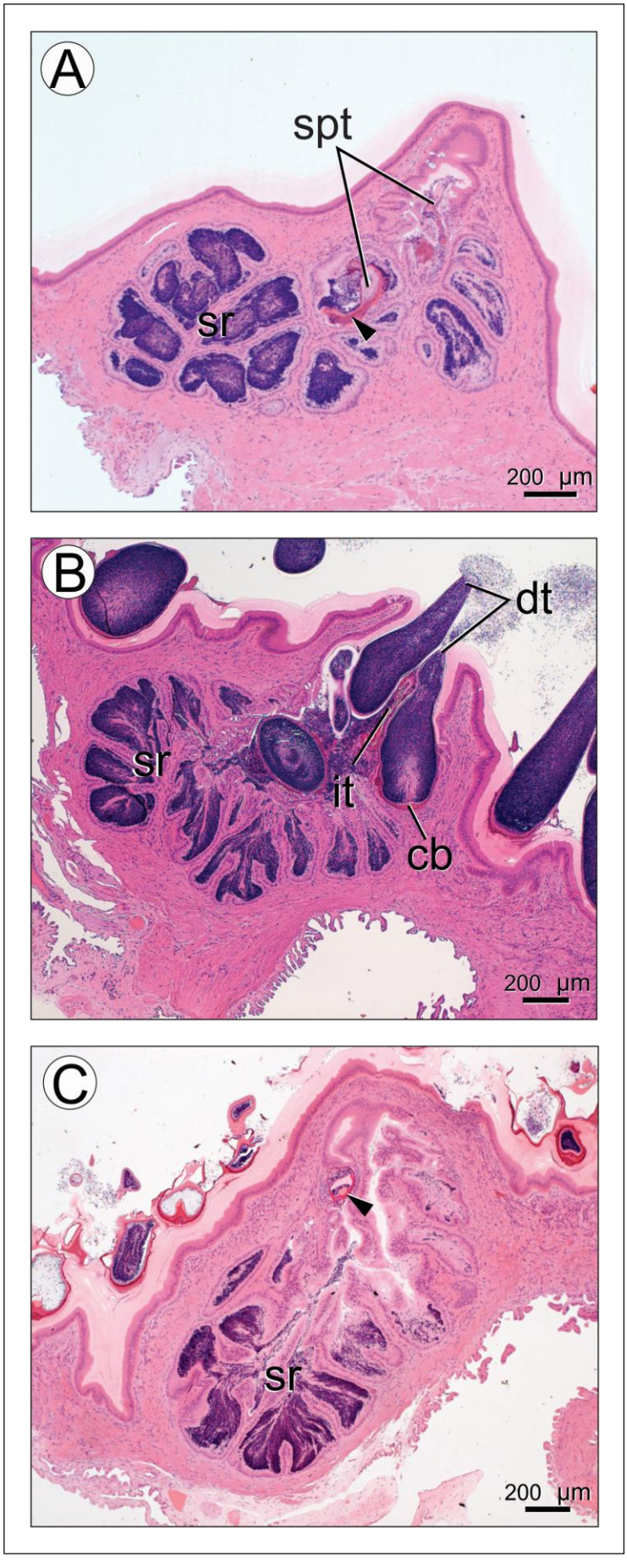


Figure 2 - General structure of spermatangia attached on the buccal membrane and plugged within the seminal receptacle. **A)** MicroCT scan showing the anterolateral view of the seminal receptacle. Spermatagia are attached externally, at the cuticle layers, and internally, passing through the seminal receptacle opening. Notice the club-like shape of the external spermatangia; *volren* module. **B)** Sagittal section through spermatangia attached externally on the buccal membrane. Spermatangia are attached at the cuticle layers by the cement body, and the inner tunic envelops the sperm mass. Sperm are released at the distal tip. AB-PAS-H staining. **C)** Lateral view of the seminal receptacle opening, showing the distal tip of a plugged spermatangium (arrow) protruding through the opening. **D-E)** MicroCT scans with transparency intensified, showing three plugged spermatangia within the seminal receptacle (marked in blue, green and pink). Notice the convoluted and intertwined form of two spermatangia; lateral views. Abbreviations: dt, distal tip; cb, cement body; cu, cuticle; it, inner tunic; op, opening; sm, sperm mass, spt, spermatangia; sr, seminal receptacle;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dt
sr
it
cb
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C
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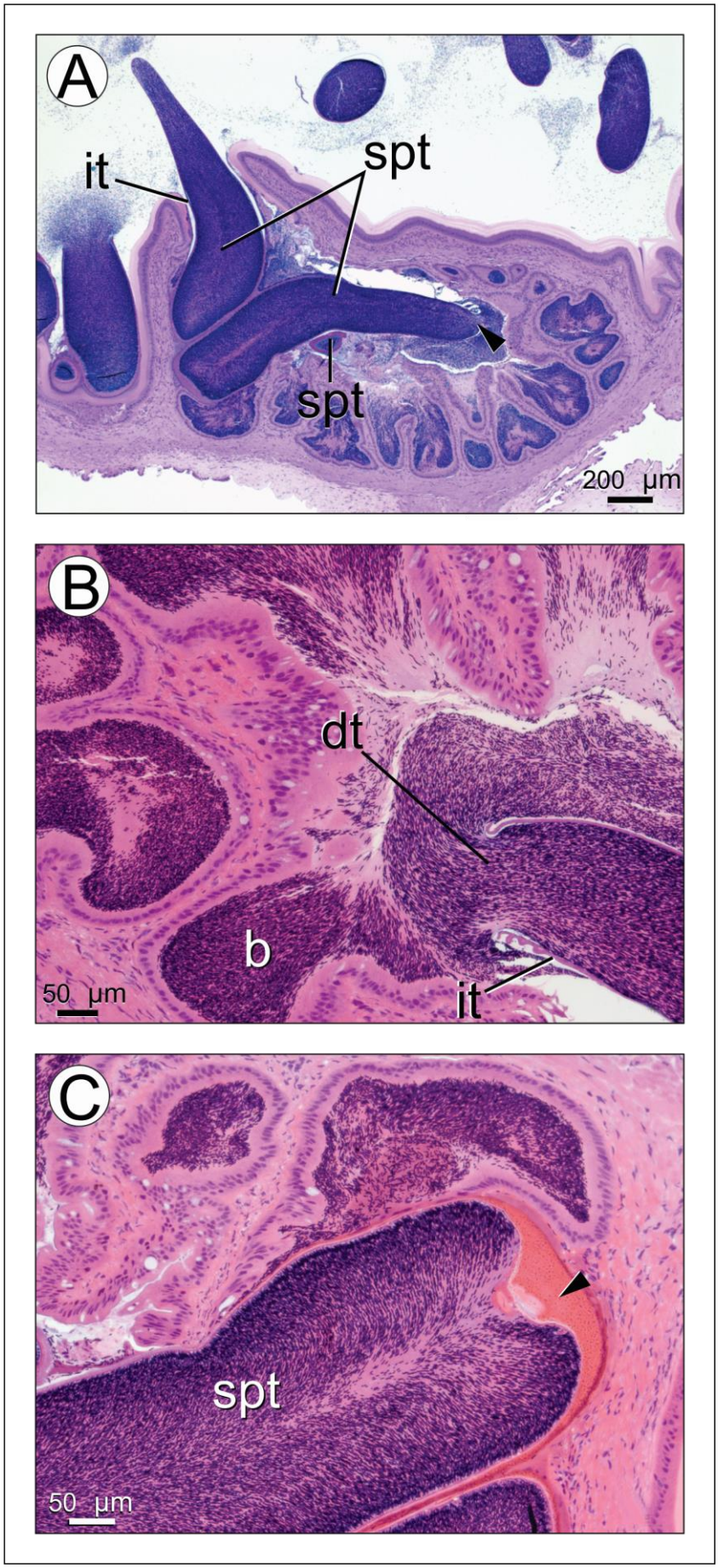
Figure 3 - General view of the seminal receptacle of experimental females with plugged spermatangia. **A)** Sagittal section through the seminal receptacle from a female from Group 1 ("before mating"), showing an empty and convoluted spermatangium. The spermatangium cement body is attached at the seminal receptacle (arrowhead). H&E staining. **B)** Sagittal section through the seminal receptacle from a female from Group 2 ("after recent mating"), showing at least two very turgid and fresh plugged spermatangia, also attached internally by the cement body. Two plugged spermatangia are evidently blocking the seminal receptacle opening. The plugged spermatangium at right is releasing sperm outside the organ but with its distal tip inside. The plugged spermatangium at left is convoluted, helping to block the seminal receptacle opening; its distal tip passes through the opening of the organ and release sperm to the exterior. H&E staining. **C)** Sagittal section through the seminal receptacle from a female from Group 3 ("after spawning"), showing the remnants of a spermatangium, with only the cement body recognizable (arrowhead). Notice that the seminal receptacle anterior bulbs are empty. H&E staining. Abbreviations: dt, distal tip; cb, cement body; it, inner tunic; spt, spermatangia; sr, seminal receptacle.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it
C
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Figure 4 - Sagittal sections through the seminal receptacle of females from Group 2 ("after recent mating"), showing freshly plugged spermatangia. **A)** Seminal receptacle with 3 plugged spermatangia (2 fresh, 1 empty). One of the fresh spermatangia has its distal tip pointing to the internal bulbs, releasing sperm inside the organ (arrowhead). The other one is releasing sperm to the exterior, while blocking the seminal receptacle opening. AB-PAS-H staining. **B)** Detail of the plugged spermatangium from A releasing sperm inside the organ. H&E staining. **C)** Detail of the cement body (arrowhead) of a plugged spermatangium. Notice that near the attachment of the plugged spermatangium, the epithelium is injured and the cement body is in direct contact with the underlying connective tissue. H&E staining. Abbreviations: b, internal bulbs; dt, distal tip; it, inner tunic; spt, spermatangia.

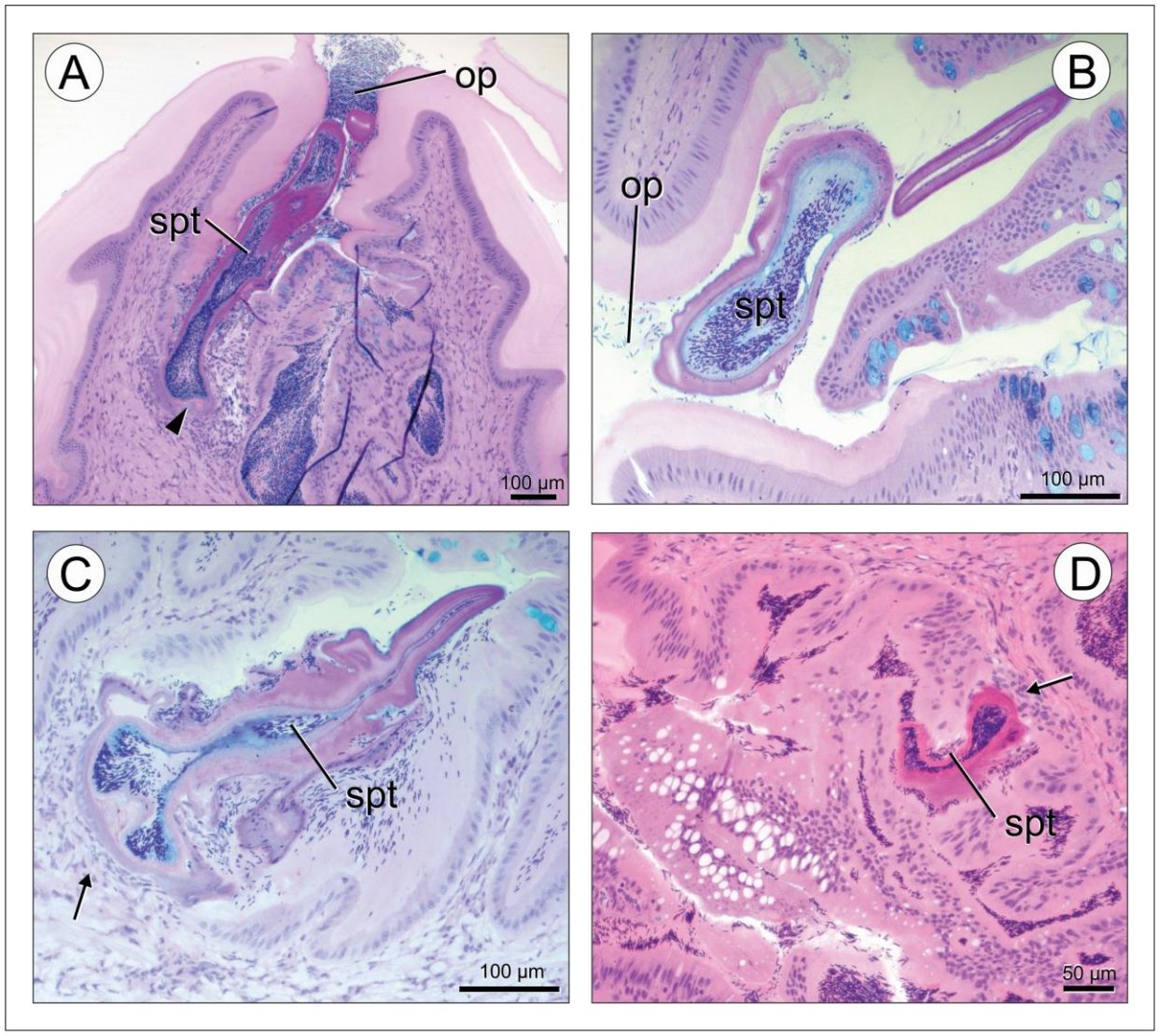

Figure 5 - Sagittal sections through the seminal receptacle of females from experimental groups 1 and 3, showing plugged spermatangia. **A)** Intermediate spermatangium partially obstructing the seminal receptacle opening in a Group 2 female. The cement body is implanted within seminal receptacle (arrowhead). AB-PAS-H staining. **B)** Intermediate spermatangium partially obstructing the seminal receptacle opening in a Group 3 female. AB-PAS-H staining**. C)** Empty spermatangium attached within the seminal receptacle in a Group 3 female. Notice the missing epithelium and the damage to the underlying connective tissue (arrow). AB-PAS-H staining. **D)** Remnant of a plugged spermatangium in a Group 3 female, with only part of the inner tunic present. The tissue were the spermatangium is attached seems to be regenerating (arrow). H&E staining. Abbreviations: op, seminal receptacle opening; spt, spermatangia.

Considerações Finais

A presente dissertação objetivou compreender a função do receptáculo seminal da lula costeira *Doryteuthis plei* e investigar se esse órgão de armazenamento de espermatozoides está envolvido em mecanismos pós-copulatórios de seleção sexual. Apesar de haver estudos morfológicos acerca do receptáculo seminal de cefalópodes (WILLIAMS, 1909; DREW, 1911; OORDT, 1938; LUM-KONG, 1992; IKEDA et al., 1993; HANLON et al., 1999; NAUD et al., 2005; SATO et al., 2010; BUSH et al., 2012; MARIAN, 2012), a estrutura desse órgão é em geral pouco detalhada, dificultando a compreensão de mecanismos de captação, armazenamento e liberação de espermatozoides pelo órgão, bem como sua influência em aspectos de seleção sexual, como escolha críptica da fêmea e competição espermática.

Dessa forma, a primeira parte da dissertação consistiu em analisar detalhadamente a morfologia do receptáculo seminal a partir da integração de diversas técnicas de microscopia (óptica, eletrônica, confocal e microCT) e pela análise da estrutura do órgão em três situações distintas (i.e., antes e após cópula recente e após a desova) obtidas a partir de manipulações experimentais. Os resultados revelaram enorme complexidade do receptáculo seminal com relação à diversidade de células secretoras e de fibras musculares associadas. Constatou-se que há alteração das células secretoras após a cópula recente e após a desova, com liberação do conteúdo de parte dessas células. Com base nos resultados obtidos, discute-se o papel dessas secreções na captação, armazenamento e liberação de espermatozoides. Além disso, a estrutura dos sistemas nervoso e muscular associados ao receptáculo seminal sugere que a fêmea tenha controle sobre deformações precisas do órgão relacionadas à captação e liberação de espermatozoides. Entretanto, outros mecanismos seriam igualmente possíveis, como atração química, natação ativa dos espermatozoides e ação ciliar do órgão (OORDT, 1938; HANLON et al., 1999; SATO et al., 2010). Neste trabalho, foi possível ainda observar que o volume de espermatozoides armazenados apresentou grande variação dentro dos experimentos “após cópula recente” e “após a desova”, sugerindo algum mecanismo de seleção de espermatozoides para armazenamento e fertilização, respectivamente. Como diversas questões acerca do funcionamento e papel do órgão permanecem enigmáticas, sugere-se a expansão desses estudos a outras espécies de lulas e sépias que apresentam receptáculos, bem como a continuidade das investigações baseadas em manipulações experimentais, associadas também a análises de paternidade.

Durante o estudo da morfologia do órgão, alguns receptáculos seminais foram encontrados com espermatângios bloqueando total ou parcialmente sua abertura, possivelmente obstruindo a entrada ou saída de espermatozoides. A segunda seção da dissertação abordou essa questão com base em análises de microCT e análises histológicas de espécimes oriundos das manipulações experimentais da seção anterior. Devido à frequência, posição e morfologia dos espermâtangios dentro do receptáculo seminal, propõe-se a hipótese de que espermatângios de machos *sneaker* poderiam atuar como plugues copulatórios. Esses plugues seriam mais eficientes nas primeiras 24 horas, sua eficiência sendo gradualmente reduzida com o tempo após a cópula, devido ao esvaziamento do conteúdo espermático e consequente redução de sua turgidez. A ocorrência de espermatângios bloqueando a abertura do receptáculo seminal é frequente, inclusive em diferentes situações experimentais (antes e após cópula recente e depois de desova), sugerindo que esse fenômeno deva influenciar inúmeros aspectos pós-copulatórios (e.g., competição espermática, conflito sexual). Embora plugues copulatórios sejam conhecidos em inúmeras espécies de animais (e.g., PARKER, 1970; DEVINE, 1975; BAUMGARD et al., 1982; TONER et al., 1987; BAUER & MIN, 1993; LUNG & WOLFNER, 2001; DIXSON & ANDERSON, 2002; BRETMAN et al., 2010), este é o primeiro registro para cefalópodes, sendo, portanto, marco importante nos estudos de seleção sexual para o grupo. Futuros estudos devem focar na confirmação do papel e eficiência desses espermatângios como plugues, a partir, por exemplo, de investigações com base em experimentação associada a análises de paternidade.

Em suma, o receptáculo seminal de *D. plei* revelou-se mais complexo do que o que havia sido documentado até então para cefalópodes. Isso se deve, possivelmente, à aplicação de distintas ferramentas de análise morfológica e ao caráter experimental deste trabalho. Cefalópodes são considerados modelos interessantes para o estudo de seleção sexual e os resultados desta dissertação contribuem para compreensão dos complexos mecanismos pós-copulatórios em lulas, como escolha críptica da fêmea e competição espermática.

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Resumo

A seleção sexual atua de forma significativa na evolução de características reprodutivas. Os machos apresentam diversas estratégias para garantir a fertilização da fêmea, a qual, por sua vez, possui papel crucial em processos pré- e pós-copulatórios. Nesse contexto, cefalópodes apresentam aspectos reprodutivos peculiares, apresentando transferência de espermatozoides via espermatóforos e, em alguns casos, armazenamento desses gametas em órgãos especializados (i.e., receptáculos seminais). Entretanto, pouco se sabe sobre a morfologia, funcionamento e mecanismos de captação, armazenamento e liberação dos espermatozoides pelos receptáculos seminais. Neste contexto, a presente dissertação teve como objetivo investigar a estrutura e função do receptáculo seminal de *Doryteuthis plei*, espécie de lula adotada como modelo, como base para compreensão de mecanismos pós-copulatórios de seleção sexual em cefalópodes. Para atingir esse objetivo, a morfologia do receptáculo seminal foi analisada com base em microscopia integrativa (microscopia óptica com emprego de técnicas histoquímicas, microscopia eletrônica de varredura e de transmissão, microscopia confocal e microCT), visando a uma caracterização detalhada da estrutura do órgão. Além disso, a morfologia e histologia/histoquímica do receptáculo seminal foi analisada em três situações experimentais distintas: (1) antes de cópula recente, (2) após cópula recente (e antes da desova) e (3) após a desova, para investigar alterações morfológicas no órgão e no volume de espermatozoides armazenados, como base para compreensão dos mecanismos de captação, armazenamento e liberação de gametas masculinos pela fêmea. Os resultados revelaram enorme complexidade do receptáculo seminal com relação à diversidade de células secretoras e de fibras musculares associadas. Constatou-se que há alteração das células secretoras após a cópula recente e após a desova, com liberação do conteúdo de parte dessas células. Com base nos resultados obtidos, discute-se o papel dessas secreções na captação, armazenamento e liberação de espermatozoides. Além disso, a estrutura dos sistemas nervoso e muscular associados ao receptáculo seminal sugere que a fêmea tenha controle sobre deformações precisas do órgão relacionadas à captação e liberação de espermatozoides. Entretanto, outros mecanismos seriam igualmente possíveis, como atração química, natação ativa dos espermatozoides e ação ciliar do órgão. Alguns receptáculos seminais foram encontrados com espermatângios (i.e., espermatóforos evertidos) bloqueando total ou parcialmente sua abertura. Devido à

frequência, posição e morfologia dos espermâtangios dentro do receptáculo seminal, propõe-se a hipótese de que essas estruturas poderiam atuar também como plugues copulatórios. Esses plugues seriam mais eficientes nas primeiras 24 horas, sua eficiência sendo gradualmente reduzida com o tempo após a cópula, devido ao esvaziamento do conteúdo espermático e consequente redução de sua turgidez. Embora plugues copulatórios sejam conhecidos em inúmeras espécies de animais, este é o primeiro registro para cefalópodes, sendo, portanto, marco importante nos estudos de seleção sexual para o grupo. Cefalópodes são considerados modelos interessantes para o estudo de seleção sexual e os resultados desta dissertação contribuem para compreensão dos complexos mecanismos pós-copulatórios em lulas, como escolha críptica da fêmea e competição espermática.

Abstract

Sexual selection exerts a significant pressure on the evolution of reproductive attributes. Males show a diverse array of strategies to gain advantage in mating and fertilization success, but females also play a crucial role in pre- and post-copulatory sexual selection processes. Within this context, cephalopod mollusks show peculiar reproductive strategies, including sperm transfer via spermatophores, and the presence of female sperm-storage organs (seminal receptacles). However, the knowledge of the functioning of the cephalopod seminal receptacles is scarce, the mechanisms involved with sperm uptake, storage and release being unknown. To shed light on post-copulatory mechanisms in cephalopods, the present dissertation aimed at investigating the structure and function of the seminal receptacle of the squid *Doryteuthis plei*. To achieve this goal, the morphology of the seminal receptacle was thoroughly analyzed applying integrative microscopy (light microscopy including histochemical techniques, scanning & transmission electron microscopy, confocal microscpy and microCT). Moreover, to investigate morphological and sperm volume changes associated with possible mechanisms of sperm uptake, storage and release by the organ, the morphology and histology/histochemistry of the seminal receptacle was also analyzed under three distinct experimental manipulations: (1) before recent mating (2) after recent mating (and before egg release) and (3) after egg release. The results show a complex and striking diversity of secretory cells and associated muscle fibers in the seminal receptacle. There were changes in the composition and predominance of secretory cells between major reproductive events, suggesting a secretory activity associated with mating and spawning. The possible roles of these secretions in sperm uptake, storage and release are discussed in light of our data. Moreover, the structure of the nervous and muscular systems associated with the receptacle suggests that females have control over precise deformations of the organ, possibly related to sperm uptake and release. However, other mechanisms would be possible, such as chemical attraction, sperm active swimming, and ciliary action. Seminal receptacles were sometimes found with spermatangia (i.e., everted spermatophores) blocking totally or partially their openings. Given their frequency, position, and morphology, we hypothesize spermatangia might function as copulatory plugs that physically obstruct the female storage organ. Plug efficiency should be high within the first 24 hours after mating, gradually decreasing its efficiency with time, when spermatangia lose their turgidity by releasing part of their sperm content. Although copulatory plugs have been reported for

numerous taxa, this is the first record for cephalopods, and as such this finding has an impact for sexual selection studies based on these mollusks. Cephalopods are considered interesting models to investigate sexual selection, and this study has cast some light on the understanding of complex post-copulatory mechanisms in squids, such as cryptic female choice and sperm competition.