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Tese apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de Título de Doutor em Ciências, na Área de Zoologia.

Orientadora: Profa. Dra. Sônia Godoy Bueno Carvalho Lopes

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Ao meu querido sobrinho, Guilherme.

"I believe that in approaching our subject with the sensibilities of statisticians and dissectionists, we distance ourselves increasingly from the marvelous and spell-binding planet of imagination whose gravity drew us to our studies in the first place. This is not to say that we should cease to establish facts and to verify our information, but merely to suggest that unless those facts can be imbued with the flash of poetic insight then they remain dull gems; semi-precious stones scarcely worth the collecting."

"Until we transform our mere sightings into genuine visions; until our ear is mature enough to order a symphony from the shrill pandemonium of the aviary; until then we may have a hobby, but we shall not have a passion."

"A scientific understanding of the beautifully synchronized and articulated motion of an owl's individual feathers during flight does not impede a poetic appreciation of the same phenomenon. Rather, the two enhance each other, a more lyrical eye lending the cold data a romance from which it has long been divorced."

Alan Moore, *Blood from the Shoulder of Pallas*

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Introdução Geral

INTRODUÇÃO GERAL

1. Panorama sobre a estrutura e o funcionamento do sistema reprodutor dos cefalópodes

A biologia reprodutiva dos moluscos da classe Cephalopoda[*] é peculiar, devido ao curto ciclo de vida das espécies, geralmente de um a dois anos (Boyle & Rodhouse, 2005), ao evento reprodutivo freqüentemente terminal[†], concentrado na fase final do ciclo de vida (Rocha *et al.*, 2001), e ao desenvolvimento direto, sem fase larval (Boletzky, 2003). Cefalópodes apresentam comportamento sexual complexo, incluindo corte, comunicação por estímulos visuais e transferência de espermatóforos através de apêndices modificados nos machos (Hanlon & Messenger, 1996). Aspecto importante da reprodução desses animais é o fato de que sempre há proteção aos gametas (Mangold, 1987). Cefalópodes são dióicos e os sistemas reprodutores feminino e masculino dispõem de complexas especializações do gonoduto, contribuindo nas fêmeas para a produção de cápsulas protetoras para óvulos e embriões (Boletzky, 1998), e nos machos, para a formação de espermatóforos muito elaborados (Mann, 1984). Nos casos em que não ocorre formação de envoltórios protetores aos ovos, há cuidado parental (Boletzky, 2003).

Grande parte do conhecimento acerca da anatomia funcional do sistema reprodutor de cefalópodes se deve a trabalhos clássicos publicados no século XIX e início do século XX. Apesar do significativo aumento no conhecimento da biologia reprodutiva dos cefalópodes durante as últimas décadas do século passado, determinados processos envolvidos na reprodução não foram elucidados.

1.1. Plano básico do sistema reprodutor dos cefalópodes

Para entender o papel funcional do sistema reprodutor dos cefalópodes, é necessário conhecer primeiramente o plano básico de seu celoma. Nos cefalópodes, diferentemente de em outros moluscos, o celoma é expandido e dividido em três cavidades principais: pericárdica, renal e genital (Mangold *et al.*, 1989). Em Nautiloidea, a cavidade pericárdica envolve apenas o coração, a genital contém a gônada e parte do sistema digestivo, adentrando o canal do sifúnculo; ambas as cavidades são separadas por um

[*] Caso julgue necessário, o leitor do texto poderá consultar o apêndice "Classificação dos Cefalópodes Viventes", onde são resumidas as características gerais de cada táxon abordado no presente texto e ao longo da Tese.

[†] Há um consenso geral de que em Coleoidea o evento reprodutivo é terminal, apesar da grande variabilidade da duração do mesmo dentre as espécies dessa subclasse (5-50% do ciclo de vida). Sugere-se a leitura do artigo de Rocha *et al.* (2001) para conhecimento da variabilidade e flexibilidade das estratégias reprodutivas do grupo.

septo membranoso contendo três pontos de comunicação (Mangold *et al.*, 1989). Em Coleoidea, há fusão entre essas duas cavidades, criando um amplo espaço celômico perivisceral ou víscero-pericárdico, contendo os corações sistêmico e acessórios, as glândulas pericárdicas, grande parte do sistema digestivo e a gônada (Mangold *et al.*, 1989). Importante exceção a esse padrão é encontrada em Octopoda, nos quais o coração sistêmico está localizado fora do celoma, este último reduzido e envolvendo apenas a gônada e as glândulas pericárdicas (Mangold *et al.*, 1989).

A gônada e o celoma genital localizam-se na região posterior e dorsal da massa visceral. A região proximal do(s) gonoduto(s) origina-se como expansões do celoma (origem mesodérmica), enquanto a região distal origina-se a partir de invaginações ectodérmicas (Mangold, 1987). Nos machos, determinados setores da região distal do gonoduto sofrem especializações que originam as glândulas formadoras dos espermatóforos. Nas fêmeas, essa mesma região dá origem às glândulas formadoras de toda ou de parte da cápsula dos ovos. Na maioria dos táxons, glândulas nidimentares, não conectadas diretamente ao sistema reprodutor, contribuem também para a formação dessa cápsula.

Tendo em mente esse plano básico, fica mais fácil entender a diversidade morfológica e funcional do sistema reprodutor dentre os Cephalopoda, como exposto a seguir.

1.2. Sistema reprodutor feminino, cápsulas dos ovos e armazenamento de espermatozóides

O sistema reprodutor feminino dos cefalópodes é composto por (Haven, 1977; Wells & Wells, 1977; Arnold, 1984; Mangold, 1989a): 1) ovário ímpar, localizado na região posterior da massa visceral; 2) oviduto proximal, sítio de armazenamento de óvulos maduros ou simplesmente uma comunicação entre o ovário e o oviduto distal; os ovidutos direito e esquerdo podem estar presentes, dependendo do grupo; 3) oviduto distal ou glândula oviducal, responsável pela secreção de toda ou de parte da cápsula do ovo; 4) glândulas nidimentares, responsáveis pela formação do restante da cápsula do ovo; podem estar ausentes; 5) glândulas nidimentares acessórias, presentes em poucos grupos; e 6) receptáculo seminal. As variações desse sistema básico são resumidas na seqüência para os principais táxons da classe.

Nautiloidea. A gônada feminina apresenta abertura cônica e localizada tão próxima à abertura do oviduto proximal que ambas estão funcionalmente conectadas e não há liberação de ovócitos no celoma (Haven, 1977). Somente o gonoduto direito é funcional e no lado esquerdo há uma bolsa piriforme interpretada como gonoduto vestigial. A região distal

do oviduto é glandular e, na superfície ântero-ventral da massa visceral, há um par de glândulas nidimentares (Haven, 1977), entretanto são escassas as informações a respeito da contribuição das mesmas para a formação da cápsula dos ovos. Os ovos de *Nautilus* são recobertos por uma cápsula branco-leitosa com parede dupla, a interna de forma ovalada e superfície lisa, e a externa irregular e pregueada (Arnold, 1984; Mangold, 1987; Mangold, 1989b). O receptáculo dos espermatóforos das espécies de *Nautilus* é constituído por uma superfície lamelar, localizada abaixo da massa bucal, e denominado órgão de Valenciennes (Haven, 1977; Mangold, 1989a).

Coleoidea. O oviduto é pareado nos coleóides dos táxons Oegopsida, Incirrata e Vampyromorpha (Pickford, 1949; Mangold, 1989a); salvo raras exceções, o restante dos coleóides apresenta somente o oviduto esquerdo. Em Sepiida e Octopoda, o oviduto proximal é apenas um canal de comunicação entre o ovário e a glândula oviducal, os óvulos sendo armazenados no celoma genital até o momento que precede a postura (Nesis, 1987). No restante dos coleóides, o oviduto proximal tem a função de gradativamente coletar e armazenar os óvulos até o momento da postura (Nesis, 1987). Com exceção de Incirrata, a glândula oviducal dos demais secreta substância mucilaginosa que forma aparentemente o primeiro envoltório da cápsula do ovo (Boletzky, 1986). Em Incirrata, a glândula oviducal contribui com o material cimentante que fixa os ovos no substrato (Froesch & Marthy, 1975). Nesse caso, dois setores distintos da glândula, o central e o periférico, produzem substâncias quimicamente diferentes que, quando misturadas, reagem mudando suas propriedades. A glândula periférica secreta uma "muco-proteína" que é polimerizada pelo "mucopolissacarídeo" secretado pela glândula central, formando o material cimentante (Froesch & Marthy, 1975). Em Incirrata, não há envoltórios suplementares, porém as fêmeas em fase de postura procuram abrigos para a desova (ex. fendas em rochas e conchas vazias), cuidando dos ovos durante todo o período de desenvolvimento embrionário, protegendo-os de predadores e mantendo-os limpos e oxigenados (Boletzky, 2003).

A superfamília de polvos epipelágicos (Incirrata: Argonautoidea) apresenta as mais variadas estratégias reprodutivas dentre os cefalópodes. Fêmeas de espécies de *Argonauta* fixam os ovos no interior de uma delicada concha calcária secretada pelo primeiro par de braços e carregam-na junto ao corpo, enquanto fêmeas de *Tremoctopus* secretam um bastonete calcário e/ou orgânico pelo mesmo par de braços, onde prendem e carregam as desovas até a eclosão; em *Ocythoe*, as fêmeas incubam os ovos no interior do oviduto (ovoviviparidade) (Wells & Wells, 1977; Boletzky, 1986; Mangold, 1987).

Polvos da subordem Cirrata depositam grandes ovos encapsulados em um envoltório rígido e resistente produzido pela glândula oviducal (Boletzky, 1986, 1998); esse envoltório é homólogo à substância cimentante produzida pelos Incirrata para fixar seus

ovos (Mangold, 1987). Em Vampyromorpha aparentemente não há envoltórios protetores dos ovos, estes sendo encontrados flutuando em águas abertas; Boletzky (1986), entretanto, sugere que as glândulas oviducais devam secretar algum tipo de envoltório protetor, que seria frágil e facilmente perdido durante a coleta com redes.

Glândulas nidimentares estão presentes em todos os Decapodiformes. Aparentemente essas glândulas formam envoltórios protetores adicionais aos ovos em forma de camadas concêntricas, porém o processo e o sítio de encapsulamento são ainda desconhecidos (Boletzky, 1986). Aparentemente todos os “sepióides” (Sepiida, Sepiolida e Spirulida) depositam ovos individualizados, embebidos por secreções da glândula oviducal e envoltos externamente por camadas concêntricas de secreções das glândulas nidimentares (Boletzky, 1986; Mangold, 1987). Nos sepiolídeos (Sepiolida), o envoltório mais externo é freqüentemente rígido e colorido (ou ao menos opaco), os ovos sendo postos muito próximos ou até mesmo fixos uns aos outros (Boletzky, 1986, 1998). Geralmente são encontrados em conchas vazias ou fixos em organismos sésseis como esponjas e tunicados (Boletzky, 1986, 1998; Mangold, 1987). Em alguns casos, grãos de areia e pequenos fragmentos de corais são incorporados à massa de ovos (Mangold, 1987). Em *Sepia* spp. (Sepiida), o método de fixação é mais elaborado: dois prolongamentos da cápsula do ovo são enrolados no substrato (algas, tubos de poliquetos) e, em seguida, fundidos (Boletzky, 1986, 1998; Mangold, 1987). Além disso, secreções da glândula da tinta podem ser incorporadas, conferindo camuflagem à cápsula (Boletzky, 1986).

Em Idiosepiida, a fêmea, fixa à superfície de uma macroalga por meio do órgão adesivo localizado na região dorsal do manto, deposita os ovos individualmente no substrato, com auxílio dos braços e tentáculos (Kasugai & Ikeda, 2003). O envoltório interno, aparentemente oriundo da glândula oviducal, apresenta camadas concêntricas; o externo, aparentemente secretado pelas glândulas nidimentares, é comum a toda massa de ovos; a superfície dessa massa é recoberta por areia lodosa, contendo uma diversidade de organismos (ex. diatomáceas, nematódeos, crustáceos) que não invadem a massa de ovos.

As cápsulas de ovos dos loliginídeos (Myopsida) são alongadas e contém um número variável de ovos dispostos em espiral (Jecklin, 1934; Boletzky, 1986, 1998; Mangold, 1987). Existe um consenso na literatura de que o envoltório interno que recobre os ovos deva ser secretado pela glândula oviducal e o externo, composto por camadas concêntricas, pelas glândulas nidimentares (e.g., Lum-Kong, 1992). Fields (1965) e Arnold & Williams-Arnold (1977) sugeriram que a região da abertura genital seja o sítio de encapsulamento dos ovos, e Boletzky (1983) propôs que a superfície côncava das glândulas nidimentares acessórias deva servir como molde durante o revestimento em espiral dos ovos pela substância gelatinosa secretada pelas glândulas nidimentares. Entretanto, permanece desconhecido como e onde ocorre o processo de encapsulamento dos ovos

(Boletzky, 1986; Mangold, 1987). As cápsulas gelatinosas são fixadas diretamente a algas, grãos de areia ou organismos sésseis (Mangold, 1987).

Em muitas espécies de Oegopsida, as massas de ovos são de grande tamanho, gelatinosas e amorfas (Boletzky, 1986; Mangold, 1987). Aparentemente a fêmea produz, na cavidade do manto, uma mistura de secreções das glândulas oviducais e nidimentares, na qual ficam imersos óvulos e espermatóforos de modo disperso e aleatório (O'Dor, 1983). Após ser liberada ao meio externo, essa massa de ovos aumenta rapidamente de volume, podendo atingir diâmetro de até 4 metros e conter de 100 mil a 2 milhões de ovos dependendo da espécie (Durward *et al.*, 1980; O'Shea *et al.*, 2004; Staaf *et al.*, 2008). Outras espécies dessa subordem apresentam massas mais organizadas; em *Thysanoteuthis rhombus*, a massa tem a forma de bastão e pode atingir 1.8 metros de comprimento e 30 cm de diâmetro, contendo ovos dispostos em arranjo espiral (Nigmatullin *et al.*, 1995). Nas espécies de *Gonatus*, as fêmeas carregam a desova entre os braços até a eclosão (Seibel *et al.*, 2000), de forma semelhante às fêmeas de polvos epipelágicos. Em outras espécies de Oegopsida não há glândulas nidimentares e os ovos são postos individualizados, sendo encontrados flutuando na superfície oceânica (e.g., Enoploteuthidae; Nesis, 1995; Boletzky, 1998).

Glândulas nidimentares acessórias estão presentes em todos os Decapodiformes, com exceção de Oegopsida (Young *et al.*, 1998). Em fêmeas imaturas, essas glândulas apresentam-se brancas, porém mudam para alaranjado a vermelho concomitantemente à maturação sexual. Isso porque os túbulos que compõem essas glândulas são colonizados por bactérias simbiontes, as quais produzem carotenóides a partir de substâncias secretadas pelas glândulas acessórias maduras (Bloodgood, 1977; Lum-Kong & Hastings, 1992; Barbieri *et al.*, 2001). A função dessas glândulas é outro tema ainda não elucidado. Sua ultraestrutura indica que tenham função secretora (Bloodgood, 1977), o que foi confirmado por Lum-Kong (1992), que constatou a presença de "vesículas secretoras" e a ocorrência de um ciclo secretor associado à maturação da fêmea, a exemplo do que havia verificado para as glândulas oviducal e nidimentares principais. Com base em tais evidências, essa autora sugeriu que as glândulas nidimentares acessórias devam contribuir para a formação da cápsula de ovos.

A presença de bactérias simbiontes nas glândulas nidimentares acessórias sugeriu outras hipóteses para sua função. Barbieri *et al.* (1996) propuseram que as bactérias devam ser transferidas para a cápsula externa do ovo durante a secreção desta pelas glândulas nidimentares principais, embora o exato processo de transmissão seja ainda desconhecido. Essas bactérias evitariam a proliferação de microorganismos nocivos em redor do ovo, como comprovaram Barbieri *et al.* (1997) ao constatarem propriedade fungicida dessa comunidade microbiana. Barbieri *et al.* (2001) demonstraram que essas glândulas abrigam

praticamente as mesmas bactérias encontradas na cápsula dos ovos, sugerindo a transmissão da simbiose. Neste último trabalho, os autores constataram também a presença de bactérias produtoras de tetrodotoxina, potente neurotoxina, reforçando a idéia de que essa colonização bacteriana é vantajosa por prover proteção aos ovos. De fato, alguns animais marinhos evitam contato com as nutritivas cápsulas de ovos de *Loligo reynaudii* (Sauer & Smale, 1991; Smale *et al.*, 1995).

O sítio de deposição de espermatóforos e aquele de armazenamento de espermatozóides variam muito dentre os coleóides. Em Sepiida, o macho transfere espermatóforos ao receptáculo seminal localizado na membrana peribucal da fêmea (Hanlon *et al.*, 1999). Em alguns Sepiolida, os espermatóforos são depositados em uma bolsa copulatória, localizada na cavidade palial (Hoving *et al.*, 2008b). Em algumas espécies de Myopsida, o mesmo indivíduo apresenta dois sítios de deposição, um sendo a região do receptáculo seminal, localizado na membrana peribucal, e o outro sendo dentro da cavidade do manto, próximo à base das brânquias e à abertura genital. A opção por um desses sítios pode depender da maturação sexual da fêmea (i.e., se está ou não em fase de postura de ovos), do tamanho do macho e das interações intraespecíficas à época do acasalamento (Hanlon & Messenger, 1996). Em Oegopsida, dependendo da espécie, os espermatóforos podem ser transferidos para a membrana peribucal (próximo a receptáculos seminais, quando presentes), para a cavidade do manto na região da abertura genital, ou ainda para bolsas especializadas localizadas na região “nucal” (Nesis, 1995). Em Octopoda, o espermatóforo é depositado próximo à abertura do oviduto, os espermatozóides sendo armazenados nas espermatecas localizadas internamente às glândulas oviducais (e.g., subfamílias Octopodinae e Bathypolypodinae; Froesch & Marthy, 1975; O’Dor & Macalaster, 1983). Em alguns Incirrata, os espermatóforos podem alcançar o celoma genital (subfamília Eledoninae; Fort, 1937, 1941), os espermatozóides podendo inclusive ser armazenados no filamento apical dos ovócitos (Perez *et al.*, 1990). As fêmeas de Vampyromorpha possuem duas espermatecas localizadas próximo aos olhos (Pickford, 1949).

Receptáculos seminais *sensu stricto* ou espermatecas são estruturas especializadas no armazenamento de espermatozóides, embora estes termos sejam utilizados de forma mais abrangente na literatura, incluindo por vezes os receptáculos de espermatóforos. No caso de lulas e sépias, o receptáculo seminal *s. str.* trata-se de uma invaginação da membrana peribucal, ventral à boca, circundada por musculatura circular e secretora de uma substância que mantém os espermatozóides em estado inativo (Drew, 1911; Oordt, 1938). O receptáculo armazena espermatozóides até o momento da postura dos ovos (Lum-Kong, 1992), mas são desconhecidos os mecanismos de atração destes para o interior do receptáculo seminal, uma vez que os espermatóforos são depositados fora deste órgão. Nos polvos, quando presente, a espermateca localizada dentro de cada glândula oviducal pode

armazenar espermatozóides por muitos meses, até a maturação sexual da fêmea (Froesch & Marthy, 1975).

1.3. Sistema reprodutor masculino e a produção e transferência de espermatóforos

O sistema reprodutor masculino dos cefalópodes pode ser dividido em cinco seções (Haven, 1977; Wells & Wells, 1977; Arnold, 1984; Mangold, 1989a): 1) testículo ímpar, localizado na região posterior da massa visceral; 2) vaso deferente (=gonoduto proximal), cujos cílios captam os espermatozóides produzidos e liberados no celoma pela gônada; 3) órgão espermatofórico, uma complicada série de especializações glandulares do gonoduto distal, as quais são responsáveis pela formação dos espermatóforos; 4) saco espermatofórico (ou de Needham), onde são armazenados os espermatóforos; e 5) órgão terminal (ou "pênis"), um prolongamento do saco espermatofórico que se comunica com a cavidade palial. Geralmente só o gonoduto esquerdo está presente e funcional, mas algumas espécies conservam o par de gonodutos masculinos originado durante a embriogênese.

Um ou mais apêndices circum-orais modificados são responsáveis pela transferência dos espermatóforos na grande maioria dos cefalópodes (Mangold, 1989a). Em alguns casos, como exposto adiante, outras estruturas são responsáveis pelo referido processo. As variações desse sistema básico são resumidas a seguir para os principais táxons da classe.

Nautiloidea. Nas espécies de *Nautilus*, o testículo apresenta uma abertura ventral em forma de fenda, adjacente à abertura proximal do único vaso deferente (direito), de modo que os espermatozóides passam diretamente para esse vaso, não sendo liberados no celoma (Haven, 1977). Esse vaso deferente tem continuidade no gonoduto distal no lado direito da massa visceral; como nas fêmeas, no lado esquerdo, há apenas uma vesícula piriforme, considerada como gonoduto vestigial. Em Nautiloidea o órgão espermatofórico, denominado de "órgão acessório", é mais simples do que aquele dos demais cefalópodes e apresenta três regiões distintas (Haven, 1977; Arnold, 1984; Mangold, 1989a): glândula espermatofórica I, glândula espermatofórica II e vesícula seminal. Ambos o saco espermatofórico e o "pênis" apresentam-se compartimentados internamente por um septo longitudinal. Ainda não foi possível estabelecer com clareza homologias entre os diferentes setores do gonoduto dos nautilóides com aqueles dos coleóides, havendo dúvidas se a origem da região distal do gonoduto, que contém a glândula acessória nos primeiros, é ectodérmica como nos coleóides (Mangold, 1989a).

O espermatóforo dos nautilóides é composto por uma longa massa espermática (15-35 cm de comprimento), muito enovelada, inclusa em uma cápsula ovóide, sendo simples

quando comparado ao dos coleóides (Haven, 1977; Arnold, 1984; Mann 1984). Apesar de haver evidências de que grande parte do espermatóforo é formada na glândula espermatofórica I (Haven, 1977; Mangold, 1989a), pouco se sabe a respeito da formação dos espermatóforos em Nautiloidea e qual a exata função dos diferentes setores do gonoduto nesse processo. O mesmo pode ser dito a respeito da transferência dos espermatóforos para a fêmea, embora o espádice, órgão derivado da hipertrofia de quatro tentáculos circum-orais, deva ser o responsável por grande parte desse processo (Haven, 1977; Arnold, 1984; Mangold, 1987). Apenas um espermatóforo é transferido por cópula e a forma de fixação do mesmo e o sítio de armazenamento dos espermatozóides são desconhecidos (Mangold, 1987).

Coleoidea. Neste grupo o único gonoduto persistente é o esquerdo, salvo raras exceções em que ambos estão presentes (*Stigmatoteuthis* spp., *Selenoteuthis scintillans* e *Lycoteuthis* spp.; Nesis, 1987; Young *et al.*, 2008). Nos coleóides há liberação de espermatozóides no celoma genital (Arnold & Williams-Arnold, 1977). Os mesmos são captados por cílios da superfície interna da parede desse celoma e conduzidos até a abertura proximal do vaso deferente, que se conecta ao primeiro setor glandular do órgão espermatofórico (Arnold & Williams-Arnold, 1977; Wells & Wells, 1977; Mangold, 1989a). Este órgão, mais complexo que o dos nautilóides, é composto por oito setores especializados, que contribuem para a formação dos espermatóforos (Drew, 1919a; Badenhorst, 1974; Wells & Wells, 1977; Mangold, 1989a). Esses setores localizam-se internamente a uma bolsa, denominada saco genital, que possui comunicação com a cavidade palial (Badenhorst, 1974).

A estrutura dos espermatóforos dos coleóides é igualmente mais complexa quando comparada aos dos nautilóides, e apresenta, essencialmente, quatro partes (Drew, 1919a; Mann, 1984): (1) massa espermática, contendo os espermatozóides; (2) corpo cimentante, responsável pela fixação da massa espermática na fêmea; (3) aparato ejaculatório, um complexo de membranas e túnicas que é evertido durante a cópula; e (4) túnicas que recobrem todas as estruturas enumeradas de 1 a 3, mais os espaços preenchidos por líquidos (a complexa estrutura dos espermatóforos dos coleóides é explicada com mais detalhes adiante). Exceção a esse padrão estrutural é encontrada nos polvos da subordem Cirrata, cujo espermatóforo consiste simplesmente de uma cápsula com opérculos nas extremidades, não apresentando aparato ejaculatório (Villanueva, 1992).

A formação dos espermatóforos foi descrita brevemente por Drew (1919a), para *Doryteuthis pealeii* (Myopsida), e por Blancquaert (1925), baseado principalmente em *Sepia officinalis* (Sepiida). No lúmen do órgão espermatofórico, o espermatóforo em formação é mantido em rotação em torno de seu eixo longitudinal. A partir da rotação, causada por cílios

no epitélio do lúmen, cada estrutura secretada é torcida em espiral à medida que o espermatóforo em formação é deslocado para o setor seguinte. Na fase final do processo de formação, a túnica externa passa por um processo de "encolhimento" permanente, causando pressão sobre o conteúdo interno e mantendo o espermatóforo pronto para o processo de evaginação (Drew, 1919a; Blancquaert, 1925; Peterson, 1959). Muitas questões permanecem não resolvidas com relação à produção dos espermatóforos nos coleóides, persistindo dúvidas sobre o exato local de formação de diversas estruturas, como o corpo cimentante e determinadas membranas e túnicas, bem como sobre a função exata de determinados setores do órgão espermatofórico. Por exemplo, um duto ciliado, não glandular, presente entre o quarto e quinto setores, permanece com função desconhecida. Duas hipóteses principais foram propostas: 1) o duto pode se tratar de um canal de evacuação para secreções residuais, ou para espermatozóides não incorporados no espermatóforo em formação (Blancquaert, 1925, com base em *S. officinalis*); 2) ou funcionar como um canal de entrada de água do meio externo via saco genital, para facilitar a movimentação do espermatóforo no interior do órgão (Kerr, 1931, com base em *Spirula spirula*).

Badenhorst (1974) complementou o trabalho de Drew (1919a) e de Blancquaert (1925) ao apresentar detalhes da histologia do órgão espermatofórico de *Loligo reynaudii*, compreendendo um dos estudos anatômicos mais completos deste órgão. Abdul-Rahim & Chandran (1984a, 1984b, 1994) estudaram a formação de espermatóforos no loliginídeo *Uroteuthis duvaucelii*, porém não acrescentaram dados que pudessem elucidar as dúvidas apontadas pelos trabalhos precedentes. Em revisão da literatura referente à anatomia microscópica do sistema reprodutor de cefalópodes, Budelmann *et al.* (1997) ressaltaram a necessidade de dados histológicos detalhados de todo o órgão espermatofórico dos coleóides.

Nos coleóides, um ou mais apêndices circum-orais estão modificados (hectocótilos), apresentando redução ou ausência de ventosas, papilas ou outras estruturas para transferência dos espermatóforos à fêmea (Mangold, 1989a). Nos polvos da subordem Incirrata, além dessas modificações da extremidade de um dos braços, geralmente no terceiro esquerdo, ocorre a formação de um sulco marginal que, por movimentos peristálticos, força o deslizamento do espermatóforo até que este alcance a extremidade distal (Wodinsky, 2008). Os machos de espécies de polvos epipelágicos (Incirrata: Argonautoidea) são sempre anões e produzem um único espermatóforo; um elaborado hectocótilo, contendo o espermatóforo, sofre autotomia durante a cópula, ficando retido na cavidade palial da fêmea (Vérany & Vogt, 1852; Müller, 1853). Em Oegopsida, há casos em que não há hectocótilo e, para algumas espécies, acredita-se que o macho transfere os espermatóforos com auxílio do longo "pênis" (e.g., Nesis, 1995).

2. Os espermatóforos e a "reação espermatofórica" dos coleóides

Durante transferência à fêmea, os elaborados espermatóforos dos coleóides sofrem drásticas modificações na chamada "reação espermatofórica", complexo processo de evaginação do aparato ejaculatório que exterioriza a massa espermática e o corpo cimentante (e.g., Mann, 1984). O primeiro registro do espermatóforo dos coleóides e do fenômeno da reação espermatofórica foi realizado pelo naturalista holandês Jan Swammerdam (1637-1680) e publicado na obra póstuma *Biblia Naturae* (Swammerdam, 1738). Com base nos espermatóforos de uma espécie de *Sepia*, Swammerdam, com os limitados recursos de microscopia óptica de sua época, não só reconheceu e ilustrou as principais regiões dos espermatóforos da sépia, como também observou que os mesmos sofriam drásticas modificações morfológicas autônomas, quando retirados do sistema reprodutor masculino e deixados por algum tempo em água do mar.

Para uma melhor compreensão e acompanhamento das fases dessa “reação” é apresentado um esquema didático (Fig. 1), baseado principalmente no estudo de Drew (1919a). No CD anexo à Tese, há também uma animação feita com base esse modelo (Apêndice S1). Para entender o funcionamento desse processo, é necessário primeiramente compreender a complexa morfologia do espermatóforo, o qual é composto pelas seguintes estruturas (Fig. 1a): (1) massa espermática na região aboral; (2) corpo cimentante, conectado à massa espermática por um cilindro conectivo; (3) três membranas (interna, mediana e externa) envolvendo um filamento espiralado (ou "filamento espiral"); (4) três túnicas (interna, mediana e externa), que recobrem todas ou parte das estruturas enumeradas de 1 a 3, mais os espaços preenchidos por líquidos; e (5) extremidade oral fechada pelo capuz e respectivo filamento do capuz, sendo ambas as estruturas continuidade da túnica externa. Ao conjunto formado pelo filamento espiral, pelas três membranas que o envolvem e pela túnica interna é dado o nome de aparato ejaculatório (Drew, 1919a).

O espermatóforo extraído de animais vivos é túrgido e elástico (Drew, 1919a; Takahama, *et al.* 1991). Isso se deve à túnica externa (Fig. 1a), que é resistente e elástica, apesar de apresentar espessura relativamente fina. Internamente à tunica externa, localiza-se a túnica mediana (Fig. 1a), de espessura maior na região aboral, reduzindo-se de forma gradual em direção à região oral após o corpo cimentante. Entre as túnicas mediana e interna há um espaço evidente em praticamente toda a extensão do espermatóforo e preenchido por líquido (no esquema didático da Fig. 1, este espaço corresponde à área em branco no interior do espermatóforo).

Na região aboral do corpo cimentante inicia-se a túnica interna[‡] e a membrana externa (Fig. 1a), a qual recobre este corpo. A membrana externa é fortemente unida à túnica interna, sendo por vezes difícil distinguí-las. Próximo às suas extremidades, a túnica interna e a membrana externa terminam formando um espessamento (Fig. 1a). Ambas, membrana e túnica formam, estruturalmente, um tubo aberto e com borda espessada na extremidade oral, porém de base fechada, pois esta envolve e se une à extremidade aboral do corpo cimentante.

A membrana mediana, situada entre as membranas interna e externa (Fig. 1a), forma um tubo que se estende desde a base da metade oral do corpo cimentante, até a extremidade oral do espermatóforo. Estruturalmente, a membrana mediana forma um tubo aberto na extremidade aboral situada ao redor do corpo cimentante e fechada na extremidade oposta onde se funde ao capuz (Fig. 1a). A membrana interna (Fig. 1a) acompanha a membrana mediana ao longo de sua extensão. O filamento espiral (Fig. 1a) é fortemente unido à esta membrana, estendendo-se desde a extremidade oral do corpo cimentante até aproximadamente a extremidade oral do espermatóforo. Tendo em mente esse plano estrutural básico, torna-se mais simples a compreensão da reação espermatofórica, descrita a seguir.

A reação tem início com uma tração sobre o filamento do capuz, a qual se transmite para o capuz, rompendo-o na região de inserção do filamento (Fig. 1b). Com o rompimento do capuz, o aparato ejaculatório abre-se para o exterior, iniciando-se o processo de evaginação (Fig. 1c). Este acontece, a princípio, devido a uma pressão inicial, pré-ruptura, pelo fato do espermatóforo intacto ser altamente túrgido. Concomitantemente, a túnica mediana torna-se gradualmente entumescida pela entrada de água no espermatóforo por absorção (Fig. 1c). O entumescimento da túnica mediana, associado à resistência natural à distensão da túnica externa, gerariam a pressão necessária para provocar a expulsão da massa espermática e do corpo cimentante (Drew, 1919a). A propriedade absortiva da membrana mediana é aparentemente resultado de sua hipertonia em relação à água do mar, o que geraria um gradiente osmótico no momento em que o espermatóforo é extraído do gonoduto e exposto à água do mar durante a cópula (nos polvos essa função é desempenhada pelo fluido ou plasma espermatofórico; Mann *et al.*, 1970).

O processo de evaginação continua e, no momento em que a extremidade oral do corpo cimentante projeta-se para o meio externo (Fig. 1d), o filamento espiral e a membrana interna se destacam do mesmo e a reação sofre um breve retardo, quando então a massa espermática é forçada contra a região aboral do corpo cimentante. Nesta fase, e após concluído o processo de evaginação da túnica interna e membrana externa, o tubo por elas formado recobre a massa espermática, a qual, a partir da constante pressão interna, continua a ser impelida para o interior desse tubo (Fig. 1d). O contato íntimo entre entre a extremidade aboral da membrana mediana

[‡] Drew (1919a) acreditava que a túnica interna estendia-se em direção aboral, recobrindo também a massa espermática; essa informação foi omitida do esquema didático (Fig. 1) objetivando maior clareza.

com a região mediana do corpo cimentante cria uma área de resistência que aparentemente retarda ao máximo o destacamento entre ambas estruturas. Esse fator permite que haja tempo para que toda a massa de espermatozóides seja impelida para dentro do envoltório formado pela túnica interna e a membrana externa.

No momento em que a membrana mediana se destaca do corpo cimentante, este último se rompe (Fig. 1e) e a massa espermática, envolta pela tubo constituído pela túnica interna e membrana externa, é lançada por completo para fora do espermatóforo (Fig. 1f). O conjunto recém-expelido, denominado de “reserva espermática” (Drew, 1919a, Takahama *et al.* 1991) ou “espermatângio” (Fort, 1937; Nesis *et al.*, 1998), tem a base aboral maior e revestida externamente pelo conteúdo adesivo do corpo cimentante, e uma extremidade livre e aberta, por onde os espermatozóides são continuamente liberados (Fig. 1g).

A duração da reação espermatofórica é variável entre as espécies de cefalópodes, ocorrendo em média em 10 segundos em *D. pealeii* (Drew, 1919a; Austin *et al.*, 1964), e em uma a duas horas em *Enteroctopus dofleini*, o polvo gigante do Pacífico, cujo espermatóforo pode atingir 1 metro de comprimento (Mann *et al.*, 1966, 1970). O gatilho exato para iniciar a reação é tema controverso; alguns autores propuseram que a tração sobre o filamento do capuz, a qual se transmite para o capuz, rompendo-o na região de inserção do filamento, é necessária para iniciar o processo (e.g., Drew, 1919a). Entretanto, foi sugerido que, em algumas espécies, o contato com a água do mar é suficiente para iniciar a reação (e.g., Takahama *et al.*, 1991).

3. Motivações para estudo do tema

Poucos trabalhos abordam com detalhes a morfologia e anatomia funcional dos espermatóforos dos cefalópodes. Apesar das descrições taxonômicas atuais incluírem a morfologia do espermatóforo (e.g., Roper & Voss, 1983), em geral a descrição morfológica e respectivas ilustrações do espermatóforo são demasiadamente simples, feitas com base na limitada ampliação de um estereomicroscópio. Poucos estudos detalhados foram efetuados, grande parte do conhecimento acerca da estrutura do espermatóforo tendo sido gerada por trabalhos clássicos do século XIX e início do século XX (e.g., Milne-Edwards, 1842; Racovitza, 1894; Marchand, 1913; Drew, 1919a; Blancquaert, 1925; Weill, 1927). Recentemente, descrições mais detalhadas foram feitas apenas por Austin *et al.* (1964), os quais empregaram cortes seriados e coloração histológica ao estudo dos espermatóforos de *Doryteuthis pealeii*, e por Takahama *et al.* (1991), que incluíram a microscopia eletrônica de varredura para investigar o espermatóforo de *Todarodes pacificus*. Embora sem o mesmo nível de detalhamento, Hess (1987) apresentou descrição dos espermatóforos de diversas

espécies de lulas, compondo, dessa forma, o estudo mais abrangente acerca dos espermatóforos dos teutóides.

Investigações acerca do funcionamento dos espermatóforos dos coleóides são consideravelmente mais raras, estando o conhecimento básico sobre a reação espermatofórica restrito a apenas 20 espécies de coleóides: *Doryteuthis pealeii* (Drew, 1919a; Austin *et al.*, 1964), *D. opalescens* (Fields, 1965), *Loligo vulgaris* (Milne-Edwards, 1842; Marchand, 1913), *Illex coindetii* (Marchand, 1913), *Octopoteuthis sicula* (Hoving *et al.*, 2008a), *Onykia ingens* (Hoving & Laptikhovsky, 2007), *Rossia macrosoma* (Racovitza, 1894; Marchand, 1913), *R. moelleri* (Hoving *et al.*, 2009), *Sepia officinalis* (Milne-Edwards, 1842; Marchand, 1913), *Sepiola atlantica* (Weill, 1927), *S. rondeleti* (Marchand, 1913), *Todarodes pacificus* (Takahama *et al.*, 1991), *Eledone cirrhosa* (Fort, 1937), *E. moschata* (Milne-Edwards, 1842; Marchand, 1913; Fort, 1941; Orelli, 1962), *Enteroctopus dofleini* (Mann *et al.*, 1966, 1970; Hanson *et al.*, 1973), *Octopus defilippi* (Marchand, 1913), *O. macropus* (Marchand, 1913), *O. tehuelchus* (Pujals, 1978) e *O. vulgaris* (Drew, 1919b). Descrições detalhadas da reação espermatofórica, indicando o papel dos componentes do espematóforo no funcionamento do processo, são escassas (e.g., Racovitza, 1894; Drew, 1919a; Weill, 1927).

Diversas questões relacionadas ao complexo processo de evaginação do espermatóforo e de fixação do espermatângio permanecem não resolvidas. Por exemplo, nos loliginídeos, o conteúdo adesivo e aparentemente alcalino do corpo cimentante é considerado responsável pela fixação da base dos espermatângios no corpo da fêmea (Drew, 1919a; Austin *et al.*, 1964). Entretanto, considerando que os espermatângios são depositados externamente, tanto na membrana peribucal como na cavidade do manto próximos à abertura genital, ambas as áreas desprotegidas e expostas à considerável turbulência[§], é no mínimo intrigante o fato dos espermatângios fixaram-se da fêmea instantaneamente somente a partir de substâncias adesivas. Em lulas da família Ommastrephidae (subordem Oegopsida), além da ação de substâncias adesivas, acredita-se que a extremidade em forma de seta do corpo cimentante tenha papel importante na fixação do espermatângio (e.g., Takahama *et al.*, 1991).

Diversos componentes do espermatóforo dos coleóides têm função enigmática. O intrincado filamento espiral, por exemplo, foi proposto por Drew (1919a), com base em *Doryteuthis pealeii*, como sendo uma estrutura capaz de evitar o colapso do aparato ejaculatório durante o processo de evaginação. Investigando a mesma espécie, Austin *et al.* (1964) encontraram numerosos corpúsculos microscópicos de forma estelar ("stellate

[§] Cefalópodes coleóides são nadadores muito ativos, que continuamente bombeiam água para dentro e para fora da cavidade palial a partir de contrações do manto. Essa condição é aqui considerada como causadora de "turbulência" nas superfícies externas do corpo, incluindo as da cavidade do manto.

particles") embebidos na matriz do filamento espiral. Austin *et al.* (1964) sugeriram que essas estruturas contribuiriam à função proposta por Drew (1919a) por proverem resistência ao filamento espiral. Esses corpúsculos haviam sido descobertos muito tempo antes por Duvernoy (1853) em *Sepiola rondeleti*, sua presença tendo sido confirmada posteriormente em outros Decapodiformes (Marchand, 1913; Blancquaert, 1923 e 1925; Weill, 1927; Badenhorst, 1974; Takahama *et al.*, 1991; Hoving *et al.*, 2009), porém pouco se sabe sobre sua exata função. Weill (1927) supôs, com base em *Sepiola atlantica*, que os corpúsculos estariam envolvidos de alguma forma no processo de fixação dos espermatângios na fêmea. Takahama *et al.* (1991) sugeriram que os mesmos poderiam auxiliar na formação do contato entre o espermatóforo e o corpo da fêmea em *Todarodes pacificus*.

A extraordinária complexidade estrutural e funcional observada nos espermatóforos das lulas (e.g., Marchand, 1913; Drew, 1919a; Hess, 1987) contrasta com a aparente simples função do todo o processo de evaginação, i.e., exteriorização e fixação superficial da massa espermática por meio de substâncias adesivas. Drew (1919b), em reconhecimento desse quadro intrigante, constatou: “[…] it is very difficult to arrive at the reasons for the formation of such complicated structures as these spermatophores to perform functions where simpler arrangements would seem do as well”.

Recentemente, diversos estudos foram efetuados sobre o fenômeno de "implante intradérmico" ou "implante profundo", comum a algumas espécies de lulas oceânicas e de águas profundas (e.g., Norman & Lu, 1997; Nesis *et al.*, 1998; Jackson & Jackson, 2004; Hoving & Laptikhovsky, 2007; Hoving *et al.*, 2008a; Hoving *et al.*, 2009). Diferentemente do que ocorre com outras lulas, nas quais a base do espermatângio fixa-se ao corpo da fêmea a partir das substâncias adesivas do corpo cimentante (Drew, 1919a), no implante profundo o espermatângio penetra inteiramente no tegumento da fêmea. Apesar dos grandes avanços proporcionados pelos estudos supracitados ao conhecimento da biologia reprodutiva dos cefalópodes oceânicos, importantes questões permanecem não resolvidas, como o mecanismo responsável pelo implante profundo. Algumas hipóteses foram propostas para explicar esse fenômeno, mas até o momento nenhuma ganhou ampla aceitação. Alguns autores são favoráveis à hipótese de que o longo órgão terminal ("pênis") de algumas espécies serviria para implantar hidraulicamente os espermatóforos no corpo da fêmea (e.g., Murata *et al.*, 1982; Norman & Lu, 1997; Jackson & O’Shea, 2003; Jackson & Jackson, 2004). Outras hipóteses envolvem a ação de ganchos ou ventosas dos braços e/ou tentáculos, bem como das mandíbulas (bicos), com os quais o macho faria incisões no corpo da fêmea para inserir os espermatóforos (e.g., Murata *et al.*, 1982; Nesis, 1995; Nesis *et al.*, 1998). Outra hipótese recorrente na literatura envolve a ação de supostas enzimas histolíticas presentes nas túnicas ou no corpo cimentante do espermatóforo (e.g.,

McSweeny, 1978; Nesis *et al.*, 1998; Hoving *et al.*, 2009), embora até o momento não haja registro de tais substâncias nas estruturas componentes desses espermatóforos.

O fato é que, recentemente, confirmou-se que o implante profundo é um processo autônomo, ou seja, o espermatóforo é capaz de implantar-se no corpo da fêmea sem a ação de agentes externos (Hoving & Laptikhovsky, 2007; Hoving *et al.*, 2009). Além disso, confirmou-se, em uma espécie que apresenta implante profundo (*Rossia moelleri*), que a força produzida pela aceleração e massa do espermatóforo em evaginação não é suficiente para perfurar mecanicamente o tecido da fêmea (Hoving *et al.*, 2009). O implante profundo dos espermatângios seria, portanto, um processo muito diferente daquele observado nos nematocistos dos cnidários, nos quais a perfuração da parede do corpo das presas é aparentemente garantida pelo estreito diâmetro e extraordinária aceleração atingida pelo túbulo interno, que é evaginado durante a "explosão" do nematocisto (e.g., Özbek *et al.*, 2009).

Dessa forma, a hipótese mais aceita atualmente para explicar o fenômeno de implante profundo é a que envolve a ação de supostas enzimas histolíticas liberadas pelo corpo cimentante (Hoving *et al.*, 2009), embora diversas questões acerca desse processo permaneçam enigmáticas. O fato é que o conhecimento acerca da morfologia funcional dos espermatóforos dos coleóides é tão escasso que dificulta inclusive a criação de hipóteses robustas para tentar explicar processos difíceis de serem observados *in vivo* ou *in vitro*. Como o funcionamento extracorpóreo dos espermatóforos depende exclusivamente da intrincada estrutura e organização de seus componentes (membranas, túnicas etc.), somente investigações detalhadas dessas estruturas proverão as bases para entender o funcionamento e a exata função do complexo espermatóforo dos coleóides.

4. Estabelecimento e desenvolvimento do projeto de pesquisa

Em face das diversas lacunas no conhecimento acerca da estrutura e funcionamento dos sistemas reprodutores masculino e feminino dos cefalópodes, o projeto de Doutorado iniciou-se com o objetivo geral de investigar a anatomia e o funcionamento destes sistemas, em uma tentativa de contribuir para a solução dos problemas expostos. Para se alcançar os objetivos propostos à época do início do projeto, decidiu-se investigar os elementos da anatomia macro e microscópica e respectivo funcionamento *in vivo* dos sistemas reprodutores da lula *Doryteuthis plei* (Blainville, 1823)[**], espécie comum no litoral do Estado de São Paulo, adotada como modelo.

[**] A realocação da espécie ao gênero *Doryteuthis* foi consenso do artigo de Vecchione *et al.* (2005). Embora seja comum encontrar citações atuais empregando o nome genérico "*Loligo*" para essa espécie, no presente trabalho optou-se pela proposta de Vecchione *et al.* (2005).

No caso do sistema reprodutor masculino, com o objetivo de investigar o seu papel na elaboração dos espermatóforos, além da análise morfológica detalhada do órgão espermatofórico, foi necessário incluir a investigação dos espermatóforos de *D. plei*. Apesar da morfologia e funcionamento dos espermatóforos de Loliginidae serem em geral considerados como bem conhecidos na literatura, somente o estudo detalhado do "produto final" (espermatóforo) é que poderia fornecer as bases para compreender sua formação. Portanto, foi incluída no projeto a análise minuciosa da estrutura do espermatóforo e da reação espermatofórica de *D. plei*. Pode-se dizer o mesmo a respeito do estudo do papel do sistema reprodutor feminino, que incluiu análise morfológica tanto dos órgãos reprodutivos femininos como das cápsulas dos ovos.

Com o avanço do projeto, percebeu-se que o conhecimento acerca da morfologia e funcionamento dos espermatóforos de Loliginidae, um tema tido como bem resolvido na literatura especializada, era pequeno em face das descobertas que estavam sendo realizadas no âmbito do presente trabalho (vide capítulos 1, 2 e 3). Dessa forma, uma seção do projeto que originalmente tinha caráter auxiliar, acabou por tomar grande parte da Tese à medida que os dados obtidos acarretavam mudanças conceituais importantes no conhecimento acerca da biologia reprodutiva dos teutóides. Com o acúmulo das evidências coligidas ao longo do Doutorado, tanto aquelas obtidas dentro do âmbito do projeto (capítulos 1 a 3), como aquelas reunidas pela consulta da literatura específica, gerou-se um arcabouço de dados (capítulos 4 e 5) que permitiu a proposta de um modelo teórico (capítulo 4) para tentar explicar o fenômeno de implante dos espermatóforos dos coleóides. Devido à importância dessa proposta, elaborou-se na seqüência uma revisão e reavaliação da função da reação espermatofórica nos cefalópodes, bem como uma discussão acerca da evolução desse fenômeno no grupo (capítulo 5).

5. Objetivos

1) Descrever com detalhes a estrutura do espermatóforo de *Doryteuthis plei* utilizando diversas ferramentas de análise morfológica;

2) Descrever todas as fases da reação espermatofórica do espermatóforo de *D. plei*, incluindo as modificações morfológicas envolvidas, bem como o papel dos componentes do espermatóforo no funcionamento do mesmo e no processo de fixação do espermatângio na fêmea;

3) Revisar e compilar a literatura referente aos mecanismos envolvidos na transferência de espermatozóides nos cefalópodes (e.g., espermatóforo, reação espermatofórica, armazenamento de espermatozóides), como base para uma reavaliação dos mesmos à luz das evidências reunidas ao longo do projeto;

4) Com base nas evidências coligidas, elaborar um modelo teórico para explicar o funcionamento dos espermatóforos dos coleóides, especificamente os mecanismos envolvidos na fixação dos mesmos no corpo da fêmea;

5) Discutir a evolução do espermatóforo e da reação espermatofórica, a partir de mapeamentos de caracteres reprodutivos feitos com base em recentes hipóteses de relacionamento filogenético dentre os Cephalopoda.

6. Metodologia básica

Por se tratar de um projeto pioneiro na área de biologia reprodutiva de cefalópodes, e por envolver uma série de abordagens distintas, eram esperadas muitas dificuldades em vista, principalmente, da ausência de tradição ou experiência em questões específicas, desde a coleta dos animais até o preparo adequado das amostras para aplicação das diferentes técnicas. Sendo assim, foi necessário grande esforço no estabelecimento de protocolos metodológicos para manutenção, fixação, histologia, histoquímica, microscopia eletrônica, entre outras técnicas, que em muitos casos somente foi possível com a utilização da infra-estrutura de outros laboratórios (Lab. de Biologia Celular do IBUSP, Lab. de Parasitologia e Lab. de Biologia Celular do Instituto Butantan, entre outros). Como conseqüência, detemos hoje os conhecimentos técnicos necessários para desenvolver estudos nessa área específica. Um resumo da metodologia empregada neste estudo é apresentado a seguir, sendo os detalhes específicos de cada técnica apresentados nos demais capítulos.

Foram realizadas coletas nos meses de verão de 2005 a 2008 ao largo das praias Pacuíba, Jabaquara e Fome (Ilha de São Sebastião), bem como ao largo da praia de Barequeçaba (Município de São Sebastião), Litoral Norte do Estado de São Paulo. Espécimes de *Doryteuthis plei* foram capturados com emprego de técnicas de pesca artesanal de lulas utilizando o "zangarelho", instrumento composto por anzóis chumbados. Após a captura, os espécimes foram acondicionados em uma bombona com capacidade para 200 L, contendo água do mar, sendo mantida aerada com bombas de ar a pilha e renovada com freqüência enquanto se procedia a pesca a partir da embarcação. Nos laboratórios do CEBIMar (Centro de Biologia Marinha da USP), o qual dispõe de um sistema de captação e distribuição de água do mar, as lulas foram alojadas em tanques com água do mar circulante de dimensões em centímetros 110×90×60 (comprimento × largura × altura) e capacidade de 500 L. Para anestesia, cada espécime foi retirado do tanque com auxílio de um puçá raso e transferido para uma cuba de plástico contendo duas partes de água do mar e uma parte de solução de cloreto de magnésio a 7.5% em água destilada. Após constatação de ausência de movimentos e reações ao toque (aproximadamente 10

minutos), os espécimes foram dissecados para análise e fixação dos espermatóforos. Os métodos de coleta, transporte e manutenção temporária dos espécimes vivos mostraram-se bastante adequados para a espécie em estudo e seguiram as orientações da literatura especializada, sumarizada em Moltschaniwskyj *et al.* (2007).

Espermatóforos frescos extraídos do saco espermatofórico de machos vivos anestesiados foram transferidos para cubas de dissecção ou lâminas e analisados sob esteremicroscópio e microscópio óptico, por vezes com emprego de solução saturada de cloreto de magnésio a 25% em água do mar, para retardar a reação espermatofórica e permitir o acompanhamento de algumas de suas fases (Drew, 1919a). Durante análise sob microscopia óptica, foi realizado registro de cada fase por meio de desenhos, fotos e filmagem. O emprego de recursos como Contraste de Interferência Diferencial e Microscopia de Luz Polarizada mostraram-se excelentes no estudo na estrutura dos espermatóforos da espécie. Experimentos *in vitro* realizados com amostras de tentáculos e manto das lulas foram muito úteis para se observar o comportamento dos espermatóforos em evaginação ante um substrato.

Espermatóforos em diferentes fases da "reação" e espermatângios, bem como amostras do corpo da fêmea contendo espermatângios depositados naturalmente pelos machos em cativeiro, foram fixados apropriadamente para diversas análises de microscopia. Para fixação, utilizou-se solução de paraformaldeído a 4% (em tampão PBS a 0.1M) ou fixador de Karnovsky (paraformaldeído a 2% e glutaraldeído a 2.5% em tampão cacodilato de sódio a 0.1M, pH 7.4, CaCl2 2.5 mM, ajustado a 1000 mOsm com sacarose). A maior parte das amostras foi desidratada e incluída em glicol metacrilato (historresina), embora inicialmente alguns testes tenham sido feitos também com parafina. Cortes de 2-4µm foram corados com os seguintes métodos (de acordo com Humanson, 1962; Bancroft & Stevens, 1982; e Pearse, 1985): Hematoxilina de Mayer e Eosina aquosa; Tricrômico de Mallory e de Gomori, para detecção de fibras colágenas; Ácido Periódico e Reativo de Schiff (PAS), para detecção de polissacarídeos; Azul de Alcian, para detecção de polissacarídeos ácidos; Azul de Bromofenol, para detecção de proteínas; e Alizarina Sódica, para detecção de depósitos de cálcio. Como complemento, foram realizadas ainda análises com Microscopia Eletrônica de Varredura das superfícies externa e interna dos espermatóforos e espermatângios de *D. plei*, bem como investigações empregando Microscopia de Varredura Laser Confocal.

No âmbito do presente projeto, mais de 200 espécimes de *D. plei* foram coletados. Para cada tipo de análise empregado no presente trabalho, centenas de espermatóforos e espermatângios foram utilizados, totalizando mais de 1000 lâminas coradas com diversos métodos histoquímicos, dezenas de *stubs* de Microscopia Eletrônica, aproximadamente três horas de filmagem da reação espermatofórica, e um arquivo digital contendo milhares de

fotos de lâminas e de amostras frescas e fixadas, bem como imagens de microscopia eletrônica e confocal dos espermatóforos e espermatângios de *D. plei*.

7. Organização da Tese

Além desta "Introdução" e das "Considerações Finais", a Tese está organizada em cinco capítulos estruturados como artigos científicos e redigidos em inglês. Precedendo cada capítulo, há uma breve apresentação redigida em português, sumarizando as idéias principais de cada artigo. Cada manuscrito está de acordo com a formatação exigida pelos respectivos periódicos aos quais serão submetidos. As respectivas tabelas, figuras e apêndices são apresentados independentemente ao final de cada capítulo. Os materiais suplementares eletrônicos citados ao longo da presente Tese (*electronic supplementary data/material*; e.g., vídeos) encontram-se no CD anexo. Embora já estejam em formato pronto para submissão, nenhum dos manuscritos está publicado ou no prelo, somente um deles tendo sido submetido para publicação. Dessa forma, todas as sugestões, correções e críticas dos membros da Banca poderão ser incorporadas aos mesmos antes de sua publicação.

O capítulo 1, “Unraveling the structure of squids’ spermatophores: a combined approach based on *Doryteuthis plei* (Cephalopoda: Loliginidae)”, apresenta descrição detalhada do espermatóforo de *D. plei* com base na combinação de diferentes ferramentas de análise, revelando uma maior complexidade estrutural do espermatóforo dos loliginídeos (e possivelmente de outros decapodiformes). Esse manuscrito será submetido ao periódico internacional *Acta Zoologica*.

O capítulo 2, “Might as well get deeper: perforating potential of loliginid spermatophores”, é uma ramificação do capítulo 2 e compreende o relato do potencial de perfuração ou penetração dos espermatóforos de *D. plei*. Sob condições artificiais, os espermatóforos em evaginação são capazes de penetrar musculatura exposta, de forma semelhante aos espermatóforos de algumas lulas oceânicas e de águas profundas. Esse manuscrito foi submetido ao periódico internacional *Biological Bulletin* em fevereiro de 2010.

O capítulo 3, “Spermatophoric reaction reappraised: novel insights into the functioning of the loliginid spermatophore based on *Doryteuthis plei* (Mollusca: Cephalopoda)”, apresenta a descrição da reação espermatofórica de *D. plei* com base em diferentes técnicas de análise, evidenciando que o processo de fixação do espermatóforo é mais complexo do que se pensava, envolvendo a ação de outras estruturas além do corpo cimentante. Esse manuscrito será submetido ao periódico internacional *Journal of Morphology*.

Com base nas evidências apresentadas nos capítulos precedentes e em evidências da literatura, o capítulo 4, “The evaginating-helix perforating mechanism: a theoretical model to explain spermatophore implantation in cephalopod mollusks” apresenta um modelo teórico para explicar como os espermatóforos de alguns cefalópodes são capazes de penetrar e implantar-se no tegumento das fêmeas. Esse manuscrito será submetido ao periódico internacional *The American Naturalist*.

O capítulo 5 “The cephalopod spermatophoric reaction and its evolution”, apresenta uma revisão da literatura acerca dos mecanismos de transferência e armazenamento de espermatozóides nos cefalópodes, bem como uma discussão acerca da evolução do espermatóforo e da reação espermatofórica em Cephalopoda, feita com base em mapeamentos de caracteres reprodutivos sobre topologias resultantes de recentes análises filogenéticas. Esse manuscrito será submetido ao periódico internacional *Zoologica Scripta*.

Ao final da Tese, é apresentado o artigo "Ethical and welfare considerations when using cephalopods as experimental animals" (Anexo 1), publicado em 2007 no periódico *Reviews in Fish Biology and Fisheries*, do qual o aluno foi co-autor com mais outros 12 pesquisadores internacionais. Esse trabalho revisa a literatura acerca das metodologias empregadas na experimentação com cefalópodes (e.g., captura, transporte, manutenção, alimentação, anestesia), como base para uma discussão acerca de questões éticas envolvidas na utilização de espécimes vivos em experimentos científicos. O trabalho é referente ao Workshop “Cephalopods as Experimental Animals”, do qual o aluno participou em fevereiro de 2006 na Tasmânia, Austrália, durante o Cephalopod International Advisory Council Symposium (CIAC). Como foi resultado, em parte, do esforço em reunir, para o presente projeto, bibliografia referente à manutenção de loliginídeos em cativeiro, decidiu-se por inserir este artigo como parte integrante da Tese. Dezenas de espécimes foram, de alguma forma, objeto de experimentação no presente estudo e houve grande esforço no sentido de reduzir o estresse e o sofrimento desses animais durante todo o processo desde a captura.

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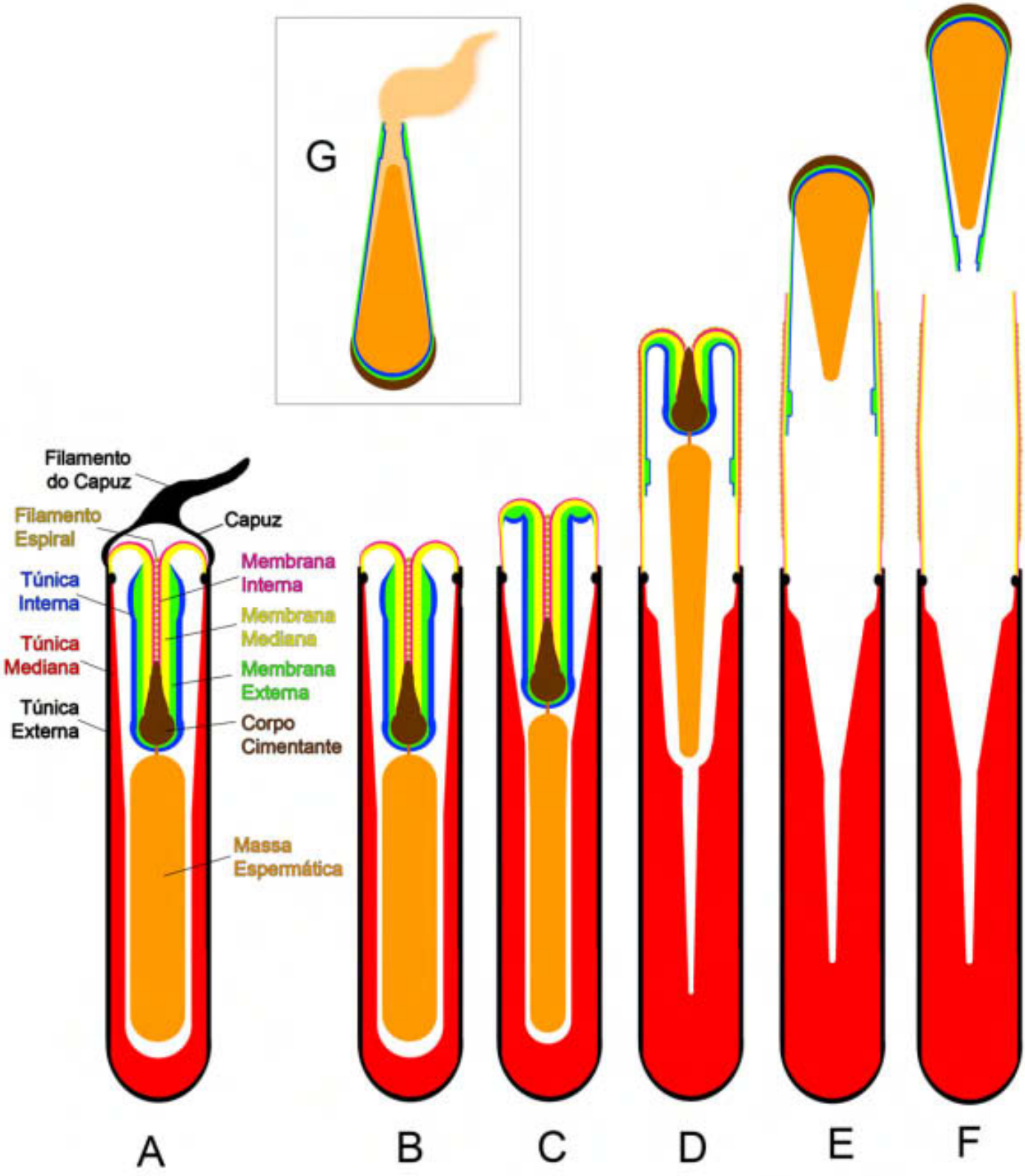
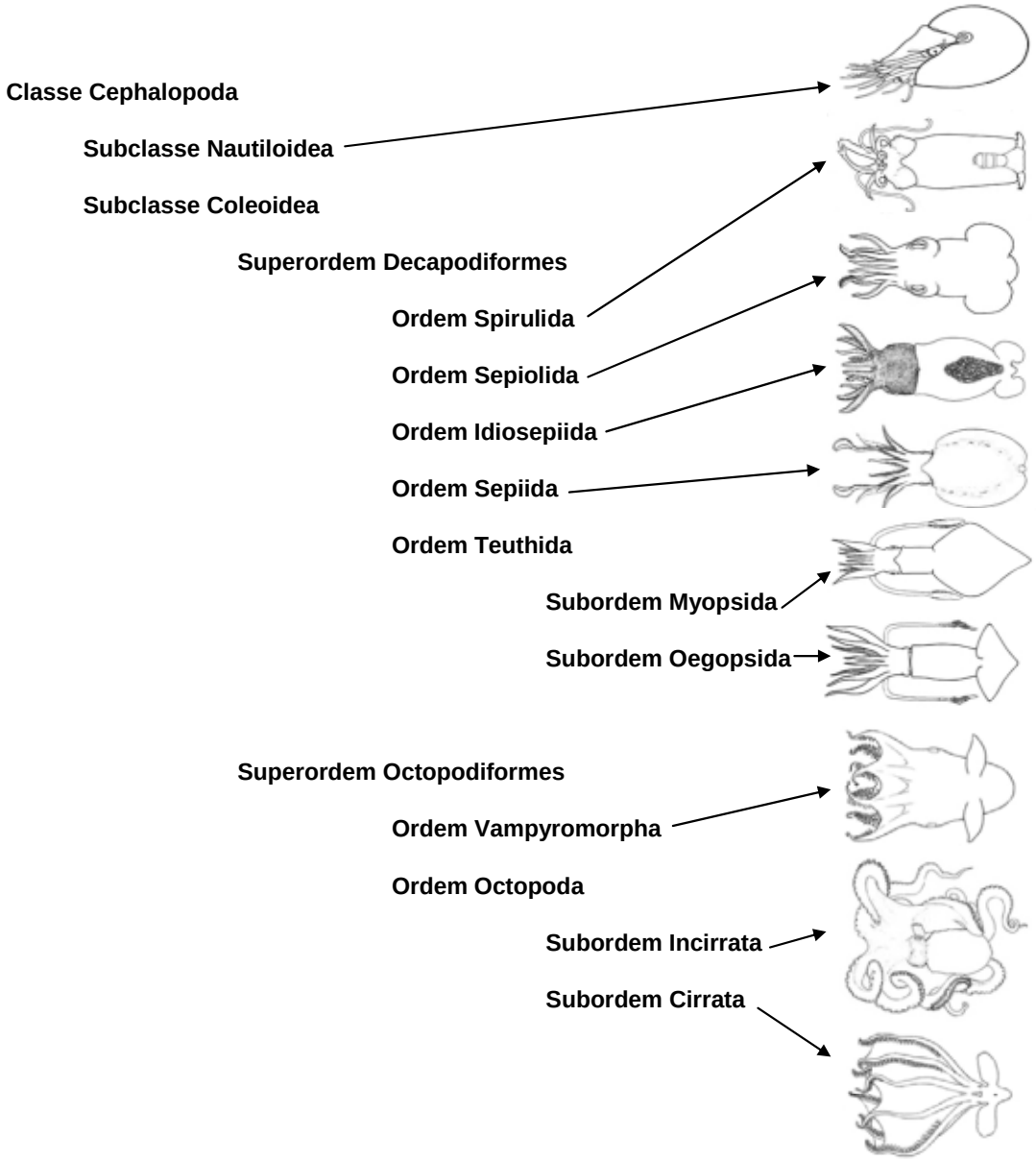


Figura 1. Esquema didático resumindo os principais passos envolvidos na reação espermatofórica (baseado principalmente no estudo de Drew, 1919a). Alguns passos foram omitidos e algumas estruturas simplificadas objetivando maior clareza. A-G) Seqüência da reação espermatofórica. Vide texto para detalhes.

APÊNDICE I

CLASSIFICAÇÃO DOS CEFALÓPODES VIVENTES

(modificada de Young *et al.*, 2008; figuras extraídas de Jereb & Roper, 2005)



De acordo com a classificação acima, ligeiramente modificada de Young *et al.* (2008), os cefalópodes viventes estão reunidos nas Subclasses **Nautiloidea** (os náutilos) e **Coleoidea** (todos os demais). Nautiloidea é representado por apenas dois gêneros (*Nautilus* e *Allonautilus*) e seis espécies viventes, endêmicas do Indo-Pacífico. Coleoidea compreende as superordens **Decapodiformes** e **Octopodiformes**. Os Decapodiformes são caracterizados pela posse de cinco pares de apêndices cefálicos (I-V), sendo os pares I, II, III e V (braços) de comprimento constante, e o par IV (tentáculos preensores) extensível. As espécies de Decapodiformes estão agrupadas em cinco ordens: **Spirulida**, monoespecífica

(*Spirula spirula*); **Sepiolida**, reunindo lulas de manto curto e com a extremidade posterior arredondada; **Idiosepiida**, representada por um único gênero (*Idiosepius*); **Sepiida**, reunindo espécies de sépias ou sibas; e **Teuthida**, agrupando as diversas espécies de lulas. É comum encontrar propostas de classificação reunindo alguns ou todos os "sepióides" (Sepiida, Sepiolida, Idiosepiida e Spirulida) na ordem "Sepioidea". Octopodiformes reúne as ordens **Vampyromorpha** e **Octopoda**. Vampyromorpha é monoespecífica e seu representante, a lula-vampiro (*Vampyroteuthis infernalis*), possui quatro pares de braços (I, III, IV e V) de comprimento constante, e o par II representado por filamentos sensoriais que se retraem completamente para dentro de bolsas em sua base. Os **Octopoda** apresentam apenas apêndices de comprimento constante, que correspondem aos pares de braços I, III, IV e V de Vampyromorpha. Octopoda reúne as Subordens **Cirrata** (polvos com nadadeiras no manto e cirros flanqueando as ventosas) e **Incirrata**.

Principais características de cada táxon:

Nautiloidea: concha externa, calcária e septada; dois pares de brânquias; sifão bilobado; até 94 apêndices circum-orais; apenas dois gêneros (*Nautilus* e *Allonautilus*) e seis espécies viventes, endêmicas do Indo-Pacífico;

Coleoidea: concha interna; um par de brânquias; 8-10 apêndices circum-orais.

Decapodiformes: cinco pares de apêndices cefálicos (I-V), sendo os pares I, II, III e V (braços) de comprimento constante, e o par IV (tentáculos preensores) extensível/retrátil.

Spirulida: concha interna, enrolada, calcária e septada; monoespecífica (*Spirula spirula*).

Sepiolida: concha rudimentar, quitinosa; nadadeiras semicirculares localizadas na região mediana do manto.

Idiosepiida: concha ausente; presença do órgão adesivo na superfície dorsal do manto, cuja função é fixar o animal na superfície de macroalgas; um único gênero (*Idiosepius*); contém as menores espécies de cefalópodes.

Sepiida: concha interna, reta, calcária e septada; “sibas”.

Teuthida: concha interna e reduzida a uma lâmina quitinosa; “lulas”.

Myopsida: olhos protegidos por membrana transparente; gonoduto ímpar; lulas costeiras.

Oegopsida: olhos sem membrana transparente; gonoduto pareado; lulas oceânicas.

Octopodiformes: quatro pares de braços (I, III, IV e V) de comprimento constante; o par II reduzido ou ausente; presença de cápsula adicional externa para abrigar o estatocisto.

Vampyromorpha: concha quitinosa e delgada; quatro pares de braços (I, III, IV e V) de comprimento constante, e o par II representado por filamentos sensoriais completamente retráteis para dentro de bolsas em sua base; monoespecífica (*Vampyroteuthis infernalis*); meso e batipelágicos.

Octopoda: apêndices de comprimento constante, que correspondem aos pares de braços I, III, IV e V de Vampyromorpha; celoma reduzido; “polvos”.

Incirrata: concha rudimentar ou ausente; nadadeiras no manto ausentes; cirros nos braços ausentes.

Cirrata: nadadeiras no manto; cirros flanqueando as ventosas; habitam as profundezas marinhas.

Capítulo 1

Revelando a estrutura do espermatóforo das lulas com base em *Doryteuthis plei*

CAPÍTULO 1

Revelando a estrutura do espermatóforo das lulas com base em *Doryteuthis plei*

Com o objetivo de se estudar a fundo a organização estrutural dos espermatóforos de *Doryteuthis plei*, diversas técnicas de análise morfológica foram testadas e empregadas. Além de se ter explorado de forma mais ampla recursos já empregados anteriormente em investigações acerca dos espermatóforos dos coleóides, novas técnicas foram introduzidas para se obter um quadro consistente da morfologia desta complexa estrutura. Como resultado da combinação de diferentes ferramentas de análise, a estrutura do espermatóforo revelou-se ainda mais complexa, como exposto no presente manuscrito.

As principais descobertas referem-se à: 1) complexa estrutura da membrana mediana, organizada em camadas e apresentando um segmento aboral quimicamente distinto que envolve parte do corpo cimentante; 2) presença de um característico material reticulado preenchendo o espaço entre a túnica interna e a membrana mediana (discute-se a possibilidade do mesmo consistir em um fluido viscoso em espermatóforos intactos); 3) presença de espículas intimamente associadas à membrana interna na região do corpo cimentante (além das espículas embebidas no filamento espiral); 4) presença de extensões membranosas que delimitam uma câmara pré-oral na região do capuz; e 5) complexa organização estrutural do corpo cimentante, delimitado por duas camadas e contendo substâncias de distintas propriedades químicas.

Uma avaliação cuidadosa da literatura permite sugerir que pelo menos parte dessas características deva ser comum aos espermatóforos de outros loliginídeos, e, em alguns casos, de outros grupos de lulas. As possíveis implicações funcionais dessa complexa organização estrutural são discutidas com base no conhecimento acerca do funcionamento da reação espermatofórica.

O manuscrito contendo essas informações em detalhe será submetido ao periódico internacional *Acta Zoologica.*

Unraveling the structure of squids' spermatophores: a combined approach based on *Doryteuthis plei* (Cephalopoda: Loliginidae)

Abstract

Coleoid cephalopods produce elaborate spermatophores, which function autonomously outside the male body during copulation, undergoing a complicated process of evagination. In order to contribute to the understanding of this unique structure, the present study investigated the morphology of the spermatophore of *Doryteuthis plei* applying several microscopy techniques. A hitherto unreported, much more complex structural arrangement was revealed for the loliginid spermatophore, the most striking findings being: 1) the complex, layered structure of the middle membrane, which bears an additional, chemically distinct segment surrounding part of the cement body; 2) the presence of a space between the inner tunic and middle membrane filled with a fine reticulated material, presumably a viscous fluid in the fresh state; 3) the presence of stellate particles not only embedded in the spiral filament, but also closely applied to the inner membrane at the level of the cement body; 4) the presence of a pre-oral chamber in the cap region; and 5) the complex organization of the cement body, formed by two distinct layers encompassing contents of different chemical and textural properties. Careful literature reassessment suggests several of these features are common to loliginids, and to some extent to other squids. Their possible functional implications are discussed in light of our knowledge on the spermatophoric reaction mechanics.

Key Words: Cephalopoda; Squids; Loliginidae; Reproduction; Spermatophore; Functional morphology.

Introduction

Male coleoid cephalopods (e.g., squids, cuttlefishes and octopods) produce elaborate spermatophores, which are transferred to the female during mating (Nesis 1987). The coleoid spermatophore is capable of functioning autonomously and extracorporeally, undergoing complicated changes during the so-called spermatophoric reaction (Mann 1984). This reaction comprises a complex process of evagination of the spermatophoric tunics and membranes, which leads to the extrusion and attachment of the sperm mass on the female's body (Drew 1919a). The spermatophores of cirrate octopods are an exception to this

complex coleoid plan, being apparently simple opercula-bearing sperm packets (Villanueva, 1992).

Although taxonomic descriptions of several cephalopod species have included the general morphology of the spermatophore (see guidelines of Roper and Voss 1983), these are usually too simplistic, as well as their accompanying illustrations, generally based on low magnification light microscopy. Few detailed morphological studies have yet been conducted, and much of the knowledge on this unique structure was generated by classical studies of the 19th and early 20th centuries (e.g., Milne-Edwards 1842; Racovitza 1894; Marchand 1913; Drew 1919a; Blancquaert 1925; Weill 1927). More recently, detailed descriptions were provided by Austin *et al.* (1964), who applied serial sectioning and staining to study the spermatophore of *Doryteuthis pealeii*, and by Takahama *et al.* (1991), who included scanning electron microscopy to investigate the spermatophore of *Todarodes pacificus*. Hess (1987), although not providing the same deep insight into spermatophore morphology, has given a comprehensive account on the spermatophore of several teuthoids (i.e., squids), composing the broadest study on coleoid spermatophores to date.

Investigations on the functioning of the coleoid spermatophore are considerably rarer, the knowledge on the general processes involved in the spermatophoric reaction being restricted, at present, to 12 decapodiforms (i.e., squids and sepioids; Milne-Edwards 1842; Racovitza 1894; Marchand 1913; Drew 1919a; Weill 1927; Austin *et al.* 1964; Fields 1965; Takahama *et al.* 1991; Hoving and Laptikhovsky 2007; Hoving *et al.* 2008; Hoving *et al.* 2009), and 7 octopodiforms (i.e., octopods; Milne-Edwards 1842; Marchand 1913; Drew 1919b; Fort 1937; Fort 1941; Orelli 1962; Mann *et al.* 1966, 1970; Hanson *et al.* 1973; Pujals 1978). As seen for the case of spermatophore structure, detailed descriptions of the spermatophoric reaction are also restricted to a handful of classical monographs (e.g., Racovitza 1894; Drew 1919a; Weill 1927).

Recent attempts to understand the functioning of the spermatophore of coleoids focused on the enigmatic process of deep intradermal implantation of spermatophores, a reproductive strategy common to some oceanic and deep-sea decapodiforms (e.g., Nesis *et al.* 1998; Hoving and Laptikhovsky 2007; Hoving *et al.* 2009). Despite several efforts, the mechanics behind this process remains unknown. The fact is, the knowledge on the structure of the coleoid spermatophore is still so poorly known that it is difficult to postulate hypotheses to explain its mechanics. Since the extracorporeal functioning of coleoid spermatophores must rely entirely on the intricate structure and organization of the tunics, membranes and other structures composing the spermatophore, only detailed investigations of these components will provide the basis for comprehending its mechanics.

In order to contribute to the knowledge on the coleoid spermatophore morphology, this paper describes in detail the spermatophore structure of the loliginid squid *Doryteuthis*

plei (Blainville, 1823), combining distinct microscopy techniques, such as light microscopy (including serial sectioning and several staining protocols), scanning electron microscopy and confocal laser scanning microscopy. The data presented herein reveal a much more complex structural organization of squids' spermatophores. Building on these results and on earlier studies, this paper attempts to provide a more comprehensive picture of the general morphology of this unique cephalopod structure.

Materials and methods

Animals

During the summers of 2005-2008, mature specimens of *Doryteuthis plei* were captured by hand-jigging off Barequeçaba Beach (São Sebastião municipality), between 23º50'00'' S and 45º26'20'' W, and off the northern littoral of São Sebastião Island (Ilhabela municipality), between 23º44'00'' S and 45º17'50'' W (São Paulo state, Brazil). Specimens were collected under IBAMA/MMA permit Nº 02027.000248/2005-11. Voucher specimens were deposited in the collection of the Museum of Zoology, University of São Paulo (MZSP).

Before dissection, male specimens were submerged and over-anesthetized in an isotonic solution of $MgCl_2$ (Moltschaniwskyj *et al.* 2007). Intact spermatophores removed from the spermatophoric sac were fixed for the following purposes.

Light microscopy

Spermatophores were 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. The material was dehydrated and embedded in glycol methacrylate (Leica's historesin), following the instructions furnished with the kits.

Due to the almost null permeability of the outer tunic to the embedding media, spermatophores had to be punctured or cut at the level of the connecting cylinder with fine dissecting scissors for complete infiltration (for a detailed protocol, see Appendix 1). Serial transverse and longitudinal 2-4 µm sections were subjected to the following staining procedures (according to Humanson 1962; Bancroft and Stevens 1982; and Pearse 1985): Hematoxylin-Eosin (H&E), Mallory's Triple Stain and Gomori's Trichrome (identification of collagen), Periodic Acid–Schiff (PAS - identification of neutral polysaccharides), Alcian Blue pH 2.5 (identification of acidic polysaccharides), Mercury-Bromophenol Blue (identification of

proteins), and Alizarin Red S (identification of calcium deposits). Solutions A and B of Mallory's Triple Stain were also experimentally applied separately. To obtain control sections for the Periodic Acid–Schiff test, the Schiff's reagent was applied also without any pretreatment. Other staining protocols and dyes were tested (e.g., Nynhidrin/Schiff, Sudan Black B), but, given their inconclusive results, these data were not considered for chemical composition analysis. Each spermatophore component showed a very specific affinity for some of the stains. Furthermore, a combination of the PAS and H&E techniques was tested on some sections, and produced reasonable staining of all spermatophore components.

Fresh spermatophores and stained slides were investigated applying several light microcopy techniques, such as Differential Interference Contrast and Polarized Light Microscopy.

Morphometry

Precise measurements of selected spermatophore regions were taken after fixation with the aid of the Zeiss AxioVision 4.5 Software, based on pictures taken under a Zeiss Stemi 2000-C stereomicroscope equipped with a Zeiss AxioCam MRc digital camera. Ten intact spermatophores removed from eight males each (ML 19.5–34cm) were measured (n = 80). Measurements included (Fig. 1): total spermatophore length (SpL), ejaculatory apparatus length (EAL), cement body length (CBL), sperm mass length (SML), spermatophore diameter at the approximate midpoint of the length of the ejaculatory apparatus (EAD), spermatophore diameter at the approximate midpoint of the length of the cement body (CBD), and spermatophore diameter at the approximate midpoint of the length of the sperm mass (SMD); see Hess (1987) for definitions. These data were used to compute seven indices, as defined by Hess (1987): SpLI = (SpL/ML)(100); EALI = (EAL/SpL)(100); CBLI = (CBL/SpL)(100); SMLI = (SML/SpL)(100); EADI = (EAD/SpL)(100); CBDI = (CBD/SpL)(100); SMDI = (SMD/SpL)(100). Data are presented herein as mean and standard deviation (for raw data, see Appendix S1[1] in Supporting Information).

Scanning electron microscopy

Spermatophores were fixed in Karnovsky's fixative (see above), washed in buffer solutions, and post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, adjusted to 1000 mOsm with sucrose. After additional rinsing in buffer solution, samples were dehydrated with acetone, critical point dried, mounted on stubs and gold coated. Some

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spermatophores were broken after critical point drying to expose and gold coat their internal structures. Subsequently, samples were examined in a Zeiss DSM 940 electron microscope.

Confocal scanning laser microscopy

Karnovsky fixed samples, mounted on slides without the application of special stains, were visualized by autofluorescence on a Zeiss Confocal LSM 510 META microscope. Digital projections were made using the accompanying software.

Nomenclature

Nomenclature is used according mainly to Drew (1919a), and also to the few terms introduced by Austin *et al.* (1964) and Takahama *et al.* (1991). As will be seen, however, it is not possible to strictly follow the present nomenclature available for squids' spermatophores. To avoid confusion, the authors have strived to maintain the basic terminology introduced by Drew (1919a) and subsequent authors, but either some spermatophore components could not be correlated to any of the available terms (e.g., "sperm mass casing"), or the correlation could not be done without uncertainty (e.g., inner membrane and outer membrane). Clearly, this issue is far from fully resolved and additional morphological studies are required if a stable nomenclature is to be attained. Each case of new-coined term or confuse terminology correlation is thoroughly discussed within the text.

Results

General structural organization

Spermatophores of *Doryteuthis plei* (Figs 1 and 2) are cylindrical structures of approximately 10mm long (SpL 9.38 ± 1.09mm; SpLI 3.41 ± 0.43), thicker at the aboral region (SMD 0.39 ± 0.04mm; SMDI 4.27 ± 0.44), tapering to the oral end (CBD 0.25 ± 0.01mm; CBDI 2.7 ± 0.22; EAD 0.21 ± 0.01mm; EADI 2.27 ± 0.19). The spermatophore consists of a sperm mass (SML 6.26 ± 0.87mm; SMLI 66.64 ± 3.44), a cement body (CBL 0.83 ± 0.07mm; CBLI 8.9 ± 0.67), and an ejaculatory apparatus (EAL 1.35 ± 0.16mm; EALI 14.43 ± 1.14), being enveloped partially by the middle tunic, and completely by the outer tunic (Figs 1 and 2). Orally, the spermatophore is closed by the cap, which bears a long cap thread (Figs 1 and 2A). The ejaculatory apparatus tube describes three loops (Fig. 2A) before reaching the cap region.

To avoid confusion, a brief terminological discussion is required at this point, before proceeding to the detailed morphological description. By definition (e.g., Drew 1919a), the ejaculatory apparatus is formed, from the outside to the median axis, by the inner tunic, the three membranes (outer, middle and inner), the spiral filament, the "amorphous material" (Austin *et al.* 1964), and the hyaline core. As will be presented, however, it is not possible to strictly follow this system. Based on the presented lines of evidence from light microscopy (serial sectioning and staining) and also from electron and confocal microscopy, the following system will be used herein. From the outside to the median axis of the spermatophore, the ejaculatory apparatus is composed of (respective abbreviation used throughout all figures given in parentheses): inner tunic (it), space filled with very fine reticulated material (*), middle membrane (mm), inner membrane (im), spiral filament membrane (sfm), spiral filament (sf), amorphous material (am), and hyaline core (hc).

Outer and middle tunics

The elastic, multi-layered (Fig. 3E) outer tunic surrounds the entire spermatophore (Figs 1, 2 and 3). The middle tunic is thicker at the region occupied by the sperm mass (Fig. 3A), tapering after the region of the cement body (Fig. 2B-D), and ending orally at the level of the thickening of the inner tunic (Fig. 7A). The middle tunic is divided internally by fibrous septa (Figs 3C,D); these septa connect the inner surface of the outer tunic to the outer surface of a thin membrane, which will be called herein as the "middle tunic inner layer" (Figs 3C,D). Within the space delimited by the middle tunic inner layer, the fibrous septa are interspersed with globular material (Figs 3B,F). Near to its oral ending, the middle tunic bears a constriction around the ejaculatory apparatus tube (Figs. 2B and 8). At this constriction, the middle tunic is longitudinally compressed, describing several short folds (Fig. 8A). The morphology of the middle tunic inner layer changes at this level from a solid membrane to an interwoven structure (Figs 8B,C,D). In some sections, there appeared to be a strong connection between the constricted region of the middle tunic and the outer surface of the inner tunic (Fig. 8D). The space between the middle tunic and the internal spermatophore structures is apparently fluid filled (Figs 2 and 3A).

Outer and middle tunics exhibited similar affinity for the applied stains (Table 1), although the middle tunic presented a higher PAS-positivity when compared to the Schiff-control, having also a higher affinity for Bromophenol and Alcian Blue (Table 1). In addition, the outer tunic stained slightly stronger with the Gomori's Trichrome (Table 1).

Cap region

At the oral-most region of the spermatophore, the outer tunic continues as the cap (Figs 2A, 4 and 5). Throughout its extension, the cap appears to be a solid, homogenous structure, except for its most oral part, which exhibits a slightly different staining intensity when compared to its aboral counterpart (Figs 5B,C,D). The cap thread (Figs 1 and 2A) starts as a sinuous thickening at the cap surface (Figs 4A,B). At one side of the cap periphery, this thickening continues as a large triangular flat thread (Figs 4C,D), which thins abruptly to form a slender, long filament.

At the cap region, the outer tunic thickens to form a ridge (Fig. 5) to which part of the oral end of the middle membrane attaches (Figs 5B,C,D). The outer tunic ridge is asymmetrically developed and diagonally disposed within the cap region (Figs 5B,C,D). On one half of the cap region periphery, the ridge is strongly developed and forms a prominent crest (Figs 5B,C,D). This crest gradually thins to form a slender ridge on the opposed side (Figs 5B,C,D). A membranous expansion (Fig. 5) originating from the outer tunic ridge extends towards the central axis of the spermatophore, delimiting a "pre-oral chamber" (Figs 5A,B,C). There is a discontinuity in the membranous expansion when the ejaculatory apparatus tube is reached, forming an aperture surrounded by a thickened rim (Fig. 5D). The ejaculatory apparatus tube passes along through this opening to reach the oral extremity of the spermatophore (Fig. 5D). Similar to the outer tunic ridge, the membranous expansion exhibits a diagonal arrangement (Fig. 5). See also the supplemental video (Appendix S2[2] in Supporting Information) showing successive focal planes throughout the spermatophore oral region, obtained under confocal laser scanning microscopy.

Inner tunic

The inner tunic is treated herein as a single unit, despite the fact that, depending on the staining technique, two layers are discernible at some places (e.g., Figs 7B,D; 10C; 17A,E). However, these are probably firmly cemented together, acting like a single solid tube. The inner tunic composes the outer covering of the whole ejaculatory apparatus tube. Orally, the inner tunic ends within the first loop of the ejaculatory apparatus (Fig. 6). Near to its oral ending, this tunic bears a prominent thickening (Figs 2B; 5,A,B; 7A-D; 8B), which thins abruptly and continues aborally as a thin-walled tube (Figs 8A-D; 9E-K; 10A-D). When this tube reaches the aboral region of the cement body, the inner tunic exhibits another prominent thickening (Figs 12A-B; 15A,D; 18A,C,E), which gradually thins aborally (Figs

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16A-D) to cover the base of the cement body (Figs 16A; 18B,D; 19A,B) and form an outer casing for the connecting cylinder (Figs 16A; 19A,B). There is at present no convincing evidence to state that the inner tunic continues aborally to cover the sperm mass (see below), as believed by Drew (1919a). Therefore, the inner tunic and the herein called "sperm mass membrane" are considered as separate entities, although they might be continuous and form a single structure.

The inner tunic stains strongly with hematoxylin and considerably with the dyes used for collagen demonstration (Table 1). The reaction is less intensive but also considerable with the Bromophenol Blue method (Table 1). Although this tunic exhibited also a strong PAS staining (Table 1; Figs 7B,D; 10B; 17C,D; 18A,B), the Schiff-control test revealed its PAS-positivity was actually low.

Fine reticulated material

Filling the space formed by the inner tunic and the middle membrane there is a characteristic material which deserves a separate description. This material is presumably dissolved during the ethanol baths required by some of the applied staining techniques, such as H&E (Figs 5B,C,D; 6B,C; 7C; 8B,C; 9E-K; 10A; 12A-C; 13A-I; 14B; 15A-D), Gomori's (Fig. 11C) and Mallory's Triple Stains (Fig. 17D), resulting in an artefactual empty space between the inner tunic and the middle membrane. However, a distinct material is preserved and brightly stained when PAS and Alcian Blue are applied to the sections (Figs 7B,D; 10B,C; 17A,C,E; 18A,E). This material exhibits a reticulated aspect in stained sections observed under light microscopy (Fig. 7D; 17E). When investigated under scanning electron microscopy, the reticulated material is revealed to be composed of a dense mesh of very delicate threads (Fig. 17F). This component is PAS-positive and stains strongly with Alcian Blue (Table 1). Interestingly, the reticulated material is preserved in the sections subjected to the combination of PAS and H&E (Fig. 8D). The reticulated material starts aborally at the beginning of the middle membrane (Figs 12A-C; 15A; 18A,C,E) and ends orally together with the inner tunic within the first loop of the ejaculatory apparatus (Figs 6A-C).

Middle membrane

The thick middle membrane extends from the intermediate region of the cement body (Fig. 12A-C) to the oral end of the spermatophore, where it stretches over and is closely applied to the inner surface of the cap, reaching the outer tunic ridge (Figs 5B,C,D). Throughout its length, the middle membrane consists of distinct layers (Fig. 7D) identifiable according to their affinity for the applied stains. Although at some regions there appears to be

several layers composing the middle membrane (Fig. 7D), generally three layers are present. The outer layer, the one in direct contact with the reticulated material, is generally thin and irregularly shaped (Figs 7B,D; 10B; 14B), and exhibits the most distinct staining affinities. While the other layers stained considerably with hematoxylin and strongly with Alcian Blue, the outer layer had no affinity for these stains (e.g., Fig. 17A), staining brightly with eosin (Table 1; Figs 13A,C; 14B). Moreover, while all middle membrane layers showed some PAS-positivity, the outer layer exhibited much stronger reactivity (Table 1; Figs 7B,D; 10B; 17C,E). At some places, it is very difficult to detect the outer layer (e.g., Figs 8B; 10A), as if this layer was artefactually removed, similar to the condition observed for the reticulated material, but convincing evidence is lacking. The thick middle and inner layers are easily distinguishable from one another, for the generally thicker inner layer has always a weaker affinity for hematoxylin (Table 1; Figs 6B,C; 7C; 8B; 9E-K; 10A; 13A-I).

At the intermediate region of the cement body, the middle membrane bears an additional layer, herein called "aboral segment" of the middle membrane, revealed in sections by its distinct affinity to the applied stains, most notably the very strong affinity for hematoxylin (Fig. 12A-C; 15A-D). Otherwise, it is very difficult to detect the middle membrane aboral segment, as its affinity for other stains is similar to the rest of the middle membrane (Table 1; Figs 18A,C,E). The aboral segment has the exact length of the intermediate region of the cement body, and is separated from it by the inner membrane (Figs 15A,B). Orally, the aboral segment ends wedged between the inner membrane and the inner layer of the middle membrane (Fig. 15A,B). At this region, the outer edge of the aboral segment is cut into several strips (Fig. 15B), a feature sometimes discernible in intact spermatophores. In fresh spermatophores, this region evidently marks a strong constriction at the level between the intermediate and oral regions of the cement body (Fig. 2C). As will be seen, the aboral segment of the middle membrane fills in the space created by the invaginations of the cement body intermediate region (Fig. 15C).

Inner membrane

The inner membrane covers the inner surface of the middle membrane throughout its length, extending from the intermediate region of the cement body (Figs 15A,B; 18A) to apparently the oral region of the spermatophore. The inner membrane is easily discernible in sections subjected to the PAS technique (Figs 7B,D; 10B; 17C,E; 18A); otherwise, however, it is very difficult to detect this membrane. Investigations using polarized light and differential interference contrast microscopy (Fig. 14C,D) helped identifying this membrane in the cases of negative staining. The inner membrane has the strongest affinity for PAS, together with the outer layer of the middle membrane (Table 1).

At the level of the intermediate region of the cement body (Figs 15A,B), the inner membrane thickens and is more easily discernible even in H&E stained sections, in this case also due to the dark blue staining of the aboral segment of the middle membrane (Figs 15A,B). Although at first sight it may appear that this membrane is a continuation of the inner layer of the middle membrane (see Fig. 15B), investigations using the PAS reaction indicate this membrane is the continuation of the strong PAS-positive inner membrane seen orally (Figs 7B,D; 10B; 17C,E; 18A).

At the level of the oral region of the cement body, the inner membrane is thrown into several longitudinal folds (Figs 13A,E-H; 14C). At this level and throughout the length of the cement body oral region, numerous tightly organized, minute stellate particles are closely applied to the inner surface of the inner membrane (Fig. 14). These particles have a strong affinity for acid stains, such as eosin, acid fuchsin and Orange G (from Mallory's Triple Stain), chromotrope 2R (from Gomori's Trichrome), and also Alizarin Red S. If a fresh spermatophore is broken and the cement body region isolated and viewed under high magnifications applying differential interference contrast microscopy, these particles are clearly discernible (Figs 14A). Due to their minute size, these particles are not always so easily detectable (e.g., Fig. 14B), but an excellent result was obtained combining Mallory's Solution B (which contains Orange G) applied alone, and interference contrast microscopy (Figs 14C,D). Fractured spermatophores examined under scanning electron microscopy revealed very fine stellate particles embedded within the space between the inner membrane and the outer layer of the cement body (Figs 14E,F). These particles are thicker than those embedded within the spiral filament matrix (described below).

Spiral filament membrane

The "spiral filament membrane" (see Discussion for nomenclatural issues) is closely applied to the inner surface of the inner membrane and covers the spiral filament throughout its length (Figs 7A,B,D; 8A-D; 9A; 10A,B; 11A,C; 13C). The discrimination between the spiral filament membrane and inner membrane may be very difficult without sectioning and staining (e.g., Figs 7A; 8A; 9A). Depending on the staining method, however, the spiral filament membrane is easily detectable for its bright red staining with eosin (Table 1; e.g., Fig. 10A) or bright blue with the Gomori's Trichrome (Table 1; Fig. 11C), to name a few. This membrane starts aborally at the oral extremity of the cement body (Fig. 13C), and apparently reaches the oral ending of the ejaculatory apparatus.

Spiral filament complex

The spiral filament lies within the tube formed by the spiral filament membrane (Fig. 7A,B,D; 8A-D; 9A; 10A-D; 11A,C), extending from the oral end of the cement body (Fig. 13C) to the loops of the ejaculatory apparatus (Fig. 2A; 6A), from where it gradually thins, ending before the ejaculatory apparatus reaches the cap (Fig. 5B-D). The spiral filament has a strong affinity for all of the employed acid dyes (Table 1; Figs 5B-D; 6B,C; 7C; 8B,C; 9E-K; 10A; 11C), exhibiting also some PAS-positivity (Table 1; Figs 7B,D; 10B).

When investigated through serial sectioning and with the application of acid stains, and viewed under high magnifications, the spiral filament is revealed to be composed of numerous minute stellate particles tightly packed together (Fig. 11C). If fresh spermatophores are tightly compressed under a cover glass and observed under differential interference contrast microscopy, these particles may also be discernible (Figs 11A,B). They are more easily seen if a trichrome method is employed, since the hyaline core stains with a different color, which results in a sharper contrast (Fig. 11C). Even with the application of this technique, only the particles which have dissociated from the spiral filament matrix may be more clearly discernible (Fig. 11C). When fractured spermatophores are examined under scanning electron microscopy, several minute (about 1.5 µm), sharp spines are seen embedded within the spiral filament matrix (Fig. 11D). They are much finer than those closely applied to the inner membrane at the level of the cement body (see above). Apparently, the spiral filament's stellate particles are thicker near to the connection with the cement body (Fig. 11B).

Throughout its length the strongly acidophilic spiral filament is interspersed with a strong basophile amorphous material (Table 1; Figs 7D; 8B-D; 10A-C). The term "amorphous material" introduced by Austin et al. (1964) is justifiable only if this material is compared to the solid, well-delimited spiral filament. However, the so called "amorphous material" has a well defined spiral organization (Fig. 10A). Although this material does fill in the space not occupied by the spiral filament (as defined by Austin et al. 1964), it also has its own structural identity. Besides staining strongly with hematoxylin, this material exhibited a very high affinity for the Alcian Blue dye (Table 1; Fig. 10C); when subjected to PAS reaction, the amorphous material stains a light purple color (Table 1; Figs 7B,D; 10B).

The hyaline core (Figs 5B,C; 7D; 8B; 10A-D; 11C) builds the longitudinal axis of both "spirals" (i.e., the spiral filament and the amorphous material), extending from the oral end of the cement body to the cap region (Figs 5B,C). Although the hyaline core exhibited staining affinities similar to the spiral filament (Table 1), the application of trichrome stains clearly separated them (Table 1; Fig. 11C) into very distinct classes of chemical substances.

The tube formed by the spiral filament complex changes from a laterally flattened tube at the level near to the thickening of the inner tunic (Figs 7C; 9E), to a more cylindrical shape at the middle region of the ejaculatory apparatus (Fig. 9F). Near to its attachment to the cement body, the cylindrical spiral filament tube rearranges itself to form three longitudinal folds (Fig. 9A-D,I-J). These folds become gradually thinner (Fig. 9J,K) towards the connection with the cement body. See also the supplemental videos (Appendixes S3[3] and S4[4] in Supporting Information), showing successive focal planes throughout the spermatophore at the level of the connection between the spiral filament and cement body, obtained under confocal laser scanning microscopy.

Cement Body

The cement body (Figs 2C,D; 12A-C) is divided into three distinct but continuous regions: the lanceolate oral region (Figs 13; 17), the cylindrical intermediate region (Figs 15; 18A,C,E) and the bulbous aboral region (Figs 16; 18B,D,F). The first division coincides with the constriction performed by the aboral segment of the middle membrane at the level of the strips (Figs 12A-C; 15A,B; 18A,C,E), and the second division with the aboral thickening of the inner tunic (Figs 12A-C; 15A; 18A,C,E).

The cement body bears its own outer casing (Figs 13A,B; 14B-D; 15B; 16B; 17D,E; 18A), which consists of two distinct layers (Fig. 14D). The inner layer (Fig. 14D) is thinner and composed of long juxtaposed fibrous material; the outer layer (Fig. 14D) is thicker and, depending on the applied method, shows either a spongy or a granular aspect (Figs 14D; 17C,D; 18A). The inner layer covers the cement body oral and intermediate regions (Figs 14B-D; 15B; 16B; 17D,E; 18A); however, at the aboral region, the inner layer separates from the outer layer and extends aborally for a short distance into the contents of the aboral cement body (Figs 16A,B; 18A,B,F). The outer layer apparently covers the entire cement body (Figs 16B; 18F), with the exception of the oral-most slender part. The whole cement body, including its layers and internal contents, exhibited strong affinity for all the applied acid dyes (Table 1).

The oral extremity of the cement body bears a distinct structure, which is lozenge shaped in longitudinal section (Figs 13A,C) and pleated in cross-section (Figs 13D). This structure shows a spongy aspect (Figs 13C,D), and bears a central canal apparently continuous with the matrix of the cement body (Figs 13A,D,E). The extremity also bears a distinct pointed structure at its oral end (Figs 11B; 13C).

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[4] Ver CD em anexo à Tese, contendo material suplementar.

Except for the region where the inner membrane is thrown into folds, the oral cement body is generally circular in cross-section (Figs 13F-I). The intermediate region, however, bears several longitudinal folds, which start as numerous short crests at its aboral region (Fig. 15D), and develop into few but prominent invaginations of the cement body walls (Fig. 15C) towards the oral region. The aboral segment of the middle membrane fills in the space created by these invaginations (Fig. 15C). Throughout its length, the aboral region of the cement body is circular in cross-section (Fig. 16C-D). At this region, the outer layer of the cement body shows an intimate connection with the inner tunic, bearing small projections that penetrate the inner surface of this tunic (Figs 16B; 18F).

The whole cement body encloses a core of coarsely granular acidophilic material embedded in an acidophilic homogeneous matrix (Table 1; Figs 13A-I; 14B; 15A-D; 16A-D; 17B,D; 18C,D,F). The granules differ in size, texture, refractive properties and chemical composition. The granular material of oral and intermediate regions are heterogeneous in size and sparsely organized (Figs 13A,B,I; 14B; 15A-B; 17C-E; 18A); besides staining strongly with acid stains (Table 1), they showed a very strong affinity for Bromophenol Blue (Table 1; Figs 17B, 18C), being also PAS-negative (Table 1; Figs 17C,E; 18A). The granular material of the aboral region is homogenous in size and densely organized (Figs 16A-D; 18A,B,D,F); besides staining strongly with acid stains (Table 1), they are PAS-positive (Table 1; Fig. 18B), and showed a much weaker affinity for Bromophenol Blue (Table 1; Fig. 18D). At the level near to the connection between aboral and intermediate regions of the cement body, the aboral granules appear isolated from the central area by the cement body's inner layer (Figs 16A-C; 18A,B,D,F). Interestingly, these isolated granules showed a much weaker PAS-positivity (if any at all) than their aboral counterparts (Figs 18A,B); except for this difference, however, the staining affinities, texture and light refraction characteristics are the same.

Connecting cylinder and sperm mass

The connecting cylinder attaches the aboral component of the cement body to the oral region of the sperm mass (Figs 2D; 16A; 19A,B). This structure starts as a spirally twisted cylinder at the aboral-most region of the cement body (Figs 16A; 19A,B); at this level, the connecting cylinder is completely enveloped by the thickened inner tunic (Figs 16A; 19A,B). When the cylinder leaves the cement body aboral region, the inner tunic envelope is difficult to trace (Figs 19B; 20B). At the sperm mass oral region, the connecting cylinder apparently develops into the "sperm mass membrane" (Fig. 19A; 20B). The connecting cylinder exhibited staining affinities very similar to those of the cement body (Table 1).

The sperm mass is primarily composed of a thin, spirally and tightly coiled sheet (Figs 20A,D) of densely organized spermatozoa (Figs 20C-E). The whole sperm mass is enveloped externally by the sperm mass membrane (Figs 20B,C) and internally by a casing of staining affinities very similar to the cement body (Table 1; Figs 19A,B; 20A,B). The sperm mass casing is more pronounced at the oral region (Figs 19A,B; 20A,B), although it may be traced as a finer casing towards the aboral region (Fig. 20A). This casing is primarily composed of granular acidophilic material (Table 1; Fig. 20B,C). The sperm mass casing is covered by the thin sperm mass membrane (Fig. 20B,C) throughout its length. Spermatozoa lie with their heads directed outwardly from the sperm mass longitudinal axis (Figs 20C-D), being embedded in a homogenous matrix (Figs 20A,B). At the aboral region of the sperm mass (Fig. 3A), both sperm mass membrane and casing gradually fade away (Fig. 20F), rendering the aboral-most region devoid of a solid covering.

Although the inner tunic was untraceable at the level of the connecting cylinder (see above), this tunic may well continues as the sperm mass membrane, since both of them exhibited very similar staining affinities (Table 1); however, convincing evidence is lacking on this subject.

Discussion

General remarks

The general morphology of the spermatophore of *Doryteuthis plei* is very similar to those of other loliginids and, to some extent, other decapodiforms described in the literature (e.g., Williams 1909; Drew 1919a; Austin *et al.* 1964; Fields 1965; Hess 1987). However, the techniques applied in the present study, notably thin longitudinal sectioning and the application of various stains, revealed a more complex structural organization of the teuthoid spermatophore. The most striking findings compared to earlier descriptions are: 1) the structure and complex organization of the middle membrane, which bears an additional, chemically distinct segment surrounding part of the cement body; 2) the presence of a space between outer and middle membranes filled with a fine reticulated material; 3) the presence of a membranous expansion delimiting a pre-oral chamber at the spermatophore oral region; 4) the presence of stellate particles not only embedded in the matrix of the spiral filament, but also closely applied to the inner side of the inner membrane at the oral region of the cement body; and 5) the complex organization of the cement body, formed by two distinct layers encompassing contents of different chemical and textural properties. As will be discussed, although these questions would only be confirmed with further comparative studies, careful investigation of the literature revealed evidence suggesting that at least some of these

characteristics may represent a common trait for loliginids, and some even for other decapodiforms.

Nomenclatural issues

Based on fresh and fixed spermatophores of *D. pealeii* investigated under light microscopy, Drew (1919a) applied the term "inner membrane" to the single membrane surrounding the spiral filament. Based on serial sectioning and staining of the spermatophores of the same species, Austin *et al.* (1964) have identified Drew's "inner membrane" as the single, PAS-positive membrane surrounding the spiral filament. However, at least in the case of *D. plei*, there are two membranes surrounding the spiral filament. Without serial sectioning and the application of several stains, both membranes may be very difficult to discern between one another (e.g., Figs 7A; 8A; 9A). This fact led the present authors to hypothesize that maybe the presence of two membranes may have passed unnoticed to previous authors. Since it is not possible to know exactly to which membrane Drew (1919a) was referring to, the authors decided to apply the term "inner membrane" *sensu* Austin *et al.* (1964), i.e., the PAS-positive membrane.

The fine reticulated material filling in the space between the inner tunic and middle membrane could well be the "outer membrane" defined by Drew (1919a), since its location and hyaline aspect in intact spermatophores (e.g., Figs 7A; 8A) match with the original description of the term. However, given that the reticulated material may constitute a viscous fluid in fresh spermatophores (see discussion below), which would be contrary to the original concept of an "outer membrane", the authors decided not to use this term for the reticulated material to avoid confusion.

Chemical composition analyses

The chemical tests results obtained herein are in accordance with the data presented on the general chemical composition of spermatophore components by previous works (Blancquaert 1925; Hamon 1939a and 1939b; Austin *et al.* 1964; Mann *et al.* 1966; Mann *et al.* 1970; Hanson *et al.* 1973; Takahama *et al.* 1991), but contribute for the first time to the knowledge on the chemical nature of specific spermatophore components (Table 1). The results obtained herein suggested a possible collagen-like nature for some tunics and membranes (e.g., outer and inner tunics, spiral filament membrane) given the staining reactions obtained with trichrome methods and PAS and Schiff techniques. The middle membrane is mostly composed of acidic polysaccharides given the Alcian Blue positivity (Table 1); its outer layer, however, is composed mainly by neutral polysaccharides (PAS-

positive; Alcian Blue negative; Table 1), maybe associated to proteins (Bromophenol Blue positive; Table 1). The inner membrane showed staining reactions similar to those of the thicker layers of the middle membrane (Table 1), but its PAS reactivity was much higher (Table 1). The fine reticulated material filling the space between the inner tunic and middle membrane also showed a similar strong alcianophilia (Table 1). In general, the spiral filament, stellate particles and the cement body showed a strong acidophilia given the results obtained with eosin and most acid dyes. These data could mean that their contents are caustic, as speculated by some authors (e.g., Hoving et al. 2009), but further experimentation is required to answer this question. The fact that the cement body had a strong affinity for Bromophenol Blue suggest its contents bear a high proportion of proteins in their composition, but the fact that this dye is acid and that the cement body is highly acidophilic prevents further discussion, since in this case the positivity might have come from the acidophilia alone. The same might be said about the results obtained with the acid dye Alizarin Red S (which is used to detect calcium deposits).

Immunocytochemical techniques should prove useful in the future for identification and localization of specific substances composing the spermatophore structures, and may help refine the knowledge on their chemical nature.

The cap region

The act of pulling the cap thread, which presumably occurs during spermatophore transfer when the male retrieves a bundle of spermatophores from the spermatophoric sac (Drew 1919a), is believed to rupture the cap and consequently trigger the spermatophoric reaction, although the contact with seawater alone is capable of doing so (Drew 1919a; Hoving *et al.* 2009). In *D. plei*, the cap thread is tightly cemented at the surface of the cap, on which it bears a sinuous thickening. The act of pulling the cap thread must transmit tension to the cap presumably through this thickening.

The presence of a membranous extension delimiting a pre-oral chamber at the spermatophore oral region is certainly striking. To the authors' knowledge, this is the first documentation of such component on a cephalopod spermatophore. The detection of this structure in *D. plei* is very difficult without the preparation of sections and staining of the spermatophores. Therefore, if the pre-oral chamber is present in other squids, they might have easily passed unnoticed to earlier authors. It should be mentioned, however, that a complexly organized cap region was described by Knudsen (1957) for the spermatophore of the giant squid *Architeuthis*, although the author did not mention the presence of an oral chamber.

The outer and middle tunics

The importance of the outer tunic for the spermatophoric reaction was already discussed and highlighted by previous authors (e.g., Racovitza 1894; Drew 1919a; Weill 1927; Mann *et al.* 1970). The elasticity and resistance of the outer tunic must be responsible for maintaining the necessary pressure for ejaculation (Drew 1919a; Mann *et al.* 1970). Besides this important function, the outer tunic is semi-permeable, allowing passage of seawater into the spermatophore, and preventing the passage of macromolecular substances (e.g., Mann *et al.* 1970), which is vital to permit seawater uptake by the middle tunic (or the spermatophoric plasma in the case of octopods). The data presented herein for *D. plei* show a stratified, laminated outer tunic, similar to that already described for *Todarodes pacificus* by Takahama *et al.* (1991), who postulated that these morphology is responsible for firmness and flexibility, both necessary for the outer tunic to withstand the inner pressure during the spermatophoric reaction.

As the outer tunic, the middle tunic has already received great attention regarding its important function of seawater absorbency (e.g., Racovitza 1894; Drew 1919a; Weill 1927) during the spermatophoric reaction. The middle tunic presumably functions as the spermatophoric plasma of octopods, which is hypertonic relative to the external milieu, generating an osmotic gradient (e.g., Mann *et al.* 1970). The vacuolated structure described herein for the middle tunic of *D. plei* appears to match this absorbent function. During seawater uptake, the vacuoles must assure space for the absorbed liquid. They might also be elastic to withstand the swelling of the middle tunic during the spermatophoric reaction (e.g., Drew 1919a). The globular material found inside the middle membrane is surely enigmatic and is worthy of a closer investigation.

The fact that the middle tunic constricts around the ejaculatory apparatus near its oral ending might indicate that this tunic performs an additional function during the spermatophoric reaction, but data is lacking on this subject. Maybe the reduced diameter of the middle tunic at this region results in elevated outflow of the internal structures being extruded, like the cement body and sperm mass. Williams (1909, p. 49 and pl. 1 fig. 5) also described and illustrated the middle tunic constriction in *D. pealeii*, which he attributed to the encirclement of "contractile fibers" around the middle tunic at this region. Although Drew (1919a) did not illustrate nor describe this peculiar morphology in *D. pealeii*, Austin *et al.* (1964, pl. 3 fig. 4) did show the constriction in a photograph of the ejaculatory apparatus of the same species. Interestingly, the spermatophore of the ommastrephid *Todarodes pacificus* also bears a similar constriction of the middle tunic around the ejaculatory apparatus (Takahama *et al.* 1991, fig. 5a), so this characteristic may well be common, at least among teuthoid spermatophores.

Fine reticulated material: a viscous fluid in fresh spermatophores?

The space between the inner tunic and middle membrane was revealed to be filled with fine reticulated alcianophilic material. This material is presumably dissolved and removed in sections subjected to ethanol washes; although historesin embedded sections do not need to be washed through a graded ethanol series, some staining techniques require immersion in ethanol solutions. Unaware of the existence of this reticulated material at the beginning of the study when investigating H&E stained sections, the authors were at first of the opinion that this empty space was artefactual, and that the inner tunic was closely applied to the middle membrane in intact spermatophores. Indeed, this empty space has been proven later to be artefactual when other staining methods were applied, since they have shown that inner tunic and middle membrane are not into contact, being clearly separated by the reticulated material.

The dissolution of this material by ethanol is apparently prevented when the periodic acid Schiff reaction is applied to the sections; see Fig. 8D showing a section that was stained first by the PAS method, then stained with H&E, which requires ethanol washing. This property could presumably be the result of the chemical treatment with periodic acid, which oxidises carbohydrates into aldehyde groups (which in turn are demonstrated with the Schiff's reagent; e.g., Bancroft and Stevens 1982). The formation of aldehyde groups could thus presumptively prevent the dissolution of the reticulated material when subject to ethanol washes.

The fact that 1) this material is soluble and easily washable in ethanol, 2) that its composition consists mainly of acidic polysaccharides (alcianophilia), and 3) that its aspect under scanning electron microscopy is that of a dense mesh of delicate threads, suggest that maybe this material constitutes a viscous, mucus-like fluid in fresh spermatophores. Williams (1909), on his monograph on squid anatomy, also believed that a fluid-filled space was present in this area of the spermatophore of *D. pealeii*, but he did not identify the middle membrane, which is definitely present in this species (Drew 1919a; Austin *et al.* 1964). Austin *et al.* (1964, fig. 14) image of the sectioned spermatophore of *D. pealeii* is suggestive of the presence of such space. Takahama *et al.* (1991) also mentioned the presence of a fluid filled space between the inner membrane and inner tunic in *Todarodes pacificus* (which they have called "middle membrane"), but gave no convincing evidence to their statement.

This presumably fluid-filled space between the outer and middle membranes would match with the functioning of the spermatophore during the spermatophoric reaction, in which the inner tunic glides through the middle membrane after evagination (e.g., Drew

1919a). This viscous fluid could presumptively facilitate gliding between both tubes by acting like a lubricant. Clearly, this material is worthy of a closer investigation.

Middle membrane

The complex structure and organization of the middle membrane revealed herein is certainly striking. There is evidence suggesting, however, that other loliginids share a similar morphology in respect to this membrane. Milne-Edwards (1842), studying the spermatophore of *Loligo vulgaris*, described and illustrated similar middle membrane morphology, i.e., layered and intricately organized at the region between the oral and intermediate regions of the cement body. While not deliberately reported by Austin *et al.* (1964), their photographs and description of cross sections of the spermatophore of *D. pealeii* matches the herein description for *D. plei*. Austin *et al.* (1964, pl. 4, figs. 11, 12 and 14) clearly evidence a layered middle membrane, as well as the possible presence of a space between the inner tunic and middle membrane. Fields (1965, fig.15) photograph of the oral half of the spermatophore of *D. opalescens* is suggestive of the presence of the aboral segment of the middle membrane.

The middle membrane also takes part in the evagination process, but, as opposed to the inner tunic, remains attached to the outer tunic after the spermatophoric reaction (Drew 1919a). Given its complicated morphology, this membrane may be more important to the reaction than ever thought. Drew (1919a) described that during the final phases of the spermatophoric reaction, the cement body is burst and smeared over the base of the spermatangium. Although this process remains enigmatic, the fact that the middle membrane exhibits different structure and chemical composition at the region of the cement body (i.e., the aboral segment and the constricted region between the oral and intermediate regions of the cement body), suggests this membrane might play a role in this complicated process. Maybe differences in density and elasticity in this region of the middle membrane could be decisive in bursting and smearing the cement body.

Inner membrane, spiral filament and stellate particles

In *D. plei*, stellate particles were found not only in the matrix of the spiral filament, but also closely applied to the inner side of the inner membrane at the oral region of the cement body. Austin *et al.* (1964) description and respective photographs of a cross section at the oral region of the cement body suggests that stellate particles are also present in *D. pealeii* on the inner membrane at this region: "The wall of the cement body consists of two eosinophilic layers, the outer coarsely granular and the inner of a more homogenous texture"

(Austin *et al.* 1964, p. 147). This "coarsely granular layer" of the wall of the cement body might be the inner membrane with the particles attached to its inner side. Their figures 14 and 15 on plate 4 are more evident on this respect, as in some places the stellate particles are actually visible. Besides this information, and similarly as in *D. plei*, Austin *et al.* (1964) stated that at approximately the middle of the cement body, its wall is thinner and simpler, i.e., without the "coarsely granular layer" (possibly the stellate particles), which could correspond to the absence of stellate particles at the inner membrane at this region of the spermatophore, similar to the condition seen in *D. plei*. The ommastrephid *Todarodes pacificus* possibly bears a similar morphology, since numerous stellate particles are apparently associated with the inner membrane at the region of the cement body (Takahama *et al.* 1991).

Stellate particles were already recorded embedded in the spiral filament's core of other loliginids (e.g., Austin *et al.* 1964), as well as several other coleoid species (e.g., *Sepiola atlantica*, Weill 1927; *T. pacificus*, Takahama *et al.* 1991; *Rossia moelleri*, Hoving *et al.* 2009). Drew (1919a), Austin *et al.* (1964) and Fields (1965) agreed in that the spiral filament located inside the inner membrane tube would help maintaining the potency of the extruding ejaculatory apparatus; Austin *et al.* (1964) believed the stellate particles would contribute to this function by providing stiffness to the spiral filament. Weill (1927) and Takahama *et al.* (1991) proposed the stellate particles would somehow take part in the attachment process. The fact that the inner membrane is everted during the spermatophoric reaction (Drew 1919a) implies that its respective stellate particles are most probably exposed during spermatophore evagination. The exposure of these sharp particles could possibly aid in the attachment process.

Cement body

The lozenge-shaped structure at the oral extremity of the cement body is apparently another feature widespread in loliginids. Drew (1919a, figs. 11-15) represented this structure in his illustrations of the ejaculating spermatophore of *D. pealeii* as the point of rupture of the inner membrane. Hess (1987, figs. 44a,c,d,e and 46c,f,g,i) also presented some evidences that this component might be present in the spermatophore of other loliginids. This structure is probably homologous to the sharp arrowhead-like component present at the same region in the spermatophores of ommastrephids (Marchand 1913; Hess 1987; Takahama *et al.* 1991; Nigmatullin *et al.* 2003).

The function of the cement body appears to be the final attachment of spermatangia by chemical (adhesive) substances (e.g., Drew 1919a), and maybe physically, since a thrust action involving a pointed structure was supposed for *Todarodes pacificus* (Takahama *et al.*

1991). The granules embedded within the matrix of the cement body showed an organized arrangement as well as distinct chemical properties, suggesting that the attachment of the spermatophore possibly involves a complex chemical process. Refining the knowledge on these components will certainly provide the basis for understanding how the spermatophore is finally attached on the female's body.

Conclusions

Coleoid spermatophores are among the most elaborate reproductive structures among metazoans (Mann 1984) and their complex structural organization has been recognized since their first record in *Biblia Naturae* (Swammerdam 1738). A handful of outstanding studies built our knowledge on the morphology of this unique coleoid character, and our understanding on its structure remains considerably deficient. Accordingly, our comprehension of the spermatophoric reaction mechanics is still very limited.

Here, following an in-depth study, several hitherto unknown characteristics of the decapodiform spermatophore were revealed, and they undoubtedly bear important functional implications during the spermatophoric reaction. These findings strengthen the need of more studies regarding the functional morphology of the coleoid spermatophore. Effort should be directed at the identification of simple, but efficient, protocols for whole-mount preparation of spermatophores. Coleoid spermatophores are generally a difficult material to handle and prepare for analysis (e.g., Hess 1987; see also Appendix 1), hence the need for a specific, standardized protocol. An expanded account of the spermatophore should also be included in the guidelines for the description of cephalopod species. Besides analyzing the general organization of the spermatophore under the low magnifications of a stereomicroscope, cephalopod taxonomists should, whenever possible, give a brief description of its structure at a minimal magnification of 100× to 200× (i.e., under a microscope; compare Figs 1 and 2). Drawings or photographs should accompany this description, which should include the main regions of the spermatophore (e.g., cap region; ejaculatory apparatus mid-region; connection between ejaculatory apparatus and cement body; cement body region; connection between the cement body and sperm mass; aboral-most region).

Besides possibly providing cephalopod systematists with novel characters to work with, this effort will certainly provide a more robust picture of the coleoid spermatophore structure, and hence broaden our basis for understanding the spermatophoric reaction, as well as coleoid general reproductive biology.

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Supporting Information – Legends of the electronic Appendixes

Appendix S1 Precise measurements of selected spermatophore regions taken with the aid of the Zeiss AxioVision 4.5 Software, based on pictures taken under a Zeiss Stemi 2000-C stereomicroscope equipped with a Zeiss AxioCam MRc digital camera. Ten intact spermatophores removed from eight males each (ML 19.5–34cm) were measured (n = 80). Measurements were taken after fixation and included: total spermatophore length (SpL), ejaculatory apparatus length (EAL), cement body length (CBL), sperm mass length (SML), spermatophore diameter at the approximate midpoint of the length of the ejaculatory apparatus (EAD), spermatophore diameter at the approximate midpoint of the length of the cement body (CBD), and spermatophore diameter at the approximate midpoint of the length of the sperm mass (SMD); see Hess (1987) for definitions. These data were used to compute seven indices, as defined by Hess (1987): SpLI = (SpL/ML)(100); EALI = (EAL/SpL)(100); CBLI = (CBL/SpL)(100); SMLI = (SML/SpL)(100); EADI = (EAD/SpL)(100); CBDI = (CBD/SpL)(100); SMDI = (SMD/SpL)(100).

Appendix S2 Video showing successive focal planes throughout the spermatophore oral region, obtained under confocal laser scanning microscopy. Several structures are evident; please refer to text for details.

Appendix S3 Video showing successive focal planes throughout the spermatophore at the region near to the attachment of the spiral filament to the cement body, obtained under confocal laser scanning microscopy. Note the three longitudinal folds of the spiral filament tube. Please refer to text for details.

Appendix S4 Video showing successive focal planes throughout the spermatophore at the region of the attachment of the spiral filament to the cement body, obtained under confocal laser scanning microscopy. Two longitudinal folds of the spiral filament tube are discernible, as well as the oral region of the cement body. Please refer to text for details.

Table 1. Results from the staining reactions. – Not stained; + weakly stained; ++ moderately stained; +++ brightly stained. H&E: Hematoxylin and Eosin; Mallory: Mallory's Triple Stain; Gomori: Gomori's Trichrome; Schiff: Schiff Reagent applied without any pretreatment; PAS: Periodic Acid / Schiff Reaction; Bromo: Mercury-Bromophenol Blue; Alcian 2.5: Alcian Blue at pH 2.5; Aliz Red S: Alizarin Red S.

	H&E	Mallory	Gomori	Schiff	PAS	Bromo	Alcian 2.5	Aliz Red S
Outer Tunic	++ Blue	+ Blue	++ Blue	++ Magenta	++ Magenta	+[1]	+[2]	–
Middle Tunic	++ Blue	+ Blue	+ Blue	+ Magenta	++ Magenta	++	++	–
Inner Tunic	+++ Blue	++ Blue	+++ Blue	++ Magenta	++ Magenta[3]	++	+[4]	–
Fine Reticulated Material[5]	–	–	–	–	++ Magenta	+	+++	–
Middle membrane								
Outer Layer	++Red	+Blue	+Blue	–	+++ Magenta	++	–	–
Middle Layer	++Blue	–	–	+ Magenta	+ Magenta	+	+++	–
Inner Layer	+Blue	–	–	–	++ Magenta	+	+++	–
Aboral segment	+++Blue	–	–	–	+++ Purple/Magenta	+	+++	–
Inner Membrane	–	+Blue	+Blue	+ Magenta	+++ Magenta	–	+++	–
Spiral Filament Membrane	+++Red	+Blue	+++ Blue	–	+ Magenta	++	–	–
Spiral Filament & Stellate Particles	+++Red	+++Red +++Orange[6]	+++Red	–	++ Magenta	++	–	+++ Reddish Orange
Amorphous Material	+++Blue	–	–	–	++ Light purple	–	+++	–

	H&E	Mallory	Gomori	Schiff	PAS	Bromo	Alcian 2.5	Aliz Red S
Hyaline Core	++Red	+Blue[7]	+++Blue	+ Magenta	++ Magenta	++	–	–
Cement Body								
1.Extremity	+++ Red	+++ Orange	+++ Red	–	–	++	–	+++ Reddish Orange
2.Outer Layer	+++ Red	+++Red ++Orange[6]	+++ Red	–	–	+++	–	+++ Reddish Orange
3.Inner Layer	+++ Red	+++Red +++Orange[6]	+++ Red	–	– and ++ Magenta	++	–	+++ Reddish Orange
4.Contents								
a.Matrix	+++ Red	+++ Red & Blue	+++ Red & Blue	–	+ Magenta	+++	–	++ Reddish Orange
b.Granules								
Oral	+++ Red	+++ Red	+++ Red	–	–	+++	–	++ Reddish Orange
Aboral	+++ Red	+++ Red	+++ Red	–	++ Magenta	++	–	++ Reddish Orange
Connecting cylinder	+++ Red	+++ Orange	+++ Red	–	++	++	–	+ Reddish Orange
Sperm mass								
Membrane	+++ Blue	+++ Blue	+++ Blue	+ Magenta	++ Magenta	++	+	–
Casing	+++ Red	+++ Red	+++ Red	–	++ Magenta	++	–	+ Reddish Orange
Matrix	++Red	–	+++ Blue	–	++ Magenta	+	–	–
								–

1. Only the outer-most layer stained moderately with Bromophenol Blue; the remaninig layers exhibited weak staining.
2. Only the outer-most layer stained moderately with Alcian Blue; the remaninig layers exhibited weak staining.
3. The inner tunic inner surface stained strongly with PAS.
4. The inner tunic inner surface stained strongly with Alcian Blue.
5. Very difficult to detect in sections subjected to ethanol washes.
6. When stained only with Mallory's Solution B.
7. In some sections interspersed with reddish orange staining.

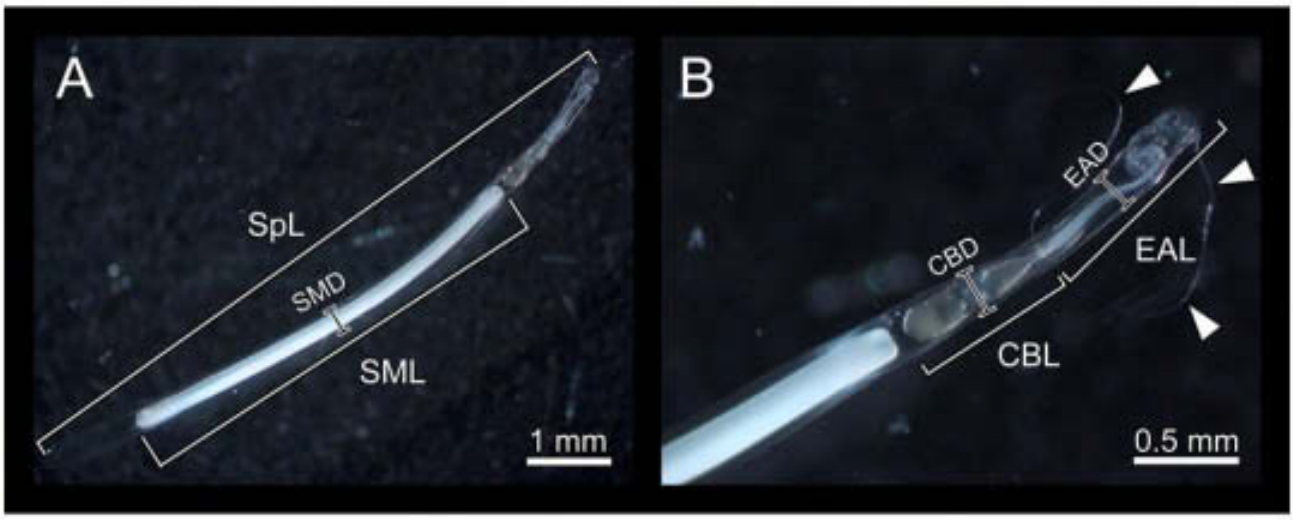

Fig. 1 – General view of preserved spermatophores of *Doryteuthis plei* and definition of the measurements taken for this study (after Hess 1987). **–A.** Whole spermatophore observed under the stereomicroscope (20x). Total spermatophore length (SpL), sperm mass length (SML) and spermatophore diameter at the approximate midpoint of the length of the sperm mass (SMD) are indicated. **–B.** Detail of the oral region observed under the stereomicroscope (50x). Ejaculatory apparatus length (EAL), cement body length (CBL), spermatophore diameter at the approximate midpoint of the length of the ejaculatory apparatus (EAD), and spermatophore diameter at the approximate midpoint of the length of the cement body (CBD) are indicated. The cap thread (arrowheads) is also discernible. These kinds of image were used to take precise measurements of the selected spermatophore regions using specific software (see Materials and methods).

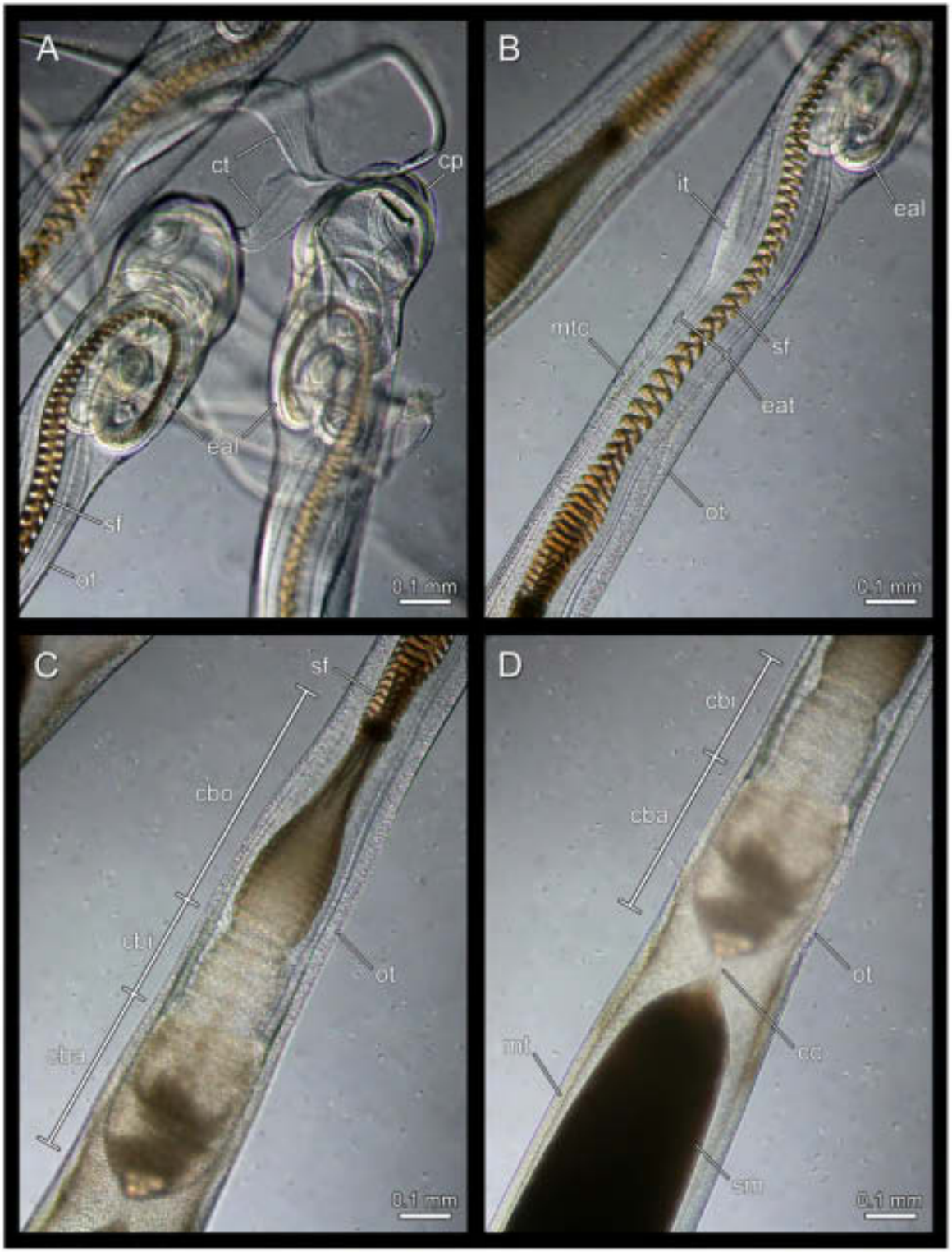

Fig. 2 – General structural organization of *Doryteuthis plei* fresh spermatophores observed under differential interference contrast microscopy (DIC). **–A.** Oral region of two spermatophores showing the cap (cp), cap thread (ct), outer tunic (ot), spiral filament (sf), and the loops of the ejaculatory apparatus (eal). Note that each spermatophore is shown in a different view, permitting the individual identification of some of the loops of the ejaculatory apparatus. **–B.** Region between the cap and the cement body, showing the ejaculatory apparatus tube (eat), the spiral filament (sf), the thickening of the inner tunic (it), the constricted region of the middle tunic (mtc), and the outer tunic (ot). **–C.** Region of the cement body, showing its basic tripartite division: aboral (cba), intermediate (cbi) and oral (cbo) regions. **–D.** Region of the connection between the sperm mass (sm) and cement body, both linked by the connecting cylinder (cc). The middle tunic (mt) is thicker and conspicuous at this region.

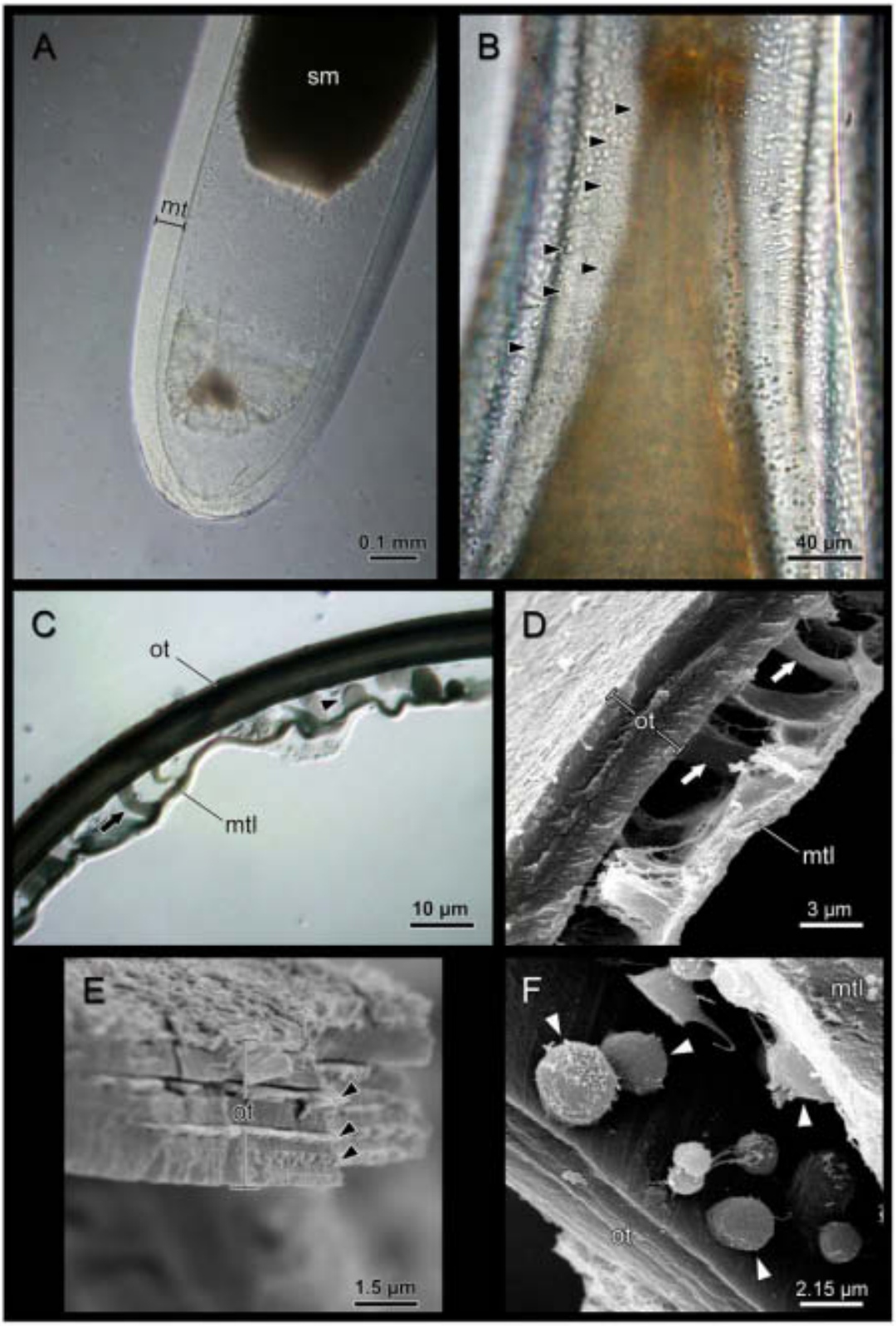

Fig. 3 – Outer and middle tunics. **–A.** Aboral region of the spermatophore showing the thick middle tunic (mt) and the base of the sperm mass (sm). DIC. **–B.** Enlarged view of the spermatophore at the region of the cement body. The middle tunic's globular material (arrowheads) is evident on the plane of focus. The cement body is visible on the background. DIC. **–C.** Detail view of a cross-section through the spermatophore showing the multilayered outer tunic (ot), and the middle tunic, which is divided internally by septa (arrow) interspaced with globular material (arrowhead). The middle tunic internal layer (mtl) separates these contents from the fluid filled internal space. Sudan Black B staining. **–D.** Scanning electron micrograph of a fractured spermatophore showing the outer tunic (ot) and the middle tunic, which is divided internally by fibrous septa (arrows) and delimited by a thin inner layer (mtl). **–E.** Scanning electron micrograph of a fractured spermatophore showing the layers (arrowheads) composing the outer tunic (ot). **–F.** Scanning electron micrograph of a fractured spermatophore evidencing the globular material (arrowheads) occupying the space between the outer tunic (ot) and the inner layer of the middle tunic (mtl).

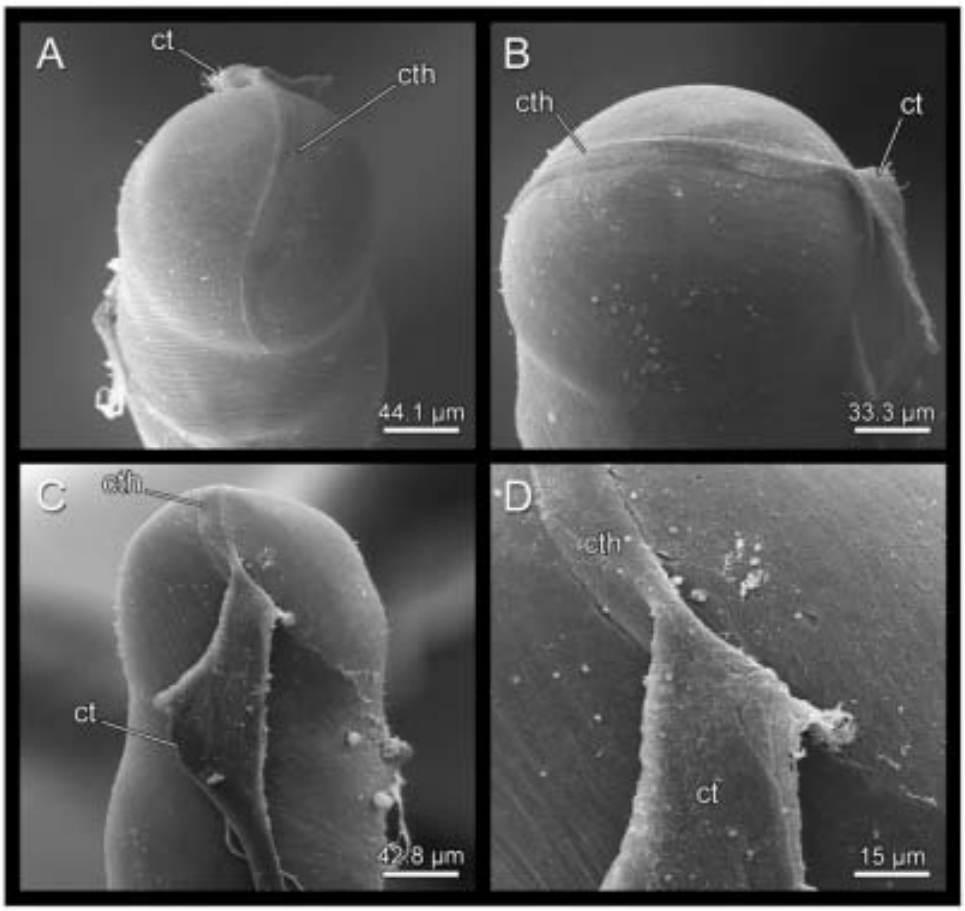

Fig. 4 – Cap thread morphology investigated under scanning electron microscopy. **–A.** Lateral-superior view of the cap region showing the sinuous thickening (cth) on the cap surface. This thickening is continuous with the cap thread (ct). **–B.** Same spermatophore, different view, showing the cap thread (ct) continuous with the thickening (cth) on the cap surface. **–C.** Same spermatophore, lateral view, showing the cap thread (ct) beginning as triangular flat thread, and thinning abruptly to form a slender filament. **–D.** Detail of the previous image, showing the connection between the base of the cap thread (ct) and the thickening (cth) on the cap surface.

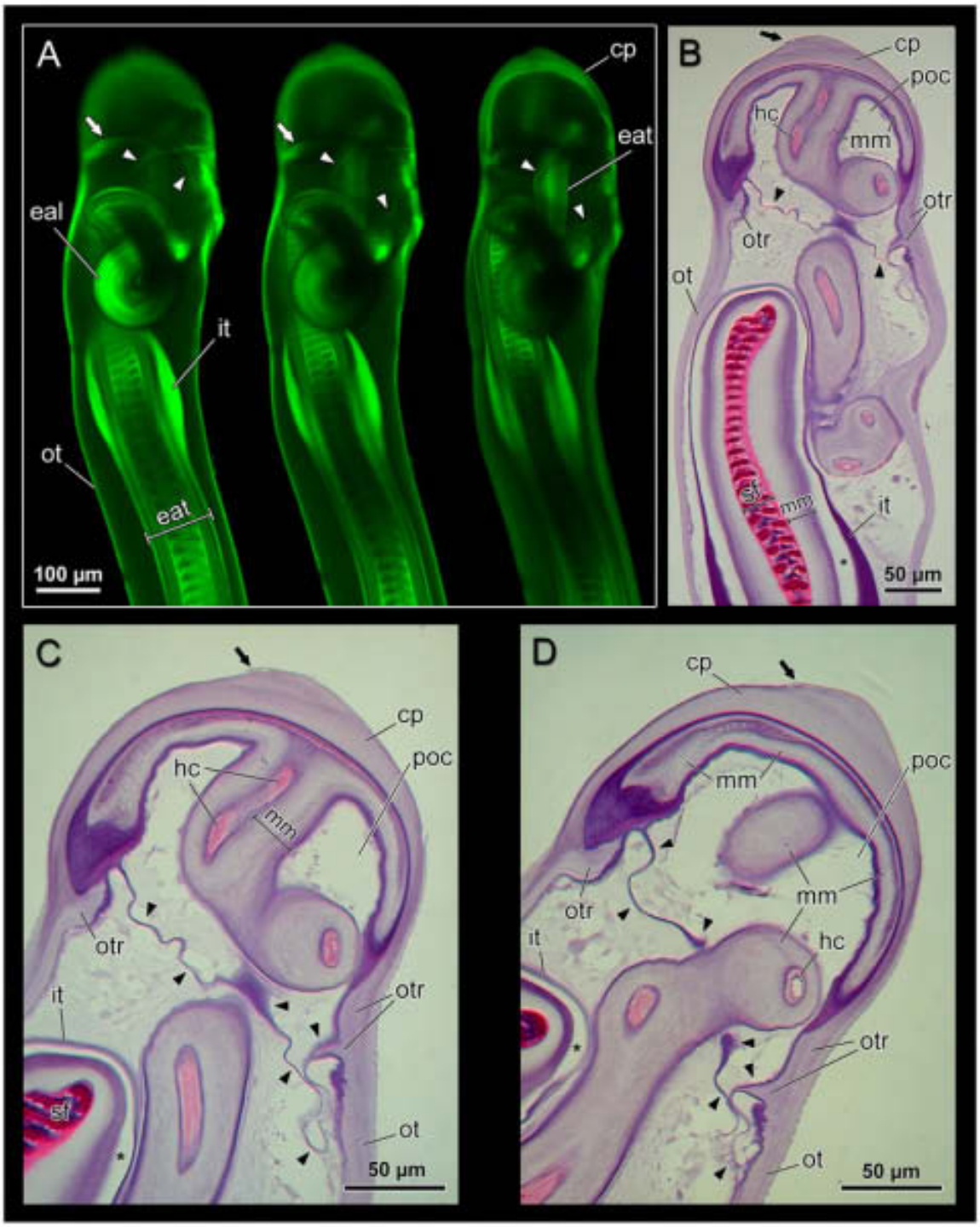

Fig. 5 – Structural organization of the cap region. **–A.** Successive focal planes throughout the spermatophore oral region, obtained under confocal laser scanning microscopy. The membranous expansion (arrowheads) begins at the level of the outer tunic ridge (arrows) and encircles the ejaculatory apparatus tube (eat) at the oral region, forming the herein called "pre-oral chamber" (compare with B–C). See also the supplemental video in Appendix S2. **–B.** Longitudinal section through the spermatophore oral region. The arrow points to the region where the cap thread (not visible) connects to the cap. The cap (cp) is continuous with the outer tunic (ot) and is apparently divided transversally into two distinct parts (the oral-most is apparently continuous with the cap thread). The middle membrane (mm) extends to the oral region, stretching over the inner surface of the cap, and reaching the outer tunic ridge (otr). From the region of the outer tunic ridge, a membranous expansion (arrowheads) isolates part of the oral region into the "pre-oral chamber" (poc). See also C–D. H&E staining. **–C.** Detail of the previous image. **–D.** Longitudinal section through the spermatophore oral region at the level of the aperture of the pre-oral chamber. There is an opening in the center of the membranous expansion through which the ejaculatory apparatus tube passes along to reach the oral region. H&E staining. Abbreviations: *, space between middle membrane and inner tunic (artefactually empty); cp, cap; eal, ejaculatory apparatus loops; eat, ejaculatory apparatus tube; hc, hyaline core; it, inner tunic; mm, middle membrane; ot, outer tunic; otr, outer tunic ridge; poc, pre-oral chamber; sf, spiral filament.

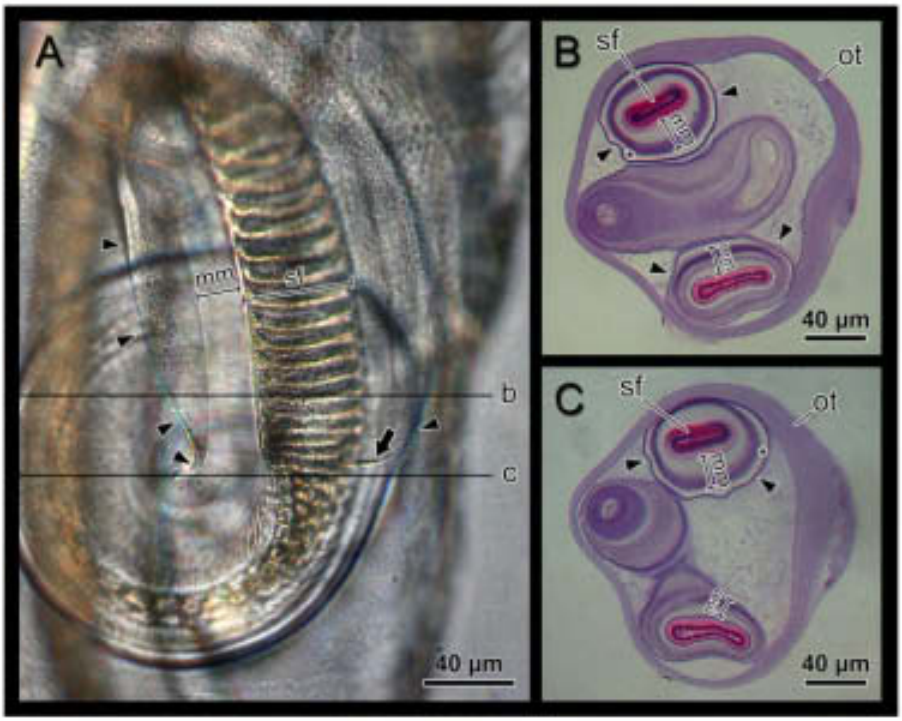

Fig. 6 – Structural organization of the loops of the ejaculatory apparatus, and the terminal region of the inner tunic. **–A.** Detail of the spermatophore at the region of the loops of the ejaculatory apparatus. The inner tunic (arrowheads) ends up (arrow) within the first loop of the ejaculatory apparatus. The middle membrane (mm) and spiral filament (sf) continue towards the oral region of the spermatophore. The lettered lines "b" and "c" indicate the approximate region of the cross-sections seen in B and C, respectively. DIC. **–B.** Cross-section through the point indicated by line "b". The inner tunic (arrowheads) is still present in the beginning of the first loop of the ejaculatory apparatus tube, seen in this section as the two most peripheral "discs" inside the outer tunic (ot). However, in the second loop (the tangentially cut tube), the inner tunic is not present. H&E staining. **–C.** Cross-section through the point indicated by line "c". The inner tunic (arrowheads) is still present in the beginning of the first loop of the ejaculatory apparatus tube, seen in this section as the upper peripheral "disc" inside the outer tunic (ot). However, at the ending of the first loop (the lower peripheral "disc") the inner tunic is not present. H&E staining. Abbreviations: *, space between middle membrane and inner tunic (artefactually empty); mm, middle membrane; ot, outer tunic; sf, spiral filament.

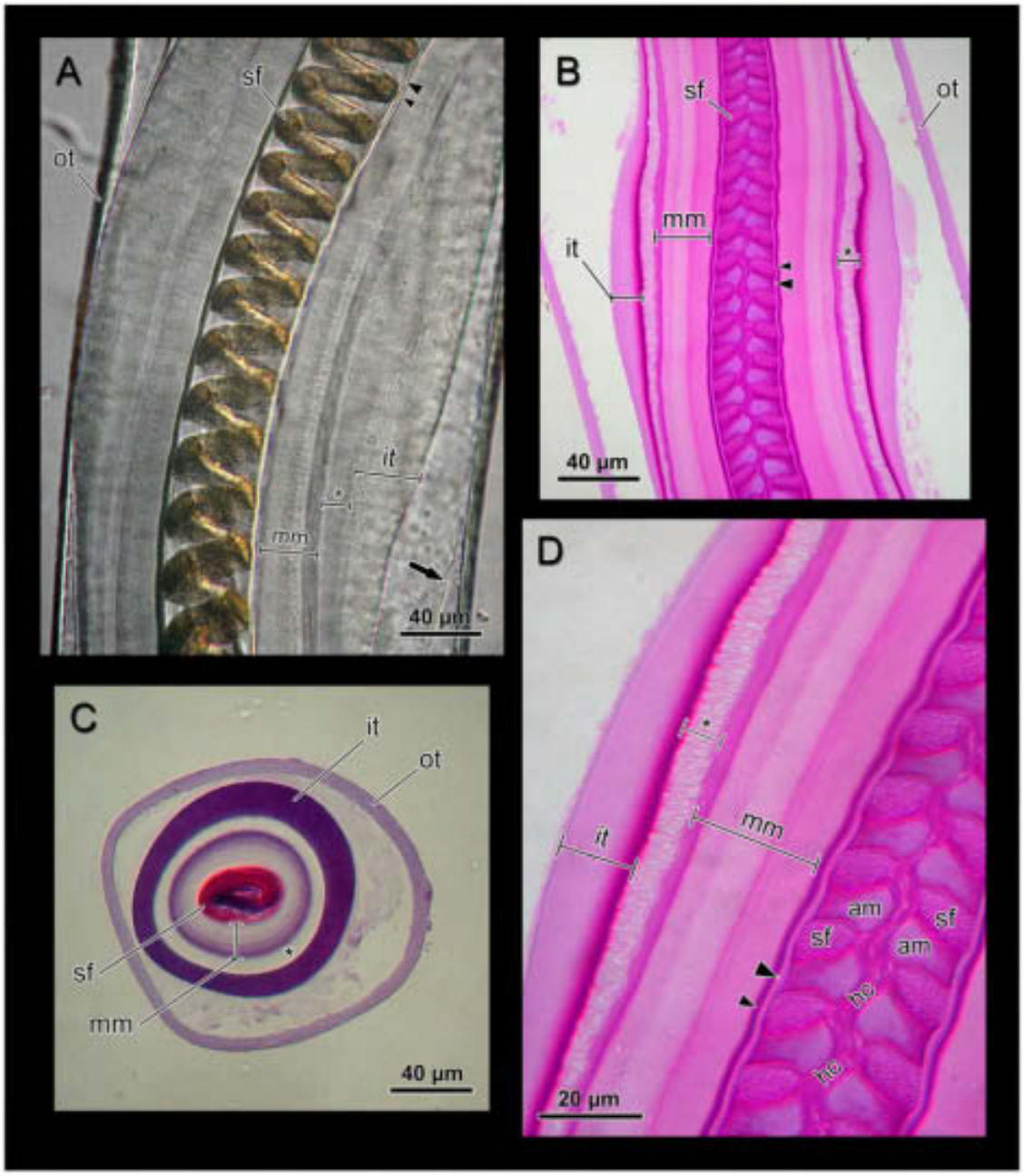

Fig. 7 – Ejaculatory apparatus morphology at the level of the thickening of the inner tunic. **–A.** Differential interference contrast micrograph of the spermatophore at the region of the thickening of the inner tunic (it). The spiral filament (sf) is encompassed by two membranes: the "spiral filament membrane" (large arrowhead) and the inner membrane (small arrowhead). The broad middle membrane (mm) is clearly composed of distinct layers and is separated from the inner tunic by a space (asterisk), which will be shown to be filled with fine reticulated material. The arrow points to the ending of the middle tunic. **–B.** Longitudinal section through the spermatophore at the region of the thickening of the inner tunic (it). The separation between the "spiral filament membrane" (larger arrowhead) and "inner membrane" (smaller arrowhead) is evident. The structures encompassed by these membranes, e.g. the spiral filament (sf), are clearly discernible (these are described in detail in D). The middle membrane (mm) consists of several layers of distinct reactivity to the applied stain. Between the middle membrane (mm) and the inner tunic, there is a space (asterisk) filled with a fine reticulated PAS-positive material. The middle tunic is no longer applied to the outer tunic (ot) at this level. PAS staining reaction. **–C.** Cross-section through the same region, showing the thickening of the inner tunic (it), the layered middle membrane (mm), and the laterally flattened spiral filament (sf). Note that the space (asterisk) between the middle membrane and the inner tunic, which was so clearly seen and stained in B, is artefactually empty in this case. The material filling this space was presumably washed up during the ethanol baths required by the applied staining protocol. H&E. **–D.** Detail of the image seen in B. Note the distinct hyaline core (hc) building the longitudinal axis of the spiral filament (sf), which is interspersed with some "amorphous material" (am). Abbreviations are the same as in B. PAS staining reaction.

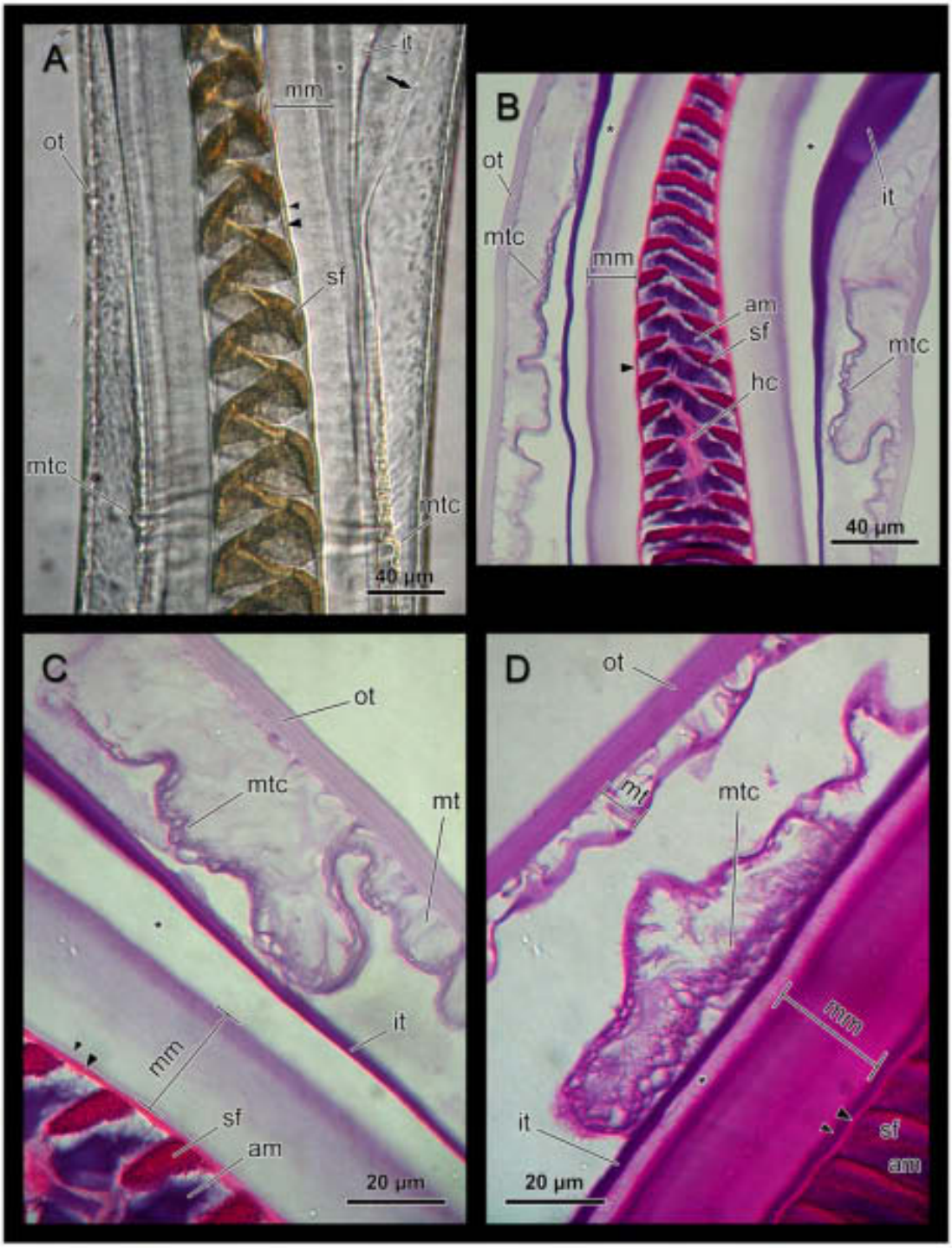

Fig. 8 – Middle tunic constriction around the ejaculatory apparatus tube. **–A.** Detail of the spermatophore at the region of the constriction of the middle tunic (mtc). Note the characteristic undulation of the middle tunic around the ejaculatory apparatus tube. The middle tunic ends (arrow) shortly after its constriction. The "spiral filament membrane" (large arrowhead) and inner membrane (small arrowhead) are distinguishable from one another (see also C and D). DIC. **–B.** Longitudinal section through the same region, showing the interwoven aspect of the middle tunic at the level of the constriction (mtc). Due to the applied technique, the spiral filament membrane (arrowhead) is clearly discernible, but the inner membrane was not stained. H&E. **–C.** Detail of a longitudinal section through the same region. Note the interwoven aspect of the middle tunic at the level of the constriction (mtc); compare to the solid middle tunic (mt) just aboral to the constriction. H&E. **–D.** Detail of a longitudinal section through the same region. In this case there seems to be a strong connection between the middle tunic constricted region (mtc) with the inner tunic (it). Note the interwoven aspect of the middle tunic at the level of the constriction; compare to the normal solid middle tunic (mt) on the left. Please note that this arrangement of the middle tunic constriction inside the normal middle tunic is artefactual; compare to the normal condition seen in A-C. PAS-H&E staining. Abbreviations: *, space between middle membrane and inner tunic filled with a fine reticulated material (artefactually empty in B and C); am, amorphous material; cp, cap; hc, hyaline core; it, inner tunic; mm, middle membrane; mtc, middle tunic constriction; ot, outer tunic; sf, spiral filament.

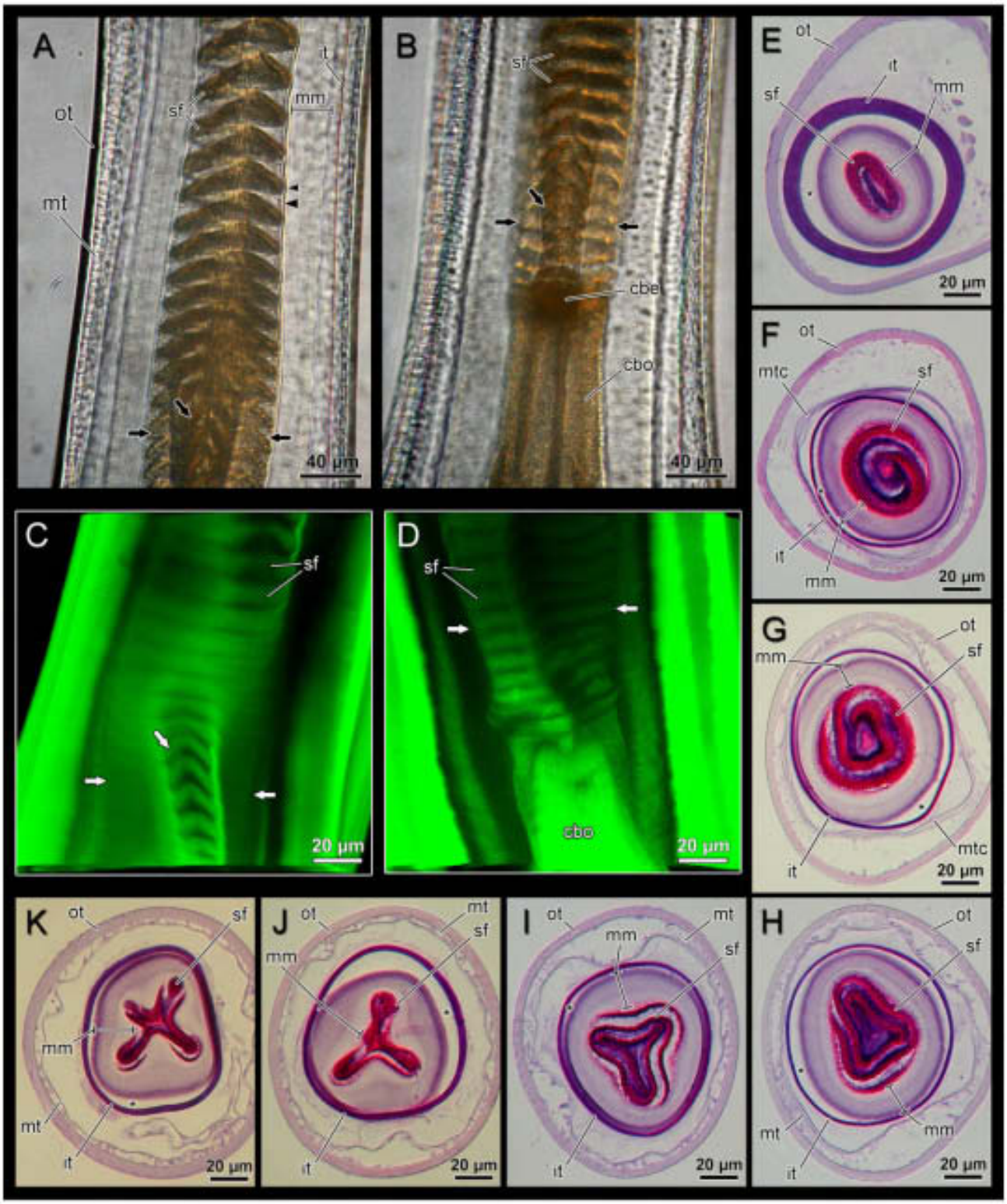

Fig. 9 – Spiral filament morphology. **–A.** Detail of the spiral filament near its attachment to the cement body, showing that the cylindrical spiral filament (sf) tube rearranges itself to form three longitudinal folds (the three large arrows). The large arrowhead points to the spiral filament membrane, and the smaller one to the inner membrane. DIC. **–B.** Detail of the spiral filament at the region of its attachment to the cement body, showing the three longitudinal folds (arrows) and their connection with the cement body oral region (cbo). The oral extremity of the cement body (cbe) is discernible as a dark amber spot. DIC. **–C.** Tridimensional reconstruction based on successive focal planes throughout the spermatophore at the region shown in A, obtained under confocal laser scanning microscopy. The cylindrical spiral filament (sf) tube rearranges itself to form three longitudinal folds (arrows). See also the supplemental video in Appendix S3. **–D.** Region immediately aboral to C, showing the connection of the spiral filament (sf) with the oral region of the cement body (cbo). Two folds (arrows) of the spiral filament tube are visible. See also the supplemental video in Appendix S4. **–E–K.** Series of cross-sections through the spermatophore, showing the structure of the spiral filament tube, which changes from a laterally flattened tube at the level near to the thickening of the inner tunic (E), to a more cylindrical shape at the middle region of the ejaculatory apparatus (F). Aborally, this tube then passes through a transition (G and H) to finally collapse into three concave faces (I). These folds become gradually thinner (J and K) towards the connection with the cement body. The section in K depicts the region immediately oral to the connection of the spiral filament with the cement body. H&E. Abbreviations: *, space between middle membrane and inner tunic (artefactually empty); cbe, oral extremity of the cement body; cbo, oral region of the cement body; it, inner tunic; mm, middle membrane; mt, middle tunic; mtc, middle tunic constriction; ot, outer tunic; sf, spiral filament.

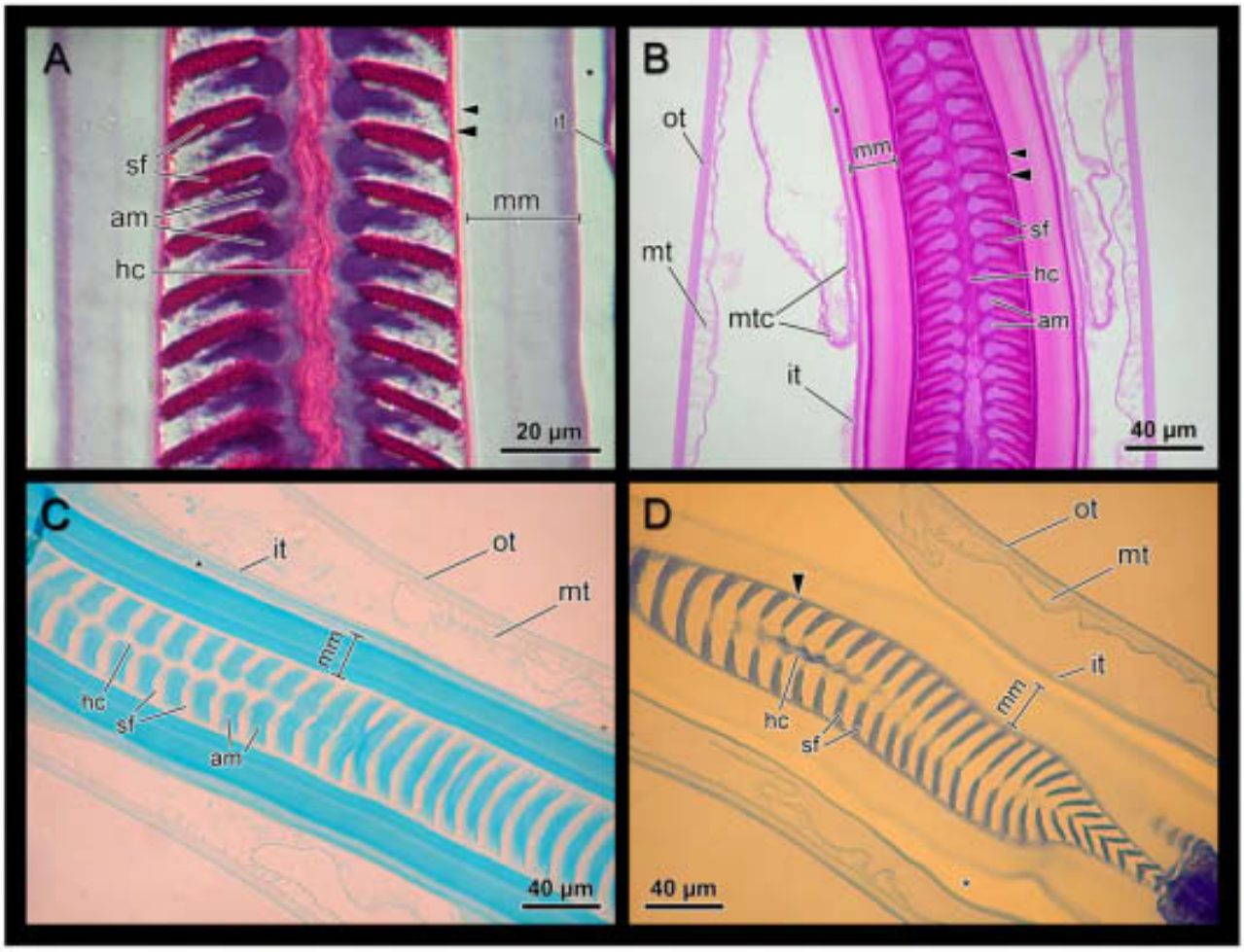

Fig. 10 – Structural organization of the spiral filament and staining reactions of the spermatophore at the level of the ejaculatory apparatus. **–A.** Detail of a longitudinal section through the spermatophore showing the organization of the spiral filament tube. The strong eosinophilic spiral filament (sf) is interspersed with some amorphous material (am), which stained strongly with haematoxylin. The eosinophilic hyaline core (hc) composes the longitudinal axis of both materials. H&E. **–B.** Results of the Periodic Acid – Schiff (PAS) reaction applied to a longitudinal section through the spermatophore at the level of the ejaculatory apparatus. **–C.** Same as B, but stained with Alcian Blue. **–D.** Same as B and C, but stained with the Mercury-Bromophenol Blue method. Abbreviations: *, space between middle membrane and inner tunic (artefactually empty in A and D); am, amorphous material; hc, hyaline core; it, inner tunic; mm, middle membrane; mt, middle tunic; mtc, middle tunic constriction; ot, outer tunic; sf, spiral filament. In all figures, the large arrowhead points to the spiral filament membrane and the small arrowhead to the inner membrane (when discernible through the applied method).

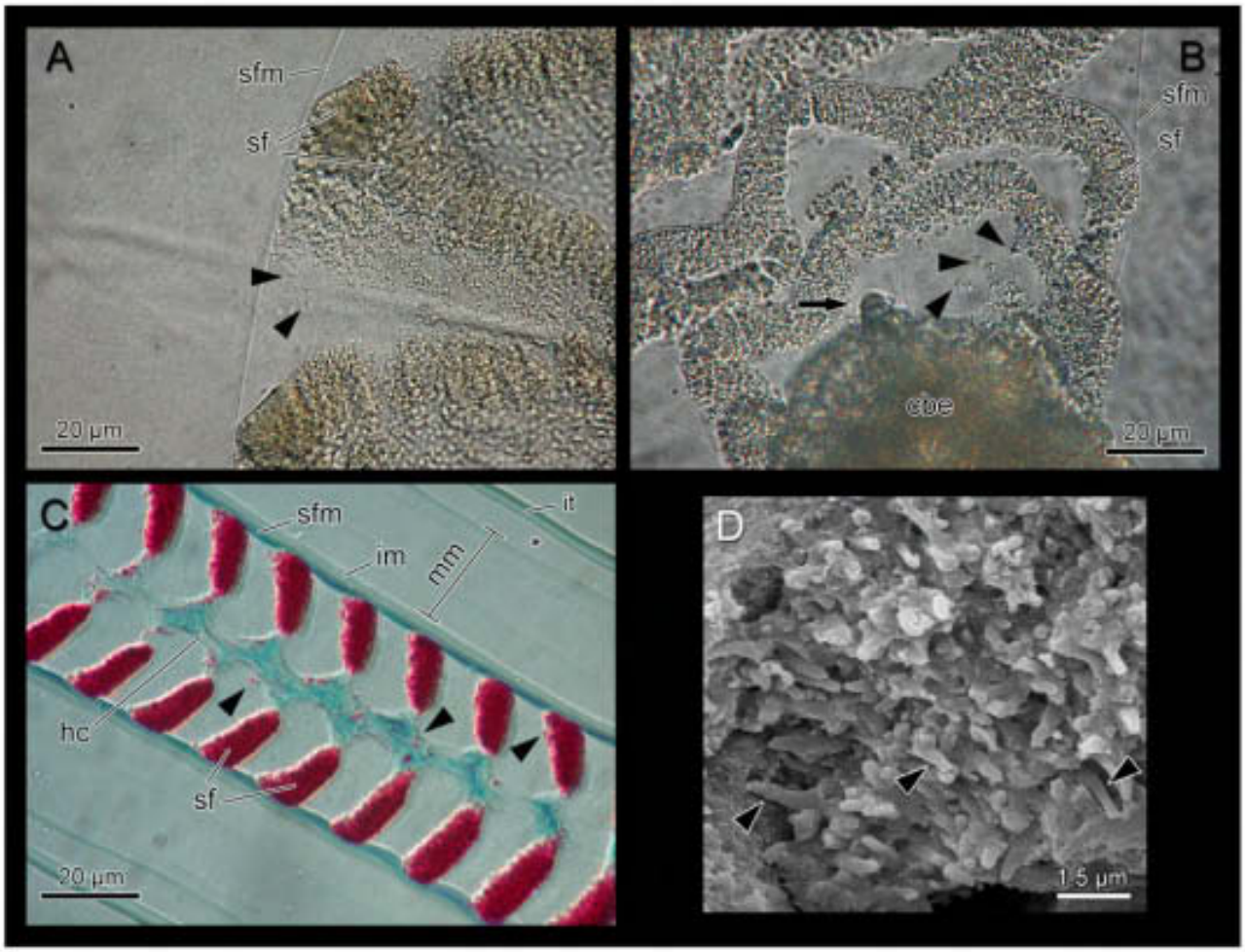

Fig. 11 – Spiral filament stellate particles. **–A.** View of the spiral filament (sf) of a spermatophore, which was compressed under a cover glass and observed under differential interference contrast microscopy. Minute stellate particles (arrowheads) compose the matrix of the spiral filament. **–B.** Same as A, but viewed at the level of the connection between the spiral filament (sf) and the oral extremity of the cement body (cbe). The stellate particles (arrowheads) are thicker at this region. The oral extremity of the cement body bears a pointed ending (arrow). **–C.** Detail of a longitudinal section through the ejaculatory apparatus. The stellate particles (arrowheads) are more clearly discernible when dissociated from the spiral filament matrix (sf). Gomori's Trichrome Stain. **–D.** Scanning electron micrograph of a spermatophore, which was fractured at the approximate mid-level of the ejaculatory apparatus. Several sharp spines (arrowheads) are seen embedded inside the spiral filament matrix, suggesting they are the stellate particles observed under light microscopy. Abbreviations: *, space between middle membrane and inner tunic (artefactually empty); cbe, cement body extremity; hc, hyaline core; im, inner membrane; it, inner tunic; mm, middle membrane; sf, spiral filament; sfm, spiral filament membrane.

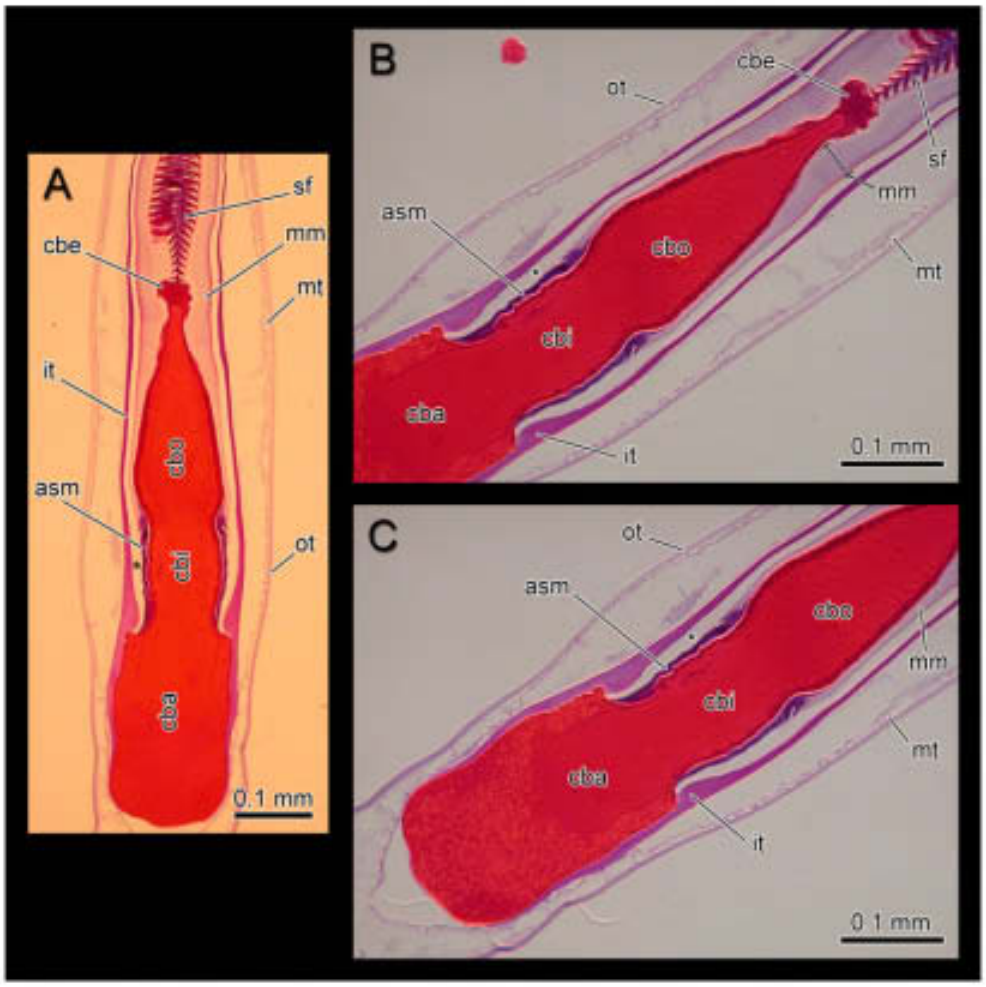

Fig. 12 – Cement body general organization. **–A.** Longitudinal section through the spermatophore at the region of the cement body. H&E. **–B.** Detail of the previous image. The cement body is divided into three main regions: oral (cbo), intermediate (cbi) and aboral (cba) regions. The whole cement body encloses a core of coarsely granular acidophilic material embedded in an acidophilic homogeneous matrix. The granular materials of oral and aboral regions are evidently distinct (compare to C). At the intermediate region of the cement body, the middle membrane (mm) bears an additional layer, the "aboral segment of the middle membrane" (asm), which stains strongly with haematoxylin. H&E. **–C.** Detail of the image seen in A, showing the distinct nature of the coarse granular acidophilic materials embedded within aboral and oral segments of the cement body. H&E. Abbreviations: *, space between middle membrane and inner tunic (artefactually empty); asm, aboral segment of the middle membrane; cba, aboral region of the cement body; cbe, cement body extremity; cbi, intermediate region of the cement body; cbo, oral region of the cement body; it, inner tunic; mm, middle membrane; mt, middle tunic; ot, outer tunic; sf, spiral filament.

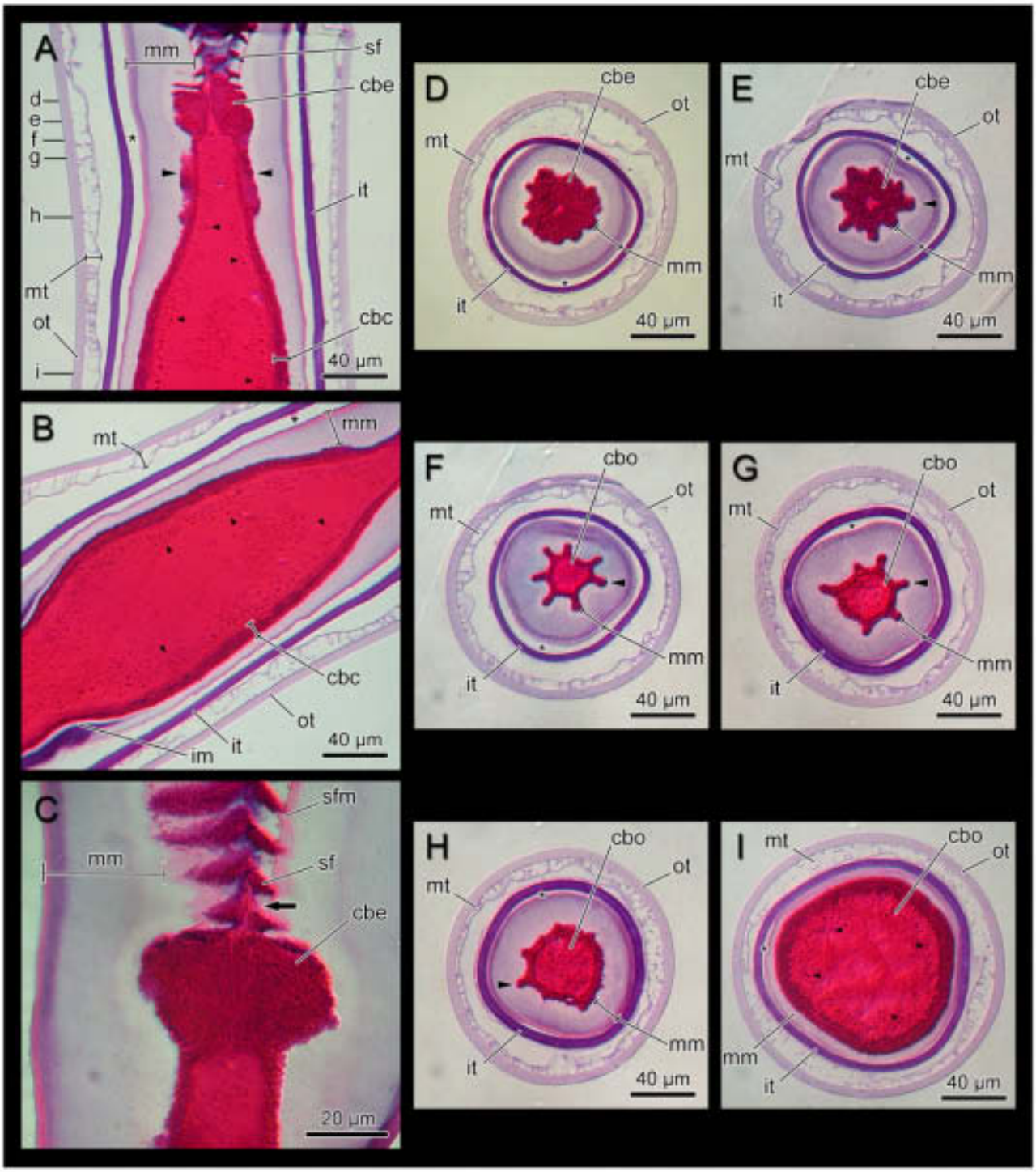

Fig. 13 – Oral region of the cement body. **–A.** Longitudinal section through the spermatophore at the oral region of the cement body, which consists of an outer casing (cbc) encompassing granular acidophilic material (small arrowheads) embedded in a highly acidophilic homogeneous matrix. The cement body extremity (cbe) is a lozenge shaped structure which apparently bears a central canal continuous with the matrix of the cement body. Large arrowheads point to the longitudinal folds of the inner membrane (see also E-H). Lettered lines "d" to "i" indicate the approximate region of the cross-sections seen in figures D to I, respectively. H&E. **–B.** Longitudinal section similar to A, but showing the aboral limit of the oral region of the cement body. H&E. **–C.** Detail of a parasagittal section through the cement body extremity (cbe), showing its pointed ending (arrow). **–D–I.** Series of cross-sections through the cement body oral region at the points indicated in figure A. Note that immediately aboral to the cement body extremity (D), the inner membrane gradually expands into several longitudinal folds (arrowheads in E-H). See also the longitudinal section in figure A. H&E. Abbreviations: *, space between middle membrane and inner tunic (artefactually empty); cbc, cement body outer casing; cbe, cement body extremity; cbo, oral region of the cement body; im, inner membrane; it, inner tunic; mm, middle membrane; mt, middle tunic; ot, outer tunic; sf, spiral filament; sfm, spiral filament membrane.

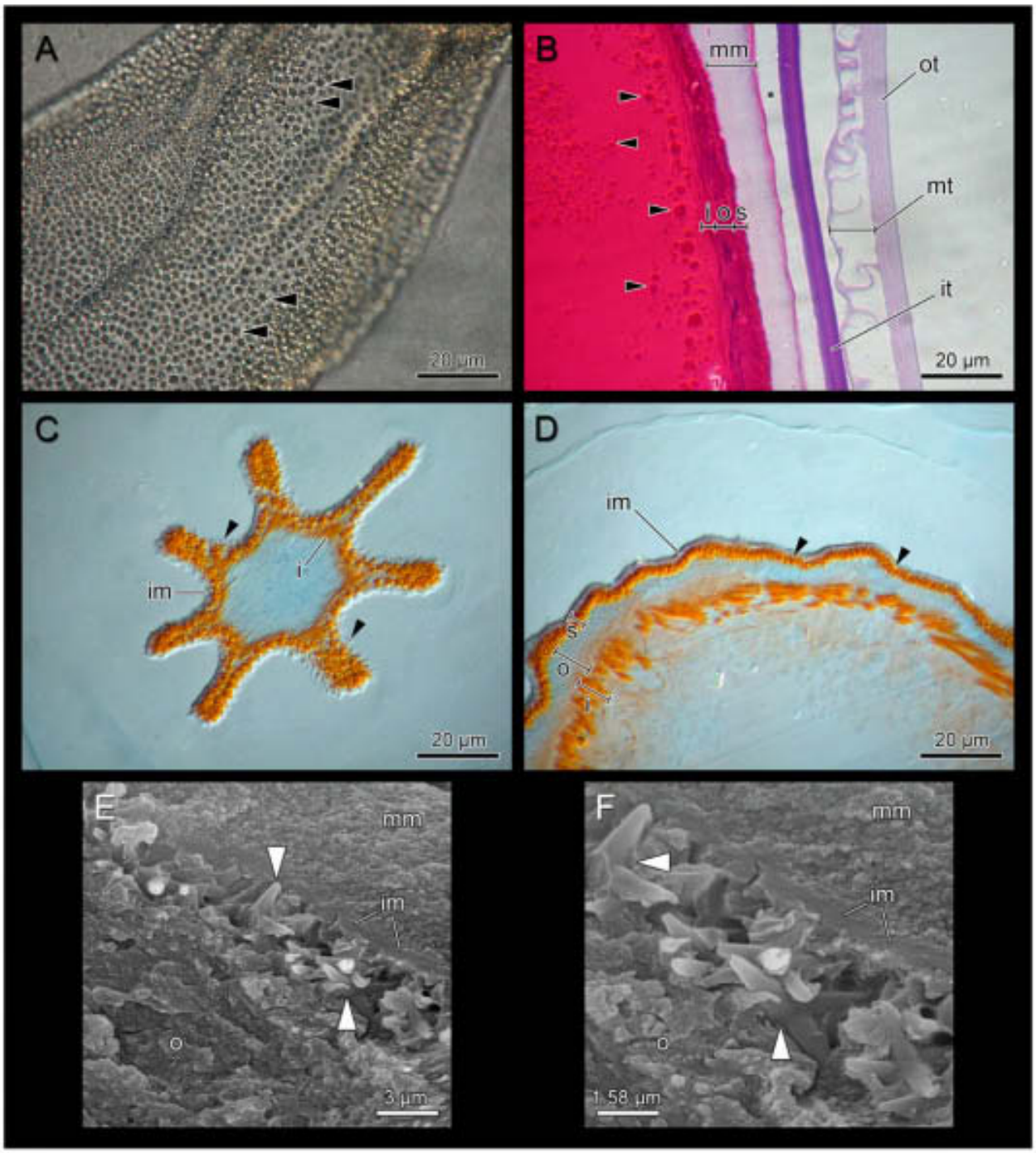

Fig. 14 – Stellate particles located between the inner membrane and the cement body oral region, and the structure of the cement body walls. **–A.** View of the cement body of a spermatophore, which was broken when compressed under a cover glass. Numerous minute stellate particles (arrowheads) are discernible; note that they are thicker than the particles embedded within the spiral filament. DIC. **–B.** Detail of a longitudinal section through the oral region of the cement body. At this region, the cement body outer casing consists of two layers; the inner layer (i) is thinner and fibrous, and the outer layer (o) thicker and of spongy or granular appearance. Numerous stellate particles (s), not individually discernible in this section, are tightly packed in an additional layer between the outer cement body layer (o) and the inner membrane (not stained in this case). Arrowheads point to granular acidophilic material. H&E. **–C.** Cross-section through the cement body oral region at the level of the folds of the inner membrane (see figure 13F for comparison). At this level, the outer layer of the cement body casing is not present; the fibrous inner layer (i) is evident, as well as the minute stellate particles (arrowheads) located internally to the inner membrane (im). Mallory's Trichrome Solution B (structures stained with Orange G). **–D.** Cross-section through the median part of the oral region of the cement body (see figure 13I for comparison and figure B depicted above). At this level both layers of the cement body are present, the fibrous inner layer (i), and the thicker outer layer (o). Numerous stellate particles (arrowheads) are tightly packed in an additional layer (s) between the cement body outer layer (o) and the inner membrane (im). Mallory's Trichrome Solution B (structures stained with Orange G). **–E.** Scanning electron micrograph of a spermatophore, which was fractured at the approximate mid-level of the cement body oral region. The minute stellate particles (arrowheads) are discernible embedded between the cement body outer layer (o) and the inner membrane (im). **–F.** Detail of the previous image. Abbreviations: *, space between middle membrane and inner tunic (artefactually empty); i, cement body inner layer; im, inner membrane; it, inner tunic; mm, middle membrane; mt, middle tunic; o, cement body outer layer; ot, outer tunic; s, layer formed by tightly packed stellate particles.

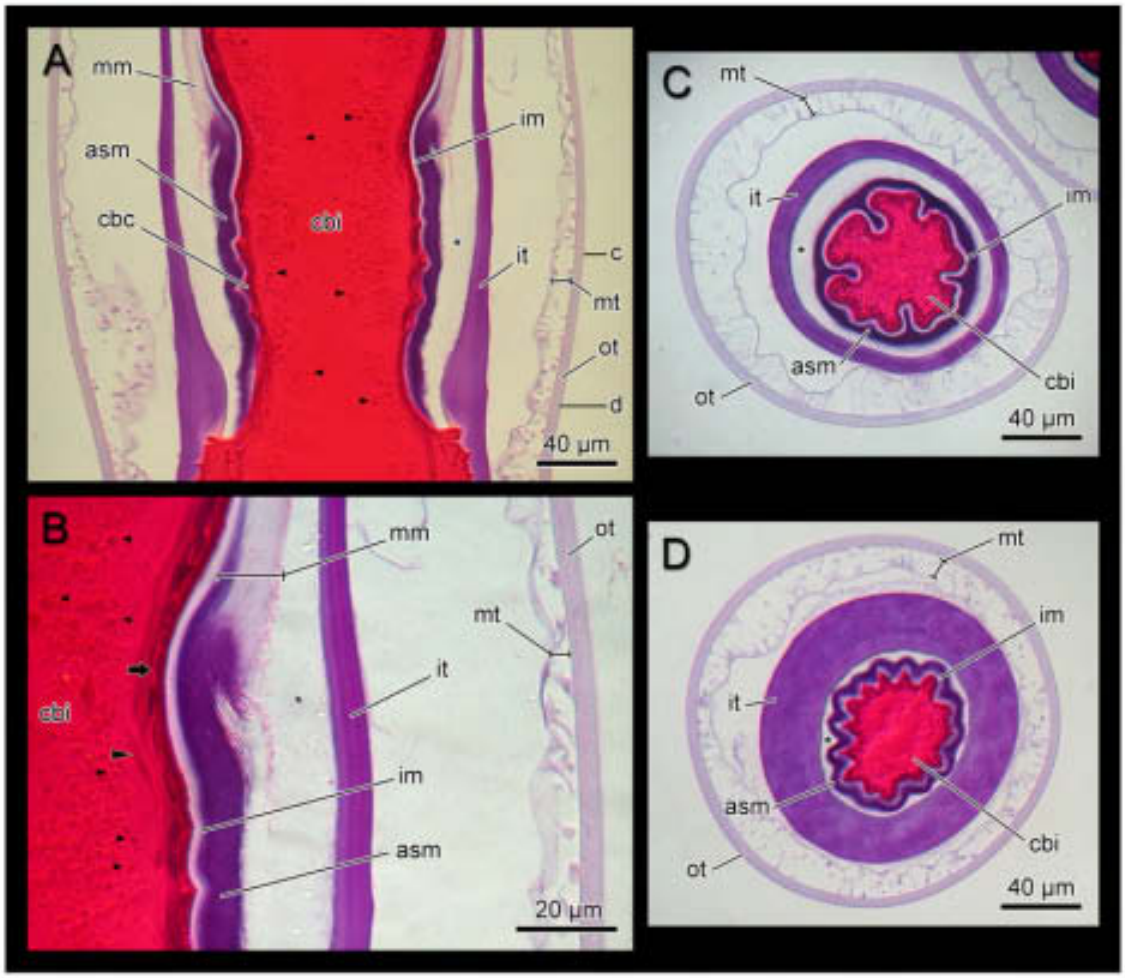

Fig. 15 – Intermediate region of the cement body. **–A.** Longitudinal section through the spermatophore at the intermediate region of the cement body, which consists of an outer casing (cbc) encompassing granular acidophilic material (arrowheads) embedded in a highly acidophilic homogeneous matrix. At this level the middle membrane (mm) bears an additional layer, the "aboral segment of the middle membrane" (asm), which stains strongly with hematoxylin. Lettered lines "c" and "d" indicate the approximate region of the cross-sections seen in figures C and D, respectively. H&E. **–B.** Detail of the previous image, showing that the casing of the cement body at the intermediate region bears both outer (arrow) and inner (large arrowhead) layers. The granular acidophilic material (arrowheads) is also discernible. H&E. **–C.** Cross-section through the cement body at the point "c" indicated in figure A. At this level the intermediate region bears prominent invaginations of the cement body walls. The aboral segment of the middle membrane (asm) fills in the space created by these invaginations. H&E. **–D.** Cross-section through the cement body at the point "d" indicated in figure A. At this level the intermediate region bears several longitudinal, short crests. Note also the thickening of the inner tunic (it). Abbreviations: *, space between middle membrane and inner tunic (artefactually empty); asm, aboral segment of the middle membrane; cbc, cement body outer casing; cbi, intermediate region of the cement body; im, inner membrane; it, inner tunic; mm, middle membrane; mt, middle tunic; ot, outer tunic.

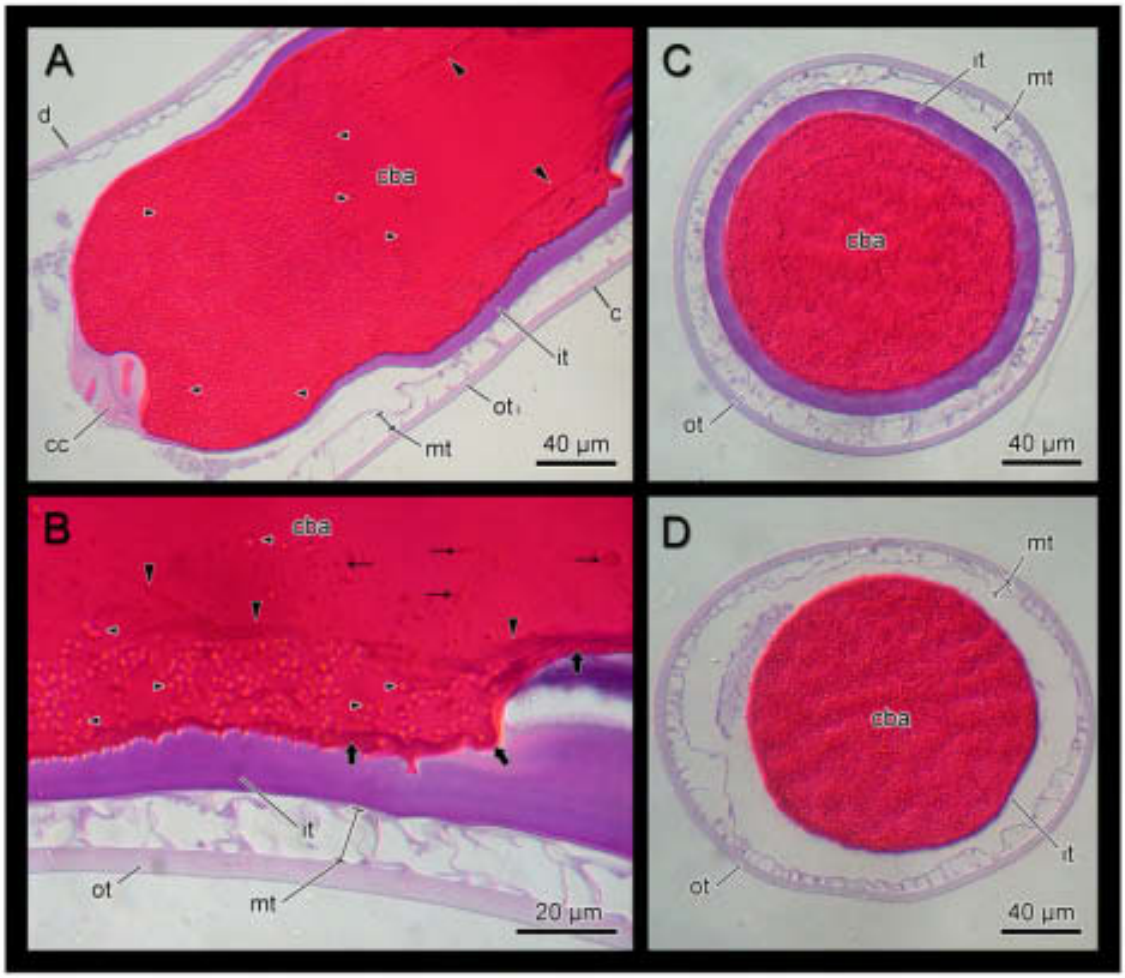

Fig. 16 – Aboral region of the cement body. **–A.** Longitudinal section through the spermatophore at the aboral region of the cement body, which encompasses distinct granular acidophilic material (small arrowheads) embedded in a highly acidophilic homogeneous matrix. These granules are different from those of the intermediate and oral regions. Note that the cement body fibrous inner layer (large arrowheads) separates from the outer layer and extends aborally into the contents of the aboral cement body. Lettered lines "c" and "d" indicate the approximate region of the cross-sections seen in figures C and D, respectively. H&E. **–B.** Detail of the previous image, showing that the casing of the cement body at the aboral region bears only the outer layer (thicker arrows), the inner layer (large arrowheads) separating from the outer layer and extending aborally into the contents of the aboral cement body. Both types of granular acidophilic material can be compared in this photograph: granules from the aboral region (small arrowheads) and those from the intermediate region (slender arrows). H&E. **–C.** Cross-section through the cement body at the point "c" indicated in figure A. Note the thick inner tunic (it). H&E. **–D.** Cross-section through the cement body at the point "d" indicated in figure A. Note that at this level the inner tunic (it) is very thin. Abbreviations: cba, aboral region of the cement body; cc, connecting cylinder; it, inner tunic; mt, middle tunic; ot, outer tunic.

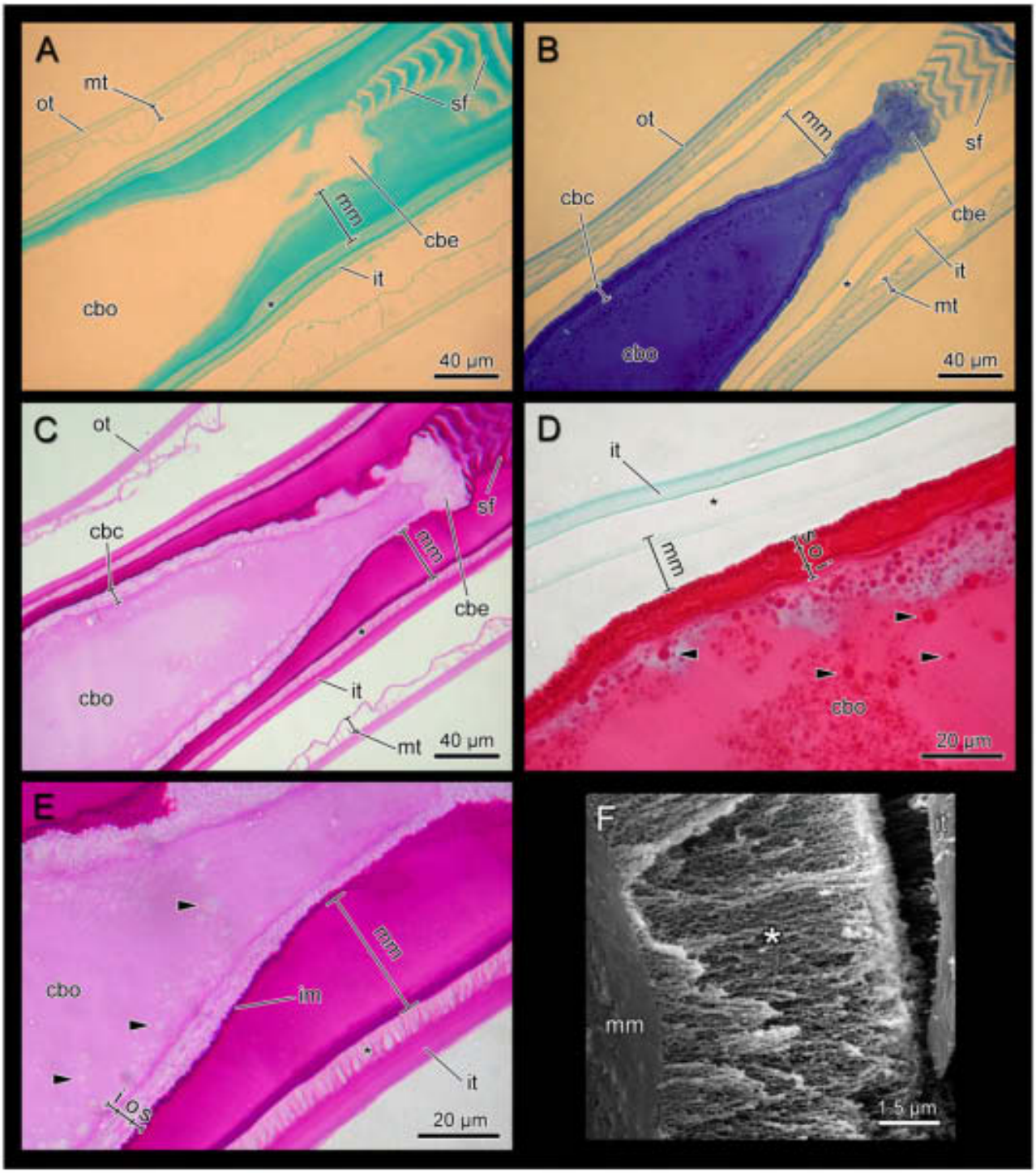

Fig. 17 – Staining reactions of the spermatophore at the level of the oral region of the cement body, and the nature of the reticulated material filling in the space between the inner tunic and middle membrane. **–A.** Longitudinal section through the spermatophore stained with Alcian Blue at pH 2.5. **–B.** Longitudinal section through the spermatophore stained with the Mercury-Bromophenol Blue method. **–C.** Longitudinal section through the spermatophore stained with the Periodic Acid–Schiff method. **–D.** Detail of a longitudinal section through the spermatophore stained with Mallory's Triple Stain. Arrowheads point to the granular material embedded within the cement body matrix. **–E.** Detail of the image seen in C. Arrowheads point to the granular material embedded within the cement body matrix. PAS. **–F.** Scanning electron micrograph of a spermatophore, which was fractured at a level near to the cement body oral region, showing the space between the inner tunic (it) and middle membrane (mm) filled with fine reticulated material (*), which consists of a dense mesh of very delicate threads. Abbreviations: *, space between middle membrane and inner tunic filled with reticulated material; cbc, cement body outer casing; cbe, cement body extremity; cbo, oral region of the cement body; i, cement body inner layer; im, inner membrane; it, inner tunic; mm, middle membrane; mt, middle tunic; o, cement body outer layer; ot, outer tunic; s, layer formed by tightly packed stellate particles; sf, spiral filament.

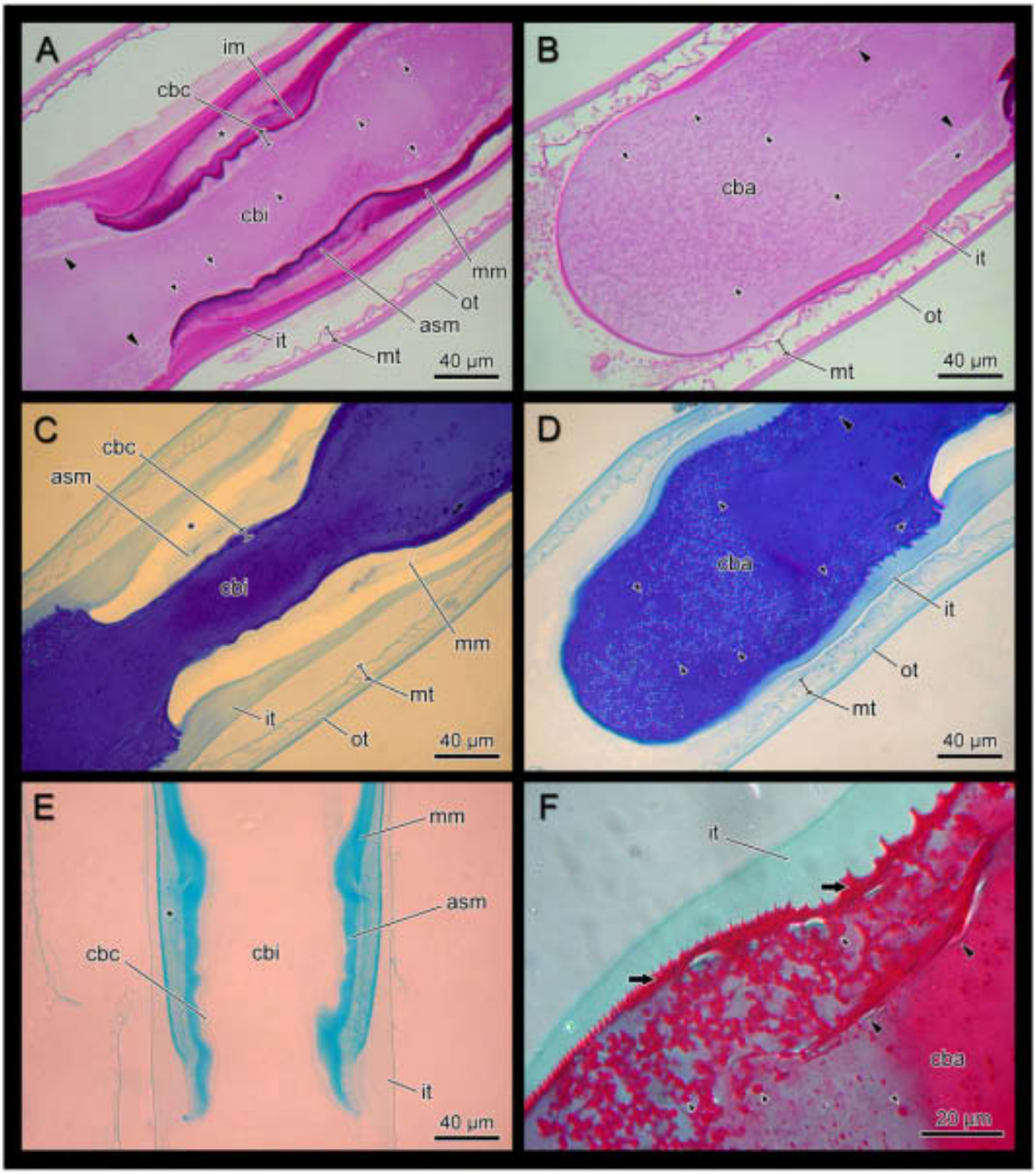

Fig. 18 – Staining reactions of the spermatophore at the level of the intermediate and aboral regions of the cement body. **–A.** Longitudinal section through the spermatophore at the intermediate region of the cement body stained with the Periodic Acid–Schiff method. Small arrowheads point to the granular material embedded within the cement body matrix. Large arrowheads point to the inner layer of the cement body outer casing, which separates from the outer layer and extends aborally into the contents of the aboral cement body. **–B.** Longitudinal section through the spermatophore at the aboral region of the cement body stained with the Periodic Acid–Schiff method. Small arrowheads point to the granular material embedded within the cement body matrix (compare with those indicated in A). Large arrowheads point to the inner layer of the cement body outer casing, which separates from the outer layer and extends aborally into the contents of the aboral cement body. **–C.** Same as A, but stained with the Mercury-Bromophenol Blue method. **–D.** Same as B, but stained with the Mercury-Bromophenol Blue method. **–E.** Same as A, but stained with Alcian Blue at pH 2.5. **–F.** Detail of a longitudinal section through the spermatophore at the aboral region of the cement body stained with Gomori's Trichrome. Small arrowheads point to the granular material embedded within the cement body matrix. Large arrowheads and arrows point to the inner and outer layers of the cement body outer casing, respectively. Abbreviations: *, space between the middle membrane and inner tunic filled with reticulated material; cbc, cement body outer casing; cbi, intermediate region of the cement body; cba, aboral region of the cement body; im, inner membrane; it, inner tunic; mm, middle membrane; mt, middle tunic; ot, outer tunic.

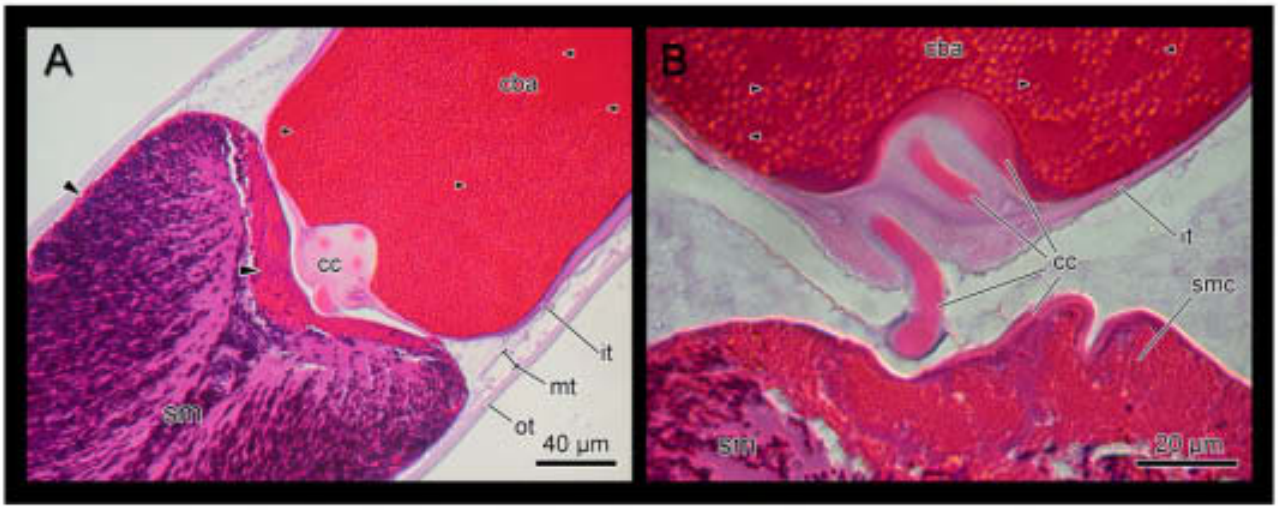

Fig. 19 – Connecting cylinder. **–A.** Longitudinal section through the spermatophore at the level of the connecting cylinder. Large arrowheads point to the sperm mass casing, and small arrowheads to the granular material embedded within the cement body matrix. H&E. **–B.** Detail of a longitudinal section through the spermatophore at the level of the connecting cylinder. Arrowheads point to the granular material embedded within the cement body matrix. H&E. Abbreviations: cba, aboral region of the cement body; cc, connecting cylinder; it, inner tunic; mt, middle tunic; ot, outer tunic; sm, sperm mass; smc, sperm mass casing.

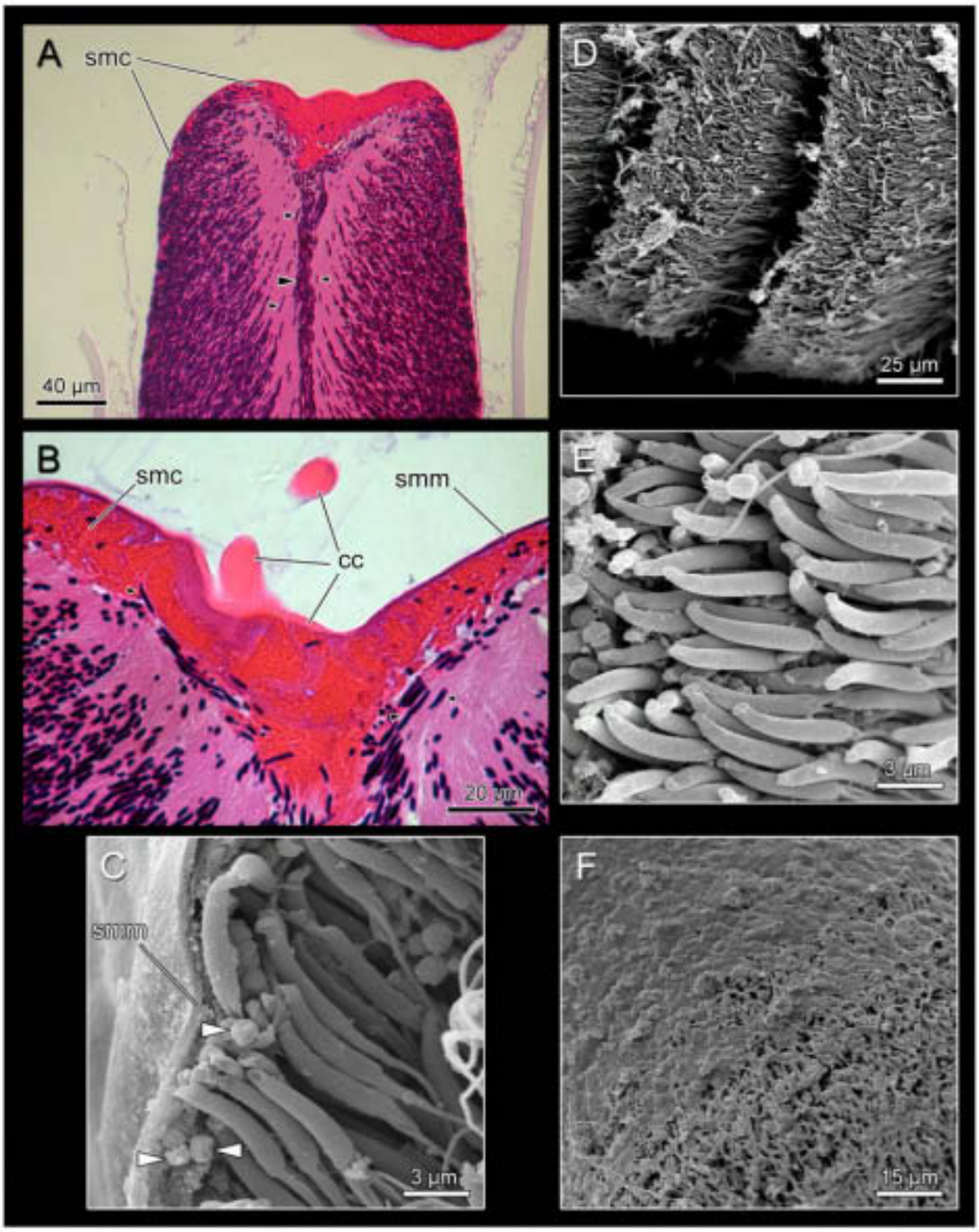

Fig. 20 – Sperm mass. **–A.** Longitudinal section through the sperm mass. The large arrowhead points to the axis of the spirally twisted sheet of spermatozoa. Small arrowheads point to some spermatozoa. H&E. **–B.** Detail of a longitudinal section through the oral region of the sperm mass. Small arrowheads point to some spermatozoa. H&E. **–C.** Scanning electron micrograph of a fractured sperm mass, showing the sperm mass membrane (smm), and some of the granular material (arrowheads) that compose the sperm mass casing. **–D.** Scanning electron micrograph of a sperm mass from which the sperm mass membrane was artificially removed. Two whorls of the spirally twisted sheet of spermatozoa are visible. **–E.** Detail of the previous image, showing the densely organized spermatozoa. **–F.** Scanning electron micrograph of the sperm mass aboral region. The sperm mass membrane gradually fades away rendering the aboral-most region devoid of a solid covering (bottom right). Abbreviations: cc, connecting cylinder; smc, sperm mass casing; smm, sperm mass membrane.

Appendix 1 Protocol used for processing *Doryteuthis plei* spermatophores enabling satisfactory embedding media infiltration.

Several authors attempting to study squids' spermatophores have realized how difficult these structures are to prepare for microscopic investigation (e.g., Hess 1987; Austin *et al.* 1964). Certainly, this peculiarity comes from the semi-permeable outer tunic (e.g., Mann 1984), which presumably hampers infiltration of the embedding media (e.g., historesin, paraffin), or, in the case of whole preparations, of the mounting media (e.g., Canada balsam). The present authors have experienced similar issues when preparing the spermatophores of *D. plei* for serial sectioning. Several dehydration and infiltration times were tested, but it soon became clear that, without a chemical or physical treatment, satisfactory embedding would not be achieved. Several physical abrasions were tested (e.g., puncturing, cutting the spermatophore at the aboral region), but the most satisfactory results were obtained when transversally cutting the spermatophore with fine dissecting scissors at the level just aboral to the connecting cylinder, but leaving also a small piece of the oral region of the sperm mass connected to the rest of spermatophore.

To avoid the internal structures from being exteriorized after cutting the spermatophore, the following procedures are suggested concerning which solution to use when applying this method. Since Karnovsky fixed samples are generally preserved in a buffer solution before processing, and paraformaldehyde samples in ethanol 70%, the instructions described below start from these solutions.

The best procedure is to prepare several samples to assure a good number will be appropriate for infiltration. The remaining pieces of the sperm masses may be used also for microscopic investigation. Applying this method provided excellent infiltration; this protocol, however, must be considered as tentative, and may be useful until another specific methodology is established.

A) Karnovsky fixed samples preserved in cacodylate buffer:

1) With the samples still in the buffer solution, cut the spermatophores as described above;
2) Proceed to dehydration in ethanol 50%, 70% and 80% (30 min. each) and 95% (overnight);
3) Follow standard infiltration procedures for historesin.

B) Paraformaldehyde fixed samples preserved in ethanol 70%:

1) Place samples into ethanol 50% solution for about a minute (to allow some rehydration);
2) With the samples still in ethanol 50%, cut the spermatophores as described above;
3) Proceed to dehydration in ethanol 50%, 70% and 80% (30 min. each) and 95% (overnight);
4) Follow standard infiltration procedures for historesin.

Capítulo 2

Descoberta do potencial de perfuração dos espermatóforos de *Doryteuthis plei*

CAPÍTULO 2

Descoberta do potencial de perfuração dos espermatóforos de *Doryteuthis plei*

Ao realizar a experimentação *in vitro* com os espermatóforos de *Doryteuthis plei*, um fenômeno inusitado foi observado durante a reação espermatofórica: o potencial de perfuração ou de penetração dos espermatóforos da espécie. Como exposto no manuscrito, experimentos foram realizados utilizando fragmentos de tentáculos, manto e outras partes do corpo, extraídos dos próprios animais recém-sacrificados para obtenção dos espermatóforos. Orientando espermatóforos em evaginação em direção a estes fragmentos, foi possível observar o comportamento dos mesmos ante um substrato tecidual, o que proporcionou a obtenção de resultados interessantes acerca da reação espermatofórica da espécie (vide Cap. 3).

Quando, por um equívoco do experimentador, um espermatóforo em evaginação foi direcionado para a musculatura artificialmente exposta do fragmento de tentáculo (a região onde este fora seccionado com a tesoura), surpreendentemente o aparato ejaculatório em eversão foi capaz de penetrar a musculatura, como se houvesse pouca resistência contra esse processo. Outros experimentos similares foram realizados, direcionando propositalmente os espermatóforos à face seccionada da amostra, e o resultado foi semelhante: houve penetração da musculatura artificialmente exposta. Esses espermatóforos, entretanto, não completavam a reação e não produziam espermatângios, o aparato ejaculatório permanecendo implantado no tecido.

A princípio, esses dados seriam descartados como simples artefatos, já que o fenômeno fora observado em condições completamente artificiais. Entretanto, havia grande semelhança entre o processo observado e o fenômeno de "implante profundo", que ocorre naturalmente em algumas espécies de lulas oceânicas e de águas profundas (e.g., Norman & Lu, 1997 [12]; Nesis *et al.*, 1998 [6]; Jackson & Jackson, 2004 [7]; Hoving & Laptikhovsky, 2007 [10]; Hoving *et al.*, 2009 [9]). Compare, por exemplo, a figura 1A do presente artigo com a figura 1C de Jackson & Jackson (2004 [7]). A possibilidade dessa descoberta poder auxiliar no entendimento desse enigmático processo estimulou a elaboração do presente manuscrito. Devido à sua relevância, sobretudo se considerarmos a quantidade de trabalhos publicados recentemente sobre o tema, decidiu-se por comunicá-la em um artigo independente, curto e de rápida divulgação. Optou-se pelo modelo de "Rapid Communication" do periódico internacional *Biological Bulletin*. O manuscrito foi submetido, no presente formato, em 22 de fevereiro de 2010.

De forma semelhante ao constatado recentemente em algumas espécies com implante profundo (Hoving & Laptikhovsky, 2007 [10]; Hoving *et al.*, 2009 [9]), no fenômeno observado em *D. plei* o aparato ejaculatório era a estrutura que efetivamente penetrava a musculatura. Dada a similaridade estrutural do espermatóforo das lulas (e.g., Hess, 1987 [15]), propõe-se no presente artigo que um mecanismo de penetração deva ser intrínseco à estrutura do espermatóforo dos Decapodiformes, embora, por razões desconhecidas, o mesmo nem sempre resulte em implante profundo.

Diversas questões permanecem não resolvidas neste tópico. Por exemplo, por que o espermatóforo de *D. plei* não é capaz de implantar-se no tecido se direcionado à epiderme intacta? Propõe-se, nesse caso, que a diferença possa residir nas propriedades intrínsecas dos tecidos: a epiderme, um tecido de revestimento e proteção, ofereceria mais resistência à perfuração que as fibras musculares, as quais, pelo menos em teoria, poderiam ser mais facilmente separadas pelo espermatóforo em evaginação. É importante ressaltar que, como este manuscrito foi preparado de forma independente daquele do terceiro Capítulo (acerca da reação espermatofórica de *D. plei*), não há menção sobre a capacidade do espermatóforo da espécie de naturalmente romper a barreira do epitélio e alcançar o tecido conjuntivo (vide Cap. 3 para mais detalhes). É importante lembrar que, até o momento, esse fato descrito no Capítulo 3 é desconhecido na literatura especializada: acredita-se que os espermatângios de Loliginidae fixem-se superficialmente à fêmea somente por meio de substâncias adesivas, sem haver nenhum tipo de perfuração. De qualquer forma, ambas as condições são muito distintas entre si: no presente relato, o espermatóforo efetivamente penetra e implanta sua região oral evertida no tecido muscular; em condições naturais, como exposto no Capítulo seguinte, somente a base do espermatângio penetra superficialmente o corpo da fêmea.

Curiosamente, em algumas lulas que apresentam implante profundo, como Onychoteuthidae (Bolstad, 2008 [16]) e Octopoteuthidae (Hoving *et al.*, 2010 [17]), acredita-se que durante a cópula os machos façam incisões no corpo da fêmea com auxílio das mandíbulas (bicos) ou das ventosas e ganchos dos tentáculos. Esses cortes seriam feitos antes da transferência dos espermatóforos, pois, nesses casos, os espermatângios são encontrados implantados nos sulcos dessas incisões. Acredita-se que esses entalhes sirvam para facilitar o implante dos espermatângios, embora esteja claro que não são pré-requisitos para o implante profundo (Hoving *et al.*, 2010 [17]). Apesar de ainda ser necessária muita experimentação para confirmar o que no momento são apenas especulações, é no mínimo intrigante que uma condição similar a esta (i.e., incisão seguida de implante) tenha sido reproduzida *in vitro* com os espermatóforos de *D. plei*.

Might as Well Get Deeper: Perforating Potential of Loliginid Spermatophores

Abstract

During mating, spermatophores of male coleoid cephalopods undergo the "spermatophoric reaction", which culminates in the attachment of the everted spermatophores (spermatangia) on the female's body. In squids, this attachment is basically of two types: 1) superficial attachment, in which the tip of the spermatangium attaches to the female (e.g., loliginids), or 2) implantation, in which the whole spermatangium is embedded into tissue (some oceanic and deep-sea squids). As part of an ongoing investigation of the spermatophore of the loliginid *Doryteuthis plei*, a seminal finding is reported herein from the in vitro analysis of the spermatophoric reaction: under artificial conditions, the loliginid spermatophore was able to partially implant itself autonomously, almost resembling natural deep implantation. As expected, everting spermatophores directed towards the epithelium of small pieces of squid's appendages ended up attached superficially, like those naturally found in the buccal membrane and mantle cavity of females. If, otherwise, directed towards the incised region of the tissue, the everting ejaculatory apparatus of the spermatophore was able to readily penetrate the artificially exposed musculature. Therefore, the still unknown mechanism involved in deep implantation may be inherent to the spermatophore structure of all squids, although not always leading to the same type of attachment.

Key Words: Cephalopoda; Squids; Loliginidae; Reproduction; Spermatophore; Functional morphology; Spermatophoric reaction; Spermatangia implantation.

Male coleoid cephalopods possess elaborate spermatophores, which are transferred to the female during copulation (1). During this process, the spermatophores undergo the so-called spermatophoric reaction (2), a complex process of evagination of its tunics and membranes that leads to the extrusion and attachment of the sperm mass on various sites of the female body (1). There seems to be a consensus in the literature that two types of attachment of spermatangia (i.e., everted spermatophores) occur in decapodiforms (i.e., squids and sepioids). In one of them, after the spermatophoric reaction, the base of the spermatangia ends up attached on the surface of the female's body, by means of the adhesive contents of the cement body (2); this type is found in several groups of decapodiforms, like loliginids (2), sepiids (3), and ommastrephids (4). The other type of attachment involves deep implantation of the spermatangia into the female's body, i.e., the

spermatangia penetrate the integument, embedding themselves completely into the female tissue; this strategy is common to some oceanic and deep-sea species, like the giant squid *Architeuthis* (5), cranchiids (6), onychoteuthids (7), octopoteuthids (8), and some sepiolids (9), among others. Despite great advances provided by recent studies (6, 9, 10), the mechanics behind deep implantation remains unknown.

In order to further investigate the coleoid spermatophore functional morphology, a research project was established in 2005 to investigate spermatophore structure and functioning in the loliginid squid *Doryteuthis plei* (Blainville, 1823), applying different microscopy techniques, as well as *in vitro* analysis of the spermatophoric reaction. The results of this investigation are being organized in a more comprehensive manuscript (Marian, in prep.[1]), but, given the significance and immediacy of a striking finding resulting from this investigation, the present communication was prepared.

Experimentation was based on mature male specimens collected by jigging off the northern coast of São Paulo State, Brazil. After anesthesia and dissection, spermatophores were retrieved from the spermatophoric sac and immersed in filtered seawater. Under a stereomicroscope, spermatophoric reaction was induced by rapidly pulling the spermatophore cap thread with the aid of fine dissecting forceps. Triggered spermatophores were then directed towards small pieces of the male's donor body samples (e.g., tentacles, arms, spermatophoric sac). Although the natural spermatangia deposition sites in *D. plei* are either the ventral buccal membrane or the vicinity of the genital opening of females, these experimental samples were useful for observing the behavior of everting spermatophores upon a substrate.

As expected, if directed towards the epithelium of the samples, the spermatophore was able to attach itself autonomously on the surface of the targeted tissue (Fig. 1f). However, by a mistake of the present author, one spermatophore was inadvertently directed towards the region where a small piece of tentacle was sectioned. Surprisingly, in this case the everting ejaculatory apparatus of the spermatophore was able to effectively penetrate into the artificially exposed muscular tissue (Fig. 1 and Supplemental Video, http://www.biolbull.org/supplemental/[2]). After this incident, similar experiments were performed and the result was always the same: the everting ejaculatory apparatus perforated and partially embedded itself inside the exposed musculature, as if there was little resistance for this process.

Although having the ability to penetrate exposed musculature, spermatophores subjected to these experiments were not able to complete the spermatophoric reaction, and the spermatozoa were not released, indicating that this phenomenon in *D. plei* is artificial and

[1] Marian in prep.: Capítulo 3.
[2] Ver CD em anexo à Tese, contendo material suplementar.

does not occur naturally. In all cases, the final spermatangia (sperm masses encased by the inner tunic and outer membrane, with the cement body smeared on the base) were not completely formed, but remained connected to the rest of the spermatophore. In one case, however, the reaction proceeded to an advanced level, with almost all the sperm mass having been pushed out of the spermatophore (Fig. 1c) into the forming spermatangium. Nonetheless, even in this case the reaction was far from complete.

These data would have been discarded as artifacts except for the resemblance of this process to that of deep implantation (e.g., 5-9), and the possibility that these results will help unravel the mechanics behind this kind of spermatophore attachment. None of the hypotheses proposed to explain deep spermatangia implantation have gained wide acceptance. Some attribute the main role of implantation to the terminal organ (“penis”), which would hydraulically pump and insert spermatangia into the female tissue (7, 11, 12, 13). Others postulate that tentacle and arm hooks or suckers, or the beaks, would be used by the male to make cuts in the female body, assisting in spermatangia insertion (e.g., 1, 6, 11). However, spermatangia autonomous implantation was recently demonstrated for some decapodiform species (9, 10). How the spermatophore alone would be capable of piercing the female body, however, has remained enigmatic. The action of proteolytic enzymes and other substances from the cement body has been suggested to play this role in deep implantation (e.g., 6, 9, 14), but evidence of such chemical properties is lacking.

In all experiments, the everting ejaculatory apparatus, not the cement body, was the first structure to come into contact with the targeted tissue, and the one that effectively penetrated musculature. Similarly, some evidence in oceanic and deep-water decapodiforms indicates that the first structure to perforate the female tissue is the ejaculatory apparatus (9, 10). Therefore, the mechanism behind deep implantation may reside in the ejaculatory apparatus, and not in the cement body. Given the overall structural similarity of spermatophores (15) and the results seen here with *D. plei*, the mechanism of tissue penetration may be intrinsic to the decapodiform spermatophore. For unknown reasons, however, this possible inherent mechanism does not always lead to deep implantation in natural conditions.

Why in *D. plei* is the everting spermatophore able to penetrate artificially exposed musculature, resembling deep spermatangia implantation of some oceanic and deepwater squids, but is not capable of doing so if directed towards the intact epidermis? Presumably, in this case, the properties and resistance for perforation of epithelial and muscular tissues differ. The epithelium, being a tissue that lines surfaces and cavities of structures throughout the body, generally has intrinsic qualities that confer protection to the underlying tissue, and might offer more resistance to perforation. However, muscular tissue is composed of long

juxtaposed muscle fibers, which, at least in theory, could be more easily separated by the everting ejaculatory apparatus of *D. plei*.

If this is so, how is it that the epidermis can be penetrated in oceanic squids with deep implantation? In some Onychoteuthidae (1, 16) and Octopoteuthidae (1, 17) with deep implantation, apparently the male – with the aid of beaks or hooks – makes incisions on the female body prior to spermatangia implantation, since spermatangia are generally found implanted within the groove of these incisions. In these cases, however, the incision is apparently not a prerequisite for successful implantation, since implanted spermatangia are not always associated with special cuts (17).

The similarity in spermatophore structure among decapodiforms but the differences in spermatangia attachment (surface attachment or deep implantation) have been perplexing. The discovery that a surface-attachment species (*D. plei*) shows some deep implantation capability raises new questions about a common underlying mechanism that should stimulate future studies regarding coleoid spermatophore functional morphology and evolution.

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Video Supplement. – Experiment from the *in vitro* analysis of the spermatophoric reaction of the loliginid *Doryteuthis plei*, conducted under a Zeiss Stemi SV11 APO stereomicroscope equipped with a Sony DCR HC1000. With the spermatophore immersed in filtered seawater, spermatophoric reaction was induced by rapidly pulling the cap thread with the aid of fine dissecting forceps. Clouds of spermatozoa from spermatangia of previous experiments are visible in the background. Visible at the bottom of the video is a small piece of the male's donor tentacle. The everting spermatophore in the video was purposively directed towards the incised part of the appendage. The everting ejaculatory apparatus readily perforates the exposed musculature, as if there was little resistance for this process. At the final seconds of the movie, the part that penetrated the tissue is visible by translucence. Intact spermatophores and spermatangia from previous experiments are also visible in the video.

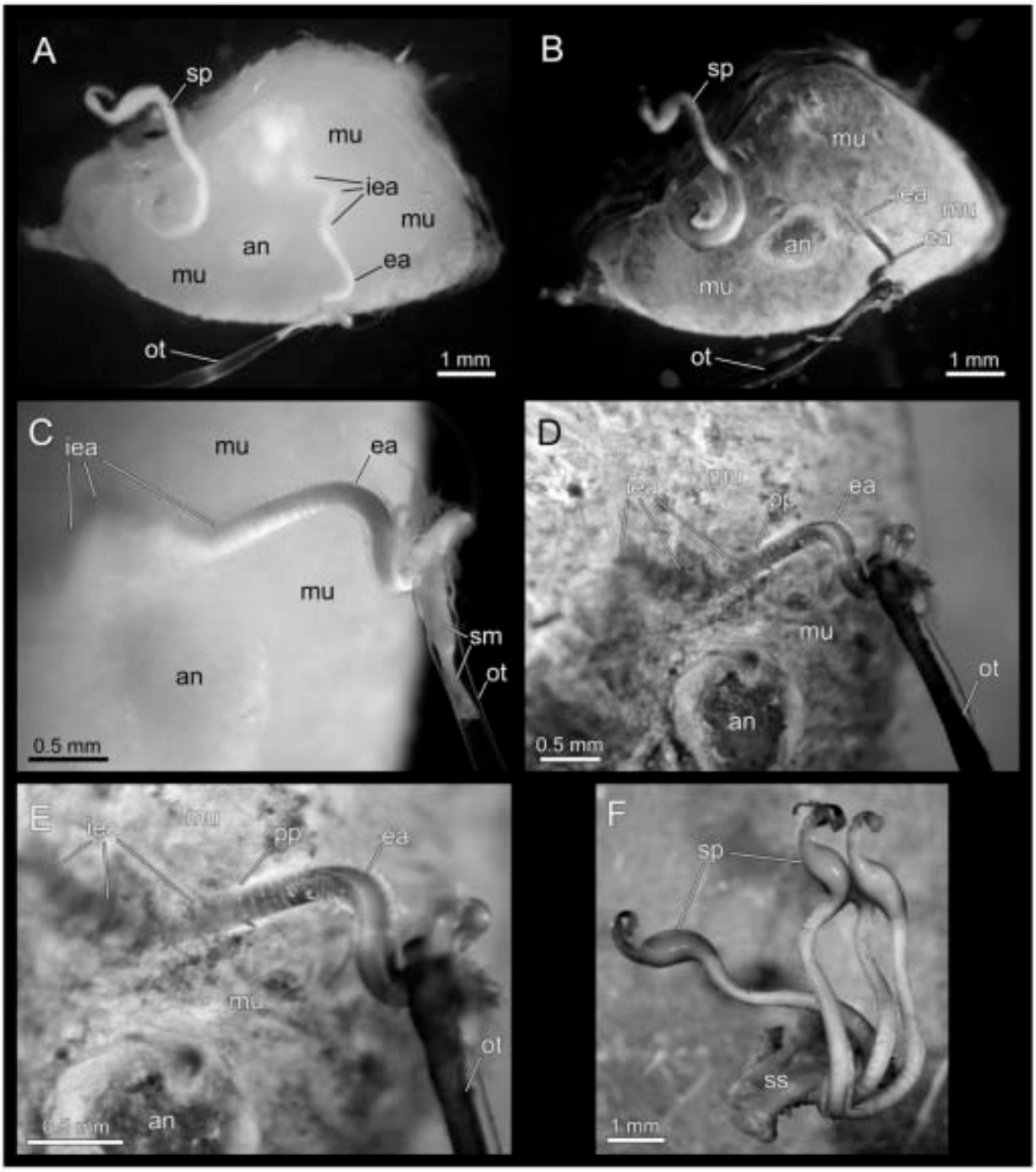

Figure 1. Experiments from the *in vitro* analysis of the spermatophoric reaction of *Doryteuthis plei*. (A) Piece of a tentacle with a spermatophore partially implanted into the incised musculature (right to the axial nerve); the implanted region is visible by translucence (compare with B). In this case the everting ejaculatory apparatus was directed towards the incised part of the sample, and readily penetrated the exposed musculature, resembling deep implantation naturally observed in some oceanic and deep-sea squids. A spermatangium normally attached to the tentacle is also visible at left. (B) Same as A, but stained with methylene blue. (C) Enlarged view of the implanted spermatophore of A; the implanted region is visible by translucence (compare with D). Note that almost all the sperm mass has been pushed out of the spermatophore into the implanted region. (D) Same as C, but stained with methylene blue. (E) Enlarged view of D. (F) Spermatangia normally attached to the surface of a small piece of the spermatophoric sac (stained with methylene blue). In this case the everting spermatophores were directed towards the epithelium of the sample, and the spermatangia ended up with their bases attached superficially, like those naturally found in the buccal membrane and mantle cavity of females. All samples were fixed in Karnovsky's fixative after the experiments. Specimens' mantle lengths: aprox. 300 mm. *an*, axial nerve cord from tentacle; *ea*, everted ejaculatory apparatus; *iea*, implanted region of the everted ejaculatory apparatus; *mu*, artificially incised and exposed tentacle musculature; *ot*, outer spermatophore tunic; *pp*, point of penetration; *sm*, sperm mass; *sp*, spermatangium; *ss*, piece of the spermatophoric sac.

Capítulo 3

Funcionamento dos espermatóforos de *Doryteuthis plei*: a descoberta de um complexo processo de fixação dos espermatângios

CAPÍTULO 3

Funcionamento dos espermatóforos de *Doryteuthis plei*: a descoberta de um complexo processo de fixação dos espermatângios

Dando continuidade às análises *in vitro* da reação espermatofórica e ao estudo da morfologia funcional dos espermatóforos de *Doryteuthis plei*, o presente manuscrito apresenta resultados muito interessantes e certamente impactantes acerca do processo de fixação dos espermatângios. As evidências reunidas a partir da investigação do funcionamento do espermatóforo, da morfologia dos espermatângios obtidos *in vitro* e daqueles naturalmente fixados na fêmea, permitem afirmar que o processo de fixação compreende distintas fases desempenhadas por diversos componentes do espermatóforo, contrariamente a um conceito anterior, bastante difundido, de que a fixação seria realizada somente por substâncias adesivas do corpo cimentante.

Durante a reação espermatofórica, o aparato ejaculatório e respectivo filamento espiral são capazes de perfurar superficialmente o tecido-alvo. Não ocorre implante profundo, mas a barreira do epitélio pode ser efetivamente rompida e o espermatângio pode alcançar o tecido conjuntivo; no caso de espermatângios fixados na membrana peribucal, a cutícula protetora do receptáculo seminal é igualmente perfurada. Subseqüentemente ao processo perfuração ou escarificação da superfície do tecido-alvo, o corpo cimentante sofre drástica modificação estrutural, resultando na extrusão de parte do conteúdo cimentante, o qual é injetado diretamente sobre o tecido perfurado. Além disso, o corpo cimentante é exteriorizado com uma extremidade afilada que, em alguns casos, foi encontrada firmemente implantada no tecido da fêmea, juntamente com as substâncias cimentantes. Concomitantemente ao processo de reconfiguração do corpo cimentante, a região da membrana interna que contém as espículas no espermatóforo intacto é evertida e estirada sobre a base do espermatângio, sugerindo um papel auxiliar no processo de fixação. O funcionamento do espermatóforo é discutido e reavaliado com base nas evidências apresentadas.

O manuscrito contendo essas informações em detalhe será submetido periódico internacional *Journal of Morphology*.

Spermatophoric reaction reappraised: novel insights into the functioning of the loliginid spermatophore based on *Doryteuthis plei* (Mollusca: Cephalopoda)

Abstract

During copulation, spermatophores produced by male coleoid cephalopods undergo the spermatophoric reaction, a complex process of evagination, which culminates in the attachment of the sperm mass on the female's body. In order to better understand this complicated phenomenon, the present study investigated the functional morphology of the spermatophore of the squid *Doryteuthis plei* applying *in vitro* analysis of the reaction, as well as light and electron microscopy investigation of spermatangia (everted spermatophores containing the sperm mass) obtained either *in vitro* or naturally attached on the female. Hitherto unnoticed functional features of the loliginid spermatophore revealed herein required a reappraisal of some important processes involved in the spermatophoric reaction, as well as the proposal of new hypotheses to explain their mechanics. The most striking findings concern to the attachment mechanism, which is not carried out solely by cement adhesive material, as previously believed, but rather by a complex process performed by multiple structures that lead to the implantation of the base of the spermatangium into the female body. The everting ejaculatory apparatus possesses a perforating ability, and is presumably able to superficially puncture the female tissue. Subsequently to this process, the cement body passes through a complex structural rearrangement, which leads to the injection of both its viscid cement contents and pointed oral region through the puncture into the female tissue. The inner membrane at the oral region of the cement body contains numerous minute stellate particles attached at its inner side, so that when the inner membrane is everted, these sharp structures are exposed, presumptively adhering to the scarified tissue and augmenting attachment by assuring the injection of the cement material inside the superficial hole. The functioning of the loliginid spermatophore is revisited in light of these findings.

Keywords: Loliginidae, *Doryteuthis plei*, reproduction, spermatophore, functional morphology, spermatophoric reaction.

Introduction

Male cephalopods encase spermatozoa within spermatophores, transferring them to the female during copulation (Nesis, 1987). In coleoid cephalopods, spermatophores are highly complex, and are composed of specialized structures that play specific roles in the so-called spermatophoric reaction (Mann, 1984). During spermatophore transfer, the spermatophoric reaction leads to the extrusion and attachment of the sperm mass on specific sites of the female's body (e.g., Drew, 1919a).

Although spermatophore general morphology has been studied in several cephalopod species due to its systematic relevance (e.g., Hess, 1987), the knowledge of its functional morphology is restricted, on a varying level of detail, to 12 decapodiforms (i.e., squids and sepioids): *Doryteuthis pealeii* (Drew, 1919a; Austin et al., 1964), *D. opalescens* (Fields, 1965), *Loligo vulgaris* (Milne-Edwards, 1842; Marchand, 1913), *Illex coindetii* (Marchand, 1913), *Octopoteuthis sicula* (Hoving et al., 2008), *Onykia ingens* (Hoving and Laptikhovsky, 2007), *Rossia macrosoma* (Racovitza, 1894; Marchand, 1913), *R. moelleri* (Hoving et al., 2009), *Sepia officinalis* (Milne-Edwards, 1842; Marchand, 1913), *Sepiola atlantica* (Weill, 1927), *S. rondeleti* (Marchand, 1913), *Todarodes pacificus* (Takahama et al., 1991), and seven octopodiforms (i.e., octopods): *Eledone cirrhosa* (Fort, 1937), *E. moschata* (Milne-Edwards, 1842; Marchand, 1913; Fort, 1941; Orelli, 1962), *Enteroctopus dofleini* (Mann et al., 1966 and 1970; Hanson et al., 1973), *Octopus defilippi* (Marchand, 1913), *O. macropus* (Marchand, 1913), *O. tehuelchus* (Pujals, 1978) and *O. vulgaris* (Drew, 1919b). Detailed descriptions of the spermatophoric reaction, providing information on the role of the spermatophore components in the mechanics of the process, are considerably rarer (e.g., Racovitza, 1894; Drew, 1919a; Weill, 1927).

Several questions regarding the complex process of extrusion of the sperm mass and the exact process of attachment of the spermatangium (everted spermatophore containing the sperm mass) remain unanswered. In loliginids, the adhesive contents of the cement body have been stated to attach spermatangia on the female (Drew, 1919a; Austin et al., 1964). However, the hypothesis that spermatangia would instantly become firmly attached solely by adhesive substances is quite intriguing, especially when considering that spermatangia are deposited externally in loliginids, either within the mantle cavity in the vicinity of the genital opening, or on the buccal membrane near the seminal receptacle, both areas being constantly exposed to substantial water turbulence.

The extraordinary structural complexity of coleoid spermatophores (e.g., Marian and Domaneschi, in prep.[1]), and its apparent simple functioning (*i.e.*, extrusion of the sperm

[1] Marian and Domaneschi in prep.: Capítulo 1.

mass out of the spermatophore, and finally its “gluing” on the female’s body) have been perplexing scientists for over a century. In the words of Gilman A. Drew (1919b): “[…] it is very difficult to arrive at the reasons for the formation of such complicated structures as these spermatophores to perform functions where simpler arrangements would seem do as well.” Conversely, recent evidence suggests that the picture is much more complex than this traditional view, as the loliginid spermatophore was demonstrated to have some tissue implantation capability under artificial conditions (Marian, submitted[2]).

In order to contribute to the knowledge on the coleoid spermatophore functional morphology, this paper describes the spermatophoric reaction in the squid *Doryteuthis plei* (Blainville, 1823) applying *in vitro* analysis of the reaction, and microscopical investigation of spermatangia obtained either *in vitro* or naturally attached on the female. The data presented herein demonstrate that the structural intricacy of the loliginid spermatophore is intimately related to a hitherto unknown complex, efficient process of attachment.

Materials and Methods

Animals

During the summers of 2005-2008, mature specimens of *Doryteuthis plei* were captured by hand-jigging off Barequeçaba Beach (São Sebastião municipality), between 23º50’00’’ S and 45º26’20’’ W, and off the northern littoral of São Sebastião Island (Ilhabela municipality), between 23º44’00’’ S and 45º17’50’’ W (São Paulo state, Brazil). Aboard the boat specimens were kept in a large container (200 l), and part of the seawater was changed continuously along the way to the Marine Biology Center of the University of São Paulo (CEBIMar-USP), where animals were transferred to 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 live shrimps (*Farfantepenaeus paulensis* and *F. brasiliensis*) once a day. Before dissection, male specimens were submerged and over-anesthetized in an isotonic solution of $MgCl_2$ (Messenger et al., 1985).

Experiments with live animals followed the recommendations given by Moltschaniwskyj et al. (2007). Specimens were collected under IBAMA/MMA permit Nº 02027.000248/2005-11. Voucher specimens were deposited in the collection of the Museum of Zoology, University of São Paulo (MZUSP). The nomenclature herein adopted for the description of the structure of the spermatophore and spermatangium is that of Drew (1919a), Austin et al. (1964) and Takahama et al. (1991)

[2] Marian, submitted: Capítulo 2.

In vitro analysis of the spermatophoric reaction

After dissection, spermatophores were removed from the spermatophoric sac of mature males, and placed on a Petri dish filled with filtered seawater. To slow down and register some stages of the normally instantaneous spermatophoric reaction, the technique introduced by Drew (1919a) was applied. This technique consisted of the following steps: spermatophores were first immersed in a hypertonic solution made of one-fourth saturated solution of magnesium chloride in seawater for a couple of minutes, then transferred back to filtered seawater and analyzed as described below. However, this technique has shown to be useful only for the analysis of the initial stages, as it yielded an inevitable artefact in the final phases of the reaction (see “Results” and “Discussion”).

Spermatophoric reaction was induced by rapidly pulling the cap thread with the aid of fine dissecting forceps while the spermatophore was held still, and observed under a Zeiss Stemi SV11 APO stereomicroscope or a Zeiss Axioplan2 microscope. Digital filming of the reaction was made by equipping both microscopes with a Sony DCR HC1000.

Experiments including a substrate for spermatangia attachment were also performed. After triggering the reaction, spermatophores were directed towards small pieces of the male's donor body samples (e.g., mantle, arms, tentacles, spermatophoric sac). Although the natural spermatangia deposition sites in *D. plei* are either the ventral buccal membrane or the vicinity of the genital opening of females, these experimental samples were useful for observing the behavior of everting spermatophores upon a substrate.

Spermatophores in distinct phases of the reaction, as well as spermatangia naturally attached near either the seminal receptacle or the genital opening of captive females, were properly fixed for the following purposes.

Light microscopy

Samples were 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. The material was dehydrated and embedded in glycol methacrylate (Leica’s historesin), following the manufacturer's protocol. Serial transverse and longitudinal 2-4 µm sections were subjected to the following staining procedures (according to Humanson, 1962; Bancroft and Stevens, 1982; and Pearse, 1985): Hematoxylin-Eosin (H&E), Mallory's Triple Stain and Gomori’s Trichrome (identification of collagen), Periodic Acid–Schiff (PAS – identification of neutral polysaccharides), Alcian Blue pH 2.5 (identification of acidic polysaccharides), Mercury-Bromophenol Blue (identification of proteins), and Alizarin Red S

(identification of calcium deposits). The staining affinities of each spermatangium component were compared to those of intact spermatophores (Marian and Domaneschi, in prep.) to attempt to confirm their identity. A combination of the PAS and H&E techniques was tested on some sections rendering excellent results, particularly when staining the delicate empty remaining capsules.

Fresh spermatangia and empty capsules, as well as stained slides, were also investigated applying Differential Interference Contrast and Polarized Light Microscopy.

Scanning electron microscopy

Samples were fixed in Karnovsky's fixative (see above), washed in buffer solutions, and post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, adjusted to 1000 mOsm with sucrose. After additional rinsing in buffer solution, samples were dehydrated with acetone, critical point dried, mounted on stubs, and gold coated. Subsequently, samples were examined in a Zeiss DSM 940 electron microscope.

Results

Spermatophoric reaction without a substrate for attachment

The spermatophoric reaction of *Doryteuthis plei* takes about 30 to 40 seconds to be completed, and is divided into four stages: I) rupturing of the cap, and evagination of the convoluted part of the ejaculatory apparatus; II) evagination of the whole ejaculatory apparatus tube; III) extrusion and reconfiguration of the cement body (and concomitant exposure of the inner membrane); and IV) detachment of the middle membrane and liberation of the spermatozoa. The general description provided in this section comes from observations made under the stereomicroscope; please refer to the other sections for details on spermatangia morphlogy. The spermatophoric reaction proceeding without a substrate for attachment is illustrated in Figs. 1-3; see also the supplemental video (Appendix S1[3] in Supporting Information).

Stage I (Figs. 1A-C). The spermatophoric reaction is triggered by rapidly pulling the cap thread while the spermatophore is held still (Fig. 1A). The tension is transmitted to the cap, which then ruptures and opens a breach for the initiation of the reaction (Fig. 1B). In *D. plei*, placing freshly removed spermatophores in sea water is not sufficient to trigger the reaction alone, although very few spermatophores do initiate the reaction with no physical

[3] Ver CD em anexo à Tese, contendo material suplementar.

tension applied to the cap thread. Subsequently to the rupture of the cap, the oral-most convoluted part of the ejaculatory apparatus tube is exposed to the external milieu, and initiates the evagination process instantaneously (Figs. 1B-C), describing a spiral of nearly one turn (Fig. 1C). During this very rapid process, the cement body is stretched (Fig. 1B) as it is pulled along by the evaginating ejaculatory apparatus. Then, there is a pause of about two seconds in the evagination process (Fig. 1C), and the cement body and sperm mass advance orally. At the aboral region, the middle tunic starts to swell (see Fig. 8F) and, thus, begins to propel the sperm mass towards the spermatophore's oral end, apparently providing high internal pressure for the evagination process.

Stage II (Figs. 1D-H; 4A,B). After the two-second pause, the evagination process continues to the very rapid second stage. Throughout this stage, the spiral filament is completely everted, i.e, as the ejaculatory apparatus tube evaginates, the spiral filament comes to join its external walls. Still images of an everting spermatophore subjected to magnesium chloride treatment (otherwise the process is too fast to be followed) shows that the everting spiral filament is exposed one whorl at a time, first as a small diameter whorl, which enlarges in diameter as the evagination proceeds (Figs. 4A,B; see also the supplemental video in Appendix S2[4] in Supporting Information). During its evagination, the diameter of the spiral filament tube increases to about four to five times its diameter in the intact spermatophore (Figs. 4A,B).

Stage III (Figs. 1I-L). This is the most difficult stage to analyze, as it happens very quickly and does not permit the use of hypertonic salt solutions to slow down the reaction. In the spermatophores of *D. plei*, the application of the magnesium chloride solution technique (Drew, 1919a) resulted in a preparation artefact, i.e., the whole cement body was extruded without any structural modification (see Appendix S3[5] in Supporting Information), a condition not resembling natural spermatophoric reactions. As will be seen, in natural reactions the cement body suffers a complex structural reorganization.

The third stage begins when the evagination process reaches the level of the cement body (Fig. 1I); at this stage, the cement body is evidently compressed longitudinally (Fig. 1I), as if there were considerable resistance for its extrusion. The subsequent phase is too rapid to be followed (Fig. 1J), but at the end of this process three important modifications occur at the oral region of the everting spermatophore. First, the cement body is extruded as a cap-like structure (Figs. 1K-L; 2A), with exception of its oral slender part, which is projected apparently without modification. Second, some cement substances are extruded seemingly from the base of the exposed cement body (Figs. 1K-L; 2A). Third, immediately after this process, an opaque membrane is seen covering the base of the forming spermatangium

[4] Ver CD em anexo à Tese, contendo material suplementar.
[5] Ver CD em anexo à Tese, contendo material suplementar.

(Fig. 2A). As will be discussed, this membrane corresponds to the region of the inner membrane, which was covering the cement body in the intact spermatophore.

Stage IV (Figs. 2 and 3). After the third stage, there is again a pause of about five seconds. Subsequently to this pause, a marked constriction appears at the level of the base of the cement body (Fig. 2B). As will be discussed, this constriction is most probably the constricted region of the middle membrane at the level of the junction between the cement body oral and intermediate regions. This constriction slowly glides through the spermatophore towards the aboral region (Figs. 2B-G), accelerating after it passes the region of the cement body (Figs. 2H-I). This process continues until the final spermatangium is formed (Figs. 3A-F). At the end of this process, spermatozoa are released through the opening opposed to the cement body (Fig. 3F).

Spermatophoric reaction with a substrate for attachment

The spermatophoric reaction proceeding with a substrate for attachment is illustrated in Figs. 5-6; see also the supplemental video (Appendix S4[6] in Supporting Information). The general functioning of the spermatophoric reaction in this case seems to be essentially as described above. However, three seminal findings resulted from these investigations. First, the attachment process was entirely autonomous, i.e., the spermatophore alone was able to attach itself onto the surface of the experimental tissue sample (Figs. 5-6). Second, the contact of the everting ejaculatory apparatus and spiral filament with the targeted tissue is apparently a precondition for final attachment (Figs. 5-6). In the numerous experiments performed, the everting spiral filament had obligatorily to enter into contact with the targeted tissue for the attachment to occur. Fully formed spermatangia obtained *in vitro* after the spermatophoric reaction without a substrate, when pressed with their bases against the tissue sample, were not able to attach or establish a firm connection with the targeted tissue. The cement substances, however, do show remarkable adhesive properties, as several spermatangia remained with their bases attached to the bottom of the Petri dish apparently due to the adhesive cement material. Nonetheless, the results obtained herein indicate that, at least under *in vitro* conditions, the adhesive properties alone are not sufficient to firmly attach the spermatangia on the targeted tissue.

A third finding, intimately associated with the others, is that the everting ejaculatory apparatus demonstrated a limited, but effective, perforating ability when in contact with the surface of the targeted tissue. Note on Fig. 5C the point of penetration of the ejaculatory apparatus into the tissue sample. On Figs. 5D-J the tip of the everting ejaculatory apparatus

[6] Ver CD em anexo à Tese, contendo material suplementar.

penetrates and advances parallel through the surface of tissue (apparently below the epithelium) until the level of the cement body is reached. At this stage, the part that had implanted superficially is pushed backwards and extruded from the targeted tissue (Figs. 5J-K). The tip of the everting spermatophore (containing the cement body) remains attached onto the surface of the sample (Fig. 5L; 6A). After this phase the functioning of the spermatophoric reaction is essentially as described above for the reaction without the substrate (Fig. 6), i.e., the constricted region of the middle membrane glides aborally (Figs. 6B-F) until the formation of the final spermatangium and the liberation of spermatozoa.

Spermatangia morphology – light microscopy

Fully formed spermatangia obtained during *in vitro* experimentation (Figs. 7; 9; 10; 11; 13; 14) consist of 1) the sperm mass enclosed by the everted inner tunic (Figs. 7A,B; 9A-C); 2) the restructured cement body, which covers their bases (Figs. 7A-C; 9A-C; 13A); and 3) a thin opaque membrane that is stretched over the base of the spermatangium (Figs. 7A,B). This membrane apparently corresponds to the region of the inner membrane, which was covering the cement body in the intact spermatophore (see Marian and Domaneschi, in prep.). When investigated under higher magnifications, this membrane is revealed to bear several minute stellate particles (Figs. 9C; 10F), to be treated later.

The reconfigured cement body covering the base of the spermatangium is basically composed of a cap-like base (partially covered with cement substances), and of a pointed structure which corresponds to the former slender oral-most region of the intact cement body (Figs. 7A-C; 9A-C; compare to Marian and Domaneschi, in prep.). At the extremity of this pointed end lies the characteristic lozenge shaped structure (Figs. 7A-C; 9A-C; 10A,B,D) exactly as seen in the intact cement body (Marian and Domaneschi, in prep.). Internally, the cement body is connected to the sperm mass by the connecting cylinder (Figs. 7D; 9A-C). When the spermatangium is investigated through serial sectioning and staining, the reconfiguration of the cement body and the morphology of its cap-like base are revealed to be the result of an intricate folding of cement body outer casing (Figs. 9A-C; 10C). This folding involves both outer and inner layers of the cement body casing (Fig. 10C). Externally, the granular outer layer and the fibrous inner layer fold at least twice onto each other (Fig. 10C). Internally, the outer layer from the intermediate region of the cement body describes also a prominent fold (Fig. 10C).

The cement body homogeneous matrix (Marian and Domaneschi, in prep.) is apparently extruded during the reconfiguration of the cement body, emerging as the cement substances covering the surface of the cement body after the spermatophoric reaction (Figs. 7A,C; 9A-C; 10A-E). There is no discrete opening for the release of such material; most

probably they are extruded through the cement body layers, which are evidently permeable (Fig. 10A-E; see also the results obtained under scanning electron microscopy, described below). The two types of granular contents of the cement body, so easily discernible between one another in the intact spermatophore (Marian and Domaneschi, in prep.), are difficult to distinguish in the spermatangium, even when applying several staining protocols. In some cases, the oral region granular material is evident, and found embedded within the pointed region of the cement body (Fig. 10D). Except for their aboral location, however, the aboral granules (Fig. 10 A,C,E) are apparently indistinguishable from the oral ones. Moreover, in some places, a fibrous-like material was found interspersed with the aboral granules (Figs. 10C; 11C,F); this material was not observed in intact spermatophores (Marian and Domaneschi, in prep.). Clearly, there is a chemical and textural modification of the cement body granular material during the spermatophoric reaction.

The region of the inner membrane that was covering the cement body in the intact spermatophore was shown to be in intimate contact with several minute stellate particles (Marian and Domaneschi, in prep.); this membrane exhibited also strong PAS-positivity. Coupling these results with the data obtained herein, the membrane observed covering the base of the spermatangium (Figs. 7A,B; 9C) most probably corresponds to this region of the former intact inner membrane. Besides being PAS-positive, this membrane bears several minute stellate particles over its surface (Fig. 10F; see also the results obtained under scanning electron microscopy, described below). During the reaction, this region of the inner membrane presumably ruptures from the part that was closely applied to the spiral filament tube (see also Marian and Domaneschi, in prep.).

Two slightly distinct spermatangia configurations were also obtained *in vitro* (Figs. 7B; 11A-F). As will be discussed, these configurations apparently represent successive steps in the formation of the final spermatangium. These spermatangia are exactly as described above, except for the degree of 1) eversion of the inner tunic, and 2) rupturing of the cement body. Considering the spermatangium described above and depicted in Figs. 9A-C as the presumable first step in this process, the spermatangium on Figs. 11A-C as the second step, and the spermatangium on Figs. 7B and 11D-F as the third step, these configurations are more easily understood. Note that in step one (Figs. 9A-C) the cement body is located at the base of the spermatangium, and also closely applied to the connecting cylinder. In steps two and three (Figs. 11A-F), the cement body is located gradually apart from the base of the spermatangium, and also from the connecting cylinder. This is apparently the result of the degree of eversion of the inner tunic, which is relatively more everted on step two (compare Figs. 9A-C to Figs. 11A-C), and completely everted on step 3 (Figs. 11D-F). This eversion results in the rupturing of the cement body on steps two and three (Figs. 11A-F), and also in the rupturing of the inner membrane on step three (Figs. 11E-F). Note that in step two the

inner membrane containing the stellate particles is still intact and covering the surface of the cement body (Figs. 11B-C). On step three, the inner membrane is ruptured, and the cement contents from the aboral region of the cement body are spread over the base of the spermatangium (Figs. 11E-F). Throughout *in vitro* experimentation, all these spermatangia configurations were equally frequent.

Spermatangia morphology – electron microscopy

Figures 13 and 14 depict the aspect of *in vitro* obtained spermatangia under scanning electron microscopy. On the cap-like base of the cement body (i.e., the intricately folded region) the granular outer layer of the cement body is clearly discernible and interspersed with some cement material (Figs. 13A,B), in some places suggesting these substances could be extruded through the cement body layers. The slender oral region of the cement body (Figs. 13A,C) is clearly composed of the fibrous inner layer (compare also with Figs. 10A,B,D,E). The lozenge-shaped extremity of the cement body (Figs. 13A,D-F) was revealed to be a complicated structure, bearing three longitudinal crests on its apex (Figs. 13E,F) and six longitudinal crests located on its periphery (Figs. 13D,E).

The inner membrane covering the base of the spermatangium bears numerous minute stellate particles on its surface (Fig. 14A). The stellate particles have a variable number of acute branches (from 7 to 12, but generally 7) and range in size from 2 to 6 microns (Figs. 14B-E). They are firmly connected to the inner membrane through one of their branches (Figs. 14D,E) and their occurrence is limited to the inner membrane, i.e., where the inner membrane is interrupted, the stellate particles are absent (Fig. 14C).

Remaining empty case morphology – light microscopy

The discharged empty case from the spermatophoric reaction is composed of the outer tunic (Figs. 8E,F; 12A), the middle tunic (which occupies most of the space inside the outer tunic; Figs. 8F; 12A), and by the evaginated ejaculatory apparatus tube (obviously without the inner tunic). The ejaculatory apparatus tube is evidently formed by the everted middle membrane and spiral filament (Figs. 8A-E; 12B,C). The presence of the inner membrane, however, is difficult to trace, and is only detectable with the PAS method (Fig. 12B,C). In such sections, the inner membrane consists of a layer strongly stained with a magenta color, and situated between the broad middle tunic and the thin spiral filament membrane (Fig. 12C). The everted spiral filament (Figs. 8C,D; 12B,C) contains numerous minute stellate particles, which are exposed to the external milieu, but remain tightly packed and closely applied to the spiral filament membrane, even after several washing in distinct

reagents used during sample preparation. They are very fine and generally difficult to discern under light microscopy (see also the results obtained under scanning electron microscopy, described below).

The region of the middle membrane at the level the cement body in the intact spermatophore (which corresponds to the terminal unornamented portion of the empty case; Figs. 8A-C; 12D-F) exhibits a peculiar morphology in the empty case that is worthy of a separate description. Firstly, all empty cases showed a marked constriction near their terminal portion (Figs. Figs. 8A,B; Figs. 12D-F), which corresponds to the middle membrane constriction dividing oral and intermediate regions of the intact cement body (Marian and Domaneschi, in prep.). Second, in several empty cases, the region corresponding to the "aboral segment of the middle membrane" (Marian and Domaneschi, in prep.) was folded outwardly (Figs. 8B; 12D-E). However, not all empty cases exhibited such folding (Fig. 8A), and the degree of folding was quite variable: the folding could start either at the tip of the aboral segment (Fig. 12D), at the median level of segment (Fig. 12E), or even at the level of the constriction of the middle membrane (Fig. 12F).

Remaining empty case morphology – electron microscopy

When investigated under scanning electron microscopy, the everted spiral filament (Figs. 15A-C) adhered to the evaginated ejaculatory apparatus tube was revealed to be composed of several tightly packed, minute stellate particles, interspersed with some unidentified material, not detected under light microscopy. The spiral filament's stellate particles are much finer than those of the inner membrane (Figs. 14A-E). Except for this difference, however, the morphology of both spiral filament's and inner membrane's stellate particles appears to be very similar.

Morphology of spermatangia naturally attached on mated females

Spermatangia were found attached externally either on the cuticle of the female seminal receptacle located on the ventral region of the buccal membrane (Figs. 16; 17B), or on the membranous expansions (“lips”) that border the female genital opening (Figs 16; 18C), located inside the mantle cavity. During mating occurring in the "head-to-head" position (Fig. 17A), males apparently place the spermatophores on the buccal membrane (Fig. 17B), although this process could not be accurately verified. During mating occurring in the "male-parallel" position, males effectively place spermatophores inside the mantle cavity of females with the aid of the hectocotylus (Fig. 18A).

Spermatangia attached on the cuticle of the buccal membrane's seminal receptacle were found with their bases implanted into the cuticle layers (Figs. 17C-F). The most superficial cuticle layers were found ruptured (Fig. 17D) at the site of implantation. The implantation depth varied from a more superficial embedding (Figs. 17C,D), to a profound implantation of the base of the spermatangia into the deposition site. In some cases, the cement substances were found occupying the space between some ruptured cuticle layers (Fig. 17D). The implanted spermatangium base consisted of the cement body casing and the cement body extremity, all structures tightly compressed between the adjacent cuticle layers (Fig. 17F). The layers in direct contact with the spermatangium base exhibited a stronger affinity for eosin (Fig. 17F), which also suggests the presence of cement material. Implanted spermatangia never reached the seminal receptacle or the buccal membrane epithelium.

Spermatophores attached on the lips bordering the genital opening of females were also found with their bases embedded into tissue (Figs. 18C-F). In all observed cases the epithelium was ruptured, and the underlying connective tissue reached by the spermatangia bases. Compare the normal condition of the intact genital opening lips (Fig. 18B), with the evidently damaged condition of the lips with implanted spermatangia (Figs. C-F). The implanted spermatangium base consisted of the cement body casing and the cement body extremity, which in some cases was found deeply thrust into the connective tissue (Fig. 18F). Cement substances were found on the surface of the injured connective tissue (Figs. 18C-F), and sometimes apparently interspersed with some of the connective tissue fibroblasts (Figs. 18D,E). The inner membrane containing the stellate particles was found stretched over the base of the spermatangium (Fig. 18G), frequently with some material attached to the stellate particles (Fig. 18G). This material includes what appears to be connective tissue fibroblasts (Fig. 18G). The general aspect of the genital opening lips with implanted spermatangia suggests an inflammatory response, in which the fibroblasts concentrate around the injured area of the tissue (Figs. 18C-F; compare to the normal condition in Fig. 18B). The general morphology of naturally attached spermatangia (on the buccal membrane and genital opening lips) matches with the morphology of spermatangia obtained *in vitro*.

Discussion

As expected, the general functioning of the spermatophoric reaction of *D. plei* is very similar to that of other loliginids already described in the literature (Drew, 1919a; Austin et al., 1964; Fields, 1965), as well as to that of other decapodiforms (e.g., Racovitza, 1894; Weill, 1927; Takahama et al., 1991). However, novel functionalities were revealed by the present study, and they require a reappraisal of some important processes involved in the spermatophoric reaction, as well as the proposal of new hypotheses to explain their

mechanics. One of the most striking findings is concerned to the attachment mechanism, which is carried out by a complex process performed by multiple structures, leading to a "shallow" implantation of the spermatangium into the female body (i.e., the base of the spermatangium becomes implanted into female tissue). Previously, the attachment of the loliginid spermatangium on the female's body was believed to be carried out solely by the cement adhesive material (e.g., Drew, 1919; Austin et al., 1964; Fields, 1965).

To facilitate the discussion of the results, in the following sections each spermatophore structure is presented separately, with a reassessment of its function based on the results obtained herein and by earlier authors.

Cap and cap thread. Although it was stated that in other species just placing the spermatophores in seawater was sufficient to trigger the spermatophoric reaction (e.g., Drew, 1919a; Fields, 1965), in *D. plei* this was not often, the act of pulling the cap thread being necessary to rupture the cap and initiate the reaction. The cap thread is tightly cemented at the surface of the cap, on which it bears a sinuous thickening (Marian and Domaneschi, in prep.). Pulling the thread must transmit tension to the cap through this thickening. Since the whole spermatophore is under high internal pressure, a slight tension should be enough to provoke cap rupture.

The function of the cap and cap thread most probably is to trigger the spermatophoric reaction during mating, regardless if seawater alone is sufficient to begin the process. As the male abruptly retrieves a bunch of spermatophores from the terminal organ (probably through the funnel; Drew, 1911), aboral regions first, the respective cap threads are the last structures to leave the terminal organ. The threads might be stretched as the aboral part of the spermatophore is rapidly pulled by the hectocotylus, since they are deeply entangled inside the spermatophoric sac (e.g., Drew, 1919a); the muscular contraction of the walls of the terminal organ (e.g., Hoving & Laptikhovsky, 2007) could also hold the cap threads. This tension, transmitted to the cap at the region of the thread thickening, could open a slit in the cap, and a breach for the reaction to occur. Earlier authors (e.g., Drew, 1919a; Weill, 1927) had already put forward the importance of the cap as a region of less resistance and as an evacuation pathway, imperative for the success of the reaction. Although in other loliginids and also other cephalopod species the contact of the spermatophore with seawater is sufficient to trigger the reaction by uptake of seawater (e.g., Drew, 1919a; Weill, 1927; Takahama et al., 1991), this tension transmitter mechanism must be important to ensure that the reaction is triggered exactly as the male had pulled the spermatophores out of the terminal organ (i.e., during mating).

Outer and middle tunics. A complete discussion on the function of these tunics is provided by Marian and Domaneschi (in prep.).

Inner tunic. The inner tunic takes part in the evagination process during the spermatophoric reaction; at the end of this process it comes to lie surrounding the sperm mass. Physical protection for the sperm mass is a function that could be ascribed to this tunic, but the inner tunic could also be important to maintain a certain pressure inside the spermatangium, to assure a continuous flow of spermatozoa. The elasticity of the inner tunic could be responsible for this function. Also, an important feature of the spermatangium is that the flow of the spermatozoa is continuous but not too fast. This might be the result of the reduced diameter of the spermatangium opening, which corresponds to the oral ending of the inner tunic in the intact spermatophore (Marian and Domaneschi, in prep.). Also, Drew (1919a) proposed that the thickening of the inner tunic at the region just before the loops of the intact ejaculatory apparatus could diminish the diameter of the lumen at the evacuation pathway, retarding the escape of sperm. A long and continuous supply of spermatozoa must be important to assure fertilization of the eggs (if the spermatangia are deposited inside the mantle cavity, which generally occurs in spawning females), or to assure that much of the spermatozoa are sucked inside the seminal receptacle (if the spermatangia are attached to the buccal membrane; Oordt, 1938).

Middle membrane. The fact that the middle membrane exhibits different structure and chemical composition at the region of the cement body (Marian and Domaneschi, in prep.), and that this same region (i.e., the aboral segment of the middle membrane) was found folded after the spermatophoric reaction, suggest that this membrane might play a vital role in the complicated reconfiguration of the cement body (see below). Certainly, the middle membrane constricted region between the oral and intermediate regions of the cement body is also involved in this process, but additional experimentation is necessary to answer these questions.

Inner membrane. The region of the inner membrane bearing stellate particles in the intact spermatophore (Marian and Domaneschi, in prep.) is exactly the part of this membrane that detaches from the rest of the inner membrane and comes to lie stretched and covering the base of the spermatangium after the spermatophoric reaction. Since this process is concomitant with the rearrangement of the cement body, and that some connective cells were found attached to the stellate particles, the function of this section of the inner membrane is assumed to be to optimize attachment, presumably by assuring that most of the cement material is injected inside the puncture perforated by the everting ejaculatory apparatus (see below). Possibly, when the inner membrane is everted and spread over the base of the spermatangia, its sharp stellate particles are exposed, continuously adhering to the scarified tissue, and therefore preventing the spermatangia from being pushed backwards during the cement body reconfiguration process (see below). The stellate

particles of the stretched inner membrane might also optimize fixation in the female tissue by physical anchorage.

Spiral filament. Drew (1919a), Austin et al. (1964) and Fields (1965) agreed in that the spiral filament located inside the inner membrane tube would help maintaining the potency of the extruding ejaculatory apparatus. However, at least in *D. plei*, but possibly in other loliginids, the everting spiral filament has apparently an important function during the spermatophoric reaction: the perforation of a superficial puncture into the female tissue for the implantation of the spermatangia. The results obtained herein and by Marian (submitted) suggest the everting ejaculatory apparatus bears impressive tissue implantation capabilities. Since the spiral filament is the structure exposed to the external milieu during the spermatophoric reaction, and the one directly into contact with the female tissue during penetration, most probably this structure is involved in the perforation process. How this structure is able to perforate tissue, however, remains enigmatic.

Regardless of the mechanics of perforation, besides penetrating female tissue, the everting spiral filament is able to anchor on the targeted tissue, always maintaining the tip of the everting spermatophore into contact with the tissue until it reaches the level of the cement body. This anchorage capacity is most probably conferred by its numerous sharp stellate particles.

Stellate particles. Stellate particles were already recorded embedded in the spiral filament's core of several other coleoid species (e.g., Duvernoy, 1842; Marchand, 1913; Blancquaert, 1923 and 1925; Weill, 1927; Badenhorst, 1974; Takahama et al., 1991). It is difficult to compare the morphology of the stellate particles of *D. plei* with that of other cephalopods already described, since they were not analyzed under the same methodology; the only exception being Takahama et al. (1991), who applied scanning electron microscopy analysis to study these structures. Apparently the number of branches is quite variable, between species and even within the same animal (Duvernoy, 1842; Marchand, 1913; Blancquaert, 1923 and 1925; Weill, 1927; Badenhorst, 1974; Takahama et al., 1991). In *Sepiola rondeleti* they are composed of 4 to 7 branches (Duvernoy, 1842; Marchand, 1913; Blancquaert, 1923 and 1925), reaching sizes of 10 microns (Blancquaert, 1923 and 1925). In *Sepiola atlantica* they are composed of 6 branches, reaching sizes of 10 microns (stellate particles from the spiral filament) and 2 microns (stellate particles from the base of the spermatangia, or “reserve spermatique II”; Weill, 1927). According to Badenhorst (1974)'s illustration of the histological section through the mucilaginous gland II of *L. reynaudii*, the stellate particles have 5 to 8 branches, reaching sizes of 6-8 microns. In *T. pacificus*, the particles are apparently composed of 5 to 7 branches, reaching sizes of possibly 6 microns (Takahama et al., 1991). The lack of detailed data for these and other species prevents further comparisons, but this character should be investigated more closely in future studies.

Cement body. During the spermatophoric reaction, the cement body passes through a complex structural rearrangement, which apparently leads to the injection of its adhesive contents and pointed oral region through the superficial puncture into the female tissue. This rearrangement could be the result of the structure of the middle membrane (see above), but certainly it depends also on the velocity and impulse of the reaction, as the application of magnesium chloride to retard the reaction produces an inevitable artefact. The function of the cement body appears to be the final attachment of the spermatangium by chemical substances, and maybe physically by a pointed structure (the cement body extremity). A thrust action involving a pointed structure was reported for *Todarodes pacificus* (Takahama et al., 1991). In *D. plei*, this probably homologous structure is apparently involved in a similar thrust action, but the cement body extremity was not always implanted into tissue in natural conditions. The chemical attachment is certainly performed by at least adhesive substances; in *D. plei* these substances are able to adhere to the bottom of the Petri dish, as observed in other species (e.g., Drew, 1919a; Weill, 1927). If the cement substances have other chemicals involved in attachment, it is not known. Another type of attachment that could be performed by cement substances, and is likely to take part, is the perfusion of the cement substances through the female tissue, and subsequent hardening of them. It was seen in *D. plei* that this cement material apparently hardens after being extruded from the cement body. So perfusion into the female tissue through the superficial puncture, followed by hardening, could be another type of fixation provided by the cement body. Further studies are required to confirm this hypothesis. Whatever mechanisms of chemical attachment are involved, the eversion of the inner membrane and exposure of its stellate particles must guarantee that the cement contents are injected inside the puncture on the surface of the female body (see above).

The possibility that the cement contents are mixed during the cement body reconfiguration should also be investigated, since, as presented herein, there is a chemical and textural modification of the cement body contents during the spermatophoric reaction. Another feature worthy of a closer investigation is the distinct morphology found within the *in vitro* obtained spermatangia (e.g., Figs. 9 and 11), since these results suggest a two-phase attachment process occurs. Firstly, the cement body would attach itself onto the female tissue through the pointed oral region and cement substances extruded at its base (e.g., Fig. 9). Subsequently, the inner tunic would completely evert and consequently burst the cement body aboral region, spreading the cement material of this region over the base of the spermatangium (Fig. 11), providing another means of attachment. Additional experimentation is necessary to confirm these hypotheses.

Conclusions

On these grounds, it is proposed that the final attachment into the female body by the loliginid spermatangium is achieved by a complex process that involves: 1) physical superficial perforation performed by the everting ejaculatory apparatus, and 2) chemical (and possibly physical) attachment provided by the cement body, and augmented by the inner membrane stellate particles. Future studies should focus on the process of rearrangement of the cement body during the spermatophoric reaction. It is almost impossible to follow this process at its natural speed, and the application of hypertonic solutions is a source of methodological artefacts. Maybe a high-speed camera running at several frames per second should prove useful in investigating this and other stages of the spermatophoric reaction. The enigmatic penetration capabilities of the ejaculatory apparatus and spiral filament should also be investigated more closely.

Ninety years after the publication of Gilman A. Drew's monograph on the loliginid spermatophoric reaction in this very same journal (Drew, 1919a), we are still far from understanding the whole mechanics behind the coleoid spermatophoric reaction, as well as the evolution of this complex structure within the Cephalopoda.

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Supporting Information – Legends of the electronic Appendixes

Appendix S1. *Doryteuthis plei* spermatophore undergoing the spermatophoric reaction without a substrate for attachment. Please refer to text for details.

Appendix S2. Spermatophoric reaction of a spermatophore of *Doryteuthis plei*, which was previously subjected to magnesium chloride treatment. Please refer to text for details.

Appendix S3. Artefact of preparation produced after subjecting the spermatophore of *Doryteuthis plei* to magnesium chloride treatment. In this case, during the spermatophoric reaction, the cement body was extruded intact, i.e., without the deep structural reconfiguration seen under normal conditions. Please refer to text for details. Abbreviations: cba, aboral region of the cement body; cbe, cement body extremity; cbi, intermediate region of the cement body; cbo, oral region of the cement body; it, inner tunic; sm, sperm mass.

Appendix S4. *Doryteuthis plei* spermatophore undergoing the spermatophoric reaction with a substrate (i.e., piece of the male's donor tentacle) for attachment. Please refer to text for details.

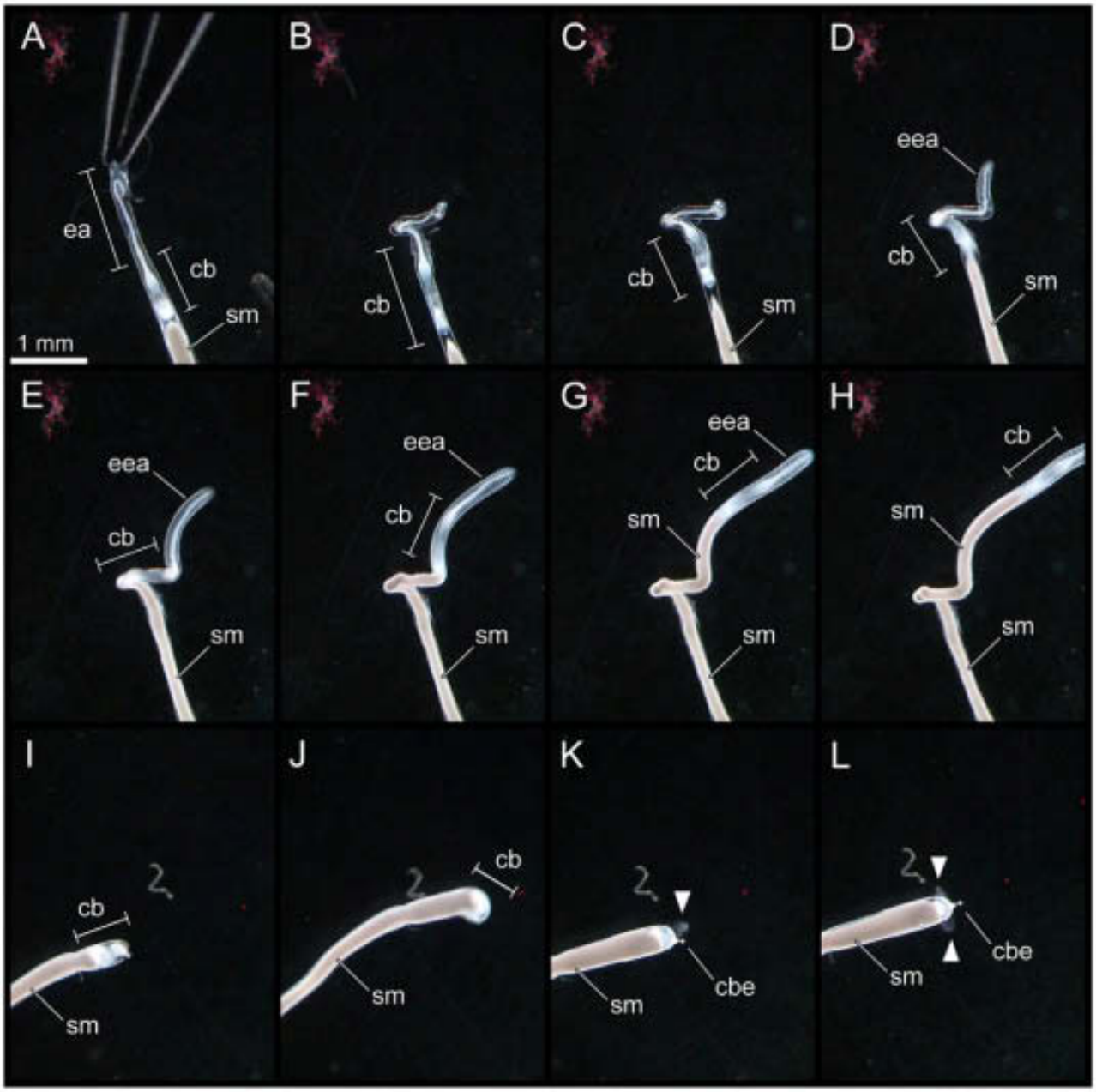

Fig. 1. Series of still images of a spermatophore undergoing the spermatophoric reaction without a substrate for attachment. **A-C:** Stage I. Rupturing of the cap and evagination of the convoluted part of the ejaculatory apparatus. **D-H:** Stage II. Evagination of the whole ejaculatory apparatus tube. **I-L:** Stage III. Extrusion and reconfiguration of the cement body and concomitant exposure of the inner membrane (difficult to discern in this case; see Fig. 2A). Arrowheads point to cement substances being extruded from the cement body. Please refer to text for additional details. See also the supplemental video (Appendix S1 in Supporting Information). Abbreviations: cb, cement body; cbe, cement body extremity; ea, ejaculatory apparatus; eea, everting ejaculatory apparatus; sm, sperm mass.

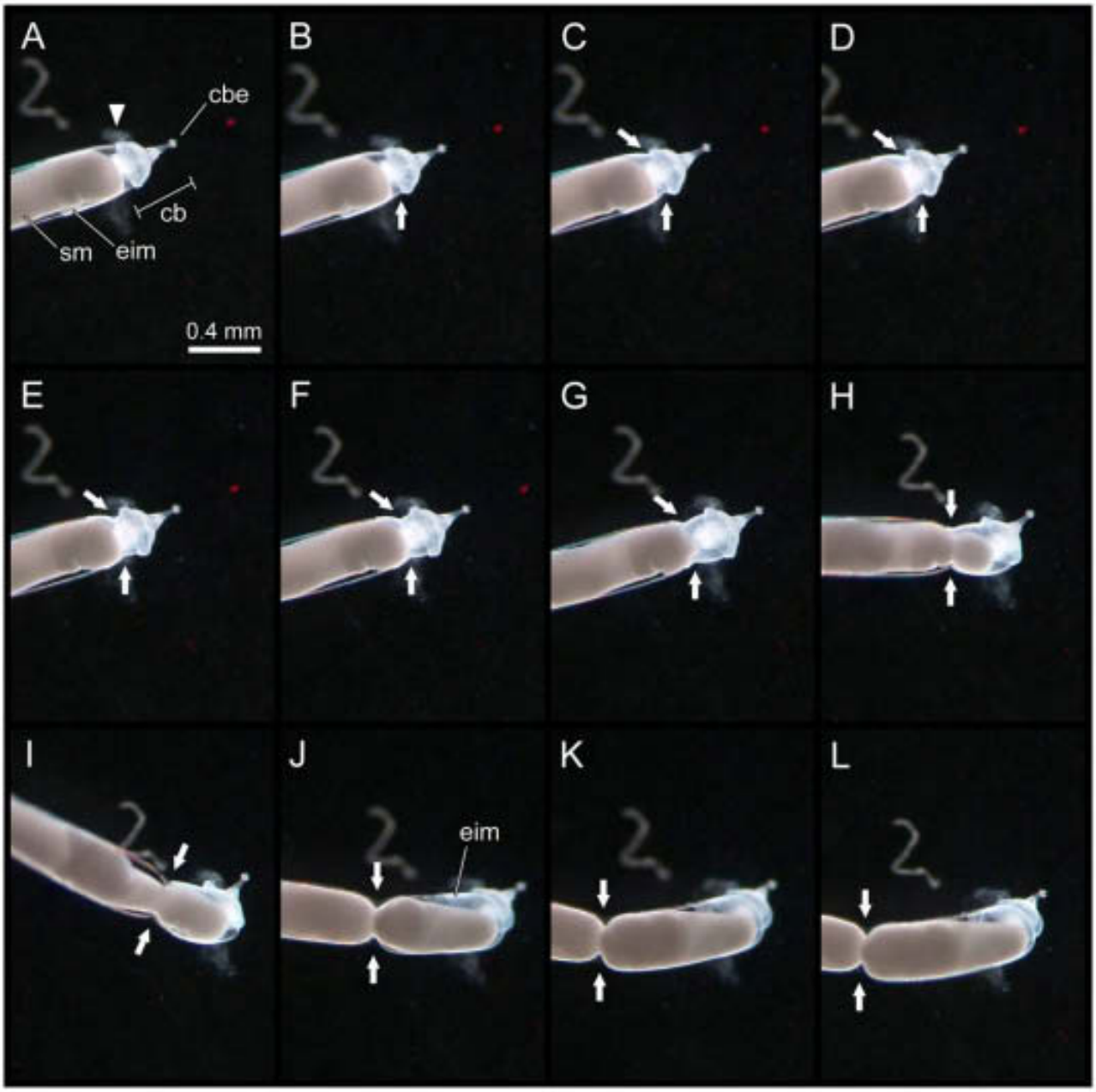

Fig. 2. Series of still images of a spermatophore undergoing the spermatophoric reaction without a substrate for attachment. **A-L:** Stage IV. Detachment of the middle membrane. Arrowheads point to cement substances extruded from the cement body. Arrows point to the constricted region of the middle membrane. Please refer to text for additional details. See also the supplemental video (Appendix S1 in Supporting Information). Abbreviations: cb, cement body; cbe, cement body extremity; eim, everted inner membrane; sm, sperm mass.

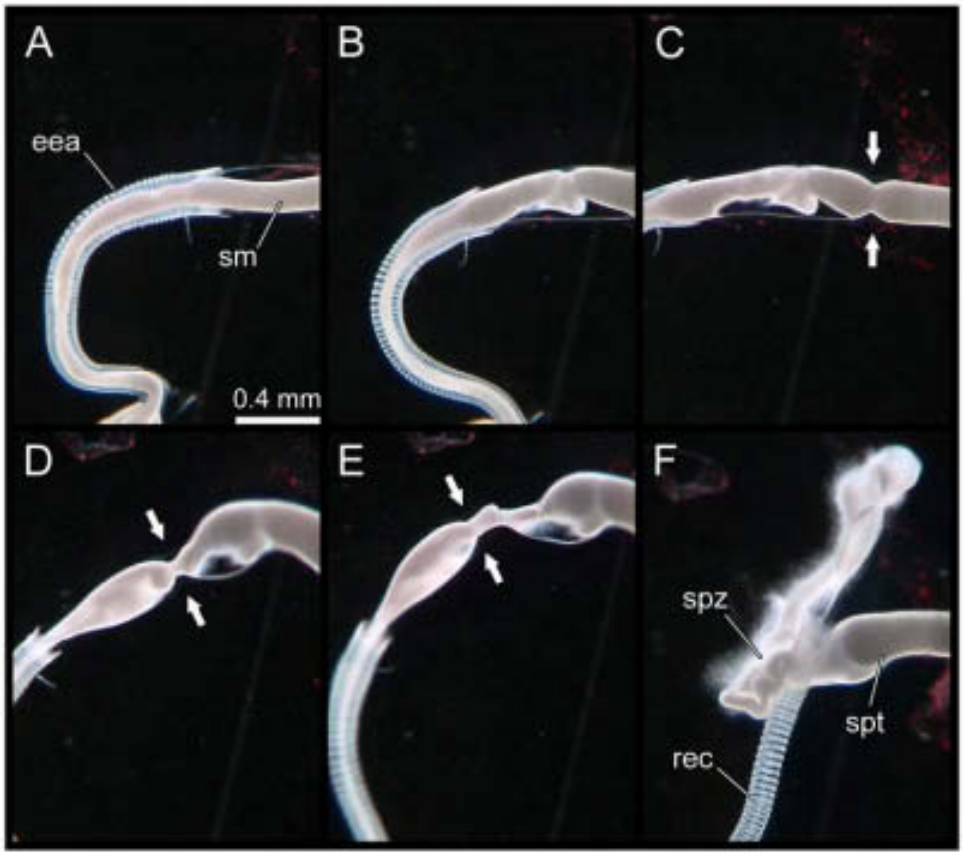

Fig. 3. Series of still images of a spermatophore undergoing the spermatophoric reaction without a substrate for attachment. **A-F:** Stage IV. Complete detachment of the middle membrane and liberation of the spermatozoa. Arrows point to the constricted region of the middle membrane. Please refer to text for additional details. See also the supplemental video (Appendix S1 in Supporting Information). Abbreviations: eea, everted ejaculatory apparatus; rec, remaining empty case; sm, sperm mass; spt, spermatangium; spz, spermatozoa.

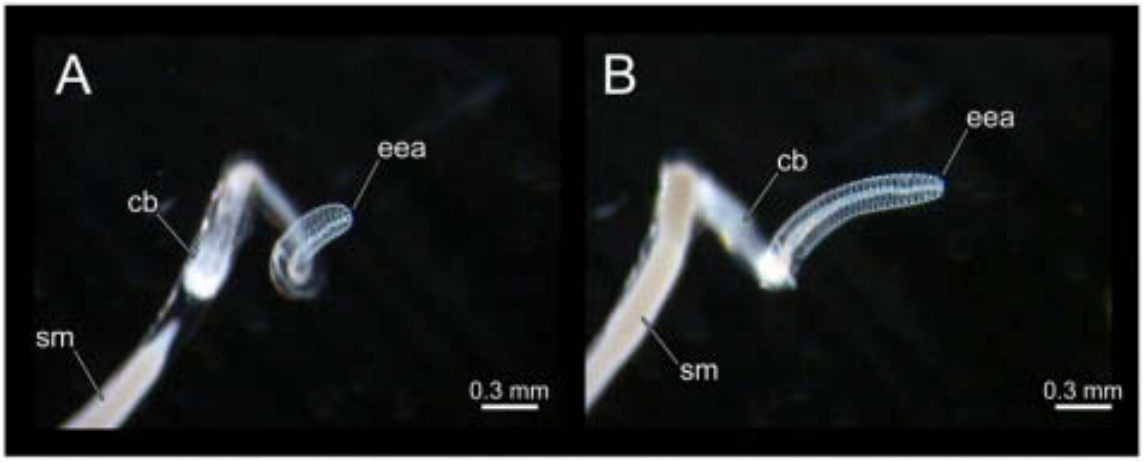

Fig. 4. Still images of an everting spermatophore subjected to magnesium chloride treatment. **A-B:** Stage II. The everting spiral filament is exposed one whorl at a time, first as a small diameter whorl, which enlarges in diameter as the evagination proceeds. During its evagination, the diameter of the spiral filament tube increases to about four to five times its diameter in the intact spermatophore. Please refer to text for additional details. See also the supplemental video (Appendix S2 in Supporting Information). Abbreviations: cb, cement body; eea, everting ejaculatory apparatus; sm, sperm mass.

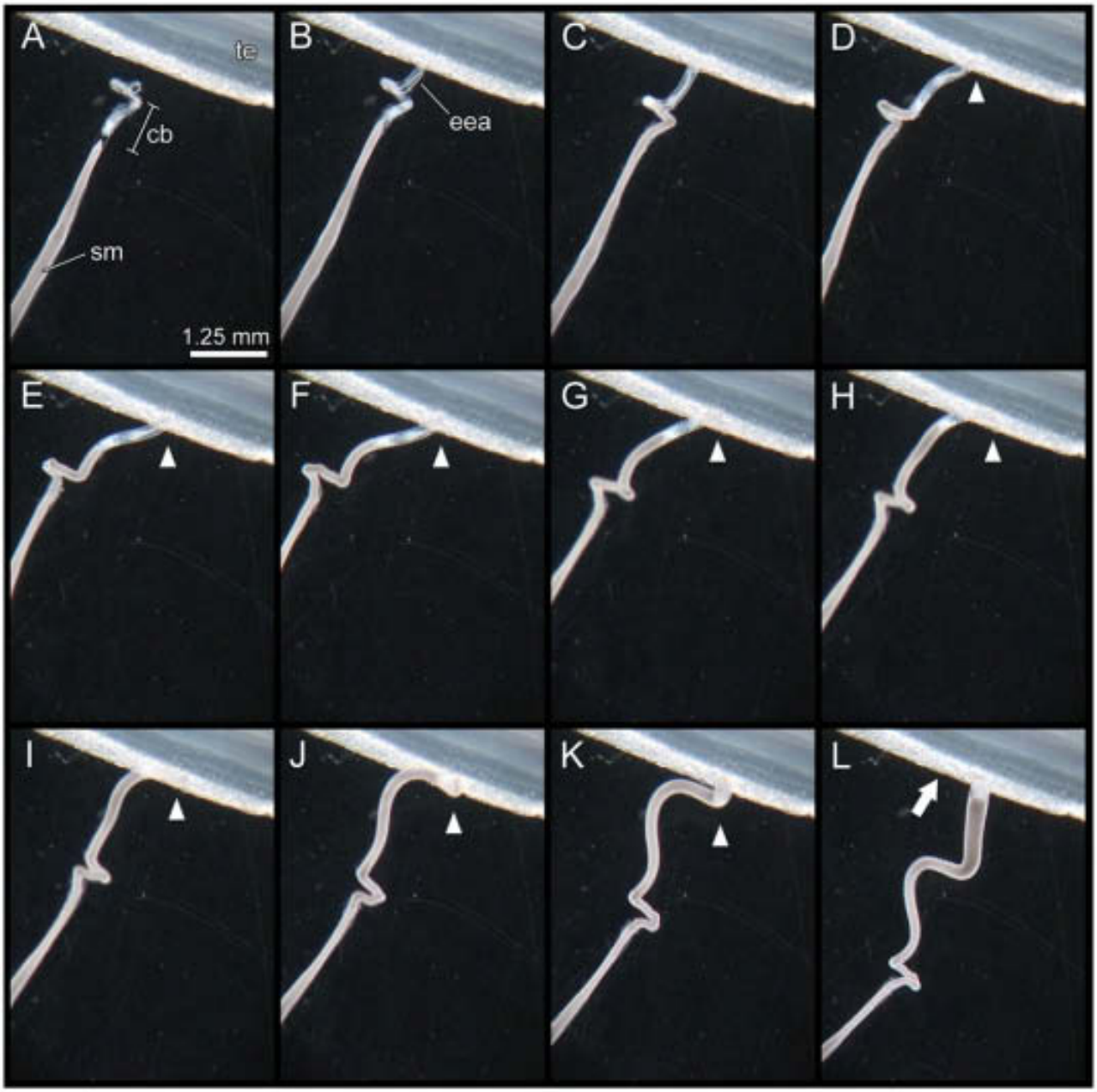

Fig. 5. A-L: Series of still images of a spermatophore undergoing the spermatophoric reaction with a substrate (i.e., piece of the male's donor tentacle) for attachment. The attachment process is entirely autonomous, i.e., the spermatophore alone is capable to attach itself onto the surface of the experimental tissue sample. Moreover, the everting ejaculatory apparatus demonstrates a limited, but effective, perforating ability when in contact with the surface of the targeted tissue (C-J). In D-J, the tip of the everting ejaculatory apparatus penetrates and advances parallel through the surface of tissue (apparently below the epithelium) until the level of the cement body is reached. Compare the point of penetration of the ejaculatory apparatus into the tissue sample on C to the advancing tip of the everting spermatophore indicated approximately by the arrowheads on D-K. When the level of the cement body is reached, the part that had implanted superficially is pushed backwards and extruded from the targeted tissue (J-K). The tip of the everting spermatophore (containing the cement body) remains attached onto the surface of the sample (L). The arrow in L indicates the approximate point of initial penetration of the everting spermatophore (compare to C). Please refer to text for additional details. See also the supplemental video (Appendix S4 in Supporting Information). Abbreviations: cb, cement body; eea, everting ejaculatory apparatus; sm, sperm mass.

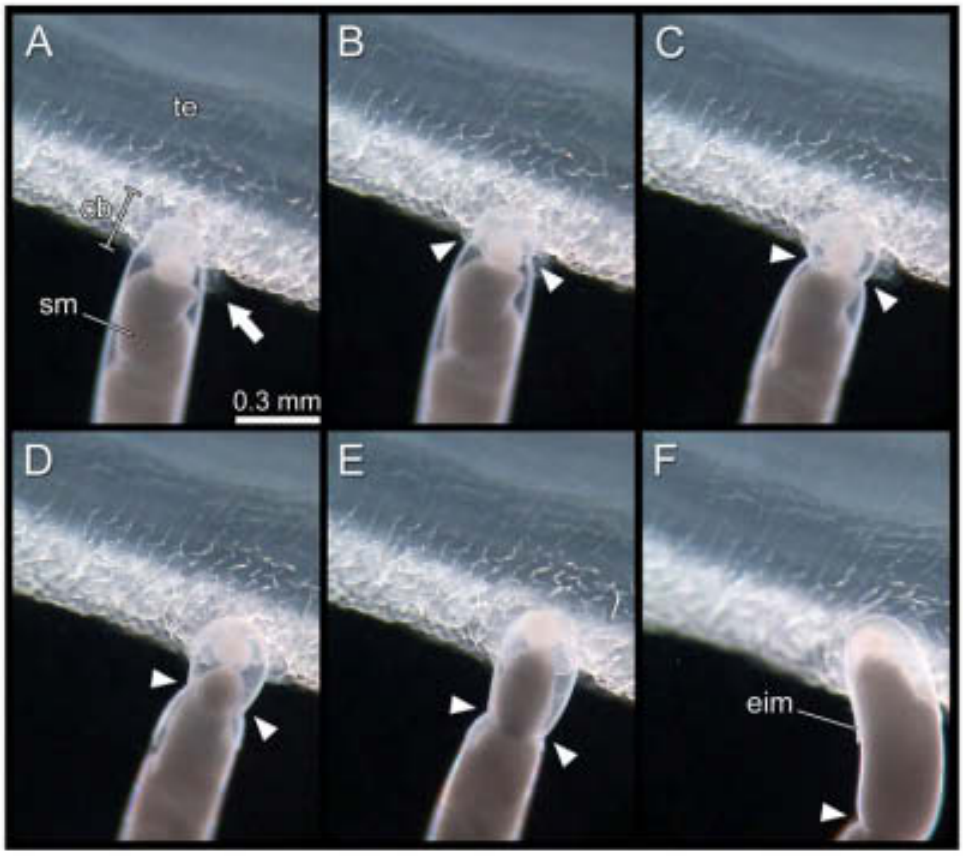

Fig. 6. A-F: Series of still images of a spermatophore undergoing the fourth stage of the spermatophoric reaction already attached onto a substrate (i.e., piece of the male's donor tentacle). This series is the continuation of Fig. 5. During the fourth stage the middle membrane is detached from the cement body. Arrow points to some cement substances extruded from the cement body. Arrowheads point to the constricted region of the middle membrane. Please refer to text for additional details. See also the supplemental video (Appendix S4 in Supporting Information). Abbreviations: cb, cement body; eim, everted inner membrane; sm, sperm mass.

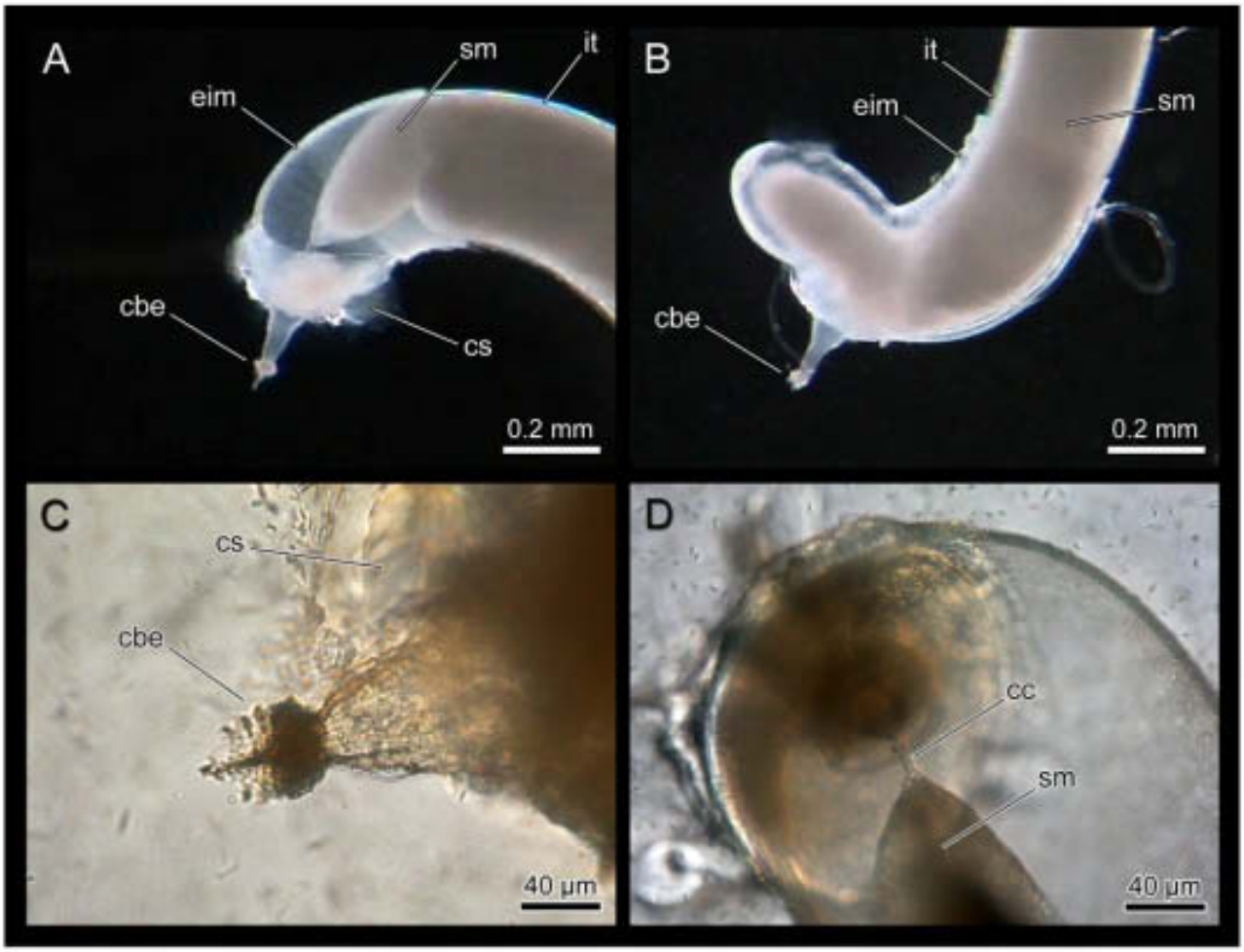

Fig. 7. Spermatangia obtained *in vitro*. Light microscopy. **A-B:** Detail of the base of the two types of spermatangia obtained *in vitro*. **C:** Detail of the cement body extremity of a spermatangium. **D:** Same material, but viewed from the side opposed to the cement body extremity, showing the connecting cylinder (cc). Abbreviations: cbe, cement body extremity; cc, connecting cylinder; cs, cement substances; eim, everted inner membrane; sm, sperm mass.

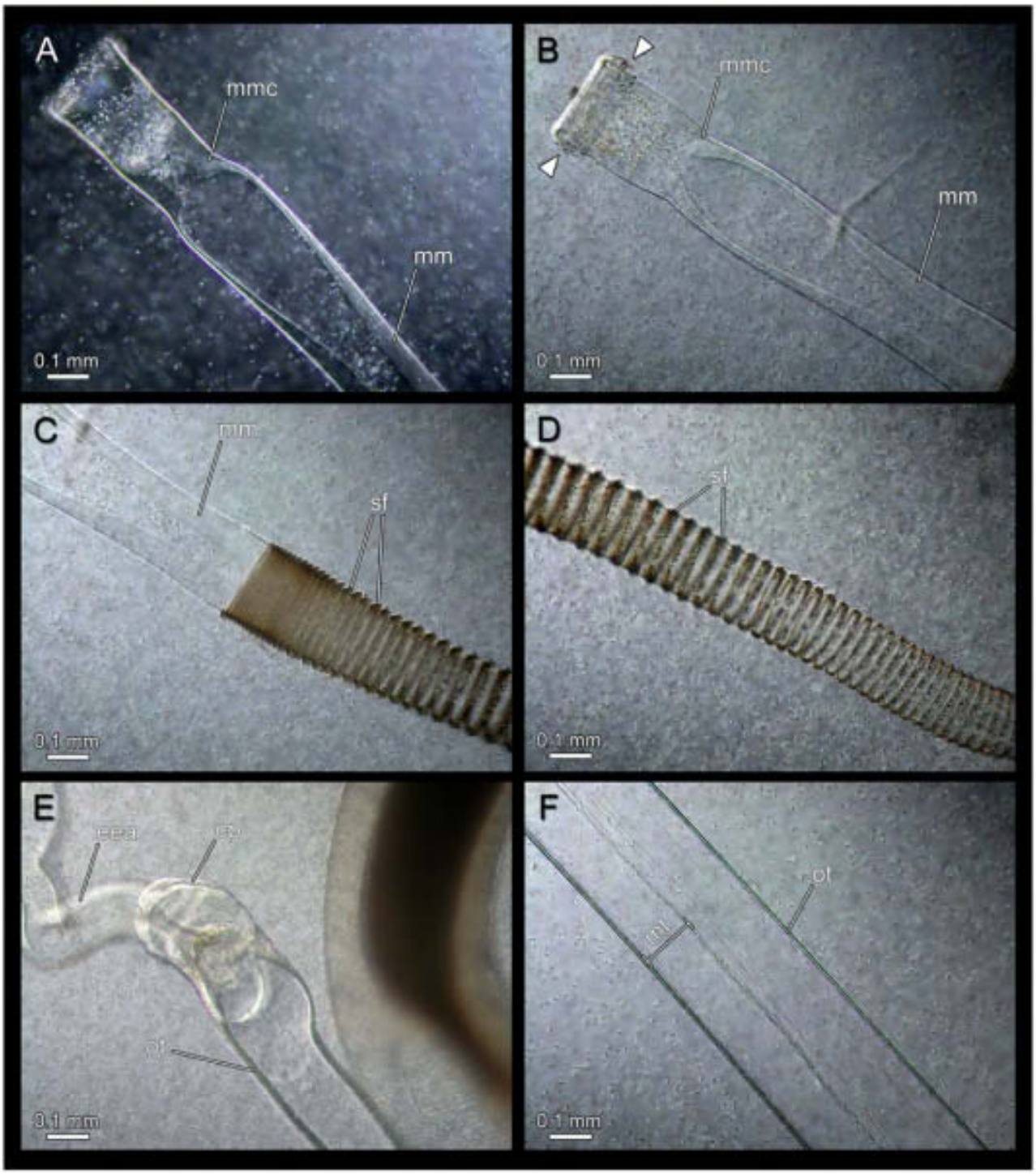

Fig. 8. Fresh remaining empty cases investigated under differential interference contrast microscopy (DIC). **A:** Terminal portion of the empty case. **B:** Terminal portion of the empty case. Arrowheads point to the folded portion of the middle membrane (mm). **C-D:** Same material as B, but showing the everted spiral filament (sf). **E:** Same material as B, but showing the cap region (cp) and the proximal part of the everted ejaculatory apparatus (eea). **F:** Same material as B, but showing the proximal region of the empty case. Note that the middle tunic (mt) occupies most of the space inside the outer tunic (ot). Abbreviations: cp, cap; eea, everted ejaculatory apparatus; mm, middle membrane; mmc, middle membrane constriction; mt, middle tunic; ot, outer tunic; sf, spiral filament.

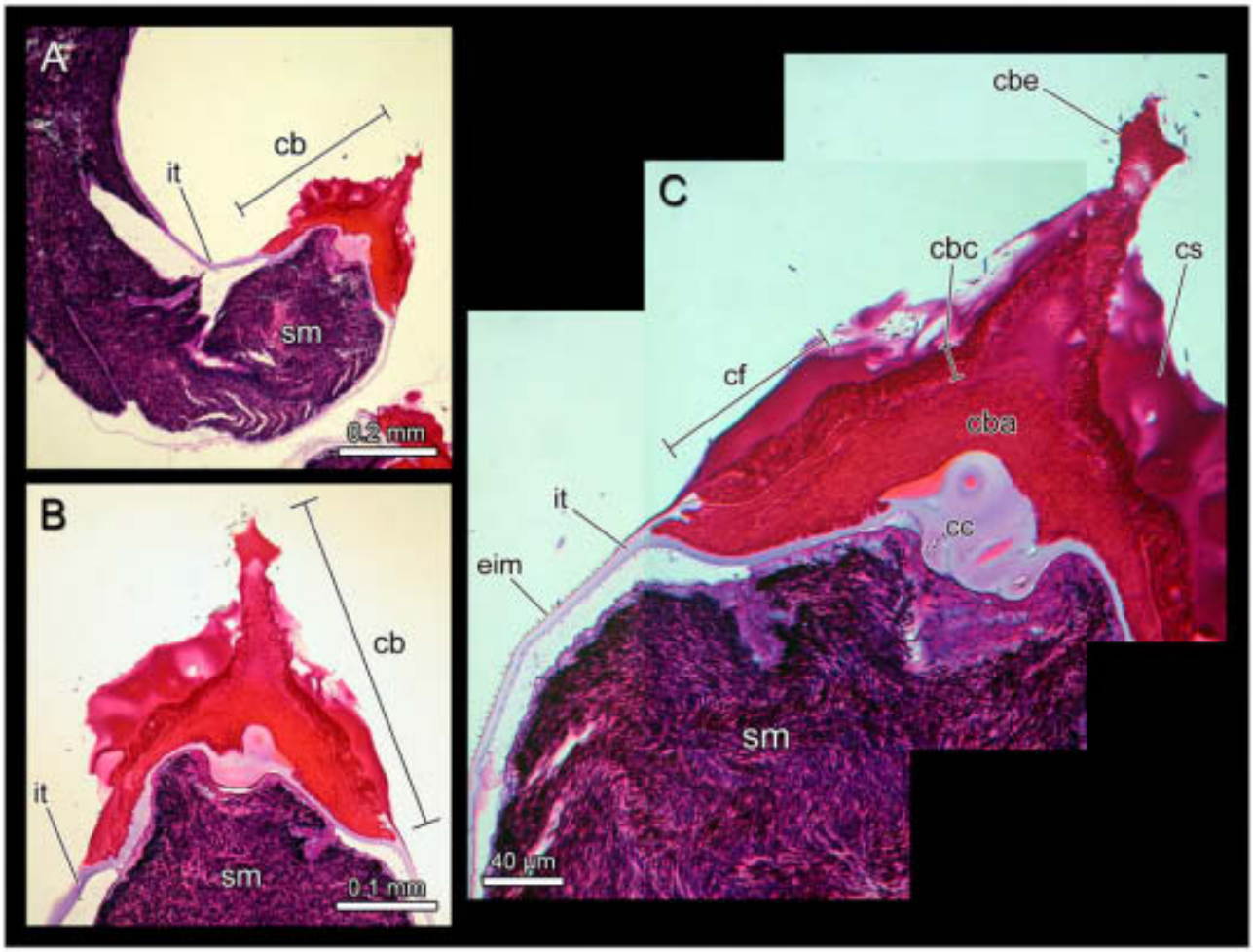

Fig. 9. Spermatangia obtained *in vitro* and subjected to serial sectioning and H&E staining. **A-B:** General views. **C:** Montage of three detailed views of the base of the spermatangium. Abbreviations: cb, cement body; cba, cement body aboral region; cbc, cement body casing; cbe, cement body extremity; cc, connecting cylinder; cf, intricately folded region of the cement body outer casing; cs, cement substances; eim, everted inner membrane; it, inner tunic; sm, sperm mass.

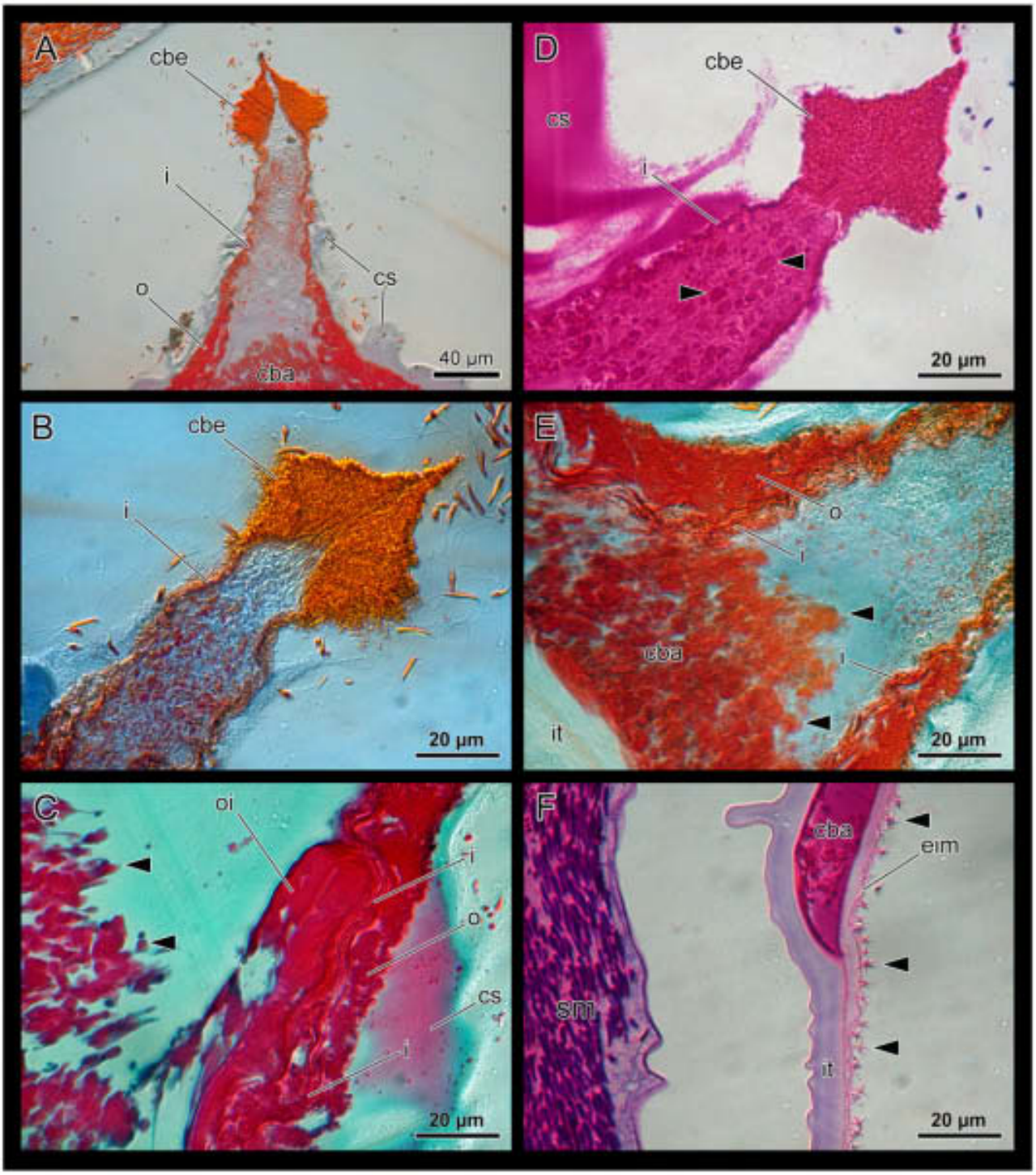

Fig. 10. Spermatangia obtained *in vitro* and subjected to serial sectioning and staining; detail views. **A-B:** Pointed region of the cement body. Mallory's Triple Stain. **C:** Detail view of the cap-like base of the reconfigured cement body from the spermatangium. This process is apparently the result of an intricate folding of cement body outer casing. Externally, the granular outer layer (o) and the fibrous inner layer (i) fold at least twice onto each other; internally, the outer layer from the intermediate region of the cement body (oi) describes also a prominent fold. Arrowheads point to the granular material from the cement body aboral region. Gomori's Trichrome. **D:** Pointed region of the cement body. Arrowheads point to the granular material from the cement body oral region. H&E. **E:** Detail view of the aboral region of the cement body. Arrowheads point to the granular material from the cement body aboral region. Mallory's Triple Stain. **F:** Detail view of the inner membrane spread over the base of the spermatangium. Arrowheads point to minute stellate particles covering the surface of the inner membrane. H&E. Abbreviations: cba, cement body aboral region; cbe, cement body extremity; cs, cement substances; eim, everted inner membrane; i, cement body inner layer; it, inner tunic; o, cement body outer layer; oi, cement body outer layer at the intermediate region of the cement body; sm, sperm mass.

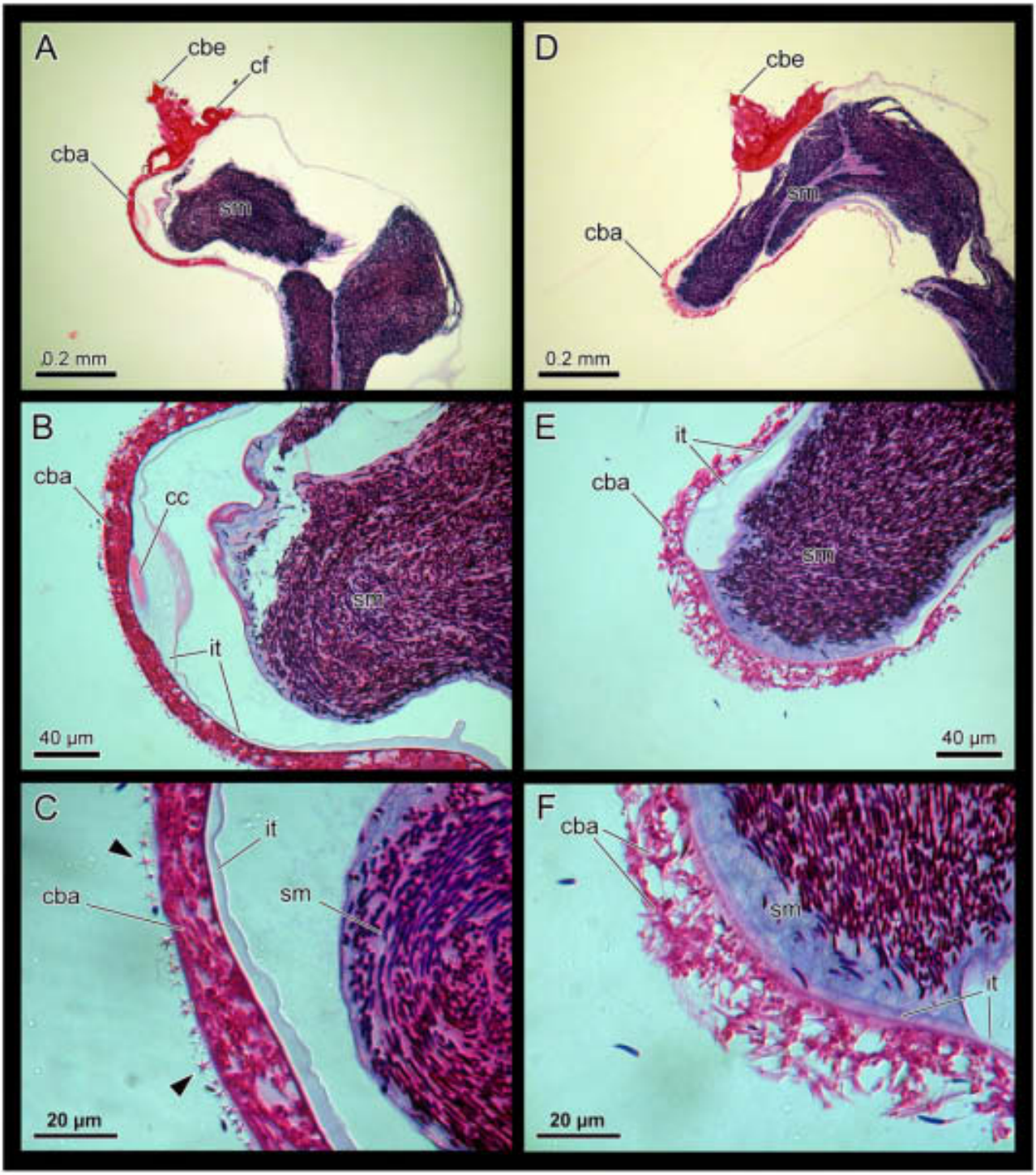

Fig. 11. Distinct spermatangia configurations obtained *in vitro*, possibly representing successive steps in the formation of the final spermatangium. **A:** Spermatangium "step two" (as defined in the text). **B-C:** Detail views of A, showing the higher degree of eversion of the inner tunic (when compared to the spermatangium on Fig. 9), and the beginning of the rupturing of the cement body. The everted inner membrane containing the stellate particles (arrowheads in C) is still intact. **D:** Spermatangium "step three" (as defined in the text). **E-F:** Detail views of D, showing the complete eversion of the inner tunic, and the complete rupturing of the cement body. The everted inner membrane containing the stellate particles has been ruptured. Please refer to text for additional details. Abbreviations: cba, cement body aboral region contents; cbe, cement body extremity; cc, connecting cylinder; cf, intricately folded region of the cement body outer casing; it, inner tunic; sm, sperm mass.

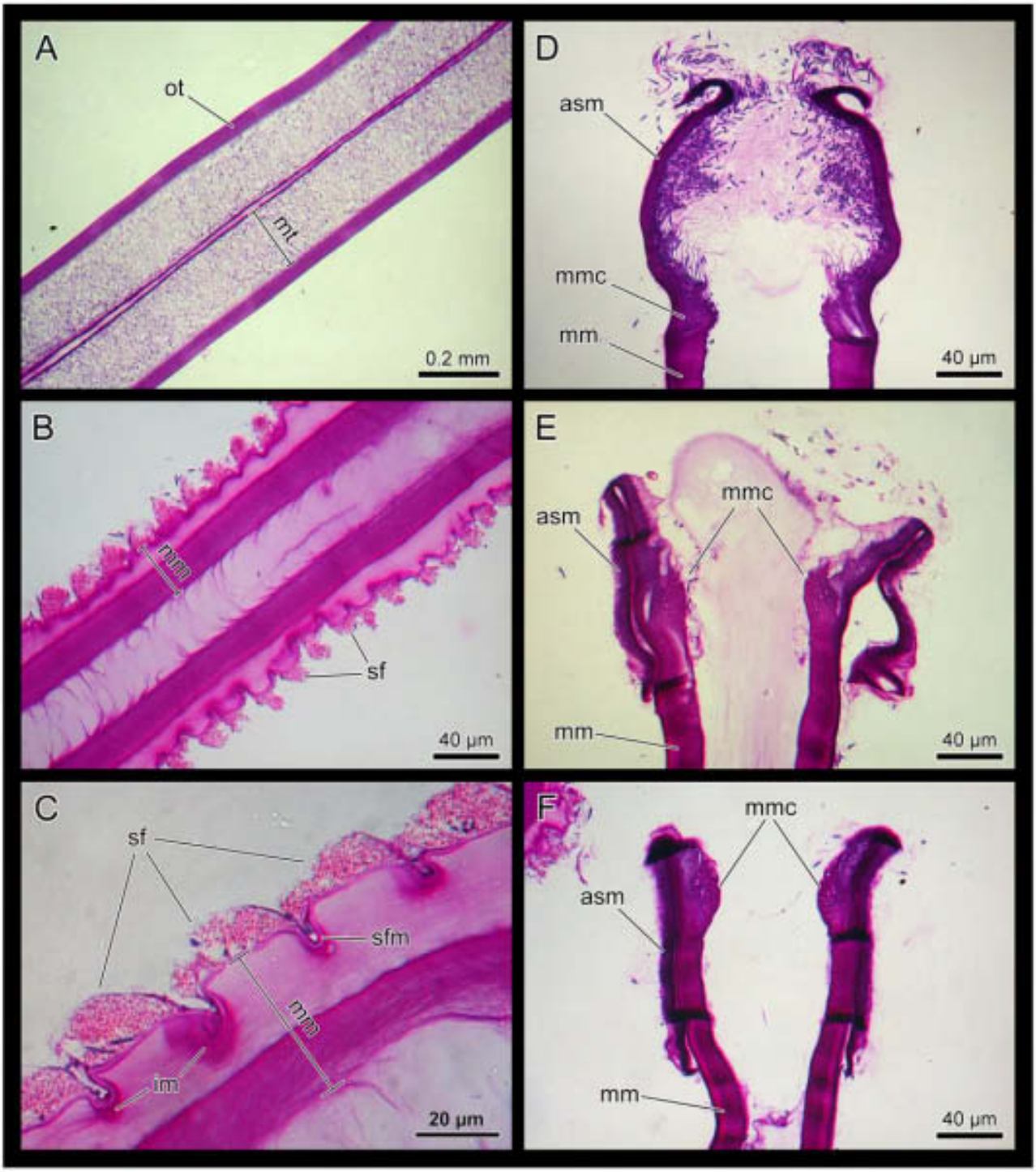

Fig. 12. Remaining empty cases obtained *in vitro*, and subjected to serial sectioning and PAS-H&E staining. **A:** Proximal region of the empty case. Note that the middle tunic (mt) occupies most of the space inside the outer tunic. **B:** Everted spiral filament (sf) adhered to the surface of evaginated ejaculatory apparatus. **C:** Detail of the everted spiral filament (sf) adhered to the surface of evaginated ejaculatory apparatus. **D-F:** Empty cases terminal portion, showing the folded part of the region corresponding to the "aboral segment of the middle membrane" (asm) of intact spermatophores. Abbreviations: asm, aboral segment of the middle membrane; im, inner membrane; mm, middle membrane; mmc, middle membrane constriction; mt, middle tunic; ot, outer tunic; sf, spiral filament; sfm, spiral filament membrane.

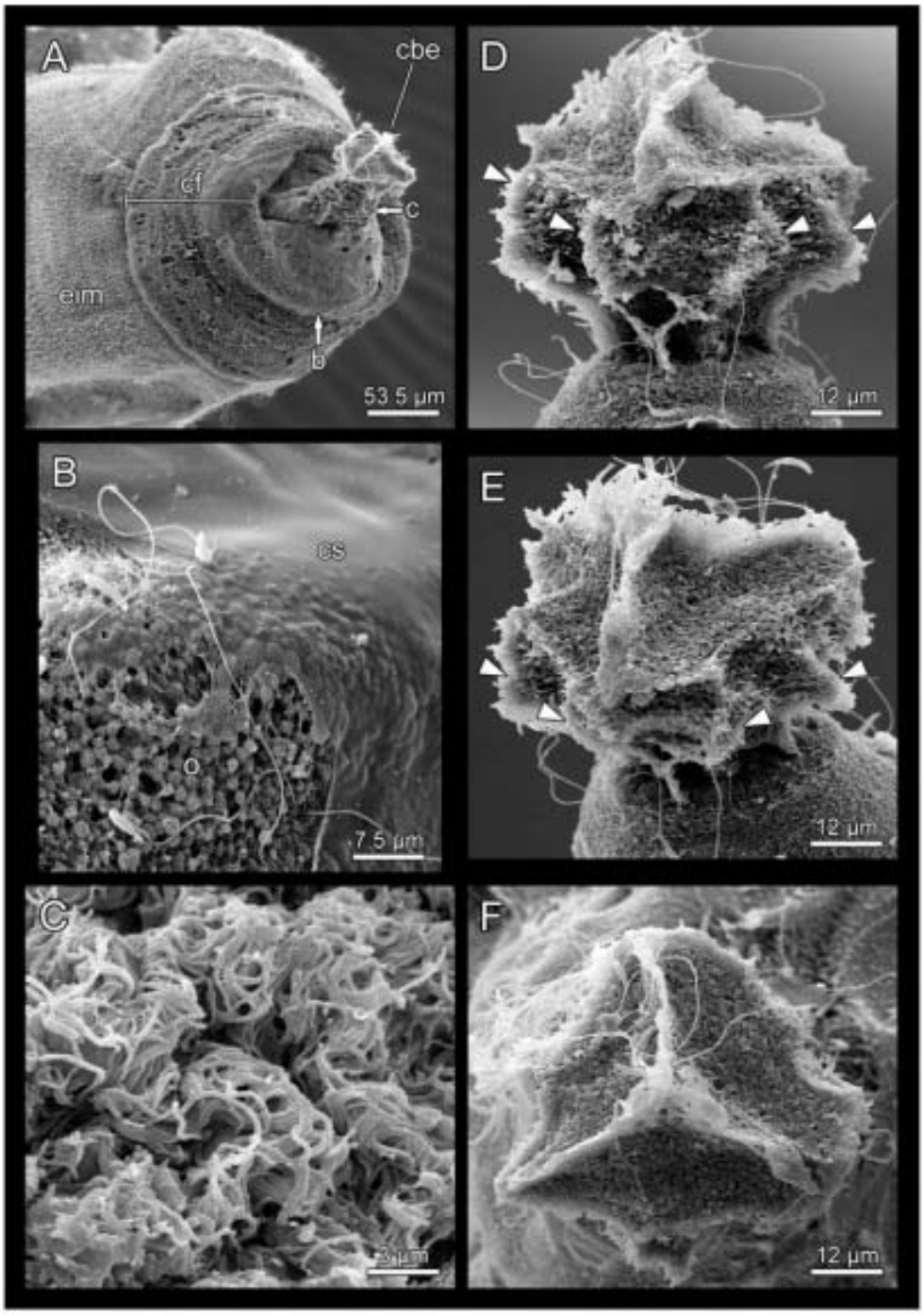

Fig. 13. Scanning electron micrographs of the spermatangium cement body. **A:** General view of the base of a spermatangium. Letters "b" and "c" indicate the approximate regions of the images depicted in B and C, respectively. **B:** Detail view of the region indicated by "b" on figure A. **C:** Detail view of the region indicated by "c" on figure A. **D:** Lateral view of the cement body extremity. Arrowheads point to four (from six) peripheral longitudinal crests. **E:** Lateral frontal view of the cement body extremity. Arrowheads point to four (from six) peripheral longitudinal crests. **F:** Frontal view the cement body extremity; the three apical crests are evident. Abbreviations: cbe, cement body extremity; cf, intricately folded region of the cement body outer casing; cs, cement substances; eim, everted inner membrane; o, cement body outer layer.

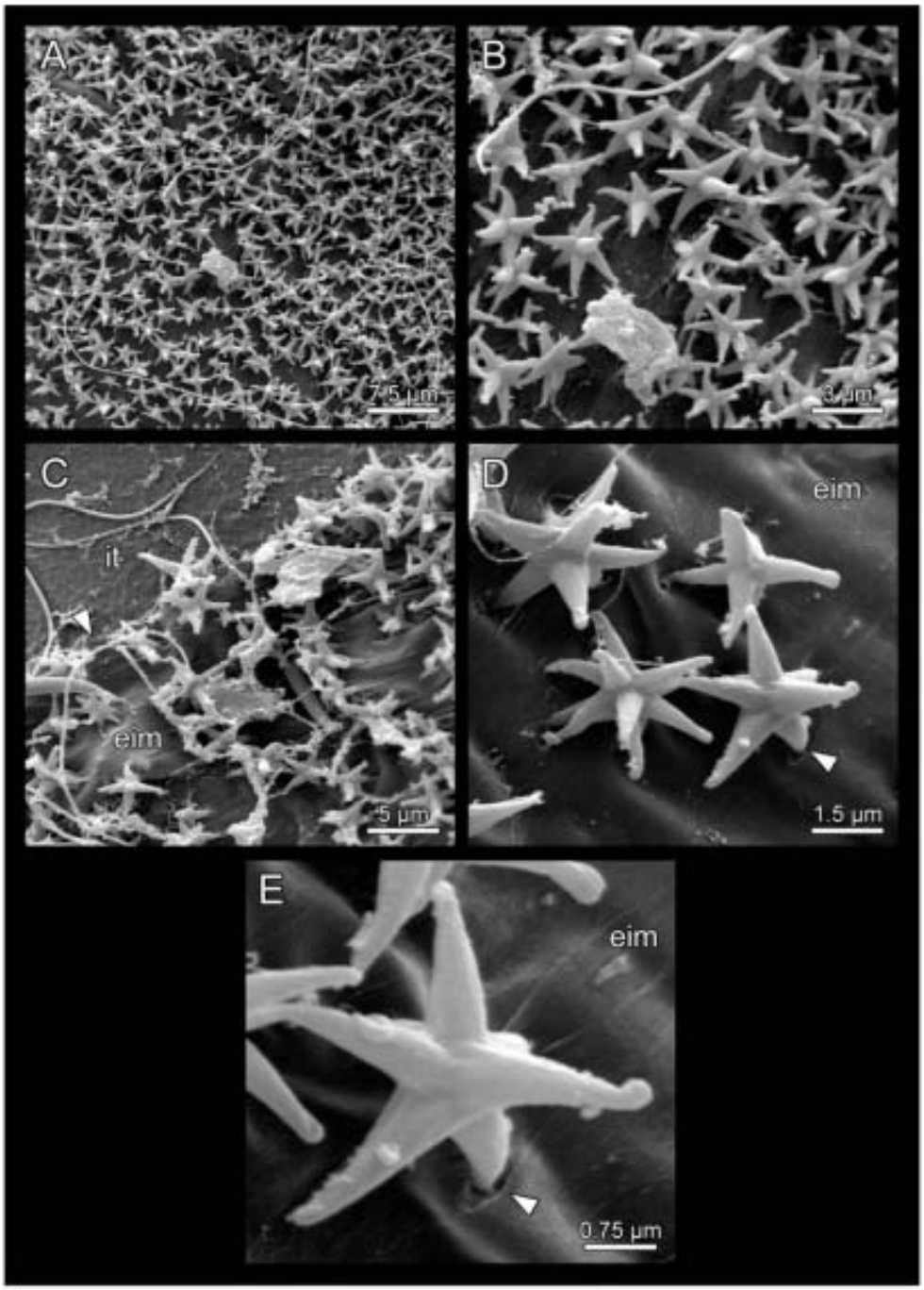

Fig. 14. Scanning electron micrographs of the spermatangium inner membrane and stellate particles. **A:** View of the inner membrane spread over the base of the spermatangium. **B:** Detail of the previous image. **C:** View of the terminal region of the inner membrane on the base of the spermatangium. Where the inner membrane is interrupted, the stellate particles are absent. **D:** Detail view of the stellate particles connected to the inner membrane. The arrowhead points to the branch of the stellate particle that is embedded and firmly attached to the inner membrane. **E:** Detail of the previous image. Abbreviations: eim, everted inner membrane; it, inner tunic.

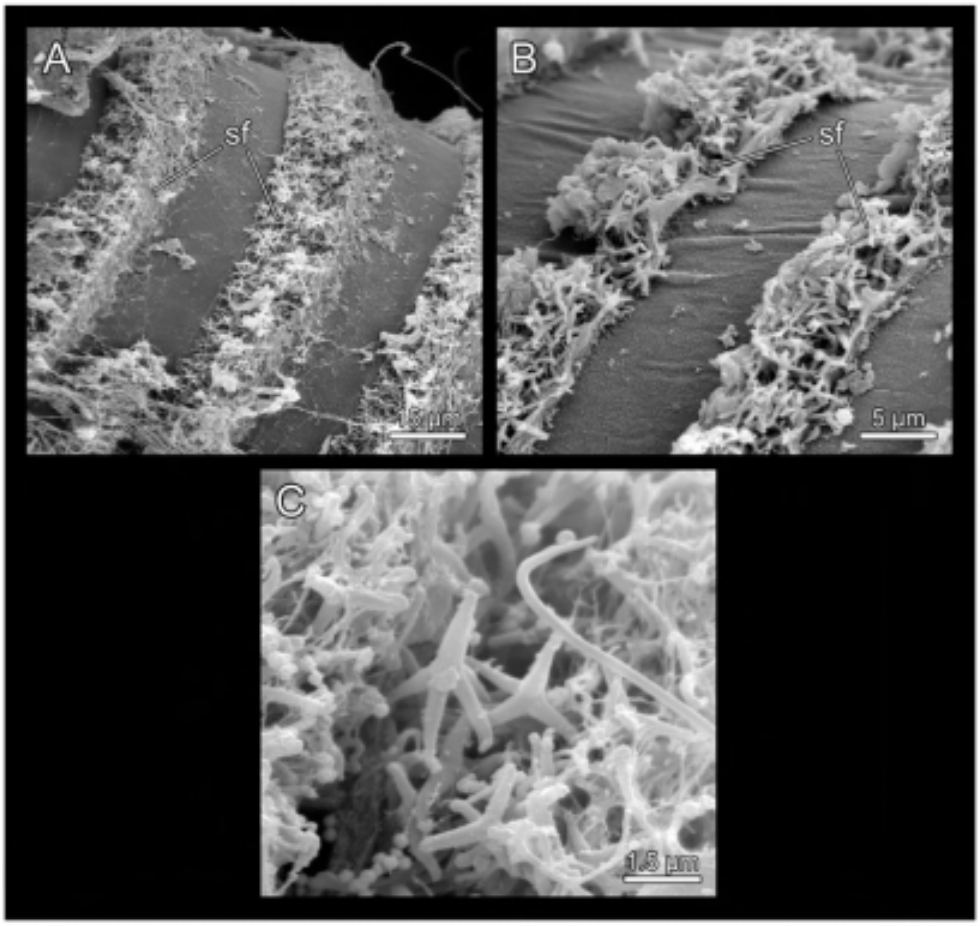

Fig. 15. Scanning electron micrographs of the everted spiral filament and its stellate particles. **A-B:** General views of the everted spiral filament (sf) adhered to the evaginated ejaculatory apparatus tube. **C:** Detail view of the spiral filament's stellate particles, tightly packed and interspersed with some unidentified material. Note that the spiral filament's stellate particles are much finer than those of the inner membrane (compare with Figs. 14A-E). Abbreviation: sf, spiral filament.

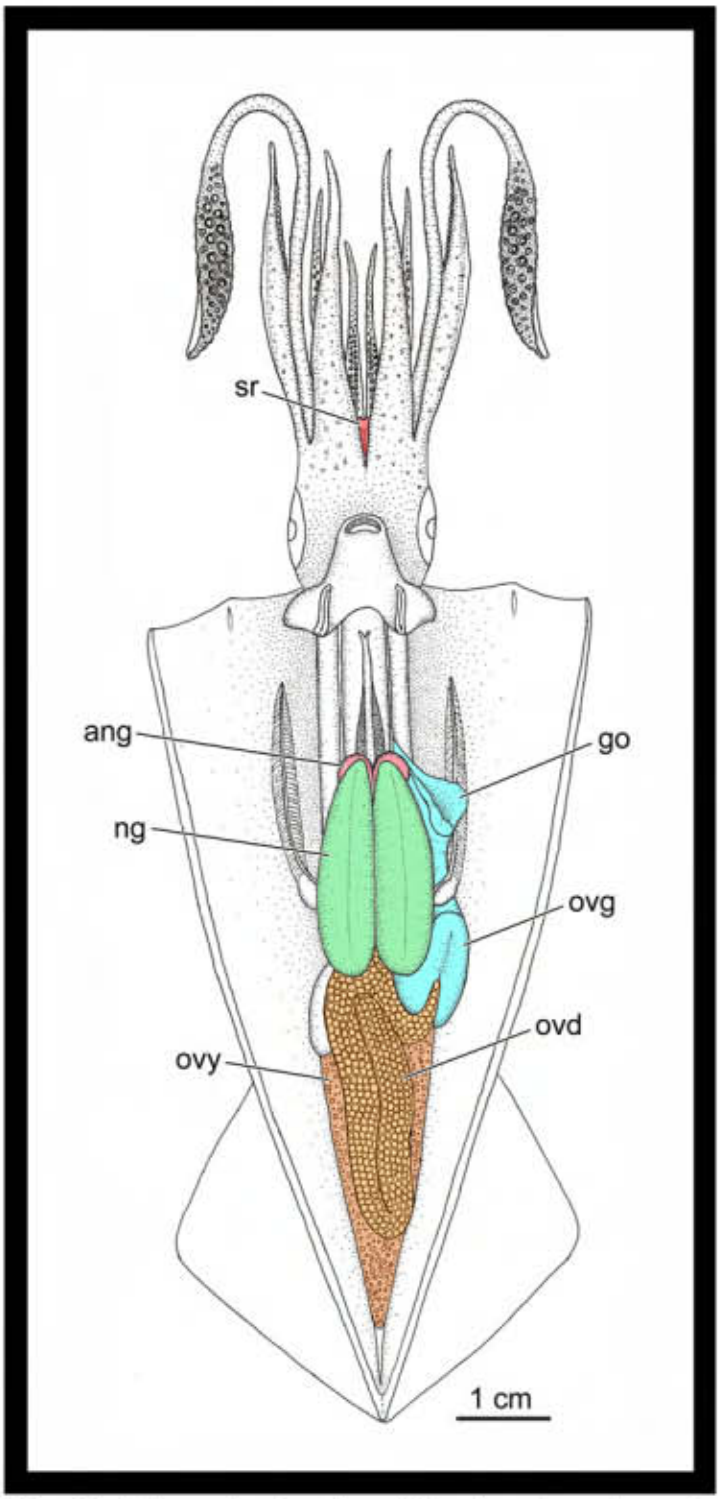

Fig. 16. Schematic drawing of the female anatomy in *Doryteuthis plei*, highlighting the reproductive system and the spermatophore deposition sites. In D. plei, the spermatophore may be placed on the buccal membrane near the seminal receptacle (sr), or within the mantle cavity on the lips bordering the genital opening (go). Abbreviations: ang, accessory nidamental glands; go, genital opening; ng, nidamental glands; ovd, oviduct; ovg, oviducal gland; ovy, ovary; sr, seminal receptacle.

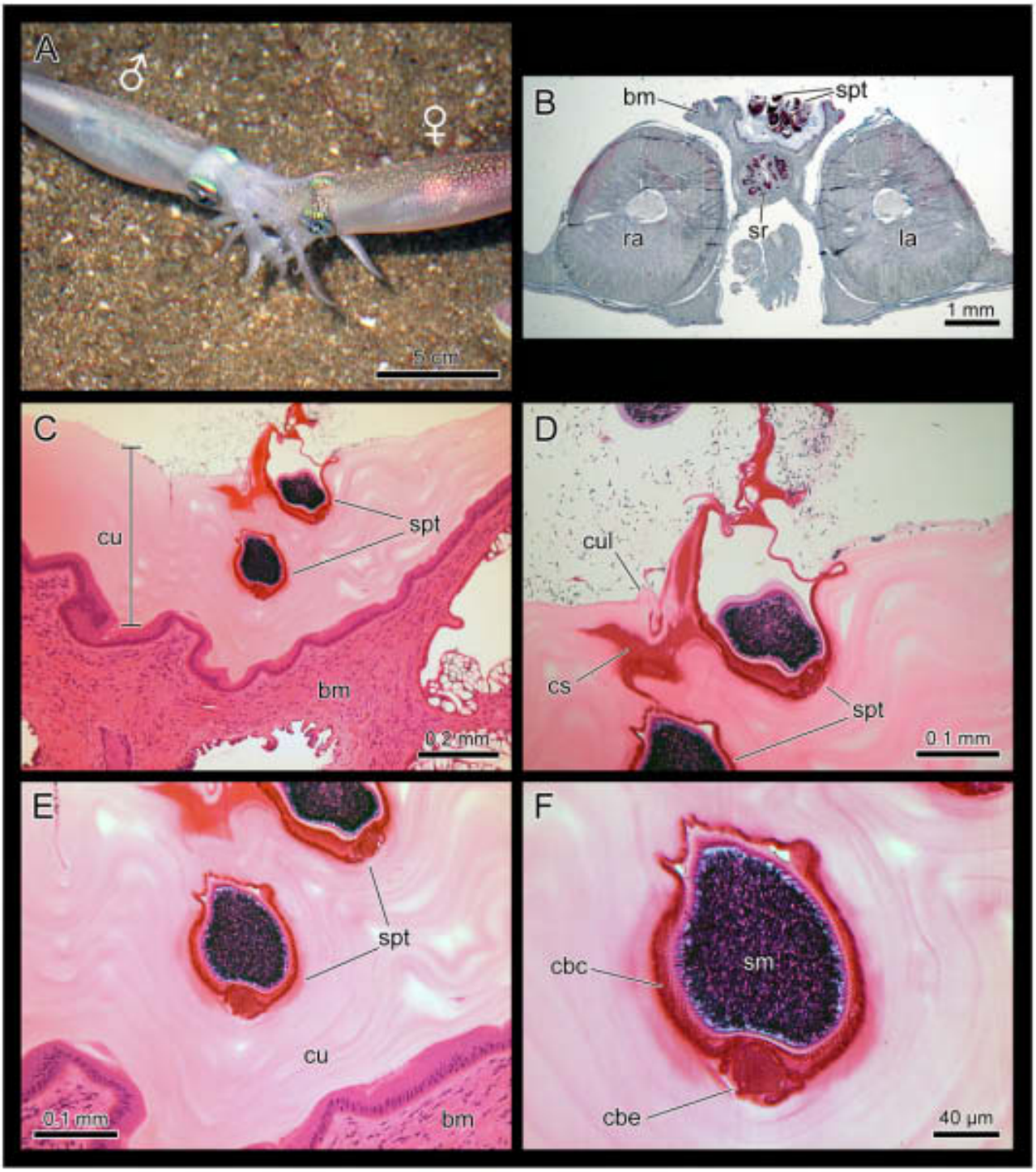

Fig. 17. Spermatangia naturally implanted on the female buccal membrane near the seminal receptacle. **A:** Mating occurring in the "head-to-head" position, during which males apparently place the spermatophores on the buccal membrane. **B:** Cross-section through the buccal region of a female, showing the seminal receptacle (sr) located on the ventral buccal membrane (bm), between both ventral arms. Some spermatangia (spt) are implanted on the cuticle of the seminal receptacle. **C:** Detailed view of a cross-section through the buccal membrane near the seminal receptacle, showing two implanted spermatangia. **D-F:** Detail views of the previous image. Please refer to text for additional details. Abbreviations: bm, buccal membrane; cbc, cement body outer casing; cbe, cement body extremity; cs, cement substances; cu, cuticle of the seminal receptacle; cul, cuticle individual layer; la, left ventral arm; ra, right ventral arm; sm, sperm mass; spt, spermatangium; sr, seminal receptacle.

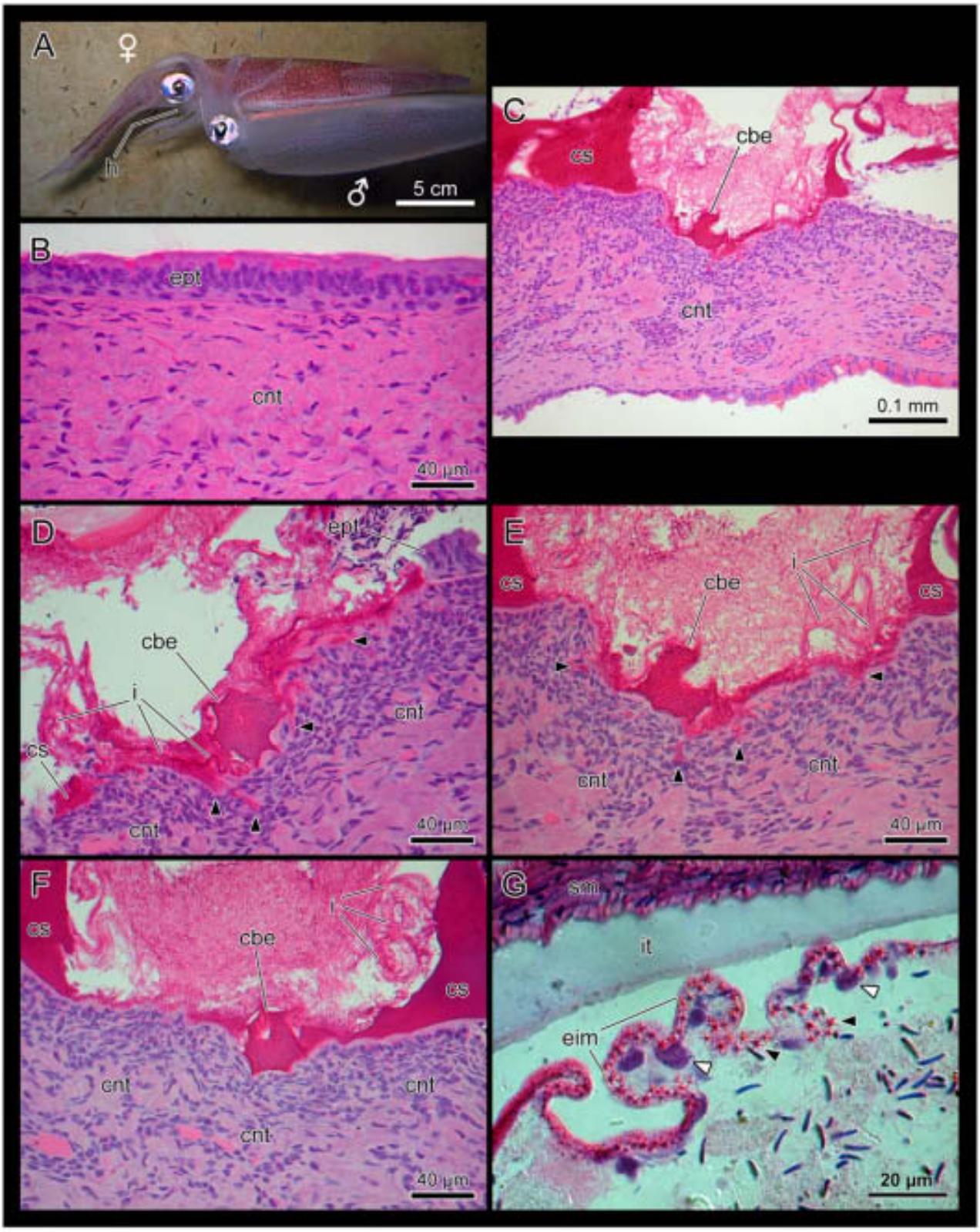

Fig. 18. Spermatangia naturally implanted on the female genital opening lips. **A:** Mating occurring in the "male-parallel" position, during which males place the spermatophores within the mantle cavity with the aid of the hectocotylus (h). **B:** Cross-section through the intact genital opening lips, showing the normal epithelium (ept) and underlying connective tissue (cnt). **C-F:** Detailed views of cross-sections through the genital opening lips, showing implanted spermatangia. Arrowheads point to cement substances interspersed with some connective tissue cells. **G:** Detailed view of a cross-section through a spermatangium implanted on the genital opening lips, showing the inner membrane stretched over the base of the spermatangium. The inner membrane contains numerous minute stellate particles (black arrowheads). White arrowheads indicate some external material (apparently connective tissue fibroblasts) attached to the stellate particles. Please refer to text for additional details. Abbreviations: cbe, cement body extremity; cnt, connective tissue; cs, cement substances; ept, epithelium; h, hectocotylus; i, cement body inner layer.

Capítulo 4

Proposta de um modelo teórico para explicar o fenômeno de implante de espermatóforos observado nos coleóides

CAPÍTULO 4

Proposta de um modelo teórico para explicar o fenômeno de implante de espermatóforos observado nos coleóides

A descoberta do potencial de penetração dos espermatóforos de *Doryteuthis plei* em condições artificiais (Capítulo 2) e a confirmação de que em condições naturais os mesmos são capazes de perfurar superficialmente o sítio de deposição na fêmea (Capítulo 3) estimularam a busca por evidências para desvendar o mecanismo responsável por este processo.

Como exposto no Capítulo 2, evidências apontavam para um mecanismo comum de perfuração, intrínseco ao espermatóforo dos Decapodiformes. A revisão e reavaliação da literatura especializada à luz dessas descobertas permitiram sugerir, de fato, que dois tipos básicos de fixação envolvendo perfuração de tecido ocorrem nos espermatângios dos Decapodiformes: implante profundo (ou intradérmico) e implante superficial (ou "raso"). No implante profundo, comum em alguns grupos de lulas oceânicas e de águas profundas, os espermatângios implantam-se inteiramente no corpo da fêmea (e.g., Architeuthidae, Cranchiidae, Octopoteuthidae, Sepiolidae). No implante superficial, somente a base dos espermatângios é implantada no tecido-alvo (e.g., Loliginidae, Sepiidae, Ommastrephidae). É importante ressaltar que, até a elaboração da presente Tese, um conceito amplamente difundido na literatura acerca do modo de fixação dos espermatóforos em muitos Decapodiformes era o de "adesão" da base dos espermatângios no corpo da fêmea, i.e., não haveria perfuração de tecido. Entretanto, além dos resultados obtidos com base em *D. plei* (Capítulo 3), diversos trabalhos anteriores oferecem elementos corroborando o conceito de "perfuração superficial" aqui defendido (este tópico é revisado com mais detalhes no Capítulo 5).

Outro resultado importante apresentado nos Capítulos 2 e 3 está relacionado ao papel do aparato ejaculatório, o qual, durante sua eversão, tem capacidade de perfuração, embora a mesma seja limitada quando comparada àquela das espécies com implante profundo. Soma-se a essa descoberta o fato de Hoving & Laptikhovsky (2007) e Hoving *et al.* (2009) apresentarem evidências de que o aparato ejaculatório está envolvido no processo de perfuração em espécies que apresentam implante profundo (*Onykia ingens* e *Rossia moelleri*, respectivamente)[1]. Com base no exposto, assume-se, na presente Tese, que o aparato ejaculatório é a estrutura envolvida na perfuração do tecido da fêmea nos dois

[1] Entretanto, esses autores, aparentemente tão concentrados no conceito tradicional de que o corpo cimentante seria a estrutura responsável pelo implante profundo, não discutiram essa importante descoberta.

tipos de implante (i.e., superficial e profundo), compondo a primeira premissa do modelo teórico apresentado neste Capítulo. A partir dessa assunção, buscaram-se elementos do aparato ejaculatório que pudessem auxiliar no entendimento de como ocorreria o processo de perfuração nos Decapodiformes.

Como exposto na Introdução desta Tese, a hipótese mais disseminada atualmente para explicar o fenômeno de implante profundo é aquela que envolve ação enzimática. Apesar de não haver, até o momento, nenhuma evidência de que enzimas histolíticas estejam presentes nos espermatóforos dos Decapodiformes, a presença de um conceito teórico anterior baseado nessa suposição (e.g., McSweeny, 1978; Nesis *et al.*, 1998; Hoving *et al.*, 2009) requer que qualquer nova proposição dentro do tema envolva ao menos uma discussão sobre essa possibilidade. Por essa razão, uma discussão a respeito desse tópico foi incluída no manuscrito. O presente autor não é contra a hipótese de ação histolítica, porém a princípio não pode incluí-la em seu modelo teórico sem que haja evidência de presença de enzimas nos espermatóforos dos coleóides, ou de ação histolítica no tecido-alvo. Além disso, como discutido no manuscrito, diversos fatores sugerem, na opinião do presente autor, que o fenômeno de perfuração não deva ser mediado por ação enzimática, pelo menos não inteiramente.

Buscaram-se então elementos da estrutura e morfologia funcional dos espermatóforos que pudessem auxiliar no entendimento desse processo. As evidências obtidas a partir da investigação morfológica de *D. plei*, bem como aquelas reunidas pela reavaliação da literatura, eram as seguintes:

a) O aparato ejaculatório é composto por um sistema de túnica e membranas envolvendo um filamento espiralado;
b) Esse sistema é evaginado durante a reação espermatofórica (i.e., durante a perfuração do tecido);
c) O filamento espiral é composto por milhares de corpúsculos microscópicos de forma estelar ("espículas"), que são expostos durante a reação espermatofórica;
d) As espículas contêm braços afiados e estão presentes em grande quantidade no filamento espiral. Por essas razões, acredita-se que tenham capacidade de conferir ancoragem, como exposto no Capítulo 3;
e) Devido à organização estrutural intrínseca do espermatóforo, e ao fato dele ser altamente túrgido, o aparato ejaculatório aumenta de diâmetro e de comprimento concomitantemente à sua evaginação. Conseqüentemente, o filamento espiral acaba sendo exposto ao meio externo de modo muito singular durante a reação espermatofórica: os anéis da espiral, de pequeno diâmetro no espermatóforo intacto, alargam-se à medida que o aparato ejaculatório é evaginado. Além disso, a distância entre os anéis aumenta, devido ao estiramento do aparato

ejaculatório.

f) Ambos os processos de aumento do diâmetro dos anéis e da distância entre os mesmos devem acontecer em conjunto, isto é, à medida que o anel da espiral é alargado, o mesmo deve ser impulsionado para frente.

Propõe-se, com base nestes elementos, um modelo teórico para explicar o fenômeno de perfuração observado nos espermatóforos dos Decapodiformes. Com base neste modelo, a perfuração seria mecânica e resultado da ação conjunta do aumento gradual do diâmetro dos anéis do filamento espiral e da distância entre os mesmos, bem como do poder de ancoragem proporcionado pelas espículas. No momento em que um anel mais distal é exteriorizado, o mesmo ancora-se ao tecido-alvo da fêmea por meio das espículas. À medida que o aparato ejaculatório continua sendo evaginado, o diâmetro desse anel aumenta e o mesmo é impulsionado para frente. Propõe-se que a área de tecido na qual o anel havia se ancorado inicialmente seria dilacerada conforme é impelida para os lados e para trás. Esse processo abriria gradualmente um pequeno espaço dentro do tecido, suficiente para a ancoragem do próximo anel da espiral, e assim sucessivamente, à medida que o espermatóforo em evaginação avança dentro do tecido-alvo da fêmea.

Espermatóforos contendo aparato ejaculatório, filamento espiral e espículas são aparentemente comuns em todos os grandes grupos de Decapodiformes, existindo, portanto, a possibilidade do mecanismo proposto ser intrínseco à estrutura do espermatóforo dos Decapodiformes, o que explicaria o fenômeno observado em *D. plei* em condições artificiais. No seu presente estado, o modelo apresenta algumas limitações, como a questão da diferença entre as profundidades de perfuração entre os implantes profundo e superficial. No entanto, algumas hipóteses são levantadas para explicar essas diferenças, como a questão do tamanho relativo do aparato ejaculatório, do grau de espaçamento entre os anéis do filamento espiral, e do comprimento final do espermatângio. A degeneração tecidual (ou "degeneração gelatinosa"), processo comum em fêmeas sexualmente maduras de algumas famílias de Decapodiformes, poderia também explicar essas diferenças, pois nesses casos os implantes profundos seriam facilitados pela menor resistência à perfuração oferecida pelo tecido-alvo da fêmea.

O manuscrito contendo essas informações em detalhe será submetido ao periódico internacional *The American Naturalist.*

The "evaginating-helix" perforating mechanism: a theoretical model to explain spermatophore implantation in cephalopod mollusks

Abstract

Male coleoid cephalopods produce elaborate spermatophores, which are able to attach themselves autonomously on the female's body after a complex process of evagination called spermatophoric reaction. In some oceanic and deepwater cephalopods, like the giant squid *Architeuthis*, the reaction leads to the intradermal implantation of the spermatophore, a hitherto enigmatic phenomenon. The present paper builds upon several lines of evidence from the literature to propose a theoretical model to explain how the everting ejaculatory apparatus would be able to mechanically perforate and concomitantly implant the spermatophore in the female body during the spermatophoric reaction. It is proposed that this process is achieved chiefly through the combination of 1) an "evaginating-helix" mechanism performed by the everting ejaculatory apparatus' spiral filament, and 2) the anchorage provided by its numerous, minute sharp stellate particles. The proposed model assumes that, as it is exposed to the external milieu, each spiral filament's whorl anchors to the surrounding tissue by means of its stellate particles. Then, as the ejaculatory apparatus tube tip continues evaginating, it grows in diameter and stretches lengthwise, enlarging the diameter of the whorl and propelling it, consequently tearing and pushing the anchored tissue outwardly and backwardly, and opening space for the next whorl to attach.

Keywords: Cephalopoda, sperm transfer mechanisms, spermatophore, functional morphology, spermatophoric reaction, spermatangia implantation

Introduction

Spermatophores produced by male coleoid cephalopods are among the most elaborate reproductive structures among metazoans (Mann 1984). During copulation, coleoid spermatophores undergo the so-called spermatophoric reaction, a complex process of evagination that leads to the extrusion and attachment of the 'spermatangia' (i.e., "exploded" or everted spermatophores) on various sites of the female body (Drew 1919a; Nesis 1995). Although spermatophore morphology has been extensively used in cephalopod systematics (e.g., Hess 1987), the mechanics underlying the spermatophoric reaction remain enigmatic more than 250 years after the first record of this structure in *Biblia Naturae* (Swammerdam

1738).

Detailed descriptions of the spermatophoric reaction are rare (e.g., Racovitza 1894; Drew 1919a; Weill 1927), and the knowledge of its basic functioning is at present restricted to 20 coleoid species, 13 decapodiforms (i.e., squids and sepioids; Milne-Edwards 1842; Racovitza 1894; Marchand 1913; Drew 1919a; Weill 1927; Austin et al. 1964; Fields 1965; Takahama et al. 1991; Hoving and Laptikhovsky 2007; Hoving et al. 2008; Hoving et al. 2009; Marian *in prep.*[2]), and 7 octopodiforms (i.e., octopods; Milne-Edwards 1842; Marchand 1913; Drew 1919b; Fort 1937; Fort 1941; Orelli 1962; Mann et al. 1966, 1970; Hanson et al. 1973; Pujals 1978).

Classic monographs (e.g., Racovitza 1894; Drew 1919a; Blancquaert 1925; Weill 1927; Mann et al. 1970) built the bulk of our knowledge concerning the role of the spermatophore components during the reaction, and especially the forces mediating this intriguing phenomenon. After these studies, it became well established that the propulsion for the spermatophoric reaction is dependent primarily upon two factors: the elastic tension provided by the outer tunic, and the osmotic pressure generated by the influx of seawater. Moreover, it became clear the importance of the cap as a region of less resistance and as an evacuation pathway, imperative for the success of the reaction.

However, fewer robust conclusions have been drawn concerning the main question that has puzzled the minds of earlier naturalists (e.g., Drew 1919b): is there a functional implication for such a complicated phenomenon as the spermatophoric reaction and its evagination process? The functions ascribed to the reaction would all require much simpler arrangements than those present in coleoid spermatophores. For example, final attachment of the spermatangium on the female body is considered to be performed solely by the cement body, by means of its adhesive or enzymatic substances, or even hard structures. Theoretically, this scenario would not necessitate a complicated evagination process to achieve attachment success.

Recently, great attention has been focused on deep intradermal implantation of spermatangia, an interesting reproductive strategy common in oceanic and deep-water cephalopods, like the giant squid *Architeuthis* (e.g., Norman and Lu 1997; Nesis et al., 1998; Jackson and Jackson 2004; Hoving and Laptikhovsky, 2007; Hoving et al. 2008; Hoving et al. 2009). Despite the great advances these studies have provided to the knowledge of sperm transfer in oceanic decapodiforms, important questions remain unanswered, such as the exact process of deep implantation itself. Some hypotheses have been formulated to explain this phenomenon, but none have gained wide acceptance, in part due to insufficient evidence. Some authors have hypothesized the terminal organ (“penis”) would hydraulically

[2] Marian *in prep.*: Capítulo 3.

pump and insert the spermatangia into the female tissue (e.g., Murata et al. 1982; Norman and Lu 1997; Jackson and O'Shea 2003; Jackson and Jackson 2004), specifically in those species in which the penis is relatively long. Other hypotheses ascribed the main role of implantation to tentacle hooks or suckers, and beaks, with the aid of which the male would make cuts in the female tissue before spermatangia insertion (e.g., Murata et al. 1982; Nesis 1995; Nesis et al. 1998). However, recent evidence suggests that wounds and cuts, when present, perform an auxiliary action in the implantation process, not being a prerequisite for tissue penetration (Hoving et al. 2010). The fact is, spermatangia autonomous implantation – i.e., the spermatophore alone being capable of piercing the female body – was recently demonstrated for some species (Hoving and Laptikhovsky 2007; Hoving et al. 2009). Nevertheless, how the spermatophore achieves this goal itself has remained a mystery; the action of chemical substances (e.g., proteolytic enzymes from the cement body) has been hypothesized to play a role in implantation (e.g., McSweeny 1978; Nesis et al. 1998; Hoving et al. 2009), but yet no evidence of such properties has been found.

Histolytic substances alone would certainly not be able to assure successful tissue perforation; if present at all, they would most likely play an auxiliary role to a main mechanical perforation, as seen, for example, in several parasitic helminths, in which various types of worm-secreted enzymes assist in the mechanical perforation performed by boring teeth, spines or suckers, and also by the worm's own penetration movements (e.g., Haas and Van de Roemer 1998; Haas et al. 2005; Lee and Yen 2005; Haas and Haeberlein 2009). Theoretically, the presence of histolytic enzymes inside the spermatophore would facilitate tissue invasion, but existing evidence from observations of the spermatophoric reaction points to a rather rapid process of perforation. Within a few seconds, the spermatophore is able to perforate tissue (Hoving and Laptikhovsky 2007; Hoving et al. 2009; Marian *submitted*[3]), i.e., penetration is almost instantaneous. If tissue perforation speed is so high, and given the relatively large sizes of coleoid spermatophores, there would not be sufficient time for the presumable histolytic enzymes to breakdown enough female tissue to a degree that would facilitate spermatophore implantation. For comparison, in the fast-penetrating, minute-sized trematode cercariae, tissue perforation takes at least more than a minute for a full entry in the skin (e.g., Haas and Haeberlein 2009). Lastly, even if potent enzymes were present inside coleoid spermatophores, some unknown anchorage mechanism would be necessary to support tissue penetration. On these grounds, there is no strong support for any of the three proposed hypotheses concerning deep implantation mechanics (hydraulic pump by the "penis"; cuts by hooks, suckers or beaks; and histolytic substances).

Recent data on the functioning of the spermatophore of the coastal loliginid squid

[3] Marian *submitted*: Capítulo 2.

Doryteuthis plei revealed that the everting ejaculatory apparatus has limited, but effective, perforating ability (Marian *submitted*; Marian *in prep.*). The discovery of such perforating potential in a "surface-attachment" spermatophore raised the hypothesis that a perforating mechanism is inherent to the structure of the coleoid spermatophore, although not always leading to the same type of attachment (Marian *submitted*). Therefore, this presumably intrinsic mechanism of the ejaculatory apparatus could be responsible for deep spermatangia implantation typical of some oceanic and deep-water coleoids. Evidences found in the works of Hoving and Laptikhovsky (2007: 179) and Hoving et al. (2009: 78) also corroborate this hypothesis, since they observed that in species in which deep implantation occurs, the everting ejaculatory apparatus is the first structure of the spermatophore to come into contact with and possibly penetrate the female tissue. The traditional idea that the spermatophore would first “explode” (forming the spermatangium and exposing the cement body), and only then tissue would be perforated (e.g., Voss 1985; Nesis et al. 1998) can no longer be supported. There is strong evidence corroborating that tissue perforation occurs during the spermatophoric reaction, i.e., during the evagination process (Hoving and Laptikhovsky 2007; Hoving et al. 2009; Marian *submitted*). This evidence implies that the structures involved in perforation are strictly those from the ejaculatory apparatus. The cement body, being one of the last structures to be extruded from the spermatophore (just before the sperm mass), would have only a final role in the perforation process, if any at all.

The ejaculatory apparatus is an invaginated tube in the oral region of the spermatophore composed of a tunic and a series of membranes encompassing the spiral filament (e.g., Drew 1919a). Careful investigation of the literature provided evidence suggesting that the spiral filament is present in almost all decapodiforms, from strongly developed (e.g., architeuthids, cranchiids, octopoteuthids, spirulids; Kerr 1931; Knudsen 1957; Hess 1987) to fine and tightly coiled spiral filaments (e.g., histioteuthids, ommastrephids, onychoteuthids, sepiolids; Hess 1987; Hoving et al. 2009), sometimes detected only by a weakly developed transverse striation inside the ejaculatory apparatus (Hess 1987). Numerous minute stellate particles are embedded in the spiral filament matrix (e.g., Austin et al. 1964). These particles have already been recorded in representatives of major decapodiform groups (Duvernoy 1853; Marchand 1913; Blancquaert 1923, 1925; Weill 1927; Austin et al. 1964; Badenhorst 1974; Takahama et al. 1991; Hoving et al. 2009; Marian and Domaneschi *in prep.*[4]), suggesting that they are common in all decapodiforms.

Based on these lines of evidence, and on a unique system performed by the everting spiral filament, the mechanics of the spermatophoric reaction are revisited, and a theoretical model to explain the perforating mechanism is proposed.

[4] Marian and Domaneschi *in prep.*: Capítulo 1.

Theoretical model of the "invaginated spiny helix" perforation mechanism

The intact oral region of the coleoid spermatophore is like an elastic-walled, fluid-filled and very turgid screw, the tip of which is pressed from the outside to the inside, leading to the invagination of its tip. Consequently, the thread of the screw comes to lie inside the thin invaginated tube. In this model, the ejaculatory apparatus is the invaginated tube, its screw thread composing the spiral filament (see figs. 1*A*,*E*). Considering that the whole system is under high pressure (provided by the elastic tension of the wall of the screw, and also by its turgidness), relieving pressure at its tip causes the screw to evaginate. Basically, this is what happens during the spermatophoric reaction: the ejaculatory apparatus tube evaginates, concomitantly exposing the spiral filament to the exterior milieu, from the basal-most to the apical-most spiral whorls (fig. 1). The proposed perforation mechanism of the coleoid spermatophore, however, does not correspond to the action of a drill or that of an ordinary rotating screw, in which all the whorls of the spiral are constantly rotating around the longitudinal axis. On the contrary, in the perforation mechanism seen in the coleoid spermatophore, there is no rotation (fig. 1).

The driving forces of the spermatophoric reaction come from the elastic outer tunic of the spermatophore and osmotic pressure generated by the influx of seawater (fig. 1). The latter role is played by the middle tunic (e.g., Drew 1919a) or the spermatophoric plasma (e.g., Mann et al. 1970), by means of a process not yet fully understood (see review by Mann 1984). Basically, the elastic outer tunic is semi-permeable and permits influx of seawater due to the hypertonic spermatophoric fluid (e.g., Mann et al. 1970). The resulting high internal pressure inside the spermatophore provides the driving force necessary for evagination, and prevents the everting tube walls from collapsing. The influx of seawater is probably continuous throughout the whole spermatophoric reaction (e.g., Drew 1919a; Weill 1927). Based on this assumption, in the proposed model the high inner pressure of the spermatophore is maintained until complete evagination.

During evagination of the ejaculatory apparatus, the spiral filament is everted and exposed one whorl at a time, first as a small-diameter whorl, as seen internally in the intact spermatophore, but subsequently growing in diameter as the everting tube advances (figs. 1 and 2). Still images and illustrations of the everting ejaculatory apparatus and spiral filament are evidence of this process (Drew 1919a: pl. 1, figs. 7-8; Marian *in prep.*; see also Hoving et al. 2009: fig. 2C, and Weill 1927: pl. 2, fig. 3). The true source of perforating power in this theoretical model lies in this invaginated helix structure of the spiral filament as the distal whorls enlarge in diameter during the spermatophoric reaction. It should be mentioned also that the everting ejaculatory apparatus tube stretches lengthwise (e.g., Drew 1919a; Austin

et al. 1964), i.e., the ejaculatory apparatus is longer after the spermatophoric reaction (compare for example the length of the intact ejaculatory apparatus in fig. 1*E* to that of the everted one in fig. 1*D*). Consequently, there is a lengthening of the space between successive whorls of the spiral filament during the reaction. The forward extension between whorls most probably occurs as the lateral expansion occurs, i.e., some of the pressure that is causing the lateral expansion would result in a forward component.

The minute stellate particles, which are embedded in the spiral filament matrix (figs. 3*F-H*), also play a vital role. As the spermatophore is evaginating, its tip is formed by a constantly renewed group of spines ornamenting a whorl (figs. 3*F-H*). In the mechanism proposed herein, the exposed sharp stellate particles provide anchorage points throughout perforation, i.e. they stick to the substrate and assure anchorage at the respective point of insertion (figs. 3*F-H*). The constantly renewed stellate particles at the advancing tip of the evaginating spermatophore provide a steady supply of anchorage points throughout the perforation process. The fact of being sharp and present in large quantities is assumed to confer on them anchorage capacity during tissue perforation (the anchorage capacity of the everting ejaculatory apparatus was demonstrated by Marian *in prep.*).

The combination of the successive enlargement of the spiral filament's whorls diameter and lengthening of the space between the whorls, together with the sharpness properties of the stellate particles, probably is responsible for tissue penetration. As it is exposed to the external milieu, the oral most, small-diameter whorl anchors to the surrounding tissue by means of its stellate particles (fig. 3*F*). Then, as the ejaculatory apparatus tube tip continues evaginating, it grows in diameter and stretches lengthwise, enlarging the diameter of the whorl and propelling it (fig. 3*G*), consequently pushing the anchored tissue outwardly to the sides and backwardly (figs. 3*F*,*G*). This action must have a tearing effect in the tissue where the whorl is anchored, opening space for the next whorl to attach (figs. 3*F-H*), as the everting spermatophore tip advances (figs. 3 and 4*A*,*B*). The high internal pressure of the spermatophore, besides providing the driving force for the evagination process, prevents the already implanted tube and the advancing tip from collapsing (figs. 3 and 4*A*,*B*). During the eversion of the ejaculatory apparatus, internally the sperm mass is also being pulled along as it is attached to the everting tube; after complete evagination, the sperm mass comes to lie inside the female tissue, encompassed by the everted ejaculatory apparatus tube (i.e., the outer membrane in fig. 4*B*). As a result of the lengthening of the ejaculatory apparatus, the final perforated tube inside the tissue is longer than the length of the ejaculatory apparatus of the intact spermatophore (figs. 3 and 4).

The effectiveness of perforation in this theoretical model will depend upon the nature of the substrate: if relatively too dense or hard, the mechanism might not work. In this case, without tearing and anchoring, the eversion would simply push the everting spermatophore

away from the substrate. But, if the force provided by the internal pressure of the spermatophore is greater than tissue strength at the area where the small-diameter whorl is anchored (i.e., greater than the force necessary to tear this minute area of tissue), then perforation is possible. Also, internal pressure must be high enough to prevent the implanted ejaculatory apparatus tube from collapsing.

The proposed perforating function ascribed to the spiral filament does not refute previous hypotheses of a supportive function for this structure (Drew 1919a; Austin et al. 1964). The spiral filament certainly maintains the potency of the extruding ejaculatory apparatus tube, and might prevent also its herniation during the spermatophoric reaction. The "histolytic substances hypothesis" (e.g., McSweeny 1978; Nesis et al. 1998; Hoving et al. 2009), however, would not fit within the proposed theoretical model, at least in its present state, due to the following reasons: 1) as already discussed, the high speed of the perforation process would not permit enough time for the enzymes to breakdown tissue to a degree that would facilitate integument invasion; and 2) theoretically, the presence of enzymatic substances within the spiral filament would act against the necessary anchorage provided by the stellate particles, i.e., the tissue where the stellate particles are attached would be constantly dissolved by the enzymes, consequently hampering anchorage, which is imperative for implantation within the proposed model.

In several cases, deeply implanted spermatangia are visible externally as saliences in the site of implantation (e.g., Nesis et al. 1998; Hoving et al. 2004; Hoving et al. 2009). During implantation the everting spermatophore most likely follows the path of least resistance, i.e., the pathway that provides the least resistance to forward motion during perforation (see also Hoving et al. 2004). Presumably if one side encounters hard tissue, the expanding tube diameter will extend away from the hard surface, changing the direction of the tube. Probably this is the reason why, in most records of deep implantation, the spermatangia are found in the loose connective tissue beneath the skin (e.g., Norman and Lu 1997; Nesis et al. 1998; Hoving et al. 2004; Hoving et al. 2008; Hoving et al. 2009). However, if the tube hits "harder" tissue (e.g., muscle) at right angles, there would be no path of least resistance and the tube would penetrate the muscle (as reported for the giant squid by Hoving et al. 2004).

After the ejaculatory apparatus has been completely everted and has perforated tissue, the cement body is extruded (fig. 4*B*). Its cement contents and sharp structures, when present, possibly aid in final anchorage and attachment inside female tissue. The sperm mass, which initially occupies the aboral end of the spermatophore, is completely forced into the forming spermatangium (fig. 4*B*), which is already implanted into the female body. Jackson and Jackson (2004: fig. 1C) found empty cases still attached to the female integument and lying freely within the mantle cavity of *Onykia ingens* (Hoving et al. 2009

reported a similar case for *Rossia moelleri*; see also Marian *submitted*). These observations are interpreted to correspond to the phases depicted in figures 4*B*,*C* (or 5*B*,*C*).

The spermatophore "empty capsule" or "empty case" is then detached from the spermatangium, which releases a continuous, slow flow of sperm out of it (Nesis et al. 1998; figs. 4*C*,*D* or 5*C*,*D*). It is not clear in the proposed model how the empty case detachment would happen, because the everted spiral filament is, at least theoretically, firmly anchored in the perforated tissue. However, this process of detachment is imperative for permitting sperm release, a hypothesis also put forward by Racovitza (1894: 530). It may be supposed that internal pressure is too high, but the spermatangium can not advance forward, since the ejaculatory apparatus and spiral filament are completely everted and there is no more perforation activity. The anchored spiral filament would, then, be pushed backwards (figs. 4*C*,*D*). If this supposition is true, this process would have some tearing effect on the perforated tissue, and some parts of the spiral filament would even be left behind, adhered to female tissue. This would explain the reddish pigmentation (i.e., presumably remains of the amber spiral filament) lining the cavities perforated by the spermatangia of the giant squid (Hoving et al. 2004).

Alternatively, it may be supposed that the empty case is firmly anchored as long as it is under pressure (fig. 5). Perhaps pressure is released when the cement body is extruded (fig. 5*B*,*C*), and the spermatangium and empty case collapse (fig. 5). The anchors would then be no longer very effective (fig. 5*C*), and as the squid moves around the empty cases would be pulled off by water movement, but the spermatangium would remain anchored by the cement body inside the tissue (fig. 5*D*). Sometimes the empty cases can still be loosely attached in preserved squid (R. E. Young, pers. comm.).

After the detachment of the empty case, the released sperm might be used immediately by the female for fertilization of the eggs, or spermatangia might store sperm for some time while implanted into female tissue (e.g., Nesis et al. 1998).

The question raised by Nesis et al. (1998) of how the spermatangia retain regular and parallel disposition during implantation in *Galiteuthis glacialis* is contemplated by the "evaginating helix" hypothesis, since the ejaculatory apparatus is known to form an almost straight tube after evagination (e.g., Racovitza 1894c; Drew 1919a; Weill 1927; Austin et al. 1964; Takahama et al. 1991). In the case of the giant squid *Architeuthis*, the implanted spermatangium describes a zigzag shape inside the female tissue (Norman & Lu 1997; Guerra et al. 2004; Hoving et al. 2004). This condition is very similar to the secondary coiling of the ejaculatory apparatus from the architeuthid intact spermatophore (Knudsen 1957; Hess 1987; Hoving et al. 2004), suggesting that the zigzag structure of the implanted spermatangium could be the consequence of the original coiled shape of the ejaculatory apparatus tube.

Marian (*submitted*) proposed that a perforation mechanism is probably inherent to the structure of the decapodiform spermatophore, although not always leading to the same type of spermatangium attachment. Indeed, recent evidence suggests that spermatophore attachment in squids always involves some kind of tissue perforation (Marian *in prep.*), the differences residing in the degree of spermatangia implantation among decapodiforms. In some groups, a "shallow" implantation is present, i.e., only the base of the spermatangium is implanted in female tissue (e.g., loliginids, Marian *in prep.*; sepiids, Hanlon et al. 1999). If the perforation mechanism is intrinsic to the spermatophore structure of all squids, the same system leading to deep implantation in some species would presumably be responsible, at least in part, to the shallow implantation seen in other decapodiforms. Considering that the mechanics behind shallow and deep implantation are the same, one must explain the reason of this depth difference between the two types of implantation. One explanation could reside in the length of the ejaculatory apparatus and spiral filament. Theoretically, longer ejaculatory apparatuses and spiral filaments would have more perforating power than shorter ones, and would have the potential to implant spermatangia more deeply. The giant squid *Architeuthis* has a very long ejaculatory apparatus with numerous loops (Hess 1987), and spermatangia implantation in this case can be very deep (Norman and Lu 1997; Hoving et al. 2004). Another important factor to be considered is the tightness of the spiral coils: a tighter spiral would have more whorls, and consequently more perforating potential. The spermatophores of octopoteuthids have a relatively tight coiled spiral filament (Hess 1987) and are known to implant spermatangia deeply in the female (Nesis 1995; Hoving et al. 2008). In some cases, final spermatangium length might also serve to explain deep implantation. For example, in some sepiolids and octopoteuthids, the spermatangium is a bulbous, relatively short structure (e.g., Racovitza 1894; Hoving et al. 2008; Hoving et al. 2009). Hence, perforation does not have to be so deep to completely implant spermatangia in these cases.

These implantation depth differences may also be explained by external factors. For example, the "gelatinous degeneration" undergone by sexually mature females of some oceanic and deepwater coleoids (e.g., Onychoteuthidae, Histioteuthidae, Cranchiidae; Nesis 1995; Nesis et al. 1998) could also explain deep implantation, since in this case the everting spermatangium would encounter less resistance for perforation. Another example is that, in some octopoteuthids and onychoteuthids, deep implantation is apparently facilitated by cuts and wounds made by the male beaks or tentacle/arm hooks on the female body (e.g., Murata et al. 1982; Nesis 1995; Nesis et al. 1998; Hoving et al. 2009), since spermatangia are generally found implanted within the groove of these incisions.

Conclusions

The ability of the spiral filament to perforate during eversion, as demonstrated herein in the theoretical model, could serve to explain how the spermatophores are deeply implanted in the female tissue in oceanic and deep-sea decapodiforms, and would require no hydraulic pump by the penis, nor cuts by hooks and suckers, nor histolytic substances. However, the present model imposes an important limitation: the length of the perforated tube inside the female tissue must be less than or equal to the length of the everted ejaculatory apparatus tube containing the spiral filament. This condition evidently fits some of the cases of deep implantation exemplified herein (e.g., Architeuthidae, Sepiolidae, Octopoteuthidae), but checking this relation is only possible when the morphology of spermatophores and spermatangia are known in some detail, which is at present still very rare for several decapodiforms.

The intradermal implantation as well as the whole spermatophoric reaction imposes a series of difficulties for their investigators. These processes are usually too rapid to be followed under a stereomicroscope, and almost impossible under a compound microscope, given the relatively large size of the coleoid spermatophore. The application of hypertonic salt solutions to retard the reaction has been successful in some cases, but might result in unwanted preparation artifacts (Marian *in prep.*). Investigations on the functional morphology of the coleoid spermatophore have been rare, and knowledge on its structure and functioning is still very limited. Therefore, the proposal of robust hypotheses based on solid evidence, describing all the steps involved as well as their premises, is a useful way to attempt answering these intriguing questions, and to guide future studies. Clearly, the matter of how the coleoid spermatophore manages to implant itself autonomously inside female tissue is far from fully resolved, additional investigation and experimentation being necessary to test the proposed model and to gain insight into this unique process.

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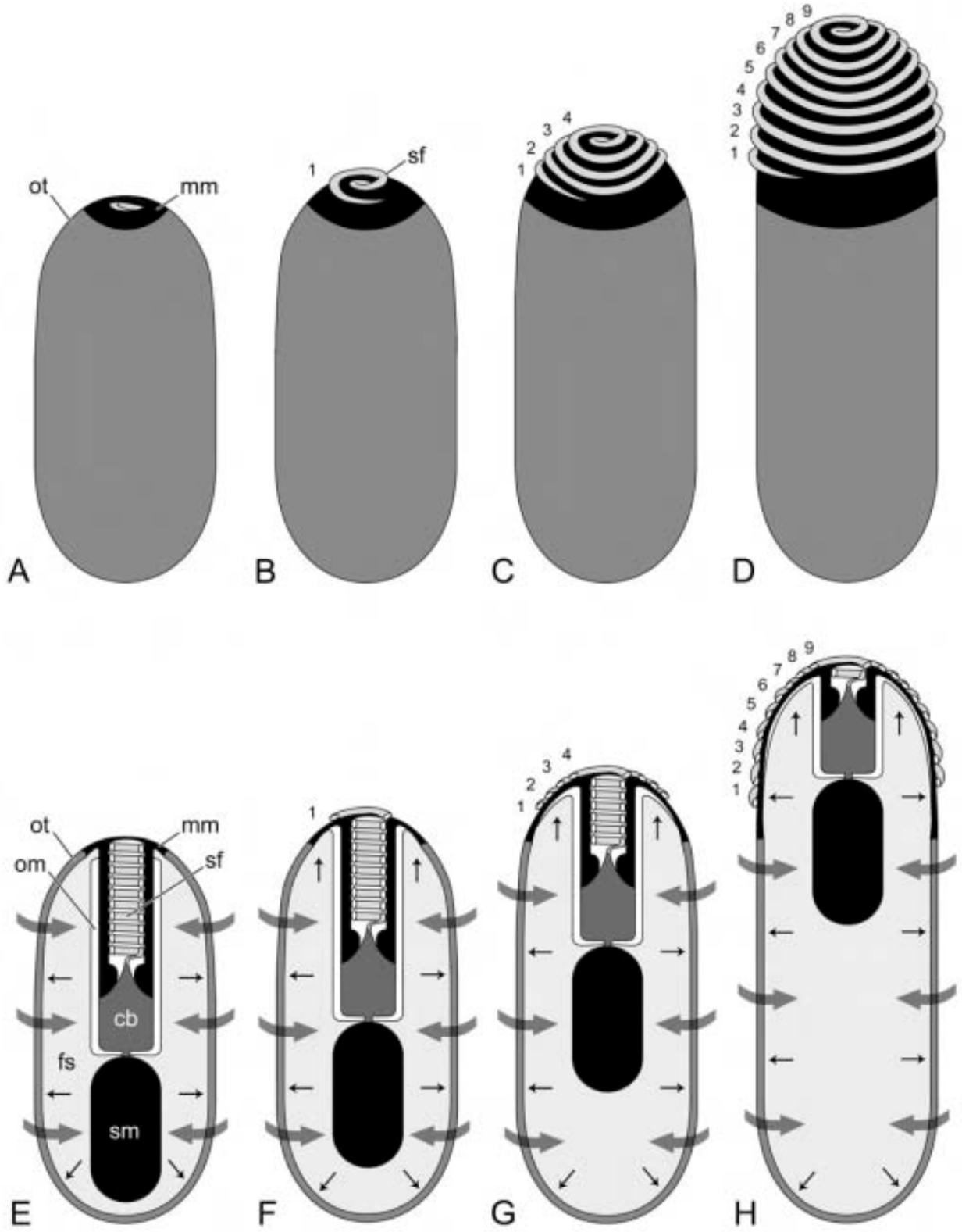

Figure 1: *A-H*, Diagrams of the external (*A-D*) and internal (*E-H*) views of a generalized coleoid spermatophore undergoing the initial phases of the spermatophoric reaction. During this process, the ejaculatory apparatus (represented by the middle and outer membranes) and spiral filament are everted due to osmotic pressure and elasticity of the outer tunic, after rupture of the cap (not depicted). Spiral filament whorls are numbered consecutively to facilitate comparison between different stages. Note that after eversion each whorl grows in diameter and is propelled forwardly as the everting tip advances. Gray translucent arrows represent the influx of seawater into the spermatophore through the outer tunic. Black solid arrows represent the pressure inside the spermatophore. Some structures were omitted for the sake of clarity. For simplicity the middle tunic is not represented in the diagram, but its role (i.e., generating an osmotic gradient) is fulfilled by the hypertonic spermatophoric fluid in this model. Abbreviations: *cb*, cement body; *fs*, spermatophoric fluid; *mm*, middle membrane; *om*, outer membrane; *ot*, outer tunic; *sf*, spiral filament; *sm*, sperm mass.

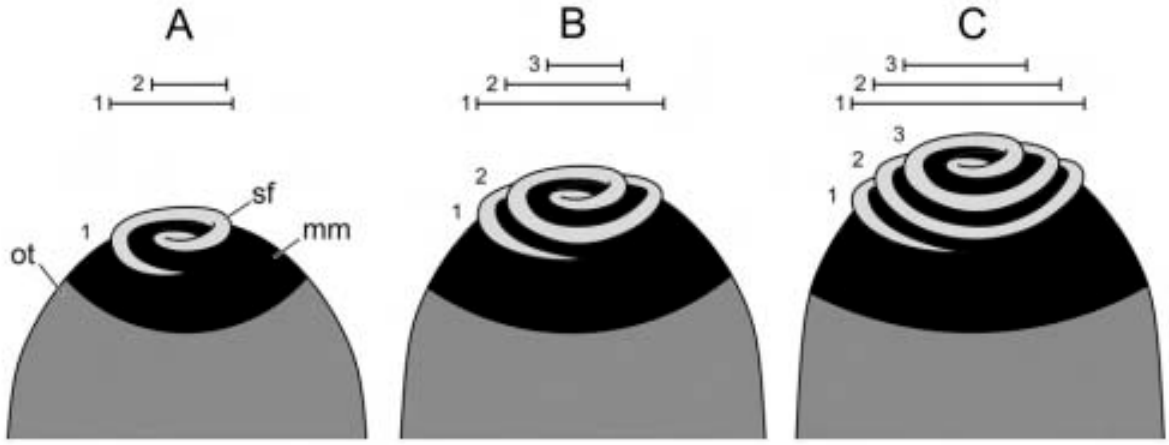

Figure 2: *A-C*, Diagrams of the advancing tip of a generalized coleoid spermatophore undergoing the spermatophoric reaction. The ejaculatory apparatus is everted and the spiral filament exposed one whorl at a time. Spiral filament whorls are numbered consecutively and their change in diameter depicted on the top of each figure. As the spermatophore everting tip advances, the whorls enlarge in diameter and are propelled forward. Abbreviations: *mm*, middle membrane; *ot*, outer tunic; *sf*, spiral filament.

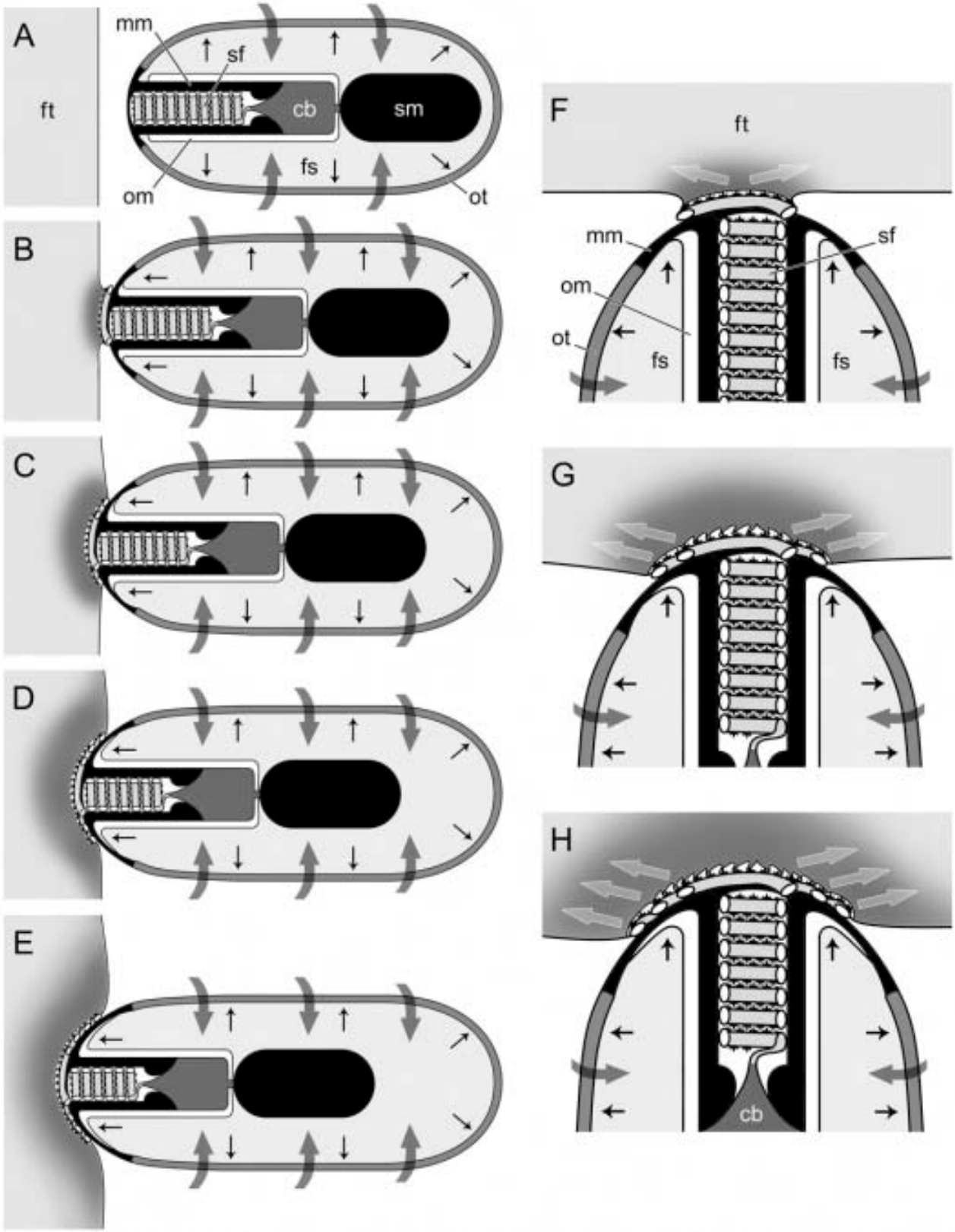

Figure 3: *A-H*, Diagrams of a hypothetic, generalized coleoid spermatophore undergoing the spermatophoric reaction close to female tissue. Some structures were omitted for the sake of clarity. *A-E*, Successive steps in the eversion of four spiral filament whorls. *F-H*, Detail of the implantation process seen in *B-D*. During evagination, the spiral filament is everted and exposed one whorl at a time, first as a small-diameter whorl, as seen internally in the intact spermatophore, but subsequently growing in diameter as the everting tube advances. As it is exposed to the external milieu, the oral most, small-diameter whorl anchors to the surrounding tissue by means of its stellate particles (visible in *F-H*). Then, as the ejaculatory apparatus tube tip continues evaginating, it grows in diameter and stretches lengthwise, enlarging the diameter of the whorl and propelling it, consequently pushing the anchored tissue outwardly and backwardly (translucent light gray arrows in *F-H*). This action has a tearing effect in the tissue where the whorl is anchored, opening space for the next whorl to attach, as the everting spermatophore tip advances. Influx of seawater (gray translucent arrows) into the spermatophore generates high internal pressure (black solid arrows), providing the driving force for the evagination process, and preventing the already implanted tube and the advancing tip from collapsing. The middle tunic is not represented, but its role is fulfilled by the spermatophoric fluid in this model. During the eversion of the ejaculatory apparatus, internally the cement body and sperm mass are being pulled. Abbreviations: *cb*, cement body; *fs*, spermatophoric fluid; *ft*, female tissue; *mm*, middle membrane; *om*, outer membrane; *ot*, outer tunic; *sf*, spiral filament; *sm*, sperm mass.

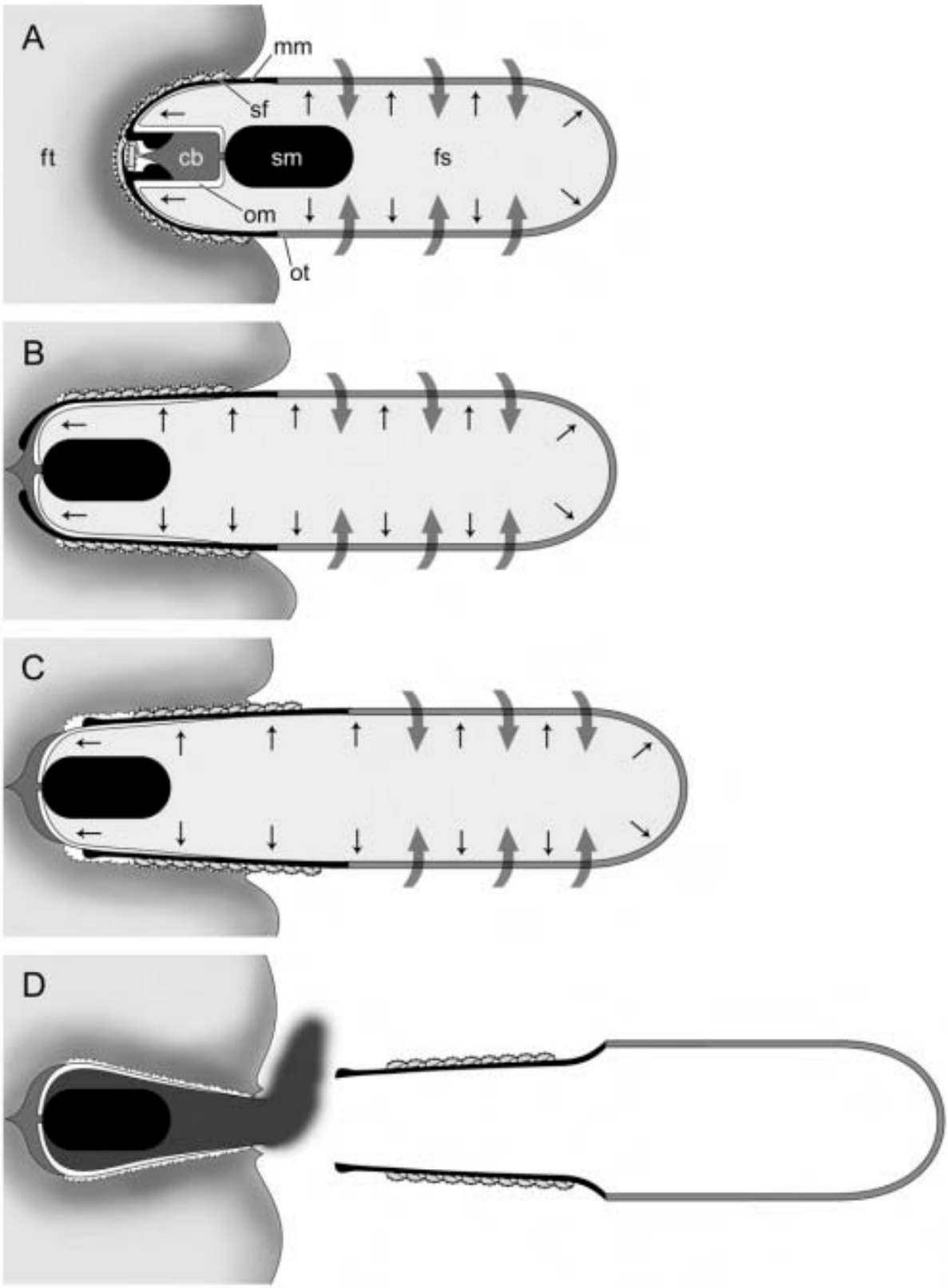

Figure 4: *A-D*, Diagrams of a hypothetic, generalized coleoid spermatophore undergoing advanced stages of the spermatophoric reaction, implanting itself into the female tissue. Gray translucent arrows represent the influx of seawater into the spermatophore, and black solid arrows the internal pressure inside the spermatophore. Some structures were omitted for the sake of clarity. The middle tunic is not represented, but its role is fulfilled by the spermatophoric fluid in this model. *A*, Spiral filament almost completely everted. *B*, Cement body extruded, providing anchor inside female tissue (through chemical and/or mechanical means). The sperm mass is forced into the forming spermatangia, which is already implanted into the female body. *C*, Internal pressure is still high, but the forming spermatangium can not advance forward, since the ejaculatory apparatus and spiral filament are completely everted and there is no more perforation activity. As a result, the anchored ejaculatory apparatus and spiral filament are pushed backwards. The cement body is firmly anchored inside the female tissue, and the middle membrane detaches from it. *D*, The spermatophore "empty case" is detached from the spermatangium, which releases a continuous, slow flow of sperm out of it. Finally, the spermatangium is completely implanted inside the female tissue, enclosing the sperm mass. Abbreviations: *cb*, cement body; *fs*, spermatophoric fluid; *ft*, female tissue; *mm*, middle membrane; *om*, outer membrane; *ot*, outer tunic; *sf*, spiral filament; *sm*, sperm mass.

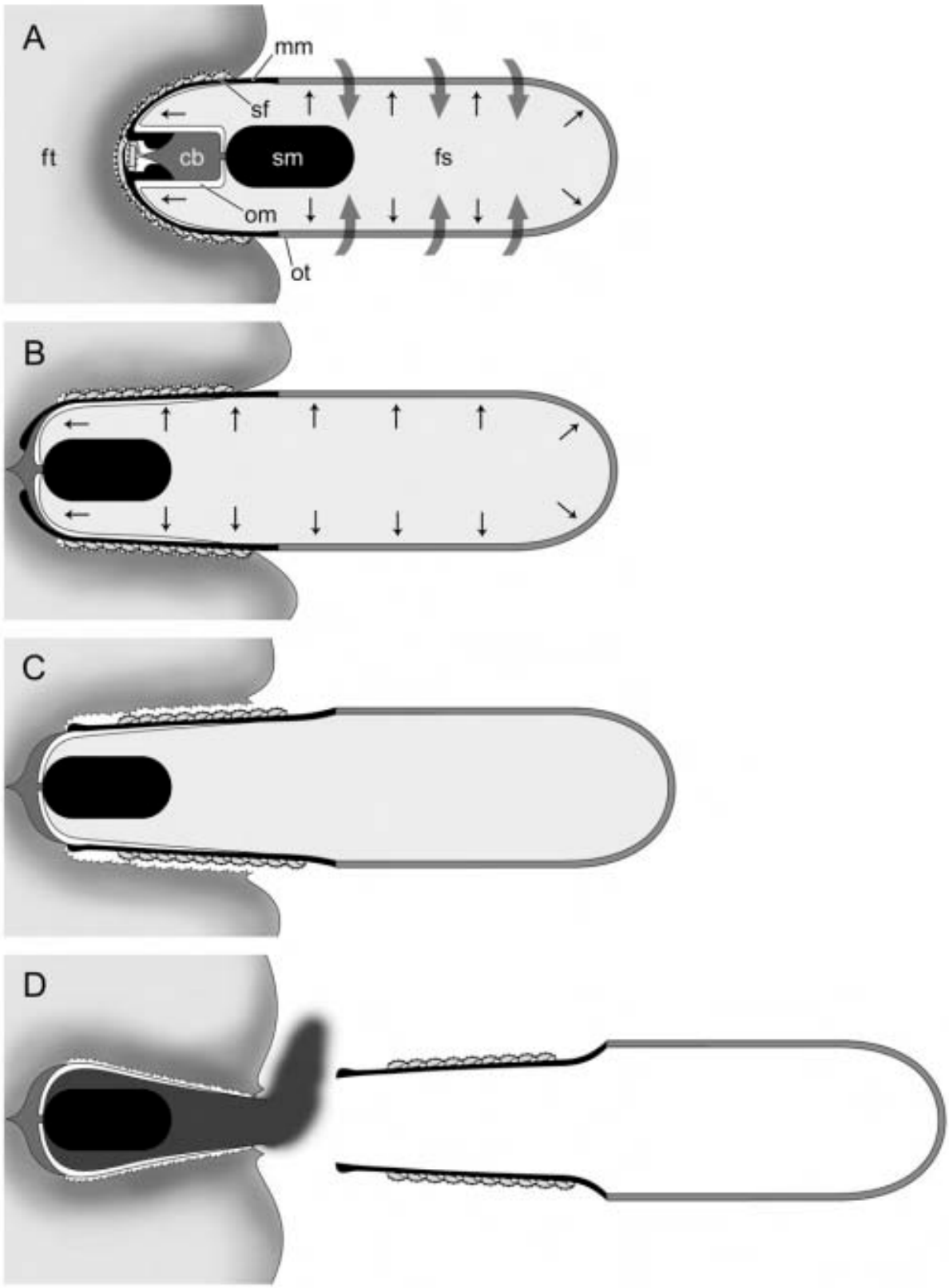

Figure 5: Alternative hypothesis to the process presented in figure 4, supposing that the empty case is firmly anchored as long as it is under pressure. *A*, Spiral filament almost completely everted. *B*, Cement body extruded, providing anchor inside female tissue (through chemical and/or mechanical means). The sperm mass is forced into the forming spermatangia, which is already implanted into the female body. Osmotic equilibrium is reached, and there is no more influx of seawater. Inner pressure is still high. *C*, Pressure is released when the cement body is extruded and, as a result, the spermatangium and empty case collapse. The anchors are no longer very effective. *D*, As the squid moves around, the empty case is pulled off by water movement, but the spermatangium remains anchored by the cement body inside the tissue. Subsequently to the detachment of the empty case, the spermatangium releases a continuous, slow flow of sperm out of it. Abbreviations: *cb*, cement body; *fs*, spermatophoric fluid; *ft*, female tissue; *mm*, middle membrane; *om*, outer membrane; *ot*, outer tunic; *sf*, spiral filament; *sm*, sperm mass.

Capítulo 5

Evolução da reação espermatofórica

CAPÍTULO 5

Evolução da reação espermatofórica

A proposta do modelo teórico apresentado no Capítulo 4 suscitou diversas questões importantes acerca do fenômeno de implante de espermatóforos nos coleóides. Por exemplo, quão aplicável seria o modelo proposto? Como teria se dado a evolução de um mecanismo de perfuração tão complexo? Quais as pressões seletivas que teriam direcionado a evolução desse fenômeno?

Na tentativa de fornecer elementos para nortear a discussão dessas questões, o presente manuscrito foi elaborado. Dando continuidade ao trabalho de revisão e reavaliação da bibliografia iniciado nos capítulos precedentes, ampliou-se a compilação de dados com base em extensa literatura acerca dos mecanismos de transferência de espermatozóides nos cefalópodes. A aplicabilidade do modelo teórico pôde ser então discutida com base em elementos da morfologia do espermatóforo. No caso de Incirrata, uma reavaliação da função da reação espermatofórica é apresentada. Propõe-se que o espermatóforo dos polvos deva alcançar o lúmen da glândula oviducal durante a reação espermatofórica, após ser introduzido na abertura do oviduto distal. A eversão do filamento espiral garantiria ancoragem ao longo do canal do oviduto à medida que seus anéis se fixariam à parede do mesmo com o auxílio de estruturas homólogas às espículas (espinhos ou dentes) e/ou substâncias adesivas. Dessa forma, a formação do espermatângio dar-se-ia dentro da glândula oviducal, onde estão localizadas as espermatecas em algumas espécies. O mecanismo de "implante" do espermatóforo dos polvos seria, portanto, muito similar ao dos Decapodiformes, sem haver, entretanto, perfuração de tecido. Essa nova proposta é baseada inteiramente em evidências da literatura acerca da biologia e anatomia reprodutiva dos polvos, principalmente em observações realizadas durante dissecção de fêmeas recém-copuladas.

Com base no levantamento de diversos caracteres reprodutivos, foi possível testar hipóteses de evolução da estrutura do espermatóforo e do sistema de implante dos espermatângios, bem como hipóteses de co-evolução de estruturas envolvidas no processo de transferência e armazenamento de espermatozóides. Esses testes foram realizados por meio de otimização parcimoniosa de caracteres sobre topologias resultantes de recentes análises filogenéticas. A reconstrução dos estados ancestrais dos caracteres morfológicos permitiu sugerir que o espermatóforo da linhagem ancestral de Coleoidea possuía aparato ejaculatório completo, contendo um filamento espiral. Ainda não é possível testar a hipótese da presença de espículas no espermatóforo ancestral, havendo necessidade da

continuidade da investigação morfológica detalhada do espermatóforo de diversos grupos de Coleoidea.

Duas hipóteses acerca da evolução do sistema de implante dos espermatângios são propostas. Uma delas assume que o sistema emergiu como "implante profundo" na linhagem ancestral de Coleoidea, possivelmente funcionando como uma espermateca e permitindo, por exemplo, separação temporal entre os eventos de cópula e fertilização. O "implante superficial" teria surgido depois, mais de uma vez independentemente, associado à evolução de receptáculos seminais ou receptáculos de espermatóforos. A segunda hipótese defende que o sistema emergiu como "implante superficial", o "implante profundo" tendo evoluído depois, possivelmente diversas vezes de forma independente, associado à adoção de modo de vida oceânico. Ambas as hipóteses são sustentadas dependendo da filogenia utilizada no mapeamento de caracteres.

Coleóides são moluscos muitos ativos que surgiram aparentemente no período Devoniano ou no início do período Carbonífero (e.g., Packard 1972; Aronson 1991; Nishiguchi & Mapes 2008). Muitos autores defendem que a intensa predação e competição, impostas por eficientes predadores emergindo nesses períodos, teriam constituído pressões seletivas importantes que modelaram profundamente a evolução dos coleóides (e.g., Packard 1972; O'Dor & Webber 1986; Aronson 1991). Essas pressões seletivas, somadas às limitações do *bauplan* morfológico, fisiológico e bioquímico dos moluscos, teriam influenciado a evolução de mecanismos e características muito peculiares dos coleóides, como curtos ciclos de vida e rápidas taxas de crescimento, sistemas nervoso e sensorial complexos, sistema raptorial e digestório altamente eficientes, sistema de camuflagem sofisticado e mediado via sistema nervoso, entre outros (Packard 1972; O'Dor & Webber 1986).

Nesse contexto, o surgimento de um mecanismo otimizando a fixação do espermatóforo na fêmea teria sido presumivelmente vantajoso. Primeiramente, coleóides são em geral nadadores muito ativos que dependem de um sistema de jato-propulsão mediado por contrações do manto; hipoteticamente, essa condição criaria limitações para deposição de espermatóforos na superfície do corpo e na cavidade do manto da fêmea, devido à questão da "turbulência" já comentada na Introdução. O sistema de fixação por implante observado nos coleóides é, de fato, eficiente a ponto de suportar a resistência imposta pelo modo de vida do animal. Além desse fator, observa-se que o processo de cópula nos coleóides, principalmente os Decapodiformes, é em geral muito rápido, provavelmente devido à pressão seletiva exercida pela predação intensa[1]. Como exposto nos Capítulos precedentes, o sistema de implante visto nos coleóides é um processo

[1] Se considerarmos a cópula como um evento reprodutivo que cria um momento de maior vulnerabilidade por expor os animais a maior risco de predação, cópulas mais rápidas diminuiriam esse risco.

extremamente rápido, durando de poucos segundos a poucos minutos. Somando-se a isso o fato do implante ser um processo inteiramente autônomo, a duração da cópula pode ser ainda mais curta: após retirar o espermatóforo do saco espermatofórico, o macho deve simplesmente transferi-lo ao sítio de deposição na fêmea; o implante se dá sem a necessidade de manuseio ou sustentação do espermatóforo pelo hectocótilo (veja, por exemplo, os vídeos referentes aos Capítulos 2 e 3[2], bem como as evidências de Hoving & Laptikhovsky 2007). Dessa forma, acredita-se que a vantagem desse sistema estaria na rapidez com que o processo de cópula pode ser executado, reduzindo presumivelmente o risco de predação. Finalmente, a separação entre os eventos de cópula e fertilização, possibilitada pelo implante do espermatóforo, é considerada como um fator igualmente importante no contexto da evolução desse mecanismo.

O manuscrito contendo essas informações em detalhe será submetido ao periódico internacional *Zoologica Scripta.*

[2] Ver CD em anexo à Tese, contendo material suplementar.

The cephalopod spermatophoric reaction and its evolution

Abstract

Spermatophores from coleoid cephalopods are complex reproductive structures capable of functioning outside the male body during mating. Through the so-called spermatophoric reaction, the spermatangium (everted spermatophore containing the sperm mass) is attached on the female body. In decapodiforms, this attachment involves some varying degree of spermatangium implantation into female tissue, which has been tentatively divided into two types: shallow implantation (the base of the spermatangium is implanted into female integument) and deep implantation (all the length of the spermatangium is implanted into tissue). This paper reviews the literature concerning the phenomenon of implantation of spermatophores in decapodiforms, and presents evidence corroborating a previous theoretical model ascribing the role of implantation to the mechanical perforation performed by the spiral filament, a structure found inside the spermatophore ejaculatory apparatus. The mechanisms of spermatophore transfer are also reviewed for octopodiforms, and a reinterpretation of the function of the spermatophoric reaction in this case is provided. In light of parsimonious character optimizations performed onto recently published phylogenetic trees, a complete ejaculatory apparatus with a spiral filament, as well as the spermatophoric reaction, apparently emerged once and early in the evolution of the Coleoidea. This novelty possibly provided an efficient attachment mechanism and presumably countered the changes associated with the adoption of an active mode of life by coleoids, augmenting fertilization success. Two hypotheses for the evolution of the complex spermatophore within Coleoidea and Decapodiformes are proposed. One hypothesis assumes that spermatangia deep implantation arose first, functioning as a spermatheca and allowing temporal separation between copulation and fertilization events; shallow implantation evolved later associated with the emergence of seminal and spermatophore receptacles. The second hypothesis assumes that shallow implantation emerged first, deep implantation arising later associated with the adoption of oceanic and deepwater lifestyles. Support for each hypothesis is dependent upon the phylogenetic tree under consideration.

Keywords: Cephalopoda, evolution, sperm transfer mechanisms, spermatophore, functional morphology, spermatophoric reaction, spermatangia implantation

Introduction

All living cephalopods provide some kind of protection to their gametes: males enclose spermatozoa within spermatophores, and females coat their eggs with gelatinous protective envelopes, or, in the case of incirrate octopods, provide them with parental care (Mangold 1987). Spermatophores of cephalopods of the subclass Coleoidea are among the most structurally and functionally elaborate spermatophores in the animal kingdom (Mann 1984). During mating, which might involve a series of complex color and postural displays (Hanlon & Messenger 1996), the coleoid spermatophore is transferred to the female by a modified appendage, called hectocotylus, except for some oceanic and deep-sea species, for which this role is ascribed to the long terminal organ (i.e., "penis") (Nesis 1995). During transfer, the spermatophore functions autonomously outside the male body (Drew 1919a), undergoing precise structural changes in the so-called spermatophoric reaction. This osmotic-mediated process comprises the evagination of the spermatophoric tunics and membranes, leading to the extrusion and attachment of the spermatangium (i.e., everted spermatophore containing the sperm mass) on the female's body (Drew 1919a).

More than 250 years after its first record in *Biblia Naturae* (Swammerdam 1738), the mechanics underlying the spermatophoric reaction remain poorly known, the knowledge of its basic functioning being restricted to only 20 species: *Doryteuthis pealeii* (Drew 1919a; Austin *et al.* 1964), *D. opalescens* (Fields 1965), *Loligo vulgaris* (Milne-Edwards 1842; Marchand 1913), *Illex coindetii* (Marchand 1913), *Octopoteuthis sicula* (Hoving *et al.* 2008a), *Onykia ingens* (Hoving and Laptikhovsky 2007), *Rossia macrosoma* (Racovitza 1894; Marchand 1913), *Rossia moelleri* (Hoving *et al.* 2009), *Sepia officinalis* (Milne-Edwards 1842; Marchand 1913), *Sepiola atlantica* (Weill 1927), *S. rondeleti* (Marchand 1913), *Todarodes pacificus* (Takahama *et al.* 1991), *Eledone cirrhosa* (Fort 1937), *E. moschata* (Milne-Edwards 1842; Marchand 1913; Fort 1941; Orelli 1962), *Enteroctopus dofleini* (Mann *et al.* 1966 and 1970; Hanson *et al.* 1973), *Octopus defilippi* (Marchand 1913), *O. macropus* (Marchand 1913), *O. tehuelchus* (Pujals 1978) and *O. vulgaris* (Drew 1919b). Even worse, detailed descriptions of the spermatophoric reaction are considerably rarer (e.g., Racovitza 1894c; Drew 1919a; Weill 1927).

Recently, this subject has regained considerable attention, mainly focused on deep intradermal implantation of spermatophores, an enigmatic reproductive strategy common in oceanic and deep water cephalopods (e.g., Norman & Lu 1997; Nesis *et al.*, 1998; Jackson & Jackson 2004; Hoving & Laptikhovsky, 2007; Hoving *et al.* 2008a; Hoving *et al.* 2009). New findings on the structure (e.g., Marian & Domaneschi *in prep.*[3]) and functioning (e.g.,

[3] Marian & Domaneschi *in prep.*: Capítulo 1.

Hoving & Laptikhovsky 2007; Hoving *et al.* 2009; Marian *submitted*[4]; Marian *in prep. A*[5]) of the coleoid spermatophore have provided new insights into this complex process, which culminated in the proposal of a theoretical model to explain spermatophore implantation (Marian *in prep. B*[6]), involving a mechanical perforation performed by the everting spiral filament, a structure found inside the spermatophore ejaculatory apparatus.

This paper summarizes our present state of knowledge on the spermatophoric reaction based on a review of the literature. It then focuses on one aspect of the spermatophoric reaction, implantation, and particularly the recent model of implantation (Marian *in prep. B*) to determine: 1) how broadly applicable this model may be from our present knowledge of implantation throughout the Coleoidea, and 2) the evolutionary history of the cephalopod spermatophore based on the anatomical features associated with the implantation model, to gain insight into the timing and ecological pressures that may have directed this evolution.

Materials and methods

Literature review and analysis

Original works on cephalopods, containing information on at least one of the following items, were examined: 1) the presence of a spiral filament inside the tube of the ejaculatory apparatus; 2) the presence of stellate particles in the spermatophoric organ of the male reproductive tract, and/or embedded in the spiral filament of the spermatophore; 3) the type of spermatangia attachment, which were divided into four categories: "shallow implantation" (i.e., only the base of the spermatangium is implanted into female tissue; Fig. 1C); "deep implantation" (i.e., all or nearly all the length of the spermatangium is implanted into female tissue; Figs 1A,B); "plugged" attachment (i.e., the spermatangium is “plugged” into the distal oviduct, finding its way through it into the oviducal gland or into the ovary; Fig. 1D); or "not implanted into female tissue"; 4) the site of spermatangia attachment on the female body; and 5) the presence of seminal receptacles *s. str.*, i.e., specialized organs bearing morphological and histological modifications which enable them to store spermatozoa released from the spermatangia. The resulting information is summarized in Table 1. Conclusions drawn from the literature review are highlighted in the table and discussed in the text.

[4] Marian *submitted*: Capítulo 2.
[5] Marian *in prep*. *A*: Capítulo 3.
[6] Marian *in prep*. *B*: Capítulo 4.

Character mapping

To estimate possible evolutionary scenarios for the coleoid spermatophore, and to attempt to reconstruct the spermatophore pattern of the stem lineages of both Coleoidea and Decapodiformes, the distribution of selected characters' states were analyzed based on published phylogenetic trees using Mesquite 2.71 (Maddison & Maddison 2009).

A species-character matrix (39 terminal taxa, 8 unordered characters; Appendices 1 and 2) was built based on information obtained from the literature (Table 1). Five different trees were constructed in Mesquite 2.71 (Figs 2 and 3) in accordance with the topologies resulting from the following studies: 1) the direct optimization analysis using POY from Lindgren *et al.* (2004); 2) the direct optimization analysis using POY from Strugnell & Nishiguchi (2007); 3) the Bayesian analysis of sequences aligned by eye from Strugnell & Nishiguchi (2007); 4) the maximum parsimony analysis of sequences aligned by eye from Lindgren (2008); and 5) the maximum likelihood analysis of sequences aligned by eye from Lindgren (2008). These topologies were chosen because they suggest different hypotheses for coleoid and decapodiform evolution, consequently corroborating different hypotheses for the evolution of the spermatophore within both the Coleoidea and Decapodiformes.

To allow comparison between the chosen topologies, all genera and species names were replaced by their respective family names. The parsimonious character optimizations were performed using the "Trace character history" function of Mesquite 2.71. The phylogenetic trees were modified as follows:

1) From the topology of Lindgren *et al.* (2004), the terminals *Opisthoteuthis* sp. and *Histioteuthis hoylei* were excluded for the sake of clarity, because they did not nest within Cirrata and Histioteuthidae, respectively. Their exclusion did not result in any changes in final character optimization. Other modification involved the coding of Bathyteuthidae as a single terminal, even though it was rendered paraphyletic in the original analysis. Again, this modification did not cause any changes in the conclusions about character evolution.

2) Although the analyses of Strugnell & Nishiguchi (2007) recovered Nautiloidea, Octopodiformes and Decapodiformes in a polytomy, in the present study these topologies were modified to render Coleoidea monophyletic. This modification was made based on several lines of evidence corroborating the monophyly of Coleoidea (e.g., Young & Vecchione 1996; Lindgren *et al.* 2004; Strugnell & Nishiguchi, 2007; Nishiguchi & Mapes 2008).

3) The maximum likelihood topology of Lindgren (2008), in which the Chiroteuthidae was paraphyletic, is modified here to render this group monophyletic in conjunction with strong morphological evidence (Young 1991). There was no change in the final optimization after this modification.

Results

1. Overview of the coleoid spermatophoric reaction

Based on evidence present in the literature, the coleoid spermatophoric reaction is apparently composed of the following steps:

1) Male retrieves a group of spermatophores (aboral ends first) from the terminal organ (= penis) with the aid of the hectocotylus (when present), transferring them near to the site of attachment on the female body; with this maneuver the oral end is directed toward the female tissue (e.g., Drew 1911). In octopods, the terminal organ is responsible for reversing the spermatophore and placing the oral end into the spermatophoric groove of the hectocotylus (Wodinsky 2008); the spermatophore travels along the groove by peristalsis and is held near or inserted into the distal end of the distal oviduct.

2) The act of pulling the spermatophores from the penis stretches the cap thread, loosening or rupturing the cap region, and triggering the spermatophoric reaction (e.g., Drew 1919a); the uptake of seawater is capable alone of triggering the reaction (e.g., Drew 1919a; Takahama *et al.* 1991). The action of the penis might also play a role in this process (e.g., Hoving & Laptikhovsky 2007; Wodinsky 2008). Being a region of less resistance and an evacuation pathway, the cap is imperative for the success of the reaction (e.g., Drew 1919a; Weill 1927).

3) There is generally a pause in the spermatophoric reaction subsequently to its initiation (e.g., Drew 1919a; Mann *et al.* 1970; Marian *in prep. A*). This pause is apparently common to all coleoid spermatophores, although its duration seems variable. This pause is believed to be an important prerequisite for successful spermatophore transfer, since it provides the male sufficient time to appropriately position the spermatophores on the female body, and also for the spermatophore to accumulate sufficient inner pressure from the inflowing seawater (see step 4; Marian *in prep. A*).

4) The propulsion for the spermatophoric reaction is dependent upon the elastic tension provided by the outer tunic, and the osmotic pressure generated by the influx of seawater (e.g., Drew 1919a; Weill 1927; Mann *et al.* 1970). This latter role is played by the middle tunic (e.g., Drew 1919a) or the spermatophoric plasma (e.g., Mann *et al.* 1970), which are hypertonic relative to seawater, and by the semi-permeable outer tunic (e.g., Mann *et al.* 1970). There is evidence also of an auxiliary mechanism of osmoregulation maintaining the high osmolality of the spermatophoric plasma throughout the spermatophoric reaction, even with the continuous influx of seawater (Mann *et al.* 1970; Hanson *et al.* 1973; Mann *et al.* 1973; Mann *et al.* 1981).

5) The ejaculatory apparatus and its spiral filament are everted, perforating the

female tissue (Marian *in prep. B*). This perforation may result in complete implantation of the spermatangium into the female body (e.g., Nesis *et al.* 1998; Figs 1A,B), or only in the formation of a shallow puncture that allows injection of the cement body contents and sharp structures inside the female tissue (e.g., Marian *in prep. A*; Fig. 1C). In octopods, a similar mechanism apparently assists in the advance of the spermatangium through the distal oviduct (see "Spermatophore implantation in the Octopodiformes" below; Fig. 1D), either into the oviducal gland (e.g., Froesch & Marthy 1975), or into the ovary (e.g., Perez *et al.* 1990).

6) The cement body is extruded. Its adhesive, cement contents (e.g., Drew 1919a) and sharp structures (e.g., Takahama *et al.* 1991), when present, are injected into the shallow puncture (Marian *in prep. A*); in the cases where a deep channel is perforated, the cement body might also aid in final anchorage and attachment inside female tissue (e.g., Hoving *et al.* 2004). Although the cement body has been stated to be absent in octopods (e.g., Drew 1919b), at least in some species the region of the octopod spermatophore homologous to this structure is occupied by a bulbous enlargement (e.g., Milne-Edwards 1842; Mann *et al.* 1970).

7) The sperm mass, which initially occupies the aboral end of the spermatophore, is completely forced into the forming spermatangium that is already attached on or implanted into the female body (e.g., Drew 1919a; Hoving *et al.* 2009; Marian *in prep. A*).

8) The spermatophore "empty capsule" or "empty case" is detached from the spermatangium, which releases a continuous, slow flow of sperm out of it (e.g., Drew 1919a), or might be closed and dependent on an external factor to release spermatozoa (Durward *et al.* 1980; O'Dor 1983). The "empty case" is generally composed proximally by the outer and middle tunics and distally by the everted middle and inner membranes and spiral filament; the outer membrane and inner tunic compose the outer coating of the spermatangium, the burst cement body covering its base (e.g., Drew 1919a); not all tunics and membranes are present in some species. In the cases where deep implantation occurs, it is not clear how the "empty case" detachment happens, with two hypotheses having been proposed to explain this process (Marian *in prep. B*). The detachment of the "empty case" from the spermatangium can take a long time to be completed depending on the species (e.g., Racovitza 1894c; Orelli 1962). In octopods the spermatangium might either not be closed (Drew 1919b), releasing the sperm rope after complete evagination, or might be closed and form the spermatophoric bladder, which eventually ruptures and releases the sperm mass into the oviducal gland (e.g., Mann 1984). The detachment of the "empty case" is apparently not universal in octopods (e.g., Drew 1919b; Pujals 1978).

9) The released sperm might be used immediately by the female for fertilization of the eggs (e.g., Drew 1911), or spermatangia might store sperm for some time while attached (e.g., O'Dor 1983) or implanted into female tissue (e.g., Nesis *et al.* 1998). In some cases,

sperm released by spermatangia are stored for long periods of time in specialized seminal receptacles or spermathecas, which have specific morphological and histological modifications to immobilize and properly store spermatozoa (e.g., Oordt 1938; Froesch & Marthy 1975). In coleoids that deeply implant spermatangia, seminal receptacles for spermatozoa storage are often absent, and spermatangia are generally implanted externally (on the mantle, arms and head surface), but also within the mantle cavity not too far from the genital opening (Table 1). In contrast, in the cases where the implants are shallow, spermatangia are either deposited within the mantle cavity near the genital opening, or in special spermatophore receptacles (e.g., nuchal receptacles in enoploteuthid families; Table 1), or near specialized seminal receptacles, when these are present (e.g, on the buccal membrane in some oegopsids, and in loliginids and sepiids; Table 1). In octopods, specialized seminal receptacles ("spermathecas" inside the oviducal gland) are generally present (Table 1).

2. Spermatophore implantation in the Decapodiformes

In decapodiform species for which data was found in the literature (Table 1), spermatophore attachment apparently always involves some kind of spermatangium implantation, i.e., the spermatangium penetrates the female integument. In some groups, "shallow" implantation (Fig. 1C; Table 1) is present, as seen in loliginids (e.g., Lum-Kong 1992; Marian *in prep. A*), enoploteuthids (e.g., Burgess 1998; Tsuchiya & Okutani 1990), ommastrephids (e.g., Durward *et al.* 1980), and sepiids (e.g., Hanlon *et al.* 1999; Wada *et al.* 2005), in which only the base of the spermatangium is implanted into female tissue. Although previously stated that in loliginids the spermatangia are attached on the female tissue solely by the cement adhesive substances (e.g., Drew 1919a), histological sections of female reproductive organs (e.g., Drew 1911; Lum-Kong 1992; Marian *in prep. A*) indicate that the base of the spermatangium lies and fits in a superficial depression in the female tissue. Moreover, there is evidence of a break in the epithelium of the female integument in loliginids and sepiids (Hanlon *et al.* 1999; Marian *in prep. A*). Hence, there is perforation, but limited to the surface tissue. This probably holds true also for other such groups for which such histological sections are not available, since in several cases the base of the spermatangium is actually thrust into female tissue (e.g., Tsuchiya & Okutani 1990; Takahama *et al.* 1991; personal observations). The mechanism for perforation, however, has only recently been suggested (see below).

In other species, specially deep-water and oceanic decapodiforms, deep implantation (Figs 1A,B; Table 1) takes place; in this type all or nearly all the length of the spermatangium is implanted into female tissue. This is the case, for example, of some sepiolids (e.g., Hoving

et al. 2009), octopoteuthids (e.g., Hoving *et al.* 2008a), onychoteuthids (Jackson & Jackson 2004; Hoving & Laptikhovsky 2007), cranchiids (Nesis *et al.* 1998), and the giant squid *Architeuthis* (e.g., Norman & Lu 1997; Hoving *et al.* 2004).

Until recently the mechanism that enables deep implantation has been uncertain. In several decapodiforms that deeply implant spermatangia (e.g., octopoteuthids and onychoteuthids, among several others), males lack hectocotylization, i.e., a morphological modification of one or a group of arms, used by the male to transfer spermatophores to the female. In the majority of these cases, males possess a relatively long penis (Nesis 1995), which has been suggested to act as a hydraulic pump to aid deep implantation (e.g., Murata *et al.* 1982; Norman & Lu 1997; Jackson & O'Shea 2003; Jackson & Jackson 2004). Others have suggested that the action of hooks, suckers teeth, or beaks would be responsible, at least in part, for this phenomenon, by wounding and cutting female tissue prior to insertion of spermatangia (e.g., Murata *et al.* 1982; Nesis 1995; Nesis *et al.* 1998; Hoving *et al.* 2010). Wounds and cuts, when present, apparently perform an auxiliary action in the implantation process, but are not a prerequisite for deep penetration (e.g., Hoving *et al.* 2010).

Recently, Hoving & Laptikhovsky (2007) and Hoving *et al.* (2009) confirmed that spermatophore deep implantation is an autonomous process, i.e., performed by the spermatophore itself. A mechanism, then, intrinsic to the spermatophore had to be responsible for this phenomenon. Indeed, McSweeny (1978) and Nesis *et al.* (1998) had previously postulated that proteolytic enzymes from the spermatophore tunics or the cement body, would be, at least in part, responsible for spermatangia penetration, but Nesis *et al.* (1998) recognized some limitations of such a postulation, and yet no evidence of such chemical properties have been documented in the literature.

Hoving *et al.* (2009) demonstrated that the direct force produced by the acceleration and mass of the ejaculating spermatophore was not sufficient for spermatangia implantation into tissue, and thought that a combination of mechanical (forward moving of the spermatophore by uptake of seawater) and chemical (proteolytic lysis) factors would explain deep implantation.

Recent data from the loliginid squid *Doryteuthis plei* revealed that the mechanism for both shallow and deep penetration is similar. In this squid the everting ejaculatory apparatus has perforating and penetrating ability under artificial conditions (Marian *submitted*), being able to implant itself readily into artificially exposed musculature; this phenomenon, however, does not occur naturally. In natural conditions, the everting ejaculatory apparatus apparently creates the first contact of the spermatophore with the female body, leading to the formation of a shallow puncture on the surface of the female tissue (Marian *in prep. A*), opening a breach for injection of cement body contents. The spermatophoric reaction thus leaves the base of the spermatangium implanted and cemented on the female (i.e., "shallow

implantation"). The perforating/penetrating ability in this shallow-implanting spermatophore led Marian (*in prep. B*) to propose a common mechanism to explain both shallow and deep implantation.

***The hypothesis for implantation of decapodiform spermatophores*.** Marian (*in prep. B*) suggests that deep implantation involves the action of the everting spiral filament, a structure within the ejaculatory apparatus. The combination of the successive enlargement of the everting spiral filament's whorls diameter and lengthening of the space between the whorls, together with the sharpness properties of the stellate particles, was proposed to be responsible for tissue penetration (Marian *in prep. B*). The "evaginating-helix perforating mechanism" hypothesis assumes that, as the evaginating whorls of the spiral filament contact the female tissue they are anchored by the contained stellate particles. Then, as the ejaculatory apparatus tube tip continues evaginating, it grows in diameter and stretches lengthwise, enlarging the diameter of the whorl and propelling it forward while pushing the anchored tissue outwardly and backwardly. This action is believed to be responsible for a tearing effect in the tissue where the whorl is anchored, opening space for the next whorl to attach (Marian *in prep. B*). Evidence found in the work of Hoving & Laptikhovsky (2007) and Hoving *et al.* (2009) also corroborates the hypothesis of the ejaculatory apparatus and spiral filament as a perforating device. Hoving & Laptikhovsky (2007) stated that, in *Onykia ingens*, “the everting ejaculatory apparatus has the first contact with the tissue and may facilitate adhesion or the first penetration into the tissue, perhaps by mechanical means”. For *Rossia moelleri*, Hoving *et al.* (2009) stated that "the frontal part of the [everting] spermatangium penetrated the tissue and moved into it". This information matches with the explanation of a perforating ejaculatory apparatus and spiral filament, which are the first structures to have contact with the female body and to perforate its tissue.

For this model to be broadly applicable, spermatophores of different taxa must possess an ejaculatory apparatus, a spiral filament and embedded stellate particles. The extent to which the hypothesis might be applicable is examined below based on the literature of the spermatophore structure in decapodiform cephalopods (Table 1).

Evidence for the hypothesis based on the structure of decapodiform spermatophores. The spiral filament is present in the spermatophore of apparently the majority of described teuthoids (Table 1), from strongly developed (e.g., architeuthids, octopoteuthids, some cranchiids; Knudsen 1957; Hess 1987) to fine and tightly coiled spiral filaments (e.g., histioteuthids, ommastrephids, onychoteuthids; Hess 1987), sometimes detected only by a weakly developed transverse striation inside the ejaculatory apparatus (Hess 1987). In most "sepioids" this structure is also present, being clearly evident in *Spirula spirula* (Kerr 1931). In sepiids and sepiolids, however, there is evidence of a very fine and tightly coiled spiral filament, which is not always quite conspicuous. For example, in *Sepia*

officinalis, Blancquaert (1923, 1925) described a tight spiral formed by the arrangement of the containing stellate particles. Hoving *et al.* (2009: figs. 2C and 4C) evidence the presence of a tightly coiled spiral filament in the everting spermatophore of *Rossia moelleri*. In the morphological description of *Neorossia leptodons*, Reid (1991: 805) described a finely coiled "middle tunic of ejaculatory apparatus". Marchand (1913: pl. 21, fig. 6) illustrations of the ejaculatory apparatus of the spermatophore of *Sepiola rondeleti* are suggestive of transverse striation. Weill (1927: pl. 1, fig. 3, and pl. 2, fig. 3) schematic illustrations of *S. atlantica* spermatophore suggest a spiral arrangement for the stellate particles. Racovitza (1894c: pls. 20 and 21, figs. 4, 9 and 10) illustrations of the spermatophore of *R. macrosoma* provide evidence of a spiral arrangement inside the tube of the ejaculatory apparatus of intact and everted spermatophores. Also, Racovitza (1894c) stated that the extremity of the everting spermatophore described a "spiral movement", which could be the reflection of the evagination of a helical structure such as the spiral filament.

Stellate particles, which can be very difficult to detect, have been recorded either embedded in the spiral filament matrix or in the spermatophoric organ of three loliginids (*Doryteuthis pealeii*, Austin *et al.* 1964; *Loligo reynaudi*, Badenhorst 1974; and *D. plei*, Marian & Domaneschi *in prep.*; Marian *in prep. A*), an ommastrephid (*Todarodes pacificus*, Takahama *et al.* 1991), two sepiids (*Sepia officinalis* and *S. elegans*; Blancquaert 1923, 1925), and three sepiolids (*Sepiola rondeleti*, Duvernoy 1853, Marchand 1913, and Blancquaert 1923, 1925; *S. atlantica*, Weill 1927; and *Rossia moelleri*, Hoving *et al.* 2009). Being present in representatives of major decapodiform groups (Myopsida, Oegopsida and "Sepioidea"; Table 1) suggests that these particles are common to all decapodiforms.

3. Spermatophore implantation in the Octopodiformes

Except for cirrate octopods, which do not possess an ejaculatory apparatus (Villanueva 1992), apparently all octopodiform cephalopods possess a complete ejaculatory apparatus with a spiral filament, or at least a spiral structure encompassed by the ejaculatory apparatus tube (e.g., Milne-Edwards 1842; Marchand 1913; Drew 1919b; Mann *et al.* 1970; Pujals 1978; Table 1). Stellate particles are apparently absent (Blancquaert 1923; Table 1), but there is evidence for the presence of modified, probably homologous structures, recorded for some species, such as *Eledone cirrhosa* (Fort 1937; Orelli 1962; Mangold-Wirz 1963). In this species, numerous sharp spines are embedded in the spiral filament matrix and “cement body”, and cover the spermatangia surface after the spermatophoric reaction (Fort 1937; Orelli 1962; Mangold-Wirz 1963). Fort (1937) proposed these spines are homologous to the stellate particles found in decapodiforms. A similar morphology is seen in *Octopus aegina*, which has "inward pointing teeth" ornamenting the spiral filament in intact spermatophores

(Norman & Sweeney 1997), and in *Enteroctopus dofleini*, which bear minute spines in the everted spiral filament (Mann *et al.* 1970: fig. 17). In other species, viscous and adhesive substances were already reported in the lumen of the ejaculatory apparatus and spiral filament (e.g., "*O. americana*", Drew 1919b); these substances adhere to the spiral filament and are spread out during ejaculation.

Unlike decapodiforms, however, implantation through tissue perforation is not a common reproductive strategy among octopods, although a few exceptions may exist (e.g., Young 1978; Gonzalez *et al.* 2008; see Table 1). In general, in octopods the mechanics and functioning of the spermatophoric reaction are quite similar to that seen in decapodiforms, but apparently in octopods this process leads to another kind of spermatangia "implantation"; in this case, after the spermatophoric reaction, the spermatangia end up "plugged" directly inside the lumen of the female reproductive tract (Fig. 1D), in some cases reaching the ovary.

In the Octopodinae (a subfamily of Octopodidae) it is still enigmatic how exactly the spermatangia (also called “spermatophoric bladder” in octopods; Mann *et al.* 1970) end up "plugged" into the oviducts and oviducal glands of females. Sperm are usually found inside the oviducal glands cavity, and always stored in specialized spermatheca located inside them (e.g., Froesch & Marthy 1975). Remnants of the spermatophoric bladders, along with masses of spermatozoa, were also found inside the oviduct lumen by Mann *et al.* (1970) in *E. dofleini*, and by Racovitza (1894a, 1894b) in *O. vulgaris*. In a female of *Pteroctopus tetracirrhus*, an intact spermatangium that “just fitted the cavity of the gland” was found by Froesch & Marthy (1975). Wells & Wells (1972) stated for *Octopus vulgaris* and *O. cyanea* that “if a female is removed and examined immediately after copulation she is found to have sperm in the oviducts and, sometimes, spermatophore cases actually protruding from the openings of the oviducts”; a similar condition was observed by Mann *et al.* (1970) in *Enteroctopus dofleini*, and by O'Dor & Macalaster (1983) in *Bathypolypus arcticus* (subfamily Bathypolypodinae). These statements strongly suggest that the ejaculating spermatophore is held by the hectocotylus near the opening of the oviduct or most probably with the ejaculatory apparatus end inside the distal oviduct (Racovitza 1894a, 1894b; Mann *et al.* 1970; Wells & Wells 1972; Wodinsky 2008), and eventually finds its way through the distal oviduct into the oviducal gland. However, how exactly the spermatangium manages to reach the lumen of the oviducal gland remains to be confirmed. The fact is, the spermatophore leaves the terminal organ and travels the spermatophoric groove of the hectocotylized arm intact (Wodinsky 2008), so the spermatophoric reaction must occur inside the female mantle cavity, or inside the oviduct. It is difficult to visualize the hectocotylus actually penetrating the entire length of the distal oviduct, inserting and placing the spermatophore or spermatophoric bladder inside the oviducal gland. Also, it would be more difficult to insert an inflated, bulbous

spermatophoric bladder than to insert a thin, intact spermatophore inside the oviduct or oviducal gland. Based on this and on the fact that spermatophore cases were found protruding from the openings of the oviducts (Wells & Wells 1972), most probably the hectocotylus inserts the intact spermatophore oral region inside the distal end of the oviduct, and then the spermatophoric reaction occurs inside the lumen of the oviduct.

Considering that a spiral filament is evidently present in most octopods of the subfamily Octopodinae (Table 1), perhaps the everting ejaculatory apparatus and spiral filament use the walls of the lumen of the distal oviduct itself to advance towards the oviducal gland, leading to the formation of a spermatophoric bladder inside the gland cavity. Presumably, the spines or adhesive substances, present in some Octopodinae, would aid the penetration by anchoring the everting tube of the spermatophore as its tip concomitantly advances towards the oviducal gland. This mechanism, therefore, would be essentially the same to that described for perforation in decapodiforms (Marian *in prep. B*).

Racovitza (1894b) suggested that the spermatophore, after being inserted in the opening of the oviduct by the hectocotylus, would penetrate and advance towards the oviducal gland by means of the spermatophoric reaction, i.e. by its evagination, although he did not propose a mechanism to explain this process. Mann (1984) was also of the opinion that the spermatophoric reaction would serve to transport the sperm mass over a long distance; he suggested also that the spermatophoric reaction would probably occur inside the oviduct.

In octopods of the subfamily Eledoninae, the process is apparently the same, but the spermatangia reach the ovarian cavity (Fort 1937; Orelli 1962; Mangold-Wirz 1963; Perez *et al.* 1990), where the sperm may be stored in the apical filaments of the oocytes until female maturity (Perez *et al.* 1990; this group lacks spermathecas in the oviducal glands). Fort (1937) and Orelli (1962) have speculated that the spermatophore in *Eledone* spp. is placed by the male inside the female mantle cavity (probably inside the oviduct), and that the spermatangium passes through the female reproductive tract to finally lie within the ovary. Fort (1937) believed the spermatangia travelled down the reproductive tract by means of oviduct peristalsis; he attributed such ability to the exposed spines from the ejaculatory apparatus (present in *E. cirrhosa*), which would prevent the spermatangia from being expelled during oviduct contractions. The fact is, these spines are not present in all eledonids (Fort 1941; Orelli 1962; Mangold-Wirz 1963); moreover, no spermatangia were found along the female reproductive tract, except for the ovary itself. Therefore, as in the case of Octopodinae, much remains to be clarified concerning spermatophore functioning in eledonids.

In the epipelagic octopods of the superfamily Argonautoidea (e.g., *Argounauta*, *Tremoctopus*, *Ocythoe*), mating, spermatophore transfer and fertilization processes are

poorly known. Males of these species are dwarf and apparently produce only one large spermatophore (Vérany & Vogt 1852); the very elaborate hectocotylus remains enclosed in a protective sac until mating. In several occasions this hectocotylized arm was found detached inside the female mantle cavity, carrying the spermatophore (e.g., Vérany & Vogt 1852; Müller 1853; Naef 1921-23). Due to its elaborate morphology, the hectocotylus was originally mistaken for a parasitic organism or even for the whole octopod male (see review in Mann 1984). Apparently, just prior to copulation, the hectocotylus everts from its sac, retrieves the spermatophore from the male terminal organ, autotomizes itself and comes to lie inside the mantle cavity of the female, in some cases reaching the distal oviducts and the ovarian cavity (Müller 1853; Naef 1921-23). Vérany & Vogt (1852), studying the dwarf male of *O. tuberculata*, described a spiral filament inside the ejaculatory apparatus of its spermatophore, and Müller (1853) went further presenting evidence for the spermatophoric reaction occurring in argonautoids. Certainly, there is still much to discover regarding argonautoid reproductive biology.

Cirrate octopods possess a highly modified spermatophore (or "sperm packet"), consisting of a simple capsule, the poles of which are provided with opercular structures (Villanueva 1992). No ejaculatory apparatus is present, and the functioning of the sperm packets is enigmatic. Aldred *et al.* (1983) found sperm packets inside the oviducal gland of *Cirrothauma murrayi*, so at least this characteristic is similar to other octopods.

4. Evolution of the cephalopod spermatophore

The discovery of the perforation system performed by the everting spiral filament during the spermatophoric reaction (Marian *submitted*; Marian *in prep. B*), and the possibility that this mechanism is widespread (and possibly modified) across coleoid cephalopods (Table 1) raise important questions regarding the origin and evolution of such a complex structure. The present study proposes two conflicting hypothesis to explain the evolution of coleoid spermatophores:

Hypothesis 1 (H1) assumes that the ejaculatory apparatus and spiral filament emerged as a mechanism that implants spermatangia deeply into the surface of the female's body, forming a temporary functional spermatheca after mating. This ability was vastly explored by coleoids, and spermatangia implantation in different regions of the female's body also evolved (e.g., within the mantle cavity, inside the ovary). Shallow implantation emerged later (more than once), associated with the evolution of female special spermatophore and seminal receptacles.

Hypothesis 2 (H2) assumes that the ejaculatory apparatus and spiral filament emerged as a mechanism that efficiently attaches spermatangia by shallowly implanting and

cementing them onto the female's body near the genital opening. These spermatangia would provide a continuous flow of spermatozoa to oocytes leaving the oviducts during spawning. Spermatophore and seminal receptacles eventually evolved associated with this kind of implantation. Deep implantation strategies emerged later, possibly independently, as a distinct reproductive strategy, maybe correlated with the adoption of oceanic and deep-water lifestyles. Because of its presumed derived nature, the "plugged" attachment type of octopods is considered in both hypotheses to have evolved secondarily.

Attempting to test these different hypotheses of spermatophore evolution in Coleoidea and also Decapodiformes, character mapping onto recently published phylogenies was employed. Phylogenetic relationships within the Cephalopoda remain somewhat unresolved, the recent proposed phylogenetic hypotheses being incongruent between one another (e.g., Lindgren *et al.* 2004; Strugnell *et al.* 2005). Moreover, many groupings are highly dependent upon the chosen DNA sequences alignment criterion, and upon the adopted method of phylogenetic analysis (Lindgren & Daly 2007; Strugnell & Nishiguchi 2007). Therefore, many distinct hypotheses of coleoid and decapodiform relationships are available in recent literature, making it almost impossible to construct minimally resolved consensus trees.

Considering only the resulting trees from studies that combined multiple sources of data, there are 9 distinct topologies for coleoid relationships (1 from Lindgren *et al.* 2004; 1 from Strugnell *et al.* 2005; and 7 from Strugnell & Nishiguchi 2007) and 5 for decapodiform relationships (Lindgren 2008). Tests were performed with all of them, but, for the sake of conciseness, only five topologies are presented herein (Figs 2 and 3; also, see methodological section). Evaluated characters (Appendix 1) are related to spermatophore structure (character 1), type of spermatophore attachment (character 2), and to the presence of special spermatophore receptacles (characters 3 to 6) and seminal receptacles (characters 7 and 8).

Spermatophore evolution within Coleoidea. The parsimonious character optimization on the basis of each phylogenetic tree unambiguously yields that a complete ejaculatory apparatus, i.e., that with a spiral filament or a spiral structure inside its tube, was already present in the stem lineage of Coleoidea (Figs 2A-C). This result reinforces the hypothesis that a complex spermatophore (= with an ejaculatory apparatus) is a synapomorphy of the Coleoidea (e.g., Young & Vecchione 1996), and indicates that the ejaculatory apparatus and spiral filament evolved together in the early evolutionary history of the Coleoidea.

The results of the two hypotheses tested were dependent upon the chosen phylogenetic tree. Both topologies from Strugnell & Nishguchi (2007) corroborate hypothesis H1, since the ancestral condition of the attachment type was unambiguously reconstructed

as being “deep implantation” in both trees (Figs 2B-C). In these phylogenetic scenarios, “shallow implantation” evolved later, twice independently on the “Bayesian” tree (Fig. 2C), or one or more times independently in the “direct optimization” tree (Fig. 2B). On the basis of the topology from Lindgren *et al.* (2004), the ancestral condition of the spermatophore attachment type was rendered equivocal, both hypothesis being equally parsimonious (Fig. 2A). In this phylogenetic scenario, shallow implantation evolved twice independently in the evolution of the Coleoidea, and deep implantation at least four times independently.

In accordance to the Bayesian phylogenetic hypothesis of Strugnell & Nishiguchi (2007), the evolution of “shallow implantation” might have been intimately associated with the evolution of seminal receptacles in coleoids (Fig. 2C), which also reinforces hypothesis H1. For example, “shallow implantation” attachment type and a seminal receptacle in the ventral region of the buccal membrane have possibly evolved together in the stem lineage of a clade comprising Spirulidae, Loliginidae, Idiosepiidae, and Sepiidae (Fig. 2C). A similar case of co-evolution between shallow implantation and seminal receptacles is seen in the Illicine + Ommastrephinae clade (Fig. 2C). The “direct optimization” phylogenetic tree of Strugnell & Nishiguchi (2007) also supported the hypothesis of associated evolution between shallow implantation and seminal receptacles (Fig. 2B), but the presence of many equivocal character states in important stem lineages within Coleoidea prevents further discussion. Character mapping onto Lindgren *et al.* (2004) phylogenetic hypothesis do not recover robust associations between the evolution of shallow attachment and specialized receptacles (Fig. 2A).

Spermatophore evolution within Decapodiformes. Character mapping onto both Decapodiformes phylogenetic hypotheses evaluated recovered the ejaculatory apparatus containing a spiral filament as the ancestral condition (Figs 3A-B), supporting the hypotheses already discussed above about the early emergence of a complete ejaculatory apparatus at the base of clade comprising all coleoids. Testing hypotheses H1 and H2 on the basis of these phylogenetic trees yielded completely distinct scenarios. In accordance to the parsimony topology of Lindgren (2008), shallow implantation emerged first in the stem lineage of Decapodiformes, and deep implantation arose later at least three times independently (Fig. 3A). This evolutionary scenario is in more accordance with hypothesis H2, which assumes that shallow implantation is the ancestral condition within coleoids.

Contrarily, character reconstruction onto the maximum likelihood topology of Lindgren (2008) unambiguously yields that the attachment type of the ancestral decapodiform lineage was “deep implantation” (Fig. 3B). According to this evolutionary scenario, shallow implantation evolved later three times independently: in the stem lineages of 1) “Enoploteuthoidea” (Enoploteuthidae, Lycoteuthidae, Pyroteuthidae), 2) Ommastrephidae (Ommastrephinae and Illicinae), and 3) a clade comprising Thysanoteuthidae,

“Bathyteuthoidea”, Idiosepiidae, Loliginidae, Sepiolidae, Spirulidae, and Sepiidae. The evolution of these independent events was intimately associated with the emergence of seminal and spermatophores receptacles, as follows: nuchal receptacles in “Enoploteuthoidea”, buccal seminal receptacles in Ommastrephidae, and buccal seminal receptacles in the third group, all having evolved independently. Therefore, this whole scenario is in more agreement with hypothesis H1, which assumes that deep implantation is the ancestral condition, shallow implantation arising later in association with the evolution of specialized receptacles.

Discussion

Based on the character-mapping analyses, the ejaculatory apparatus emerged complete in the stem lineage of Coleoidea, i.e. as an inverted "tube" (composed of membranes and tunics) encompassing a spiral filament in the oral region of the spermatophore, this "tube" being evaginated during copulation in the so-called spermatophoric reaction. There are still few records of stellate particles or spines ("teeth") within the spiral filament of coleoids (see Table 1). However, they are known to occur in most major taxa (Myopsida, Oegopsida, Sepioidea and possibly Octopoda), suggesting that future will show them to be common. If the presence of such structures is proven to be widespread within Coleoidea there might be enough evidence to test if the ejaculatory apparatus has emerged with an ornamented spiral filament (= one with spines or stellate particles).

Assuming that the ejaculatory apparatus emerged complete with a spiral filament, and was capable of evaginating, one is left with questions about the function of the ancestral coleoid spermatophore. Would the “everting helix perforating mechanism” of the ejaculatory apparatus have emerged as a system that deeply implanted spermatangia into the surface of the female body (H1), similar to the condition seen, for example, in architeuthids and octopoteuthids? The emerging of a spermatophore that implants itself almost completely into the body surface of the female would have brought important adaptive advantages as an efficient attachment mechanism, storing spermatozoa until the time of spawning, augmenting the basic function of a spermatophore, and maximizing chances of fertilization. Moreover, the deeply implanted spermatangia could have acted also like temporary functional spermathecas, allowing temporal separation between copulation and fertilization events.

Alternatively, would the ejaculatory apparatus have emerged as a mechanism that efficiently attached the spermatangia to the female body through shallow perforation (H2)? Superficial perforation opens a breach allowing the penetration of cement substances, possibly augmenting attachment (Marian *in prep. A*). The emerging of a spermatophore that has the ability to effectively attach itself on the female’s body (e.g., near the genital opening)

would also have brought important adaptive advantages. These firmly attached spermatangia could provide a continuous flow of spermatozoa to the oocytes during spawning (like in loliginids; Drew 1911), or they could store spermatozoa for some time after mating, also acting like temporary functional spermathecas (like in *Illex* spp.; e.g., O'Dor 1983).

Based on the presented character-mapping analyses, both hypotheses H1 and H2 are phylogenetically plausible (Figs 2 and 3). Theoretically, the basic mechanics of this complex structure leads to tissue perforation, by means of the evagination of a tube containing a spiral filament ornamented with sharp structures (Marian *in prep. B*). Therefore, being its basic nature to perforate and implant into tissue, initially the author was of the opinion that this mechanism would have arisen in the evolutionary history of Coleoidea as a "spermatangium implantation device", similar to deep implantation seen in recent coleoids. However, as shown above, support for this hypothesis is dependent upon the phylogenetic tree under consideration.

Spermatophores and controlled fertilization are widely present in the animal kingdom and increase the likelihood of successful fertilization (e.g., Mann 1984). Recent nautiloids have a relatively simple spermatophore composed of a coiled sperm mass enclosed in a spherical capsule, which is transferred to the female by modified and fused appendages, called the spadix (Arnold 1984; Mann 1984). No ejaculatory apparatus is present, and, hence, there is neither spermatophoric reaction nor tissue perforation. During mating, the capsule is shed and the spermatophore becomes attached to the female organ of Valenciennes by an unknown mechanism (Haven 1977; Arnold 1984; Mann 1984). The nautiloid spermatophore performs the basic spermatophore function of minimizing sperm loss during mating, and optimizing the chances of fertilization by acting as a “storage container and transport vehicle for spermatozoa”, also protecting them from the hostile external environment (Mann 1984).

By contrast, coleoids are very active animals that have emerged presumably in response to the evolutionary pressure created by efficient and active predators emerging and rising in the Devonian and early Carboniferous (Packard 1972; Aronson 1991; Nishiguchi & Mapes 2008). The intense predation and competition pressures to which they were presumptively subjected to during the course of their evolutionary history are believed to have deeply molded coleoid evolution (e.g., Packard 1972; O'Dor & Webber 1986; Aronson 1991). The limitations of the molluscan morphological, physiological and biochemical bauplan have presumably favored the evolution of the typical coleoid rapid growth rates and short life cycles (i.e., rapid turnover of the population; O'Dor & Webber 1986). Possibly intimately associated with these evolutionary paths are the coleoid highly developed nervous and sensorial systems (Nixon & Young 2003), voracious feeding and efficient raptorial and

digestive organs (Bidder 1966), and a sophisticate camouflage mechanism (Hanlon & Messenger 1996), to name a few (see Packard 1972 for a detailed account).

The emergence of several locomotory abilities associated with the adoption of an active mode of life could presumably have brought a limitation to spermatophore transfer and attachment. Coleoids are constantly and vigorously pumping water in and out of their mantle cavity, as well as skillfully and rapidly swimming. As a consequence, superficial areas of the female body, including the surfaces within the mantle cavity, are exposed to considerable water turbulence, creating substantial resistance against attachment in such exposed areas. Hypothetically, a nautiloid-like spermatophore (presumably plesiomorphic within Cephalopoda) would most likely be ineffective and probably be lost during or after mating in this case. A mechanism augmenting spermatophore attachment, as seen in extant decapodiforms, would have been evolutionary advantageous to coleoids, and would have countered the changes associated with a very active mode of life.

Mating in decapodiforms can be very fast, lasting about few seconds in several teuthoids (Hanlon & Messenger 1996). This condition may be another response to high predation pressures, since copulation involves a distraction from the surrounding environment (Hoving *et al.* 2009). Fast mating is made possible by the spermatangia implantation mechanism, which is indeed a high-speed process (e.g., Hoving & Laptikhovsky 2007; Hoving *et al.* 2009; Marian *in prep. A*) requiring only some seconds or few minutes to accomplish its task. Moreover, this mechanism is an autonomous process (e.g., Marian *in prep. B*), which implies that copulation may be even faster: after retrieving a group of spermatophores from the terminal organ with the aid of the hectocotylus, the male has simply to transfer them near to the site of attachment on the female body. The implantation process is evidently not dependent upon the handling of the spermatophore by the hectocotylus (see videos[7] provided by Marian *submitted* and Marian *in prep. A*, as well as Hoving & Laptikhovsky 2007), consequently the duration of the mating embrace may be shorter than spermatangia implantation.

Finally, present evidence indicates that, by acting like temporary functional spermathecas, coleoid spermatangia provide sperm storage, which may lead to delayed fertilization. For example, in the cases where shallow implantation occurs, the sperm continuously flowing out of the spermatangia (e.g., Drew 1919a) is either stored for long periods in specialized seminal receptacles located on the buccal membrane (e.g., Drew 1911; Ikeda *et al.* 1993a; Nigmatullin *et al.* 1995; Hanlon *et al.* 1999; Nigmatullin & Markaida 2009; Table 1), or accessed in the hours or days following mating, during egg encapsulation (e.g., Drew 1911; Fields 1965; Hanlon & Messenger 1996). Sperm release is a process that

[7] Ver CD em anexo à Tese, contendo material suplementar.

may last relatively long periods of time depending on the species and environmental temperature, lasting up to one day in *D. pealeii* (Drew 1919a), and up to eight days (at 8 °C) in *Sthenoteuthis pteropus* (Zuyev *et al.* 2002). A specific mechanism preventing the escape of spermatozoa was postulated to be present in *Illex illecebrosus*, since intact, full spermatangia have been recovered from females at least five days after copulation (O'Dor 1983). In several cases where deep implantation occurs, the presence of an escape route for the spermatozoa from the embedded spermatangia was reported (e.g., Racovitza 1894c; Nesis *et al.* 1998; Hoving *et al.* 2004; Hoving *et al.* 2008a), similar to spermatangia that are shallowly implanted. Therefore, in this case the sperm might also be used in the hours or days following copulation, during the flow of spermatozoa out of the spermatangia. However, the fact that submature females, of distinct species, were found with deeply implanted spermatangia (e.g., Norman & Lu 1997; Hoving *et al.* 2004; Jackson & Jackson 2004; Hoving *et al.* 2008a) suggests also that some unknown mechanism that prevents the escape of the spermatozoa might be present, permitting later access to the sperm by the female. Considering the high predation pressure to which coleoids are (and have been) subjected to, this temporal separation of reproductive events would allow more control over two seemingly dangerous procedures, possibly reducing the risk of predation during copulation and/or spawning, which may, in turn, have an effect on sexual selection. Individuals would be allowed to mate whenever they get the chance, even if not at the proper time (i.e., immature females). Also, with delayed fertilization, the female is allowed to wait for favorable conditions to spawn.

The herein tested character mappings must be considered as a first step in the study of the evolution of the cephalopod spermatophore. The matrixes contained a high number of missing data, which reflect our general lack of knowledge on spermatophore morphology (see Table 1). Moreover, some characters are likely to be divided into more states as the spermatophore morphology and reproductive biology of several species are assessed. For instance, the character “attachment type” is probably composed of more than four states, because “deep implantation” could refer to two spermatangia types: deeply implanted bulbous spermatangia (e.g., sepiolids and octopoteuthids; Fig. 1B), and deeply implanted elongated spermatangia (e.g., cranchiids and architeuthids; Fig. 1A). Further morphological investigations are needed to elucidate these issues.

Continued collection of detailed morphological data (descriptive and functional) of the spermatophore of various coleoid species, together with the expected advancement of the resolution of the higher level cephalopod phylogeny, will certainly provide a more robust picture on the origin and evolution of this unique and striking feature of coleoid reproduction.

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Table 1 Distribution of spermatophore-related characters across extant cephalopod families. Literature is referenced by numbers in parentheses, and listed below the table.

Family / Subfamily	Spiral filament	Stellate particles	Type of attachment	Site of attachment	Seminal receptacle *s. str.*
Loliginidae	+ (1)	+ (2-4)	Shlw (4, 5)	VBM; MC (6-8)	+ VBM (6-9)
Australiteuthidae	+ (10)	Unk	Unk	Unk	Unk
Bathyteuthidae	+ (1)	Unk	Unk	Unk	Unk
Chtenopterygidae	+ (1)	Unk	Unk	Unk	Unk
Architeuthidae	+ (1, 11)	Unk	Deep (12)	MOS; A; T; H (12)	– (12)
Brachioteuthidae	+ (1)	Unk	? (13)	BM (13, 14)	Unk
Batoteuthidae	Unk	Unk	Unk	Unk	Unk
Chiroteuthidae	? (1)	Unk	Deep (15)	Ov (15)	Unk
Joubiniteuthidae	? (1)	Unk	Unk	Unk	Unk
Magnapinnidae	? (16)	Unk	Unk	Unk	Unk
Mastigoteuthidae	? (1)	Unk	Unk	Unk	Unk
Promachoteuthidae	Unk	Unk	Unk	Unk	Unk
Cranchiidae					
Cranchiinae	– (1)	Unk	Deep? (13, 14, 17)	MOS; MC; H (13, 14, 17)	– (14)
Taoniinae	+ (1, 18)	Unk	Deep (13, 14, 18, 19, 20)	MOS; MC (13, 18, 19, 20)	? (14, 18)
Cycloteuthidae	Unk	Unk	Deep (21)	MOS (21)	Unk
Ancistrocheiridae	+ (1)	Unk	Shlw? (22)	NR (22)	– (22)
Enoploteuthidae	+ (1, 23)	Unk	Shlw (23-25)	MC or NR (23-25)	– (23-25)
Lycoteuthidae	+ (1)	Unk	Shlw (26)	NR (26)	– (26)
Pyroteuthidae	+ (1)	Unk	Shlw? (22)	NR (22)	– (22)
Gonatidae	+ (1, 27)	Unk	? (14, 28, 29)	BM or MC (14, 28, 29)	Unk
Histioteuthidae	+ (1, 30, 31)	Unk	Deep (14, 31)	MC; MOS; A; BM (14, 31)	– (14, 31)
Psychroteuthidae	Unk	Unk	Unk	Unk	Unk
Lepidoteuthidae	Unk	Unk	Unk	Unk	Unk
Octopoteuthidae	+ (1)	Unk	Deep (14, 32, 33)	MOS; A; H (14, 32, 33)	– (32, 33)
Pholidoteuthidae	? (1)	Unk	Deep (34)	MOS (34)	– (34)

Family / Subfamily	Spiral filament	Stellate particles	Type of attachment	Site of attachment	Seminal receptacle *s. str.*
Neoteuthidae	+ (1)	Unk	Unk	Unk	Unk
Ommastrephidae					
Illicinae	+ (1)	Unk	Shlw (35, 36)	MC (35, 36)	– (35, 36)
Ommastrephinae	+ (1)	Unk	Shlw (13, 37, 38)	BM (13, 37-40)	+ BM (39-41)
Todarodinae	+ (1)	+ (42)	Shlw (43, 44)	BM (43, 44, 45)	+ BM (44, 45)
Onychoteuthidae	+ (1, 46)	Unk	Deep (14, 47, 48, 49)	MOS; MC (14, 29, 47, 49)	– (14, 29)
Thysanoteuthidae	+ (1)	Unk	Shlw? (14, 50)	BM (14, 50)	+ BM (51)
Idiosepiidae	Unk	Unk	? (52)	VMB; A; H; MOS (52-54)	+ VMB (55)
Sepiidae	+ (56, 57)	+ (57, 58)	Shlw (59, 60)	VMB (59-61)	+ VMB (59-61)
Sepiolidae					
Heteroteuthinae	Unk	Unk	Deep or NI (62)	H & A or PSS (62)	– (62)
Rossiinae	+ (63-65)*	+ (65)	Deep (62, 63, 66, 67)	MOS; H; MC (62)	– (62)
Sepiolinae	+ (56, 68)*	+ (56-58, 68, 69)	? (62, 68)	BC (62, 70)	– (62, 70, 71)
Sepiadariidae	? (30)	Unk	Unk	VMB (14)	+ VMB (14, 72)
Spirulidae	+ (73)	Unk	Unk	VMB (14, 72)	+ VMB (14, 72)
Amphitretidae	Unk	Unk	Unk	Unk	+ Ovg (74)
Bolitaenidae	Unk	Unk	Deep? (13)	A (13)	+ Ovg (74)
Vitreledonellidae	Unk	Unk	Unk	Ovd (14)	+ Ovg (74)
Alloposidae	Unk	Unk	Unk	Hct (14)	+ Ovg (74)
Argonautidae	+? (75, 76)	Unk	Unk	Hct (14, 75, 76, 77)	+ Ovg (74)
Ocythoidae	+ (76)	Unk	Unk	Hct (14, 76, 77)	+ Ovg (74)
Tremoctopodidae	+? (75, 76)	Unk	Unk	Hct (14, 75, 76, 77)	+ Ovg (74)
Octopodidae					
Bathypolypodinae	+ (78, 79)	Unk	Plug (80)	Ovd & Ovg (80)	+ Ovg (80)
Eledoninae	+ (56, 81-87)	Spines (56, 82, 88)	Plug (82, 88, 89)	Ov (82, 88, 89)	– (89, 90), AFO (89)
Graneledoninae	+ (91)	Unk	Unk	Unk	Unk
Octopodinae	+ (56, 92-99)	–(58); teeth (95,100)	Plug (90,95,101); Shlw?(102)	Ovd & Ovg (90,95,101); Ov?(102)	+ Ovg (90) or – (102,103)
Cirroteuthidae	– (79, 104)	Unk	Unk	Ovg (104)	? (104)

Family / Subfamily	Spiral filament	Stellate particles	Type of attachment	Site of attachment	Seminal receptacle *s. str.*
Opisthoteuthidae	– (79, 105, 106)	Unk	Unk	Unk	Unk
Stauroteuthidae	– (107)	Unk	Unk	Unk	Unk
Vampyroteuthidae	+ (1)	Unk	? (108)	SRE (108, 109)	? (108)
Nautilidae	– (110, 111)	–	NI (112)	Val (112)	– (112)

Symbols and abbreviations: +, present; **–**, absent; **?**, uncertain; **Unk**, Unknown; **Shlw**, shallow implantation (*i.e.*, only the base of the spermatangium is implanted into the female tissue); **Deep**, deep implantation (all or nearly all the length of the spermatangium is implanted into the female tissue); **Plug**, the spermatangium is "plugged" into the distal oviduct, finding its way through it into the oviductal gland or into the ovary; **NI**, spermatangia are not implanted into the female tissue; **VBM**, ventral region of the buccal membrane; **MC**, within the mantle cavity in the vicinity of the genital opening; **MOS**, mantle outer surface; **A**, arms; **T**, tentacles; **H**, head region; **BM**, buccal membrane; **NR**, nuchal region; **BC**, bursa copulatrix; **PSS**, posterior seminal sac; **Hct**, spermatophore is placed inside the elaborate hectocotylus, which is autotomized and deposited within the female mantle cavity; **Ovd**, oviducts; **Ovg**, oviducal glands; **Ov**, ovary; **AFO**, sperm stored in the apical filament of the oocytes; **SRE**, spermatophore receptacles, large subcutaneous pouches located on either side of the head, in front of the eye; **Val**, organ of Valenciennes; *****, indirect evidences for the presence of the spiral filament in sepiolids (see text for discussion). **References: (1)** Hess, 1987; **(2)** Austin *et al.* (1964); **(3)** Badenhorst (1974); **(4)** Marian (*in prep. A*); **(5)** Lum-Kong (1992); **(6)** Drew (1911); **(7)** Fields (1965); **(8)** Hanlon & Messenger (1996); **(9)** Oordt (1938); **(10)** Lu (2005); **(11)** Knudsen (1957); **(12)** Hoving *et al.* (2004); **(13)** Young (1978); **(14)** Nesis (1995); **(15)** Young (1972); **(16)** Vecchione & Young (2008); **(17)** Young (1975); **(18)** Voss (1985); **(19)** Nesis *et al.* (1998); **(20)** Laptikhovsky & Arkhipkin (2003); **(21)** Clarke (1980); **(22)** Young & Harman (1998); **(23)** Burgess (1991); **(24)** Burgess (1998); **(25)** Tsuchiya & Okutani (1990); **(26)** Hoving *et al.* (2007); **(27)** Pearcy & Voss (1963); **(28)** Kristensen (1981); **(29)** Laptikhovsky *et al.* (2007); **(30)** Dell (1952); **(31)** Voss *et al.* (1998); **(32)** Hoving *et al.* (2008a); **(33)** Hoving *et al.* (2010); **(34)** O'Shea *et al.* (2007); **(35)** O'Dor (1983); **(36)** Durward *et al.* (1980); **(37)** Nesis (1983); **(38)** Snÿder (1998); **(39)** Arkhipkin *et al.* (1998); **(40)** Zuyev *et al.* (2002); **(41)** Nigmatullin & Markaida (2009); **(42)** Takahama *et al.* (1991); **(43)** Ikeda *et al.* (1993a); **(44)** Ikeda *et al.* (1993b); **(45)** Nigmatullin *et al.* (2002); **(46)** Nesis & Nikitina (1986); **(47)** Jackson & Jackson (2004); **(48)** Hoving & Laptikhovsky (2007); **(49)** Bolstad (2007); **(50)** Nigmatullin *et al.* (1991); **(51)** Nigmatullin *et al.* (1995); **(52)** Kasugai (2000); **(53)** Voss (1963); **(54)** Nabhitabhata & Suwanamala (2008); **(55)** Kasugai *et al.* (2004); **(56)** Marchand (1913); **(57)** Blancquaert (1925); **(58)** Blancquaert (1923); **(59)** Hanlon *et al.* (1999); **(60)** Wada *et al.* (2005); **(61)** Hall & Hanlon (2002); **(62)** Hoving *et al.* (2008b); **(63)** Reid (1991); **(64)** Racovitza (1894c); **(65)** Hoving *et al.* (2009); **(66)** Cuccu *et al.* (2007); **(67)** Laptikhovsky *et al.* (2008); **(68)** Weill (1927); **(69)** Duvernoy (1853); **(70)** Singley (1983); **(71)** Jereb & Roper (2005); **(72)** Nesis (1987); **(73)** Kerr (1931); **(74)** Voight (1997); **(75)** Müller (1853); **(76)** Vérany & Vogt (1852); **(77)** Naef (1921-23); **(78)** Toll (1981); **(79)** Voss & Pearcy (1990); **(80)** O'Dor & Macalaster (1983); **(81)** Meyer (1911a, 1911b); **(82)** Fort (1937); **(83)** Voss (1964); **(84)** Palacio (1978); **(85)** Lu & Stranks (1991); **(86)** Allcock (2005); **(87)** Palacio (1977); **(88)** Orelli (1962); **(89)** Perez *et al.* (1990); **(90)** Froesch & Marthy (1975); **(91)** Kommritz (2000); **(92)** Drew (1919b); **(93)** Pujals (1978); **(94)** Hochberg *et al.* (2006); **(95)** Mann *et al.* (1970); **(96)** Norman & Finn (2001); **(97)** Norman *et al.* (2004); **(98)** Voss (1975); **(99)** Norman *et al.* (2005); **(100)** Norman & Sweeney (1997); **(101)** Wells & Wells (1972); **(102)** Gonzalez *et al.* (2008); **(103)** Morales (1973); **(104)** Aldred *et al.* (1983); **(105)** Meyer (1906); **(106)** Villanueva (1992); **(107)** Collins & Henriques (2000); **(108)** Pickford (1946); **(109)** Pickford (1949); **(110)** Arnold (1984); **(111)** Mann (1984); **(112)** Haven (1977).

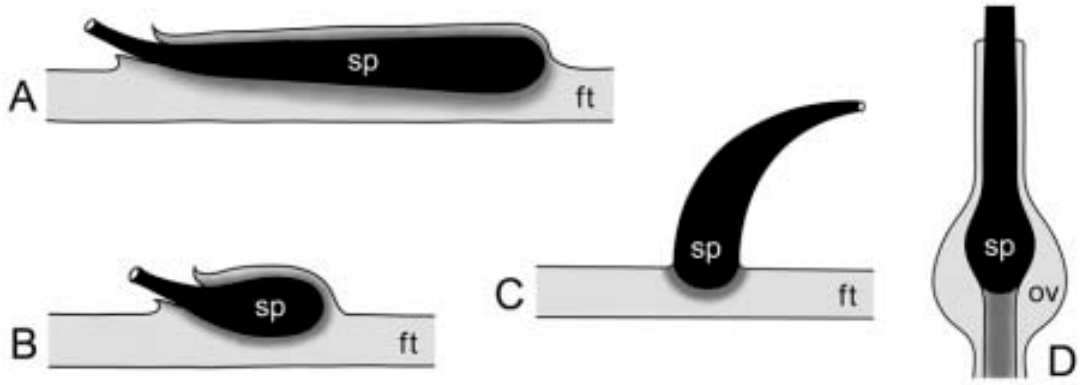

Fig. 1 A-D. Types of spermatophore attachment in coleoids. – A. Deep implantation (elongated spermatangium; e.g., Architeuthidae, Cranchiidae). – B. Deep implantation (bulbous spermatangium; e.g., Octopoteuthidae, Sepiolidae). – C. Shallow implantation (e.g., Loliginidae, Ommastrephidae, Enoploteuthidae). – D. Plugged attachment (e.g., incirrate octopods). Abbreviations: ft, female tissue; ov, oviducal gland; sp, spermatangium.

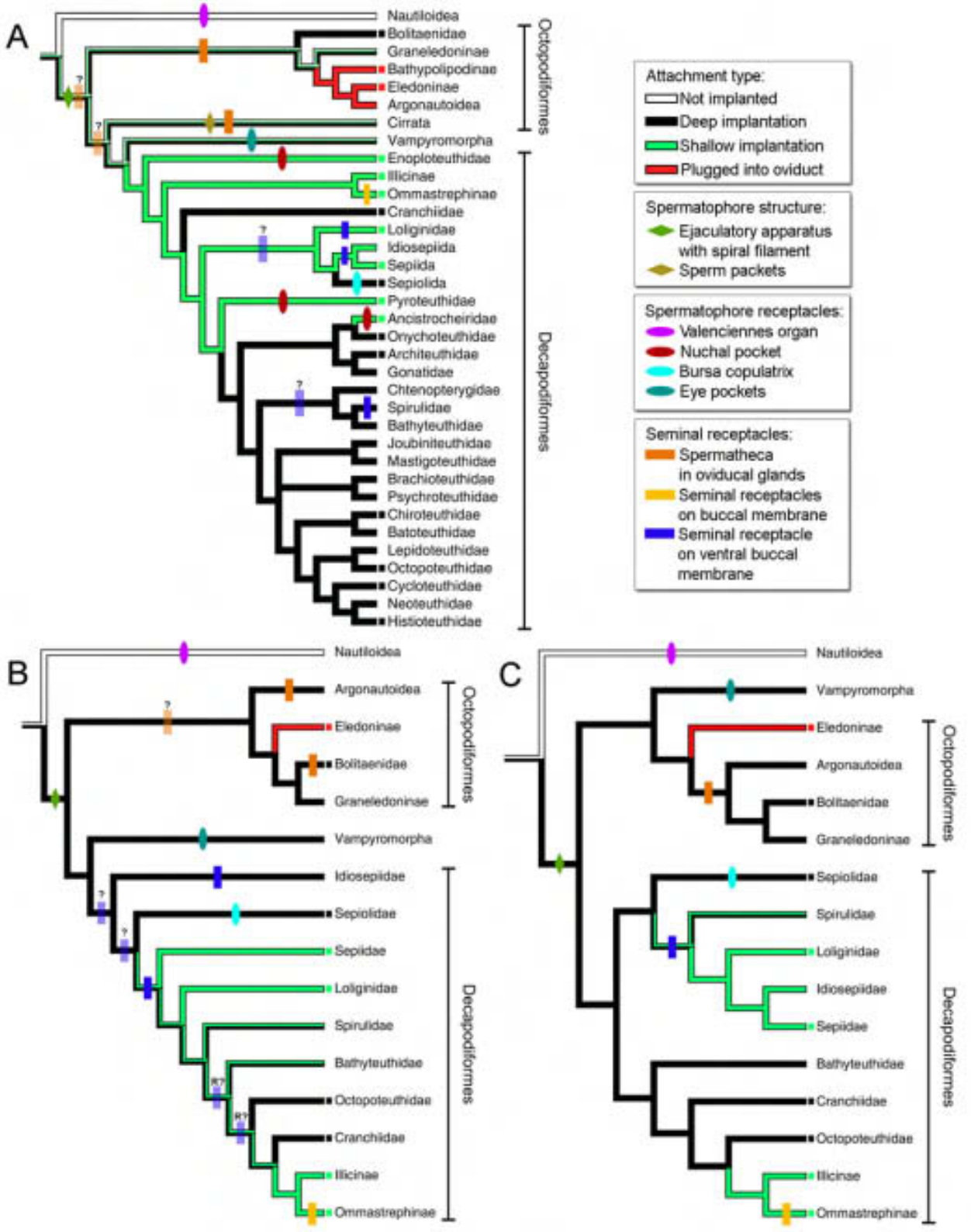

Fig. 2 A-C. Spermatophore related characters parsimonious optimization using different hypothesis for the internal relationships of Coleoidea. Character states are traced and indicated by shape and color codes; colored branches (and colored boxes to the left of taxon names) refer to the spermatophore attachment type, and colored symbols to the remaining characters. Presence of more than one color in branches or rectangles, and the presence of a question mark both indicate equivocal character states. "R" refers to reversal events. – A. Tree according to the direct optimization analysis of Lindgren *et al.* (2004). – B. Tree according to the direct optimization analysis of Strugnell & Nishiguchi (2007). – C. Tree according to the Bayesian analysis of Strugnell & Nishiguchi (2007).

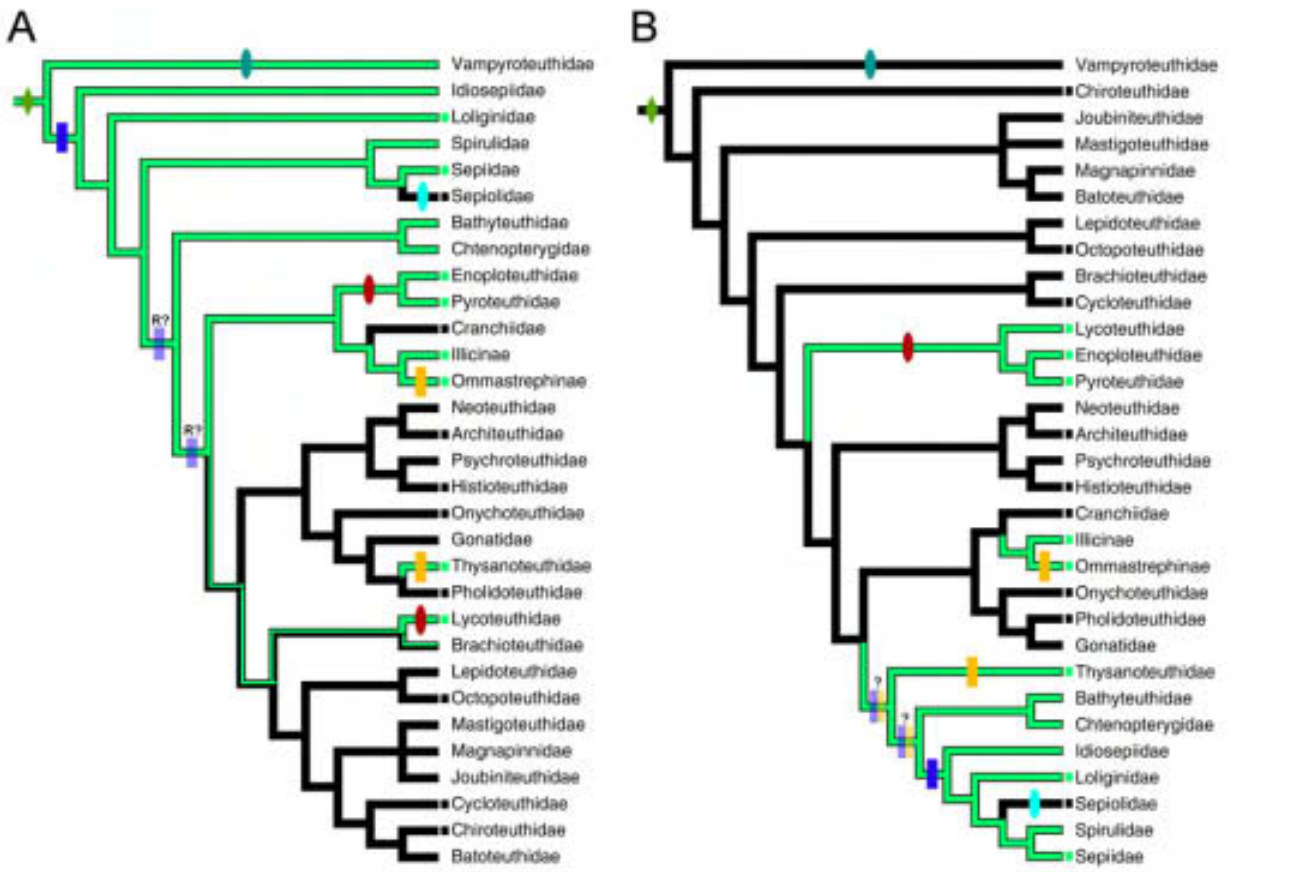

Fig. 3 A-B. Spermatophore related characters parsimonious optimization using different hypothesis for the internal relationships of Decapodiformes. Character states are traced and indicated by shape and color codes; colored branches (and colored boxes to the left of taxon names) refer to the spermatophore attachment type, and colored symbols to the remaining characters (see shape and color legends on Fig. 2). Presence of more than one color in branches or rectangles, and the presence of a question mark both indicate equivocal character states. "R" refers to reversal events. – A. Tree according to the parsimony analysis of Lindgren (2008). – B. Tree according to the maximum likelihood analysis of Lindgren (2008).

Appendix 1 Characters and character state descriptions.

Coding of the character states for the included species is based on several studies (see Table 1 for references).

I. Ejaculatory apparatus – existence and structure: (0) absent; (1) absent, spermatophores modified as opercular sperm packets; (2) present, without a spiral filament; (3) present, with a spiral filament. Put simply, an ejaculatory apparatus is an invaginated tube present in the oral region of the spermatophore of most coleoid cephalopods, and absent in nautiloids. It is composed of a complex of membranes and tunics which are everted during the so-called spermatophoric reaction. Cirrata are an exception, as they present spermatophores modified as opercular sperm packets. The term “spiral filament” is applied herein in the broader sense, i.e. referring to a spiral construction or structure inside the tube of the ejaculatory apparatus. Apparently, this structure is quite variable among coleoids, varying from conspicuous and thick (e.g., octopoteuthids, some cranchiids) to fine and tightly coiled (e.g., most oegopsids).

II. Spermatangia attachment – type: (0) not implanted; (1) shallowly implanted; (2) deeply implanted; (3) “plugged” (into the oviduct and oviducal gland, or into the ovary). In nautiloids, spermatophores are simply deposited on the female spermatophore receptacle, the Valenciennes organ. In coleoids, a range of attachment types occurs. In the “shallow implantation” attachment type, only the base of the spermatangium is implanted (and cemented) into the female tissue (e.g., loliginids, ommastrephids, sepiids; Fig. 1C). In deep implantation, all or nearly all the length of the spermatangium is implanted (e.g., architeuthids, octopoteuthis, cranchids, sepiolids; Figs 1A,B). In incirrate octopods, the spermatophore is “plugged” into the distal oviduct, finding its way through it into the oviducal gland (Fig. 1D), or even into the ovary (herein considered as the same state). Deep implantation could actually refer to more than one state (e.g., those that form bulbous bodies inside female tissue, and those that maintain the elongated form; see Figs 1A,B), but the lack of information on this subject prevents the inclusion of such information in the analysis.

III. Valenciennes organ – existence: (0) absent; (1) present. The organ of Valenciennes is a lamellated and glandular spermatophore receptacle located on the ventral head region of the female in nautiloids.

IV. Nuchal spermatophore receptacle – existence: (0) absent; (1) present. Spermatophore receptacles are present in the nuchal region of squids of the enoploteuthid families (Ancistrocheiridae, Enoploteuthidae, Lycoteuthidae, and Pyroteuthidae).

V. Bursa copulatrix (pharetra) – existence: (0) absent; (1) present. The bursa copulatrix is considered a unique specialization of Sepiolinae, a subfamily of Sepiolidae; it involves the modification of the distal oviduct for spermatophore reception. The subfamily

Heteroteuthinae presents another modification, the posterior seminal sac, which also functions as a spermatophore receptacle. Species of the subfamily Rossiinae apparently do not bear any special modification for spermatophore reception. Given the scope of the present study, and for the sake of clarity, the character "bursa copulatrix" was coded as "present" for the terminal "Sepiolidae", although not all representatives of the family bear this modification.

VI. Spermatophore receptacles located in front of the eyes – existence: (0) absent; (1) present. In the vampire squid *Vampyroteuthis infernalis*, spermatophore receptacles are present as large subcutaneous pouches located in front of the eyes.

VII. Seminal receptacles on the buccal membrane – existence, number and location: (0) absent; (1) several receptacles distributed circularly on the buccal membrane; (2) paired or single receptacle located in the ventral region of the buccal membrane. Seminal receptacles *stricto sensu* are specialized sperm storage organs, which bear morphological and histological modifications enabling them to storage spermatozoa released from the spermatangia. In some oegopsids, numerous seminal receptacles occur in a ring around the buccal membrane. In loliginids and some "sepioids" there is only one main organ (single or paired) located ventrally on the buccal membrane.

VIII. Spermatheca located in the oviducal glands: (0) absent; (1) present. In octopods, seminal receptacles *stricto sensu*, when present, are located inside the oviducal glands. Octopods from the genus *Eledone* lack spermatheca in the oviducal glands and store spermatozoa inside the ovary; in some species of this genus, the apical filaments of the oocytes serve as a sperm storage site (Perez *et al.* 1990). Given that only two species have been confirmed to bear this feature, this state was not coded in the present study.

Appendix 2 Species character matrix.

	CHARACTERS							
TAXA:	I	II	III	IV	V	VI	VII	VIII
Nautiloidea	0	0	1	0	0	0	0	0
Cirrata	1	?	0	0	0	0	0	1
Bolitaenidae	?	2	0	0	0	0	0	1
Graneledoninae	3	?	0	0	0	0	0	?
Bathypolipodinae	3	3	0	0	0	0	0	1
Eledoninae	3	3	0	0	0	0	0	0
Argonautoidea	3	?	0	0	0	0	0	1
Vampyromorpha	3	?	0	0	0	1	0	0
Idiosepiida	?	?	0	0	0	0	2	0
Loliginidae	3	1	0	0	0	0	2	0
Sepiida	3	1	0	0	0	0	2	0
Sepiolida	3	2	0	0	1	0	0	0
Spirulidae	3	?	0	0	0	0	2	0
Bathyteuthidae	3	?	0	0	0	0	?	0
Chtenopterygidae	3	?	0	0	0	0	?	0
Architeuthidae	3	2	0	0	0	0	0	0
Brachioteuthidae	3	?	0	0	0	0	?	0
Batoteuthidae	?	?	0	0	0	0	?	0
Chiroteuthidae	?	2	0	0	0	0	?	0
Joubiniteuthidae	?	?	0	0	0	0	?	0
Magnapinnidae	?	?	0	0	0	0	?	0
Mastigoteuthidae	?	?	0	0	0	0	?	0
Cranchiidae	2&3	2	0	0	0	0	?	0
Cycloteuthidae	?	2	0	0	0	0	?	0
Ancistrocheiridae	3	1	0	1	0	0	0	0
Enoploteuthidae	3	1	0	1	0	0	0	0
Lycoteuthidae	3	1	0	1	0	0	0	0
Pyroteuthidae	3	1	0	1	0	0	0	0
Gonatidae	3	?	0	0	0	0	?	0
Histioteuthidae	3	2	0	0	0	0	?	0
Psychroteuthidae	?	?	0	0	0	0	?	0
Lepidoteuthidae	?	?	0	0	0	0	?	0
Octopoteuthidae	3	2	0	0	0	0	0	0
Pholidoteuthidae	?	2	0	0	0	0	0	0
Neoteuthidae	3	?	0	0	0	0	?	0
Illicinae	3	1	0	0	0	0	0	0
Ommastrephinae	3	1	0	0	0	0	1	0
Onychoteuthidae	3	2	0	0	0	0	0	0
Thysanoteuthidae	3	1	0	0	0	0	1	0

Considerações Finais

CONSIDERAÇÕES FINAIS

As evidências obtidas e reunidas pela presente Tese de Doutorado permitiram reavaliar os processos envolvidos na reação espermatofórica dos cefalópodes, acarretando em mudanças conceituais importantes relacionadas a este tema (Tabela 1). Em primeiro lugar, propõe-se que a principal função da reação espermatofórica (i.e., do processo de evaginação do espermatóforo) é fixar ou implantar o espermatângio no corpo da fêmea. Igualmente importante, é a proposição de que a fixação ou implante dar-se-iam durante a reação espermatofórica, em contraposição a um conceito anterior bastante difundido de que esses processos ocorreriam somente após a reação.

Outra mudança conceitual importante está relacionada ao mecanismo de fixação ou implante. Propõe-se que tal mecanismo seria intrínseco à estrutura do espermatóforo dos Decapodiformes e responsável pela perfuração do tecido-alvo, podendo resultar em dois tipos básicos de implante de espermatângio: profundo ou superficial. Em ambos os tipos de implante, a perfuração seria executada por um mecanismo de "hélice invertida" executado pelo filamento espiral e suas espículas durante a eversão do aparato ejaculatório. No implante superficial, a ação sinérgica dessas e outras estruturas do espermatóforo (e.g., corpo cimentante e membrana interna) está envolvida em um complexo processo de fixação mecânica e química do espermatângio no corpo da fêmea.

No caso dos polvos, propõe-se que a reação espermatofórica também deva ter importante papel no processo de transferência de espermatozóides, por auxiliar no avanço do espermatóforo através do canal do oviduto, resultando na formação do espermatângio no lúmen da glândula oviducal, onde está localizada a espermateca. O mecanismo responsável por esse processo seria semelhante àquele descrito para os Decapodiformes, porém sem haver perfuração de tecido.

Os resultados obtidos por meio do estudo da morfologia dos espermatóforos de *Doryteuthis plei* revelaram o quão pouco sabemos a respeito desta complexa estrutura. A combinação de diversas ferramentas de análise morfológica permitiu avanço no entendimento da organização estrutural do espermatóforo. Recomenda-se, portanto, a continuidade dos estudos morfológicos detalhados acerca dos espermatóforos dos coleóides. Considera-se igualmente importante a busca por uma metodologia simples e eficiente para processamento dos espermatóforos, os quais são, em geral, de difícil preparação para microscopia. Como já exposto na Introdução e no Capítulo 1, a utilização de espermatóforos em descrições taxonômicas é ainda muito restrita. Com a elaboração de um protocolo rápido de preparação total de espermatóforos (i.e., amostras inteiras montadas e coradas em lâminas), outros pesquisadores (e.g., sistematas) poderiam usufruir mais

facilmente das informações morfológicas disponíveis na complexa estrutura dos espermatóforos dos coleóides. Em contrapartida, o produto gerado por esses estudos (i.e., lâminas depositadas em museus científicos), bem como a própria informação resultante da investigação (i.e., descrições morfológicas), estariam amplamente disponíveis para estudos de morfologia funcional e evolução dos espermatóforos. A experimentação realizada no âmbito da presente Tese permitiu solucionar o problema da semi-permeabilidade da túnica externa, a qual dificulta a infiltração de diversos reagentes necessários durante a preparação dos espermatóforos. Testes de diversas técnicas de preparação e coloração disponíveis na literatura já estão sendo efetuados pelo presente autor, de modo que se espera que a proposta desse protocolo metodológico ocorra em pouco tempo.

Como apontado acima para a estrutura do espermatóforo, recomenda-se a continuidade dos estudos relativos à reação espermatofórica dos coleóides. Ainda sabemos muito pouco sobre esse fenômeno, tanto em espécies costeiras como oceânicas. Os resultados da presente Tese permitiram avanço no entendimento desse processo, porém as necessidades de investigações e de refinamento da análise são amplas. Por exemplo, o processo de fixação química do espermatângio aparenta ser altamente complexo, envolvendo evidentemente distintos componentes, os quais devem atuar em diferentes fases da reação. Refinar o conhecimento acerca dessas substâncias, tanto no espermatóforo intacto como nos espermatângios fixados na fêmea, deverá fornecer elementos importantes para avaliar seu papel funcional.

Igualmente complexa e necessitando de estudos de refinamento é a fixação física do espermatângio, complexo processo que envolve: 1) estabelecimento de contato e perfuração ou escarificação superficial do tecido-alvo pelo filamento espiral e respectivas espículas; 2) fixação primária e otimização da fixação química pela membrana interna e respectivas espículas; e 3) possivelmente, ação de ancoragem pela ponta afilada do corpo cimentante. Além disso, o rearranjo estrutural do corpo cimentante durante a reação espermatofórica, envolvido na exteriorização de distintas substâncias químicas, é um processo extremamente rápido e de difícil acompanhamento. Novas abordagens no estudo da reação espermatofórica, como o emprego de câmeras de vídeo de alta freqüência, para registrar a reação a milhares de quadros por segundo, poderão auxiliar na investigação desses processos.

Faz-se necessária, portanto, a continuidade da experimentação *in vitro* da reação espermatofórica, bem como das investigações acerca da morfologia de espermatóforos em evaginação e espermatângios fixados, devendo-se ampliar esse estudo para outras espécies de coleóides. A recente descoberta de espermatóforos funcionais em espécimes que haviam sido congelados após a coleta (Hoving & Laptikhovsky, 2007; Hoving *et al.*,

2009) permite afirmar que as perspectivas na área são excelentes, pois torna-se factível a expansão das investigações *in vitro* para espécies oceânicas e de águas profundas.

O modelo teórico proposto na presente Tese para explicar o fenômeno de implante de espermatângios carece ainda de testes. Estes poderiam ser feitos a partir de modelagem computacional, traduzindo-se o modelo para equações matemáticas, associado à experimentação *in vitro*, para tentar calcular as forças resultantes do mecanismo proposto e compará-las às forças necessárias para penetração no tecido. Dependendo dos resultados obtidos pela continuidade das investigações acerca da reação espermatofórica, o modelo proposto está sujeito ao refinamento, à complementação ou, até mesmo, à completa refutação. Os processos que levam à penetração do espermatóforo no tecido da fêmea não são passíveis de observação direta. Dependemos, portanto, de evidências isoladas (e.g., morfologia, composição química etc.) para compreensão desse fenômeno. Nesse contexto, a proposição de hipóteses e modelos teóricos é imprescindível para auxiliar no entendimento do processo e nortear estudos futuros. É importante, entretanto, não ficarmos presos a essas hipóteses e modelos, de forma a não limitar nossa capacidade de avaliação das evidências.

Finalmente, com relação ao estudo da evolução do espermatóforo e da reação espermatofórica em Cephalopoda, prevê-se igualmente grandes avanços nesta área no futuro próximo. Alguns dos dados faltantes da tabela apresentada no Capítulo 5 deverão ser preenchidos rapidamente à medida que especialistas de distintos grupos tomem conhecimento da mesma. Outros dados faltantes poderão ainda ser coletados diretamente por meio de consulta e análise de materiais de coleções científicas. Somando-se esses fatores às excelentes perspectivas de aumento da resolução das relações filogenéticas dos cefalópodes (Nishiguchi & Mapes, 2008), espera-se poder, em pouco tempo, repetir o mapeamento de caracteres com uma base de dados mais completa e utilizando uma filogenia mais robusta de Cephalopoda.

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Tabela 1. Mudanças conceituais propostas pela presente Tese, relacionadas à reação espermatofórica dos cefalópodes.

Processo	Conceitos anteriores à Tese	Conceitos defendidos pela presente Tese
Função da reação espermatofórica nos Decapodiformes	Exteriorização da massa espermática e corpo cimentante no momento da cópula (e.g., Drew 1919). Obs.: A reação espermatofórica está INDIRETAMENTE ligada ao processo de fixação/implante. O corpo cimentante exteriorizado conteria substâncias adesivas responsáveis pela adesão do espermatângio (e.g., Drew, 1919) ou enzimas responsáveis pela dissolução do tecido-alvo (e.g., Hoving *et al.*, 2009).	Fixar ou implantar o espermatângio no corpo da fêmea. Obs.: A reação espermatofórica está DIRETAMENTE ligada ao processo de fixação/implante, que ocorre durante a reação. Obs.: A "exteriorização da massa espermática e corpo cimentante no momento da cópula" ocorre concomitantemente ao processo de fixação/implante; este conceito não é modificado com a nova proposta.
Função da reação espermatofórica nos Octopodiformes (Incirrata)	Transporte da massa espermática por uma longa distância (Mann, 1984). Alcançar o lúmen da glândula oviducal, onde está localizada a espermateca (Racovitza, 1894).	Complementando o conceito de Racovitza (1894): alcançar o lúmen da glândula oviducal, onde está localizada a espermateca, por meio da evaginação do aparato ejaculatório e filamento espiral.
Momento da fixação ou do implante do espermatângio	APÓS a reação espermatofórica (e.g., Drew, 1919; Nesis *et al.*, 1998).	DURANTE a reação espermatofórica.

Processo	Conceitos anteriores à Tese	Conceitos defendidos pela presente Tese
Distinção do mecanismo responsável pela fixação dos espermatângios nos Decapodiformes	A "fixação superficial" e o "implante profundo" dos espermatângios seriam mediados por mecanismos completamente distintos (e.g., Hoving *et al.*, 2009).	Um mecanismo similar, aparentemente intrínseco à estrutura do espermatóforo, seria responsável por parte do processo fixação do espermatângio (perfuração).
Estruturas responsáveis pela "fixação superficial" do espermatângio no corpo da fêmea (e.g., Loliginidae, Sepiidae, Ommastrephidae)	Conteúdo adesivo e alcalino do corpo cimentante (e.g., Drew, 1919; Austin *et al.*, 1964); ponta afilada do corpo cimentante (e.g., Takahama *et al.*, 1991). A base do espermatângio é aderida ao sítio de deposição na fêmea.	Aparato ejaculatório, filamento espiral, espículas, conteúdo adesivo do corpo cimentante, ponta afilada do corpo cimentante, membrana interna com espículas. A base do espermatângio é implantada e cimentada no sítio de deposição na fêmea ("implante superficial").
Estruturas responsáveis pelo "implante profundo" do espermatângio no corpo da fêmea (e.g., Architeuthidae, Octopoteuthidae, Onychoteuthidae, Sepiolidae)	A perfuração do tecido da fêmea seria realizada por meio de enzimas histolíticas liberadas pelas túnicas ou pelo corpo cimentante (e.g., Hoving *et al.*, 2009).	A perfuração do tecido da fêmea seria realizada durante a evaginação do aparato ejaculatório, por meio de um mecanismo de "hélice invaginada" desempenhado pelo filamento espiral e garantido pela ancoragem proporcionada por suas espículas.
Mecanismo responsável pelo avanço do espermatângio através do oviduto em Incirrata	Evaginação do aparato ejaculatório (Racovitza, 1894).	No caso dos polvos, um mecanismo semelhante ao dos Decapodiformes auxiliaria no avanço do espermatóforo em evaginação ao longo do canal do oviduto, até a formação do espermatângio final no lúmen da glândula oviducal.

Resumo

Cefalópodes coleóides (lulas, sépias e polvos) produzem espermatóforos muito elaborados, os quais são transferidos à fêmea durante a cópula por meio de um apêndice modificado nos machos. Durante a transferência à fêmea, os espermatóforos sofrem, de forma autônoma, drásticas modificações na chamada "reação espermatofórica", complexo processo de evaginação do aparato ejaculatório, que conduz à exteriorização da massa espermática e corpo cimentante. Poucos trabalhos abordam com detalhes a morfologia e anatomia funcional dos espermatóforos dos cefalópodes, grande parte do conhecimento acerca da estrutura do espermatóforo tendo sido gerada por trabalhos clássicos do século XIX e início do século XX. Investigações acerca do funcionamento dos espermatóforos são consideravelmente mais raras, estando o conhecimento básico sobre a reação espermatofórica restrito a apenas 20 espécies de coleóides. Como o funcionamento extracorpóreo dos espermatóforos depende exclusivamente da intrincada estrutura e organização de seus componentes (e.g., membranas e túnicas), somente investigações detalhadas dessas estruturas proverão as bases para a compreensão do funcionamento e da exata função do complexo espermatóforo dos coleóides. Nesse contexto, a presente Tese, organizada em cinco capítulos, teve como objetivo principal investigar a estrutura e o funcionamento dos espermatóforos da lula *Doryteuthis plei* (Blainville, 1823). No primeiro capítulo, com o objetivo de se estudar a fundo a organização estrutural dos espermatóforos da espécie, diversas técnicas de microscopia foram testadas e empregadas. Como resultado da combinação de diferentes ferramentas de análise, a estrutura do espermatóforo revelou-se ainda mais complexa, sendo as principais descobertas referentes à: 1) elaborada estrutura da membrana mediana, organizada em camadas e apresentando um segmento aboral quimicamente distinto, que envolve parte do corpo cimentante; 2) presença de um material reticulado preenchendo o espaço entre a túnica interna e a membrana mediana (discute-se a possibilidade do mesmo consistir em um fluido viscoso em espermatóforos intactos); 3) presença de espículas intimamente associadas à membrana interna na região do corpo cimentante (além das espículas embebidas no filamento espiral); 4) presença de extensões membranosas que delimitam uma câmara pré-oral na região do capuz; e 5) complexa organização estrutural do corpo cimentante, delimitado por duas camadas e contendo substâncias de distintas propriedades químicas. Uma avaliação cuidadosa da literatura permite sugerir que pelo menos parte dessas características deva ser comum aos espermatóforos de outros loliginídeos, e, em alguns casos, de outros grupos de coleóides. Como parte da investigação acerca da reação espermatofórica e dos mecanismos envolvidos na fixação da massa espermática no corpo da fêmea, constatou-se que, sob

condições artificiais, espermatóforos em evaginação são capazes de penetrar musculatura exposta, de forma similar ao fenômeno de "implante profundo" observado naturalmente em algumas lulas oceânicas. Esse resultado foi descrito no segundo capítulo, no qual foi levantada a hipótese de que um mecanismo de perfuração seria inerente à estrutura dos espermatóforos dos coleóides. Dando continuidade ao estudo da morfologia funcional dos espermatóforos de *D. plei*, o terceiro capítulo apresenta os resultados obtidos a partir da investigação do funcionamento do espermatóforo e da morfologia dos espermatângios (i.e, espermatóforos evertidos) obtidos *in vitro*, bem como daqueles naturalmente fixados na fêmea. As evidências reunidas permitem afirmar que o processo de fixação compreende distintas fases desempenhadas por diversos componentes do espermatóforo, contrariamente a um conceito anterior de que a fixação seria realizada somente por substâncias adesivas do corpo cimentante. Durante a reação espermatofórica, o aparato ejaculatório e respectivo filamento espiral são capazes de perfurar superficialmente ou escarificar o tecido-alvo. Subseqüentemente, o corpo cimentante sofre drástica modificação estrutural, resultando na extrusão de parte do conteúdo cimentante, o qual é injetado diretamente sobre o tecido perfurado. Além disso, o corpo cimentante é exteriorizado com uma extremidade afilada que, em alguns casos, foi encontrada firmemente implantada no tecido da fêmea, juntamente com as substâncias cimentantes. Concomitantemente ao processo de reconfiguração do corpo cimentante, a região da membrana interna que contém as espículas no espermatóforo intacto é evertida e estirada sobre a base do espermatângio, sugerindo um papel auxiliar no processo de fixação. Com base em evidências da literatura, bem como nas obtidas no âmbito da presente Tese, no quarto capítulo propõe-se um modelo teórico para explicar como o aparato ejaculatório em evaginação seria capaz de perfurar e implantar-se no corpo da fêmea durante a reação espermatofórica. Sugere-se que a perfuração seria mecânica e resultado da ação conjunta do aumento gradual do diâmetro dos anéis do filamento espiral e da distância entre os mesmos, bem como do poder de ancoragem proporcionado pelas respectivas espículas. Finalmente, o quinto capítulo apresenta uma revisão da literatura acerca do fenômeno de implante de espermatóforos em Decapodiformes, e reúne evidências que corroboram o modelo teórico proposto. Neste capítulo, é apresentada também uma reinterpretação da função da reação espermatofórica em Octopodiformes. Com base no levantamento de diversos caracteres reprodutivos, foi possível testar hipóteses de evolução da estrutura do espermatóforo e do sistema de implante dos espermatângios, bem como hipóteses de co-evolução de estruturas envolvidas no processo de transferência e armazenamento de espermatozóides. Duas hipóteses principais acerca da evolução do sistema de implante dos espermatângios são propostas.

Abstract

Male coleoid cephalopods produce elaborate spermatophores, which are transferred to the female during mating. These spermatophores are capable of functioning autonomously and extracorporeally, undergoing complicated changes during the so-called spermatophoric reaction, i.e., a complex process of evagination of the spermatophoric tunics and membranes that, ultimately, leads to the extrusion and attachment of the sperm mass on the female's body. Few detailed morphological studies regarding this structure have yet been conducted, and much of the knowledge on the coleoid spermatophore was generated by classical studies of the 19th and early 20th centuries; furthermore, investigations on the functioning of this structure are even rarer. Since the extracorporeal functioning of coleoid spermatophores must rely entirely on the intricate structure and organization of the tunics, membranes, and other structures composing the spermatophore, only detailed investigations of these components would provide the basis for comprehending its mechanics. On these grounds, the present five-chapter Thesis aimed to provide solid evidence that could allow for postulating hypothesis on the functioning and evolution of this unique structure. In the first chapter, an investigation of the morphology of the spermatophore of *Doryteuthis plei* (Blainville, 1823) applying several microscopy techniques was carried on. A much more complex structural arrangement was revealed for the loliginid spermatophore, the most striking findings being: 1) the complex, layered structure of the middle membrane, which bears an additional, chemically distinct segment surrounding part of the cement body; 2) the presence of a space between the inner tunic and middle membrane filled with a fine reticulated material, presumably a viscous fluid in the fresh state; 3) the presence of stellate particles not only embedded in the spiral filament, but also closely applied to the inner membrane at the level of the cement body; 4) the presence of a pre-oral chamber in the cap region; and 5) the complex organization of the cement body, formed by two distinct layers encompassing contents of different chemical and textural properties. Careful literature reassessment suggests several of these features are common to loliginids, and to some extent to other squids. Their possible functional implications are discussed in light of our knowledge on the spermatophoric reaction mechanics. As part of the investigation on the spermatophoric reaction, and the mechanisms involved in the attachment of the sperm mass on the female's body, it was found that the everting spermatophore, when directed towards the incised region of an experimental tissue sample, was able to readily penetrate the artificially exposed musculature, almost resembling natural deep implantation observed in some oceanic and deep-sea squids; this finding is reported in the second chapter, where it was hypothesized that the mechanism involved in deep implantation could be inherent to the

spermatophore structure of all squids. The third chapter investigated the functional morphology of the spermatophore of the squid *D. plei* applying *in vitro* analysis of the reaction, as well as light and electron microscopy investigation of spermatangia (everted spermatophores containing the sperm mass) obtained either *in vitro* or naturally attached on the female. Hitherto unnoticed functional features of the loliginid spermatophore revealed herein required a reappraisal of some important processes involved in the spermatophoric reaction, as well as the proposal of new hypotheses to explain their mechanics. The most striking findings concern to the attachment mechanism, which is not carried out solely by cement adhesive material, as previously believed, but rather by a complex process performed by multiple structures that lead to the implantation of the base of the spermatangium into the female body. Firstly, the everting ejaculatory apparatus is presumably able to superficially puncture the female tissue. Subsequently to this process, the cement body passes through a complex structural rearrangement, which leads to the injection of both its viscid cement contents and pointed oral region through the puncture into the female tissue. When the inner membrane at the oral region of the cement body is everted, its sharp stellate particles are exposed, presumptively adhering to the scarified tissue and augmenting attachment by assuring the injection of the cement material inside the superficial hole. The functioning of the loliginid spermatophore is revisited in light of these findings. The forth chapter, building upon evidence from the literature along with evidence from these experiments, proposes a theoretical model to explain how the everting ejaculatory apparatus would be able to mechanically perforate, and concomitantly implant the spermatophore into the female body during the spermatophoric reaction. It is proposed that this process is achieved chiefly through the combination of 1) an "evaginating-helix" mechanism performed by the everting ejaculatory apparatus' spiral filament, and 2) the anchorage provided by its numerous, minute sharp stellate particles. Finally, the fifth chapter reviews the literature concerning the phenomenon of implantation of spermatophores in decapodiforms, and presents evidence corroborating the proposed theoretical model ascribing the role of implantation to the mechanical perforation performed by the spiral filament. The mechanisms of spermatophore transfer are also reviewed for octopodiforms, and a reinterpretation of the function of the spermatophoric reaction in this case is provided. In light of parsimonious character optimizations performed onto recently published phylogenetic trees, a complete ejaculatory apparatus with a spiral filament, as well as the spermatophoric reaction, apparently emerged once and early in the evolution of the Coleoidea. This novelty possibly provided an efficient attachment mechanism and presumably countered the changes associated with the adoption of an active mode of life by coleoids, augmenting fertilization success. Two main hypotheses for the evolution of the complex spermatophore within Coleoidea and Decapodiformes are proposed.

Anexo 1

Ethical and welfare considerations when using cephalopods as experimental animals

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RESEARCH PAPER

Ethical and welfare considerations when using cephalopods as experimental animals

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Abstract When using cephalopods as experimental animals, a number of factors, including morality, quality of information derived from experiments, and public perception, drives the motivation to consider welfare issues. Refinement of methods and techniques is a major step in ensuring protection of cephalopod welfare in both laboratory and field studies. To this end, existing literature that provides details of methods used in the collection, handling, maintenance, and culture of a range of cephalopods is a useful starting point when refining and justifying decisions about animal welfare. This review collates recent literature in which authors have used cephalopods as experimental animals, revealing the extent of use and diversity of cephalopod species and techniques. It also highlights several major

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issues when considering cephalopod welfare; how little is known about disease in cephalopods and its relationship to senescence and also how to define objective endpoints when animals are stressed or dying as a result of the experiment.

Keywords Animal welfare · Animal ethics · Capture · Cephalopods · Cuttlefish · Handling · Housing · *Nautilus* · Octopus · Squid

Introduction

There is a long tradition of using cephalopods as experimental animals, particularly squid and octopus, in the field of neurophysiology (e.g. Young 1971; Wells 1978). However, more recently, the use of a range of cephalopod species for field and laboratory experiments has increased, largely due to their importance in fisheries and their potential in aquaculture. Experiments using cephalopods range from use of neural and optic material (e.g. Eyman et al. 2003), to tank experiments that manipulate biotic and abiotic growth conditions (e.g. Sykes et al. 2003), to tagging individuals and releasing them into the wild to obtain information about their ecology, biology, and behaviour (e.g. Nagasawa et al. 1993; Gilly et al. 2006). These experiments all seek to obtain information that will contribute to an accurate and informative picture of biology and/or ecology of the animal.

When considering the welfare of vertebrate animals in experiments it is recommended that the three R's (reduction, replacement, and refinement) be considered. This involves ensuring that the number of animals used in the experiments is valid (reduction), considering alternatives to live animals in experiments (replacement), and adoption of experimental methods that minimize distress to the animals (refinement). We recommend that the three R's should be a major consideration when using cephalopods in experiments. When addressing the question of whole animal biology there are limited opportunities to replace animals with alternatives, such as computer simulations and cell cultures. However, replacement is worth considering for species groups which have juveniles that are currently impossible to rear (e.g. ommastrephids and Idiosepiidae). Given the 100% mortality rates of these juveniles in culture conditions, it may be worth considering using alternative cephalopod species, for which we have developed culture techniques, as ''model species''.

Reduction and refinement are important areas to address when planning and designing experiments. Given the need to provide good water quality and live food when maintaining many cephalopods (Table 1), it is typically difficult to hold large numbers of adult cephalopods. As a result logistics will restrict the numbers of animals, however, smaller individuals (small adults and juveniles) can be potentially held in larger numbers. Therefore, reduction in the number of animals can be addressed a number of ways including, ensuring that animals in research facilities are held to answer direct biological results and not only to see if they can be held. For many commercially important species details of maintenance and culture are readily available (Tables 2–8). The number of animals needed in an experiment to determine differences among treatments is a function the variability among replicate animals. Such information is often difficult to obtain, however, an increasing number of publications (Tables 2–8) are presenting results of rigorous experiments. These studies provide estimates of inter-animal variability that is readily usable as a guide for future experimental designs. Refining the methods associated with all aspects of using cephalopods as experimental animals is one of the three R's that experimenters can readily address and should be given full consideration. Refinement based on past experience (as reported in the literature) is the major consideration of this paper.

We have identified existing expertise and literature that outlines techniques for handling and holding cephalopods in different life stages, from embryo to adults (e.g. Teuthoids and Sepioids in Boletzky and Hanlon 1983; Hanlon 1990). This depth of knowledge provides an informed basis upon which cephalopod biologists can justify decisions about the welfare of animals used in scientific endeavours. However, this realization needs to be tempered with an understanding and recognition that cephalopods have unique biological characteristics that need consideration (Table 1). In particular the very short lifespan of most species means that death in captivity

Table 1 General biological characteristics of cephalopod of particular relevance when handling and holding

Biological characteristics	Special consideration
External protection	No protection (except for Nautilus) and the delicate skin is readily damaged by physical contact e.g. handling and contact with the side of tanks. All species are marine and most have limited capacity to tolerate changes to salinity changes.
Mobility	Active species e.g. pelagic species will need to swim constantly, may jet out of tank, and repeatedly hit side of tank. Some benthic species need shelter or hides.
Response to stress e.g. attack, toxic substances, disease	Cephalopods will display a startle and escape response. The copious volumes of black ink should be removed. Self damage can occur through eating of arms and repeated contacting with the side of tanks. Lesions will be evident where damage to the skin has occurred, these lesions may be an early sign of senescence.
Food	All species are carnivores and most require live prey, especially during early life history. Artificial food suitable for cephalopod is not yet available. Cannibalism can occur when insufficient food provided. Prey size is limited by body size rather than mouth size. Prey items up to 150% larger than the juvenile can be captured and eaten.
Life span	Many inshore cephalopod species live for 12 months or less, so adults brought into the laboratory are likely to die in captivity due to natural senescence. Some species will die shortly after egg production or after hatching of juveniles.
Reproductive biology	Range of reproductive traits, with some species producing a single batch of eggs at end of life while others will produce numerous batches. Most cephalopod species produce external eggs that are sheathed and well protected by a mucilaginous coat. A suitable substrate or the presence of an egg mass often needs to be provided to encourage egg deposition. Egg size varies with species, with large egg species being easier to culture in captivity. Larger eggs have longer development times, but can be vulnerable to fungal infections the longer they are held. Removal of the parents is not necessary and in the case of *Octopus* species the females must be left in the tank to care for eggs. Eggs with no maternal care will need to be oxygenated using a gentle flow of water across the eggs.
Respiration	Oxygen is taken up via the gills and skin.
Social behaviour	Some species naturally school, particularly squid, and space and water quality needs to be carefully monitored. Some species are non-aggregative and keeping high densities of mixed genders can cause problems with constant interactions resulting in death.
Early life history	Cephalopods have a short (ca. 5 days) endogenous feeding period during which they must learn to capture prey. Many species suffer extremely high juvenile mortality in a laboratory setting, possibly due to starvation when predation behaviour fails to develop. There is variation in the size and life style of cephalopod hatchlings; small-egged species usually produce planktonic young while large-egged species produce benthic young. The latter have been easier to rear, because the juveniles look and behave as miniature adults capable of jetting, inking and prey capture.
Behaviour	Complex and diverse (Hanlon and Messenger 1996). There will need to an awareness of ''typical'' behaviours for the species being held in captivity. In particular the need for objects to hide under or in, and the interaction among individuals held in the same tanks.

(not initiated by the experimenter) is the norm not the exception, particularly following spawning (e.g. octopus species).

In most countries ethical guidelines for the use of animals in experiments are restricted to vertebrate species. As invertebrates, ethical guidelines for the use and handling of animals in science do not include cephalopods. However, cephalopods have a well developed nervous system (Young 1971; Wells 1962; Budelmann 1995) and display advanced behaviours (Hanlon and Messenger 1996), suggesting that welfare guidelines for these animals are needed. The presence of free nerve endings in the skin suggests that perception of pain is possible and behavioural responses suggest that many cephalopods do respond to pain (Mather and Anderson in press). The concept of pain can be extended to include psychological suffering, and the appropriateness of culture conditions, including behavioural enrichment should be considered (Mather 1986, 2001). Currently, there is no universal standard or legislation concerning the welfare of cephalopods, and the adoption of

Table 2 Examples of recent literature that has used Nautilidae as experimental animals

Species	Collection, Handling, Transport	Housing	Food and Feeding	Eggs, embryos and juveniles	Anaesthetics and Euthanasia	Behaviour	Treatment of Diseases	Tagging and Tracking
N. macromphalus	1, 2, 3	2, 4	2, 5, 6, 7	2, 6, 7		1, 2		3, 4
N. belauensis	2	2	2	2, 8, 9		1, 10, 11		
N. pompilius	1, 2, 12, 13, 14	2, 4, 12, 15, 16, 17, 18	2	2, 17, 19		1, 12, 15, 17		4, 12, 20
Unnamed N. species	12, 16	12, 16, 21		22		12, 22, 23, 24		12, 22, 24

(1) Ward (1987); (2) Hamada et al. (1980); (3) Saunders (1983); (4) Hamada (1987); (5) Ward and Wicksten (1980); (6) Ward (1983); (7) Mikami and Okutani (1977); (8) Okubo et al. (1995); (9) Arnold et al. (1990); (10) Carlson et al. (1984); (11) Saunders (1984); (12) Saunders and Landman (1987); (13) Saunders and Spinosa (1978); (14) Wells and Wells (1985); (15) Zann (1984); (16) Carlson (1987); (17) Fields (2006); (18) Westermann et al. (2004); (19) Arnold et al. (1993); (20) O'Dor et al. (1990a); (21) Spinosa (1987); 22. Boyle and Rodhouse (2005); (23) O'Dor et al. (1993); (24) Ward et al. (1984)

Table 3 Examples of recent literature that has used Sepiida as experimental animals

Life Stage	Collection, handling, and transport	Housing	Feeding	Reproduction	Behaviour	Anaesthetics	Health, disease, and treatment	Tagging and tracking
Eggs	1, 2, 3, 4, 5	6, 7, 4, 8					2, 3	
Juveniles		4, 9, 10, 11, 12, 13, 14	14, 15, 16	17, 18, 19, 20, 21	22			
Adults	23	9, 10, 22, 24, 25, 26, 27	28, 29	5, 9, 19, 30, 31, 32, 33	19, 34, 35, 36, 37, 38	39	21, 24, 40, 41, 42, 43,	44, 45, 46

(1) Blanc and Daguzan (1998); (2) Boletzky (1998); (3) D'Aniello et al. (1990); (4) Minton et al. (2001); (5) Oka (1993); (6) Bouchaud and Daguzan (1989); (7) Cronin and Seymour (2000); (8) Paulij et al. (1991); (9) Correia et al. (2005); (10) Forsythe et al. (2002); (11) Hanley et al. (1998); (12) Koueta and Boucaud-Camou (1999); (13) Sykes et al. (2003); (14) Domingues et al. (2003); (15) Blanc et al. (1998); (16) Moltschaniwskyj and Martínez (1998); (17) Boal and Ni (1996); (18) Boletzky and Roeleveld (2000); (19) Crook et al. (2002); (20) Hanlon and Messenger (1996); (21) Warnke (1994); (22) Sherrill et al. (2000); (23). Forsythe et al. (1991); (24) Hanley et al. (1999); (25) Loi and Tublitz (1999); (26) Sykes et al. (2006); (27) Domingues et al. (2002); (28) Castro and Lee (1994); (29) Domingues et al. (2005); (30) Boletzky (1987); (31) Corner and Moore (1980); (32) Hall and Hanlon (2002); (33) Nabhitabhata and Nilaphat (1999); (34) Adamo et al. (2000); (35) Boal et al. (1999); (36) Castro and Guerra (1989); (37) Lipiński et al. (1991); (38) Quintela and Andrade (2002); (39) Aitken et al. (2005); (40) Castro et al. (1992); (41) Halm et al. (2000)l; (42) Sangster and Smolowitz (2003); (43) Reimschuessel et al. (1990); (44) Ezzedine-Najai et al. (1995); (45) Jackson et al. (2005); (46) Watanuki and Iwashita (1993)

Table 4 Examples of recent literature that has used Sepiolida as experimental animals

Species	Collection, handling, and transport	Housing	Feeding	Eggs, embryos and juveniles	Behaviour
Sepietta spp, *Rossia* spp, *Sepiola* spp, *Euprymna scolopes*	1, 2, 3	1, 2, 3, 4	2, 3, 4, 5	1, 2, 6, 7, 8, 9, 10	2, 5, 11, 12

(1) Summers (1985); (2) Summers and Colvin (1989); (3) Hanlon et al. (1997); (4) Claes and Dunlap (2000); (5) Bergström (1985); (6) Salman (1998); (7) Anderson and Shimek (1994); (8) Boletzky (1975); (9) Yau and Boyle (1996); (10) Arnold et al. (1972); (11) Moynihan (1982); (12) Shears (1988)

Table 5 Examples of recent literature that has used Idiosepiidae as experimental animals

Species	Capture, Handling, Transport	Housing	Feeding	Eggs, embryos and juveniles	Behaviour
Idiosepius biserialis			1		
I. notoides	2, 3	3	2, 3, 4		
I. macrocheir					
I. paradoxus			5	6, 7, 8, 9	
I. picteti					
I. pygmaeus	5, 10, 11		12, 13	6, 8, 14, 15	17, 18
I. thailandicus	19, 20	19, 20	19, 20	20	

(1) Hylleberg and Nateewathana (1991); (2) English (1981); (3) Tracey et al. (2003); (4) Eyster and Van Camp (2003); (5) Kasugai (2001); (6) Kasugai (2000); (7) Kasugai and Ikeda (2003); (8) Natsukari (1970); (9) Yamamoto (1988); (10) Jackson (1992); (11) Moynihan (1983); (12) Jackson (1989); (13) Semmens (1993); (14) Jackson (1993); (15) Lewis and Choat (1993); (16) Van Camp (1997); (17) Roberts (1997); (18) Sasaki (1923); (19) Nabhitabhata (1994); (20) Nabhitabhata (1998)

legislation by scientists is patchy. For example, in the UK *Octopus vulgaris* is included in the legislation, Canada includes all cephalopods, and in Australia and USA legislation is in place for some research institutes in some states. The future implementation of legislation for the ethical use of cephalopods is unclear and research scientists will be responsible for determining the legal requirements for their country and/or research institute when using cephalopods in experiments. For example, the status of cephalopods in the EU legislation for animal ethics is currently under review, and one recommendation is that all cephalopods are included in the legislation.

Legislation aside, there are a suite of reasons that cephalopod research scientists may need to consider the welfare of their study animals. These include moral and ethical issues associated with experimental manipulations and the need to ensure that obtaining information in experiments is with minimal distress to the animals. This becomes important for scientists with limited experience or restricted access to experienced researchers, who may have difficulty obtaining guidance about what may be the ''right'' or suitable method to manage cephalopods, and is critical to maximising the scientific value of animals used in experiments.

On a pragmatic level, experiments obtain information about the biology and ecology of cephalopods that cannot be obtained directly from wild animals (e.g. factors affecting growth and reproduction, movement, and behaviour), by either holding animals in captivity or handling animals prior to release into the wild. In these cases, it is essential that experiments accurately reflect the biology of the species, as there is often an explicit interest in extrapolating these results to wild populations, or at least understanding the processes that shape wild populations. To do this it is vital that the handling and maintenance conditions of these experiments are as close to ''natural'' as possible, therefore animal welfare will be an important element of these experiments. However, cephalopod researchers must also be aware of, and sensitive to, the public perception of their experiments and the animals upon which these experiments are conducted. The natural charisma of cephalopods draws significant public interest (e.g. Anderson 2000), and their complex behaviours are subject to frequent anthropomorphism.

Table 6 Examples of recent literature that has used Loliginidae as experimental animals

Life stage	Capture Handling Transport	Housing	Feeding	Anaesthetics	Treatment of diseases	Artificial fertilization	Tagging and tracking	Behaviour
Eggs/ Embryos	1, 2, 3, 4	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 22, 23, 42, 44, 46, 47			1, 4, 20	21		
Hatchlings/ Paralarvae		1, 2, 4, 5, 6, 9, 10, 16, 17, 18, 19, 24	1, 2, 4, 6, 9, 10, 16, 17, 19, 24, 25, 26, 27, 28, 43	18, 19				
Juveniles/ Adults	29, 30, 31, 32	1, 2, 4, 10, 29, 31, 32, 33, 34, 45	1, 2, 4, 10, 27, 31, 32, 33, 35, 43	1, 31, 36, 41	20, 31, 37, 38		29, 39, 40	32, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58

(1) Lee et al. (1994); (2) Lee et al. (1998); (3) Steer et al. (2003); (4) Walsh et al. (2002); (5) Nabhitabhata et al. (2001); (6) Cardoso et al. (2005); (7) D'Aniello et al. (1989); (8) Gowland et al. (2003); (9) Ikeda et al. (2005); (10) Nabhitabhata (1996); (11) Omar et al. (2001); (12) Oosthuizen et al. (2002a); (13) Oosthuizen et al. (2002b); (14) Paulij et al. (1990); (15) Sen (2004a); (16) Vidal et al. (2002a); (17) Vidal et al. (2002b); (18) Villanueva (2000a); (19) Villanueva (2000b); (20) Forsythe et al. (1990); (21) Crawford (2002); (22) Ikeda et al. (2004a); (23) Fagundez and Robaina (1992); (24) Mladineo et al. (2003); (25) Akiyama et al. (1997); (26) Navarro and Villanueva (2000); (27) Segawa (1993); (28) Villanueva (1994); (29) Gonçalves et al. (1995); (30) Ikeda et al. (2004b); (31) Oestmann et al. (1997); (32) Porteiro et al. (1990); (33) Hanlon et al. (1991); (34) Nabhitabhata and Nilaphat (2000); (35) DiMarco et al. (1993); (36) Garcia Franco (1992); (37) Hanlon and Forsythe (1990); (38) Hanlon et al. (1988); (39) Estacio et al. (1999); (40) Sauer et al. (2000); (41) Messenger et al. (1985); (42) Sen (2005); (43) Segawa (1990); (44) Ito and Sakurai (2001); (45) Segawa (1995); (46) Sen (2004b); (47) Sen (2004c); (48) King et al. (2003); (49) Dimarco and Hanlon (1997); (50) Sauer et al. (1997); (51) Jantzen and Havenhand (2003a); 52. Boal and González (1998); (53) Buresch et al. (2004); (54) Hanlon et al. (2002); (55) Hanlon et al. (1999); (56) Cornwell et al. (1997); (57) Jantzen and Havenhand (2003b)

Table 7 Examples of recent literature that has used Ommastrephidae as experimental animals

Species	Collection, handling, transport	Housing	Feeding	Eggs, embryos and juveniles	Anaesthetics and Euthanasia	Treatment of Disease	Tagging and Tracking
Dosidicus gigas				1			2,3,4
Illex spp (I. argentinus, I. coindetii, I. illecebrosus)	5	5, 6, 7, 8, 9	5, 10	11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21	8, 22		23, 24, 25, 26, 27, 28
Nototodarus spp (N. sloanii and gouldi, hawaiiensis)					29		30, 31
Ommastrephes bartramii				32			33, 34, 35, 36
Sthenoteuthis oualaniensis				32			
Todarodes pacificus	37, 38	39, 40, 41	42	43, 44	45		46

(1) Yatsu et al. (1999a); (2) Bazzino et al. (2005); (3) Markaida et al. (2005); (4) Yatsu et al. (1999b); (5) O'Dor et al. (1977); (6) Bradbury and Aldrich (1969a); (7) Bradbury and Aldrich (1969b); (8) Portner et al. (1993); (9) O'Dor et al. (1982a); 10. O'Dor et al. (1980); 11. Sakai and Brunetti (1997); (12) Sakai et al. (1997); (13) Sakai et al. (1998); (14) Sakai et al. (2004); (15) Sakai et al. (1999); (16) Boletzky et al. (1973); (17) O'Dor et al. (1982b); (18) Durward et al. (1980); (19) O'Dor et al. (1985); (20) Balch et al. (1985); (21) O'Dor et al. (1986); (22) O'Dor et al. (1990b); (23) Brunetti et al. (1996); (24) Brunetti et al. (1997); (25) Dawe et al. (1981); (26) O'Dor et al. (1979); (27) Webber and O'Dor (1985); (28) Webber and O'Dor (1986); (29) Lykkeboe and Johansen (1982); (30) Sato (1985); (31) Yamada and Kattho (1987); (32) Sakurai et al. (1995); (33) Nakamura (1991); (34) Nakamura (1993); (35) Tanaka (2000); (36) Tanaka (2001); (37) Bower et al. (1999); (38) Flores et al. (1976); (39) Flores et al. (1977); (40) Mikulich and Kozak (1971a); (41) Mikulich and Kozak (1971b); (42) Soichi (1976); (43) Ikeda et al. (1993); (44) Ikeda and Shimazaki (1995); (45) Sakurai et al. (1993); (46) Mori and Nakamura (2001)

Table 8 Examples of recent literature that has used Octopodidae and other octopods as experimental animals

	Collection, handling and transport	Housing	Feeding	Eggs, embryos and planktonic stages	Anaesthetics and Euthanasia	Health, disease, and treatment	Tagging and tracking
Coastal octopods	1, 2, 3	4, 5, 6, 7, 8, 9, 10, 11, 12	5, 7, 12, 13, 14, 15	16, 17, 18, 19, 20, 21, 22, 23, 24	10, 25, 26, 27, 28, 29	30, 31, 32, 33, 34	35, 36, 37
Deep-sea and cold-adapted octopods	38, 39, 40	38, 39, 40	38, 39, 40, 41	38, 42			
Pelagic octopods	43	44, 45, 46, 47	44, 48				

(1) Voight (1988); (2) Sánchez and Obarti (1993); (3) Smale and Buchan (1981); (4) Van Heukelem (1977); (5) Forsythe and Hanlon (1980); (6) Boyle (1981); (7) Forsythe (1984); (8) Hanlon and Forsythe (1985); (9) Forsythe and Hanlon (1988); (10) Anderson (1996); (11) Mather and Anderson (1999); (12) Anderson and Wood (2001); (13) Joll (1977); (14) Garcia Garcia and Cerezo Valverde (2006); (15) Segawa and Nomoto (2002); (16) Itami et al. (1963); (17) Marliave (1981); (18) Snyder (1986); (19) Boletzky (1989); (20) Forsythe and Toll (1991); (21) Villanueva (1995); (22) Iglesias et al. (2004); (23) Villanueva et al (2004); (24) Okumura et al (2005); (25) Batham (1957); (26) Dew (1959); (27) Brough (1965); (28) Forsythe and Hanlon (1985); (29) Seol et al. (2007); (30) Hanlon et al. (1984); (31) Budelmann (1988); (32) Adams et al (1989); (33) Forsythe et al (1990); (34) Pascual et al. (2006); (35) Robinson and Hartwick (1986); (36) Anderson and Babcock (1999); (37) Domain et al. (2002); (38) Wood et al. (1998); (39) Daly and Peck (2000); (40) Hunt (1999); (41) Collins and Villanueva (2006); (42) Boletzky (1994); (43) Seibel et al. (1997); (44) Lacaze-Duthiers (1892); (45) Boletzky (1983b); (46) Bello and Rizzi (1990); (47) Packard and Würtz (1994); (48) Young (1960)

On a moral basis, as scientists and biologists using these animals in experiments, we have a responsibility to be proactive about minimizing the distress of individuals in our care and to better understand how we can care for welfare of the animals that we are using in experiments. We recommend that collecting cephalopods humanely as possible, e.g. basket traps for cuttlefish, jigs for squid, and pots for octopuses. An anaesthetic that is commonly and successfully used on cephalopods is magnesium chloride (Messenger et al. 1985; Scimeca and Forsythe 1999). The best method for euthanising cephalopods varies among species, but the pool of information is limited. Boyle (1991) recommends decapitation; however, this is not always easy with very small or large animals, or onboard on a rolling ship. Decapitation is completely unsuitable when intact specimens are need for museum collections or collection of statoliths, as these sit at the back of the head. Chilling is suitable for tropical and warm temperate species (Anderson 1996; Moltschaniwskyj and Semmens 2000), but is not useful for cold-water species. Recent studies on fish demonstrate that live chilling is stressful (Lambooij et al. 2006). Over-anaesthesia is useful when decapitation or chilling are not logistically possible. Several anaesthetic agents have been used with success, including an isotonic solution of $MgCl_2$ (e.g. Messenger et al. 1985; Cornwell et al. 1997; Bartol 2001; Thompson and Kier 2001), although this does not work on some *Octopus* species (Anderson 1996). An alternative is ethanol with increasing concentrations from 1 to 5% over a period of hours (Anderson 1996), or clove oil (Seol et al. 2007). Whatever the end use of the animals, euthanising cephalopods should be done as rapidly as possible and decapitation and overdose of anaesthetics are suitable methods.

Given that welfare considerations of cephalopods is relatively new, particularly in relation to capture and culture (this includes handling, housing, maintenance, and rearing), this review has compiled literature that provides details of using cephalopods as experimental animals. Cephalopods are a diverse group of animals and it is not possible to provide a single set of guidelines or rules with respect to capture and culture of cephalopods as a group, or even across all life stages. Furthermore, it is not possible to provide strict guidelines about techniques or methods that minimize distress and maximize welfare, especially since limited knowledge is available or published. Published work provides a starting point, with techniques and approaches that have worked, but these techniques may be further refined especially as more information about a species comes to light. The aim of this paper is to identify welfare issues that researchers using cephalopods in experiments should consider (Table 1), and to provide sources of information that can help in making the best possible decision (Tables 2–8). The information is designed to help those scientists with limited experience or with little access to experienced researchers. It will also help research scientists needing to apply for permission from ethics committees to use cephalopods and be able to provide evidence to justify welfare decisions.

Nautilidae

Three species of *Nautilus* have been maintained in captivity over the past 30–40 years (Table 2). As a result there is a reasonable volume of information about collection, handling, and housing of individuals, as well as basic aspects of their biology (Table 2). Saunders and Landman (1987) provide the best overview of collection, maintenance, and rearing of *Nautilus* in aquariums, including housing systems. A newer publication that provides detailed specifics on the conditions and infrastructural requirements for optimum rearing in aquarium settings and for general aspects of *Nautilis pompilius* husbandry is provided by Fields (2006). Boyle and Rodhouse (2005) provide a good general summary of many facets of *Nautilus* species biology, covering form and function, ecology, predation, growth, reproduction, activity, and fisheries.

Natural growth rates of *Nautilus* are available for both wild and lab/aquarium held animals (e.g. Saunders 1983). Juvenile *N. belauensis* have the capacity to add 0.1 mm of shell per day at the ventral circumference, but this rate decreases rapidly as individuals approach maturity. Individual chamber formation is estimated to take >100 days to >1 year. Similarly, the period required for an individual animal to reach sexual maturity varies across species and individuals with a range of 2.5–15 years. Other studies by Ward (1983) and Westermann et al. (2004) review laboratory growth rates for *N. macromphalus*

and *N. pompilius*, respectively and relate them to wild caught animals for age estimation.

A clear gap in our knowledge of maintaining *Nautilus* is assessment of health, besides using discoloration of the mantle and loss of ability to maintain buoyancy little is known about disease and health issues. Research is required on the best methods for euthanizing individuals in apparent poor health.

Sepiida

Due to their benthic ecology and tolerance of handling cuttlefish adapt well to life in captivity (Hanlon 1990). Methods of transport, housing, and culture of cuttlefish are reasonably well established (Forsythe et al. 1991, Table 3). One species in particular, the European common cuttlefish *Sepia officinalis*, has been studied and maintained in captivity for many years and cultured through multiple generations (Schröder 1966; Pascual 1978; Boletzky 1979; Forsythe et al. 1994). More recently, several other species have also been cultured through multiple generations, including the needle cuttlefish *Sepiella inermis* (Nabhitabhata 1997) and pharoah cuttlefish *Sepia pharaonis* (Minton et al. 2001).

Due to the ease of maintaining cuttlefish in captivity, much information on sepiid growth and behaviour has been obtained from captive individuals (Boal et al. 1999; Domingues et al. 2006). Unfortunately, few studies have related their captive conditions to the likely field environments experienced by wild individuals and this remains a significant area needing further investigation to ensure that laboratory studies are relevant to natural ecology and life history of species. Boletzky (1983a) provides a thorough review of the biology and ecology of *S. officinalis* and more recent studies on wild species include juvenile growth rates (Challier et al. 2002, 2005; Minton 2004), reproductive biology (e.g. Gabr et al. 1998), life cycles (e.g Guerra and Castro 1988), and behaviour (e.g. Aitken et al. 2005). Other areas of research specifically related to ethics that require further investigation include a critical examination of appropriate euthanasia, anaesthesia and disease treatment methods and non-invasive ways to accurately assess condition, well-being, and stress levels of individuals.

Aspects of sepiid biology that are of particular relevance to maintaining captive individuals include: their social behaviour and habitat requirements, as many species show dominance hierarchies, cannibalism or territoriality especially when reproductively active or in crowded conditions (Boal et al. 1999); dietary requirements, which are poorly understood, particularly during the early life stages when individuals require live food and are voracious feeders (Sykes et al. 2006); and water quality requirements, as cuttlefish are susceptible to skin ulceration and buoyancy malfunction in response to poor water quality and bacterial infection (Forsythe et al. 1991; Sherrill et al. 2000).

Sepiolida

The sepiolids have attracted a lot of attention from a number of research teams because of the symbiotic relationship between these animals and light-producing *Vibrio* spp. hosted within the light-organ (see review McFall-Ngai 1999). Given their relatively solitary nature and benthic mode of life, large eggs and large benthic juveniles, it has been relatively easy to culture a number of sepiolid species through several generations (Sinn et al. 2006). Detailed descriptions of culture methods, including collection, transport, housing and reproduction, are available for *Euprymna scolopes* (Hanlon et al. 1997), *Rossia pacifica* (Summers and Colvin 1989), and five *Sepiola* spp. (Bolezky et al. 1975). With careful handling and correct packing live adults and eggs of many sepiolids have been flown half-way around the world with excellent results.

Details of the biology and growth of wild animals are limited, largely due to their nocturnal and cryptic behaviour. The well-hidden eggs are rarely seen in the natural environment. Estimates of growth from natural populations have yet to be obtained due to the lack of daily growth increments on the statoliths and absence of other hard structures (Moltschaniwskyj and Cappo, in press). As much of our knowledge of the biology of these animals is being obtained from captive populations it is critical to take careful consideration of culture conditions and husbandry is made if we are to extrapolate information to wild populations (Table 4).

There are several issues to consider when culturing these species. Every 24 h, at first light, sepiolids release a high number of *Vibrio* from the light organ into the seawater. As a result, in recirculating seawater systems, the *Vibrio* levels in the seawater can reach reasonably high concentrations. It is not clear what the consequences for the health of the sepiolids are, but there is no evidence in the literature that this impacts negatively on their growth and survival. If the high densities of *Vibrio* are of concern then it is possible to reduce the density of *Vibrio* in the water using UV sterilisers. However, use UV sterilisation with caution and for limited periods, as there is the possibility of killing the biofilm in the biofilter.

Another issue that needs to be considered is that these species use a sand coat adhered to the outside of the animal and bury themselves into the soft substrate during daylight hours. Although providing sand for burying may not be essential for successful culture, the lack of available burying substrate could potentially cause increased stress (e.g. Mather 1986). The addition of sand to the tanks can cause problems with maintaining water quality and cleanliness, especially from rotting debris from prey items. On the other hand, bacteria flora building up in the sand may effectively provide a type of ''biofilter' in the tank.

Idiosepiidae

There are no papers that explicitly describe handling or care of the species of *Idiosepius*. Most of the following information is from papers that have used these species in their research; to date five of the seven described species have been collected and held in aquaria. Research has focussed on aspects of the biology of both wild and captive animals, in particular growth (Jackson 1989; Pecl and Moltschaniwskyj 1997), reproduction (Table 5), and behaviour (Table 5). For the other two species, only species descriptions are available (*I. macrocheir* and *I. picteti*). A review of the biology and ecology of this genus considers the issues and specialisation of this mini-maximalist, the smallest of the cephalopod species (Boletzky et al. 2005).

It is worth noting that adults of these species are extremely amenable to collection and maintenance, and will readily mate and deposit eggs in captivity. However, the juveniles have so far proven impossible to hold for more than five days; therefore the lifecycle has not been closed for any of the species in this group.

Loliginidae

Due to their economic value to inshore fisheries and the relative ease of capture and holding of a range of life history stages, there have been a very large number of studies that have used loliginid squids as experimental animals. Three substantial overviews of maintenance of loliginid squid have been produced (Boletzky and Hanlon 1983; Hanlon 1987, 1990). These documents are a valuable starting point to anyone wishing an overview of techniques and information about handling and holding these species. The literature in Table 6 focuses on publications since 1990 that provide additional information that complements and extends the knowledge in these earlier reviews.

Over the last six years there has been an increase in the number of species that are used for experimental work. Species which have been used extensively in experiments include: *Alloteuthis subulata, Doryteuthis gahi, D. opalescens, D. pealeii, D. plei, D. sanpaulensis, Heterololigo bleekeri, Loligo forbesi, L. reynaudii, L. vulgaris, Lolliguncula brevis, Sepioteuthis australis, S. lessoniana, S. sepioidea, Uroteuthis chinensis, U. duvauceli, U. noctiluca*, and *Uroteuthis* sp. There is extensive information available on holding and maintaining loliginid squid in captivity during all life history stages. Further, as more research teams are setting up or using land-based facilities to conduct experiments involving loliginid squids, it is evident that a diversity of seawater systems, flow through and recirculating, may be successfully used (Table 6).

Research over the last decade has provided more valuable biological detail of particular value when rearing these animals. Some examples include, the role of temperature (e.g. Oosthuizen et al. 2002a), light intensity (e.g. Ikeda et al. 2004a), salinity (e.g. Sen 2005), ionic composition of seawater on embryo growth and viability (e.g. D'Aniello et al. 1989); and rates and causes of embryonic abnormalities (e.g. Oosthuizen et al. 2002b; Gowland et al. 2003). There

has also been a significant increase in the knowledge of diet and nutrition of juvenile loliginids (e.g. Vidal et al. 2002a and b).

The increase in tagging and tracking of wild loliginids means that issues of how to handle individuals are going to be critical to the success of these projects (Table 6). Such research techniques need to maximise the survival of animals upon release and ensure that data retrieved realistically represents what occurs in the wild. For example, the use of antibiotics (2–4 ml of 6 mg/ml tetracycline) at the time of tagging may benefit survival and tag retention (Moltschaniwskyj and Pecl this issue). Types of tags used include spaghetti tags inserted in the mantle or fin (Sauer et al. 2000; Moltschaniwskyj and Pecl this issue), and acoustic tags (Pecl et al. 2006).

A considerable volume of work on wild populations and individuals provides a strong base upon which experimenters can assess the performance of captive-reared animals, especially growth rates and behaviours. There is an extensive body of work on estimating growth rates for a range of loliginid squids (Jackson 2004), including measures of spatial and temporal variability. This provides a useful basis for determining what growth rates in captivity may be expected. The importance of considering captive conditions is highlighted when validation of statolith incremental structure has been conducted using captive reared animals (Jackson 2004). The use of both tagging and photographic information of wild squid has allowed an assessment of the behaviours or absence of certain behaviours in captive individuals. Hanlon and Messenger (1996) provide descriptions of behaviours associated with feeding, reproduction, and general inter-individual interactions. More recent work that is relevant to captive animals is provided in Table 6.

Ommastrephidae

The ommastrephid squids are large, oceanic species that undertake migrations between feeding and spawning grounds over thousands of kilometres. Their significant commercial importance has resulted in considerable interest in their biology and ecology (e.g. *Illex* sp.). However, their large size and continuous, active swimming make them one of the hardest groups of cephalopods to maintain in captivity. They do not hover in the water column as many sepiids and loliginids do and thus they require much larger holding tanks. Many ommastrephids also have strong cannibalistic tendencies.

Ommastrephids have not yet been successfully reared from eggs. Females produce large, gelatinous, neutrally buoyant egg masses that are difficult to find in the wild. However, techniques of artificial fertilization have allowed hatchlings of a number of ommastrephid species to be produced. Unlike many of the cephalopods discussed earlier, newly hatched ommastrephids are not functional adults; hatchlings are very small paralarvae referred to as rhynchoteuthions. Their feeding tentacles are fused into a proboscis, resulting in a diet and mode of food capture that is probably unique among cephalopods during these early stages. Feeding of these early stages has never been successful, and thus the life cycle has yet to be closed for any species.

Nevertheless, there is a significant amount of information available about certain aspects of ommastrephid life that will be of value when optimising captive conditions and conducting experiments (Table 7). This information includes details of the capture and transport of adults, the holding of eggs and observations of the rhynchotheution paralarvae, and maintenance of adults in tanks (Table 1). Researchers have successfully tagged and tracked of a number of species; details of these techniques are readily available (Table 7). For 19 of the 21 Ommastrephidae species growth rates of wild individuals has been estimated using statoliths (Arkhipkin 2004), providing a strong basis for assessing the growth rates of captive animals.

Octopodidae and other octopods

There is a long history of using coastal octopus species of the family Octopodidae for experiments in behavioural, physiological, and ecological studies, probably because this group of cephalopods is best adapted to laboratory conditions. This is due, in part, to their benthic mode of life, reclusive behaviour, and reduced swimming activity in comparison with other cephalopod groups. Recent interest in octopus as

biomedical and aquaculture species has resulted in a refinement of handling and culture methods (Table 8). Studies of wild octopus populations, exploring aspects of biology, ecology, and fisheries, use traditional fishing methods, *in-situ* observations, and mark-recapture techniques with internal and external tags. This is currently the only group of cephalopods covered by Ethic Guidelines in the UK.

Reviews of information about how to maintain, rear, and culture inshore large-egged octopus species of the family Octopodidae with benthic juveniles is a function of research done during the 1980s (Boletzky and Hanlon 1983; Forsythe 1984; Forsythe and Hanlon 1988). Inshore octopus species with small eggs have a delicate planktonic stage ranging from three weeks to six months depending on temperature and species, which requires special handling methods (Table 8). Rearing benthic juveniles to adult stages for all inshore species of Octopodidae are similar for small- and large-egg species. There are species-specific requirements, as preference for temperature ranges, diurnal or nocturnal activity patterns, size and quality of food, and breeding behaviour.

In comparison with the coastal species of the family Octopodidae, little information exists for the deep-sea, polar, and pelagic octopod species represented by more than 10 octopod families. Considerably less is known about the cirrate octopods as well as families of deep-sea and cold-adapted incirrate octopods due to the difficulties of collecting and holding these cephalopods. They have very specific requirements for food, low temperature, and light levels, and high water pressure (Table 8). Pelagic octopod species have been maintained for very short periods of time, less than two weeks, and their rearing requirements are practically unknown (Table 8).

Deep-sea cephalopods

There is relatively little information on the use of deep-sea cephalopods in laboratory studies, due to difficulties in collecting and maintaining them under suitable conditions of light and temperature. Deep-sea sepiolids have been reared (Summers and Colvin 1989); however, no deep-sea squid species has been maintained for extended periods in captivity. Methods for collection and laboratory maintenance of deep-sea octopods, under suitable conditions of low light and temperature levels are available for the cirrate (Hunt 1999) and incirrate (Wood et al. 1998) octopods. Most deep-sea cephalopods seem to be long-lived species with low fecundity rates making their populations particularly vulnerable to deep-sea fishing activities (Collins and Villanueva 2006).

Concluding thoughts

It is evident that over the past 10 years an extensive volume of work done has used cephalopods as experimental animals (Tables 2–8). As a result of this research, a strong knowledge base is developing about a diversity of species and techniques. This information should guide scientists in refining their methodologies and approaches when using cephalopods in experimental systems. Refinement of experimental techniques reduces the stress of the investigation on the animals. Such refinement is achievable through careful consideration of the experimental design and procedures, housing conditions, and handling. Experiments should also be planned keeping in mind how you will monitor, assess, and manage impacts; and what procedures can be used to identify and respond to unforeseen complications. Adequate and suitable methods of euthanasia will also be points for consideration, especially when collecting animals.

Explicitly identifying how to assess welfare, the exact cause of stress, and when to terminate experiments will pose the greatest challenge to biologists using cephalopods as experimental animals. As a result of work to date with cephalopods we are gaining significant insights into their capacity to respond in adverse ways that suggests a capacity to perceive pain, suffering, and stress (Mather and Anderson in press). In a recent review of issues in fish welfare, Huntingford et al. (2006) highlighted the fact that wild fish experience stress and will suffer damage in the natural environment. Consequently, there is a conflict between what animals experience in the wild and the assessment of welfare and condition when caring for animals in captivity; we can say the same for cephalopods. Wild animals will suffer damage and stress associated with natural processes, e.g. large scale migrations, predation, interspecific

and intraspecific interactions. However, in experimental conditions we expose animals to a different set of stressors and factors causing damage, which need to be minimised. One resource currently available to scientists for use as an assessment tool is a body of work on cephalopod behaviour derived from both wild and captive studies (reviewed by Hanlon and Messenger 1996, recent work in Tables 2–8). Therefore, while we continue to build upon other areas of knowledge which we are lacking, normal behaviours associated with locomotion, feeding and reproduction provide a powerful tool in the assessment of cephalopod welfare.

While disease does not cause senescence, disease is often associated with senescence (Anderson et al. 2002). Given the short life span of cephalopods a persistent question is how we identify and separate natural processes that result in senescence from health problems associated with captive conditions and handling. Tables 2–8 clearly highlight just how little we know about the health of and diseases states in these animals. There is some work on the octopus *Eledone cirrhosa* that suggests a linkage between stress and health in cephalopods (Malham et al. 2002). Furthermore, the interaction of stress and health in cephalopods is likely to be as complex as it is for fish (Huntingford et al. 2006). Evidence of disease is rarely seen in captive populations and because of this, some cephalopod species are thought to be resistant to disease (e.g. *Sepia pharaonis*, Minton et al. 2001). As a result, we know very little about causes of disease, disease progression, and the capacity of immune systems to deal with disease in cephalopods. There is anecdotal information about the occurrence of extensive skin lesions in squid by fishers, but is not clear what association the presence of these lesions has with natural senescence processes.

Given that cephalopods are invertebrates, their inclusion in animal welfare legislation is the exception not the rule; however this situation is seriously being re-considered by a number of countries. Once challenged by animal welfare legislation, cephalopod biologists will find it difficult to provide evidence and standards for techniques to collect, hold, and kill animals. Few studies that have used cephalopods as experimental animals have had to justify their decisions about animal welfare to an independent body. This is starting to change and it will be increasingly important that biologists can develop a set of welfare indicators against which we can justify decisions about experimental protocols and methods.

Not addressed in this review, but of equal importance is the issue of ecological or environmental ethics; this includes the taking of sustainable numbers of eggs, juvenile, and adults from wild populations in a way that minimizes impact to the ecosystem. This is an issue, given the collateral damage associated with the use of trawl gear to collect or sample cephalopods. It is estimated that 60–100% of cephalopods that manage to escape otter trawls will die (Broadhurst et al. 2006). Again there are no standards or guidelines for collection currently available and it is possible that in the future, scientists will have to argue that the numbers they plan to remove from the wild and the methods of collection not only have minimized impact on the animals, but also have minimal impact on the populations and environments. We recommend that targeted collection methods are preferentially used e.g. pots for octopus, jigs for squid, and hand nets for sepioids. These will ensure that both the stress on the animals is minimized and that there is limited damage to the environment. Estimating the population size of the most accessible of cephalopods (loliginids) is difficult, even when there is an established fishery (e.g. Lipinski and Soule this issue). Collection from populations that may be at risk of over exploitation e.g. giant cuttlefish in Australia or the mimic octopus in Indonesia, highlight the need to balance protection of wild populations and the benefits of developing culture techniques. Without a doubt it will be necessary to use existing studies and knowledge base as the starting point in justifying how biologists address issues of welfare and ethics (animal and environmental) when using cephalopods as experimental animals.

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