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Sexual selection in simultaneous hermaphrodites: sea slugs as model organisms

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Orientador: Prof. Dr. José Eduardo A. R. Marian

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Orientador

Aos meus amados pais e irmã,
e ao meu noivo Vinicius,
por serem sempre minha fortaleza
e porto seguro.

“O importante não é vencer todos
os dias, mas lutar sempre.”

(Waldemar Valle Martins).

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Introdução Geral

Seleção Sexual – Uma breve introdução

A teoria da seleção sexual pressupõe que caracteres que elevem o sucesso reprodutivo dos indivíduos são selecionados durante o processo evolutivo, mesmo que, em algumas situações, estes caracteres se mostrem custosos em termos de viabilidade e sobrevivência (Andersson 1994). Essa teoria foi originalmente proposta por Darwin (1859) para explicar como caracteres masculinos extravagantes poderiam ter evoluído, uma vez que eles iam de encontro à teoria de evolução por seleção natural, pois reduziriam a sobrevivência dos indivíduos. De acordo com Darwin (1874), a seleção sexual seria decorrente de dois processos: 1) competição entre indivíduos do mesmo sexo (geralmente machos) por indivíduos do sexo oposto (geralmente fêmeas) e 2) seleção criteriosa de parceiros, geralmente realizada pelas fêmeas. Esses processos denominam-se seleção intrassexual e intersexual, respectivamente. Entretanto, tal abordagem de Darwin contemplava apenas eventos pré-copulatórios de seleção sexual, ou seja, o que ocorre antes da cópula e é relativo à aquisição de parceiros, pois ainda não havia evidências de que essa seleção poderia continuar mesmo após esses eventos (Eberhard 2009).

Somente após o trabalho de Parker (1970), os biólogos evolucionistas passaram a reconhecer a importância dos processos que ocorrem após o início da cópula, convencionalmente chamados de processos pós-copulatórios (Eberhard 2009). Existem equivalentes pós-copulatórios para ambos os processos de seleção intra e intersexual pré-copulatória propostos por Darwin (Eberhard 2009). O mecanismo pós-copulatório de seleção intrassexual seria a competição espermática, que é a disputa entre o espermatozoides de dois ou mais machos pela fertilização de um dado conjunto de ovócitos (Parker 1970). Por sua vez, um mecanismo pós-copulatório de seleção intersexual seria a escolha críptica da fêmea (ECF) (Eberhard 2009). Esta ocorre quando há influência da fêmea favorecendo a utilização dos gametas de um ou mais machos em detrimento de outros (e.g., rejeição, incapacitação ou digestão de esperma) (Eberhard 1996).

Seleção Sexual em hermafroditas

Muito do conhecimento existente sobre seleção sexual em animais decorre de

uma extensa pesquisa com espécies dioicas (Andersson 1994), isto é, organismos que possuem sexos separados em indivíduos diferentes. Isso se deve em parte à antiga e equivocada concepção de que não ocorreria seleção sexual em animais hermafroditas, também chamados de monoicos (Angeloni et al. 2003). Hermafroditas são indivíduos capazes de produzir ambos os gametas femininos e masculinos durante suas vidas (Avise 2011). De fato, Darwin (1871) considerou que a seleção sexual seria incompatível com o hermafroditismo, pois uma vez sendo hermafrodita, não haveria evolução de caracteres sexuais secundários (e.g., estruturas para competição entre machos e dimorfismo sexual) que elevassem o sucesso reprodutivo dos indivíduos. Entretanto, essa visão inicial foi se modificando no decorrer do tempo e, nas últimas três décadas, o estudo da seleção sexual em hermafroditas tem se mostrado como um campo em crescimento (Leonard 2013).

O hermafroditismo é amplamente distribuído entre diversos grupos de animais terrestres e aquáticos, com alguns clados de invertebrados sendo compostos predominantemente ou exclusivamente por espécies monoicas, tais como esponjas, anêmonas, corais, minhocas, sanguessugas, briozoários, caramujos pulmonados e lesmas-do-mar (Baur 1998; Michiels 1998). É possível distinguir dois tipos principais de hermafroditismo: sequencial e simultâneo. Hermafroditas sequenciais são indivíduos que iniciam o ciclo de vida com um sexo e mudam para o outro posteriormente, ex., corais, alguns peixes e poliquetas (Michiels 1998). Em algumas espécies, é possível ocorrer ainda alternância na mudança dos sexos (e.g., Kuwamura et al. 1994; Nakashima et al. 1995). Hermafroditas sequenciais são relativamente bem estudados e a maioria dos trabalhos realizados com esses animais tem tentando compreender os mecanismos envolvidos na mudança do sexo, bem como as condições em que seria mais vantajoso mudar para o sexo masculino ou feminino (Michiels 1998). Por sua vez, hermafroditas simultâneos, que serão o foco desta introdução, são organismos que possuem sistemas reprodutores masculino e feminino funcionais simultaneamente durante a maior parte de seu ciclo de vida, e cujo ato de reprodução geralmente envolve tanto a função feminina quanto a masculina de cada indivíduo (Michiels 1998). Como exemplos desse segundo tipo podemos citar lesmas marinhas e terrestres, caramujos, alguns platelmintos, entre outros (e.g., Haase & Karlsson 2004; Koene 2006; Janicke et al. 2016).

Inicialmente, foi sugerido que a seleção sexual em hermafroditas simultâneos

seria mais fraca quando comparada àquela presente em organismos dioicos (e.g., Morgan 1994; Greeff & Michiels 1999a). Entretanto, atualmente é amplamente reconhecido que a seleção sexual também pode operar de forma intensa nestes hermafroditas (e.g., Charnov 1979; Michiels 1998; Anthes 2010; Schärer et al. 2015). Devido ao fato de que muitas espécies monoicas são promíscuas e têm capacidade de armazenamento de esperma, elas são afetadas por pressões similares àquelas que influenciam a evolução das estratégias de acasalamento e competição espermática observadas em espécies dioicas (Charnov 1996; Baur 1998; Michiels 1998).

Escolha do parceiro em hermafroditas simultâneos

Ao contrário do proposto em trabalhos mais antigos, os quais consideravam que mecanismos pré-copulatórios de seleção sexual seriam apenas fracamente expressados em hermafroditas simultâneos (e.g., Greeff & Michiels 1999a), trabalhos mais recentes indicam que pode haver escolha criteriosa de parceiros nesses animais (Anthes 2010). Apesar da alta frequência de cópula ser comum (e.g., Angeloni & Bradbury 1999) e dar a impressão de interações sexuais indiscriminadas, isso por si só não prova ausência de escolha criteriosa de parceiros em animais monoicos. Nesses organismos, tal escolha pode incluir uma sofisticada discriminação baseada no tamanho do corpo, histórico de cópula e parentesco (Anthes 2010).

O tamanho corporal pode influenciar os recursos disponíveis para a reprodução e estar positivamente relacionado ao tamanho dos órgãos internos responsáveis pelo armazenamento de espermatozoides (Charnov 1996). Desse modo, em um cenário onde os custos de inseminação não são triviais e a fecundidade é diretamente proporcional ao tamanho, é esperado que animais copulem preferencialmente com indivíduos de mesmo tamanho ou maiores, pois indivíduos menores produziriam menos ovos (Anthes et al. 2010). Evidências corroborando a existência de preferência do maior parceiro pelo papel feminino em cópulas unilaterais (i.e., onde um indivíduo atua apenas como "macho" e o outro apenas como "fêmea") já foram demonstradas em gastrópodes marinhos, terrestres e de água doce (e.g., DeWitt 1996; Tomiyama 1996; Sprenger et al. 2009). Por outro lado, em espécies que realizam troca recíproca de esperma, a preferência por inseminar indivíduos maiores resultaria em cópula entre parceiros de tamanhos similares (Anthes et al. 2010). Até então, entretanto, há poucas evidências que corroborem essa previsão (Michiels et al. 2001; Monroy et al. 2005;

Pal et al. 2006). Por fim, o tamanho corporal do parceiro poderia influenciar o investimento "masculino" dos indivíduos durante a cópula, onde mais espermatozoides seriam doados para parceiros mais fecundos (Wedell et al. 2002). Evidências nesse sentido já foram reportadas para algumas espécies (e.g., Angeloni 2003; Michiels et al. 2003).

Muitas espécies de hermafroditas são promíscuas e possuem órgãos capazes de estocar aloesperma (i.e., espermatozoides exógenos), havendo condições para que haja competição espermática (Baur 1988). Desse modo, o histórico de cópula de um parceiro pode ser um proeminente critério de escolha, principalmente sob a perspectiva "masculina" (Anthes 2010). Em um estudo feito com uma espécie de lesma marinha que deposita espermatóforos na parede do corpo do parceiro, foi observado que indivíduos que carregavam espermatóforos eram mais frequentemente rejeitados durante interações pré-copulatórias quando comparados a indivíduos que tiveram o espermatóforo experimentalmente removido (Haase & Karlsson 2004). Mesmo em hermafroditas que não depositam espermatóforos externamente, i.e., sem sinais externos do seu histórico de cópula, já foi evidenciada influência deste fator na escolha de parceiros e comportamento de cópula (Michiels & Bakovski 2000; Anthes et al. 2006). Tais achados sugerem a existência de outros sinais que permitem o acesso ao histórico de cópula de um parceiro (Anthes 2010).

O endocruzamento geralmente ocasiona efeitos deletérios à prole, uma vez que aumenta a quantidade de homozigose, aumentando assim a expressão de doenças recessivas e/ou reduzindo a diversidade genética. Portanto, seria vantajoso se a escolha dos parceiros levasse em conta a qualidade genética dos mesmos (Anthes 2010). Estudos com caramujos de água doce do gênero *Physa* evidenciaram que estes organismos são capazes de discriminar indivíduos mais aparentados no momento da cópula, e que isso pode resultar em diferentes comportamentos de acasalamento. Tais estudos indicaram uma preferência por parceiros com graus intermediários de parentesco (McCarthy 2004; Facon et al. 2006; McCarthy & Sih 2008), o que proporcionaria a manutenção da diversidade genética sem perda de genótipos benéficos (Anthes 2010). Por outro lado, existem espécies que não parecem evitar o endocruzamento, e diferentes hipóteses foram propostas para explicar esse comportamento (e.g., Baur & Baur 1997; Schjørring & Jäger 2007). Por exemplo, estratégias reprodutivas que envolvam uma dispersão eficiente de indivíduos podem

tornar o encontro entre irmãos tão improvável que uma escolha criteriosa de parceiros nesse sentido seria irrelevante (e.g., Peters & Michiels 1996). De qualquer modo, os mecanismos envolvidos no reconhecimento do grau de parentesco ainda são pouco compreendidos (Anthes 2010).

Mecanismos pós-copulatórios de seleção sexual em hermafroditas simultâneos

Apesar das evidências já discutidas de ocorrência de escolha criteriosa de parceiros em hermafroditas simultâneos, a importância dos mecanismos pós-copulatórios de seleção sexual nestes organismos é muito mais claramente estabelecida (Schärer & Ramm 2016). De fato, vários autores especularam que a seleção sexual nestes hermafroditas seria mais proeminente na arena pós-acasalamento, quando comparados aos animais dioicos (Janicke et al. 2016). Isto ocorreria devido a uma maior propensão à cópula, decorrente do interesse comum de ambos os parceiros em doar esperma, principalmente nos hermafroditas simultâneos, nos quais o acasalamento é geralmente recíproco. Consequentemente, haveria maior intensidade de competição espermática e maior potencial de ECF (Michiels 1998; Schärer & Pen 2013).

Como já mencionado, a promiscuidade e a presença de órgãos de armazenamento de aloesperma geram um cenário potencial para a competição espermática (Baur 1998). Muitas das adaptações pós-copulatórias sexualmente selecionadas, que são comumente conhecidas em animais dioicos, têm sido documentadas em espécies hermafroditas (Schärer & Ramm 2016). Uma resposta clássica à competição espermática é a transferência de maior quantidade de espermatozoides durante a cópula (Parker 1998). Modelos teóricos, assumindo que a produção de espermatozoides é custosa e que os espermatozoides competem numericamente, preveem que, quando a competição espermática é intensa, os machos deveriam investir mais na produção de espermatozoides para conseguir uma maior taxa de paternidade (Parker 1998). Vários estudos comparativos suportam essa predição, evidenciando que machos de espécies sujeitas à maior intensidade de competição espermática apresentam testículos relativamente maiores (e.g., Hosken 1997; Stockley et al. 1997; Byrne et al. 2002; Pitcher et al. 2005). Há evidências similares para hermafroditas simultâneos, não só em relação ao tamanho da gônada masculina, mas também à taxa de produção de espermatozoides (e.g., Schärer &

Ladurner 2003; Schärer et al. 2004; Schärer & Vizoso 2007; Ramm & Stockley 2009). Além disso, várias outras adaptações associadas à processos pós-copulatórios são conhecidas nestes hermafroditas. Um exemplo clássico é a cópula traumática, que pode ser colateral ou adaptativa (Lange et al. 2013). Cópula traumática colateral pode ser exemplificada pela a inoculação de substâncias bioativas que causam modificações nos processos reprodutivos do parceiro. Por exemplo, algumas espécies de caramujos terrestres fincam "dardos-do-amor" no parceiro durante a corte. Aqueles mais eficientes na fixação desses dardos apresentam maiores taxas de paternidade, devido a modificações no funcionamento do sistema reprodutor do parceiro induzidas pelo muco injetado com o dardo (Landolfa et al. 2001). Algumas espécies de minhocas apresentam uma estratégia similar (e.g., setas copulatórias; Koene et al. 2002). Um exemplo de cópula traumática adaptativa seria o pênis em forma de estilete de algumas espécies de lesmas marinhas (e.g., Lange et al. 2013; Schärer & Ramm 2016), que causam feridas no parceiro durante a cópula. Uma hipótese para explicar esse fenômeno é a de que, ao ferir o parceiro, os recursos energéticos do mesmo seriam preferencialmente alocados na sua recuperação, fazendo com que ele evitasse cópulas conseguintes e, consequentemente, elevasse o sucesso de fertilização em curto prazo do doador de espermatozoides (Michiels 1998).

Além de órgãos de armazenamento de aloesperma, representantes da maioria dos principais táxons de hermafroditas simultâneos com fertilização interna também possuem órgãos ou células capazes de digerir espermatozoides (Baur 1998; Michiels 1998). O comportamento reprodutivo promíscuo, juntamente com a presença desses órgãos, representam condições potenciais para que haja ECF. Os receptores de espermatozoides poderiam, por exemplo, utilizar a capacidade de digestão de aloesperma como uma forma de reduzir o sucesso de fertilização de parceiros de baixa qualidade (Birkhead et al. 1993). Alternativamente, espermatozoides excedentes poderiam ser digeridos para fins energéticos, servindo como uma fonte de nutrientes (Brandriff & Beeman 1973; Calow et al. 1979). Reabsorção de espermatozoides exógenos é conhecida, por exemplo, em alguns platelmintos (e.g., Sluys 1989) e minhocas (e.g., Grove 1925). Digestão de espermatozoides também já foi observada em lesmas marinhas (e.g., Brandriff & Beeman 1973). Entretanto, estudos sobre ECF em hermafroditas estão geralmente limitados a modelos teóricos.

Por exemplo, há vários estudos sobre as implicações de ECF em termos de alocação sexual (i.e., quantidade de recursos reprodutivos empregada nas funções feminina e masculina por um indivíduo) em hermafroditas simultâneos (e.g., Greeff & Michiels 1999b; Greeff & Parker 2000; Schärer 2009; Velzen et al. 2009).

Seleção Sexual em lesmas marinhas

Lesmas marinhas, moluscos gastrópodes pertencentes ao táxon Heterobranchia, são em sua grande maioria hermafroditas simultâneos durante a maior parte de seu ciclo reprodutivo e possuem sistemas reprodutores complexos (Hadfield & SwitzerDunlap 1984). A fertilização é interna e cruzada, embora existam algumas poucas exceções (Baur 1998). Nesses organismos, as estruturas consideradas responsáveis pelo recebimento e armazenamento de aloesperma são a bursa copulatória e o receptáculo seminal, respectivamente (Gosliner 1994). Segundo Beeman (1970), a bursa copulatória seria uma das estruturas mais enigmáticas do sistema reprodutor de lesmas marinhas, pois já foi relacionada a diferentes funções. Há, inclusive, evidência de digestão de espermatozoides por esse órgão em algumas espécies (e.g. Schmekel, 1971; Brandriff & Beeman 1973).

Uma vez que lesmas marinhas geralmente possuem órgãos armazenadores de espermatozoides que poderiam participar de processos pós-cópulatórios de seleção sexual, a atividade destes órgãos poderia influenciar resultados de experimentos, por exemplo, afetando taxas de paternidade resultantes de cópulas com parceiros qualitativamente diferentes. Desse modo, o funcionamento desses órgãos compõe uma importante fonte de informação para a elaboração de modelos teóricos sobre seleção sexual em hermafroditas simultâneos. De acordo com Eberhard (2009), o uso de caracteres genitais no âmbito taxonômico gerou uma imensa literatura sobre a morfologia dos órgãos reprodutores nos mais diferentes grupos animais, dados estes que poderiam ser empregados para ilustrar argumentos relacionados à seleção sexual pós-copulatória. Nesse contexto, estudos morfofuncionais sobre o trato reprodutivo de hermafroditas simultâneos associados a situações experimentais constituem uma abordagem promissora para inferências sobre processos de seleção sexual.

O nudibrânquio *Okenia polycerelloides* (Ortea & Bouchet 1983) é abundante no litoral de São Paulo, além de se tratar de um organismo de fácil coleta, cultivo e manutenção em laboratório. É uma espécie hermafrodita simultânea, promíscua, com

fertilização interna cruzada, possui atividade reprodutiva ao longo de todo o ano, variação no tamanho de indivíduos sexualmente maduros e sistema reprodutor complexo, possuindo tanto a bursa copulatória quanto o receptáculo seminal. Todas essas características fazem dessa espécie um potencial organismo modelo para estudos de seleção sexual em hermafroditas simultâneos.

Objetivos

Esta tese teve como objetivo geral investigar a estrutura e a função dos órgãos que abrigam espermatozoides no sistema reprodutor do nudibrânquio *O. polycerelloides*, como base para identificação de possíveis processos pós-copulatórios de seleção sexual em lesmas marinhas. Especificamente, esta tese pretendeu responder as seguintes perguntas: Quais as funções do receptáculo seminal e da bursa copulatória? Tais órgãos estão relacionados à digestão de esperma? O que ocorre com o aloesperma após a cópula? Em havendo digestão de esperma, tal comportamento está relacionado a situações de limitação de recursos alimentares? Ademais, foi estudado o comportamento de cópula dessa e de outra espécie de nudibrânquio, *Phidiana lynceus* Bergh, 1867. Todos os resultados são discutidos à luz da teoria de seleção sexual em hermafroditas simultâneos.

Organização da tese

Além desta "Introdução Geral" e das "Considerações Finais", esta tese está estruturada em três capítulos escritos em formato de artigo científico e redigidos em inglês. Respectivas figuras e referências são apresentadas ao final de cada capítulo.

O capítulo 1, intitulado "Functional morphology of the sperm-containing chambers of the reproductive system of *Okenia polycerelloides* (Ortea & Bouchet 1983)" apresenta uma descrição detalhada da ampola, receptáculo seminal e bursa copulatória da espécie em questão com base em dados de microscopia óptica, confocal e eletrônica de transmissão. Detalhes da organização geral do sistema reprodutor da espécie também são apresentados. Baseado nos resultados obtidos, possíveis funções são inferidas para esses órgãos. Esse capítulo, portanto, visa responder as questões: Quais as funções do receptáculo seminal e da bursa

copulatória? Tais órgãos estão relacionados à digestão de esperma?

O capítulo 2, intitulado "Sperm transfer, storage and digestion in a sea slug: towards understanding post-copulatory sexual selection mechanisms in simultaneous hermaphrodites" reúne dados de estudo histológico do sistema reprodutor de *O. polycerelloides* associado a manipulações de cópula e a um experimento onde os indivíduos foram submetidos à privação alimentar. Dessa forma, os resultados apresentados nesse capítulo visam responder as seguintes perguntas: O que ocorre com o aloesperma após a cópula? Em havendo digestão de esperma, tal comportamento está relacionado a situações de limitação de recursos alimentares? Ademais, o comportamento de cópula da espécie é descrito.

O capítulo 3, intitulado "Love will tear us apart: traumatic mating through consumption of body parts in a sea slug" documenta o interessante comportamento de cópula do nudibrânquio *Phidiana lynceus*, o qual consome cerata durante o acasalamento. Essa descoberta é discutida com base nas hipóteses propostas para explicar a evolução da cópula traumática. Esse manuscrito foi submetido ao periódico internacional *Ecology* e encontra-se em fase de revisão.

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Capítulo 1

Functional morphology of the sperm-containing chambers of the reproductive system of *Okenia polycerelloides* (Ortea & Bouchet, 1983)

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Abstract

Sea slugs are interesting models to study post-copulatory sexual selection in simultaneous hermaphrodites due to the enormous variation of their reproductive systems. However, the knowledge of the functional morphology of their reproductive system is limited to few species, and it is rarely discussed in the light of sexual selection. In this study, we investigated the functional morphology of the sperm-containing chambers (i.e., ampulla, seminal receptacle and bursa copulatrix) of the reproductive system of *Okenia polycerelloides* (Ortea & Bouchet 1983), based on light, confocal and transmission electron microscopy. Although the morphology of the ampulla is similar to other species – indicating it is a site for autosperm storage –, we found sperm facing the ampullar epithelium, a feature commonly regarded as characteristic of the seminal receptacle of sea slugs. The seminal receptacle of *O. polycerelloides* showed secretory activity and contained sperm with distinct orientations and distribution within its lumen, suggesting stratification of allosperm from distinct mating events. The bursa copulatrix had epithelial cells with secretory and absorptive characteristics, and contained degraded sperm and yolk granules within its lumen. Comparative analysis of the contents of each organ demonstrated that sperm digestion occurs in the bursa copulatrix and affects sperm heads first, changing their morphology from slender and curved to shorter and ellipsoid before complete lysis. Putative post-copulatory mechanisms are discussed in the light of these findings.

Keywords: post-copulatory sexual selection, sperm competition, cryptic female choice, gametolytic gland, sperm storage, sperm digestion.

Introduction

Sexual selection theory predicts that traits that elevate the reproductive success of individuals are selected during evolution, even if in some situations these characters prove to be costly in terms of viability and survival (Andersson 1994). There are both pre-copulatory and post-copulatory mechanisms of sexual selection (Eberhard 2009). Sperm competition is a kind of post-copulatory mechanism that happens when the sperm of two or more individuals compete for the fertilization of a set of ova from an individual (Parker 1970). This competition generally occurs in the female reproductive tract, which allows the female the possibility of selecting the sperm that will fertilize its ova (Wigby & Chapman 2004). "Cryptic female choice" refers to the female's influence favoring the use of sperm from one or more males to the detriment of others (e.g., by rejection, incapacitation or sperm digestion; Eberhard 1996). This kind of selection can also occur within the female reproductive tract, and therefore, the knowledge of the functional morphology of the reproductive tract is of utmost importance to understand post-copulatory sexual selection mechanisms.

Sexual selection has been widely studied in gonochoristic animals (Andersson 1994), while in hermaphrodites it has been more recently explored (Angeloni et al. 2003). Hermaphrodites usually possess organs for sperm digestion and/or storage as components of their reproductive systems (Baur 1998; Michiels 1998), and these structures can play an important role in sexual selection processes such as sperm competition and cryptic female choice. For example, when an individual mates with different partners and stores their sperm, there is a potential scenario for sperm competition (Baur 1998). Also, if an individual mates with many partners and is capable of digesting sperm, this individual could use this capacity to reduce the fertilization success of low-quality partners, which would be a case of cryptic female choice in a hermaphrodite (Birkhead et al. 1993).

Hermaphroditism is widespread among animals, some taxa being almost exclusively hermaphroditic, such as sea anemones and corals, pulmonate snails and sea slugs (Baur 1998). The vast majority of sea slugs are simultaneous

hermaphrodites, i.e., individuals that produce functional male and female gametes simultaneously for most of their lives (Baur 1998; Michiels 1998). There is great variety in arrangements of the reproductive system in sea slugs (Beeman 1977), which makes these mollusks interesting models for understanding sexual selection in simultaneous hermaphrodites. Similarly to what occurs in others taxa, there is a great amount of information available on the morphology of the reproductive system in sea slugs, but this has been mainly used to discuss taxonomy and phylogenetic relationships (e.g., Wägele & Willan 2000; Dayrat & Gosliner 2005; DaCosta et al. 2010; Alvim et al. 2011). Not only are functional morphology investigations limited to few species (e.g., Brandriff & Beeman 1973; Schmekel 1971), but also links between the morphology of the reproductive system and sexual selection are scarce.

Sea slugs may bear two or three sperm-containing chambers: the ampulla, the seminal receptacle, and the bursa copulatrix. The ampulla is generally regarded as the site in which autosperm (i.e., endogenous sperm) is stored (Gosliner 1994), but phagocytosis and sperm digestion by cells in the ampullar wall were also reported for two species of cephalaspideans (Hadfield & Switzer-Dunlap 1984). The ampullar wall shows considerable morphological variation among sea slug species (Hadfield & Switzer-Dunlap 1984), highlighting the need of more studies on its structure and function (Ghiselin 1965). The seminal receptacle is generally associated with allosperm (exogenous sperm) storage (Gosliner 1994). Moreover, reception of sperm during copulation was also proposed as a function of the seminal receptacle in *Phyllaplysia taylori* Dall 1900, as well as activation and nutrition of allosperm, and removal of the seminal fluid (Beeman 1970a, 1970b). The presence and function of the bursa copulatrix, in turn, are quite variable in sea slugs (Hadfield & Switzer-Dunlap 1984; Valdes et al. 2010), but this organ is generally regarded as the site for allosperm reception during mating (Gosliner 1994), with evidence for gamete digestion in some species (e.g., Brandriff & Beeman 1973; Schmekel 1971). It was also suggested that the bursa copulatrix would have a resorptive function of the digested products within its lumen (Brandriff & Beeman 1973), although much controversy still persists regarding its possible functions (Medina et al. 1988). Thus, as can be seen, the exact functions of these organs remain unclear.

Okenia polycerelloides (Ortea & Bouchet 1983) is a simultaneously hermaphroditic sea slug that is easily collected, cultivated, and maintained in lab

conditions. This dorid nudibranch has a promiscuous copulatory behavior and a complex reproductive system with three sperm-containing organs (Sales et al. in press). These characteristics make this species an interesting model organism for sexual selection studies. However, there is no morphofunctional information about its sperm-containing organs, and it is unknown if this species is capable of digesting sperm. Therefore, we present here a morphofunctional characterization of the ampulla, seminal receptacle and bursa copulatrix of *O. polycerelloides* based on light, confocal, and transmission electron microscopy, and we discuss our findings in the light of post-copulatory sexual selection theory.

Material and Methods

Animals

Colonies of the bryozoan *Amathia verticillata* (delle Chiaje 1822) were collected in the Araçá Bay (São Paulo state, Brazil), between 23°48'47.5" S and 45°24'31.4" W. These colonies were transported alive within buckets containing seawater to the Center for Marine Biology of the University of São Paulo (CEBIMar-USP). The colonies of the bryozoan were kept in aquaria and verified for the presence of the sea slug *Okenia polycerelloides* (Fig. 1a), which lives and feeds on these colonies. Once individuals of the sea slug were found, they were isolated, anesthetized with a mixture of menthol and isotonic magnesium chloride solution dissolved in seawater, and fixed according to the purposes of the study (light, confocal or transmission electron microscopy).

Light microscopy

Seven specimens (5-8 mm in length) were fixed in 4% paraformaldehyde (in phosphate buffered saline 0.1 M) for 8 h at 4°C. Then, they were washed in distilled water, dehydrated in a graded ethanol series, and embedded in glycol-methacrylate resin (Leica Historesin Embedding Kit, Leica Microsystems Nussloch GmbH, Germany) according to the manufacturer's protocol. Serial sagittal 3 µm sections of the entire individuals were produced on a Leica RM2255 microtome (Leica, Wetzlar,

Germany) and stained (Humason 1962; Behmer et al. 1976; Bancroft & Stevens 1982; Pearse 1985) with the following dyes: Haematoxylin-Eosin (H&E – general stain), Toluidine Blue (TB – general stain), Periodic Acid–Schiff (PAS – identification of neutral polysaccharides), Alcian Blue at pH 2.5 (AB – identification of acidic polysaccharides), Naphthol yellow (NY – identification of proteins), Mercury-Bromophenol Blue (BB – identification of basic proteins), and Gömöri Trichrome (GT – to evidence muscle fibers). The slides were photographed using a Leica DMLB microscope equipped with a Leica DFC295 camera.

Transmission electron microscopy

Three specimens (6-8mm in length) were fixed 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, and then washed three times in cacodylate buffer. Post-fixation was performed for 1 h at 4°C in 1% OsO_4 in cacodylate buffer. The samples were dehydrated in increasing concentrations of ethanol and embedded in Epoxy resin. Ultrathin sections (50-70 nm) were contrasted with uranyl acetate and lead citrate, and then observed in a Zeiss EM 900 transmission electron microscope.

Confocal microscopy

Three specimens (7-8 mm in length) were fixed in 4% paraformaldehyde in phosphate buffer (PB) 0.1 M for 2 h at 4ºC, washed in PB three times (15 minutes each), and maintained in the PB for posterior dissection. Then, the sperm-containing organs were dissected and their contents permeabilized in PB containing Triton-X 100 (PBT) overnight. The samples were immersed in a solution of 1:400 anti-α-tubulin/PBT for 24 h at room temperature in the dark. Then, they were washed three times (20 minutes each) in PB and mounted in microscope slides in ProLong Diamond Antifade Mountant with DAPI (Molecular Probes, USA). They were stored in the freezer, in the dark, before being analyzed. The samples were analyzed and photographed under a Zeiss LSM 880 confocal laser scanning microscope (Zeiss,

Germany). Image stacks were digitally merged in the software ZEN lite 2.3 (Zeiss, Germany).

Nomenclature

Anatomical nomenclature follows Hadfield & Switzer-Dunlap (1984), Gosliner (1994), Gosliner & Bertsch (2004) and Sales et al. (in press).

Results

1. Gross morphology of the reproductive system

The ampulla is a tubular and elongate organ, which generally appears glistening white because of the abundant sperm inside of it, and leads from the hermaphrodite gonad to the prostate – which leads to the vas deferens and penis – and the fertilization chamber located inside the female gland (Fig. 1b). From the fertilization chamber arises the thin uterine duct in anterolateral position to the insertion of the ampulla's duct (Fig. 1b). The uterine duct joins the connection of the short receptacle duct with the long allosperm duct (Fig. 1b). The seminal receptacle duct leads to the seminal receptacle, which is a blind and oval sac (Fig. 1b). The allosperm duct leads to the bursa copulatrix and continues as the vagina to the common genital pore (Fig. 1b). The bursa copulatrix is a thin-walled sac with the same shape of the seminal receptacle (Fig. 1b), and generally contains an internal material visible externally by translucency that can vary in color from white to brownish-red. The common genital pore includes the apertures of the vagina and the penis (Fig. 1b). The female gland connects subterminally to the vagina by the nidamental opening (Fig. 1b).

2. Light and transmission electron microscopy

2.1. Ampulla

The ampulla has a simple cuboidal epithelium, with sparse nuclei, surrounded by a layer of muscle fibers (Fig. 2a-c). In the scarce areas of the ampulla devoid of sperm content, the epithelium was revealed to bear long cilia (Fig. 2c), but it was not

possible to confirm the extension of this ciliary cover. The ampulla was always filled with a massive amount of sperm (Fig. 2a-b, d). Sperm were generally randomly oriented (Fig. 2a-d), and a few of them had their heads in contact with the ampullar epithelium (Fig. 2d). In some samples, particulate material similar to oocyte granules (hereafter called "yolk granules") was found dispersed in the lumen and close to the epithelium (Fig. 2b). The epithelium had no affinity for the applied histochemical procedures.

Under TEM, microvilli were found in some areas of the ampullar epithelium (Fig. 3a-b), as well as some kind of secretion within the luminal material (Fig. 3c). Sperm heads in contact with the ampullar wall were revealed to be embedded in indentations of the epithelium (Fig. 3a, c-d), i.e., apparently without penetrating the epithelial cells (Fig. 3d).

2.2. Seminal receptacle

The seminal receptacle has a simple cuboidal epithelium, with small nuclei (Fig. 4a-c), surrounded by a thick muscular layer (Fig. 4b-c). The epithelium is vacuolated; except for eosin, the vacuoles had no affinity for the applied stains (Fig. 4b-c). The epithelial surface was covered by a secretion that stained mostly in pink with HE and in light blue with TB (Fig. 4c). However, this secretion had also an external thin layer that stained in pink with TB (Fig. 4c) and showed a positive reaction for AB (Fig. 4d). AB staining also revealed that this secretion is scattered throughout the seminal receptacle lumen (Fig. 4d). All analyzed seminal receptacles had sperm in their lumen, and usually contained sperm with distinct orientations and distribution. They could be either randomly oriented (Fig. 4e) or oriented with their heads facing the epithelium (Fig. 4b); also, they could be either homogeneously distributed within the lumen, or with part of the sperm concentrated in a spherical mass located near the opening of the organ – observed in 1 specimen – (Fig. 4a, e). All of these configurations could be found simultaneously within the seminal receptacle of a single individual.

Under TEM, the contents of the epithelial vacuoles were more electron-lucent than the cytoplasm (Fig. 5a-c). Digitiform microvilli were also found (Fig. 5c-d), as well as secretion within the luminal contents (Fig. 5e). Sperm heads in contact with

the epithelium were either surrounded by the microvilli or embedded in invaginations of the epithelium (Fig. 5a, c-d).

2.3. Bursa copulatrix

The bursa copulatrix has a simple cuboidal epithelium, with large apical nuclei, surrounded by a thin layer of connective tissue (Fig. 6a-b). Long cilia were observed only at the opening of the organ (Fig. 6a,c). The contents of the bursa copulatrix were largely composed of sperm tails interspersed with amorphous and particulate materials (Fig. 6a, d-g). Sperm heads were not identified under light microscopy, except rarely at the opening of the organ (Fig. 6c). Part of the luminal contents was also composed of seemingly secreted substances with affinity for TB (light purple; Fig. 6b, d) and AB (Fig. 6f). This secretion could either surround (Fig. 6b, d) or be interspersed within the luminal contents (Fig. 6f). The epithelium of the bursa, however, had a weak affinity for the applied stains. The particulate material within the lumen probably corresponds to yolk granules (Fig. 6a, d, e, g), due to their morphological and histochemical similarities (i.e., eosinophilic and PAS-, NY- and BB-positive) to the oocyte's granules of the hermaphrodite gonad (Fig. 6h).

Under TEM, the surrounding connective tissue of the bursa copulatrix was revealed to bear muscle cells (Fig. 7a-b). The cytoplasm of the epithelial cells was rich in mitochondria and rough endoplasmic reticulum (RER) cisternae (Fig. 7c). There were also electron-lucent vesicles of different sizes inside the cytoplasm (Fig. 7a,d), generally smaller at the basal region and becoming larger towards the apical region (Fig. 7a). Apparently, endosomal tubules and endosomes were present near the apical plasma membrane (Fig. 7e-f). From the apical plasma membrane, numerous small protuberances apparently detached as small vesicles (Fig. 7d-e, g). Numerous large swollen vesicles were interspersed with the small ones (Fig. 7d, g). The luminal content of the bursa consisted largely of degenerating gametes and amorphous material (7g-h). Sperm heads were rarely found (Fig. 7h-inset), and, interestingly, sperm tails were more electron-lucent than sperm tails from the ampulla and seminal receptacle (Fig. 7f-h). Several sperm flagella were found embedded within small and large vesicles (Fig. 7 f, g).

3. Confocal microscopy

Confocal microscopy analysis of the contents of each organ revealed similar conditions for the sperm within the ampulla and the seminal receptacle (Fig. 8a-c). In this case, sperm morphology under confocal microscopy closely resembled the one observed in histological sections, i.e., with a long flagellum and a slender and curved head (Fig. 8a-c). Interestingly, sperm content from the bursa copulatrix was different from the other two organs. First, an evidently smaller number of sperm heads were observed in the bursa copulatrix (Fig. 8d-e). Second, sperm morphology was strikingly different, i.e., with a shorter and more ellipsoid head (Fig. 8f). Particulate material (possibly yolk granules) were also found within the contents (Fig. 8d-e).

Discussion

In sea slugs, the gonoduct is divided into coelomic and pallial regions. The coelomic gonoduct is composed of a preampullar region, the ampulla, and a postampular portion (Ghiselin 1965). The pallial gonoduct comprises all structures present between the distal end of the postampullar portion and the common genital pore (Ghiselin 1965). Although the shape of the ampulla and the proportions of the different parts of the coelomic gonoduct vary among species, the arrangement of the coelomic gonoduct is generally uniform in sea slugs (e.g., Pola 2015; Cunha & Simone 2018). In turn, the pallial gonoduct shows considerable variation regarding its subdivisions, specializations and their general arrangement (Beeman 1977; Hadfield & Switzer-Dunlap 1984).

The seminal receptacle and bursa copulatrix are specialized structures of the female pallial gonoduct that apparently develop as evaginations of the vaginal region (Hadfield & Switzer-Dunlap 1984). Although the seminal receptacle is generally found at the proximal end of the pallial gonoduct, its position and connections can vary across sea slugs (Hadfield & Switzer-Dunlap 1984). For example, in some species the seminal receptacle opens directly into the fertilization chamber (e.g., Beeman 1970b), while in others there is no direct connection between the seminal receptacle and the fertilization chamber (e.g., Marcus & Marcus 1957). *Okenia polycerelloides* shows a third configuration – also present in other species – where the

duct of the seminal receptacle is divided into two channels, one (i.e., the uterine duct) leading to the fertilization chamber and the other continuing as the allosperm duct (Fig. 1b).

Based on the reproductive system configuration, autosperm originated in the hermaphrodite gonad are directed to the ampulla, and during mating to the prostate and penis (Fig. 1b). Allosperm, in turn, would be received from the vagina, from where they could reach the bursa copulatrix and the seminal receptacle (Fig. 1b). During oviposition, oocytes would be first directed into the ampulla, then into the fertilization chamber, where they would receive allosperm coming from the uterine duct. Then, they would receive the coatings from the female gland before being released through the nidamental opening into the vagina to be extruded (Fig. 1b) (e.g. Thompson 1966; Thompson 1976).

Ampulla

The ampulla is regarded as the site of autosperm storage in sea slugs (Gosliner 1994). Similarly to other species (e.g., Beeman 1970a; Schmekel 1971), the ampulla of *Okenia polycerelloides* is always filled with sperm. Although it is not possible to differentiate autosperm from allosperm only by morphological grounds (Beeman 1970b; Hadfield & Switzer-Dunlap 1984), the position and connections of the ampulla in the reproductive system (Fig. 1b) suggest that its sperm contents correspond to autosperm. Moreover, autosperm storage in the ampulla was confirmed by radiolabeling experiments with aplysiids (Beeman 1970b). Whole oocytes were not observed in the lumen of the ampulla in *O. polycerelloides*, but possible yolk granules – similar to those found in the oocytes – were sometimes observed dispersed in the lumen of the organ and in contact with the epithelium. Similarly, Beeman (1970a) reported the presence of "various impurities" mixed with sperm in the ampulla of *P. taylori*, including yolk particles and single oocytes. Oocytes pass through the ampulla during oviposition (Schmekel 1971), thus the yolk granules observed in the ampulla could be the ones not incorporated into oocytes, but carried along with the female gametes during a previous spawning event.

Although male and female gametes pass through the ampulla, self-fertilization is not common in sea slugs (Baur 1998). The ampulla of *O. polycerelloides* has a

ciliary tract, which could carry oocytes along the organ during oviposition. Evidence for this function was observed in other sea slugs that bear a similar ciliary tract (e.g., Baudelot 1863; Ghiselin 1965; Thompson 1966), which was inferred to help reduce the contact between male and female gametes within the ampulla (Ghiselin 1965). Nevertheless, considering that the ampulla of *O. pollycerelloides* was always completely filled with autosperm, there should be some contact between both male and female gametes during oviposition. In this scenario, self-fertilization could be avoided if the autosperm in the ampulla were immature. Sperm motility is indicative of physiological maturity (Thompson 1976), and in some sea slugs the ampullar autosperm are predominantly non-motile (Thompson 1966; Thompson 1976; Thompson & Bebbington 1969; Beeman 1970a).

Although the ampullar sperm of *O. polycerelloides* were mostly randomly oriented, all analyzed individuals had some sperm with their heads facing the ampullar epithelium. Interestingly, the latter sperm orientation is characteristic of the seminal receptacle of sea slugs (Ghiselin 1965; Hadfield & Switzer-Dunlap 1984). Except for *Aplysia punctata* (Cuvier 1803), which shows a large number of sperm radially arranged within the ampulla (Thompson & Bebbington 1969), and for rare individual findings in some nudibranchs (Schmekel 1971), sperm heads generally do not face the ampullar epithelium in sea slugs (e.g., Beeman 1970a; Schmekel 1971; Thompson 1976). In *O. polycerelloides*, some sperm heads were embedded within invaginations of the ampullar epithelium, a configuration that may suggest a nutritive function (e.g., Beeman 1970a). Nevertheless, most sperm within the ampulla had a random orientation, which suggests non-motility (Schmekel 1971) and, hence, low nutritional needs (Thompson & Bebbington 1969).

Seminal receptacle

The seminal receptacle is commonly regarded as site of allosperm storage in sea slugs (Gosliner 1994). Except for *Aplysia fasciata* Poiret 1789, which has sperm oriented in swirling groups (Thompson & Bebbington 1969), sperm are consistently found with their heads facing the wall of the seminal receptacle (i.e., radial orientation; Ghiselin 1965; Beeman 1970a, 1970b; Schmekel 1971; Thompson 1976). Although this characteristic orientation was indeed observed for *O. polycerelloides*,

random alignments were also common. Experimental data for *P. taylori* indicated that allosperm achieve radial orientation in the seminal receptacle within five hours after mating (Beeman 1970b). Therefore, the random orientation found herein for *O. polycerelloides* could be due to recent mating.

The finding of sperm concentrated in a spherical mass separated from the remaining sperm content of the seminal receptacle is certainly striking. Given that the seminal receptacle is a blind sac (Fig. 1b), this conspicuous sperm distribution could indicate that these masses of sperm were received in distinct mating events, and raises the possibility of sperm stratification within the seminal receptacle.

The thick muscular wall surrounding the seminal receptacle is considered another characteristic feature of this organ when compared to the bursa copulatrix (Beeman 1977). However, a thick muscular wall is also present in the ampulla in *O. polycerelloides.* Peristaltic waves in the seminal receptacle and the ampulla were associated, respectively, with allosperm displacement to the fertilization chamber during the egg-string formation, and with autosperm displacement to the prostate during mating (Beeman 1977; Thompson 1976). In live *O. polycerelloides*, contractions of these organs could be observed due to the translucent body wall (LSO, pers. obs.).

Seminal fluid was detected in histological sections of the seminal receptacle of *P. taylori* (Beeman 1970a). As allosperm generally have their heads embedded within the epithelium of the seminal receptacle, it was hypothesized that muscular contractions of the wall of the organ could serve to remove the seminal fluid from the allosperm without displacing them (Beeman 1970a). We did not find clear evidence for seminal fluid in the seminal receptacle of *O. polycerelloides*. The alcianophilic substance found within the lumen of the seminal receptacle probably does not correspond to seminal fluid, given that an AB-positive reaction was not found in the prostate (data not shown), where we would expect to find the cells that produce the seminal fluid (Thompson 1976). At least for *O. polycerelloides*, we discard the hypothesized function of seminal fluid removal for the seminal receptacle, because just a fraction of the sperm observed inside of the seminal receptacle had radial orientation. Therefore, given that not all sperm would be attached to the epithelium, if there were contractions of the seminal receptacle, sperm would be expelled together with the seminal fluid.

Other functions hypothesized for the seminal receptacle are nourishment and capacitation of the sperm, mainly because of the proximity between the sperm and epithelium in this organ, and the presence of microvilli that could play a role in nutrient transmission (Beeman 1970a; Thompson & Bebbington 1969; Medina et al. 1988). In *O. polycerelloides*, besides proximity of sperm and epithelial cells and presence of microvilli, a possible secretion was found within the lumen that could play a role in, e.g., sperm nourishment or sperm activation. We could not find, however, histochemical correspondence between this substance and the secretory vesicles of the seminal receptacle epithelial cells. Therefore, we cannot exclude the possibility that this alcianophilic substance has been transferred by the partner during mating.

Bursa copulatrix

In all analyzed samples, the bursa copulatrix contained only degraded sperm (identifiable mainly by their tails) and yolk granules, in accordance with previous reports for other species (e.g., Beeman 1970a; Schmekel 1971; Medina et al. 1988). Although yolk granules were hypothesized to be remnants of degenerative oocytes (Thompson & Bebbington 1969; Brandriff & Beeman 1973; Medina et al 1988), they could also be granules that were not successfully incorporated into oocytes in the hermaphrodite gonad. The most intuitive route for oocytes to reach the bursa copulatrix would be from the fertilization chamber to the uterine and allosperm ducts (Fig. 1b). However, considering the thin diameter of the uterine duct in *O. polycerelloides*, it is unlikely that complete oocytes could reach the bursa through this pathway. Yolk granules, in turn, could easily fit the uterine duct. Another possible path for complete oocytes to reach the bursa would be from the nidamental opening: if not incorporated into the egg mass during oviposition, eggs could reach the bursa copulatrix backwardly through the vagina (Fig. 1b).

Sperm heads were not detected in the bursa copulatrix under conventional light microscopy, and only rarely under TEM, while confocal microscopy revealed that sperm heads were much less numerous in the bursa copulatrix than in the seminal receptacle and ampulla. Moreover, their regular morphology (i.e., slender and curved sperm head) changed to short and ellipsoid in the bursa copulatrix. These results

indicate that sperm nuclei are digested before the flagella, as already suggested for *Aplysia* spp. (Thompson 1976).

Sperm digestion in the bursa copulatrix appears to be related to the secretory activity of its epithelium (Medina et al. 1988). Brandriff & Beeman (1973) described four cell types in the bursa of the aplysiid *P. taylori*, while Schmekel (1971) and Medina et al. (1988) described only one main cell type for several nudibranch species, but with some morphological variations. Our results show that the bursa copulatrix of *O. polycerelloides* is similar to other nudibranchs (Schmekel 1971; Medina et al. 1988). For example, the epithelial cells are rich in RER and mitochondria, suggesting intense cellular activity. Also, their apical plasma membrane shows microvilli-like protuberances that apparently detach as small vesicles, suggesting apocrine secretion. We are not certain, however, if these small vesicles swell into the large ones, as hypothesized by Medina et al. (1998). The electron-lucent vesicles found within the cytoplasm could compose a second type of secretion, although we did not observe their release.

Digestion of surplus sperm and unviable eggs (e.g., Beeman 1970a, 1970b; Medina et al. 1988) has been commonly regarded as the main function of the bursa copulatrix in some sea slugs. Some authors have also proposed that the digested gametes could be used as an energetic source (Brandriff & Beeman 1973; Calow et al. 1979). Accordingly, reabsorption of digested luminal material has been suggested as another possible function of the bursa copulatrix of sea slugs (Hadfield & Switzer-Dunlap 1984). Among the four cell types of the bursa copulatrix of *P. taylori*, one of them had large numbers of vacuoles containing protein-bound lipids that could correspond to absorbed products of gametes digestion (Brandriff & Beeman 1973). Based on the fact that some mollusks can survive for relatively long periods of starvation and still maintain adequate metabolic functions (Nimitz & Giese 1964), it was suggested that the products of the digestion of sperm and oocytes could be used as an energy source (Brandriff & Beeman 1973). However, in the freshwater snail *Limnaea stagnalis*, sperm digestion and absorption in the bursa copulatrix was associated with increased rate of maturation of the individual's oocytes (Horstman 1955), which suggests another possible function, i.e., linking mating behavior to oocyte maturation (Brandriff & Beeman 1973).

Based on the characteristic morphology of the epithelial cells of the bursa copulatrix of several nudibranchs, such as dense microvillar space and high mitochondrial count, Schmekel (1971) suggested that the digested material within the lumen was probably resorbed. Moreover, Medina et al. (1988) found multivesicular bodies (a type of endosome) in the apical region of the epithelial cells of the bursa copulatrix of *Hypselodoris messinensis* [current name *Felimare villafranca* (Risso 1818)]. In *O. polycerelloides*, we found what appeared to be endosomal tubules and endosomes near the apical plasma membrane, which could be evidence of endocytosis of digested products from the lumen. Therefore, it is reasonable to suppose that the bursa copulatrix not only digests but is also capable of absorbing the products of digestion.

Probably based on Thompson (1966), the bursa copulatrix was hypothesized by some authors to be the site of allosperm deposition during copulation (Gosliner 1994; Valdes et al. 2010). However, for aplysiids, the hypothesized process is different (based on the study performed with *P. taylori* by Beeman1970b): the allosperm would be initially directed to the seminal receptacle, being only directed to the bursa copulatrix if the amount of received allosperm exceeds the capacity of the seminal receptacle (e.g., Beeman 1970b; Beeman 1977). The name "gametolytic gland" was suggested for this organ in aplysiids based on this hypothesis (Brandriff & Beeman 1973), even though several authors continued to use the term "bursa copulatrix" (e.g., Klussmann-Kolb 2001; Cunha & Simone 2018). For dorid nudibranchs, the bursa was speculated to be the organ of initial sperm reception during mating (e.g., Thompson 1966; Thompson 1976). However, the bursa of *O. polycerelloides* (also a dorid nudibranch) contained degraded sperm in all specimens analyzed herein, which means that either this organ is not the site for initial sperm reception, or that there is precise control over the digestion process, otherwise all received allosperm would be under the risk of being damaged before being transferred to the seminal receptacle.

Putative post-copulatory mechanisms of sexual selection

Post-copulatory sexual selection theory predicts that the last partner to copulate tends to have greater reproductive success if, e.g., there are strategies for sperm removal by the sperm donor (i.e., individual that donates sperm during mating), or

simply due to sperm stratification within the storage organs (Birkhead & Parker 1997). We found evidence for a possible stratification within the seminal receptacle, with a spherical sperm mass located near the opening of the organ, and some of the remaining sperm displaying radial orientation. Given that sperm may take five hours to be radially oriented (Beeman 1970b), the spherical mass could have been deposited later, presumptively by a different partner. The seminal receptacle is a blind sac, and sperm from the last partner could be moved to the fertilization chamber before the sperm from previous mating events.

If the seminal receptacle has any influence on allosperm capacitation and/or nourishment, the recipient (i.e., individual that receives sperm) could also bias the paternity of its offspring by choosing which sperm would be capacitated and/or nourished (Eberhard 1996). Another possible mechanism of cryptic female choice would be through sperm digestion in the bursa copulatrix, which could affect the fertilization success of a sperm donor, independently of the mating order (e.g., Birkhead et al. 1993).

Sperm-digesting organs are common in hermaphrodites, as well as strategies to counteract sperm digestion (e.g., Lind 1973; Rogers & Chase 2001). For example, during courtship, the snail *Helix aspersa* (Müller 1774) attempts to pierce the body wall of its partner with calcareous darts covered with mucus. . The injected mucus leads to the closure of the opening of the bursa copulatrix of the partner. Consequently, more sperm are stored in the seminal receptacle of the sperm recipient (Koene & Chase 1998). Considering the morphological characteristics of the reproductive system of *O. polycerelloides*, the long penis of this species (see Sales et al. in press) would permit sperm deposition directly into the seminal receptacle. A long penis could thus be a strategy to avoid or delay sperm digestion in the bursa copulatrix, but further studies are required to test this hypothesis.

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Figures

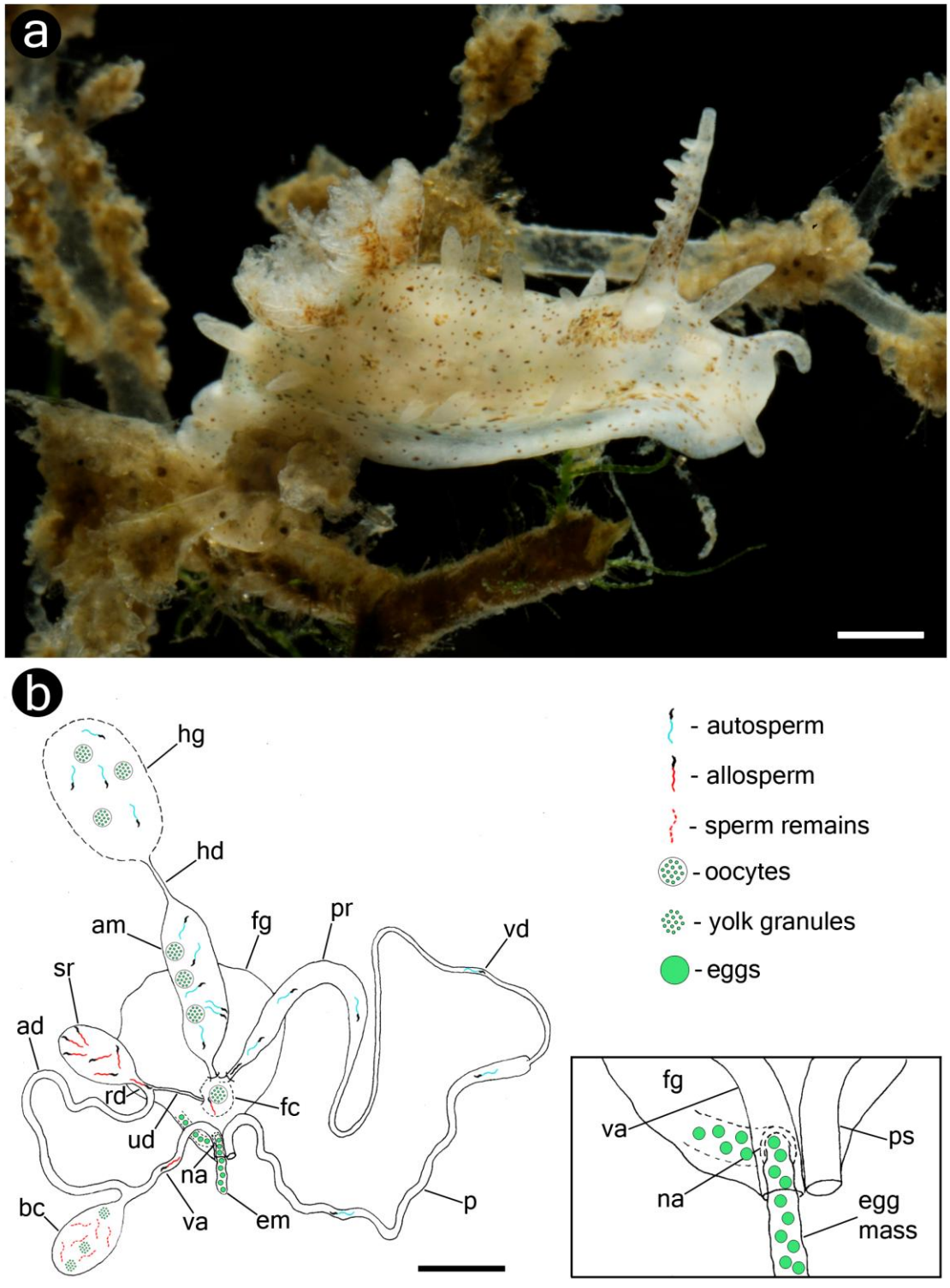

Figure 1. *Okenia polycerelloides* (Ortea & Bouchet 1983). **(a)** Live specimen. **(b)** Gross morphology of the reproductive system, and inferred pathway of oocytes, autosperm and allosperm. The inset shows the region of the common genital pore in detail. **Scale bars**: (a) 1 mm; (b) 500 µm. **Abbreviations**: ad, allosperm duct; am, ampulla; bc, bursa copulatrix; em, egg mass; fc, fertilization chamber; fg, female gland; hd, hermaphrodite duct; hg, hermaphrodite gland; na, nidamental opening; p, penis; pr, prostate; ps, penial sac; rd, seminal receptacle duct; sr, seminal receptacle; ud, uterine duct; va, vagina; vd, vas deferens.

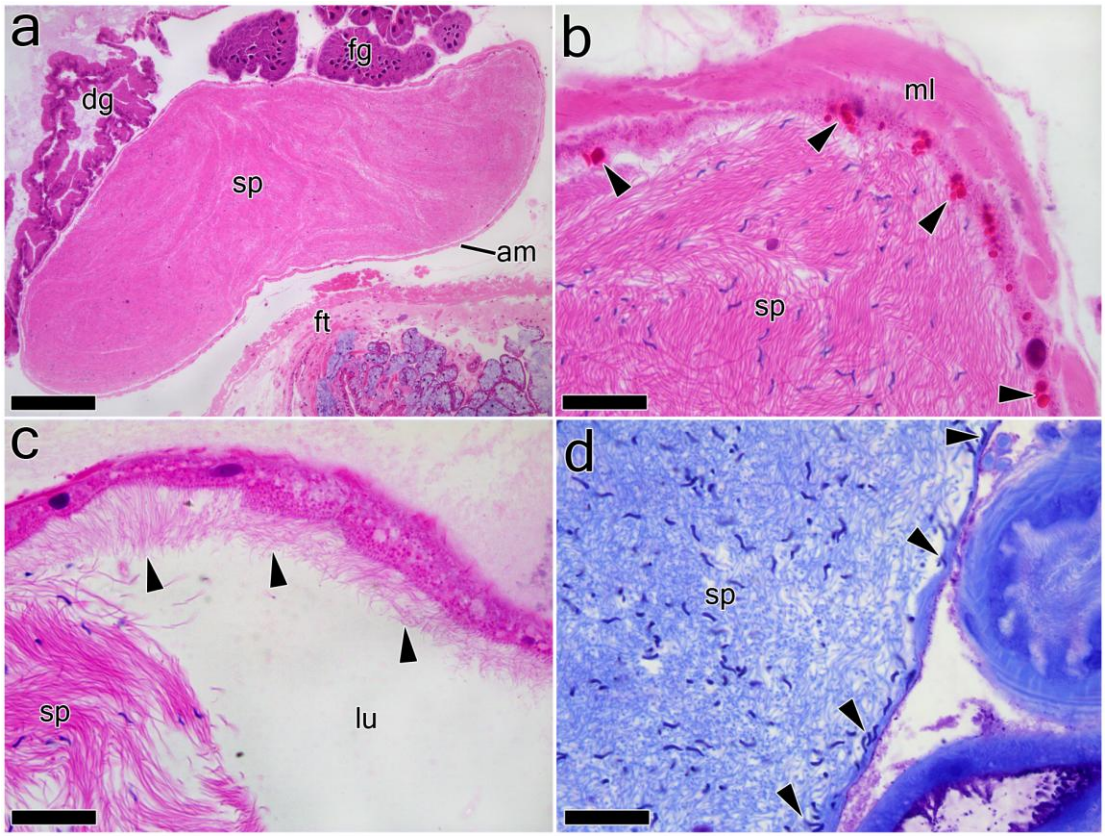

Figure 2. Histology of the ampulla of *Okenia polycerelloides* under light microscopy. **(a)** Overview of a sagittal section. HE. **(b)** Detail of the ampullar wall, showing the epithelium surrounded by a layer of muscle fibers. The lumen of the ampulla is filled with sperm, and particulate material similar to yolk granules (arrowheads) lies close to epithelium. HE. **(c)** Detail of the ampullar wall, showing long cilia (arrowheads). HE. **(d)** The ampullar lumen is filled with sperm; some sperm heads are in contact with the ampullar epithelium (arrowheads). TB. **Scale bars**: (a) 200 µm; (b-d) 20 µm. **Abbreviations**: am, ampulla; dg, digestive gland; fg, female gland; ft, foot; lu, lumen; ml, muscle layer; sp, sperm.

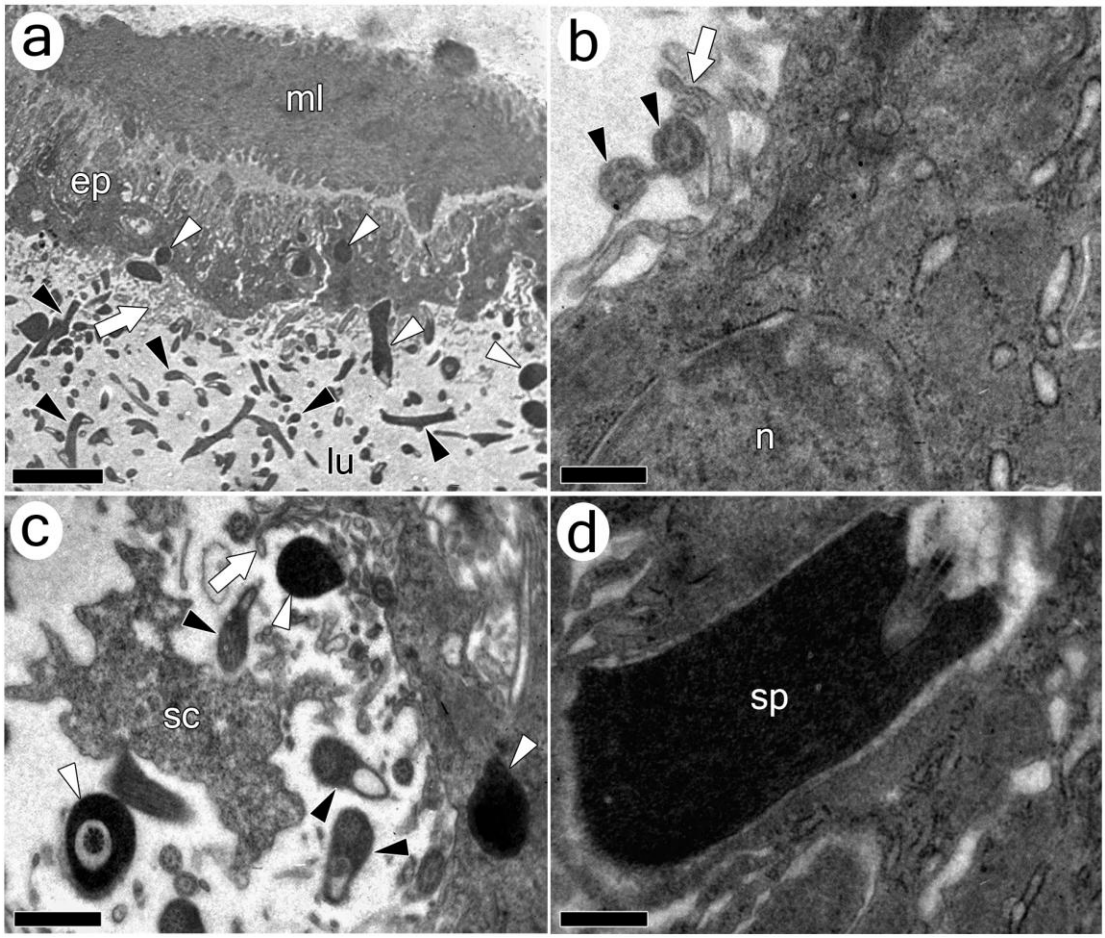

Figure 3. Histology of the ampulla of *Okenia polycerelloides* under electron microscopy. **(a)** Overview of the ampullar wall under transmission electron microscopy White arrowheads indicate sperm heads; black arrowheads indicate sperm tails; and arrows indicate microvilli. **(b)** Detail of the ampullar epithelium, showing microvilli (arrows) and sperm flagella (arrowheads). **(c)** Detail of the ampullar lumen, showing secretion interspersed with sperm heads (white arrowheads) and sperm tails (black arrowheads). Arrow points to microvilli. **d)** Sperm head immersed in the ampullar epithelium. **Abbreviations**: ep, epithelium; lu, lumen; ml, muscle layer; n, nucleus; sc, secretion; sp, sperm. **Scale bars**: (a) 5 µm; (b-d) 0,5 µm; (c) 1 µm.

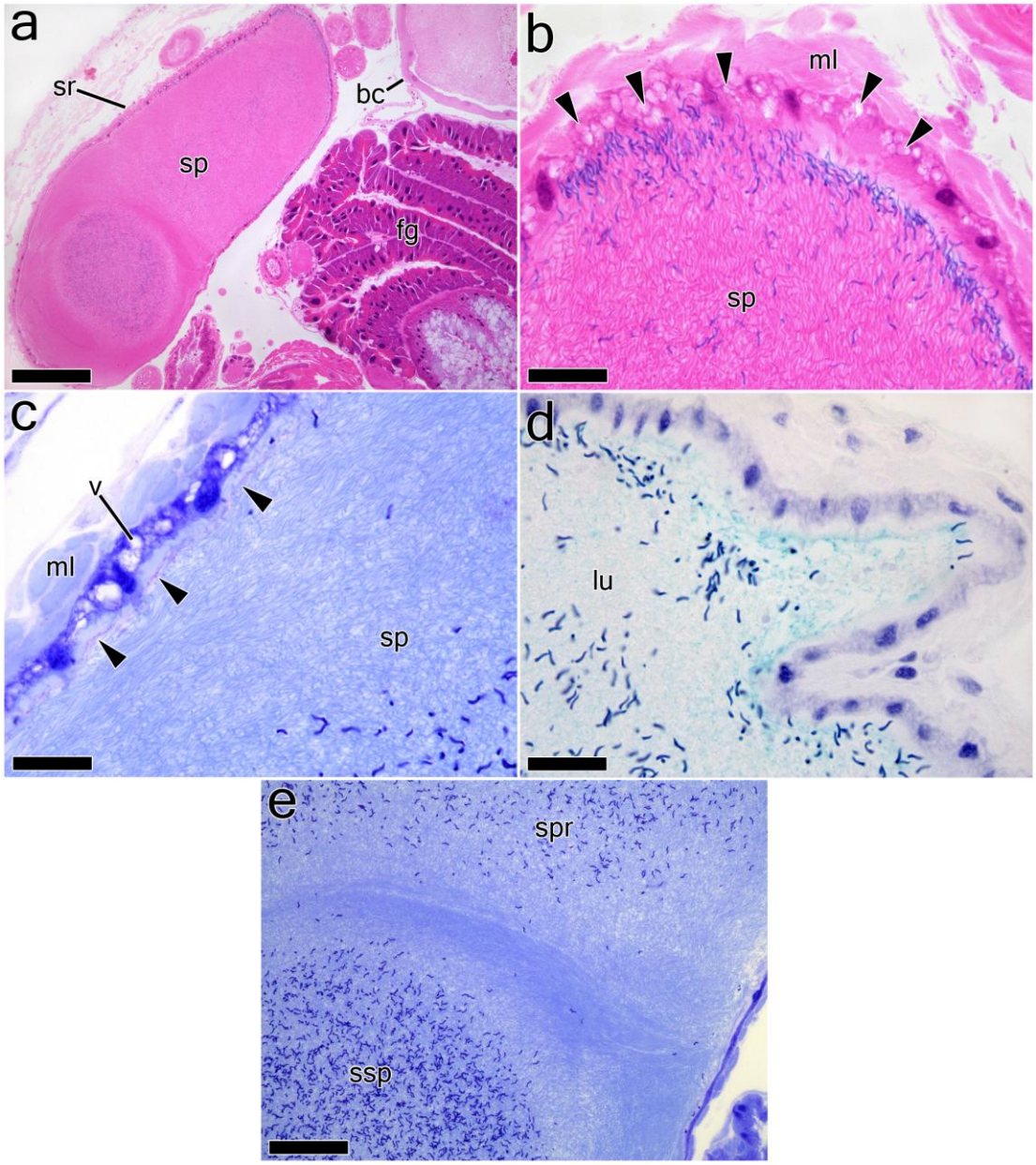

Figure 4. Histology of the seminal receptacle of *Okenia polycerelloides* under light microscopy. **(a)** Overview of a sagittal section. HE. **(b)** Detail of the seminal receptacle wall, showing the vacuolated epithelium (arrowheads) surrounded by a layer of muscle fibers. Most sperm within the lumen are oriented with their heads facing the epithelium. HE. **(c)** Detail of the seminal receptacle wall, showing the epithelial surface covered by a secretion (arrowheads) that stained mostly in light blue, except for its thin external layer that stained in pink. TB. **(d)** Alcianophilic secretion scattered throughout the seminal receptacle lumen. AB. **(e)** Detail of the lumen seen in **a**, showing sperm concentrated in a spherical mass located near the opening of the seminal receptacle. TB. **Scale bars**: (a) 200 µm; (b-d) 20 µm; (e) 50 µm. **Abbreviations**: bc, bursa copulatrix; fg, female gland; lu, lumem; ml, muscle layer; n, nucleus; sp, sperm; spr, sperm randomly oriented; spp, spherical sperm mass; sr, seminal receptacle, v, vacuole.

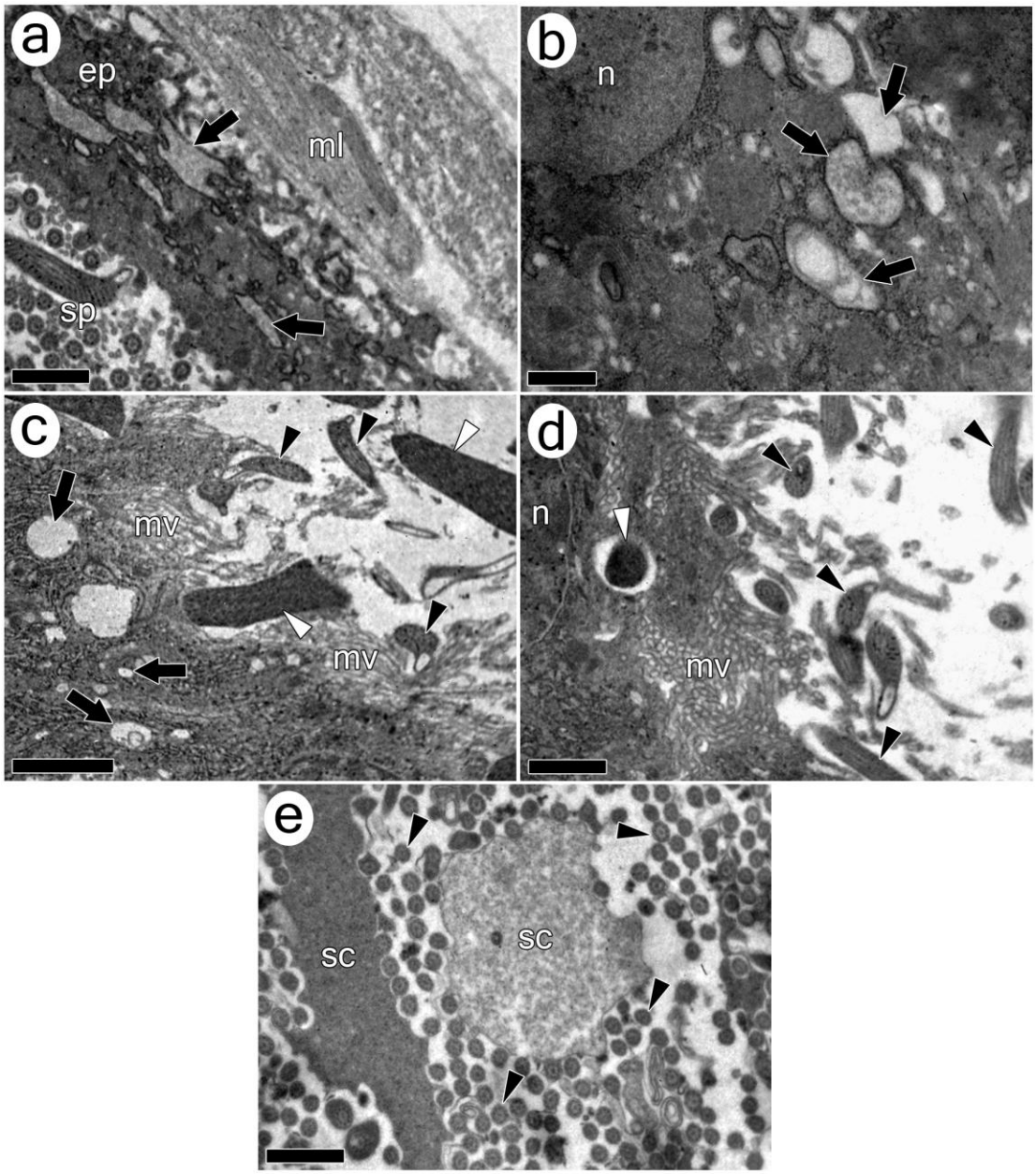

Figure 5. Histology of the seminal receptacle of *Okenia polycerelloides* under electron microscopy. **(a)** Overview of the seminal receptacle wall under transmission electron microscopy showing a thick muscular layer, an epithelium with electron-lucent vacuoles (arrows), and sperm in the lumen. **(b)** Detail of the epithelium, showing electron-lucent vacuoles (arrows). **(c-d)** Detail of the epithelial surface, showing sperm heads (white arrowheads) embedded within the microvilli. Black arrowheads indicate sperm tails and arrows point to vacuoles in the epithelium. **(e)** Secretion interspersed with sperm flagella (arrowheads) within the lumen. **Scale bars**: (a, d and e) 1 µm; (b) 0,5 µm; (c) 2 µm. **Abbreviations**: ep, epithelium; ml, muscle layer; mv, microvilli; n, nucleus; sc, secretion; sp, sperm.

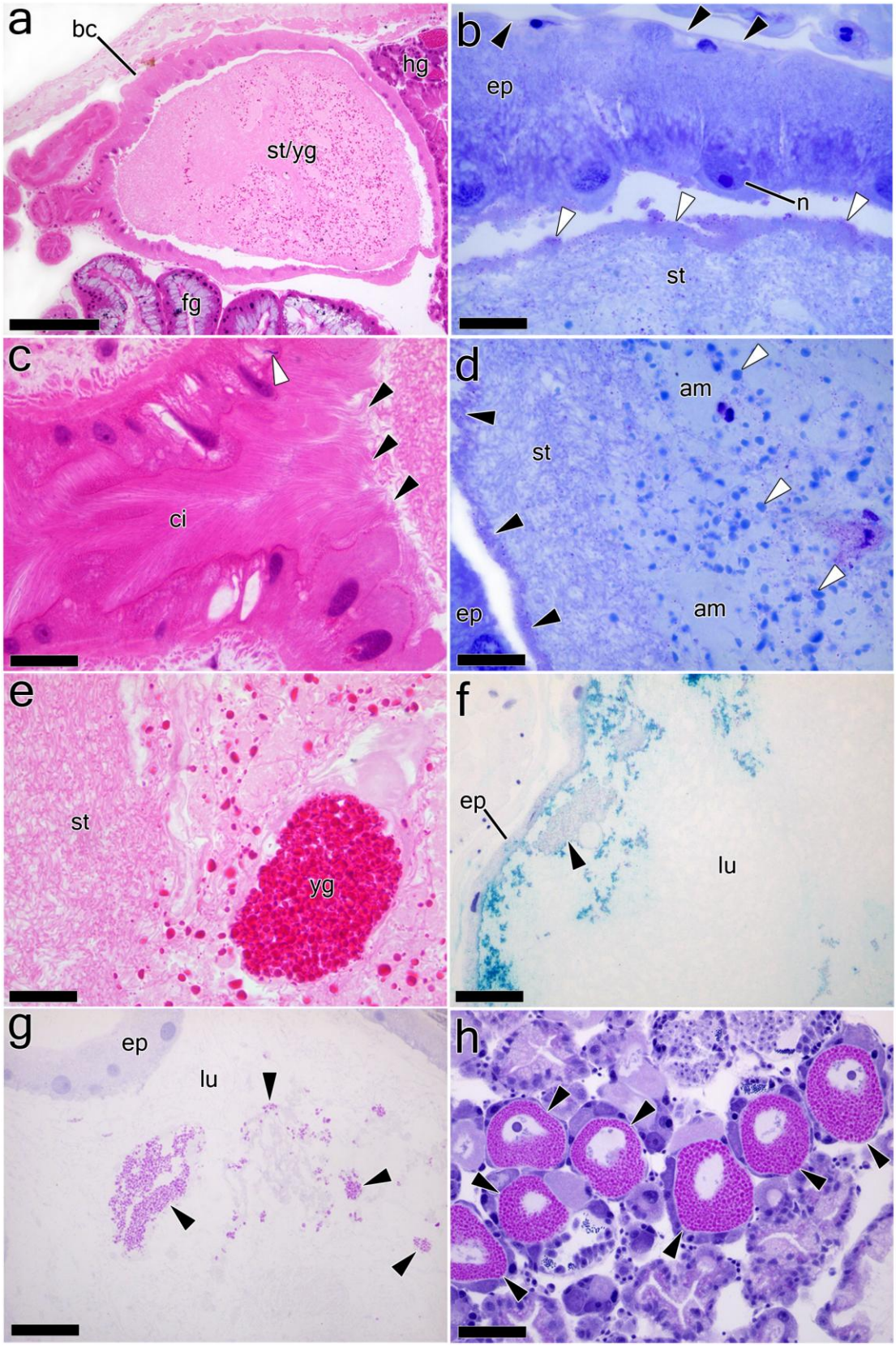
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Figure 6. Histology of the bursa copulatrix and hermaphrodite gonad of *Okenia polycerelloides* under light microscopy. **(a)** Overview of a sagittal section. HE. **(b)** Detail of the bursa copulatrix wall, showing the epithelium with large nuclei and the surrounding thin layer of connective tissue (black arrowheads). Within the lumen, a layer of apparently secreted substances (white arrowheads) surrounds the luminal contents. TB. **(c)** Detail of the region of the opening of the organ, showing long cilia (black arrowheads). A sperm head (white arrowhead) is also visible. HE. **(d)** Detail of the lumen, showing sperm tails interspersed with amorphous material and yolk granules (white arrowheads). Black arrowheads indicate the layer of possibly secreted material surrounding the luminal contents. TB. **(e)** Same as **d**, but stained with HE. **(f)** Alcianophilic substance (possibly secretion) within the lumen. Arrowhead points to yolk granules. AB. **(g)** Yolk granules (arrowheads) with affinity for PAS. **(h)** Oocytes (arrowheads) in the hermaphrodite gonad. PAS. **Scale bars**: (a) 200 µm; (b-e) 20 µm; (f-h) 50 µm. **Abbreviations**: am, amorphous material; bc, bursa copulatrix; ci, cilia; ep, epithelium of the bursa copulatrix; fg, female gland; hg, hermaphrodite gonad; lu, lumen; n, nucleus; st, sperm tails; st/yg, sperm tails mixed with yolk granules; yg, yolk granules.

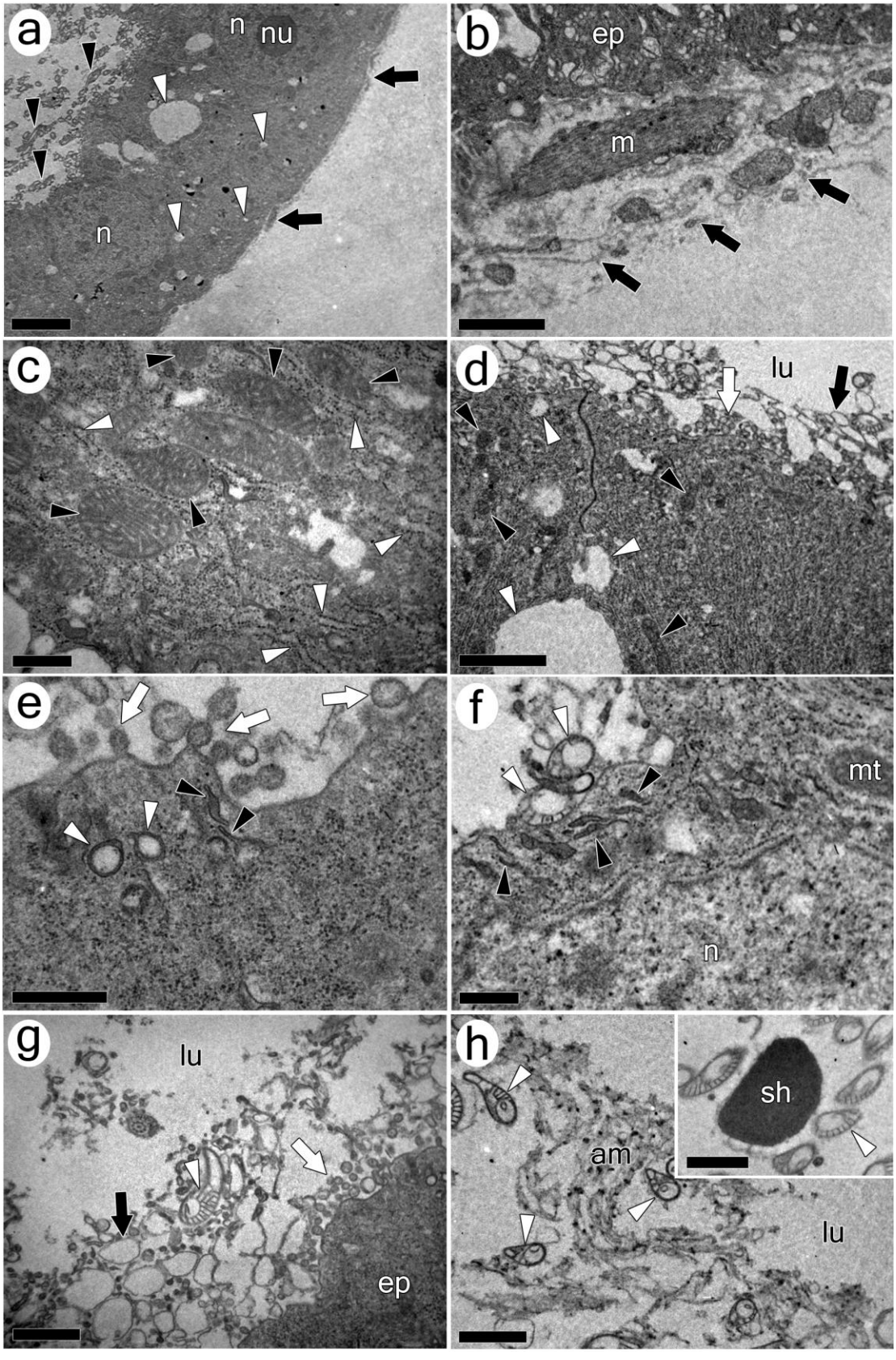
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Figure 7. Histology of the bursa copulatrix of *Okenia polycerelloides* under transmission electron microscopy. **(a)** Overview of the bursa copulatrix wall under transmission electron microscopy. White arrowheads indicate electron-lucent vesicles of different sizes; black arrowheads indicate degenerate sperm tails in the lumen; and arrows point to the surrounding connective tissue of the bursa copulatrix. **(b)** Muscle cell within the connective tissue layer (arrows) surrounding the organ. **(c)** Detail of the cytoplasm of an epithelial cell, showing mitochondria (black arrowheads) and rough endoplasmic reticulum cisternae (white arrowheads). **(d)** Apical region of the epithelial cell, showing vesicles (white arrowheads) and mitochondria (black arrowheads) within the cytoplasm, and small (white arrows) and large vesicles (black arrows) at the luminal surface. **(e)** Detail of the apical region of the epithelium, showing small protuberances detaching from the plasma membrane as small vesicles (white arrows). Within the cytoplasm, what appear to be endosomes (white arrowhead) and endosomal tubules (black arrowheads) were present near the apical plasma membrane. **(f)** Detail of the apical plasma membrane, showing what appear to be endosomal tubules (black arrowheads). Sperm tails (white arrowheads) are also visible. **(g)** Detail of the apical region of the epithelium, showing small (white arrows) and large vesicles (black arrows) and sperm tail (arrowhead). **(h)** Detail of the luminal content, showing sperm tails (arrowheads) and amorphous material (**inset**: detail of the lumen showing part of a sperm head and sperm tail indicated by arrowhead). **Scale bars**: (a) 5 µm; (b, d) 2 µm; (c, e and g) 0,5 µm; (f, h and inset) 1 µm. **Abbreviations**: am, amorphous material; ep, epithelium; lu, lumen: m, muscle cell; mt, mitochondria; n, nucleus; nu, nucleolus; sh, sperm head.

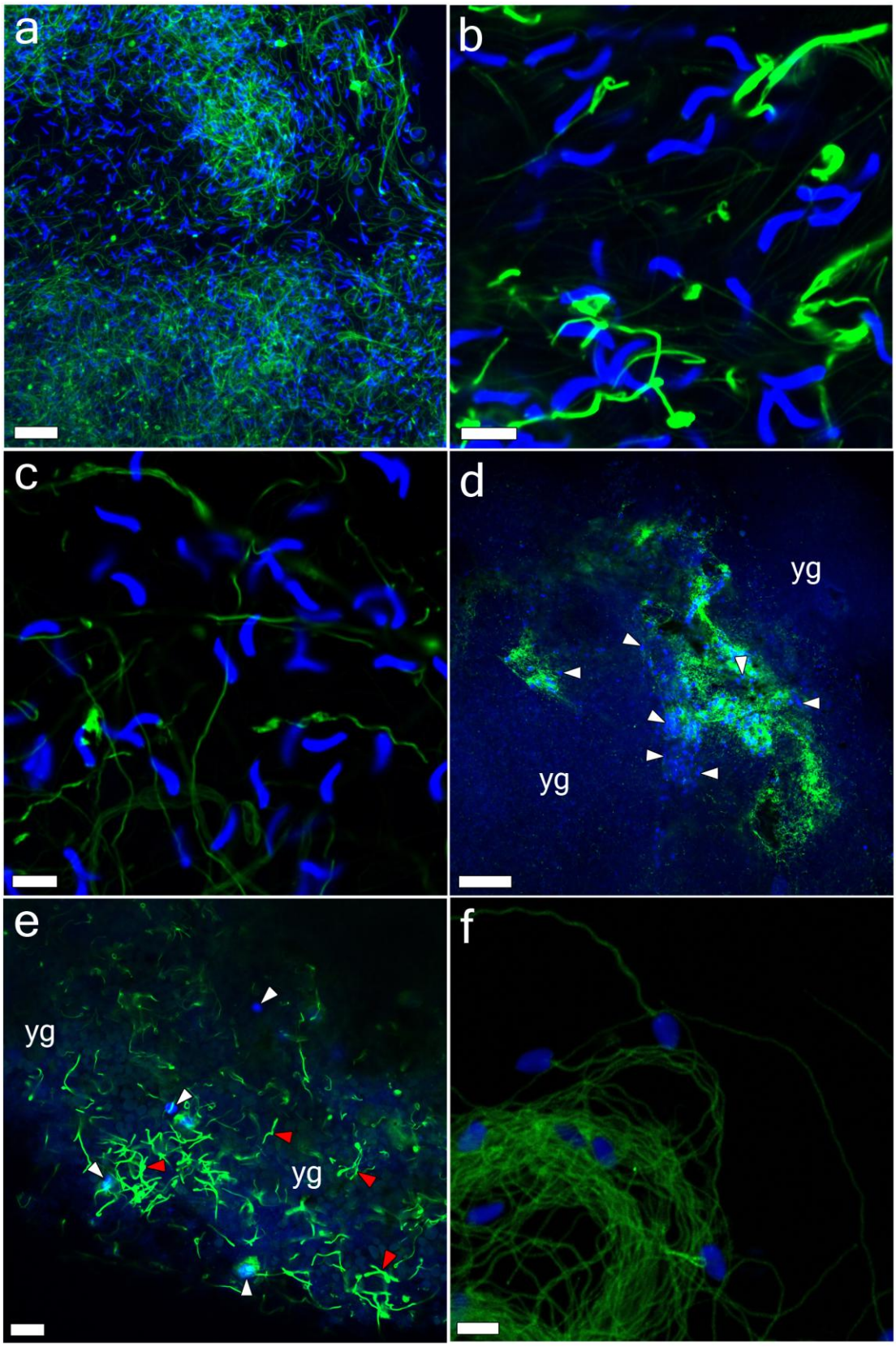
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Figure 8. Confocal microscopy analysis of the contents of the sperm-containing chambers of *Okenia polycerelloides*. **(a)** Contents of the seminal receptacle. A similar condition was observed for the ampulla. **(b-c)** Detail of sperm from the seminal receptacle **(b)** and ampulla **(c)**, showing long flagella and slender and curved heads. **(d-e)** Contents of the bursa copulatrix, showing a much smaller number of sperm heads (white arrowheads) than the other organs, and also abundant yolk granules. Red arrowheads point to sperm tails. **(f)** Detail of sperm from the bursa copulatrix, showing shorter and more ellipsoid heads. **Scale bars**: (a) 20 µm; (b, c and f) 5 µm; (d) 50 µm; (e) 10 µm**. Abbreviations**: yg, yolk granules.

Capítulo 2

Sperm transfer, storage and digestion in a sea slug: towards understanding post-copulatory sexual selection mechanisms in simultaneous hermaphrodites

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Abstract

Sea slugs are simultaneous hermaphrodites that have organs for sperm storage and digestion, which makes the fate of the sperm uncertain after it is transferred to a partner. Also, many functional implications are attributed to sperm digestion, but little is known about in which specific situations and how often digestion occurs. In the present study, we combined experimental manipulations and histological analyses of the sea slug *Okenia polycerelloides* (Ortea & Bouchet 1983) to investigate (1) its mating behavior, (2) the fate of sperm within the female reproductive tract after one and two subsequent matings, and (3) if starvation influences the occurrence of sperm digestion. Mating interactions initiated by mutual contact using the oral region, followed by body alignment, penis intromission, and sperm transfer. However, interactions could end at any of these stages, and even penis intromission did not guarantee insemination. Sperm was received and stored by the seminal receptacle. Distinct mating events could lead to sperm stratification in the seminal receptacle. Although starvation seemed to intensify sperm digestion, it did not determine its occurrence. Sperm digestion occurred continuously, and at least some amount of sperm received during each mating event was directed to the bursa copulatrix to be digested, corroborating the hypothesis of surplus sperm digestion. However, all received sperm could also be directed to the bursa copulatrix, suggesting the possibility of cryptic female choice. Gradual reduction of the contents of the bursa copulatrix with increasing post-copulation times suggests resorption of the digested materials. These finds are discussed in the light of sexual selection theory.

Keywords: post-copulatory sexual selection, cryptic female choice, sperm competition, sperm storage, sperm digestion.

Introduction

Sexual selection is pervasive and shapes reproductive traits not only in dioecious animals, but also in hermaphrodites (Charnov 1979; Andersson 1994). Many hermaphrodites mate with multiple partners and have organs for sperm storage and digestion, which are potentially involved in post-copulatory mechanisms of sexual selection such as sperm competition and cryptic female choice (Baur 1998; Michiels 1998). The former mechanism occurs when sperm from different mates compete for fertilization of a given set of ova (Parker 1970), while the latter occurs when an individual biases paternity favoring the selection or differential use of sperm of a particular mate (Eberhard 1994). Sperm competition and cryptic female choice generally occur inside the female reproductive tract. Thus, the knowledge of the functioning of the female reproductive tract during and after sperm reception can be really useful in understanding post-copulatory sexual selection.

Sperm storage organs vary in number and morphology in hermaphrodites, associated with distinct reproductive strategies. For example, oligochaetes may bear several sperm storage organs (Adiyodi 1988), while land snails have one compartmentalized organ (e.g., Haase & Baur 1995; Dillen et al. 2009) and sea slugs a blind-sac (e.g., Schmekel 1971; Sales & Marian in prep.[1]). Land snails may show intraspecific variation in the number of compartments of the storage organ (i.e., spermathecal tubules), thus there are different possibilities of sperm storage among conspecifics (Baur 1998). In this case, as well as in oligochaetes with several storage organs, differential sperm storage could occur under the sperm recipient (i.e., the individual that receives sperm during mating) control (Haase & Baur 1995; Michiels 1998; Bojat & Haase 2002). On the other hand, mixing of sperm from different matings is more likely when the storage organ is a blind-sac (Baur 1998), resulting in spatial and temporal overlap among sperm from different mating partners and, hence, intense sperm competition (Wigby & Chapman 2004).

[1] Sales and Marian (in prep.) refere-se ao capítulo 1 da presente Tese.

The majority of hermaphrodites with internal fertilization have organs or special cells that digest sperm (Baur 1998; Michiels 1998), a phenomenon that affects sperm competition and can also be involved in cryptic female choice. A common organ related to sperm digestion in many hermaphrodite taxa is the bursa copulatrix (e.g., flatworms, Ball & Reynoldson 1981; snails Barker 2001; sea slugs, Thompson 1966). Its function is usually regarded as digestion of surplus sperm and unused eggs (e.g., Beeman 1970a, 1970b; Medina 1988), but a possible role in cryptic female choice was also speculated, i.e., by reducing the fertilization success of low-quality partners (Birkhead et al. 1993). Moreover, it was hypothesized that the digested surplus sperm could be used as a nutrient source (Brandriff & Beeman 1973; Calow et al. 1979; Sluys 1989). Allosperm resorption was reported for free-living flatworms (e.g., Sluys 1989) and earthworms (e.g., Grove 1925), and suggested for sea slugs (e.g. Brandriff & Beeman 1973). Protein-bound lipid was found in the vacuoles of the bursa copulatrix epithelium of the sea slug *Phyllaplysia taylori* Dall, 1900 (Brandriff & Beeman 1973). Based on this finding and in the fact that some mollusks can survive starvation for long periods (Nimitz & Giese 1964), it was suggested that the products of sperm and eggs digestion could be used as a nutrient source in sea slugs (Brandriff & Beeman 1973). Although the occurrence of sperm digestion in the bursa copulatrix is known for several sea slugs (e.g., Beeman 1970a; Schmekel 1971; Medina et al. 1988), it remains obscure in which situations and how often sperm are digested, and whether this process really provides nutrients for the recipient.

The fact is, for most hermaphrodites the fate of sperm after they are transferred to a partner is obscure (Angeloni et al. 2003). Considering the presence of organs capable of storing and digesting sperm, and thus potentially involved in post-copulatory sexual selection mechanisms, sperm fate is uncertain. Therefore, studies that shed some light on what happens to the sperm of different partners after copulation are important to understand the roles played by such organs and detect post-copulatory mechanisms of sexual selection.

Okenia polycerelloides (Ortea & Bouchet 1983) is a dorid nudibranch that shows a promiscuous mating behavior (Sales at al. in press) and a complex reproductive system that contains both a seminal receptacle and a bursa copulatrix (Sales & Marian in prep.). In the present study, we combined experimental manipulations and histological analyses of *O. polycerelloides* to investigate: (1) its

mating behavior, (2) the fate of sperm within the female reproductive tract after one and two subsequent matings, and (3) if starvation influences the occurrence of sperm digestion.

Material and Methods

1. Animals

1.1. Collection. *Okenia polycerelloides* is commonly found associated with colonies of the bryozoan *Amathia verticillata* (delle Chiaje 1822) and *Amathia vidovici* (Heller 1867), and because of its small size (up to 10 mm) and cryptic color pattern, capture of this sea slug is carried out indirectly. Thus, colonies of the bryozoans were collected by free diving in the Araçá Bay (São Paulo state, Brazil), between 23°48'47.5" S and 45°24'31.4" W. The colonies were packed alive in plastic bags or buckets with seawater and transferred to the Center for Marine Biology of the University of São Paulo (CEBIMar-USP). The colonies were kept in aquaria with circulating seawater and verified by eye for the presence of the sea slug *O. polycerelloides.* Once individuals of the sea slug were found, they were isolated and treated according to the purpose for which they would be used. For the experimental manipulations, we have used both "field" and "cultured" individuals.

1.2. Field individuals. Field individuals were initially maintained isolated in plastic pots with seawater and food until total depletion of their stored sperm. In *Alderia modesta* (Lovén 1844) (a sea slug species), unfertilized egg masses (i.e., those with only unviable eggs) are produced by virgin specimens and also by individuals who have completely exhausted their previous sperm stock (see Angeloni 2003). Thus, before the beginning of the manipulations, field individuals were kept isolated and their egg masses were checked until they no longer had viable eggs. This procedure was adopted so that the trajectory of the received sperm could be observed in situations where the reproductive tract was free of previous sperm.

1.3. Cultured individuals. Collected animals were allowed to copulate and their egg masses were cultured until juveniles were obtained. Juveniles were isolated and kept in pots with seawater (daily changed), and with bryozoan colonies as substrate and food source, until they reached the adult stage. About 15 days after settlement,

individuals were already able to mate. As these animals were cultured in the laboratory, there would be a risk of a potential degree of kinship among them, which could influence the results of the experiments. To prevent this issue, identification codes were attributed to the progenitors, their egg masses and descending offspring. Therefore, we could avoid that mating partners and focal individuals were siblings.

2. Experimental manipulations – functional morphology

To study the route of the received sperm inside the female reproductive tract, we analyzed the histology of the reproductive system of *O. polycerelloides* in five situations: 1) without mating; 2) 30 minutes after one mating; 3) 1 h after one mating; 4) 2 h after one mating; and 5) 3 h after one mating. Eight individuals were analyzed in each manipulation (five field and three cultured individuals). They were allowed to mate and, after the specified interval, focal individuals were anesthetized and processed for analysis (see section 5). Individuals fixed without mating were used as a control group, providing information about the histological aspect of the reproductive tract when the bursa copulatrix and seminal receptacle were empty. Also, they were useful to determine whether the sperm stock of field individuals actually depletes after laying a sequence of unviable egg masses during the period of isolation in captivity.

In a second series of experimental manipulations, individuals were allowed to mate subsequently with two different partners, to determine if sperm reception and storage change in this situation. Also, we intended to analyze whether the sperm stratification previously reported in the seminal receptacle of this species (Sales & Marian in prep.) is indeed related to multiple matings. Sample size and conditions for fixation were similar to the first manipulation (i.e., 30 min, 1 h, 2 h and 3h after the second mating). Thus, considering all manipulations performed with field and cultured individuals, a total of 72 individuals were analyzed.

3. Experimental manipulations – sperm digestion during starvation

In order to avoid potential effects of the presence of remnant sperm in the reproductive tract, only cultured individuals were used in this experiment. The length

of the specimens was measured prior to and after the end of the experiment by observing them for 1 minute under a stereomicroscope equipped with an ocular micrometer. The total length was considered as the longest length observed during its movement within the interval of one minute (Angeloni 2003). The percentage of body growth in relation to the initial size of the individual was compared between control and experimental groups using t-test.

Control and experimental groups were formed by individuals of similar size, each group with an initial sample of 10 focal individuals. Each individual was allowed to copulate with only one partner during the whole duration of the experiment, to avoid or reduce the influence of post-copulatory sexual selection pressures in this experiment. As there would be sperm from only one donor within the reproductive tract, there would be no inter-ejaculate sperm competition, and we would lower the chances of cryptic female choice. Therefore, the chances of sperm digestion potentially associated with post-copulatory sexual selection would be lowered. The control group was maintained with food supply during the whole experiment, while in the experimental group the only difference was the removal of the food supply during a period of five days. Previous experience of one of us (LSO) with this species showed that it may survive longer than five days without food. Because of the death of some individuals from control and experimental groups during the experiment, the final sample was reduced to seven individuals for each group.

Individuals were randomly selected for each group, as well as focal individuals and their possible partners. For the first mating, at least two potential partners were offered to each focal individual at the same time, to give it the chance of partner choice. The individuals were observed for up to 30 minutes and, if copulation did not occur, other possible partners were randomly selected to be paired with the focal. Once the first copulation occurred, the same partner was used for the whole experiment. Focal individuals were allowed to copulate once a day. After copulation had ended, the individuals were again isolated in their respective pots. Total copulation duration per individual (i.e., the sum of the duration of all copulations performed during the 5 days) and mean copulation duration were compared between control and experimental groups using t-test. Mating duration was used as a proxy for

sperm transfer (i.e., the higher the mating duration, the higher the volume of sperm transferred).

During the experiment, pots were daily checked for the presence of egg masses, which were fixed in ethanol 70%. Later, egg masses were photographed and their length and diameter measured from the photographs using Leica Application Suite V.4. Subsequently, a subsample of each egg mass was dissected, measured as above and their number of eggs counted. As the egg mass is cylindrical, the volume of the entire mass and their subsamples were calculated using the cylinder formula ($V = h.\pi.r^2$). The total number of eggs of each egg mass was then extrapolated from its subsample. Time (in days) from first mating until first spawning, number of egg masses per individual, number of eggs per egg mass, and total number of eggs laid per individual were compared among control and experimental groups using t-test. Total number of eggs laid was used as a proxy for use of stored allosperm (i.e., the higher the number of eggs laid, the higher the number of sperm used for fertilization and, hence, not stored nor digested).

After 24 h of the last mating, the individuals of both groups were anesthetized and fixed for subsequent histological analysis (see section 5). We calculated mean and standard deviation for all numerical data.

4. Mating behavior

The mating behavior was carefully observed under the stereomicroscope during each mating interaction in all aforementioned manipulations. Additionally, the mating behavior of field individuals that were not used for any experimental manipulation (n = 10) was also analyzed. Whenever possible, the mating behavior was recorded under the stereomicroscope using a high-definition video camera.

5. Histology

Specimens (45 field and 27 cultured individuals of the mating manipulations, and 14 cultured individuals of the starvation experiment) were first anesthetized with a mixture of menthol and isotonic magnesium chloride solution dissolved in seawater and then fixed in 4% paraformaldehyde in phosphate buffered saline 0.1 M for 8 h at

4°C. The material was then washed in distilled water, dehydrated in a graded ethanol series, and embedded in glycol-methacrylate resin (Leica Historesin Embedding Kit, Leica Microsystems Nussloch GmbH, Germany). Serial sagittal 3 µm sections of the entire individuals were obtained on a Leica RM2255 microtome (Leica, Wetzlar, Germany) and stained with Hematoxylin-Eosin (H&E) and Toluidine Blue (TB). Some histochemical protocols, such as Periodic Acid–Schiff (PAS), Alcian Blue at pH 2.5 (AB), Naphthol yellow (NY) and Mercury-Bromophenol Blue (BB), were also applied and compared to the results of Sales & Marian (in prep.).

Results

Mating behavior

Okenia polycerelloides has a common genital pore at the right side of the body (Fig. 1a, b), where the openings of male and female reproductive systems are located. This sea slug possesses two short oral tentacles in dorsolateral position to the mouth (Fig. 1a). When two individuals approached, they generally made contact using the oral region including the oral tentacles, moving these structures over each other before mating (hereafter called "oral contact"). Oral contact could be either mouth to mouth, mouth to the right region of the partner's body, or both types of contact, in a random order. The oral contact on the right region of the body could either occur randomly over the whole region, specifically over the genital pore (Fig. 1c), or both. This behavior varied in duration, and one partner could invest more time in oral contact than the other. After oral contact, the partners could either separate from each other without mating, or align their bodies in a way that allowed the contact between their genital pores, achieving mating position ("body alignment"; Fig. 1d). Based on the mating manipulations performed herein (see below), when partners maintained body alignment for less than ca. four minutes, sperm exchange did not occur, even if their penises were inserted in the vagina of each other. If they maintained body alignment for more than ca. four minutes, sperm exchange did generally occur.

During mating, individuals could also feed on the bryozoans on which they live. The separation of two partners could either occur simultaneously, or only one partner moved away while the other remained in the same position. This species has a very

long penis (Fig. 1b, and see Sales & Marian in prep., fig. 1b) and, in some cases, when only one individual moved away, the penis of the other could remain in the genital pore of the moving partner for some time, until the distance surpassed the length of the penis.

Functional morphology

The bursa copulatrix and seminal receptacle of field individuals that were maintained isolated until laying unviable eggs showed the same general condition of the organs of cultured virgin individuals, i.e., both organs had no sperm (Fig. 2a–d). However, the bursa copulatrix of field individuals showed a more intense secretory activity (Fig. 2a, b). Another difference was the presence of yolk granules in the bursa copulatrix of four out of five field individuals (Fig. 2a), which was not observed in cultured virgin individuals (Fig. 2b). The aforementioned differences between field and cultured animals were observed in all manipulations (Figs. 3-5).

When partners maintained body alignment for less than ca. four minutes, the histological aspect of both organs was identical to individuals without copulation. This happened with a total of four individuals: one field individual fixed 30 min after mating, two individuals (one field and one cultured) fixed 1 h after mating, and one field individual fixed 2 h after mating. At the time of the experiments, we were not aware of this time-dependent behavior, and the partners of two of these individuals were allowed to copulate a second time and were used in the experimental group of two subsequent matings (see below).

Individuals that mated once and were fixed 30 min after copulation (Fig. 3a–d) presented two possible configurations (similar in both field and cultured individuals): 1) some sperm tails conspicuous in the bursa copulatrix (Fig. 3a), and seminal receptacle full of sperm (Fig. 3c); or 2) bursa copulatrix devoid of sperm (similar to the control group; Fig. 3b), and seminal receptacle full of sperm (Fig. 3d). In field individuals the sperm was randomly arranged in the seminal receptacle (Fig. 3c), while in some cultured individuals sperm heads were close to and faced the epithelium of the organ (Fig. 3d).

One hour after one mating, the bursa copulatrix of all analyzed individuals showed a large amount of sperm tails in the lumen (Fig. 4a, b), and in some of them,

the lumen of the organ was almost completely filled with sperm tails (Fig. 4b). The seminal receptacle remained full of sperm (Fig. 4c–e). Sperm orientation in this organ was similar to the condition found after 30 minutes, except that at this time some field individuals also showed sperm heads close to and facing the epithelium (Fig. 4e). In some field individuals, there appeared to be intense secretory activity by the seminal receptacle (Fig. 4e) and bursa copulatrix (Fig. 4f), with numerous secretory droplets.

In both field and cultured individuals, there was a decrease in the amount of sperm tails in the bursa copulatrix in two hours after one mating (Fig. 5a, b), when compared to individuals that were fixed 1 hour after mating (Fig 4a, b). The seminal receptacle, however, remained full of sperm (Fig 5c, d). The same pattern was found in individuals fixed three hours after one mating.

The main difference between one- and two-matings experimental groups was sperm organization in the seminal receptacle. In many individuals fixed after two subsequently matings, the sperm content in the receptacle was apparently divided into two sperm masses, one with the sperm concentrated in a spherical mass in the center of the lumen (sometimes near the opening, sometimes more internally), and the other randomly organized and sometimes surrounding the first mass (Fig. 6a). This sperm configuration within the seminal receptacle was observed in at least some individuals of each experimental group (i.e., 30 min, 1 h, 2 h, 3 h after the second mating), in both field and cultured animals. In some individuals – including those whose first mating lasted less than 4 minutes – only sperm randomly arranged were found (Fig. 6b).

Individuals that mated twice had sperm in the bursa copulatrix and seminal receptacle in all post-copulation times analyzed herein (in both field and cultured individuals). Although most of them had only sperm tails in the bursa copulatrix, two individuals had conspicuous sperm heads in this organ: a field (Fig. 6c, d) and a cultured individual (Fig. 7a, b) fixed 30 min and 2 hours after the second mating, respectively. In the first case, three sperm configurations were found (Fig. 6c, d): a compact sperm mass still showing sperm heads, located near the opening of the bursa copulatrix; followed by a compact sperm mass composed only of sperm tails; and more internally, sparse masses of sperm tails embedded within secretion and mixed with yolk granules. The second case, in turn, showed the precise moment when the sperm coming from the allosperm duct – which connects the seminal receptacle to the

bursa copulatrix – reached the bursa copulatrix (Fig. 7a, b). Additionally, another sperm mass composed only of sperm tails was present more internally (Fig. 7a, c). Interestingly, the seminal receptacle of this individual was almost empty (7a, d).

Histochemical tests resulted in negative reaction of the epithelium of the bursa copulatrix and seminal receptacle in all mating situations. The secretory contents of both organs had a positive reaction for AB. However, the intensity of the reaction was always more pronounced in the bursa copulatrix than in the seminal receptacle of all individuals, and in the bursa copulatrix of the field individuals when compared to the same organ of the cultured ones.

Sperm digestion during starvation

All individuals from the control (CG) and experimental (EG) groups showed sperm digestion in the bursa copulatrix. Individuals from the CG showed a uniform pattern for the seminal receptacle and bursa copulatrix, the former being always full of sperm (Fig. 8a) and the latter almost empty, having only a few sperm tails (Fig. 8b). In the EG, five out of seven individuals showed the seminal receptacle with only a few sparse sperm (Fig. 8c) and the bursa copulatrix with sperm tails and yolk granules (Fig. 8d). The other two individuals of the EG showed more sperm content in the seminal receptacle (similar to the CG; Fig. 8e), while the aspect of the bursa copulatrix was similar to the remaining individuals of the EG (Fig. 8f).

Total (TM) and mean time of mating (MM) did not differ between the groups (TM, $p = 0.8$, n = 7 for each group, CG = 126.6 ± 32 min, EG = 127.9 ± 43.2 min; MM, $p = 0.6$, n = 33 for each group, CG = 25.67 ± 11.6 min, EG = 27.12 ± 12.97 min). Only five out of seven individuals of each group spawned. The elapsed time (in days) between the first mating and the first spawning was greater in the CG than in the EG ($p = 0.0002$; n = 5; CG = 4.2 ± 0.84 d, EG = 1.4 ± 0.55 d). The number of egg masses laid per individual was greater in the EG ($p = 0.04$; n = 5; CG = 1.6 ± 0.9, EG = 4 ± 2), but the number of eggs per egg mass was lower ($p < 0.0001$; n = 8; CG = 82 ± 29.4; EG = 19.35 ± 13.4). However, the total number of eggs laid per individual did not differ significantly between the groups ($p = 0.2$; n = 5; CG = 131.2 ± 87.5; EG = 77.4 ± 33.74). Individuals from the CG grew larger than the EG, which actually

presented a reduction in size ($p < 0.0001$; $n = 7$; CG = 33 ± 11.15 %; EG = -8.57 ± 4.03).

Discussion

1. Mating behavior

Copulation has been commonly assumed to always result in insemination, which in turn has been assumed to result in fertilization (Eberhard 1985). However, these three processes generally occur separately in time and space, and the occurrence of one not necessarily leads to the other, given that in species with internal fertilization, males rarely deposit sperm directly onto the eggs (Eberhard 1985). In *Okenia polycerelloides* we observed that, in four out of 64 individuals that have mated, insemination did not occur, and in at least one individual, fertilization would certainly not occur, since the sperm recipient directed all the received allosperm for digestion in the bursa copulatrix (Fig. 7a, d).

As it is common in other nudibranchs, *O. polycerelloides* reciprocally copulates with its genital pore aligned to the genital pore of the partner, so that the penis of one partner can deposit sperm into the female reproductive tract of the other. However, we have found that, when *O. polycerelloides* finds a potential sexual partner, interactions between two individuals may end up at any of the different stages of mating (oral contact, body alignment, penis intromission, and sperm transfer), indicating a sophisticated mechanism of mate choice.

For some time, pre-copulatory mechanisms of sexual selection were hypothesized to be weaker in simultaneous hermaphrodites (e.g., Greeff & Michiels 1999). However, recent studies evidenced that mate choice can be prevalent in these animals (Anthes 2010), being associated with different factors, such as body size (e.g., Tomiyama 1996; Sprenger et al. 2009) and mating history (e.g., Haase & Karlsson 2004). Body size can influence the resources available to reproduction and be positively related to the size of the internal organs responsible for sperm storage (Charnov 1996). It is expected that, when the costs of insemination are significant and fecundity increases with size, individuals would tend to inseminate larger or similar-sized partners, because they would probably produce more eggs than small partners

(Anthes 2010). In species that mate reciprocally, the preference for inseminating larger individuals would result in mating between partners of similar sizes. However, empirical evidences for this prediction remain ambiguous in hermaphrodites (Anthes 2010). Body size could also influence investment in the male role during mating, when more sperm would be donated to larger (i.e., more fecund) partners (Wedell et al. 2002). Evidence for this prediction was found in the sea slug *Alderia modesta*, where individuals flexibly adjust mating patterns depending on the relative size of their sexual partners (Angeloni 2003). This species shows a behavior similar to that of *O. polycerelloides*, where each individual moves its oral lobes over the partner before mating. Angeloni (2003) speculated that this could be a method of size assessment in this species through mechano- or chemoreceptors. Tactile and chemical cues for body assessment are known in hermaphroditic flatworms (Vreys & Michiels 1997; Lüscher & Wedekind 2002), and this may be a common strategy in invertebrate hermaphrodites (Angeloni 2003).

Considering the presence of sperm storage organs in many hermaphrodites (Baur 1998), sperm from multiple partners are expected to compete to fertilize the eggs of the same individual (Anthes 2010). Therefore, the mating history of the partner is considered relevant for mate choice (Anthes 2010). Even in the case of internal fertilizers, in which the mating history is not externally visible, animals tend to mate preferentially with unmated partners, which suggests that either chemical cues are present and signal mating history, or that tactile receptors in the penis are able to detect rival's sperm (Anthes 2010), or even both. Accordingly, the fact that penis intromission did not always result in insemination in *O. polycerelloides* raises the possibility of mate choice through the use of the penis, e.g., by assessing the mating history or other trait of the partner.

2. Functioning of the bursa copulatrix

2.1. Mating manipulations

Although it has been proposed that the bursa copulatrix could function as the initial site for sperm reception in dorid nudibranchs (Thompson & Bebbington 1969; Hadfield & Switzer-Dunlap 1984; Gosliner, 1994), our results suggest that this organ

does not perform such function in *O. polycerelloides*. After 30 minutes from mating, all individuals had a seminal receptacle full of sperm, while their bursa copulatrix was generally empty or had only a few sperm tails, its contents increasing with 1h after mating. These results indicate, therefore, that sperm are initially received by (or are deposited by the partner in) the seminal receptacle, and some of them are then directed to the bursa copulatrix.

The original idea that the bursa copulatrix could act as the site for sperm reception in dorid nudibranchs probably came from the report made by Thompson (1966) that mentioned this function for this organ in *Doris pseudoargus* Rapp, 1827. However, it is not clear if mating manipulations were performed in that study, or if this process was presumed based on morphological characteristics, such as the position of the bursa copulatrix, which is closer to the genital pore than the seminal receptacle. The same author found only degraded sperm within the bursa copulatrix of the same species and mentioned that the functioning of the bursa copulatrix was "problematical" (Thompson 1976). Other authors also reported degraded sperm inside this organ in other dorid nudibranchs (e.g., Schmekel 1971; Medina et al. 1988). Therefore, there is the possibility that in other dorid nudibranchs sperm are also received by the seminal receptacle and not by the bursa copulatrix, as we have demonstrated for *O. polycerelloides*, and similarly to what occurs in aplysiid sea slugs (Beeman 1970a, 1970b).

Our previous study demonstrated that sperm heads are degraded before sperm tails in *O. polycerelloides* (Sales & Marian in prep.), as is the case of other sea slugs (Thompson 1976), but different from the freshwater snail *Lymnaea stagnalis* (Linnaeus, 1758), in which sperm tails are degraded before the heads (Horstman 1955). If presence of sperm tails and absence of heads are evidence of sperm digestion in *O. polycerelloides*, we infer that digestion proceeds very fast due to: (1) the presence of sperm tails in all individuals that showed any content in the bursa copulatrix, coupled with the rarity of sperm heads (found in only 2 out of 60 individuals); (2) even 30 min after mating, only sperm tails may be found in the bursa copulatrix; (3) in the two individuals in which sperm heads were found in the bursa copulatrix, the sperm were either reaching the organ (Fig. 7) or had presumably recently arrived (Fig. 6b-d). Rapid sperm digestion also tends to support that the bursa copulatrix is not the site for initial sperm deposition. Considering that the sperm

mass of *O. polycerelloides* is devoid of any capsule or protective envelope (pers. obs.), allosperm deposited in this organ would be easily damaged.

Our results also indicate that at least part of the sperm transferred during mating is always digested by the bursa copulatrix in *O. polycerelloides.* This is mainly based on the fact that, except for some individuals that mated once and were fixed 30 minutes after mating, all individuals had sperm tails in the bursa copulatrix. Two individuals that were fixed 30 minutes (Fig. 6b-d) and 2 h after the second mating (Fig. 7) also provide evidence for this process. In their bursa copulatrix, we identified at least two different sperm masses in different stages of digestion, indicating that part of the sperm transferred by each of their two partners was directed to the bursa for digestion. Sperm digestion may occur when more sperm is received during mating than the amount of sperm needed for fertilization (Michiels 1998). Thus, our data corroborate the hypothesis that the bursa copulatrix digests surplus sperm, as proposed by Beeman (1970b). However, we also found evidence that allosperm can be completely sent to the bursa copulatrix by the sperm recipient, which occurred in one individual after 2 h of the second mating (Fig. 7). Hence, other roles could be played by this organ, such as cryptic female choice. For example, sperm digestion could be used to reduce the fertilization success of low-quality partners (e.g., Birkhead et al. 1993; Eberhard 1996).

2.2. Starvation experiment

Starvation did not determine the occurrence of sperm digestion by the bursa copulatrix in *O. polycerelloides*. All individuals from control (with food) and experimental groups (without food) showed at least some sperm tails in the bursa copulatrix. Also, considering all experimental manipulations conducted herein, sperm digestion seemed rather a continuous process.

At the end of the experiment, there was a reduction in the size of the individuals of the experimental group. Reduction of body mass during starvation was reported for others invertebrates (e.g., Christa et al 2014; Fu et al. 2014), and it is considered the most obvious and common response to starvation (McCue 2010). Although there was reduction in size, our results suggest that starvation may have intensified sperm digestion. Copulation duration and total number of laid eggs per individual did not

differ between the groups, thus we assume that the amount of sperm transferred during mating and the amount of sperm used for fertilization by both groups were similar. If they were similar, then starvation may have influenced the intensity of digestion, due to the evidently lesser amount of sperm stored in the seminal receptacle of the experimental group. Considering that the contents of the bursa copulatrix were similar between both groups, a lesser amount of sperm stored in the seminal receptacle of the experimental group indicates that more sperm were digested. This finding should stimulate further studies to test if and how sperm may be used as nutritional source in sea slugs.

We did not find difference in the total number of eggs laid per individual between control and experimental groups. On one hand, the number of eggs per egg mass was higher in the control group, in accordance with previous studies that showed that number of eggs per egg mass is generally positively correlated with larger body size (Anthes & Michiels 2007). On the other hand, individuals from the experimental group spawned earlier and more frequently. Egg production is more costly than sperm production, and a classical prediction is that, when in poor conditions, simultaneous hermaphrodites should invest more resources in the male than in the female function (Janicke & Chapius 2016). This raises the question of why the total number of eggs deposited per individual did not differ between control and experimental groups. This could be related to the low size of our sample, but there are other plausible explanations. In the freshwater snail *Limnaea stagnalis* the absorption of the digested gametes in the bursa copulatrix increases the rate of oocytes' maturation (Horstman 1955). The fact that there is a progressive reduction in the sperm content of the bursa copulatrix in *O. polycerelloides* as post-copulation time increases, as demonstrated herein, suggests that the digested gametes are absorbed. If sperm digestion is a trigger for spawning in *O. polycerelloides* as it is for *L. stagnalis* (Horstman 1955), the highest intensity of sperm digestion in the experimental group could be associated with their frequent egg-laying.

Another explanation for the earlier and frequent spawning by the experimental group could reside in "emergency physiology", i.e., because of their reduced survival perspectives, resources could have been relocated to reproduction. This scenario has been originally proposed to explain traumatic mating, in which an individual could

deliberately injury its partner to induce such “emergency physiology” state and, thus, increase the chances of his sperm being used for fertilization (Michiels 1998).

3. Functioning of the seminal receptacle

We found evidence for sperm stratification in individuals that mated twice, with sperm content in the seminal receptacle divided into two sperm masses. One of these sperm masses was spherical and concentrated in the center of the lumen, sometimes located near the opening but sometimes more deeply in the organ. The same condition was previously found in a wild specimen of *O. polycerelloides* (Sales & Marian in prep.). The other sperm mass consisted in randomly organized sperm sometimes surrounding the first mass. The lack of stratification in some individuals that mated twice could be attributed to their short first mating event, which lasted less than 4 min. In these cases, there is a high probability that the first mating did not result in insemination.

The last partner to copulate tends to have higher reproductive success if there are strategies of sperm removal by the sperm donor or simply if there is sperm stratification in the storage organs (Birkhead & Parker 1997). For instance, last male sperm precedence would be expected if the morphology of the sperm storage organ favors sperm stratification, and if the site for entrance and exit of sperm is the same (Roderick et al. 2003). As in other sea slugs (e.g., Hadfield & Switzer-Dunlap 1984; Medina et al. 1988; Anthes et al. 2008), the seminal receptacle is a blind sac in *O. polycerelloides*. Considering that all sperm received from different matings are deposited in the same physical space, rival sperm come into direct contact with one another, intensifying sperm competition. However, if sperm stratification is present, different post-copulatory processes can be envisaged. For example, last mate sperm deposited near the opening of the seminal receptacle would have the advantage of being the first to be directed to the fertilization chamber (i.e., the site where fertilization occurs), but would also have a higher risk of being directed to the bursa copulatrix for digestion.

Although we are not certain which sperm mass was first or last deposited in the seminal receptacle of the individuals with sperm stratification, the spherical sperm mass is an arrangement found only in the experimental manipulations with two

subsequent matings. Therefore, we hypothesize this spherical mass was transferred by the second partner. However, given that the seminal receptacle of *O. polycerelloides* is provided with muscle fibers (Sales & Marian in prep.), we cannot exclude the possibility that the contents of the seminal receptacle are constantly mixed, affecting sperm stratification. This would also explain why in some cases we did not find sperm stratification (e.g., Fig. 6b).

In the sea slug *P. taylori* sperm become oriented with their heads close to and facing the epithelium of the seminal receptacle five hours after mating (Beeman 1970b). In *O. polycerelloides* this sperm arrangement was observed herein in several samples from different post-copulation times, from 30 minutes to 3 h after mating, but was also absent in several of them, which showed only random orientation. Accordingly, in wild specimens of *O. polycerelloides* sperm in the seminal receptacle were either randomly arranged or showed both kinds of orientation (Sales & Marian in prep.). Substances secreted by the seminal receptacle may induce sperm activation or attraction by chemical action (e.g., Beeman 1970a; Medina et al. 1988). Human spermatozoa are attracted to factors released by the eggs, but only a fraction of the sperm population responds to these factors (Ralt et al. 1991). This mechanism was proposed as a strategy for selecting more competent spermatozoa for fertilization (Ralt et al. 1991). A similar mechanism could operate in *O. polycerelloides*. In this context, response times (or even the response itself) to the seminal receptacle's secretion could differ among sperm, and this could explain the different arrangements observed in the seminal receptacle of *O. polycerelloides.* This would also have implications for sperm competition and cryptic female choice, given that sperm with faster response times would be preferentially stored for later use.

Conclusions

Mating interactions in *Okenia polycerelloides* may end at different stages of the mating process (oral contact, body alignment, penis intromission and sperm transfer), with penis intromission not necessarily resulting in insemination. Considering that *O. polycerelloides* is an internal fertilizer, we speculate that penis intromission could be used as an additional strategy for mate choice, along with oral contact. This study also demonstrated that, differently from what has been previously proposed for dorid

nudibranchs, the bursa copulatrix does not act as an organ for sperm reception in *O. polycerelloides*. Instead, sperm is initially deposited in the seminal receptacle during mating. The primordial function of the bursa copulatrix is sperm digestion, which occurs continuously, and at least part of all sperm received in different matings is directed for digestion. Thus, we corroborate the function of surplus sperm digestion by the bursa copulatrix. Although continuous digestion by the bursa copulatrix may indicate that sperm digestion occurs indiscriminately, we also found evidence for potential cryptic female choice, with one case of digestion of all received allosperm. The gradual reduction of the contents of the bursa copulatrix with increasing post-copulation times suggests resorption of the digested materials. However, although starvation seemed to intensify sperm digestion, it did not determine its occurrence. Finally, evidence for sperm stratification was found in the seminal receptacle of *O. polycerelloides* with two subsequent matings, which sheds light on how post-copulatory mechanisms may operate in this species.

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Figures

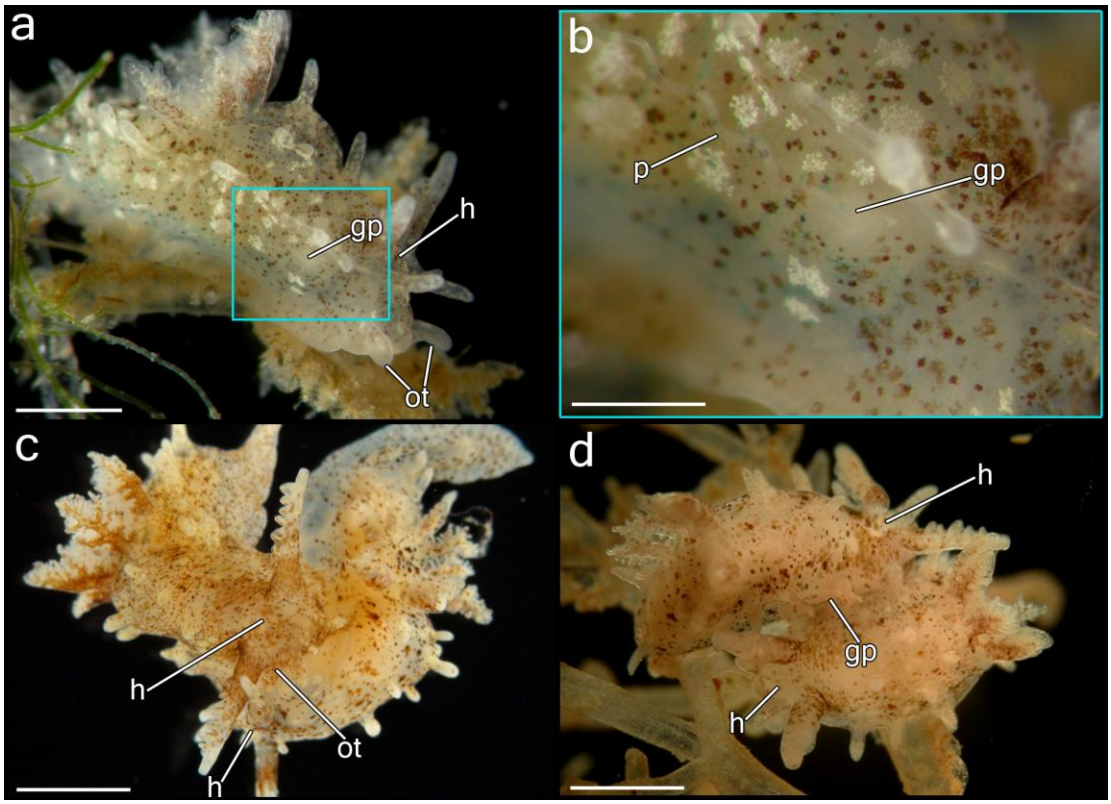

Figure 1. Mating behavior of *Okenia polycerelloides*. They live, feed and mate on the bryozoans *Amathia vidovici* (a) and *A. verticillata* (d). **(a)** General aspect of an individual crawling on the bryozoan *A. vidovici*. The genital pore lies on the right side of the animal. **(b)** Detail of the region of the genital pore, showing an everted penis. **(c)** Two individuals during initial mating interaction, showing one of them contacting the genital pore of the partner using its oral region. **(d)** Two individuals during body alignment, with their heads positioned in opposite directions, which allows contact between their genital pores. Images a-c: courtesy of Alvaro Migotto. **Scale bars**: a, c and d, 1 mm; b, 500 µm. **Abbreviations**: gp, genital pore; h, head; ot, oral tentacle; p, penis.

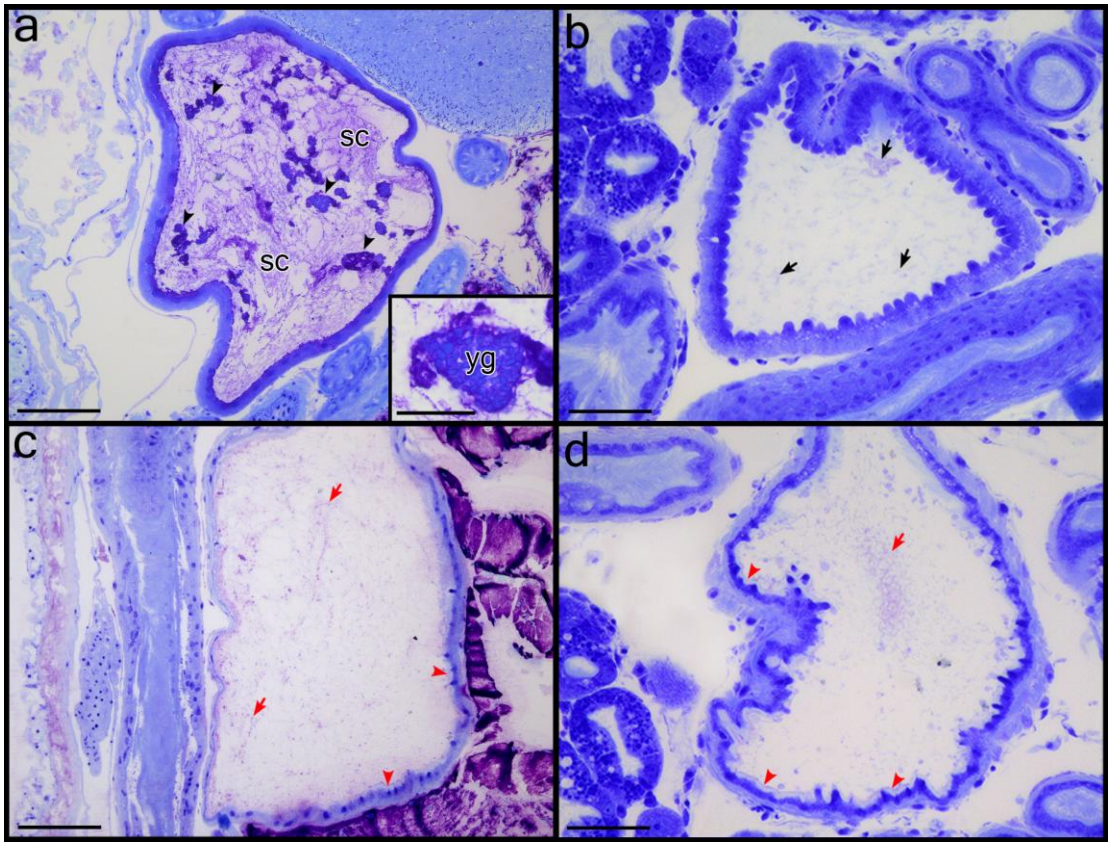

Figure 2. Histological comparison of the bursa copulatrix (a-b) and seminal receptacle (c-d) between field and cultured individuals without mating. Toluidine blue. **(a)** Bursa copulatrix of a field individual, showing copious amount of secretion in the lumen mixed with oocytes granules (arrowheads). The inset shows yolk granules in detail. **(b)** Bursa copulatrix of a cultured individual, showing some secretion (black arrows) in the lumen. Seminal receptacle of a field **(c)** and a cultured individual **(d)**, both showing some secretion dispersed within the lumen (stained in pink; red arrows), and also close to the epithelium (stained in blue; red arrowheads). **Scale bars**: (a and c) 100 µm; (b and d) 50 µm; (inset) 20 µm. **Abbreviations**: sc, secretion; yg, yolk granules.

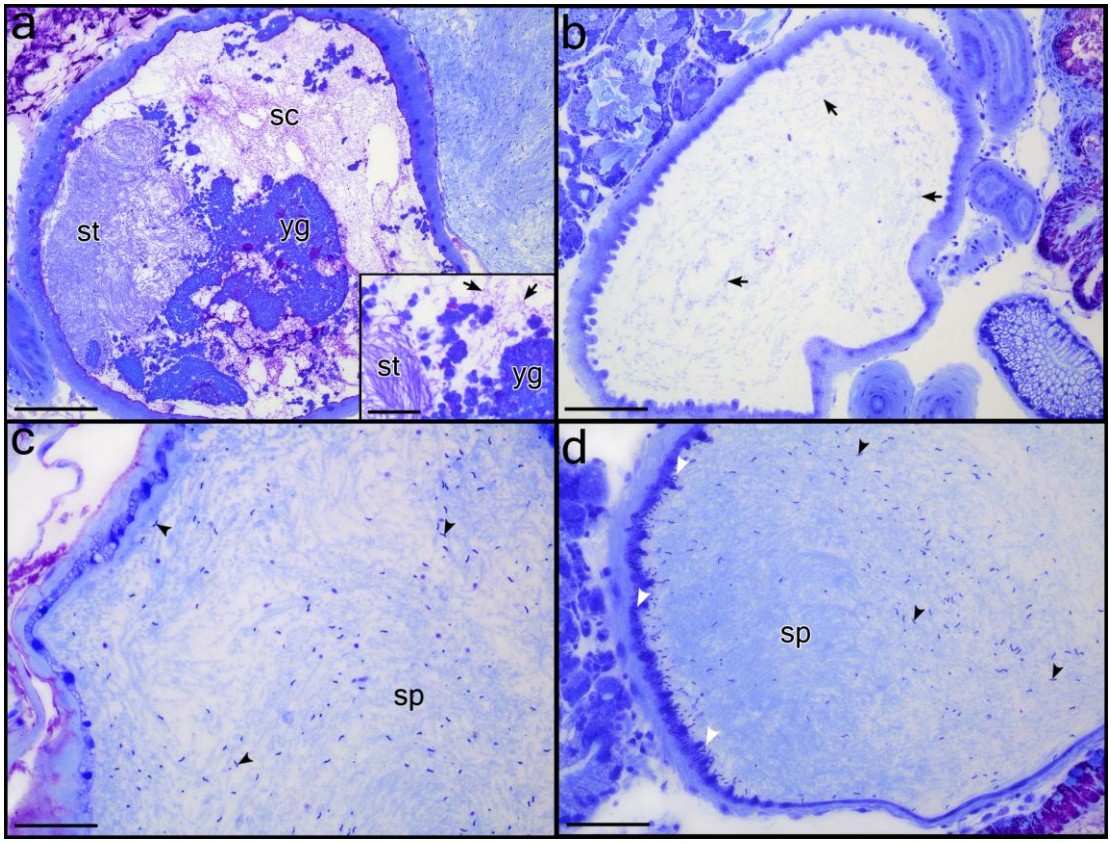

Figure 3. Histological comparison of the bursa copulatrix (a-b) and seminal receptacle (c-d) between field and cultured individuals, both fixed 30 minutes after one mating. Toluidine blue. **(a)** Bursa copulatrix of a field individual, showing some sperm tails, yolk granules and secretion (black arrows), all shown in detail in the inset. **(b)** Bursa copulatrix of a cultured individual, showing smaller amount of secretion (black arrows) in the lumen. **(c)** Seminal receptacle of a field individual, showing sperm (arrowheads) randomly arranged in the lumen. **(d)** Seminal receptacle of a cultured individual, showing both randomly arranged sperm in the lumen (black arrowheads) and sperm with their heads facing the epithelium (white arrowheads). **Scale bars**: (a-b) 100 µm; (c-d) 50 µm; (inset) 20 µm. **Abbreviations**: sc, secretion; sp, sperm; st, sperm tails; yg, yolk granules.

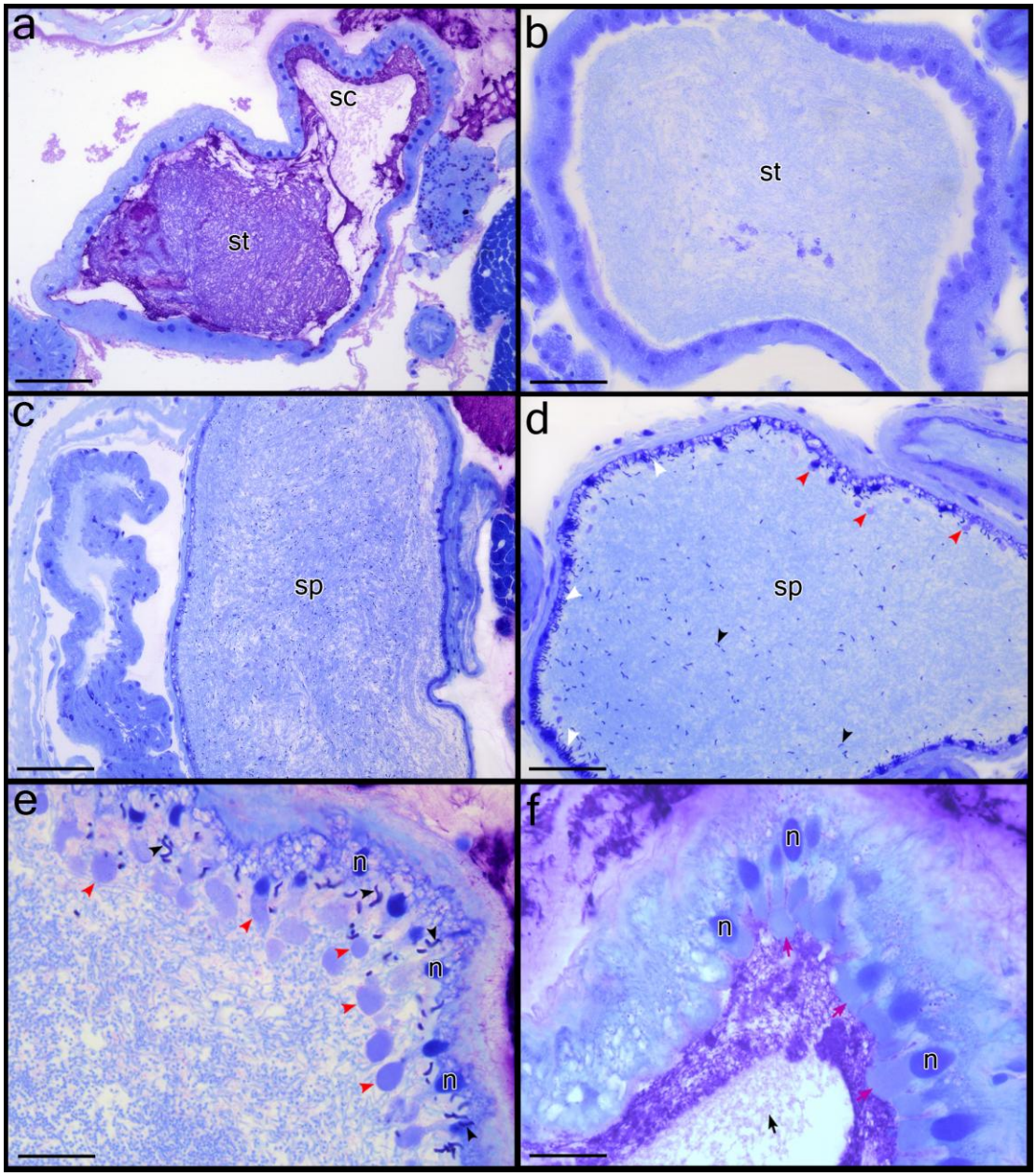

Figure 4. Histological comparison of the bursa copulatrix (a-b) and seminal receptacle (c-d) between field and cultured individuals, and detail of the secretory epithelium of both organs in field individuals (e-f). All specimens were fixed 1 hour after one mating. Toluidine blue. **(a)** Bursa copulatrix of a field individual, showing a large amount of sperm tails mixed with secretions within the lumen. **(b)** Bursa copulatrix of a cultured individual, showing a large amount of sperm tails in the lumen. **(c)** Seminal receptacle of a field individual, showing sperm randomly arranged in the lumen. **(d)** Seminal receptacle of a cultured individual, showing both randomly arranged sperm in the lumen (black arrowheads) and sperm with their heads facing the epithelium (white arrowheads). Red arrowheads point to secretory droplets. **(e)** Detail of the seminal receptacle's epithelium of a field individual, showing intense secretory activity (red arrowheads point to secretory droplets) and some sperm heads (black arrowheads) close to and facing the epithelium. **(f)** Detail of the bursa copulatrix's epithelium of a field individual, showing intense secretory activity. Black arrow points to the secretion of the bursa copulatrix in the lumen, and pink arrows to secretory droplets. **Scale bars**: (a and c) 100 µm; (b and d) 50 µm; (e and f) 20 µm. **Abbreviations**: n, nucleus; sc, secretion; sp, sperm; st, sperm tails.

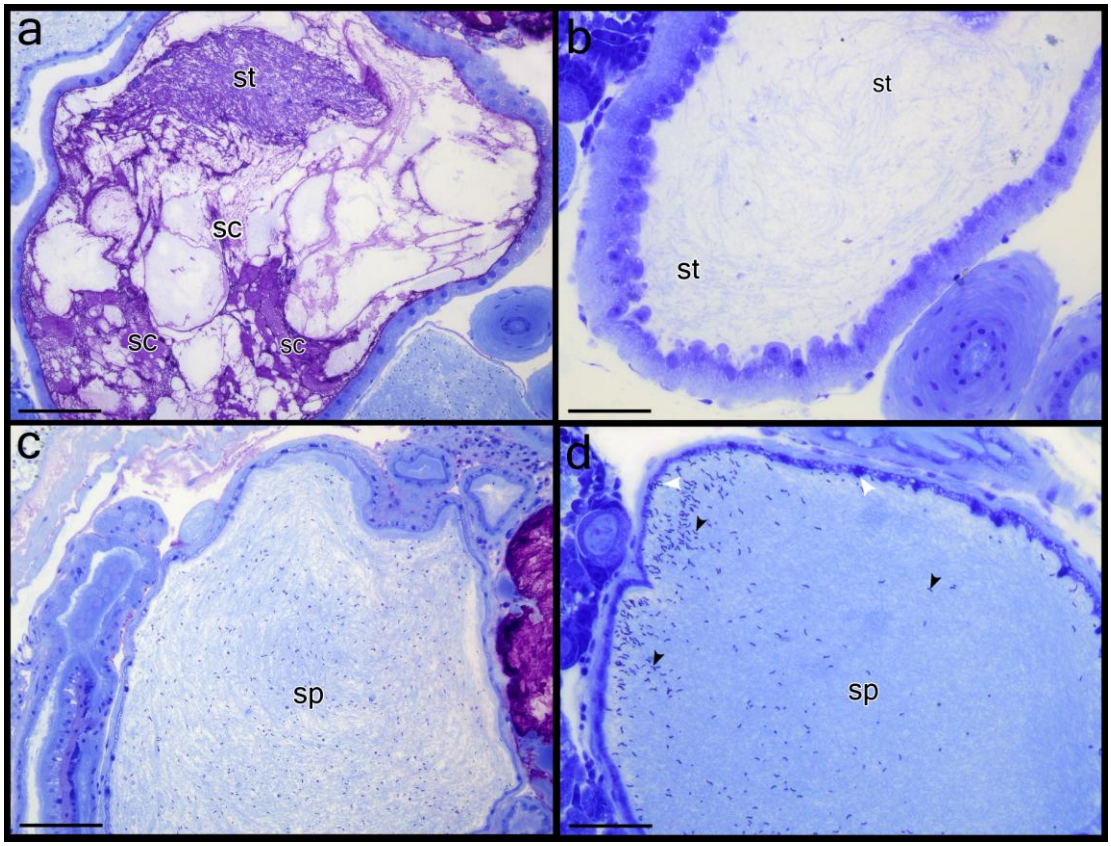

Figure 5. Histological comparison of the bursa copulatrix (a-b) and seminal receptacle (c-d) between field and cultured individuals, both fixed 2 hours after one mating. Toluidine blue. **(a)** Bursa copulatrix of a field individual, showing some sperm tails and secretion in the lumen. **(b)** Bursa copulatrix of a cultured individual, showing sparse sperm tails in the lumen. **(c)** Seminal receptacle of a field individual, showing randomly arranged sperm in the lumen. **(d)** Seminal receptacle of a cultured individual, showing both randomly arranged sperm in the lumen (black arrowheads) and sperm with their heads facing the epithelium (white arrowheads). **Scale bars**: (a and c) 100 µm; (b and d) 50 µm. **Abbreviations**: sc, secretion; sp, sperm; st, sperm tails.

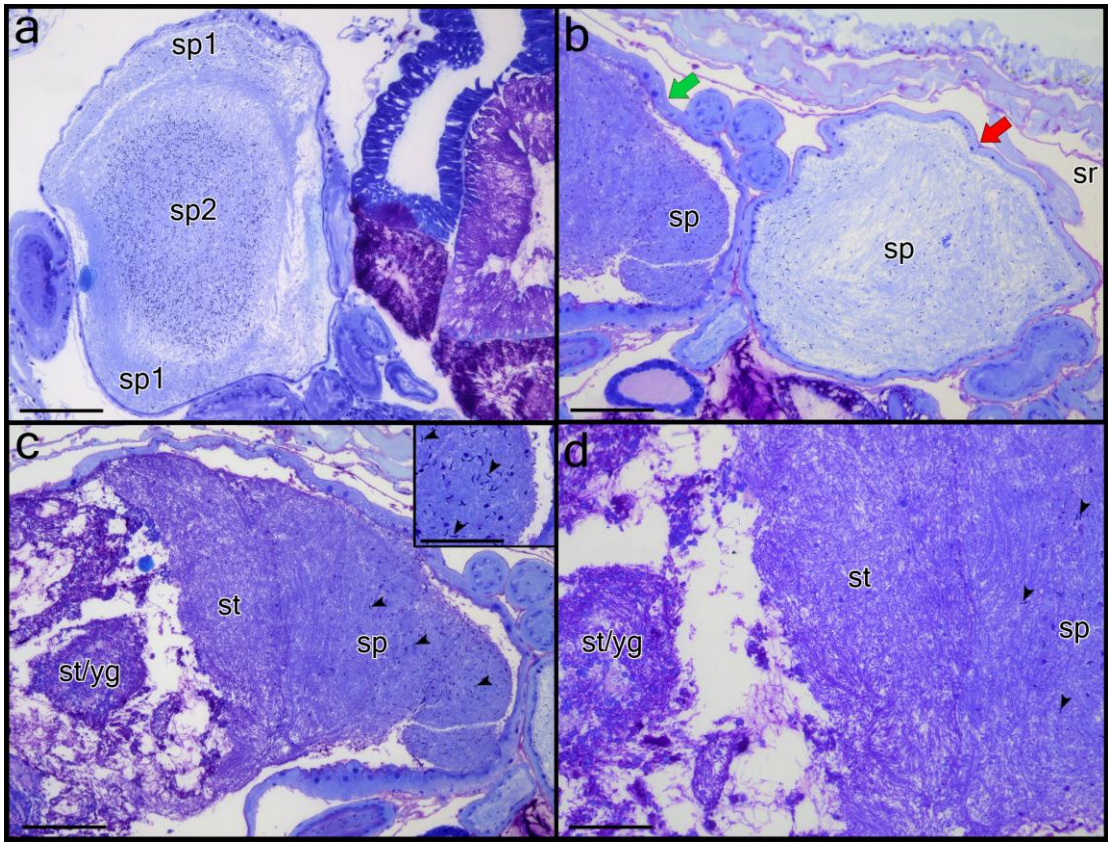

Figure 6. Histological sections showing the seminal receptacle (a-b) and bursa copulatrix (c-d) found in individuals fixed 30 min (b-d) and 1h (a) after two subsequent matings. Toluidine blue. **(a)** Seminal receptacle showing a stratified pattern of sperm masses in its lumen, each potentially corresponding to a distinct partner. **(b)** Seminal receptacle (pointed by red arrow) showing sperm randomly arranged in its lumen; green arrow points to the bursa copulatrix. **(c)** Bursa copulatrix showing different stages of sperm degradation in its lumen; from right to left, there is a compact sperm mass with sperm heads (black arrowheads) still present (detailed in the inset), then a compact sperm mass composed only of sperm tails, and finally sparse masses of sperm tails embedded within secretion and mixed with yolk granules. **(d)** Detail of **c**. **Scale bars**: (a–c) 100 µm; (d and inset) 50 µm. **Abbreviations**: sp, sperm; sp1 and sp2, sperm masses putatively transferred by different partners; st, sperm tails; st/yg, sperm tails mixed with yolk granules.

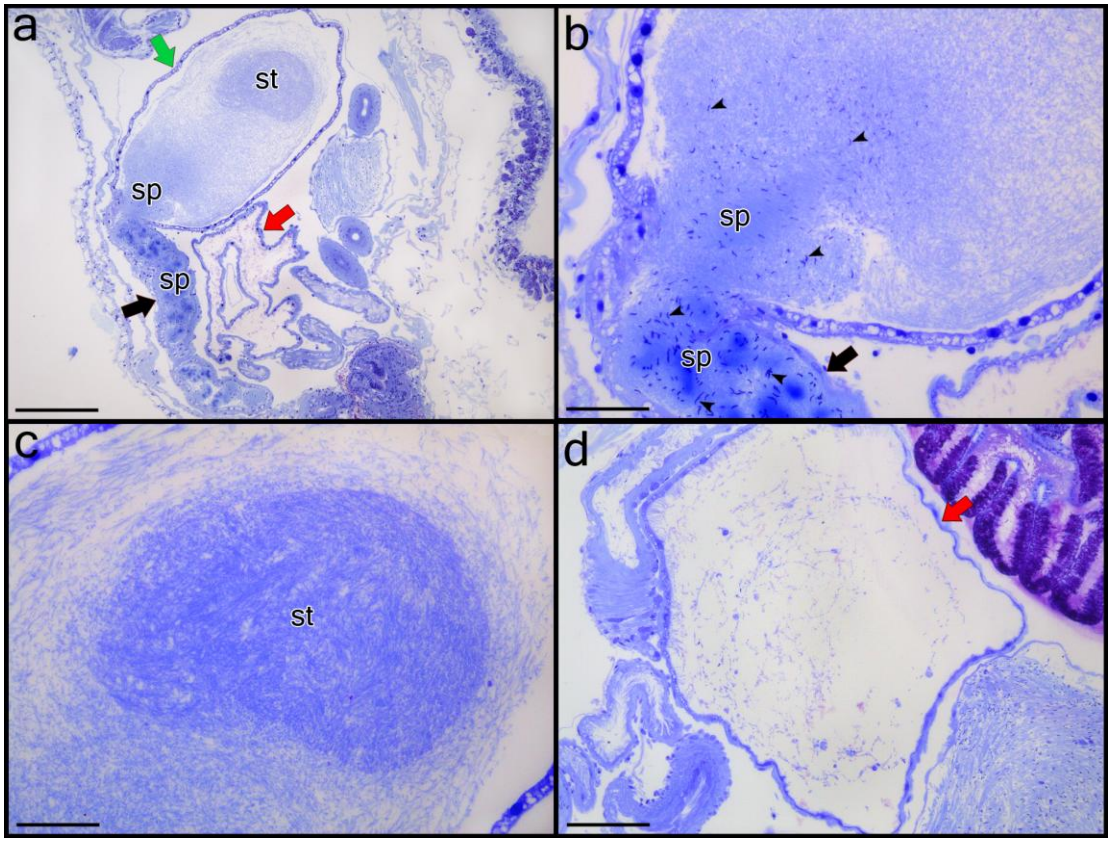

Figure 7. Histological sections showing the bursa copulatrix (a-c) and seminal receptacle (a and d) of a cultured individual fixed two hours after two subsequent matings. Toluidine blue. **(a)** Section showing the precise moment of arrival of the second sperm mass in the bursa copulatrix (green arrow) through the allosperm duct (black arrow), which connects the bursa copulatrix to the seminal receptacle. The seminal receptacle (red arrow) is empty. **(b)** Detail of **a**, showing the connection between bursa copulatrix and allosperm duct (black arrow). Sperm coming from the allosperm duct and entering the bursa copulatrix still have their heads intact (arrowheads). **(c)** Detail of a, showing the sperm mass presumably received in the first mating, and that its sperm are composed only of sperm tails. **(d)** Empty seminal receptacle (red arrow) of the same individual. **Scale bars**: (a) 200 µm; (b-c) 50 µm; (d) 100 µm. **Abbreviations**: sp, sperm; st, sperm tails.

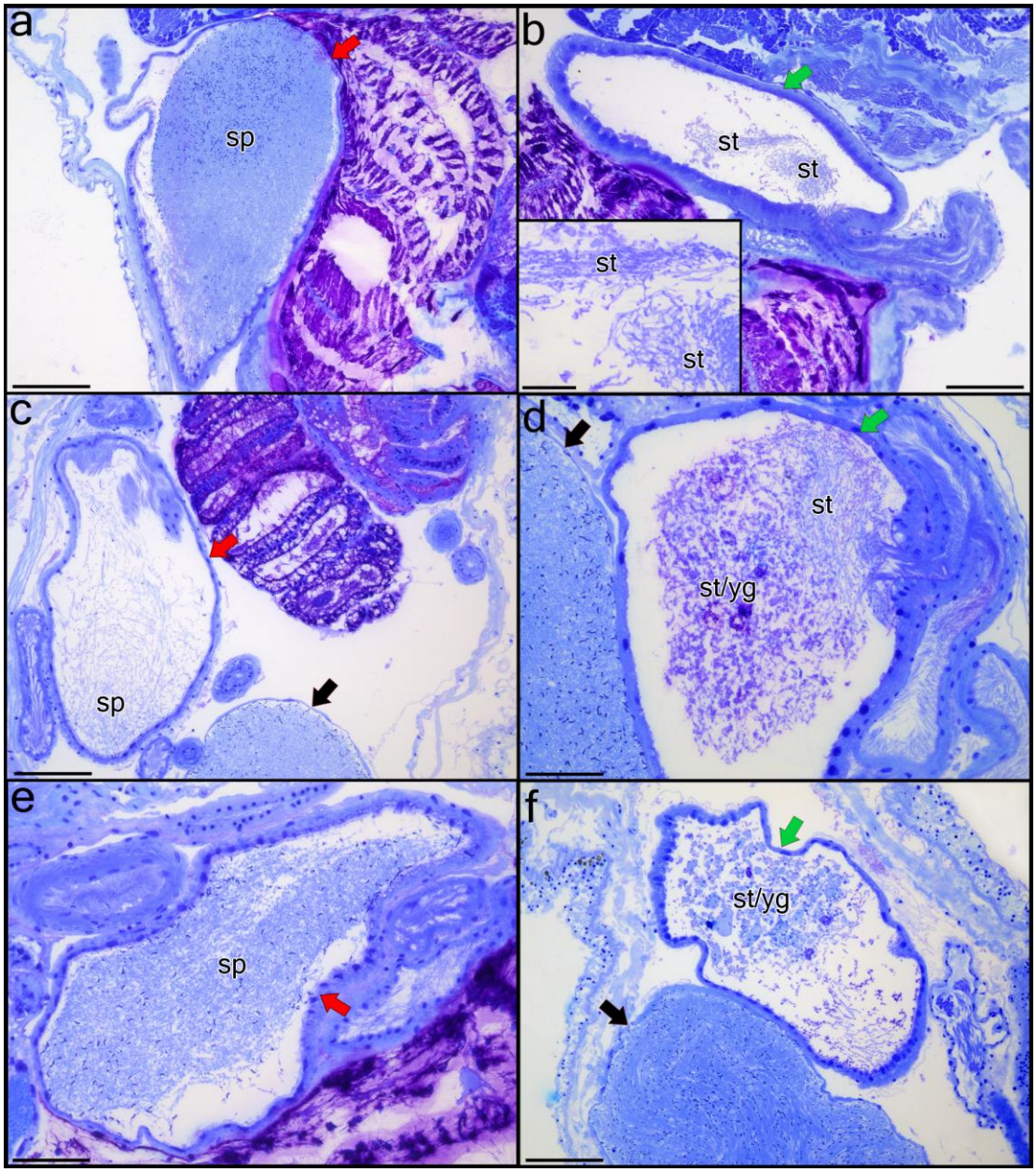

Figure 8. Histological sections comparing the seminal receptacle and bursa copulatrix of cultured individuals that were allowed to mate once a day with the same partner, during five consecutive days, one maintained with food resource (a-b), and the other two in starvation (c-f). All of them were fixed 24 h after the last mating. **(a)** Seminal receptacle full of sperm and **(b)** bursa copulatrix almost empty (inset: sperm tails), representing the general pattern observed for all seven individuals that were maintained with food supply. **(c)** Seminal receptacle showing only a few sparse sperm and **(d)** bursa copulatrix with some sperm tails and yolk granules, representing the general pattern observed in five out of seven individuals that were maintained in starvation. **(e)** Seminal receptacle with a reasonable amount of sperm, and bursa copulatrix **(f)** of the same specimen representing the pattern found in two out of seven individuals that were maintained in starvation. Red arrows point to the seminal receptacle, green arrows to bursa copulatrix, and black arrows to the ampulla. **Scale bars**: (a-c and f) 100 µm; (d-e) 50 µm; (inset) 20 µm. **Abbreviations**: sp, sperm; st, sperm tails; st/yg, sperm tails mixed with yolk granules.

Capítulo 3

Love will tear us apart: traumatic mating through consumption of body parts in a sea slug

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There is growing evidence that "traumatic mating"−a copulation that involves wounding the partner's tegument (Lange et al. 2013)−is a widespread phenomenon. Traumatic mating may have evolved associated with several distinct fitness benefits, from physical anchorage during mating to fertilization advantages (Lange et al. 2013). Currently, two hypotheses explain how traumatic mating may lead to fertilization advantages. First, the trauma inflicted during mating may be "collateral"−a by-product of another strategy associated with reproductive success (e.g., Morrow et al. 2003). For example, the injection of secretions that manipulate the partner's physiology generally involves piercing by sharp structures (e.g., love darts in snails; Chase and Blanchard 2006). Second, the trauma itself may be adaptive (Johnstone & Keller 2000; Morrow et al. 2003): the wound would induce a refractory period in the partner, which would avoid further mating and, thus, further damage. This scenario would benefit the sperm donor by increasing its short-term fertilization success (Michiels 1998; Johnstone & Keller 2000). While there is increasing empirical evidence for collateral harm in traumatic mating (e.g., Hotzy & Arnqvist 2009), the same cannot be said about adaptive harm.

Traumatic mating is prevalent in hermaphrodites (Michiels 1998; Lange et al. 2013), which will accept further copulations even when traumatic mating is costly (Michiels & Koene 2006). Most sea slugs are simultaneous hermaphrodites, i.e., individuals that produce functional male and female gametes simultaneously (Michiels 1998). While studying the reproductive behavior of sea slugs in the coast of

São Paulo state, Brazil, during May–June 2016, we found specimens of *Phidiana lynceus* Bergh 1867 (Fig. 1a), an "eolid" nudibranch with a narrow body and numerous finger-like dorsal projections called cerata (Fig. 1a). When two individuals were placed together in a Petri dish with seawater, we were astonished by their aggressive behavior, which at first seemed agonistic (Fig. 1a, b). However, at the end of the interaction, both individuals had a pack of sperm near their genital pores, a clear evidence of mating. To analyze this behavior, pairs of possible sexual partners were randomly selected from the 10 specimens collected and recorded under a stereomicroscope equipped with a high-definition video camera.

All encounters resulted in mating with reciprocal sperm transfer and the same behavioral pattern. Generally one partner (hereafter called "follower") follows and contacts the other using its oral tentacles. Then, the second turns to the follower and makes contact using the oral tentacles. Although it is difficult to observe the moment when the penises are everted due to the ceratal movement, a few seconds after the follower makes contact its penis is already everted (Video S1: 00:00:13). Subsequently, they move along the right side of each other (Fig. 1a), allowing contact between their gonopores. Once they approach, they start to pluck and ingest cerata from each other (Fig. 1a, b; Video S1: 00:00:24; 00:01:12), although sometimes the cerata are only ruptured and their contents sucked through the wound (Video S1: 00:01:47). Histology revealed the ingested cerata within the stomach of the specimens (Fig. 1c; Appendix S1: Fig. S1). Both partners direct their cerata towards the partner in an apparent attempt to defend themselves (Video S1: 00:01:00). Nevertheless, they maintain their mating alignment, and start to move in a circle, possibly because of simultaneously trying to inflict trauma while avoiding being wounded. The penises stay everted throughout the interaction, occasionally intertwining with each other, and sometimes separating and making seemingly random movements. At the beginning of one mating event, we observed the tip of the penis of one partner being thrust on the dorsum of the other for a few seconds (Video S1: 00:00:29). Under scanning electron microscopy, the tip of the penis was shown to bear a stylet (Fig. 1d), which serves as a channel for sperm transfer, as revealed by histology. It is unknown if the stylet could also inject substances into the partner's body during mating, since the penis has a glandular aspect (Fig. 1d) that deserves investigation. The interaction takes four minutes on average and ends with sperm exchange, a process lasting a few seconds

and observable due to the translucency of the penis. Sperm is deposited on the gonopore as a compact viscous mass (Video S1: 00:02:10).

A recent classification proposed three types of traumatic mating (Lange et al. 2013), including both intra- and extragenital damage. Traumatic mating through cerata consumption in *P. lynceus* can be a case of extragenital "traumatic penetration" (Lange et al. 2013), a mechanism not associated with injection of secretions ("traumatic secretion transfer") or sperm ("traumatic insemination"). Studies aiming to test adaptive vs. collateral harm have mainly used species with intragenital traumatic mating as models, and in those cases traumatic mating was associated with collateral harm (Morrow et al. 2003; Hosken et al. 2003; Hotzy & Arnqvist 2009; Hotzy et al. 2012). Choosing a model species to test the adaptive hypothesis is difficult, because most of the known cases of traumatic mating are intragenital and suspected to be associated with collateral harm (e.g., Lange et al. 2013). For example, the wound caused by the injection of substances or sperm into the partner tegument generally indicates collateral harm, because injected substances are known to manipulate the partner (Hotzy et al. 2012), or because sperm is injected nearer to the site of fertilization to counteract sperm competition (competition among male gametes from different partners for the fertilization of an individual's ova; Parker 1970) (Lange et al. 2013). The known cases of traumatic penetration of the genital tract also generally point to some degree of collateral harm (e.g., penile spines involved with an anchoring function or with displacement of previous sperm; Hotzy & Arnqvist 2009). The only type of traumatic mating for which it is difficult to envisage some obvious form of collateral harm is extragenital traumatic penetration. Being extragenital, this trauma would not be related to the direct manipulation of the female genital tract; and providing it does not involve injection of substances nor sperm, it would not be related to directly manipulating the partner's physiology nor be associated with avoiding sperm competition within the genital tract, respectively. However, this type of traumatic mating seems rare, particularly in hermaphrodites (Lange et al. 2013). The discovery of extragenital traumatic penetration in *P. lynceus*, therefore, open doors for further experimentation to test adaptive harm.

Under the adaptive harm hypothesis, cerata consumption in the context of mating would extend the remating interval of the partner (Michiels 1998; Johnstone & Keller 2000). Also, as usual for sea slugs, *P. lynceus* stores sperm from many

partners and, consequently, increases the pressure of sperm competition. Therefore, delaying remating would also benefit the sperm donor by reducing that pressure.

Cerata are blood-filled tubes (Appendix S1: Fig. S1) that contain branches of the digestive gland, playing important roles in gas exchange and digestive processes (Behrens & Hermosillo 2005). Eolid nudibranchs, which generally feed on cnidarians, can store intact nematocysts (organelles usually with stinging function) from their preys at the tip of their cerata (Appendix S1: Figure S1), discharging them in their own defense (Goodheart & Bely 2016). Therefore, under the adaptive harm scenario, the loss of cerata during mating would exceed the costs of the wounds, since the partner would also be partly deprived of important organs for gas exchange, digestion and defense.

Traumatic mating may be costly to the wound inflictor if the fecundity of the partner is reduced due to the trauma inflicted (Lange et al. 2013). Interestingly, nudibranchs are known to use ceratal autotomy as a defensive mechanism (Goodheart & Bely 2016). Moreover, they can rapidly regenerate these structures (ca. 24 days in *P. crassicornis*; Miller & Byrne 2000). Therefore, removing few cerata during mating in *P. lynceus* should not dramatically compromise the reproductive output of the partner.

Anyhow, losing cerata is certainly costly to both partners, as suggested by their behavior of repelling the mutual attacks. Further studies should test if this behavior is indeed associated with extending the partner's remating interval. If cerata consumption is an adaptive form of traumatic mating, its benefits should surpass the costs of losing cerata and reducing the reproductive output of the partner. Would there be other benefits than extending the partner's remating interval? Nuptial gifts–the donation of nutrients during mating–are generally predicted to be ineffective in simultaneous hermaphrodites, because, for example, of the potential risk of benefitting a future rival (Michiels 1998). Sexual cannibalism, too, seems not the case, since it generally implies the death of partner (Schneider 2014). It is unknown, however, if there is a difference in the number of consumed vs. lost cerata between the partners, and how this would affect their reproductive success.

Although the function of cerata consumption during mating remains uncertain, this finding clearly adds to the complexity of mating strategies in hermaphrodites. Traumatic mating is pervasive in hermaphrodites and range from traumatic

insemination to traumatic secretion transfer (Lange et al. 2013). Although occasional "apophallation" (act of severing the partner's penis) was described for terrestrial slugs (Leonard et al. 2002), the behavior reported here for *P. lynceus* is unique regarding the nonlethal ingestion of extragenital body parts of the partner during mating in hermaphrodites.

Acknowledgments

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Figure

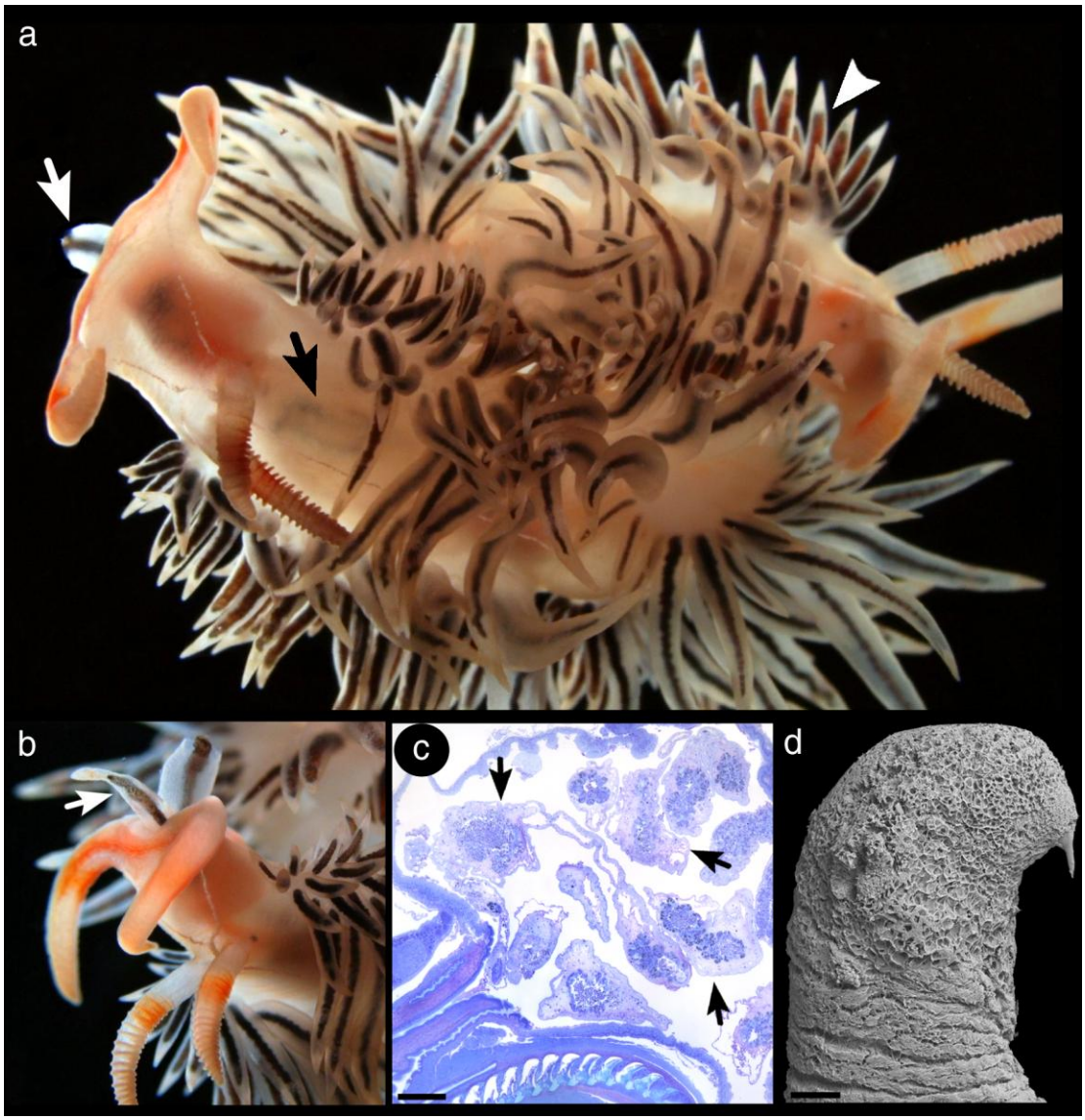

Figure 1. Traumatic mating through cerata consumption in *Phidiana lynceus*. **(a)** Two individuals (approximately 15 mm long alive) during mating. The arrows point to cerata either being ingested (white arrow) or already within the stomach and visible through translucency (black arrow); the arrowhead points to intact cerata. **(b)** Ceras being ingested through the mouth (white arrow). **(c)** Histological section stained with toluidine blue, showing cerata within the stomach (black arrows). See also Appendix S1: Fig. S1 for a comparison between the histology of intact and ingested cerata. **(d)** Scanning electron micrograph of the penis distal region, showing the stylet at the tip. **Scale bars**: (c) 500 µm; (d) 100 µm.

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Appendix S1

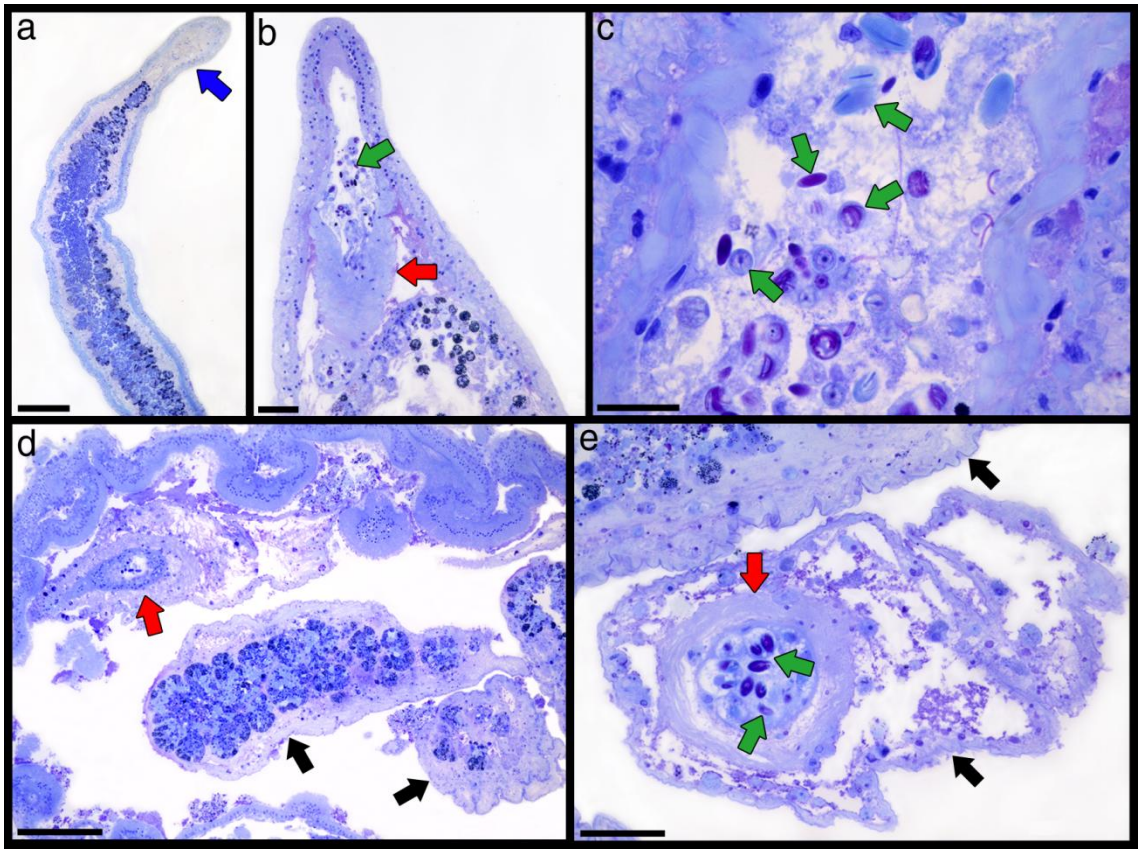

Figure S1. Histological comparison between intact (a-c) and ingested cerata (d-e). Intact cerata were removed from the dorsum of preserved specimens. For the analysis of ingested cerata, the whole stomach was dissected and processed for histology. Toluidine blue stain. **(a)** Intact ceras. **(b)** Tip of an intact ceras, showing the cnidosac, a structure that contains undischarged nematocysts obtained from the sea slug prey (i.e., cnidarians). **(c)** Detail of the cnidosac of an intact ceras, showing numerous nematocysts. **(d)** Ingested cerata within the stomach and nematocysts within a cnidosac of an ingested ceras. Blue arrow points to the region of the cnidosac; red arrows to the cnidosac; green arrows to nematocysts; and black arrows to ingested cerata. **Scale bars**: (a) 500 µm; (b) 100 µm; (c) 20 µm; (d) 200 µm; (e) 50 µm.

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Video S1 – download link

https://drive.google.com/drive/folders/1oDkMBgxrvqYxX71hB0XG7hbKGsSGGKaU?usp=sharing

Video S1 Legend

Video S1. *In vitro* recording of the mating behavior of *Phidiana lynceus*, a simultaneous hermaphrodite sea slug that ingests extragenital body parts of the partner during mating.

Considerações Finais

Embora estudos morfofuncionais sobre órgãos que contêm espermatozoides (i.e., ampola, bursa copulatória e receptáculo seminal) no sistema reprodutor de lesmas marinhas já tenham sido realizados com algumas espécies (e.g., Beeman 1970a; Schmekel 1971; Brandriff & Beeman 1973; Medina et al. 1988), há ainda muita controvérsia acerca das possíveis funções de tais órgãos (Medina et al. 1988). Por exemplo, no que diz respeito aos nudibrânquios doridídeos, a bursa copulatória já havia sido relacionada tanto à recepção quanto à digestão de esperma (e.g., Thompson 1966). Porém, se tais funções são desempenhadas pelo mesmo órgão, seria necessário um preciso controle sobre o processo de digestão, caso contrário, o esperma poderia sofrer danos antes de ser direcionado para o receptáculo seminal para armazenamento.

Devido a certas características (e.g., comportamento de cópula promíscuo, sistema reprodutor complexo, fácil coleta e manutenção em laboratório), o nudibrânquio doridídeo *Okenia polycerelloides* mostrou-se um excelente organismo modelo para estudos de seleção sexual em hermafroditas. Considerando a inexistência de dados morfofuncionais sobre seu sistema reprodutor e a importância de órgãos de armazenamento e digestão de espermatozoides em um contexto de seleção sexual, foi necessário um estudo morfológico detalhado dos órgãos que abrigam espermatozoides no sistema reprodutor dessa espécie. Tais dados, apresentados no capítulo 1 da presente Tese, revelaram que pode haver separação espacial das massas de espermatozoides no receptáculo seminal, suscitando questões sobre estratificação de espermatozoides associada a cópulas múltiplas. De modo similar, a constatação da função de digestão pela bursa copulatória levantou dúvidas sobre a função de recepção de espermatozoides hipotetizada para nudibrânquios doridídeos (e.g. Hadfield & Switzer-Dunlap 1984; Gosliner 1994; Valdes et al. 2010). Tais questões foram então investigadas e elucidadas no trabalho apresentado no capítulo 2 desta Tese.

No capítulo 2, revelou-se que o órgão responsável pela recepção de espermatozoides em *O. polycerelloides* é o receptáculo seminal, e que a função primordial da bursa copulatória é a digestão. Considerando a hipótese de que produtos resultantes da digestão de gametas poderiam ser absorvidos pela bursa

copulatória e utilizados como recurso energético em situações de restrição alimentar (Brandriff & Beeman 1973), testamos se tal situação influenciaria a ocorrência de digestão de espermatozoides. Entretanto, os resultados indicaram que a bursa copulatória está continuamente digerindo espermatozoides. Os padrões observados nas manipulações de cópula demonstraram ainda que pelo menos parte dos espermatozoides recebidos é transferida para a bursa copulatória, corroborando a hipótese de digestão de espermatozoides supérfluos por esse órgão. Porém, apesar desses resultados indicarem digestão indiscriminada de espermatozoides, houve um caso de digestão completa do aloesperma, o que sugere escolha críptica da "fêmea". Ademais, a redução progressiva no conteúdo da bursa copulatória com o aumento do tempo pós-cópula sugere absorção dos gametas digeridos. Características do epitélio da bursa copulatória descritas no capítulo 1 também sugerem tal função. Possíveis valores fisiológicos associados à reabsorção de gametas digeridos merecem futuras investigações.

Ainda no capítulo 2, foi constatado estratificação de espermatozoides apenas em situações com mais de uma cópula. Além disso, a orientação radial dos espermatozoides no receptáculo de *O. polycerelloides* pode ocorrer em diferentes tempos pós-cópula, inclusive em tempos tão curtos quanto 30 min, diferentemente do reportado para *Phyllaplysia taylori* (Beeman 1970b), que requer um tempo mínimo de 5 h. Especula-se que as diferentes orientações dos espermatozoides no receptáculo seminal de *O. polycerelloides* poderiam estar associadas a diferentes tempos de resposta a um possível sinal químico produzido pelo receptáculo seminal.

O comportamento de cópula de *O. polycerelloides* inclui contato oral, alinhamento, intromissão do pênis e transferência de espermatozoides. Tais interações podem ser interrompidas em qualquer um desses estágios, e mesmo a intromissão do pênis não resulta necessariamente em inseminação. Levando em conta que *O. polycerelloides* apresenta fertilização interna, especula-se que a intromissão do pênis poderia ser inicialmente utilizada como uma forma de acessar o histórico de cópula, sendo uma possível estratégia de escolha do parceiro. De maneira similar, o contato oral entre parceiros poderia ser uma forma de averiguar o tamanho corporal dos mesmos, podendo ser também uma estratégia associada à escolha de parceiro.

O comportamento de cópula da espécie *Phidiana lynceus*, apresentado no capítulo 3 desta Tese, possui como característica marcante o consumo de partes

corpóreas extragenitais do parceiro durante o acasalamento. Trata-se de uma cópula traumática do tipo "penetração traumática extragenital" (Lange et al. 2013). Além de sua importância para o conhecimento do comportamento de cópula de lesmas marinhas, a descoberta desse fenômeno revelou um organismo-modelo que possivelmente permitirá testar a hipótese de trauma adaptativo, que é uma das hipóteses propostas para explicar a evolução da cópula traumática. Ademais, essa espécie apresenta o pênis com aspecto glandular e que porta um estilete que serve como canal para transferência de espermatozoides. Em um dos eventos de cópula analisados, foi observado um parceiro projetando a ponta do seu pênis no dorso do outro indivíduo por alguns segundos. Existe a possibilidade, portanto, da injeção de substâncias na parede do corpo do parceiro, o que precisa ser investigado futuramente.

Por fim, ao descrever o funcionamento dos órgãos de armazenamento e digestão de espermatozoides de *O. polycerelloides*, bem como o comportamento de cópula de *O. polycerelloides* e de *Phidiana lynceus*, este trabalho suscitou questões importantes sobre seleção sexual pré- e pós-copulatória em hermafroditas simultâneos. Certamente, quando publicados, esses resultados estimularão futuras investigações sobre o tema utilizando lesmas marinhas como organismos modelo.

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Resumo

A seleção sexual atua significativamente na diversificação de atributos reprodutivos. Muitos hermafroditas possuem órgãos para armazenamento e digestão de espermatozoides, os quais estão potencialmente envolvidos em mecanismos pós-copulatórios de seleção sexual, como competição espermática e escolha críptica da "fêmea". Tais processos geralmente ocorrem no interior do sistema reprodutor feminino, portanto, o conhecimento sobre sua morfologia funcional é de extrema importância para compreensão de mecanismos pós-copulatórios. Lesmas marinhas são predominantemente hermafroditas e possuem sistema reprodutor complexo, incluindo órgãos para armazenamento e digestão de esperma. Nesse contexto, o presente estudo teve como objetivo investigar o comportamento de cópula e a morfologia funcional dos órgãos que contêm esperma no sistema reprodutor feminino do nudibrânquio *Okenia polycerelloides* (Ortea & Bouchet 1983), bem como o comportamento de cópula do nudibrânquio *Phydiana lynceus* Bergh 1867. Para o estudo morfológico, foram empregadas técnicas de microscopia óptica, confocal e eletrônica. Manipulações experimentais e análises histológicas foram combinadas para investigar o destino dos espermatozoides dentro do sistema reprodutor após uma e duas cópulas subsequentes, e se a ausência de alimento influencia a ocorrência de digestão de espermatozoides. Os resultados sugerem função de recepção e armazenamento de espermatozoides para o receptáculo seminal, e de digestão de espermatozoides para a bursa copulatória. Cópulas múltiplas podem levar à estratificação de espermatozoides no receptáculo seminal. Embora a ausência de alimento aparente intensificar a digestão de esperma, ela não determinou sua ocorrência. A digestão de espermatozoides ocorreu continuamente, e pelo menos parte dos espermatozoides recebidos em cada cópula foi direcionada para a bursa copulatória, corroborando a hipótese prévia de digestão de espermatozoides supérfluos. Entretanto, todo o aloesperma recebido pode também ser digerido, sugerindo a possibilidade de escolha críptica. Redução gradual dos conteúdos da bursa copulatória associada ao aumento do tempo pós-cópula sugere reabsorção do material digerido. Interações de cópula em *O. polycerelloides* incluem contato mútuo utilizando a região oral, alinhamento corporal, intromissão do pênis e transferência de esperma. Porém, as interações podem ser interrompidas em qualquer um desses estágios, e mesmo intromissão do pênis não implica em inseminação. *P. lynceus* exibe cópula traumática, na qual parceiros consomem cerata um do outro durante a cópula, um fenômeno inédito dentre as complexas estratégias reprodutivas de hermafroditas.

Abstract

Sexual selection is pervasive and shapes reproductive traits. Many hermaphrodites have organs for sperm storage and digestion, which are potentially involved in post-copulatory sexual selection mechanisms such as sperm competition and cryptic "female" choice. In animals with internal fertilization, these processes occur within the female reproductive tract, thus the knowledge of its functional morphology is of utmost importance for understanding post-copulatory mechanisms of sexual selection. Most sea slugs are hermaphrodites and have a complex reproductive system, with organs for sperm storage and digestion. In this context, the present study aimed to investigate the mating behavior and the functional morphology of the sperm-containing chambers of the reproductive tract of the nudibranch *Okenia polycerelloides* (Ortea & Bouchet 1983), and the mating behavior of the nudibranch *Phidiana lynceus* Bergh 1867. First, we have applied different microscopy techniques (light, confocal and transmission electron microscopy) to study the sperm-containing chambers of *O. polycerelloides*. Then, we have combined experimental manipulations and histological analyses to investigate the fate of sperm within the female reproductive tract after one and two subsequent matings, and if starvation influences the occurrence of sperm digestion. Sperm was received and stored by the seminal receptacle, while the bursa copulatrix had a digestive function. Multiple mating events could lead to sperm stratification in the seminal receptacle. Although starvation seemed to intensify sperm digestion in the bursa copualtrix, it did not determine its occurrence. Sperm digestion occurred continuously, and at least some amount of sperm received during each mating event was directed to the bursa copulatrix to be digested, corroborating a previous hypothesis of surplus sperm digestion. However, all received sperm could also be directed to the bursa copulatrix, suggesting the possibility of cryptic female choice. Gradual reduction of the contents of the bursa copulatrix with increasing post-copulation times suggests resorption of the digested gametes. Mating interactions in *O. polycerelloides* initiated by mutual contact using the oral region, followed by body alignment, penis intromission, and sperm transfer. However, interactions could end at any of these stages, and even penis intromission did not guarantee insemination. *Phidiana lynceus* exhibited traumatic mating, in which partners consume cerata from one another before sperm exchange, clearly adding to the complexity of mating strategies in hermaphrodites.