UNIVERSIDADE ESTADUAL PAULISTA
“JÚLIO DE MESQUITA FILHO”
INSTITUTO DE BIOCIÊNCIAS – RIO CLARO

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS
(ZOOLOGIA)

CARACTERIZAÇÃO DO SISTEMA REPRODUTOR, COMPORTAMENTO REPRODUTIVO E PATERNIDADE EM *CHARYBDIS HELLERII*: PODE O SUCESSO DESTE SIRI INVASOR ESTAR RELACIONADO A UM PADRÃO DE BIOLOGIA REPRODUTIVA DIFERENTE DO CONHECIDO PARA PORTUNIDAE?

TIMÓTEO TADASHI WATANABE

JANEIRO - 2020

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Tese apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutor em Ciências Biológicas (Zoologia).

RIO CLARO – SP

JANEIRO - 2020

W324c

Watanabe, Timóteo Tadashi

Caracterização do sistema reprodutor, comportamento reprodutivo e paternidade em Charybdis hellerii (A. Milne-Edwards, 1867) : Pode o sucesso deste siri invasor estar relacionado a um padrão de biologia reprodutiva diferente fo conhecido para Portunidae? / Timóteo Tadashi Watanabe. -- Rio Claro, 2020

193 p. : il., tabs., fotos

Tese (doutorado) - Universidade Estadual Paulista (Unesp), Instituto de Biociências, Rio Claro

Orientador: Fernando José Zara

Coorientador: Marcos Antonio Oliveira

1. Biologia marinha. 2. Morfologia (Animais). 3. Microscopia eletrônica. 4. Biologia molecular. 5. Decápode (Crustáceo). I. Título.

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TÍTULO DA TESE: CARACTERIZAÇÃO DO SISTEMA REPRODUTOR, COMPORTAMENTO REPRODUTIVO E PATERNIDADE EM CHARYBDIS HELLERII: PODE O SUCESSO DESTE SIRI INVASOR ESTAR RELACINADO A UM PADRÃO DE BIOLOGIA REPRODUTIVA DIFERENTE DO CONHECIDO PARA PORTUNIDAE?

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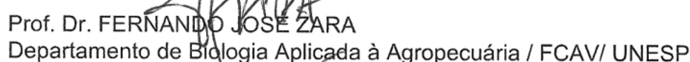

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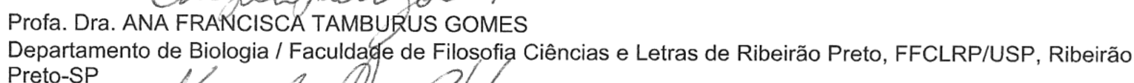

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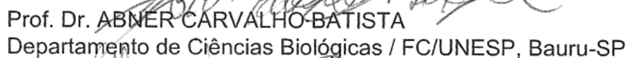

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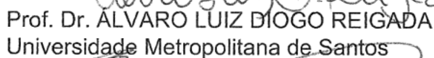

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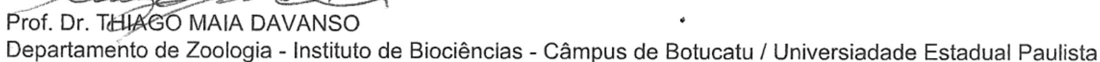

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“Mera mudança não é crescimento. Crescimento é a síntese de mudança e continuidade, e onde não há continuidade não há crescimento.”

C.S. Lewis

AGRADECIMENTOS

Aos meus orientadores prof. Dr. Fernando José Zara e prof. Dr. Marcos Antonio de Oliveira pela oportunidade e aprendizado ao longo do desenvolvimento deste projeto.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) – Código de Financiamento 001. E também através do projeto CAPES - Ciências do Mar II (FJZ Proc. #23038.004309/2014-51) concedido ao orientador, cuja bolsa de estudos fez parte do montante recebido, além do custeio deste doutorado.

À Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) pelo apoio financeiro concedido na forma de auxílio à pesquisa na linha Jovem Pesquisador (FJZ JP Proc. #2005/04707-5), do qual provém uma parte significativa dos dados para esta tese. E também ao suporte financeiro ao Laboratório de Morfologia de Invertebrados (IML) através do Biota Intercrusta (Proc. #2018/13685-5).

À técnica de laboratório do IML, Márcia Fiorese Mataqueiro, juntamente com os colegas/alunos Camila Assugeni, Maria Alice, Fernanda Salti, Bárbara Paiva, Lucas Paschoal, Guilherme Andrioli e Leo Jaime Oliveira (*in memoriam*) no qual sempre estavam à disposição para ajudar naquilo que fosse preciso. E também ao Laboratório de Biologia Molecular Estrutural (LABIMES) do Instituto de Biociências do campus da Unesp de São Vicente, e seus alunos que me ajudaram na execução de parte deste estudo.

Agradeço também à minha família que sempre me apoiou durante toda a minha formação acadêmica, em especial meu pai (*in memoriam*), minha mãe e meus irmãos. E também à minha esposa Adriany Pimentão e minha filha Ayla que foram meu alicerce durante o doutorado.

Resumo

Espécies invasoras são comuns no mundo e causam diversos impactos à fauna local. Com o aumento do tráfego naval e do uso da água de lastro, aumentam as chances de propagação de espécies aquáticas invasoras, sendo o siri *Charybdis hellerii* um exemplo deste processo, ao redor do mundo. Diversos estudos foram conduzidos aumentando o conhecimento de características que favorecem a invasão deste siri, como longo período larval, alimentação onívora, crescimento rápido e estocagem de espermatozoides. Apesar dos estudos já efetuados, pouco se conhece sobre o sistema reprodutor e o comportamento reprodutivo desta espécie, bem como se ela segue o padrão mais comum já descrito para a família Portunidae. Nas espécies nativas do gênero *Callinectes* os índices hepatossomáticos e gonadossomáticos apresentaram pouca variação. Os vasos deferentes possuem células tipicamente secretórias. A região anterior (AVD) é responsável por compactar e formar os espermatóforos. Região média (MVD) onde a maior parte dos espermatóforos formados é armazenada. Na região posterior (PVD) não é encontrado espermatóforos, somente secreção glicoproteica. A presença de grande quantidade de bolsas laterais na MVD e PVD auxilia na produção de fluido seminal. Deste modo, em *Callinectes* as espécies seguem o padrão esperado para formadores de plug espermático. No caso de *C. hellerii* os índices gonadossomáticos e hepatossomáticos foram sempre baixos e praticamente não houve variação nem por classe de tamanho ou por sazonalidade. Os espermatóforos também são formados na AVD, porém a maioria dos espermatóforos é armazenada na região distal da AVD. Os espermatóforos não possuem parede, somente uma película mucosa que sofrem deiscência em água do mar, após passar pelo gonópodo. Todas as células dos vasos deferentes são secretórias, porém a composição da secreção é mais similar à espécies que não são formadoras de plug. Este padrão exclusivo dentro de Portunidae pode ser explicado ao analisarmos o sistema reprodutor feminino. Nas fêmeas não foi encontrado em nenhum momento plug espermático. O receptáculo seminal mantém o mesmo volume independente do estágio ovariano e são encontrados somente espermatozoides livres no lúmen. Os espermatozoides são encontrados imersos em secreção rica em polissacarídeos ácidos, encontrados somente após a cópula. A cópula de *C. hellerii* ocorre em estágio intermuda e as fêmeas são capazes de copular com mais de um macho antes da ovulação. Deste modo o sistema reprodutor de *C. hellerii* é diferente do padrão mais comum registrado em Portunidae e mais similar à subfamília Thalamitinae. As características reprodutivas comportamentais e morfológicas refletem na variabilidade genética da prole, no qual há a confirmação de poliandria em *C. hellerii.* Essas características vêm a aumentar a variabilidade genética da prole, o que é mais uma característica dentre as já conhecidas para a espécie que conferem uma maior vantagem adaptativa e favorece a colonização de novas áreas.

Palavras-Chave: transferência espermática, microssatélites, paternidade, ultraestrutura, histoquímica.

Abstract

Invasive species are a common occurrence in the world and they cause several impacts to the local fauna. With the increasing naval traffic (consequently also increasing the use of ballast water), the potential of spread of aquatic invasive species rise, as the swimming crab *Charybdis hellerii* an example of this process around the world. Several studies were conducted increasing the knowledge of characteristics that favors the invasor of this crab, such as long larval period, omnivorous diet, rapid growth and sperm storage. Despite these studies, little is known abou the reproductive system of this species, as well if it has the most common pattern described to Portunidae. In the native species of the genus *Callinectes* the hepatosomatic and gonadosomatic índices had little variation. The vas deferens has typical secretory cells. The anterior region (AVD) is responsible to compact and form the spermatophores. The median region (MVD) is where the most of the spermatophores is stored. In the posterior region (PVD) there is no spermatophores, only glycoproteic secretion. The large quantity of outpockets in the MVD and PVD aid in the production of the seminal fluid. Thus, in *Callinectes* the species follows the expected patern of sperm plug producer. In the case of *C. hellerii* the gonadosomatic and hepatosomatic índices were Always low and there was barely any variation among size class or between seasons. The spermatophores are also formed in the AVD; however most of the spermatophores are stored in the distal portion of the AVD. The spermatophores have no wall, only a mucous pellicle that suffers dehiscence in seawater, after going through the gonopod. All the cells of the vas deferens are secretory; however the secretion composition is more similar to species that does not form sperm plug. This exclusive pattern in Portunidae could be explained as we analyse the female reproductive system. The sperm plug was not found in the females. The seminal receptacle has the same volume regardless of the ovarian stage and only free spermatozoa are found inside the lumen. The spermatozoa are found immersed in secretion rich in acid polysaccharides, which are only found after the copula. The mating in *C. hellerii* occurs in intermolt stage and the females are capable to mate with more than one male before ovulation. Thus, the reproductive system of *C. hellerii* is different from the most common pattern described for Portunidae and more similar to the subfamily Thalamitinae. The reproductive behavior and morphological characteristics reflects in the genetic of the offspring, which confirms the polyandry in *C. hellerii.* These characteristics increases the genetic variability which is another characteristic among others already described to the species that provides more adaptative advantage and favors the colonization of new áreas.

Keywords: sperm transfer, microsatellites, histochemistry, electron microscopy, paternity.

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Introdução

Atualmente, as invasões biológicas são muito comuns ao redor do mundo e causam diversos impactos ambientais como predação de espécies nativas ou mesmo competição por recursos (Vitousek et al.*,* 1997; Ricciardi et al., 2000). Dentre as diferentes formas de invasão, em organismos aquáticos, a água de lastro é considerada um dos principais meios de introduções de espécies não nativas em ambientes marinhos, devido principalmente à intensificação do tráfego de navios (Kerckhof et al.*,* 2007; Katsanevakis et al., 2014). Um exemplo proposto para este tipo de invasão foi o siri *Charybdis hellerii* (A. Milne-Edwards, 1867) espécie originária do Indo-Pacífico (Lemaitre, 1995), o qual invadiu e se estabeleceu em diversas partes no mundo e, ao longo de grande parte da costa brasileira (Sant'Anna et al., 2012a; Rosa 2014; Negri et al., 2018). A invasão de *C. hellerii* na costa brasileira teve como origem, em sua maioria, de indivíduos provenientes de populações que se estabeleceram no mar Mediterrâneo e somente uma pequena parcela das populações estabelecidas no Brasil foi proveniente do local nativo da espécie (Negri et al., 2018)

Charybdis hellerii mostra vários fatores que favorecem a colonização, como 1) longo período de desenvolvimento larval; 2) rápido crescimento e maturação sexual; 3) dieta variada e onívora, 4) habilidade de armazenar espermatozoides e 5) produzir várias massas de ovos com alta fecundidade (Dineen et al.*,* 2001; Sant'Anna et al., 2012; 2015). O hábitat em que *C. hellerii* vive varia de acordo com o local de invasão e é capaz de se adaptar tanto em substratos arenosos como em substratos rochosos (Lemaitre, 1995; Dineen et al., 2001; Mantelatto e Garcia, 2001; Sant'Anna et al.*,* 2012). No Brasil, inicialmente foi encontrado na costa do Nordeste (Carqueija e Gouvea, 1996; Calado, 1996) e no Sudeste (Tavares e Mendonça Jr., 1996) e se estabeleceu rapidamente na costa brasileira (Mantelatto e Dias, 1999; Mantelatto e Garcia, 2001; Sant'Anna

et al., 2012; 2015). Na costa o estado de São Paulo foi demonstrado que este siri invasor ocupa principalmente substratos consolidados, como o intertidal de costões rochosos com maior salinidade co-existindo com diversas espécies nativas (Sant'Anna et al., 2012).

Apesar de *C. hellerii* ter diversos registros no mundo e evidências de comunidades estabelecidas nos locais introduzidos (Dineen et al., 2001; Mantelatto e Garcia, 2001; Sant'Anna et al., 2012), ainda pouco se sabe a respeito de sua biologia reprodutiva, havendo majoritariamente dados ecológicos a respeito da espécie (Dineen et al., 2001; Mantelatto e Garcia, 2001; Bolaños et al., 2012; Sant'Anna et al., 2012; 2015; Watanabe et al., 2015). Tendo em vista os problemas que espécies invasoras causam, *C. hellerii* também não está isenta de exercer pressão sobre as populações nativas de Brachyura, como foi sugerido em relação ao declínio de populações de *Cronius ruber* (Lamarck, 1818) por Mantelatto et al. (2009) e demonstrado na Baía de Santos-São Vicente (Sant'Anna et al., 2012).

Além do sucesso da invasão de *C. hellerii* no Atlântico oeste (Mantelatto e Dias, 1999; Dineen et al., 2001; Tavares e Amouroux, 2003; Morán e Atencio, 2006; Sant'Anna et al., 2012; 2015, Negri et al., 2018), outras espécies do gênero *Charybdis* já são reportadas no mar Mediterrâneo como *Charybdis feriata* (Linnaeus, 1758) por Abelló e Hispano (2006) e *Charybdis lucifera* (Fabricius, 1798) em Mizzan e Vianello (2009), o que é um alerta em relação ao potencial invasor do gênero *Charybdis.* Isso torna os estudos sobre estas espécies importantes e necessários para a melhor compreensão delas em futuras tentativas de controle da invasão, sendo a prevenção sempre a estratégia mais sensata e menos honerosa.

Em razão da dificuldade de controlar todas as variáveis na natureza, o conhecimento sobre a etologia reprodutiva de Portunidae vem sendo estudada principalmente por meio de experimentos em cativeiro em condições controladas (Pinheiro e Fransozo, 1999). Entre as

temáticas científicas ainda pouco exploradas para *C. hellerii* está o comportamento reprodutivo. Para a família Portunidae, a comunicação e reconhecimento sexual são realizados principalmente por meio de estímulos táteis, visuais e/ou químicos (Hartnoll, 1969; Pinheiro e Fransozo, 1999). Nesta família, têm-se como padrão de cópula, longos períodos de "abraço" pré e pós-copulatórios simultaneamente com a muda da puberdade, estando a fêmea assim, com o exoesqueleto flácido (Hartnoll 1969, Pinheiro e Fransozo 1999). Por outro lado, nos Portunidae da mesma subfamília de *C. hellerii,* Thalamitinae, *Thalamita picta* Stimpson, 1858, *Thalamita prynma* (Herbst, 1803) e *Thalamita sima* H. Milne Edwards, 1834, as fêmeas são capazes de copularem no período intermuda, ou seja, com a carapaça rígida (Norman 1996; Norman et al., 1997; 1999).

Estudos do comportamento reprodutivo em *Charybdis* foram reportados para uma só espécie *C. feriata* (Soundarapandian et al., 2013). Segundo estes autores, a cópula é realizada com a fêmea durante a muda da puberdade (padrão mais comum para Portunidae), com longos períodos de abraço pré (quatro dias) e pós-copulatórios (12 horas), sendo a duração da cópula de 11 horas. Este tempo total de cópula é bem menor em relação à espécie nativa do Brasil como *Arenaeus cribarius* (Lamarck, 1818) que teve duração média de 30 dias de abraço pré-copulátorio, 17 horas de tempo de cópula e 30 dias de abraço pós-copulatório (Pinheiro e Fransozo, 1999). Contudo, este tempo de cópula foi semelhante ao de *Portunus pelagicus* (Linnaeus, 1758), da Oceania, *Charybdis feriata* (Linnaeus, 1758) de origem Indo-Pacífica e *Carcinus maenas* (Linnaeus, 1758), cuja origem é europeia e, também, apresenta potencial como invasor (Fielder e Eales, 1972; Berril e Arsenault, 1982; Grosholz e Ruiz, 1996; Thresher et al., 2003). *Charybdis feriata* ainda não pode ser considerado um invasor, apesar de já ter sido registrado no Mar Mediterrâneo (Abelló e Hispano, 2006). Este tipo de comportamento

reprodutivo é o esperado para seu congênere *C. hellerii* que ainda não possui nenhum estudo neste tema. Considerando outros Thalamitinae como *T. picta, T. prymna*, e *T. sima* e os congêneres *Charybdis japônica* (A. Milne-Edwards, 1861) e *Charybdis longicollis* (Leene, 1938) que diferem do padrão mais conhecido de cópula em Portunidae e realizam cópula com fêmea em estágio intermuda (Norman 1996; Norman et al., 1997; 1999; Innocenti et al., 1998; Baker et al., 2018). Adicionalmente, atividades de campo realizadas pelo grupo de pesquisa, onde esta tese foi desenvolvida, foi constatado ao menos um casal de *C. hellerii* em cópula, sendo a condição da carapaça da fêmea rígida (Timóteo Tadashi Watanabe e Bruno Sampaio Sant'Anna, observação pessoal, 2012). Apesar de ser uma única observação, tal evento sugeriu a possibilidade de ocorrência de poliandria, o que resultou em uma série de eventos que culminaram em parte nesta tese, vislumbrando a possibilidade das fêmeas aumentarem a variabilidade genética dos descendentes, sendo este um aspecto vantajoso para um organismo invasor (Norman et al., 1999; Cornell e Tregenza, 2007). Assim, o presente estudo pretende fechar lacunas importantes para a compreensão do sucesso da invasão deste animal ao redor do mundo.

Outro aspecto importante a ser estudado sobre a biologia reprodutiva de crustáceos é a morfologia do sistema reprodutor. Por meio da morfologia, histologia e ultraestrutura, podem-se obter informações detalhadas dos mecanismos da transferência espermática (Beninger et al., 1988; 1993; Zara et al., 2012; 2014). Em Brachyura, a complexidade do processo de inseminação tende a ser maior em relação a outros grupos de crustáceos, uma vez que a inseminação é interna, levando a estocagem de fluido seminal para posterior fertilização (Bauer, 1986). Logo, os aspectos morfológicos do sistema reprodutor de *C. hellerii* são de extrema importância para a compreensão da reprodução (Zara et al., 2012; Nascimento e Zara, 2013), o que pode ser chave

para o entendimento do sucesso de invasão e estabelecimento das populações. O sistema reprodutor masculino para a vasta maioria dos Brachyura e, Portunidae não é uma exceção, encontra-se localizado no cefalotórax em forma da letra "H". Este é constituído por um par de testículos, unidos por comissura central logo abaixo do coração. Cada testículo desemboca no vaso deferente, o qual termina nos dutos ejaculatórios conectados aos gonopódios por meio do gonóporo na coxa do quinto pereiópodo (Cronin, 1947; Johnson, 1980; Zara et al., 2012). Apesar da grande diversidade existente em Brachyura e, em especial a família Portunidae (Ng et al., 2008; Evans, 2018), existem poucos estudos conciliando a morfologia, histologia e, principalmente a ultraestrutura do sistema reprodutor masculino desta família com representantes de importância econômica (Cronin, 1947; Ryan, 1967; Johnson, 1980; Stewart et al., 2010; Zara et al., 2012; Nascimento e Zara, 2013). Para o gênero *Charybdis* existe somente a descrição da morfologia geral e a histologia para *Charybdis smithii* MacLeay, 1838 em Balasubramanian e Suseelan (2000), sendo os resultados semelhantes ao encontrado para *Callinectes sapidus* Rathbun, 1896 em Cronin, (1947) e Johnson, (1980), *P. pelagicus* (Stewart et al., 2010) e *Portunus hawaiiensis* Herbst, 1783 como *Portunus sanguinolentus* em Ryan (1967), porém com lóbulos testiculares incompletos. As células germinativas de *C. smithii* foram descritas como sincrônicas (Balasubramanian e Suseelan, 2000) como ocorrem para a maioria dos siris (Johnson, 1980; Stewart et al., 2010; Zara et al., 2012; Nascimento e Zara, 2013). Porém a única diferença encontrada em *C. smithii* é que o vaso deferente anterior, na sua porção proximal, possui epitélio cúbico e na porção distal, o epitélio é colunar (Balasubramanian e Suseelan, 2000) diferente do padrão para os portunídeos (Ryan, 1967; Johnson, 1980; Stewart et al., 2010; Zara et al., 2012; Nascimento e Zara, 2013). Assim, espera-se que a análise histoquímica e ultraestrutural comparativa entre o gênero *Callinectes* e *C. hellerii* possam esclarecer se o

sistema reprodutor masculino deste siri invasor segue o padrão de produção de espermatozoide, espermatóforos e fluido seminal típico de Portunidae ou variações neste padrão possam indicar prováveis adaptações para explicar o sucesso da invasão deste animal.

O sistema reprodutor feminino em Eubrachyura possui um órgão de origem ecto-mesodérmica, o receptáculo seminal, cuja função é armazenar espermatozoides para posteriores eventos de fertilização (Diesel, 1989; 1990; 1991; Guinot e Quenette, 2005; López-Greco et al., 2009; McLay e López-Greco, 2011). Este órgão é fundamental para determinar desde padrões de estoque, até evidências de competição espermática (Diesel, 1991). Na família Portunidae, assim como em outros Brachyura, o receptáculo seminal é um órgão par conectado aos ovários por meio dos ovidutos. O receptáculo seminal está posicionado no interior da cavidade cefalotorácica, abaixo do coração, circundada por parte da musculatura de locomoção associada aos apódemas internos (Pyle e Cronin, 1950).

O receptáculo seminal em Eubrachyura pode ser classificado como dorsal, ventral, ou intermediário de acordo com a posição de inserção do oviduto em relação à vagina, a qual se localiza ventralmente (Diesel, 1991; McLay e López-Greco, 2011; Zara et al. 2014). Apesar de terem provavelmente se derivado de uma característica ancestral totalmente ectodérmica, observada em Podotremata, chamada espermateca, os receptáculos seminais tipo dorsal são considerados o mais próximo da condição ancestral (McLay e López-Greco 2011). Em Portunidae o padrão do receptáculo seminal é majoritariamente dorsal, como ocorre em *A. cribarius* (Zara et al., 2014), *C. sapidus* (Johnson, 1980) e *P. pelagicus* (Bawab e El-Sherief, 1988). Porém em *P. hawaiiensis* o receptáculo seminal foi classificado como do tipo intermediário (Ryan, 1967; Diesel, 1991; McLay e López-Greco, 2011). Estas diferenças de padrão morfológico estão relacionadas às modificações morfológicas que ocorrem após a cópula,

quando ocorre a eliminação do material seminal transferido pelo macho, levando o receptáculo do tipo dorsal assumir uma posição praticamente do tipo ventral em espécies como *A. cribrarius* (Zara et al., 2014). Adicionalmente, a diferença nos tipos de receptáculos pode estar relacionada à cópula com mais de um macho, levando a competição espermática (Diesel, 1989; 1991; McLay e López-Greco, 2011).

A competição espermática normalmente é limitada em Brachyura, pois os espermatozoides são imóveis e desprovidos de flagelo (Krol et al., 1992). Esta competição torna-se ainda menor com os diversos mecanismos que impedem a poliandria como a cópula com a fêmea durante a muda (fêmea flácida), seguido pela guarda do macho o qual pode depositar um tampão de secreção denominado “plug espermático”, como descrito para *Arenaeus*, *Callinectes* e *Portunus* ou, em outros casos, quando a fêmea realiza a postura dos ovos logo após a cópula (McLay e López-Greco, 2011). Contudo, com a possibilidade da ausência destas características durante a cópula, como observado em Thalamitinae (Norman et al., 1999), podem diminuir as chances e a importância da competição entre os machos, visto que a possibilidade de poliandria torna-se plausível e vantajosa para aumento da variabilidade genética da prole. Em particular para as famílias Cancridae e Portunidae, o fluido seminal produzido no vaso deferente dos machos forma o “plug” espemático dentro do receptáculo seminal (Hartnoll, 1969; Hinsch, 1988). Este “plug” preenche o receptáculo seminal impedindo ou dificultando a introdução de material genético de outros machos (Hines et al., 2004; Hartnoll, 1969; Wolcott et al., 2005; Zara et al., 2012; 2014). Porém, com o desenvolvimento ovariano, o “plug” espermático desaparece após algum tempo e o receptáculo seminal torna-se menor e flácido, como observado em outras espécies de Portunidae como o Carcininae *C. maenas* (Spalding, 1942) e nos Portuninae *P. hawaiiensis* (Ryan, 1967), *P. pelagicus* (Bawab e El-Sherief, 1988), *Callinectes danae* Smith,

1869 (Zara et al., 2012; 2013) e *A. cribrarius* (Pinheiro e Fransozo 2002; Zara et al., 2014). Por sua vez, no Thalamitinae *C. smithii* não existe evidência de "plug" no receptáculo (Balasubramanian e Suseelan, 1998).

Sabendo-se que em Portunidae o "plug" espermático desaparece após algum tempo e que em *C. smithii* não foi detectada a presença de "plug" espermático, abre-se, então, a possibilidade das fêmeas de *C. hellerii* não possuírem o "plug" e serem poliandras. Entre as vantagens de um macho copular com várias fêmeas, a principal é o aumento do *fitness* e sucesso reprodutivo do macho (Bateman, 1942; Emlen e Oring, 1977), o que é um fato comum para Brachyura (Hartnoll, 1969). Para as fêmeas apenas uma cópula assegura o armazenamento de espermatozoides, para posterior fertilização e, além disso, o ato da cópula traz riscos como danos físicos, gasto energético e susceptibilidade à predação (Blanckerhorn et al., 2002; DiBattista et al., 2008). Porém considerando uma espécie invasora como *C. hellerii,* a poliandria poderia beneficiar a espécie com a proteção fornecida pelo macho no momento da cópula (DiBattista et al., 2008), aumentar a diversidade genética da prole, evitar incompatibilidades genéticas e consanguinidade e aumentar a chance de receber genes de maior qualidade dos machos (Jennions e Petrie, 2000; Zeh e Zeh, 2001; Johnson e Brockmann, 2013). Dado as características já conhecidas na família Portunidae, como possibilidade de estocagem de espermatozoide em longo prazo (Dineen, 1999; McLay e López-Greco 2011; Zara et al., 2014) e a possibilidade da ausência de "plug" espermático em *Charybdis,* isso permite levantar a hipótese que as fêmeas do siri invasor, *C. hellerii,* sejam poliândricas como demonstrado para as espécies da família Ucididae e Cancridae (Baggio et al., 2011; Jensen e Bentzen 2012; Rojas-Hernandez et al., 2014). Caso a hipótese da poliandria seja aceita para *C. hellerii*, esta informação pode esclarecer um ponto crucial do sucesso de invasão e estabelecimento deste Portunidae ao redor do mundo,

saído do padrão monoandrico ou quase monoandrico (12% das fêmeas de *C. sapidus* mostram dois pacotes de espermatozoide de um ou dois pais) determinado para os siris (para revisão Jivoff et al., 2007). Tais dados permitirão estudos posteriores, com outras espécies do mesmo gênero, as quais podem apresentar esta e, outras várias características, que as favorecem como invasores potenciais.

Por meio de análises genéticas pode-se esclarecer a paternidade, realizar a sexagem dos indivíduos, definir a estrutura das populações, determinar gargalos, padrões de migração, a história demográfica das populações e detectar introgressão (Frankham et al., 2010; Chambers et al., 2014). Análises nas diferenças existentes nas sequências de DNA entre indivíduos e entre populações permite explorar os processos evolutivos e eventos demográficos do passado da espécie (Nosil et al.; 2009). Por outro lado, taxas de dispersão são difíceis de estudar por observação direta, pois muitas vezes são baixas e as dispersões de longa distância são dificilmente mensuradas com precisão (Mirimin et al., 2009). A falta de dispersão faz com que os acasalamentos não sejam aleatórios e, sendo assim, ocorre déficit de heterozigosidade (Frankham et al., 2008). Com o uso de marcadores genéticos é possível fazer inferências a respeito dos padrões de dispersão da população. Informações sobre as paternidades são essenciais para o estudo do impacto do endocruzamento, e consequentemente para um maior entendimento do sucesso reprodutivo de espécies invasoras (Mirimirin et al., 2009; Barret, 2015). Além disso, o conhecimento da composição genética de uma espécie invasora e de sua estrutura populacional é fundamental para as ações de conservação de organismos endêmicos, os quais podem ter seus nichos ameaçados por espécies exóticas (Frankham et al., 2008; Mirimirin et al., 2009).

Marcadores STRs (Short Tandem Repeats), também denominados de microssatélites de DNA, estão distribuídos pelo genoma nuclear de eucariotos, e seus polimorfismos são resultados de variações no número de repetições *in tandem* (uma atrás da outra) de uma sequência repetitiva curta de DNA (Bhargava et al., 2010). No caso de STRs estas repetições possuem de 1 a 6 bases que podem se repetir de 5 a 100 vezes por *locus* (Wan et al., 2004). Em muitos casos, quando uma população possui um tamanho significativo (N) e os *loci* são bastante polimórficos, apresentando alto grau de variabilidade, encontrar as mesmas combinações de alelos em dois indivíduos é algo bastante raro. Neste contexto a analise destas regiões podem revelar heterozigose e homozigose para cada individuo, sendo assim, são muito utilizados para determinar parentesco, sexo, estrutura e classificação de uma população (Wan et al., 2004). Com o advento da técnica de reação em cadeia da polimerase (PCR - Polymerase Chain Reaction), microssatélites são utilizados como importantes marcadores genéticos, identificados a partir de um prévio sequenciamento do DNA da espécie (Bhargava et al., 2010). Muitas vezes, podem ser utilizados primers ou oligonucleotídeos de espécies relacionadas para a amplificação heteróloga de microssatélites das espécies de interesse (Farro et al., 2008). Neste contexto, as análises moleculares revelam que a poliandria, múltiplo acasalamento pelas fêmeas é altamente difundido na natureza, incluindo diversas espécies de aves e mamíferos os quais eram considerados classicamente monogâmicos (Waser et al., 2006; Soulsbury, 2010; Chambers et al., 2014,). No que concerne especificamente a invertebrados diversos trabalhos recentes também apontam que a múltipla paternidade é um evento bastante comum (Yue e Chang, 2010; Jossart et al., 2014).

Trabalhos recentes também apontam que fêmeas de diversos crustáceos possuem comportamento poliândrico, comprovado pela análise de STRs que demonstra claramente a múltipla paternidade em embriões de ovos fecundados (Baggio et al., 2011; Jensen e Bentzen,

2012; Ma et al., 2013; Rojas-Hernandez et al., 2014; Dennenmoser e Thiel, 2015). Dentre os trabalhos existentes, recentemente foram identificados em *C. feriata* 47 STRs sendo que 10 se apresentaram altamente polimórficas e com heterozigosidade (*H*) na população variando de 41-81% (Ma et al., 2013). Em razão da grande proximidade filogenética entre *C. feriata* e *C. hellerii* este trabalho abre a perspectiva da utilização de oligonucleotideos hierólogos de *C. feriata* para estudos populacionais e de paternidade múltipla de *C. hellerii*, o qual apesar de representar uma espécie invasora não só no Brasil, mas também em diversos países como a Venezuela, Colômbia, Estados Unidos e Turquia (Sant'Anna et al., 2012) ainda carece de estudos a variedade populacional e poliandria. Adicionalmente, a amplificação hieróloga das STRs permitiria clonar e sequenciar os microsatelites, permitindo a determinação de oligonucleotídeos específicos para STRs de *C. hellerii*.

Sabe-se que *C. hellerii* foi bem sucedido, tanto na invasão, como no estabelecimento de suas populações ao redor do mundo. Com isso vários estudos como Dineen et al. (2001), Mantellato e Garcia (2001), Tavares e Amouroux (2003), Sant'Anna et al. (2012), Watanabe et al. (2015) e Negri et al. (2018) aumentaram significativamente o conhecimento acerca da espécie. Nota-se que esta espécie parece influenciar espécies nativas associadas a substratos consolidados (costões rochosos) no Brasil, como *C. ruber* (Mantelatto et al., 2009; Sant'Anna et al., 2012), não afetando as espécies de substratos não consolidados como os *Callinectes* (Sant'Anna et al., 2012). Porém, ainda existem várias lacunas cruciais para o entendimento do sucesso de invasão e estabelecimento destes animais. Dentre elas, sem dúvida, o conhecimento da biologia reprodutiva é o que se destaca, e várias dúvidas ainda necessitam ser esclarecidas, como elencadas a seguir: 1) quais os principais hábitos comportamentais exibidos durante a cópula; 2) as fêmeas podem aceitar mais de um macho; 3) as fêmeas de *C. hellerii* recebem ou

não plug espermático transferido pelos machos, 4) devido a presença ou ausência de plug espermático, as fêmeas são monoândras ou poliandras; 5) se a progênie produzida é oriunda de um único ou vários machos.

Baseado nas informações disponíveis desta espécie e seu sucesso no processo de invasão, ao se investigar as questões previamente levantadas, espera-se que *C. hellerii* apresente um padrão de produção de fluido seminal, estocagem de espermatozoides, comportamento reprodutivo e genética da progênie distinto das espécies nativas já estudadas como *Arenaeus* e *Callinectes,* as quais são tipicamente formadoras de "plug" espermático. Caso isso seja confirmado, as características reprodutivas de *C. hellerii* podem ser mais um fator adicional para o sucesso da invasão, além dos já levantados por Dineen et al. (2001), fechando assim as lacunas de conhecimento para esta espécie.

Deste modo, esta tese foi dividida em capítulos, na qual o primeiro trata da morfologia e ultraestrutura do sistema reprodutor masculino de *Callinectes* sendo este gênero considerado modelo para espécies de Portunidae, e além disso, estas espécies são tipicamente formadoras de "plug" espermático. No segundo capítulo, figura a morfologia e ultraestrutura do sistema reprodutor masculino do siri invasor *C. hellerii* mostrando as diferenças para as espécies formaduoras de plug espermático e no terceiro capítulo, é apresentado a morfologia e ultraestrutura do sistema reprodutor feminino de *C. hellerii,* com a adição comportamento reprodutivo em laboratório e a análise de paternidade da progênie deste siri invasor.

Referências

Abelló, P., Hispano, C. (2006). The capture of the Indo-Pacific crab *Charybdis feriata* (Linnaeus, 1758) (Brachyura: Portunidae) in the Mediterranean Sea. *Aquatic Invasions,* 1: 13-16.

Baggio, R.A., Pil, M.W., Boeger, W.A., Patella, L.A., Ostrensky, A., Pie, M.R. (2011). Genetic evidence in the mangrove land crab *Ucides cordatus* (Decapoda: Ocypodidae). *Marine Biology Research*, 7: 520-524.

Baker, C.F., Gublin, Y., Reeve, K.A. (2018). Observations of hard-shell mating in the portunid crab *Charybdis japonica* (A. Milne-Edwards, 1861) (Decapoda: Brachyura). *Journal of Crustacean Biology*, 38: 504-508.

Balasubramanian, C.P., Suseelan, C. (1998). Reproductive biology of the female deepwater crab *Charybdis smithii* (Brachyura: Portunidae) from the Indian seas. *Asian Fisheries Science*, 10: 211-222.

Balasubramanian, C.P., Suseelan, C. (2000). Male reproductive system and spermatogenesis in the deep water crab *Charyhdis smithii* McLeay (Brachyura: Portunidae). *Indian Journal of Fisheries,* 47: 275-282.

Barrett, S.C. (2015). Foundations of invasion genetics: the Baker and Stebbins legacy. *Molecular Ecology* 9: 1927-41.

Bateman, A.J. (1948). Intra-sexual selection in *Drosophila*. *Heredity,* 2(Pt. 3): 349-368.

Bauer, R.T. (1986). Phylogenetic trends in sperm transfer and storage complexity in decapod crustaceans. *Journal of Crustacean Biology*, 6: 313-325.

Bawab, F.M., El-Sherief, S.S. (1988). Stages of the reproductive cycle of the female crab *Portunus pelagicus* (L., 1758) based on the anatomical changes of the spermatheca (Decapoda, Brachyura, Portunidae). *Crustaceana*, 54: 139-148.

Beninger, P.G., Elner, R.W., Foyle, T.P., Odense, P.H. (1988). Functional anatomy of the male reproductive system and female spermatheca in the snow crab *Chionoecetes opilio* (O. Fabricius) (Decapoda: Majidae) and the hypothesis for fertilization. *Journal of Crustacean Biology*, 8: 322-332.

Beninger, P.G., Lanteigne, C., Elner, N.W. (1993). Reproductive processes revealed by spermatophore dehiscence experiments and by histology, ultrastructure and histochemistry of the female reproductive system in the snow crab *Chionoecetes opilio* (O. Fabricius). *Journal of Crustacean Biology*, 13: 1-16.

Berril, M., Arsenault, M. (1982). Mating behavior of the green shore crab *Carcinus maenas*. *Bulletin of Marine Science,* 32: 632-638.

Bhargava, A., Fuentes, F.F. (2010). Mutational dynamics of microsatellites. *Molecular Biotechnology,* 44: 250-66.

Blanckenhorn, W.U., Hosken, D.J., Martin, O.Y., Reim, C., Teuschl, Y., Ward, P.I. (2002). The costs of copulating in the dung fly *Sepsis cynipsea*. *Behavioral Ecology*, 13: 353-358.

Bolaños, J.A., Baeza, J.A., Hernandez, J.E., Lira, C., López, R. (2012). Population dynamics and reproductive output of the non-indigenous crab *Charybdis hellerii* in the south-eastern Caribbean Sea. *Journal of the Marine Biological Association of the United Kingdom*, 92: 469-474.

Calado, T.C.S. (1996). Registro de *Charybdis hellerii* (Milne Edwards, 1867) em águas do litoral brasileiro (Decapoda: Portunidae). *Boletim de Estudos de Ciências do Mar,* 9: 175-180.

Carqueija, C.R.G., Gouvêa, E.P. (1996). A ocorrência, na costa brasileira, de um Portunidae (Crustacea, Decapoda), originário do Indo-Pacífico e Mediterrâneo. *Nauplius,* 4:165- 168.

Chambers, G.K., Curtis, C., Millar, C.D., Huynen, L., Lambert, D.M. (2014). DNA fingerprinting in zoology: past, present, future. *Investigative Genetics,* 5: 3.

Cornell, S.J., Tregenza, T. (2007). A new theory for the evolution of polyandry as a means of inbreeding avoidance. *Proceedings of the Royal Society B: Biological Sciences*, 274: 2873-2879.

Cronin, L.E. (1947). Anatomy and histology of the male reproductive system of *Callinectes sapidus* Rathbun. *Journal of morphology*, 81: 209-239.

Dennenmoser, S., Thiel, M. (2015). Cryptic Female Choice in Crustaceans. Cryptic Female Choice in Arthropods. *Springer International Publishing* pp.203-237.

DiBattista, J.D., Feldheim, K.A., Gruber, S.H., Hendry, A.P. (2008). Are indirect genetic benefits associated with polyandry? Testing predictions in a natural population of lemon sharks. *Molecular Ecology,* 17: 783-95.

Diesel, R. (1989). Structure and function of the reproductive system of the symbiotic spider crab *Inachus phalangium* (Decapoda: Majidae): observations on sperm transfer, sperm storage and spawning. *Journal of Crustacean Biology*, 9: 266-277.

Diesel, R. (1990). Sperm competition and reproductive success in a decapod *Inachus phalangium* (Majidae): A male ghost spider crab that seals off rivals sperm. *Journal of Zoology*, 220: 213-223.

Diesel, R. (1991). Sperm competition and mating behavior in Brachyura. In: Bauer, R.T. & Martin, J.W. (eds), Crustacean Sexual Biology. Columbia University Press, New York, pp. 145-163.

Dineen, J.F., Clark, P.F., Hines, A.H., Reed, S.A., Walton, H.P. (2001) Life history, larval description, and natural history of *Charybdis hellerii* (Decapoda, Brachyuran, Portunidae), an invasive crab in the western Atlantic. *Journal of Crustacean Biology,* 21: 774-805.

Emlen, S.T., Oring, L.W. (1977). Ecology, sexual selection, and the evolution of mating systems. *Science*, 197: 215-223.

Evans, N. (2018). Molecular phylogenetics of swimming crabs (Portunoidea Rafinesque, 1815) supports a revised family-level classification and suggests a single derived origin of symbiotic taxa. *PeerJ*, 6: e4260.

Farro, A.P.C., Rollo Jr, M.M., Silva Jr, J.M., Marino, C.L. (2008). Isolation and characterization of microsatellite DNA markers for spinner dolphin (*Stenella longirostris*). *Conservation Genetics*, 9: 55-59.

Fielder, D.R., Eales, A.J. (1972). Observations on courtship, mating and sexual maturity in *Portunus pelagicus* (L., 1766) (Crustacea, Portunidae), *Journal of Natural History*, 6: 273-277

Frankham, R., Ballou, J.D., Briscoe, D.A. (2010). Introduction to Conservation Genetics. Cambridge University Press.

Grosholz, E.D., Ruiz, G.M. (1996). Predicting the impact of introduced marine species: lessons from the multiple invasions of the European green crab *Carcinus maenas*. *Biological Conservation*, 78: 59-66.

Guinot, D., Quenette, G. (2005). The spermatheca in podotreme crabs (Crustacea, Decapoda, Brachyura, Podotremata)and its phylogenetic implications. *Zoosystema*, 2: 267-342.

Hartnoll, R.G. (1969). Mating in the Brachyura. *Crustaceana,* 16: 161-181.

Hines, A.H., Jivoff, P.R., Bushmann, P.J., van Montfrans, J., Reed, S,A,, Wolcott, D.L., Wolcott, T.G. (2003). Evidence for sperm limitation in the blue crab, *Callinectes sapidus*. *Bulletin of Marine Science*, 72: 287-310.

Hinsch, G.W. (1988). Morphology of the reproductive tract and seasonality of reproduction in the golden crab, *Geryon fenneri* from the eastern Gulf of Mexico. *Journal of Crustacean Biology*, 8: 254-261.

Innocenti, G., Vannini, M., Galil, B.S. (1998). Notes on the behavior of the portunid crab *Charybdis longicollis* Leene parasitized by rhizocephalan *Heterosaccus dollfusi* Boschma. *Journal of Natural History*, 32: 1577-1585.

Jennions, M.D., Petrie, M. (2000). Why do females mate multiply? A review of genetic benefits. *Biological Reviews,* 75: 21-64.

Jensen, P.C., Bentzen, P. (2004). Isolation and inheritance of microsatellite loci in the Dungeness crab (Brachyura: Cancridae: *Cancer magister*). *Genome*, 47: 325-331.

Jensen, P.C., Bentzen, P. (2012). A molecular dissection of the mating system of the dungeness crab, *Metacarcinus magister* (Brachyura: Cancridae). *Journal of Crustacean Biology,* 32: 443-456.

Jivoff, P., Hines, A.H. (1998). Female behaviour, sexual competition and mate guarding in the blue crab, *Callinectes sapidus*. *Animal Behaviour*, 55: 589-603.

Jivoff, P.R., Hines, A.H., Quackenbush, L.S. (2007). Reproduction Biology and Embryonic Development. Em: The Blue Crab: *Callinectes sapidus*. Kennedy, V.S. & L.E. Cronin (eds). 2007. College Park, Maryland Sea Grant College, University of Maryland, 774 p.

Johnson, P.T. (1980). Histology of the blue crab *Callinectes sapidus*: a model for the Decapoda. Praeger, New York. 440p.

Johnson, S.L., Brockmann, H.J. (2013). Parental effects on early development: testing for indirect benefits of polyandry. *Behavioral Ecology*, art056.

Jossart, Q., Wattier, R.A., Kastally, C., Aron, S., David, B., De Ridder, C., Rigaud, T. (2014). Genetic evidence confirms polygamous mating system in a crustacean parasite with multiple hosts. *PLoS One*, 9: e90680.

Katsanevakis, S., Wallentinus, I., Zenetos, A., Leppäkoski, E., Çinar, M.E., Oztürk, B., Grabowski, M., Golani, D., Cardoso, A.C. (2014). Impacts of invasive alien marine species on ecosystem services and biodiversity: a pan-European review. *Aquatic Invasions*, 9: 391-423.

Kerckhof, F., Haelters, J., Gollasch, S. (2007) Alien species in the marine and brackish ecosystem: the situation in Belgian waters. *Aquatic Invasions,* 2: 243-257.

Kroll, R.M., Hawkins, W.E., Overstreet, R.M. (1992). Reproductive Components. In: Harrison, FW & AG Humes (eds.). Microscopic anatomy of invertebrates. New York: Wiley-Liss Inc., 10: 295-343.

Lemaitre, R. (1995). *Charybdis hellerii* (Milne Edwards, 1867), a nonindigenous portunid crab (Crustacea: Decapoda: Brachyura) discovered in the Indian River lagoon system of Florida. *Proceedings of the Biological Society of Washington*, 108: 643-648

López-Greco, L.S., Lopez, G.C., Rodriguez, E.M. (1999). Morphology of spermatheca in the estuarine crab *Chasmagnathus granulata* Dana 1851 (Graspidae, Sesarminae). *Journal of Zoology,* 249: 490-493.

López-Greco, L.S., Fransozo, V., Negreiros-Fransozo, M.L., Santos, D.C. (2009). Comparative morphology of the seminal receptacles of *Ocypode quadrata* (Fabricius, 1787) (Brachyura: Ocypodoidea). *Zootaxa,* 2106: 41-50.

Ma, H., Zou, X., Ji, X., Ma, C., Lu, J., Jiang, W., Xia, L., Li, S., Liu, Y., Gong, Y., Ma, L. (2013). Discovery and characterization of a first set of polymorphic microsatellite markers in red crab (*Charybdis feriatus*). *Journal of genetics,* 92: e113-5.

Mantelatto, F.L.M., Dias, L.L. (1999). Extension of the known distribution of *Charybdis hellerii* (A. Milne Edwards, 1867) (Decapoda, Portunidae) along the western tropical South Atlantic. *Crustaceana* 72: 617-620.

Mantelatto, F.L.M., Garcia, R.B. (2001). Biological aspects of the nonindigenous portunid crab *Charybdis hellerii* in the western tropical South Atlantic. *Bulletin of Marine Science,* 68: 469-477.

Mantelatto, F.L., Robles, R., Schubart, C.D., Felder, D.L. (2009). Molecular Phylogeny of the Genus *Cronius* Stimpson, 1860, with Reassignment of *C. tumidulus* and Several American Species of *Portunus* to Genus *Achelous* De Haan, 1833 (Brachyura: Portunidae). In: Martin, JW, Crandall, KA, Felder, DL (eds), Crustacean Issues: Decapod Crustacean Phylogenetics. CRC Press, pp 567-579.

McLay, C.L., López-Greco, L.S. (2011). A hypothesis about the origin of sperm storage in the Eubrachyura, the effects of seminal receptacle structure on mating strategies and the evolution of crab diversity: How did a race to be first become a race to be last? *Zoologischer Anzeiger-A Journal of Comparative Zoology*, 250: 378-406.

Mirimin, L., Westgate, A., Rogan, E., Rosel, P., Read, A., Coughlan, J., Cross, T. (2009). Population structure of short-beaked common dolphins (*Delphinus delphis*) in the North Atlantic Ocean as revealed by mitochondrial and nuclear genetic markers. *Marine Biology*, 156: 821-83.

Mizzan, L., Vianello, C. (2009). First record of *Charybdis* (*Charybdis*) *lucifera* (Fabricius, 1798) (Crustacea, Decapoda, Portunidae) in the Mediterranean Sea. *Bollettino del Museo civico di Storia naturale di Venezia,* 59: 27-30.

Morán, R., Atencio, M. (2006). *Charybdis hellerii* (Crustacea: Decapoda: Portunidae), espécie invasora em La Península de Paranaguá, estado Falcón, Venezuela. *Multiciencias,* 6: 202-209

Nascimento, F.A., Zara, F.J. (2013). Development of the male reproductive system in *Callinectes ornatus* Ordway, 1863 (Brachyura: Portunidae). *Nauplius*, 21: 161-177.

Negri, M., Schubart, C.D., Mantelatto, F.L. (2018). Tracing the introduction history of the invasive swimming crab *Charybdis hellerii* (A. Milne-Edwards, 1867) in the Western Atlantic: evidences of high genetic diversity and multiple introductions. *Biological invasions*, 20: 1771-1798.

Ng, P.K.L., Guinót, D., Davie, P.J.F. (2008). Systema Brachyurorum: Part I. An Annotated Checklist of Extant Brachyuran Crabs of the World. *The Raffles Bulletin of Zoology*, 17:1-286.

Norman, C.P., Nuka, T., Miyazaki, T. (1999). Mating Behavior in *Thalamita picta* (Brachyura: Portunidae) and comparisons with congeneric species. *Crustacean Research*, 28: 16-23.

Nosil, P., Funk, D.J., Ortiz, D. (2009). Divergent selection and heterogeneous genomic divergence. *Molecular Ecology*, 18: 375-402.

Pinheiro, M.A.A., Fransozo, A. (1999). Reproductive behavior of the swimming crab *Arenaeus Cribrarius* (Lamarck, 1818) (Crustacea, Brachyura, Portunidae) in captivity. *Bulletin of Marine Science*, 64: 243-253.

Pinheiro, M.A.A., Fransozo, A. (2002). Reproduction of the speckled swimming crab *Arenaeus cribrarius* (Brachyura: Portunidae) on the Brazilian coast near 23º30'S. *Journal of Crustacean Biology,* 23: 416-428.

Pyle, R., Cronin, E. (1950). The general anatomy of the Blue Crab, *Callinectes sapidus* Rathbun. *Chesapeake Biological Laboratory*, 87: 3-40.

Ricciardi., A., Steiner, W.W., Mack, R.N., Simberloff, D. (2000). Toward a global information system for invasive species. *BioScience*, 50: 239-244.

Rosa, R.S. (2014) New records of portunid crabs (Decapoda: Brachyura: Portunidae) from Sergipe, NE Brazil. *Check List* 10: 445-447.

Rojas-Hernandez, N., Véliz, D., Pardo, L.M. (2014). Use of novel microsatellite markers for population and paternity analysis in the commercially important crab *Metacarcinus edwardsii* (Brachyura: Cancridae) *Marine Biology Research*, 10: 839–844.

Ryan, E.P. (1967). Structure and function of the reproductive system of the crab *Portunus sanguinolentus* (Hersbt) (Brachyura: Portunidae) I. The male system. *Proceedings of the Symposium on Crustacea, Marine Biology Association India Ernakulam,* 2: 506-521.

Sant`Anna, B.S., Watanabe, T.T., Turra, A., Zara, F.J. (2012). Relative abundance and population biology of the non-indigenous crab *Charybdis hellerii* (Crutacea: Brachyura: Portunidae) in a southwestern Atlantic estyuary-bay complex. *Aquatic Invasions*, 7: 347-356.

Sant'Anna, B.S., Branco, J.O., Oliveira, M.M.D., Boos, H., Turra, A. (2015). Diet and population biology of the invasive crab Charybdis hellerii in southwestern Atlantic waters. *Marine Biology Research*, 11: 814-823.

Soulsbury, C.D. (2010). Genetic patterns of paternity and testes size in mammals. *PLoS One*, 5: e9581.

Soundarapandian, P., Varadharajan, D., Ilavarasan, N., Kumar, J., Kumar, A. (2013). Mating Behaviour of Flower Crab, *Charybdis feriata* (Linnaeus). *Journal of Marine Science Research & Development,* 3: 3.

Spalding, J.F. (1942). The nature and formation of the spermatophores and sperm plug *Carcinus maenas*. *Quarterly Journal of Microscopical Science*, 83: 399-422.

Stewart, M.J., Steward, P., Soonklang, N., Linthong, V., Hanna, J.P., Duan, W., Prasert, S. (2010). Spermatogenesis in the blue swimming crab, *Portunus pelagicus*, and evidence for histones in mature sperm nuclei. *Tissue Cell*, 42: 137-150.

Tavares, M., Amouroux, J.M. (2003). First record of the non-indigenous crab, *Charybdis hellerii* (A. Milne-Edwards, 1867) from French Guyana (Decapoda, Brachyura, Portunidae). *Crustaceana,* 76: 625-630.

Tavares, M., Mendonça Jr., J.B. (1996). *Charybdis hellerii* (A. Milne Edwards, 1867) (Brachyura: Portunidae), eighth nonindigenous marine decapod recorded from Brazil. *Crustacean Research,* 25: 151-157.

Thresher, R., Proctor, C., Ruiz, G., Gurney, R., MacKinnon, C., Walton, W., Rodriguez, L., Bax, N. (2003). Invasion dynamics of the European shore crab, *Carcinus maenas*, in Australia. *Marine Biology*, 142: 867-876.

Vitousek, P.M., D'Antonio, C.M., Loop, L.L., Rejmánek, M., Westbrooks, R. (1997) Introduced species: a significant component of human-caused global change. *New Zealand Journal of Ecology,* 21: 1-16.

Wan, Q.H., Wu, H., Fujihara, T., Fang, S.G. (2004). Which genetic marker for which conservation genetics issue? *Electrophoresis*, 25: 2165-2176.

Waser, P.M., DeWoody, J.A. (2006). Multiple paternity in a philopatric rodent: The interaction of competition and choice. *Behavioral Ecology,* 17: 971-978

Watanabe, T.T., Zara, F.J., Hattori, G.Y., Turra, A., Sant'Anna, B.S. (2015). Biological associations of color variation in the Indo-Pacific swimming crab *Charybdis hellerii*. *Anais da Academia Brasileira de Ciências,* 87: 219-232.

Wolcott, D.L., Hopkins, C.W.B., Wolcott, T.G. (2005). Early events in seminal fluid and sperm storage in the female blue crab *Callinectes sapidus* Rathbun: effects of male mating history, male size and season. *Journal of Experimental Marine Biology and Ecology*, 319: 43-55.

Yeh, F.Y., Boyle, R., Ye, T., Mao, Z. (1997). POPGENE, the user-friendly shareware for population genetic analysis, version 1.31. Alberta, Canada: Molecular Biology and Biotechnology Centre, University of Alberta.

Yue, G.H., Chang, A. (2010). Molecular evidence for high frequency of multiple paternity in a freshwater shrimp species *Caridina ensifera*. *PLoS One*, 9: e12721.

Zara, F.J., Toyama, M.H., Caetano, F.H., López-Greco, L.S. (2012). Spermatogenesis, spermatophore and seminal fluid production in the adult blue crab *Callinectes danae* (Portunidae). *Journal of Crustacean Biology*, 32: 249-262.

Zara, F.J., Gaeta, H.H., Costa, T.M., Toyama, M.H., Caetano, F.H. (2013). The ovarian cycle histochemistry and its relationship with hepatopancreas weight in the blue crab *Callinectes danae* (Crustacea: Portunidae). *Acta Zoologica*, 94: 134-146.

Zara, F.J., Pereira, G.R.R., Sant'Anna, B.S. (2014). Morphological Changes in the Seminal Receptacle During Ovarian Development in the Speckled Swimming Crab *Arenaeus cribrarius. The Biological Bulletin*, 227: 19-32.

Zeh, J.A., Zeh, D.W. (2001). Reproductive mode and the genetic benefits of polyandry. *Animal Behaviour*, 61: 1051-1063.

Capítulo 1

Ultrastructure of the male reproductive system of *Callinectes* (Portunidae): filling the gaps in the histology and histochemistry

Prólogo – Capítulo 1

Este capítulo originou-se de dados do projeto Jovem Pesquisador da FAPESP do meu orientador Prof. Dr. Fernando José Zara (Proc. #2005/04707-5), no qual ele obteve dados ecológicos e morfológicos de diversas espécies do gênero *Callinectes.* No decorrer dos estudos, o laboratório observou que as modificações que o sistema reprodutor possuem conferem às espécies de *Callinectes* a produção de "plug" espermático. Ao analisar os dados, apesar de terem sido encontrados diferenças ecológicas entre as espécies deste gênero, os dados morfológicos do sistema reprodutor seguiram um padrão comum entre as espécies.

Complementado de dados coletados mais recentemente, foi realizado este estudo mais completo a respeito da morfologia, histoquímica e ultraestrutura do sistema reprodutor masculino de *Callinectes* spp, no qual segue o padrão mais comum em Portunidae das espécies formadoras de "plug" espermático.

Capítulo 1

Ultrastructure of the male reproductive system of *Callinectes* Stimpson, 1860 (Brachyura: Portunidae): filling the gaps in the histology and histochemistry

ABSTRACT

Males of the commercially important *Callinectes* produce sperm plugs. Currently, information on the ultrastructure of the male reproductive system of these crabs is lacking. Here we described the ultrastructure and histochemistry of the reproductive system to five *Callinectes* species. We also evaluated the seasonal variation in weight of the reproductive system and hepatopancreas through somatic indices. The somatic indices changed little throughout the year. In *Callinectes*, the spermatogenesis occurs inside the lobular testes and, within each lobule, the cells are at the same development stage. The spermatogenesis and spermiogenesis follows the same development pattern in all *Callinectes* studied. Mature spermatozoa are released into the seminiferous ducts through the collecting ducts. The vas deferens cells are secretory showing rough endoplasmic reticulum, Golgi complex, and secretory vesicles that produce the seminal fluid. The anterior vas deferens shows two portions: proximal and distal. In the proximal (AVDp), the spermatozoa are embedded and clustered by an electron-dense, basophilic glycoproteinaceous secretion type I. In the distal portion (AVDd), the spermatophore wall is formed by the incorporation of a less electron-dense glycoproteinaceous type II secretion. The secretion type I change to an acid polysaccharide-rich matrix that separates the spermatophores from each other. The median vas deferens (MVD) stores the spermatophores and produces the granular glycoproteinaceous seminal fluid. The posterior vas deferens (PVD) has nearly no spermatophores. Its epithelium has many mitochondria and the PVD seminal fluid changes into a liquid and homogeneous glycoprotein. Many outpocketings in the PVD and MVD help to increase the fluid production. Overall, the reproductive pattern of *Callinectes* is similar to that of other species that produce sperm plugs. The secretions of AVD, MVD and PVD are responsible for the polymerization that forms the solid, waxy plug in the seminal receptacle. The traits identified here are common to all Portunidae species studied so far.

Keywords: sperm plug, Portunoidea, blue crab, electron microscopy.

INTRODUCTION

The morphology of the Brachyura male reproductive system is highly diverse, but members of different groups have specific features. In *Callinectes* Stimpson, 1860 (Eubrachyura: Heterotremata: Portunoidea), the bilateral, H-shaped male reproductive system is composed of a pair of testes and a pair of vasa deferentia (Cronin, 1947; Johnson, 1980; Kennedy and Cronin, 2007). The testes lay on the antero-dorsal region of the cephalothoracic carapace, above the hepatopancreas, and are connected by a commissure at the posterior region of the stomach, below the heart (Cronin, 1947; Johnson, 1980; Simeó et al., 2009, 2010; Stewart et al., 2010).

From a histological point of view, there are two types of testes: tubular, found in Majoidea, Xanthoidea, and Grapsoidea, and lobular, commonly observed in most groups of Brachyura (Simeó et al., 2010). The testes of portunid crabs have been extensively studied through light microscopy, and much focus was given to the maturation of germ cells, i. e., the spermatogenesis (Ryan, 1967; Johnson, 1980; Stewart et al., 2010; Zara et al., 2012: Nascimento and Zara, 2013). However, given the high diversity of brachyurans, with ~7,000 valid species (Ng et al., 2008), there is insufficient information to allow the formulation of a robust hypothesis about the evolution of their reproductive system. In addition, studies on the ultrastructure of spermatogenesis in crabs are scarce, and portunids are not an exception. The most comprehensive studies on spermatogenesis and/or spermiogenesis focused on *Cancer borealis* Stimpson, 1859 and *Cancer irroratus* Say, 1817 in Langreth (1969), *Afruca tangeri* (Eydoux, 1835) as *Uca tangeri* in Medina and Rodriguez (1992), *Portunus pelagicus* (Linnaeus, 1758) in Stewart et al. (2010), and *Maja brachydactyla* Balss, 1922 in Simeó et al. (2010). In crabs, the sperm production begins with the proliferation of spermatogonia in the peripheral germinal center of the lobules, or near the seminiferous tubules (Johnson, 1980; Stewart et al., 2010; Zara

et al., 2012; Nascimento and Zara, 2013). In *M. brachydactyla*, however, it starts in the tubular testis (Simeó et al., 2010). These regions are delimited by accessory cells whose morphology changes during the spermatozoa production (Johnson, 1980). During the spermatogenesis and spermiogenesis, there are cells at different stages, e.g., primary and secondary spermatocytes, spermatids in different maturation stages per lobule, and mature spermatozoa. The classification of the spermatogenesis according to the number of cell stages varies among species. Initially, the classification is based on the chromosome organization, and later, on with the formation of the acrosomal vesicle and distribution of the mitochondria and lamellar complex (Langreth, 1969; Simeó et al., 2010; Stewart et al., 2010).

The vas deferens of a few groups has been studied under light microscopy, including the Majoidea (Beninger et al., 1988; Diesel, 1989; Sainte-Marie and Sainte-Marie, 1999; Sal Moyano et al., 2010), Grapsoidea (Garcia and Silva, 2006; Tiseo et al., 2014, 2017), Ocypodoidea (Castilho et al., 2008), and Portunoidea (Ryan, 1967; Johnson, 1980; Stewart et al., 2010, Zara et al., 2012 Nascimento and Zara, 2013). In brachyurans, the vas deferens consists of a series of convoluted tubules divided into three regions: anterior (AVD), median (MVD), and posterior (PVD) (Krol et al., 1992). In general, the spermatophores are produced in the AVD and stored in the MVD and PVD. The seminal fluid is produced and stored in the PVD. The sites of spermatophore and seminal fluid production and storage differ depending on the species (Adiyodi and Anilkumar, 1988; Benhalima and Moriyasu, 2000; Jivoff et al., 2007; Klaus et al 2013).

In contrast with light microscopy, a detailed ultrastructural description of the vas deferens is lacking. There is some information for the Majoidea *Libinia emarginata* Leach, 1815, *Libinia dubia* H. Milne Edwards, 1834 in Hinsch and Walker (1974), and *M. brachydactyla* (Simeó et

al., 2009), and for the Oregoniidae *Chionoecetes opilio* (Fabricius, 1788) in Benhalima and Moriyasu (2000). In these species, the vas deferens exhibits cell features related to protein synthesis, e. g., a well-developed rough endoplasmic reticulum (RER) and secretion vesicles.

Somatic indices are widely used to determine reproductive periods and/or seasonal reproductive peaks (Chu, 1999; Kyomo, 1988; López-Greco and Rodriguez, 1999). Several studies used the gonadosomatic and hepatosomatic indices to estimate the mobilization of resources from the hepatopancreas to the maturation of germ cells, but they targeted mainly the female reproductive system (López-Greco and Rodriguez, 1999; Zara et al., 2013). Recently, these indices were used to estimate the energetic investment of males of *Callinectes danae* Smith, 1869 and *Callinectes ornatus* Ordway, 1863 during the development of the reproductive system (Zara et al., 2012; Nascimento and Zara, 2013). However, there are no studies in the seasonal variation of these indices in tropical blue crabs.

The Portunidae family has many species of high economic and ecological importance, including the blue crabs *Callinectes sapidus* Rathbun, 1896, *C. ornatus*, and *C. danae*, which occur from the southern United States to southern Brazil (Melo, 1996). The genus *Callinectes* is especially important due to its ecological role (Mantelatto and Christofoletti, 2001) and economic potential, which has been relatively underexploited in the southeast of Brazil (Mantelatto and Fransozo, 1999). Species of *Callinectes* are also a bycatch of small-scale fisheries (Severino-Rodrigues et al., 2001). The reproductive biology, population structure, and sexual maturity of these species has been studied often (Mantelatto and Fransozo, 1996, 1997, 1999; Costa and Negreiros-Fransozo, 1998; Negreiros-Fransozo et al., 1999; Sforza et al., 2010; Sant'Anna et al., 2012; Watanabe et al. 2014). Despite the reasonable extant knowledge about the reproduction of *Callinectes*, there is no ultrastructural description of the male reproductive

system of blue crabs. Aiming to fill this gap, this study describes in detail the complete ultrastructure of the spermatogenesis and vas deferens of *Callinectes sapidus*, *C. bocourti* A. Milne-Edwards 1879, *C. exasperatus* (Gerstaecker, 1856), *C. ornatus*, and *C. danae*. In addition, the seasonal weight variation in the hepatopancreas and male reproductive system of *C. danae*, *C. ornatus*, and *C. bocourti* were evaluated.

MATERIAL AND METHODS

Sampling and handling of crabs

The samplings took place in the Bay/Estuary Complex of Bertioga, Santos-São Vicente, state of São Paulo, Southeast Brazil, from 2007 to 2009 and some additional samples were collected in 2017. Crabs were collected by trawling for approximately 20 minutes or through baited ring net traps in four sites of 6–15 m of depth. They were kept alive in labeled plastic boxes with water from the collection site. *Callinectes danae, C. sapidus* and *C. ornatus* were collected by trawling in the São Vicente Bay/Estuary Complex from March 2007 to February 2008, following the method described by Sant'Anna et al. (2012) and Watanabe et al. (2014). *Callinectes bocourti* was collected in the São Vicente estuary in two sites along the Branco river (23°57'45.62''S, 46°28'46.62''W and 23°56'00.62''S, 46°27'11.34'W), and in one site of the Mariana river (23°56'56.97''S, 46°25'57.76''W) from January to December 2009. The sampling was conducted with 20 baited ring net traps placed 4–5 m apart. They were checked every 15 min for 2 hours (Zara et al., 2009). *Callinectes exasperatus* were collected in the Bertioga Estuary (using the same 20 traps), in October 2009 and December 2018 in one site of the Itapanhaú river (23°50'09.8''S, 46°09'08.1''W).

In the laboratory, the crabs were identified according to Melo (1996), sexed, and classified as juveniles or adults based on the shape and adherence of the abdomen to the *cephalothoracic sternum*. They were anesthetized by chilling (-20ºC for 5 min) prior to the dissections. The dissected reproductive system was macroscopically examined to estimate the size, in comparison with the hepatopancreas, and color. Males with completely developed testes and a reproductive system/hepatopancreas weight ratio of 1:2 were considered mature (Costa and Negreiros-Fransozo, 1998; Zara et al., 2012; Nascimento and Zara, 2013). Three blue crab species with different habitat preferences were used to evaluate the weight variation of the hepatopancreas and reproductive system: *C. bocourti*, which inhabits low-salinity areas or freshwaters; *C. danae*, a migratory species that can tolerate from brackish to marine waters; and *C. ornatus*, which has a stronger preference for marine waters than the other species (Willians, 1974; Melo 1996; Sant' Anna et al., 2012; Watanabe et al, 2014). To evaluate the weight variation in the hepatopancreas and reproductive system of *C. danae* and *C. ornatus*, we used the data from our samplings in addition to those collected by Sant'Anna et al. (2012) and Watanabe et al. (2014).

Somatic indices

Due to the great volume of the vasa deferentia of *Callinectes,* it was used only fresh animals to have a more reliable representation of the actual condition of the animals. The reproductive system and hepatopancreas of mature males were weighed separately using an analytical scale (0.001 g). The gonadosomatic index (GSI) was estimated based on the weight of both testes plus the vas deferens. The GSI was calculated by the equation GSI = GW/AW x 100, where GW is the wet gonad weight and AW is the total wet body weight (Kyomo, 1988). The hepatosomatic index (HSI) was calculated by the formula HSI = HW/AW x 100, where HW is

the wet hepatopancreas weight and AW is the total body weight (Kyomo, 1988). The monthly averages and standard deviations were grouped by seasons. Differences between the indices were tested with a one-way ANOVA and a post-hoc Tukey test to detect possible significant differences between seasons. Prior to each ANOVA, the data were tested for normality (Shapiro–Wilks test) and homoscedasticity (Cochran's test) and, if necessary, were transformed to meet the ANOVA assumptions (Sokal and Rohlf, 1995).

3D modelling

Based on the macroscopic and microscopic observations of the testes and vas deferens, with also the observations of the morphology of the male reproductive system of *C. danae* (Zara et al. 2012) a 3D model of the general disposion of the male reproductive whitin the carapace and the morphology of the testes of *Callinectes* was constructed and rendered with the open source 3D creator suite Blender®.

Light microscopy

Fragments of the testes and vas deferens of five mature adult individuals of *C. exasperatus* and *C. bocourti* were fixed for 24 h in a 4% paraformaldehyde solution prepared with seawater from the sampling site. After fixation, the samples were transferred to sodium phosphate buffer 0.2 M (pH 7.2), dehydrated in an ascending ethanol series (70–95%), and embedded for 72 h at 4ºC in a glycol methacrylate resin (Leica® historesin kit). Seriated sections of 4 and 7 µm were obtained using a rotating microtome Leica RM2245. The samples were stained with the hematoxylin and eosin technique (HE) used in traditional histological descriptions (Junqueira and Junqueira, 1983). The spermatogenesis was observed in sections stained with the periodic

acid of Schiff (PAS) and hematoxylin. Xylidine ponceau (Mello and Vidal 1980) was used to observe proteins. Alcian blue (pH 2.5) and PAS were used to detected acid and neutral polysaccharides, respectively, and were applied simultaneously (Junqueira and Junqueira 1983).

Transmission electron microscopy

Tissues of mature adult individuals of *C. sapidus, C. danae, C. ornatus, C. exasperatus* and *C. bocourti* (n=5 of each species) were used in the transmission electron microscopy (TEM). The samples were fixed in 2.5% glutaraldehyde with 0.2% picric acid (Mancini, and Dolder, 2001; Zana et al., 2001) in 0.1 M seawater buffered sodium cacodylate (pH 7.6) for 24 h. The samples were rinsed twice with the same buffer, post-fixed with 1% seawater buffered osmium tetroxide for 2 h, and en bloc stained with 1% aqueous uranyl acetate. Subsequently, the samples were dehydrated in an ascending ethanol series (70–100%) and embedded in Epon-Araldite resin. Ultrathin sections were contrasted with 2% aqueous uranyl acetate (20 min) and 2% lead citrate in NaOH 0.1N (7 min). The material was photographed under a Philips CM100 operating at 80Kv transmission electron microscope.

RESULTS

Hepatosomatic and gonadosomatic indices

The mature individuals of *C. ornatus*, *C. danae*, and *C. bocourti* had a mean GSI of 4.57±1.53 (N = 80), 4.73±2.51 (N = 106), and 3.98±0.49 (N = 45), and a mean HSI of 3.99±1.28 (N = 80), 3.61±0.76 (N = 106), and 3.51±0.85 (N = 45), respectively. There was a slight seasonal variation in the somatic indices (Fig. 1). The HSI of *C. ornatus* differed between summer

(3.61±0.62) and spring (5.16±2.03), and between winter (3.65±0.54) and spring (F = 3.59, p = 0.023). The GSI of *C. danae* differed between summer (3.72±0.97) and winter (3.45±0.84) (F = 3.23, p = 0.025), and its HIS differed between summer (3.70±0.60) and autumn (3.19±0.80) (F = 3.51, p = 0.017). The GSI of *C. bocourti* differed between summer (2.19±0.55) and spring (2.64±0.79) and between autumn (3.62±0.84) and spring (F = 6.59, p = 0.001).

Testes

Spermatogenesis

Based on the gross morphology and histological observations, a 3D model was built to facilitate the morphological description of the reproductive system (Fig. 2) and the testes of *Callinectes* (Fig. 3A–C). The lanky and convoluted testes have several semiferous lobules attached to the seminiferous duct, which is an anastomosed duct (Fig. 3A). A short collector duct connects the seminiferous lobules to the seminiferous duct (Fig 3B) that has several ramifications and is very convoluted (Fig. 3C). The testes of all species of *Callinectes* have a lobular histology and each lobule is filled with germ cells undergoing spermatogenesis (Fig. 3D and E). Each lobule, or acini, is connected to the seminal duct by a very short accessory or collecting duct. This duct opens when it receives the mature spermatozoa immersed in a secretion (Fig 3B and F). The seminal ducts lead the immersed spermatozoa to the vas deferens (Fig 3F). Under TEM, each lobule is surrounded by accessory (Sertoli) cells that spread their cytoplasm like tree branches between the germ cells (Fig. 4A). The branches are easier to detect in the lobules filled with spermiogenic cells, and, at this stage, they have numerous large vesicles that form the residual body (Fig. 4B). The spermatogonia are cells with a large round nucleus with many heterochromatin blocks and one nucleolus (Fig. 4C). The cytoplasm has a poorly

developed RER and many polyribosomes (Fig. 4D). The primary spermatocytes are small, with a central nucleus occupying most of the cell (Fig. 4E). In these cells, different phases of the meiotic prophase are observed. The cytoplasm is highly homogeneous, and just a few RER and mitochondria are seen throughout the spermatogenesis (Fig. 4F). In the pachytene phase, chromosomes are extremely thick and the synaptonemal complexes are more evident (Fig. 4G). After the prophase, the chromosomes of the primary spermatocytes are aligned on the metaphase plate, and the cytonucleoplasm is homogeneously electron-dense (Fig. 4H). In the lobules with cells in prophase II, the round nucleus of the secondary spermatocytes has a condensed chromatin and granular nucleoplasm, and a small nucleus (Figs. 4I and J). The cytoplasm of the secondary spermatocytes has RER and many mitochondria (Fig. 4J).

Spermiogenesis

The spermiogenesis begins with the early spermatids, which are characterized by the formation of many proacrosomal vesicles that merge into a unique proacrosomal vesicle. In this stage, the cytoplasm is more concentrated in one of the cell poles and is narrower around the nucleus. The nucleus, in turn, occupies almost the entire cell, has a homogeneous chromatin and is enclosed by the nuclear envelope (Fig. 5A–C). As the spermiogenesis proceeds, the spermatids become surrounded by the branching accessory cells that have many residual electron-lucent vesicles and other residual electron-dense materials (Fig. 5B).

The spermatids were divided into three stages: early, intermediary, and late (or final). Early spermatids have a small proacrosomal vesicle in one of the cell poles (Fig. 5B and C) that is filled with a granular electron-dense material (Fig. 5C and D). The formation and growth of the proacrosomal vesicle begin to modify the round nucleus with granular and homogeneous

chromatin (Fig. 5C and D). The nuclear envelope begins to fragment and remains unchanged until the end of cell maturation. The cytoplasm is reduced to a thin layer, and there are a series of interconnected membranes and vesicles between the nucleus and the proacrosomal vesicle forming the lamellar complex (Fig. 5D). At higher magnifications it can be seen that some vesicles of the lamellar complex are merged with the proacrosome membrane. Near the lamellar complex, the shape of the mitochondria and their cristae is irregular, indicating the onset of the degeneration process (Fig. 5E).

The main feature of the intermediary spermatids is the formation of the perforatorial chamber in the median basal region of the acrosomal vesicle. Figures 5F and G show the formation of the perforatorial chamber in *C. danae* and *C sapidus*. Since the perforatorial chamber is the main feature of the acrosome, during spermiogenesis the proacrosomal vesicle will be henceforth referred to as acrosomal vesicle. The centriole is located at the base of the perforatorial chamber and this portion of the cytoplasm separates the lamellar complex, which is more often located laterally to the base of the acrosomal vesicle (Fig. 5F). At this stage, the formation of the electron-dense operculum in the acrosomal vesicle begins at the pole opposite to the nucleus. Next to it, a very electron-dense material is visible beneath the operculum area, which will form the acrosome ray zone (ARY) in the mature spermatozoa (Fig. 5F and G).

In late spermatids, the perforatorial chamber reaches the operculum, and its apical portion is immersed in electron-dense material that comprises the subopercula. The ARY is large and distributed from the subopercular layer to the vicinity of the perforatorial chamber base, forming two concentric layers of different electron densities in the acrosomal vesicle. The lamellar complex is small and visible in only one side of the spermatozoa. The cup-shaped nucleus has a granular chromatin and is extremely irregular (Fig. 5H and I). In the later stages, the perforatorial

chamber is narrower, with perforatorial tubules, and its apical end reaches the operculum (Fig. 5I). The ARY becomes narrow and elongated, extending from the subopercular layer to two thirds of the perforatorial chamber. The perforatorial chamber has already the morphology of the mature spermatozoa and the apical end interacts with the operculum, which becomes salient and triangular in *C. danae, C. ornatus* and *C. bocourti*. At this stage, the outermost acrosome layer is completed and is less electron-dense than the innermost layers. A thickened ring inside the acrosome vesicle lies at the base of perforatorial chamber. The cytoplasm is extremely thin and the lamellar complex is mostly concentrated in only one side of the late spermatid. A few mitochondria with few cristae are observed. The nucleus becomes smaller and nearly surrounds the acrosomal vesicle, and the chromatin varies from loose to fibrous. Nearly mature spermatozoa were found in one lobule filled with late spermatids. Compared to them, late spermatids have a less condensed chromatin and voluminous nucleus (Fig. 5J).

Mature spermatozoa are only found in the collecting and seminiferous ducts, which are delimited by a simple epithelium comprised by squamous to cubic cells (Fig. 5K). The mature spermatozoon of the species of *Callinectes* is characterized by the very thin nucleus with homogeneous and granular chromatin. The perforatorial chamber is located within the subopercular region in contact with the operculum. The acrosomal vesicle is formed by three concentric layers of different electron densities. The ARY is always the inner acrosomal layer that surrounds the perforatorial chamber. The cup- or C-shaped nucleus is small and has projections or radial arms surrounding the acrosome until the base of the operculum. The chromatin varies from granular to fibrous, but it is never as loose as in the late spermatids (Fig. 5L). The cytoplasm occupies a thin band between the nucleus and the acrosome. The lamellar

complex is almost completely absent and there are only a few membranes remaining at the base of the acrosome (Fig. 5L).

Vas deferens and spermatophore formation

The vas deferens of all *Callinectes* species is divided into three regions that differ in function and morphology: anterior (AVD), median (MVD), and posterior (PVD). Based on the density of free spermatozoa and presence of luminal secretion, the AVD is divided into two portions, proximal (AVDp) and distal (AVDd) (Fig. 6A). Taking *C. exasperatus* and *C. bocourti* as models, two types of luminal secretions are found in the AVD. In the AVDp the type I secretion is basophilic (Fig 6B), positive for neutral polysaccharides, weakly reactive to acid polysaccharides (6B and C) and intensely stained for proteins (Fig. 6D). In the AVDd, the secretion is still basophilic and glycoproteinaceous but, unlike in the AVDp, it is strongly reactive to acid polysaccharides (Fig 6E-G). The type I secretion increases the sperm density and forms small ridges between the spermatozoa mass (Fig 6A, B and D). The type II secretion is acidophilic and more abundant in the AVDd than in the AVDp (Fig 6A). This secretion is strongly reactive to neutral polysaccharides and positive for proteins (Fig. 6B and D). The type II secretion has the same histochemical features of an acidophilic glycoprotein without acid polysaccharides (Fig 6A, E–G). The spermatophore wall has chemical compounds that confirm their secretion type II origin (Fig 6E–G). In the AVDd, the inner spermatophore secretion is also positive for acid polysaccharides (Fig. 6F).

The epithelium of the AVDd and AVDp is simple and both have a similar ultrastructure. The nucleus has an irregular morphology with several nucleoli and heterochromatin (Fig. 6H).

The basal lamina is homogeneous and the AVD lies on a connective tissue with fibroblasts, collagen, and muscular fibers (Fig. 6H and I). The basal region has a short folded basal plasma membrane and several mitochondria (Fig. 6J). The cytoplasm is filled with RER and small regular Golgi complexes (Fig. 6K–M). In the trans face of the Golgi complex there are different electron-dense vesicles (Fig. 6L). In the cell apex, vesicles with a higher electron-density than trans Golgi vesicles were undergoing exocytosis. The microvilli are irregular due to the large amount of luminal secretion (Fig. 6G and L). In the lumen, in anteriormost portions of the AVDp, the secretion type I shows different electron-densities and forms large clusters that apparently break up in the lumen (Fig. 6M). In the AVDp, the spermatozoa are compacted by a less electron-dense secretion without a wall (Fig. 6N). Two secretion types are frequently observed as round masses in the AVDd lumen: an electron-dense and homogeneous type I secretion, and a less electron-dense type II secretion. The type II secretion has the same electron density of the spermatophore wall and is apparently condensed in the spermatozoa mass (Fig. 6O). Finally, the spermatophore formation is completed in the AVDd: the spermatophore wall is thicker, and the type I secretion is less electron-dense and homogeneous and distributed among the spermatophores (Fig. 6P).

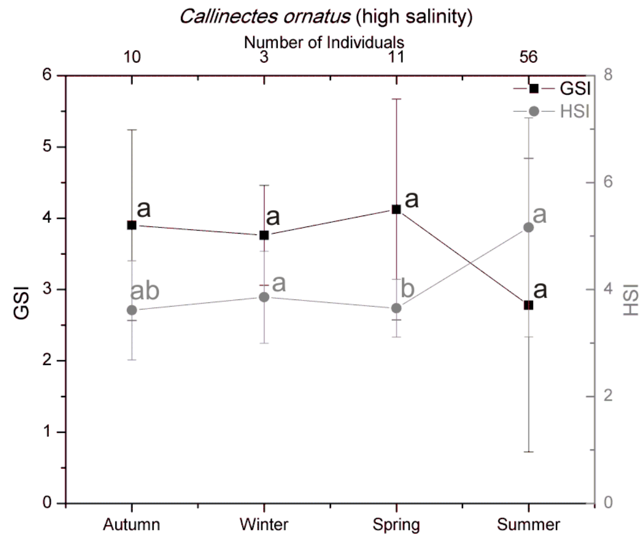
Under light microscopy, the MVD has a simple and squamous epithelium with anatomical folds called outpockets *sensu* Johnson (1980). These outpockets are comprised by cells flattened by the intense secretion in the lumen. The lumen is filled with mature spermatophores and granular secretions immersed in a homogeneous matrix — the main MVD features (Fig. 7A). The MVD secretion is positive for neutral polysaccharides and proteins (Fig. 7A and B). However, the lumen is not reactive to acid polysaccharides (Fig. 7A). The PVD also has several lateral outpockets (Fig. 7C) and the secretion in the lumen is homogeneous and liquid-gelatinous,

but does not have the granules observed in the MVD secretion. No spermatophores are found immersed in the PVD secretion (Fig. 7D and E). The PVD secretion is strongly positive for proteins (Fig. 7D) and neutral polysaccharides (Fig. 7E). The acid polysaccharides are absent in the PVD secretion (Fig. 7F). Under TEM, the MVD cells have a large and lobular nucleus with heterochromatin blocks and a single nucleolus (Fig. 7G). The cytoplasm has large quantities of RER and many mitochondria in the basal region. The aspect of the connective tissue is similar to that of the AVD, with fibroblasts subjacent to the basal lamina, and an underlying thin musculature (Fig. 7G). The apical region of the MVD cells contains large secretory vesicles that are released into the lumen by exocytosis (Fig. 7H). The MVD secretion is a matrix of electron-dense, slightly heterogeneous, and electron-lucent materials that merge into the large MVD granules that look like secretory vesicles. The spermatophores laying in the MVD are completely formed and no additional material seems to be added to the wall (Fig. 7I).

The PVD is a simple cubic-squamous epithelium whose cells nuclei have the same features as in the AVD and MVD (Fig. 7J). The main trait of the PVD cells is the enormous quantities of mitochondria (Fig, 7K and L). The basal region of the epithelium has many large electron-lucent vesicles that apparently were poorly fixed by our fixation technique. However, the same electron-lucent vesicles were observed in all studied species (Fig. 7J). The cells are very sinuous and lay on the connective and muscle tissues (Fig 7K). The cells of the PVD apical region have many mitochondria and a few small electron-dense secretion vesicles, which are released by exocytosis (Fig. 7 L-N). The secretion in the lumen is homogeneous, electron-dense and fluid during the dissections, and shows small and sparse less electron-dense bubbles (Fig 7J).

FIGURES

Figure 1. Seasonal variation of the gonadosomatic (GSI) and hepatosomatic (HSI) indices of mature males of *Callinectes.* Hypersaline, migratory, and low or even freshwater species. Values are means ± standard deviations. Different letters indicate a statistically significant difference ($p < 0.05$).


Callinectes ornatus (high salinity)
Number of Individuals
10
3
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56
GSI
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Autumn
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ab
a
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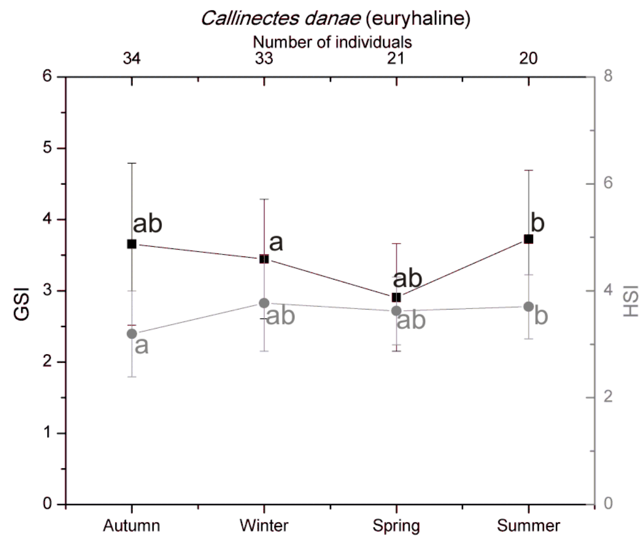
Callinectes danae (euryhaline)
Number of individuals
34
33
21
20
GSI
HSI
Autumn
Winter
Spring
Summer
ab
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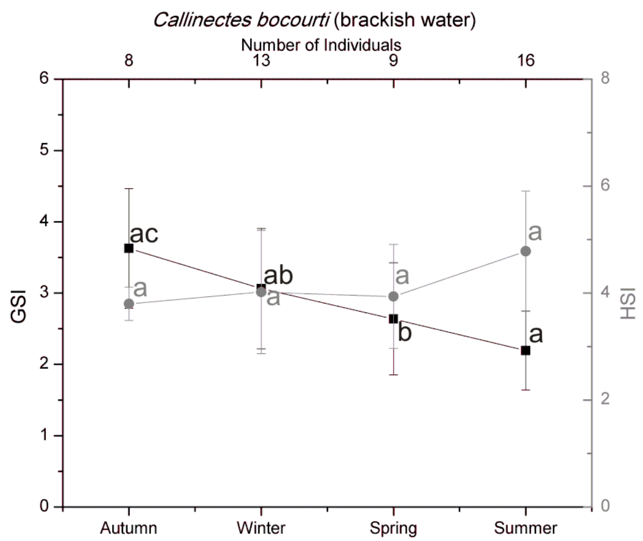
Callinectes bocourti (brackish water)
Number of Individuals
8
13
9
16
GSI
HSI
Autumn
Winter
Spring
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a
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Figure 2. 3D model of the male reproductive system of *Callinectes.* The pair of testicles are joined to each other by a commissure (arrow), near the anterior vas deferens. The vas deferens is a very convoluted structure which increases the caliber along the duct. avd, anterior vas deferens; mvd, median vas deferens; pvd, posterior vas deferens; t, testicle.

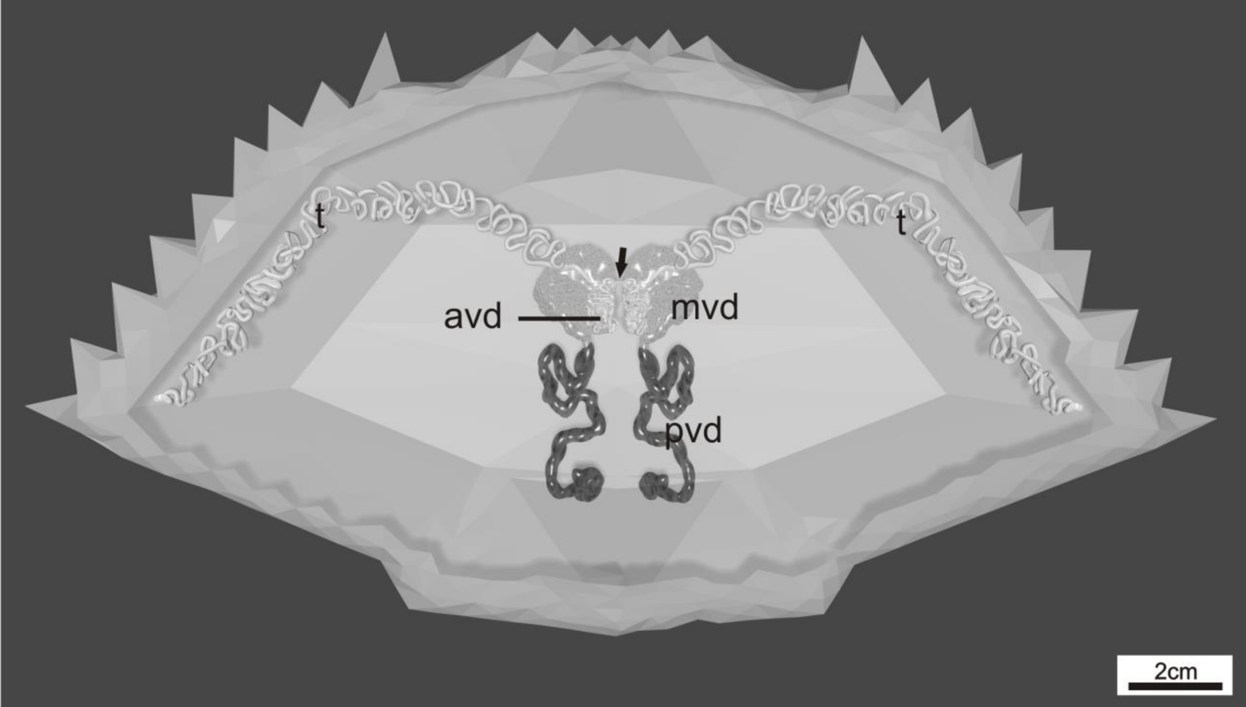
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Figure 3. Testes: (A) 3D model of the testes of *Callinectes* Stimpson, 1860 based on gross morphology and histology. Several testis lobules (arrow) are seen around the seminiferous duct; **(B)** Detail of the short collecting duct (arrow) that connects the lobule to the seminiferous duct; **(C)** The seminiferous duct, without the convoluted and anastomosed lobes; **(D)** Testis of *Callinectes exasperatus* (Gerstaecker, 1856) showing several lobules undergoing spermatogenesis and the seminiferous duct with spermatozoa, stained by PAS/hematoxilin; **(E)** Testis of *C. exasperatus* showing each lobule with a different maturation stage, stained by PAS/hematoxilin; **(F)** In detail, the collecting duct releasing the spermatozoa immersed in secretion to the seminiferous duct. The spermatogonia are filling the lobule (Hematoxilin and eosin stain). cd, collecting duct; est, early spermatid; ist, intermediate spermatid; lst, late spermatid; sd, seminiferous duct; spg, spermatogonia; spc1, primary spermatocyte; sz, spermatozoa; tl, testicular lobule.

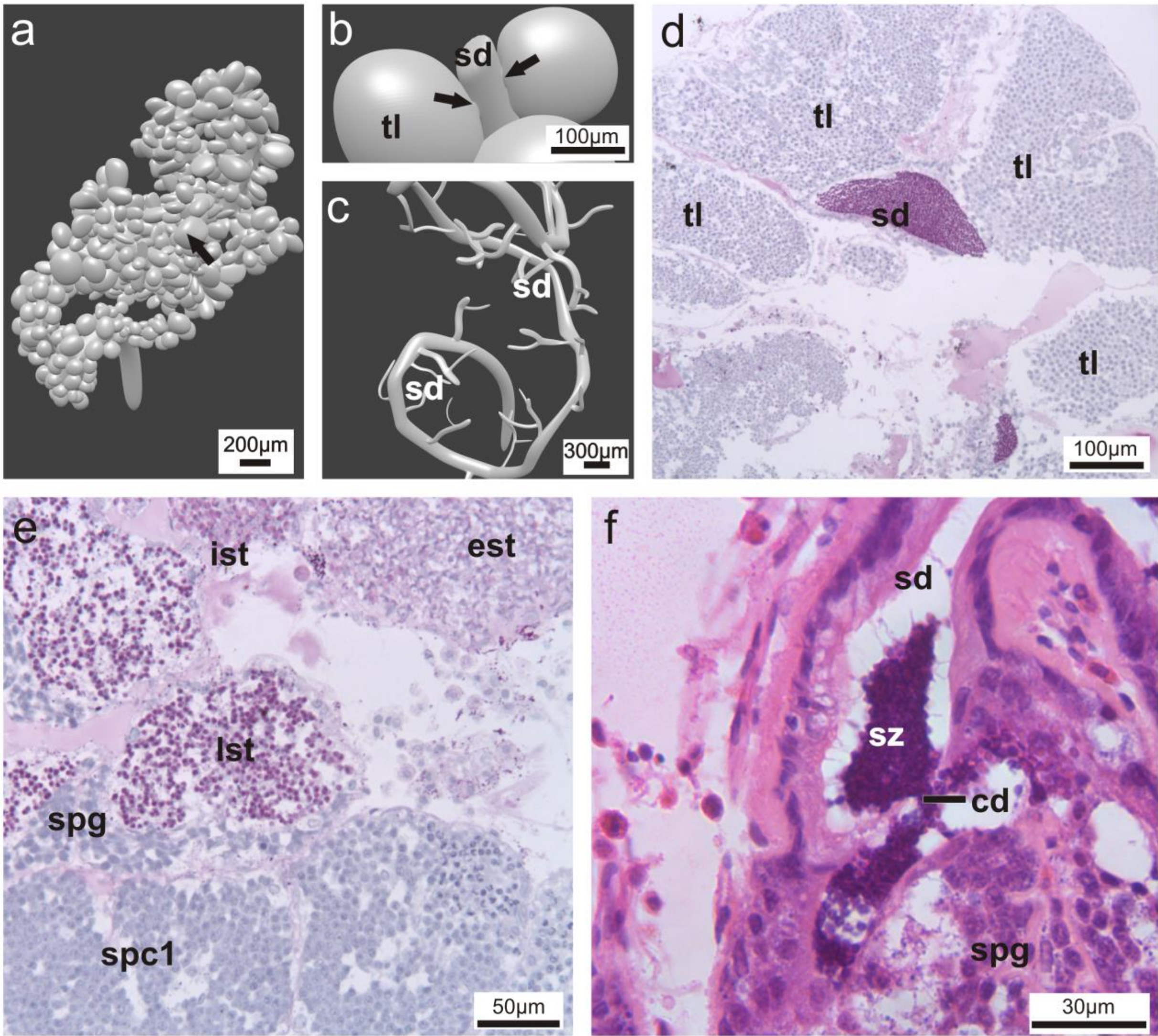
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Figure 4. Spermatogenesis: (A) Testis lobule of *Callinectes danae* Smith, 1869 showing accessory cells with nucleus and residual bodies within the branched cytoplasm (white arrow); **(B)** Detail of the accessory cells of *C. danae* showing the residual body, Golgi complex (white arrow), and rough endoplasmic reticulum (black arrow); **(C)** Testis lobule of *Callinectes sapidus* showing accessory cells lacking evident cytoplasmic branches surrounding the spermatogonia; **(D)** Spermatogonium cytoplasm of *Callinectes sapidus* Rathbun, 1896 with polysomes (white arrow); **(E)** Germinal center of *Callinectes ornatus* Ordway, 1863 with primary spermatocytes showing a central, round nucleus; **(F)** Detail of the spermatocyte of *C. ornatus* with normal mitochondria among the RER (black arrow); **(G)** Primary spermatocyte of *C. danae* in pachytene with evident synaptonemal complex (white arrow); **(H)** Metaphase plate in *C. sapidus* during the prophase I (white arrow); **(I)** Secondary spermatocyte of *C. ornatus* with round nucleus filled with heterocromatin; **(J)** Detail of the secondary spermatocyte of *Callinectes exasperatus* (Gerstaecker, 1856) showing the condensed chromatin in the nucleus and the cytoplasm with a few RER lamellae and mitochondria (white arrow). ac, accessory cell; m, mitochondria; n, nucleus; rb, residual body; rer, rough endoplasmic reticulum; spc1, primary spermatocyte; spc2, secondary spermatocyte; spg, spermatogonia.

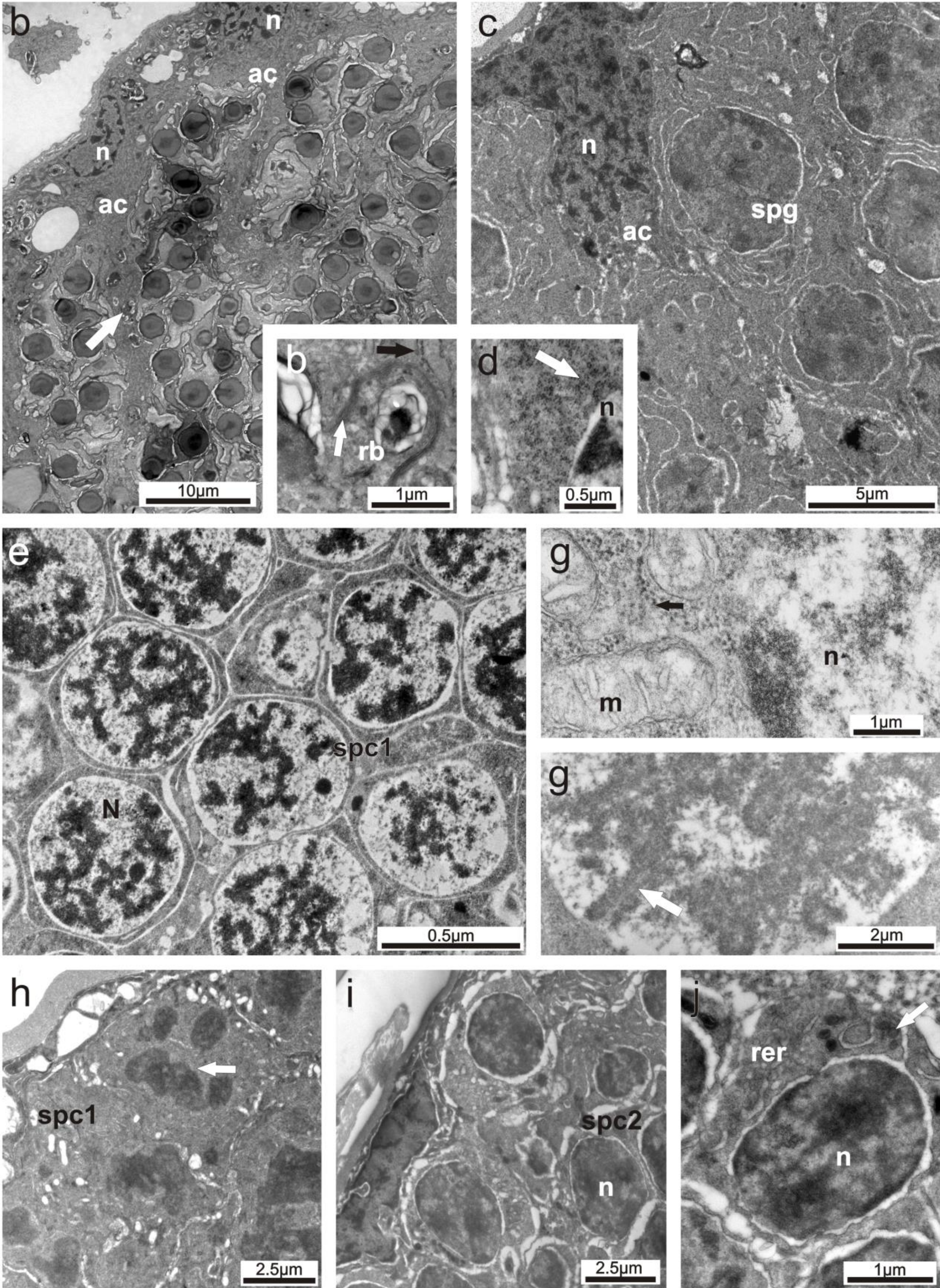
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Figure 5. Spermiogenesis: (A) Early spermatid of *Callinectes danae* Smith, 1869 with a homogeneous nucleus and proacrosomal vesicle (arrow); **(B)** In *Callinectes ornatus* Ordway, 1863, the early wrapped branching of the accessory cells, with electron-lucid (black arrow) and electron-dense (white arrow) vesicles; **(C)** *Callinectes danae* early spermatid with a uncondensed chromatin and proacrosomal vesicle (arrow); **(D)** *Callinectes ornatus* proacrosomal vesicle, small mitochondria (arrow) and lamellar complex; **(E)** Vesicles of the lamellar complex. Golgi complex with few lamella (arrow); **(F)** Intermediate spermatid of *C. danae* forming of the perforatorial chamber (white arrow) and the lamellar complex in both sides and the operculum (black arrow); **(G)** Same stage as in F, in *Callinectes sapidus* Rathbun, 1896, with operculum (black arrow) and perforatorial chamber in formation. The less electron-dense area beneath the operculum (white arrow) is likely the acrosome ray zone; **(H)** Late spermatid of *Callinectes bocourti* A. Milne-Edwards, 1879 with irregular and large C-shaped nucleus. The perforatorial chamber reaches the subopercular region. The lamellar structure and mitochondria are displaced to one side of the acrosomal vesicle. The operculum (arrow) is larger than that of the previous phase, and the acrosome ray zone occupies the upper portion of the perforatorial chamber; **(I)** *Callinectes ornatus* late spermatid showing the perforatorial chamber larger and with tubules (white arrow). The operculum is also round (black arrow). **(J)** Late spermatid of *C. danae* showing the acrosome vesicle organized in concentric layers. The perforatorial chamber is lanceolate, and the apical end is salient and triangular at the subopercular region (arrow); **(K)** Seminiferous tubule of *C. ornatus*, with simple squamous epithelium; **(L)** Mature spermatozoon of *C. danae*, with thin nucleus surrounding the acrosome (arrow). acr, acrosome; ary, acrosome ray zone; est, early spermatid; ep, epithelium; ist, intermediary spermatid; ls, lamellar complex; lst, late spermatid; m, mitochondria; n, nucleus; p, perforatorial chamber; pt, perforatorial tubules; pacr, proacrosomal vesicle; so, subopercular region; sz, spermatozoon; tr, thick ring.

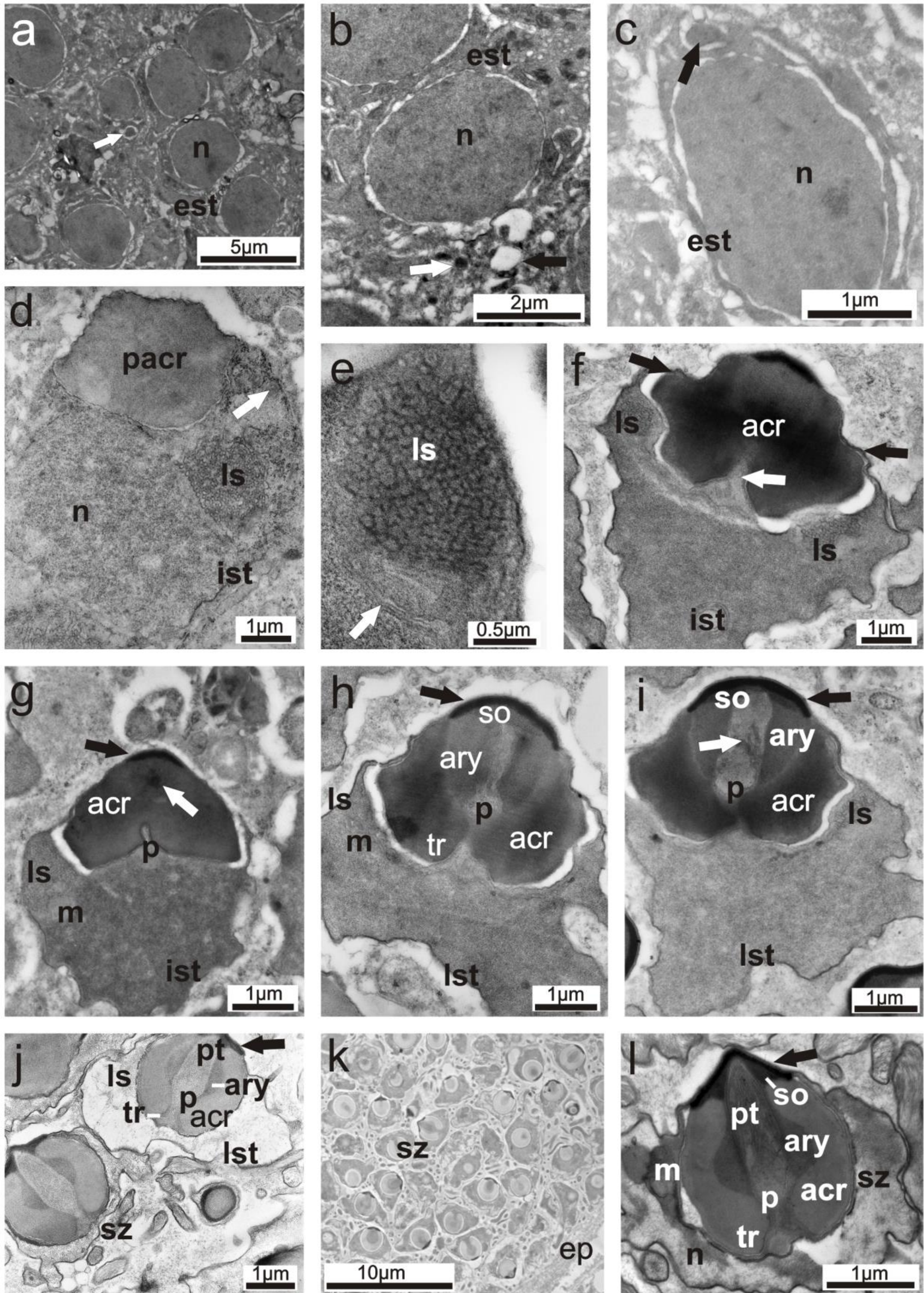
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Figure 6. Anterior vas deferens (AVD): (A) AVD of *Callinectes exasperatus* (Gerstaecker, 1856) showing the proximal (AVDp) with free spermatozoa, and the distal (AVDd) portions with spermatophores in acidophilic (black arrow) and basophilic secretion (white arrow), HE; **(B, C, D)** AVDp of *C. exasperatus* with secretion positive for neutral polysaccharides (white arrow) and strongly positive close to the cell apex (black arrow), weak reactive to acid polysaccharides (black arrow) and positive for proteins (black arrow) while the secretion strongly positive close to the cell apex (white arrow), PAS, Alcian blue, Xylidine ponceau, respectively; **(E, F, G)** AVDd of *C. exasperatus* with secretion positive for neutral (arrows) and strongly positive for acid polysaccharides (arrows) and positive for proteins (arrow), PAS, Alcian blue, Xylidine ponceau, respectively; **(H)** AVDp of *Callinectes danae* Smith, 1869 with RER, irregular nucleus and electron-dense vesicles (arrow); **(I)** Basal portion of AVDp of *Callinectes bocourti* A. Milne-Edwards, 1879 lying on connective tissue and muscle fibers; **(J)** Base of AVDd cell in *Callinectes ornatus* Ordway, 1863 depicting the mitochondria among the folded basal membrane (arrow); **(K)** *Callinectes danae* cell with RER and mitochondria. Note electron-dense vesicles in the apex (arrow); **(L)** Golgi complex releasing vesicles with different electron-densities (black and white arrow) in the AVDp; **(M)** AVDp cell apex of *C. danae* with vesicles undergoing exocytosis (arrow). In the lumen, two types of secretion showing different electron densities are noticed; **(N)** AVDp of *Callinectes sapidus* Rathbun, 1896 with spermatozoa lacking the spermatophore wall. Electron-dense secretion (white arrow) and electron-lucent vesicles (black arrow) are observed; **(O)** AVDp apex of *C. ornatus*, with electron-dense type I and less electron-dense type II secretion similar to the spermatophore wall. **(P)** AVDp secretion in *C. danae*, with less electron-dense type I secretion among the spermatophores. bl, basal lamina; col, collagen; ep, epithelium; fb, fibroblast; cg, golgi complex; m, mitochondria; mc, muscle fibers; nu, nucleolus; n, nucleus; rer, rough endoplasmic reticulum; sp, spermatophore; sw, spermatophore wall; sz, spermatozoa; si, type I secretion; sii, type II secretion.

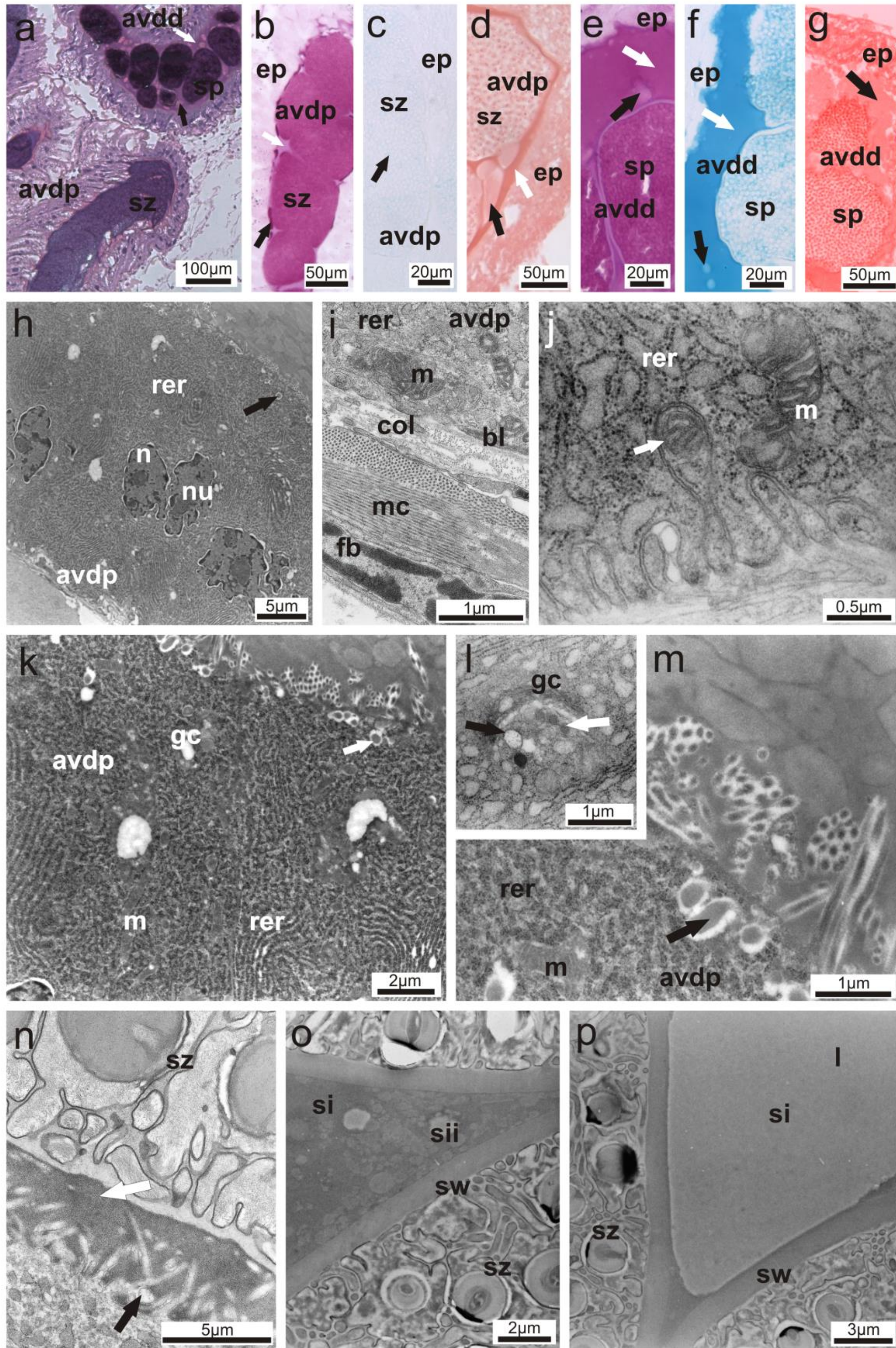
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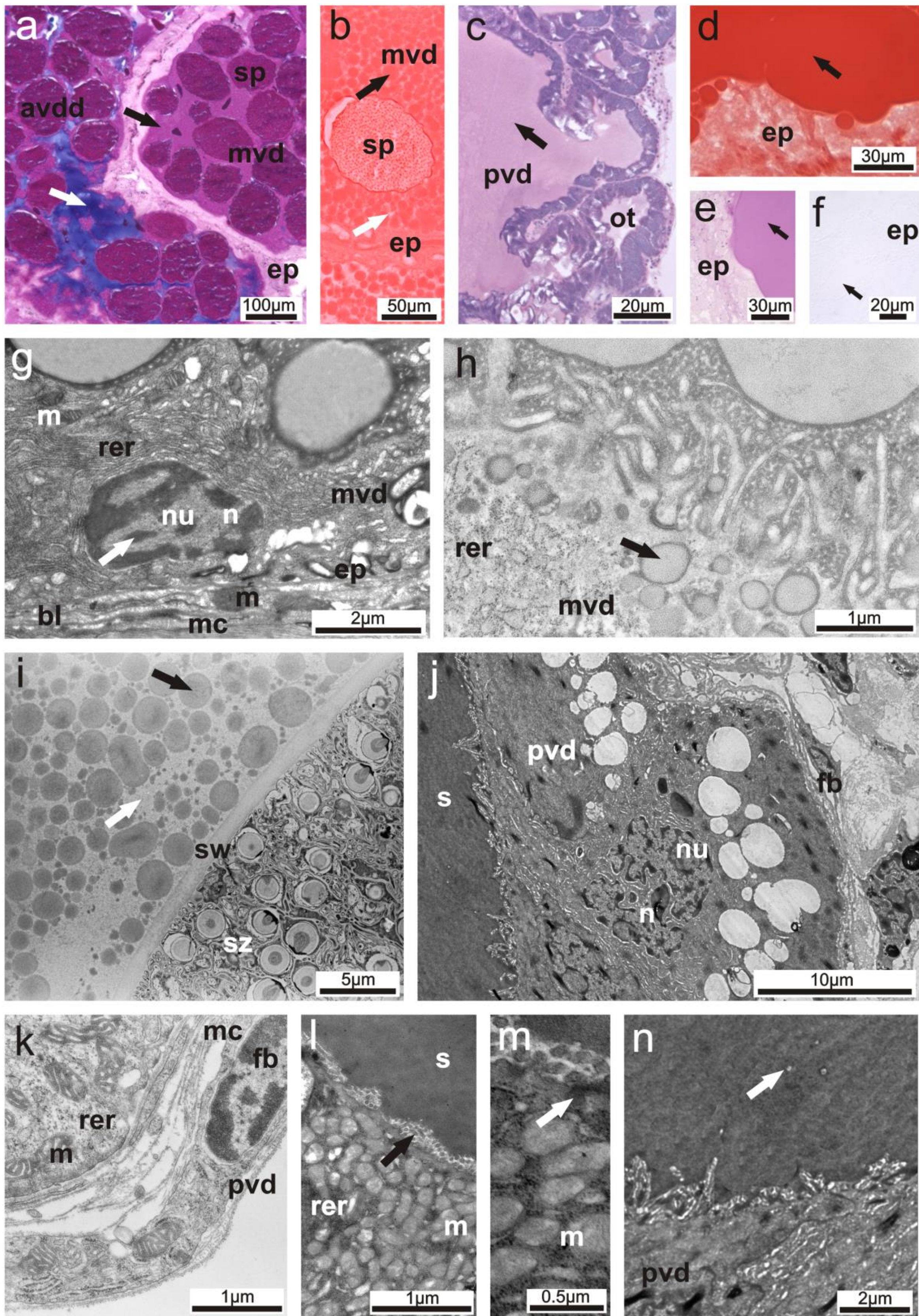
Figure 7. Median (MVD) and Posterior vas deferens (PVD): (A) Transition between the AVD and MVD of *Callinectes exasperatus* (Gerstaecker, 1856) showing the differences in the luminal secretion. The AVDd is strongly reactive for acid polysaccharides with clumps of secretion positive for neutral polysaccharides. The MVD secretion is strongly positive for neutral polysaccharides and negative for acid polysaccharides, stained with PAS\Alcian blue; **(B)** MVD of *C. exasperatus* with granular (black arrow) and matrix (white arrow) secretion strongly positive and positive for proteins, Xylidine ponceau; **(C)** PVD with lateral outpocketings and secretion is an homogenous acidophilic matrix (arrow) without spermatophores, HE; **(D-E)** PVD secretion strongly reactive for proteins (arrow), Xylidine ponceau; **(E)** PVD of *C. exasperatus* with a homogenous matrix strongly reactive for neutral polysaccharides (arrow), PAS; **(F)** PVD of *C. exasperatus*, negative for acid polysaccharides (arrow), Alcian blue; **(G)** MVD of *Callinectes danae* Smith, 1869 with lobulated nucleus and heterochromatin blocks (white arrow). The cytoplasm is filled with RER and basal mitochondria. The lumen shows a granular secretion (black arrow) among the matrix; **(H)** MVD apex with several large secretory vesicles (arrow) in *C. danae*; **(I)** Seminal fluid in the MVD of *Callinectes ornatus* Ordway, 1863, composed of a matrix (white arrow) and granules (black arrow). **(J)** PVD cells in *Callinectes bocourti* A. Milne-Edwards, 1879 with irregular nucleus laying on the connective tissue. The lumen has a homogenous electron-dense secretion (arrow). **(K)** Basal portion of the PVD cell in *C. bocourti* filled with RER and mitochondria laying on the connective tissue and muscle fiber; **(L)** PVD of *Callinectes sapidus* Rathbun, 1896 depicting many mitochondria, RER and short microvilli (arrow) in contact with the homogeneous electron-dense seminal fluid; (**M**) Cell apex of the PVD of *C. sapidus* with mitochondria and short microvilli (arrow); **(N)** PVD of *C. ornatus* showing some secretory vesicles undergoing exocytosis (arrow). bl, Basal lamina; ep, epithelium; fb, fibroblast; ot, lateral outpocketings; m, mitochondria; mc, muscle fibers; nu, nucleolus; n, nucleus; rer, rough endoplasmic reticulum; s, secretion; sp, spermatophore; sw, spermatophore wall; sz, spermatozoa.



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m
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n
pvd
2µm

DISCUSSION

This study provides the first detailed description of the ultrastructure of the male reproductive system of *Callinectes*, based on the observation of five species. Considering *C. exasperatus* and *C. bocourti* as models, the morphology of the male reproductive system of *Callinectes* and other Portunids is extremely similar, corroborating the close taxonomic proximity between the species of *Callinectes* (Robles et al., 2007; Mantelatto et al., 2009).

Along the seasons there were no evident peaks in the gonadosomatic index of mature males of *C. ornatus*, *C. danae* and *C. bocourti*, which indicates that they can reproduce continuously after maturation. Indeed, a continuous reproduction was observed for *C. ornatus* and *C. danae* in Ubatuba (Costa and Negreiros-Fransoso, 1998, Mantelatto and Fransozo 1997, 1999) and in the Santos-São Vicente Estuary/Bay complex (Sant'Anna et al., 2012, Watanabe et al., 2014), both in the southeast of Brazil. Our data confirms that, after maturation, the reproductive system varies little in weight and never reaches the values reported during the development of *C. danae* and *C. ornatus* (Zara et al., 2012; Nascimento and Zara, 2013). Thus, after an initial investment to develop the reproductive system (Zara et al., 2012; Nascimento and Zara, 2013), male crabs do not need to store a specific amount of nutrients. Besides being recorded in tropical species, this pattern has also been observed in species of temperate areas (Kyomo, 1998; Chu, 1999). Interestingly, the GSI of *C. ornatus* and *C. danae* decreased in the same period when the abundance of their respective ovigerous females was at its peak (Sant'Anna et al, 2012; Watanabe et al., 2014). Thus, at least in these species, the lower GSI indicates that males had already copulated and were recovering or replenishing the reproductive system. Moreover, we could speculate that the GSI increase seen in the five species of

Callinectes results from a rapid recovery of the reproductive system, as occurs in *C. sapidus* (Jivoff, 1997).

Testis

Based on histology, the testes of *Callinectes* are classified as lobular (sensu Nagao and Munehara, 2003; Simeó et al., 2009). Zara et al. (2012) defined the "seminiferous lobules" (acini) as the place surrounded by accessory (Sertoli) cells filled with germ cells undergoing spermatogenesis and spermiogenesis. The seminiferous duct receives the spermatozoa and sends them to the vas deferens. The term "collecting duct" has been used in different and confusing ways in many descriptions of crab testes. Adding to Zara et al. (2012), we propose a more refined description of "collecting duct", following the traditional histology nomenclature applied to compound acinar glands (for a review see Mesher, 2018). Thus, a collecting duct is a very short somatic mono-stratified epithelium that receives this name since it collects the spermatozoa from the lobule (acinus) and transfers them to the main/seminiferous duct. In *Callinectes*, the collecting duct remains closed until the lobule releases the spermatozoa into the seminiferous duct, as occurs in *C. ornatus* (Nascimento and Zara, 2013). The spermatogenesis of *Callinectes* begins in the lobules of the testes with cells at the same stage, in synchrony, until the formation of mature spermatozoa (Zara et al., 2012; Nascimento and Zara, 2013). This has also been observed in the portunids *C. sapidus* (Johnson, 1980) and *P. pelagicus* (Stewart et al., 2010; Ravi et al., 2014).

The classic description of the spermatogenesis in Portunidae, under light microscopy, has been done for the first time in *Portunus hawaiiensis* Stephenson, 1968 (as *Portunus sanguinolentus* in Ryan, 1967) and *C. sapidus* (Johnson, 1980). The spermatogenesis of *Callinectes* begins in the germinal centers with the proliferation of spermatogonia, soon after the

previously formed spermatozoa are released into the seminiferous duct lumen, resuming the formation of a new testicular lobule by mitosis (Nascimento and Zara, 2013). In *Callinectes*, the germinal centers are located in the periphery of the testicular lobules, near the seminiferous duct, as also in the portunids *P. hawaiiensis* (Ryan, 1967), *C. sapidus* (Johnson, 1980), and *Charybdis japonica* (A. Milne-Edwards, 1861) (Wong and Sewell 2015) and in the majoid *Libinia spinosa* Guérin, 1832 (Sal Moyano et al., 2010). However, in another majoid, *M. brachydactyla*, cells are organized in germinal zones arranged diametrically opposed to the evacuation zone (seminiferous tubule lumen) that characterize the tubular-type testes (Simeó et al., 2009, 2010). The tubular testes are also found in other Podotremata and Eubrachyuran crabs (Tiseo et al., 2014; Antunes et al., 2018; Garcia Bento et al., 2018a).

Under TEM, the granular aspect of the cytoplasm of spermatogonia in *Callinectes* is caused by the presence of polyribosomes, vesicular RER, and few mitochondria, as occurs in other crustaceans (Hinsch, 1993; Simeó et al., 2010; Stewart et al., 2010). Only one spermatogonia type was observed in the species of *Callinectes* studied here, and in *C. sapidus* (Johnson, 1980) and *M. brachydactyla* (Simeó et al., 2009, 2010). On the other hand, in *Metacarcinus magister* (Dana 1852) as *Cancer magister* in Fasten (1918), *Himalayapotamon koolooense* (Rathbun, 1904) as *Potamon koolooense* in Joshi and Khanna (1982), *Ucides cordatus* (Linnaeus, 1763) in Castilho et al. (2008), and *P. pelagicus* (Stewart et al., 2010), two spermatogonia types were observed, the primary, or type A, and the secondary, or type B. The spermatogonia of *Callinectes* have the characteristics of spermatogonia A, especially the nucleus structure and the heterochromatin arrangement.

In general, regarding the brachyurans, few ultrastructural studies described in detail the early stages of the prophase of meiosis. The morphology of the nucleus and the arrangement of

chromosomes varies in the primary spermatocytes, and the stages of the meiotic prophase are clearly visible, like in other brachyurans such as *Me. magister* (Fasten, 1918). In *Callinectes,* the meiotic prophase is marked by the formation of the synaptonemal complex, a feature used to identify these cells (Stewart et al., 2010; Simeó et al., 2010). In *Callinectes* and in the phylogenetically related species *P. pelagicus*, the chromosomes are associated with the nuclear envelope since the pachytene (Stewart et al., 2010). However, this organization does not occur in the majoid *M. brachydactyla* (Simeó et al., 2010). The primary spermatocytes of *C. ornatus* exhibit a thin cytoplasm and few organelles during the entire prophase, as in other portunids (Stewart et al., 2010). However, Simeó et al. (2010) describe the formation of an annulate lamellae and cytoplasmic "*Nuage*" in majoids, which seem to be lacking in *Callinectes* and other Portunidae (Stewart et al., 2010). The secondary spermatocytes of *Callinectes* still exhibit a condensed chromatin in the nucleus. In this respect, *Callinectes* is more similar to *M. brachydactyla* (Simeó et al., 2010) than to *P. pelagicus*, in which the chromatin is arranged in a clock-face pattern (Stewart et al., 2010).

The spermatids undergo three maturation stages, which seems to be a typical feature of brachyurans, including Podotremata (Stewart et al., 2010; Zara et al, 2012; Tiseo et al., 2014; Garcia Bento 2018a). Although similar in size to secondary spermatocytes (Zara et al., 2012; Nascimento and Zara, 2013), early spermatids have a nucleus with diffuse and homogeneous heterochromatin, due to the chromatin decondensation after the last meiosis (Langreth, 1969; Medina and Rodrigues, 1992; Simeó et al., 2010; Stewart et al., 2010). In addition, a single proacrosomal vesicle occurs in one of the cell poles, pushing the nucleus to the opposite pole (Langreth, 1969; Medina and Rodrigues, 1992; Medina, 1994; Simeó et al., 2010). This differs from the proacrosomal vesicle formed by small perinuclear vesicles as reported for *P. pelagicus*

(Stewart et al., 2010). The proacrosomal vesicle is formed by the fusion of vesicles from the Golgi complex in *M. brachydactyla* (Simeó et al., 2010) and *P. pelagicus* (Stewart et al., 2010). In *Callinectes*, a complex of membranes (i.e., the lamellar complex or lamellar system) occupies the cytoplasm at the base of the proacrosomal vesicle. This lamellar complex has been described in detail (Moses, 1961; Langreth, 1969; Medina and Rodrigues, 1992; Medina, 1994). In *Callinectes* it produces vesicles that fuse with the proacrosome, as observed by Simeó et al. (2010), who suggested that this complex is in fact a degenerating Golgi complex. It reduces gradually as the proacrosomal vesicle increases, and few membranes and associated mitochondria remain around the acrosome in the mature spermatozoon. This seems to be a common characteristic of decapod spermatozoa (Moses, 1961; Langreth, 1969; Medina and Rodrigues, 1992; Medina, 1994; Simeó et al., 2010).

As seen in other crabs, in *Callinectes* other organelles are also modified during spermiogenesis (Medina and Rodrigues, 1992; Simeó et al., 2010). In early spermatids, the mitochondria exhibit a typical ultrastructure, and, as the lamellar complex changes, the mitochondria exhibit fewer cristae and become scarce and degenerated. Pearson and Walker (1975) observed a total loss of the mitochondria oxidative role during the spermiogenesis in *Carcinus maenas* Leach 1814, which might explain the lack of cristae in the spermatozoa of *M. brachydactyla* (Simeó et al., 2010) and in the mature spermatozoa of *Callinectes*. The reduction of the cytoplasm to a thin layer between the nucleus and the acrosome is an event commonly observed during the spermiogenesis of decapods (Moses, 1961; Langreth, 1969; Medina and Rodrigues, 1992; Medina, 1994; Simeó et al., 2010). In *Callinectes* the reduction seems to be promoted by the branch-like accessory cells. Electron-dense vesicles similar to residual bodies, are observed in many cytoplasmic branches, as in *M. brachydactyla*, (Simeó et al., 2010).

According to Langreth (1969), accessory cells degrade the cytoplasmic remnants of spermatids, which explains the presence of electron-dense vesicles in the cytoplasm of accessory cells in *Callinectes*.

The perforatorial chamber formation begins during the reduction of cytoplasm, separating the lamellar complex into two portions, one on each side of the old proacrosomal vesicle. The perforatorial chamber is the main trait of the sperm acrosome and, upon the beginning of its formation, the proacrosomal vesicle is named acrosome or acrosome vesicle. This is a characteristic structure of intermediary spermatids, formed by the centrioles positioned in the median basal region of the proacrosome. Langreth (1969) and Medina and Rodrigues (1992) reported that a granular and fibrillar material accumulates at the base of the perforatorial chamber, and only after that, at least in *Cancer* (Langreth, 1969), the formation of the perforatorial chamber begins. In *Callinectes*, the perforatorial chamber tubules are formed as the chamber elongates.

The presence of the centriole until the complete maturation of the spermatozoon suggests the participation of tubulin from the centrosome during the formation of the perforatorial chamber, but this has not been recorded in other brachyurans. Unlike in *Cancer* Linnaeus, 1758 in Langreth (1969), *A. tangeri* (Medina and Rodrigues, 1992), *M. brachydactyla* (Simeó et al., 2010), and *P. pelagicus* (Stewart et al., 2010), the formation of the perforatorial chamber begins before electron-dense secretion granules appear under the operculum. These granules shrink until they occupy two thirds of the perforatorial chamber length, and form the acrosome ray zone of the late spermatids and spermatozoa. Although the origin of this secretion is unknown, the formation of the acrosome ray zone or other inner acrosome vesicle that occurs in *Callinectes* has been described in many other brachyurans (Langreth, 1969; Medina and Rodrigues, 1992;

Medina, 1994; Simeó et al., 2010). In the portunid *P. pelagicus*, nonetheless, the migration of electron-dense granules is distinct: they initially accumulate in the apical end of the perforatorial chamber (Stewart et al., 2010). This difference indicates either that this process is not phylogenetically conserved, or that the section is oblique.

Another organelle that undergoes important changes during spermiogenesis is the nucleus. In *Callinectes*, the nuclear envelope breaks during the early spermatid stage, after the proacrosomal vesicle becomes enlarged and electron-dense. The disintegration of the nuclear envelope occurs near the base of the proacrosome, and membrane remnants of the envelope are observed, as in other decapods (Langreth, 1969; Medina and Rodrigues, 1992; Medina, 1994; Simeó et al., 2010), but not in *P. pelagicus* (Stewart et al., 2010). Due to the contact between the chromatin and cytoplasm, Simeó et al. (2010) suggested the term "nucleus-cytoplasm complex" for spermatids. The nucleus undergoes extensive morphological modifications: it changes from circular in early spermatids, to "half-moon shaped" in intermediary, and finally, to a compact "C-shaped" or "cup-shaped" form that almost surrounds the spermatozoon. This characteristic is commonly observed in brachyurans (Langreth, 1969; Medina and Rodrigues, 1992; Medina, 1994; Simeó et al., 2010), and the cup-shaped nucleus is the most common shape seen among the mature spermatozoa in this group (Jamieson, 1994).

Vas deferens

The anterior vas deferens is divided into two portions that have distinct characteristics and roles: proximal (AVDp) and distal (AVDd). Based on light microscopy studies, this division has also been reported in many crabs, e.g. the grapsids *G. cruentata* (Garcia and Silva, 2006), *Pachygrapsus gracilis* (Saussure, 1857), and *Pachygrapsus transversus* (Gibbes, 1850) in Tiseo et al. (2014), the majid *M. brachydactyla* (Simeó et al., 2009), the gecarcinucid *Sundathelphusa*

phillippina (von Martens, 1868) in Klaus et al. (2013), and the portunids *P. pelagicus*, *C. danae* and *C. ornatus* (Zara et al., 2012; Nascimento and Zara, 2013; Ravi et al., 2014). The beginning of the spermatophore formation occurs in the AVDp, where spermatozoa are clustered before being embedded in the secretion that will compose the spermatophore wall. The spermatophore formation is completed in the AVDd. Waiho et al (2017) divided the AVD of another portunid, *Scylla olivacea* (Herbst, 1796) in three portions (=regions in the original paper). However, they pointed that that free sperm masses were only found in the proximal region, while the “central” and distal regions were filled with spermatophores. Since it is only possible to differentiate the AVD morphologically by the presence or absence of spermatophores, the recognition of two portions is more adequate in Portunidae, as seen here and probably in other crabs (Garcia and Silva, 2006; Simeó et al., 2009; Zara et al., 2012; Nascimento and Zara, 2013; Ravi et al., 2014; Tiseo et al., 2014; Antunes et al., 2018).

In *Callinectes*, the secretion released at the apex of the AVD epithelium is further condensed in the lumen to form the spermatophore, as in *Cancer* sp. (Langreth 1969). In *Ca. borealis* (Moriyasu et al., 2002), the formation of spermatophores begins in the testicular lobules, near the AVD, whereas in *Inachus phalangium* (Fabricius, 1775) in Diesel (1989) and *Ch. opilio* (Beninguer et al., 1988; Moriyasu and Benhalima, 1998) the spermatophores are formed at the transition between the AVD and MVD. Differently, in the crayfish *Cherax albidus* Clark, 1936 the spermatophore formation occurs in the proximal, median, and distal regions (Talbot and Beach, 1989), i. e., along the entire vas deferens. The same also happens in non-eubrachyuran crabs like Podotremata (Garcia Bento et al., 2018a).

The AVDp epithelium in *Callinectes* is filled with rough endoplasmic reticulum (RER), electron-dense and electron-lucent vesicles, and many invaginations in the basal membrane. In

addition, the AVDp has the same lobulated nucleus observed in the AVDd, and the epithelium is filled with RER, Golgi complex, and secretion vesicles in exocytosis between microvilli. Therefore, the epithelium cells are specialized in glycoprotein production. Similar ultrastructural characteristics associated with protein synthesis have been observed in other crabs, including portunids (Hinsch and Walker 1974; Benhalima and Moriyasu 2000; Simeó et al. 2009; Ravi et al., 2014). In the freshwater crab *Su. phillippina*, the AVDp and AVDd cells differ slightly: the AVDp shows a less electron-dense and irregular RER, lacks secretory vesicles, and has a smooth apical apex, while the AVDd shows vesicular RER and Golgi complex. Moreover, in the AVDd, the apical apex of the cell budding has large lobules forming a large secretory mass with organelles (Klaus et al., 2013). Although this does not occur in *Callinectes* and *P. pelagicus*, there are some similarities between the AVD of freshwater crabs and *Callinectes*, such as the presence of glycoprotein secretory cells. This feature of the AVD could be considered as the pattern of Eubrachyura, since it is also observed under light microscopy in many other species of different families, phylogenetically closely-related or not (Garcia and Silva, 2006; Simeó et al., 2009; Ravi et al., 2014; Tiseo et al., 2014; Antunes et al., 2018; Oliveira and Zara, 2018).

Two distinct types of luminal secretion are observed along the AVD in *C. exasperatus*, *C. bocourti*, *C. sapidus, C. ornatus*, and *C. danae*. The acidic secretion type I become positive for acidic polysaccharides only in the AVDd (Johnson, 1980; Zara et al., 2012; Nascimento and Zara, 2013), which could explain the less electron-dense aspect of the secretion type I in this portion. This electron-dense compound that groups the spermatophores was named substance I by Simeó et al. (2009) in *M. brachydactyla*, and unlike in *Callinectes*, it was stained with eosin (acidophilic) and Mallory's stain (hematoxylin/eosin/Mallory). The secretion type I is surrounding and clustering the spermatozoa. The secretion type II is less electron-dense than the

secretion type II and more abundant in AVDd. This secretion form large or small masses incorporated around groups of spermatozoa to form the spermatophore wall. This is the pattern of all Portuninae studied so far, and also occurs in the former portunid, now Carcinidae species, *Ca. maenas* (Spalding 1942; Ryan 1967; Zara et al., 2012; Nascimento and Zara, 2013; Ravi et al., 2014; Waiho et al., 2017). Our histochemical analysis of the male reproductive system of *C. exasperatus*, plus the studies on *C. danae* and *C. ornatus* (Zara et al., 2012; Nascimento and Zara, 2013) revealed that the electron-dense secretion that clusters the spermatozoa is basophilic, as in *P. pelagicus* (Stewart et al., 2010; Ravi et al., 2014) and it is composed of a glycoprotein with neutral polysaccharides that increases the amount of secretion from AVDp to AVDd. The less electron-dense secretion type II that composes the spermatophore wall of *P. pelagicus* is acidophilic but no chemical analysis was carried out (Stewart et al., 2010; Ravi et al., 2014). In all species of *Callinectes* studied here, as well as in *C. danae* and *C. ornatus* (Zara et al., 2012; Nascimento and Zara, 2013), the glycoproteins of the secretion type II have neutral polysaccharides. In *C. sapidus*, the secretion was PAS positive (Johnson, 1980). In *M. brachydactyla*, the secretion that forms the spermatophore wall or pellicle was named substance II by Simeó et al. (2009). It was stained blue with HE (basophilic), and was only observed in the AVDp. In the eriphiid *E. verrucosa*, the secretion that forms the wall contains proteins (Erkan et al., 2009).

Anilkumar and Subramoniam (1999) suggested that the spermatophore wall is composed of proteins linked to glycosaminoglycans (mucopolysaccharides), while Garcia and Silva (2006) suggested that the wall is composed of neutral polysaccharides. In our study, the secretion type II (less electron-dense) of *C. exasperatus, C. bocourti, C. danae*, and *C. ornatus* contains neutral polysaccharides and proteins, but lacks acidic polysaccharides (Zara et al., 2012; Nascimento

and Zara, 2013). This pattern, i. e., two substances with different compounds are secreted in the AVD to form the spermatophores, seems to be the most general pattern of Eubrachyura since it has been observed in several species (Cronin, 1947; Ryan 1967; Johnson, 1980; Adiyodi and Anilkumar, 1988; Sainte-Marie and Sainte-Marie, 1999; Simeó et al., 2009; Stewart et al., 2010; Zara et al, 2012; Nascimento and Zara, 2013; Tiseo et al., 2014; Ravi et al, 2014). The single exception known so far is *Su. phillippina*. In this species, at least three secretion types occur in the AVD. They blend around the spermatophore mass to form a mature spermatophore that lacks a wall (or pellicle), and it is classified as the mucous type spermatophore (Klaus et al., 2013).

In *Callinectes*, only one type of coenospermic round spermatophore filled with mature spermatozoa was observed. Accordingly, this is the most common pattern seen in Eubrachyura, as opposed to the very long coenospermic spermatophore with sperm cord observed in Podotremata Dromiidae (Garcia Bento et al., 2018a, b). In the cancrid crab *Ca. borealis* and in the oregoniid crab *Ch. opilio*, two spermatophore types are found: one with a thin and smooth wall that probably breaks easily and is used primarily for fertilization, and a rougher and more resistant one, which can be stored for longer periods in the vas deferens and in the female seminal receptacle (Moriyasu and Benhalima, 1998; Moriyasu et. al., 2002). Thus, the mechanism of spermatophore storage in the females of *Callinectes* is different than that observed in Cancridae and Majoidea. The spermatophore dehiscence should occur in synchrony with the ovary maturation, as in the portunid *A. cribrarius*, a known species that produces sperm plugs (Zara et al., 2014).

In *C. exasperatus, C. bocourti* and in all other *Callinectes,* the MVD epithelium exhibits many folds or outpocketings (sensu Johnson, 1980) that increase the size of vas deferens lumen for spermatophore storage and granular seminal fluid production. These outpocketings and the

granular seminal fluid are typical of *Callinectes.* They are found in *C. sapidus*, *C. danae* and *C. ornatus* and in other species that produce sperm plugs, like *P. pelagicus.* In *Ca. maenas* the outpocketings are named as pouch-like folds and described as "epithelium of uneven height with large bulbus" (Spalding 1942; Johnson, 1980; Zara et al., 2012; Nascimento and Zara, 2013; Ravi et al., 2014). On the other hand, the outpocketings are absent in the MVD of the portunid *S. olivacea*, which has an acidophilic granular seminal fluid (Waiho et al., 2017). The plug prevents other males from transferring their semen to a previously inseminated female (Spalding, 1942; Hartnol, 1969; Bawab and El Sherief, 1988; Zara et al., 2012). Thus, the outpocketings of *Callinectes* (and probably in *Portunus*) increase the production of granular secretion that could play a key role during the sperm transfer and sperm plug formation in Portunidae. In portunids and majoids, the MVD and AVD cells are similar under TEM, i. e., they show a large amount of RER, mitochondria, and secretion vesicles, and thus are considered secretory cells (Hinch and Walker, 1974; Simeó et al., 2009; Ravi et al., 2014). However, in the MVD cells, large apical secretory vesicles occurred in all species of *Callinectes*. This general ultrastructure of the MVD cells occurs in the portunid *P. pelagicus* (Ravi et al., 2014) and in two species of the majoid genera *Libinia* and *Maja* (Hinsch and Walker, 1974).

Unlike in *Callinectes*, the secretory vesicles of majoids are smaller and electron-dense. In *Callinectes,* as well in *M. brachydactyla* and *P. pelagicus* (Simeó et al., 2009; Ravi et al., 2014), only secretory vesicles undergoing exocytosis were noticed in the MVD. Pinocytotic vesicles, which seem to play a role in the absorption of MVD fluids in *L. dubia* and *L. emarginata*, were undetected (Hinsch and Walker, 1974). In MVD region, the secretion vesicles are associated with the formation of the less electron-dense granular glycoproteinaceous component immersed in the electron-dense, almost homogeneous matrix that modifies the seminal fluid coming from

the AVD. This MVD fluid carries the spermatophores in a dense secretion, from the AVD and MVD to the female seminal receptacle. The fluid is an important component of the wax-like secretion that forms the sperm plug inside the female seminal receptacle (Zara et al., 2012, 2014; Nascimento and Zara, 2013). However, at least in *A. cribrarius*, and probably in *P. pelagicus*, the granules are absent in the sperm plug (Bawab and El-Sherief, 1988; Zara et al., 2014). Thus, the MVD granules change during the sperm plug formation. Further specific histochemical and biochemical studies are required to understand how MVD and PVD fluids interact with each other in *Callinectes*.

In the MVD, the spermatophores are completely formed, and remain stored until the transfer to the female seminal receptacle. The spermatophore formation in the AVD and storage in the MVD also occur in *C. sapidus*, *Ca. borealis*, *M. brachydactyla* and *U. cordatus* (Johnson, 1980; Moriyasu et al., 2002; Castilho et al., 2008; Simeó et al., 2009). However, in all species of *Callinectes* studied here, as in *S. olivacea* and *P. pelagicus*, the spermatophores are almost exclusively found in the MVD (Zara et al., 2012; Nascimento and Zara, 2013; Ravi et al., 2014; Waiho et al., 2017). The presence of spermatophores almost exclusively in the MVD was reported in other species that produce sperm plugs: in the Portunidea *Ca. maenas* (Carcinidae), in which the PVD presents as a long seminal vesicle (Spalding, 1942), and in the Ovalipidae *Ovalipes trimaculatus* (de Haan, 1833), in which only a few spermatophores are found in the PVD (Vallina et al., 2014). Thus, in the species that produce sperm plugs, as well as in the species that produce sperm packets, the MVD is the main region of spermatophore storage (Diesel, 1989, 1991; Sainte-Marie and Sainte-Marie, 1998; Sainte-Marie et al., 2000; Antunes et al., 2018). This seems to be a morphological convergence found in crab species with different sperm competition strategies. In the species that produce sperm packets, the secretion of the PVD

and accessory glands (caecas) dislocate the seminal fluid from the previous mating. Thus, a stratum is formed, and the MVD secretion and spermatophores from most recent copula will likely fertilize the oocytes (Diesel, 1989,1991; Antunes et al., 2016, 2018).

The PVD of *Callinectes* is fully developed and has many outpocketings. The luminal secretion, i. e., the seminal fluid, is fluidic-gelatinous (gross morphology), acidophilic homogeneous, and glycoproteic. It is less reactive to neutral polysaccharides than the granular MVD secretion, and intensely positive for proteins, at least in *C. danae* and *C. ornatus* (Zara et al., 2012; Nascimento and Zara, 2013). The general aspect of the seminal fluid found in the PVD of species of *Callinectes* — acidic, homogeneous and gelatinous — was seen in other portunids of the genera *Portunus* and *Scylla* (Ryan, 1967; Ravi et al., 2014; Waiho et al., 2017). The epithelium is simple, as often reported in the literature, rather than pseudostratified, as in *M. brachydactyla* (Simeó et al., 2009). The PVD cells are less secretory, have only a few electron-dense vesicles but a great amount of mitochondria in both cell poles. This suggests that this region functions as a pump that uptakes compound from the hemolymph (probably water and ions) to add to the seminal fluid. In majoids, however, this process occurs along the vas deferens (Hinsch and Walker, 1974; Simeó et al., 2009). No spermatophores were found in the PVD of blue crabs under light microscopy or TEM, which seems to be the general pattern for most of portunids as reported for *Callinectes* (Zara et al., 2012; Nascimento and Zara 2013), *S. olicacea* (Waiho et al., 2017), *Portunus hawaiiensis* (Ryan 1967), and *P. pelagicus* (Ravi et al., 2014). Nonetheless, spermatophores have been found in the PVD of *P. pelagicus*, according Stewart et al. (2010).

Apparently, the PVD of *Callinectes* makes the seminal glycoprotein fluid homogeneous and more liquid to facilitate the mixing with the material from the MVD, and later, the transfer to

the female seminal receptacle where it forms the sperm plug. Indeed, this has been seen in *C. sapidus*, *C. danae* and *C. ornatus* under light microscopy (Johanson, 1980; Nascimento and Zara, 2013 Zara et al., 2012, 2014). The fluidic secretion and absence of spermatophores (or presence of a few) in the PVD seem to be characteristics of species that produce sperm plugs, including the portunids studied here, plus *P. pelagicus* and *S. olivacea* (Ravi et al., 2014; Waiho et al., 2017). In addition, this is the same trait also found in sperm packets species as some Inachidae (Diesel, 1989, ; Diesel, 1991), Inochoididae (Antunes et al., 2016, 2018), Oregoinidae, (Beninguer et al., 1988; Diesel, 1991: Sainte-Marie et al., 2000) and Trychodactylidae (Oliveira and Zara, 2018). However, in these species, the accessory gland participates in the sperm packet formation, and this gland is absent in *Callinectes*. On the other hand, in *M. brachydactyla*, few organelles are observed in the PVD, which does not have a secretory role and apparently functions as a site to combine spermatophores with the MVD secretion (Simeó et al., 2009). This ultrastructural characteristic has also been observed in other majoids like *L. dubia* and *L. emarginata* (Hinsch and Walker, 1974) that do not produce sperm plugs. Additional roles were not observed for *Callinectes* aside from the fluidification of the seminal fluid as other PVD roles have been reported for other groups, e.g., storage of seminal fluid and spermatophores in Grapsidae crabs (Tiseo et al., 2014), and phagocytosis of spermatozoa and spermatophores and storage of seminal fluid in *Ch. opilio* (Benhalima and Moriyasu, 2000).

In conclusion, the spermatogenesis and spermiogenesis in *Callinectes* follows the pattern known for brachyurans. There are three spermatid stages and active participation of accessory cells. Spermatophores produced in the AVD are compacted by two secretions of different electron densities that vary in chemical composition among brachyurans. In future species descriptions, the presence of 1) outpocketings in the MVD and PVD, 2) spermatophores stored

only in the MVD, and 3) large amounts of seminal fluid in the vas deferens, should be taken into account when inferring whether a species produces sperm plugs or not. We expect that all sperm packet species should show a low amount of seminal fluid and accessory glands between the MVD and PVD, or in the PVD. The MVD and PVD are also secretory regions, and the ultrastructure of the PVD shows that it plays an important role changing the seminal fluid into a homogeneous, fluid secretion. This is a key role during the sperm transfer since this will be the first secretion inside the female seminal receptacle. The formation of the sperm plug probably occurs by the mixing of the AVD, MVD and PVD secretions, just after mating. Then, the seminal fluid polymerizes and forms a solid, waxy material. This general pattern appears to be the same for all members of the genus *Callinectes* and probably for all Portunoidea that produce sperm plugs. In the future, we expect to see a conspicuous difference between them and species that do not produce sperm plugs.

ACKNOWLEDGEMENTS

The authors are thankful to the São Paulo Research Foundation - FAPESP (IC #2009/01348-5 to FAN, JP #2005/04707-5 to FJZ), to the Coordenação de Aperfeiçoamento de Nível Superior - CAPES - Ciências do Mar II (#1989/2014 - 23038.004309/2014-51 and #2005/2014 – 23038.004308/2014-14 granted to FJZ and FLM, and TTW PhD fellowship associated to CAPES CIMAR II #1989/2014, CAPES financial code 001), and to the Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq (PQ2 #303314/2017-6 to FJZ and PQ1A 302748/2010-5 to FLM). The authors also would like to thank the anonymous reviewers that helped to improve this manuscript; Fernanda C. Francesquini, Monika Iamonte,

Antonio Yabuki and Márcia F. Mataqueiro for their technical support; and the Laboratory of Electron Microscopy of UNESP Rio Claro and FCAV, UNESP - Jaboticabal. This research was conducted according to the Brazilian laws (FJZ - MMA SisBio permanent license #34587 for the collection of zoological material).

REFERENCES

Adiyodi, K.G., Anilkumar, R.G. (1988). Accessory sex glands. In: K.G. Adiyodi and R.G. Adiyodi, Editors, *Reproductive Biology of Invertebrates* vol. 3, John Wiley and Sons, Kerala, pp. 261–318.

Anilkumar, K.S., Subramoniam, T. (1999). Spermatophore transfer and sperm structure in the brachyuran crab *Metopograpsus messor* (Decapoda: Grapsidae). *Journal of Crustacean Biology*, 19: 361-370.

Antunes, M., Zara F.J., López-Greco, L.S., Negreiros-Fransozo, M.L. (2016). Morphological analysis of the female reproductive system of *Stenorhynchus seticornis* (Brachyura: Inachoididae) and comparisons with other Majoidea. *Invertebrate Biology,* 135: 75-86.

Antunes, M., Zara, F.J., López-Greco, L.S., Negreiros-Fransozo, M.L. (2018). Male reproductive system of the arrow crab *Stenorhynchus seticornis* (Inachoididae). *Invertebrate Biology,* 137: 171-184.

Bawab, F.M., El-Sherief, S.S. (1988) Stages of the Reproductive Cycle of the Female Crab *Portunus pelagicus* (L., 1758) Based on the Anatomical Changes of the Spermatheca (Decapoda Brachyura, Portunidae). *Crustaceana,* 54: 139-148.

Benhalima, K., Moriyasu, M. (1999). Structure and function of the posterior vas deferens of the snow crab, *Chionoecetes opilio* (Brachyura, Majidae). *Department of Fisheries and Oceans, Maritimes Region, Science Branch, Invertebrate Fisheries Division,* Canadá.

Beninger, P.G., Elner, R.W., Foyle, T.P., Odense, P.H. (1988). Functional anatomy of the male reproductive system and the female spermathecae in the snow crab *Chionoecetes* opilio (O. Fabricius) (Decapoda: Majidae) and a hypothesis for fertilization. *Journal of Crustacean Biology,* 8: 322-332.

Castilho, G.G., Ostrensky, A., Pie, M.R., Boeger, W.A. (2008). Morphohistology of the male reproductive system of the mangrove land crab *Ucides cordatus* (L.) (Crustacea, Brachyura, Ocypodidae). *Acta Zoologica* (Stockholm), 89: 157-161.

Chu, K.H. (1999). Morphometric analysis and reproductive biology of the crab *Charybdis affinis*

(Decapoda, Brachyura, Portunidae) from the Zhujiang Estuary, China. *Crustaceana,* 72: 647–658.

Costa, T.M., Negreiros-Fransozo, M.L. (1998). The reproductive cycle of *Callinectes danae* Smith, 1869 (Decapoda, Portunidae) in the Ubatuba region, Brazil. *Crustaceana,* 71: 615-627.

Cronin, L.E. (1947). Anatomy and histology of the male reproductive system of *Callinectes Sapidus* Rathbun. *Journal of Morphology,* 81: 209-239.

Diesel, R. (1989). Structure and function of the reproductive system of the symbiotic spider crab *Inachus phalangium* (Decapoda: Majidae): observations on sperm transfer, sperm storage and spawning. *Journal of Crustacean Biology,* 9: 266-277.

Diesel, R. (1991). Sperm competition and mating behavior in Brachyura. In: R.T. Bauer and J.W. Martin (eds.). Crustacean Sexual Biology, pp. 145-163. Columbia University Press, New York.

Erkan, M., Tunali, Y., Balkis, H., Oliveria, E. (2009). Morphology of testis and vas deferens in the xanthoid crab, *Eriphia verrucosa* (Forskal, 1775) (Decapoda: Brachyura). *Journal of Crustacean Biology*, 29: 458-465.

Fasten, N. (1918). Spermatogenesis of the Pacific Coast Edible Crab, *Cancer magister* Dana. *Biological Bulletin*, 34: 277-306.

Garcia, T.M., Silva, J.R.F. (2006). Testis and vas deferens morphology of the red-clawed mangrove tree crab (*Goniopsis cruentata*) (Lattreille, 1803). *Brazilian Archives of Biology and Technology*, 49: 339-345.

Garcia Bento, M.A., López-Greco, L.S., Zara, F.J. (2018a). Seminal fluid production and sperm packaging in dromiid crabs (Brachyura, Podotremata). *Zoology* (Jena), 132: 17-30.

Garcia Bento, M.A., Trentin, I.M., Mantelatto, F.M., Zara, F.J. (2018b). Comparative spermatozoal ultrastructure and molecular analysis in dromiid crabs and their phylogenetic implications for Dromiidae and Podotremata (Decapoda: Brachyura). *Arthropod Structure and Development*, 47: 627-642.

Hartnoll, R.G. (1969). Mating in the Brachyura. *Crustaceana*, 16: 161-181.

Hinsch, G.W., Walker, M.H. (1974). The vas deferens of the spider crab, *Libinia emarginata. Journal of Morphology*, 143: 1-20.

Hinsch, G.W. (1993). Ultrastructure of spermatogonia, spermatocytes, and Sertoli cells in the testis of the crayfish, *Procambarus paeninsulanus. Tissue and Cell,* 25: 737-742.

Jamieson, B.G.M. (1994). Phylogeny of the Brachyura with particular reference to the Podotremata: Evidence from a review of spermatozoal ultrastructure (Crustacea, Decapoda). *Philosophical Transactions: Biological Sciences*, 345: 373-393.

Jivoff, P. (1997). Sexual Competition among Male Blue Crab, *Callinectes sapidus*. *Biological Bulletin*, 193, 368-380.

Jivoff, P., Hines, A.H., Quackenbush, L.C. (2007). *Reproductive biology and embrionic development.* In: Cronin, L.E., Kennedy, V.S. *The Blue Crab Callinectes sapidus.* Maryland Sea Grant College, 255-286.

Johnson, P.T. (1980). *Histology of the Blue Crab (Callinectes Sapidus): A Model for the Decapoda*. Praeger Scientific, New York, 440p.

Joshi, P.C., Khanna, S.S. (1982). Structure and seasonal changes in the testes of a freshwater crab, *Potamoon koolooense* (Rathbun). *Proceedings of the Indian Academy of Sciences,* 91: 439-450.

Junqueira, L.C.U., Junqueira, L.M.M.S. (eds.). (1983). *Técnicas Básicas de Citologia e Histologia*. Ed. Santos, São Paulo.

Kennedy, V.S., Cronin, L.E. (2007). *The Blue Crab Callinectes Sapidus*. Maryland Sea Grant College.

Klaus, S., Münzner, S., Modenbach, A., Streit, B., Tudge, C.C. (2013). Spermatophore formation and sperm ultrastructure of *Sundathelphusa philippina* (Crustacea: Brachyura: Gecarcinucidae). *Acta Zoologica (Stockholm)*, 94: 267-272.

Krol, R.M., Hawkins, W.E., Overstreet, R.M. (1992). Reproductive components. In: F.W. Harrison and A.G. Humes (eds.), *Microscopic Anatomy of Invertebrates*. Decapoda Crustacea 10: 295-343. Wiley-Liss Inc., New York.

Kyomo, J. (1988). Analysis of the relationship between gonads and hepatopancreas in males and females of the crab *Sesarma intermedia*, with reference to resource use and reproduction. *Marine Biology,* 97: 87-93.

Langreth, S.G. (1969). Spermiogenesis in Cancer Crabs. *The Journal of Cell Biology*, 43: 575-603.

López-Greco, L.S., Rodríguez, E.M. (1999). Annual reproduction and growth of adult crabs *Chasmagnathus granulata* (Crustacea, Brachyura, Grapsidae). *Cahiers de Biologie Marine,* 40: 155-164.

Mancini, K., Dolder, H. (2001). Ultrastructure of apyrene and eupyrene spermatozoa from the seminal vesicle of *Euptoieta hegesia* (Lepidoptera: Nymphalidae). *Tissue and Cell,* 33: 301-308.

Mantelatto, F.L., Fransozo, A. (1996). Size at sexual maturity in *Callinectes ornatus* (Brachyura – Portunidae) from the Ubatuba region (SP), Brazil. *Nauplius*, 4: 29-38.

Mantelatto, F.L., Fransozo, A. (1997). Fecundity of the crab *Callinectes ornatus* Ordway, 1863 (Decapoda, Brachyura, Portunidae) from the Ubatuba region, São Paulo, Brazil. *Crustaceana*, 70: 214-226.

Mantelatto, F.L., Fransozo, A. (1999). Reproductive biology and moulting cycle of the crab *Callinectes ornatus* (Decapoda, Portunidae) from the Ubatuba, São Paulo, Brazil. *Crustaceana*, 72: 64-76.

Mantelatto, F.L., Christofoletti, R.A. (2001). Natural feeding activity of the crab *Callinectes ornatus* (Portunidae) in Ubatuba Bay (São Paulo, Brazil): influence of season, sex, size, and molt stage. *Marine Biology*, 138: 585-594.

Mantelatto, F.L., Robles, R.A., Schubart, C.D., Felder, D.L. (2009). Molecular phylogeny of the genus *Cronius* Stimpson 1860, with reassignment of *C. tumidulus* and several American species of *Portunus* to the genus *Achelous* de Haan, 1833 (Brachyura: Portunidae). In: J. W. Martin, K. A. Crandall, and D. L. Felder (Eds.), *Decapod Crustacean Phylogenetics.* Crustacean issues 18, 567-579. Boca Raton: Taylor and Francis/CRC Press.

Medina, A., Rodríguez, A. (1992). Structural changes in sperm from the fiddler crab, *Uca tangeri* (Crustacea, Brachyura), during the acrosome reaction. *Molecular Reproduction Development*, 33: 195-201.

Medina, A. (1994). Spermiogenesis and sperm structure in the shrimp *Parapenaeus longirostris* (Crustacea: Dendrobranchiata): comparative aspects among decapods. *Marine Biology*, 119: 449-460.

Melo, G.A.S. (1996). *Manual de Identificação dos Brachyura (Caranguejos e Siris) do Litoral Brasileiro.* São Paulo, Plêiade/FAPESP. 604p.

Mello, M.L.S., Vidal, B.C. (1980). *Práticas de Biologia Celular*. Edgar Blucher – FUNCAMP, São Paulo. 69p.

Mescher, A.L. (2018). *Junqueira's Basic Histology Text and Atlas*. 15th ed, McGraw-Hill, New York. 1136p.

Moriyasu, M., Benhalima, K. (1998). Snow crabs, *Chionoecetes opilio* (*O. Fabricius*, 1788) (Crustacea: Majidae) have two types of spermatophore: hypotheses on mechanism of fertilization and population reproductive dynamics in the southern Gulf of St. Lawrence, Canada. *Journal of Natural History,* 32: 1651-1665.

Moriyasu, M., Benhalima, K., Duggan, D., Lawton, P., Robichaud, D. (2002). Reproductive biology of male jonah crab, *Cancer borealis* Stimpson, 1859 (Decapoda, Cancridae) on the Scotian Shelf, Northwestern Atlantic. *Crustaceana*, 75: 891-913.

Moses, M.J. (1961). Spermiogenesis in the crayfish *(Procambarus clarkii)*. II. Description of stages. *The Journal of Biophysical and Biochemical Cytology*, 10: 301-333.

Nagao J., Munehara, H. (2003). Annual cycle of testicular maturation in the helmet crab *Telmessus cheiragonus*. *Fisheries Science*, 69: 1200-1208.

Nascimento, F.A., Zara, F.J. (2013). Development of the male reproductive system in *Callinectes ornatus* Ordway, 1863 (Brachyura: Portunidae). *Nauplius*, 21: 161-177.

Negreiros-Fransozo, M.L., Mantelatto, F.L., Fransozo, A. (1999). Population biology of *Callinectes ornatus* Ordway, 1863 (Decapoda, Portunidae) from Ubatuba (SP), Brazil. *Scientia Marina*, 63: 157-163.

Ng, P.K.L., Guinót, D., Davie, P.J.F. (2008). Systema Brachyurorum: Part I. An Annotated Checklist of Extant Brachyuran Crabs of the World. *The Raffles Bulletin of Zoology*, 17: 1-286.

Oliveira, L.J.F., Zara, F.J. (2018) An investigation into the male reproductive system of two freshwater crabs from the Amazon: is there a sperm plug or packet formation? *Marine and Freshwater Behaviour and Physiology*, 51: 227-249

Pearson, P.J., Walker, M.H. (1975). Alteration of cytochrome-C oxidase activity during spermatogenesis in *Carcinus maenas*. *Tissue and Cell*, 164: 401-410.

Ravi, R., Manisseri, M.K., Sanil, N.K. (2014). Structure of the male reproductive system of the blue swimmer crab *Portunus pelagicus* (Decapoda: Portunidae). *Acta Zoologica*, 95: 176-185.

Robles, R., Schubart, C.D., Conde, J.E., Carmona-Suárez, C., Alvarez, F., Villalobos, J.L., Felder, D.L. (2007). Molecular phylogeny of the American *Callinectes* Stimpson, 1860 (Brachyura: Portunidae), based on two partial mitochondrial genes. Marine Biology. 150(6):1265-74. In: Rusell, L.; Griswold, M. (eds): *The Sertoli Cells*, pp. 660–683. Cache River Press, Clearwater FL.

Ryan, E.P. (1967). Structure and function of the reproductive system of the crab *Portunus sanguinolentus* Herbst (Brachyura: Portunidae). I. The male system. Proceedings of the Symposium on Crustacea at Erkulan, Índia, 1965. Part II. Symposium, Series 2, 522-544 + IIIpl.

Sant'Anna, B.S., Turra, A, Zara, F.J. (2012). Reproductive migration and population dynamics of the blue crab *Callinectes danae* in an estuary in southeastern Brazil. *Marine Biology Research*, 8: 354-362.

Sainte-Marie, G., Sainte-Marie, B. (1998). Morphology of the spermathecal, oviduct, intermediate chamber, and vagina of the adult snow crab (*Chionoecetes opilio*). *Canadian Journal of Zoology*, 76: 1589-1604.

Sainte-Marie, G., Sainte-Marie, B. (1999). Reproductive products in the adult snow crab (*Chionoecetes opilio*). I. Observations on spermiogenesis and spermatophore formation in the vas deferens. *Canadian Journal of Zoology*, 77: 440-450.

Sainte-Marie, G., Sainte-Marie, B., and Sévigny, J.M. (2000). Ejaculate storage patterns and the site of fertilization in female snow crabs (Chionoecetes opilio; Brachyura, Majidae). *Canadian Journal of Zoology*, 78: 1902-1917.

Sal Moyano, M.P., Gavio, M.A., Cuartas, E.I. (2010). Morphology and function of the reproductive tract of the spider crab *Libinia spinosa* (Crustacea, Brachyura, Majoidea): pattern of sperm storage. *Helgoland Marine Research*, 64: 213-221.

Severino-Rodrigues, E., Pita, J.B., Graça-Lopes, R. (2001). Pesca artesanal de siris (Crustacea, Decapoda, Portunidae) na região estuarina de Santos e São Vicente (SP), Brasil. *Boletim do Instituto de Pesca,* 27: 7-19.

Sforza, R., Nalesso R.C., Joyeux, J.C. (2010). Distribution and population structure of *Callinectes danae* (Decapoda: Portunidae) in a tropical Brazilian estuary. *Journal of Crustacean Biology,* 30: 597-606.

Simeó, C.G., Kurtz, K, Chiva, M., Ribes, E., Rotllant, G. (2010). Spermatogenesis of the spider crab *Maja brachydactyla* (Decapoda: Brachyura). *Journal of Morphology*, 271: 394-406.

Simeó, C.G., Ribes, E., Rotllant, G. (2009). Internal anatomy and ultrastructure of the male reproductive system of the spider crab *Maja brachydactyla* (Decapoda: Brachyura). *Tissue and Cell*, 41: 345-361.

Sokal, R.R., Rohlf, F.J. (1995). *Biometry: the principles and practice of statistics in biological research.* 3rd edition. W. H. Freeman and Co., New York. 887p.

Spalding, J.F. (1942). The nature and formation of the spermatophore and sperm plug in *Carcinus maenas*. *Quarterly Journal of Microscopical Science*, 83: 399-423.

Stewart, M.J., Stewart, P., Soonklang, N., Linthong, V., Hanna, P.J., Duan, W., Sobhon, P. (2010). Spermatogenesis in the blue swimming crab, *Portunus pelagicus*, and evidence for histones in mature sperm nuclei. *Tissue and Cell*, 42: 137-150.

Talbot, P., Beach, D. (1989). Role of the vas deferens in the formation of the spermatophore of the crayfish (*Cherax*). *Journal of Crustacean Biology*, 9: 9-24.

Tiseo, G.R., Mantelatto, F.L.M., Zara, F.J. (2014). Is cleistospermy and coenospermy related to sperm transfer? A comparative study of the male reproductive system of *Pachygrapsus transversus* and *Pachygrapsus gracilis* (Brachyura: Grapsidae). *Journal of Crustacean Biology*, 34: 704-716.

Tiseo, G.R., Mantelatto, F.L.M., Zara, F.J. (2017). Ultrastructure of spermatophores and spermatozoa of intertidal crabs *Pachygrapsus transversus*, *Pachygrapsus gracilis* and *Geograpsus lividus* (Decapoda: Grapsidae). *Zoologischer Anzeiger*, 269: 166–176.

Vallina, M., Sal Moyano, M. P., Cuartas, E. I., Gavio, M. A. (2014). Repreductive system and size of maturity of paddle crab *Ovalipes trimaculatus* (Brachyura: Portunidae) along the Argentine coast. *Journal of Crustacean Biology*, 34: 357-366.

Waiho, K., Fazhan, H., Jasmani, S., Ikhwanuddin, M. (2017). Gonadal development in males of the orange mud crab *Scylla olivacea* (Herbest, 1796) (Decapoda, Brachyura, Portunidae).

Crustaceana, 90: 1-19.

Watanabe, T.T., Sant'anna, B.S., Hattori, G.Y., Zara, F.J. (2014). Population biology and distribution of the Portunidae crab *Callinectes ornatus* (Decapoda: Brachyura) in an estuary-bay complex of Southern of Brazil. *Zoologia*, 31: 329-336.

Williams, A.B. (1974). The swimming crabs of the genus *Callinectes* (Decapoda: Portunidae). *Fishery Bulletin*, 72: 685-798.

Wong, N.A., Sewell, M.A. (2015). The reproductive ecology of the invasive Asian paddle crab, *Charybdis japonica* (Brachyura: Portunidae), in northeastern New Zealand. *Invertebrate Biology*, 134: 303-317.

Zana, U., Lino-Neto, J., Dolder, H. (2001). Ultrastructure of spermatozoa in *Plebeia droryana* Friese (Hymenoptera: Apidae: Meliponina). *Journal of Hymenoptera Research,* 10: 261-270.

Zara, F.J., Reigada, A.L.D., Passero, L.F.D., Toyama, M.H. (2009). *Myzobdella platensis* (Hirudinida: Piscicolidae) is true parasite of blue crabs (Crustacea: Portunidae). *The Journal of Parasitology*, 95: 124-128.

Zara, F.J., Toyama, M.H., Caetano, F.H., López-Greco, L.S. (2012). Spermatogenesis, spermatophore, and seminal fluid production in the adult blue crab *Callinectes danae* (Portunidae). *Journal of Crustacean Biology*, 32: 249-262.

Zara, F.J., Gaeta, H.H., Costa, T.M., Toyama, M.H., Caetano, F.H. (2013). The ovarian cycle histochemistry and its relationship with hepatopancreas weight in the blue crab Callinectes danae (Crustacea: Portunidae). *Acta Zoologica*, 94: 134-146.

Zara, F.J., Pereira, G. R, Sant'Anna, B.S. (2014). Morphological changes in the seminal receptacle during ovarian development in the speckled swimming crab *Arenaeus cribrarius*. *The Biological Bulletin* (Lancaster), 227: 19-32.

Capítulo 2

Can a different pattern of seminal fluid production contribute to the invasion success of the non native crab *Charybdis hellerii* (Portunidae)?

Prólogo – Capítulo 2

Este capítulo também derivou de dados do projeto Jovem Pesquisador da FAPESP do meu orientador (Proc. #2005/04707-5), mas já em uma etapa mais posterior. Esta espécie foi encontrada em sua maioria em costões rochosos na baía de São Vicente e estudada por mim desde a minha Iniciação científica, onde mostrei, por meio de dados ecológicos, que a espécies está consolidada na região e é uma das mais abundantes nos costões e na faixa consolidada nas praias estudadas.

Os dados morfológicos do sistema reprodutor masculino, inicialmente causaram confusão quanto à classificação do estágio de desenvolvimento do sistema reprodutor. Os dados iniciais provêm de outros bolsistas de iniciação científica vinculado ao projeto Jovem Pesquisador, e foram complementados posteriormente dando corpo a este capítulo.

Capítulo 2

Can a different pattern of seminal fluid production contribute to the invasion success of the non native crab *Charybdis hellerii* (A. Milne-Edwards, 1867) (Brachyura: Portunidae)?

ABSTRACT

Biological invasions are an important environmental issue worldwide since, among many effects, they can affect native populations. The Portunidae crab *Charybdis hellerii* is a successful invasive marine species. In this study, we describe the male reproductive system under light and electron microscopy and evaluate the gonadosomatic index (GSI) and spermatophore dehiscence. The testis is of the lobular-type and releases the spermatozoa immersed in an acidophilic secretion that fills the vas deferens. The reproductive system of *C. hellerii* is smaller, and its GSI much lower (0.4%), than those of other crabs that produce sperm plugs. The distal portion of the anterior vas deferens is the main site of spermatophore storage, which is uncommon in Portunidae. Lateral pockets are found in the very short middle and posterior vas deferens, where no spermatophore is found. The strong muscular layer of this region participates in the sperm transfer. The usual spermatophore wall of Portunidae is absent and the round to elliptical and even cleistospermic spermatophore is enclosed by an acidic polysaccharide secretion mass. Thus, the spermatophore is of the mucous-type and undergoes dehiscence in seawater or after passing through the gonopod I. *Charybdis hellerii* also differs from portunid species that produce sperm plugs regarding the composition of the vas deferens luminal secretion, being more similar to species that lack this feature. The cells of the entire vas deferens are typically secretory. The cytoplasm is filled with rough endoplasmic reticulum and Golgi complex, and along the posterior vas deferens the cytoplasm has a high concentration of vesicles. *Charybdis hellerii* differs from other portunid species described regarding the mode of seminal fluid production and spermatophore morphology, and it does not produce sperm plugs. Therefore we suggest that *C. hellerii* can mate during the intermolt (when females are hard-shelled), and multiple times. This different new reproductive strategy found to this portunid crab can increase the offspring gene variability and could play an important role in the colonization of new habitats.

Keywords: Thalamitinae, vas deferens, dehiscence, swimming crab, histology, ultrastructure.

INTRODUCTION

The establishment of invasive species threatens the native biodiversity (Ricciardi et al., 2000; Geiger et al., 2005; Bolaños et al., 2012). The consequent intensification of cargo ship traffic is the main introduction pathways of aquatic species (Kerckhof et al., 2007; Katsanevakis et al., 2014). Not surprisingly, along the Brazilian coast, exotic brachyuran species are recorded including *Pilumnoides perlatus* (Poeppig, 1836), *Pyromaia tuberculata* (Lockington, 1877), *Cancer pagurus* Linnaeus, 1758, *Rhithropanopeus harrisii* (Gould, 1841) and the Portunidae species, *Liocarcinus navigator* (Herbst, 1794) as *Polybius navigator* in Melo and Crivelaro (2002), *Scylla serrata* (Forskål, 1775) and *Charybdis hellerii* (A. Milne-Edwards, 1867), which is one of the most well stablished in Brazil (Negreiros-Fransozo, 1996; Tavares and Mendonça, 1996; D`Incao and Martins, 1998; Melo et al., 2000; Mantelatto and Garcia, 2001; Sant'Anna et al., 2012). Aside from *C. hellerii* only *Py. tuberculata, R. harrisii* have stablished populations in the Brazilian coast (Bertini et al., 2004; Rodrigues and D'Incao, 2015), while is suspected *Pi. perlatus* is also probably stablished in Brazil since an ovigerou female was sampled (Mello et al., 2000), but there is no further population data.

Charybdis hellerii is native to the Indo-Pacific Ocean and is one of the most widespread exotic species worldwide (Dineen et al., 2001; Sant'Anna et al., 2012). The ballast water is the most likely introduction pathway of *C. hellerii* to the Western Atlantic Coast (Lemaitre, 1995; Carqueija and Gouvêa, 1996). Populations of *C. hellerii* often become well-established in the areas where they are introduced (Dineen et al., 2001; Sant'Anna et al., 2012). This species has an extended larval development (44 days) which increases their survival success while being transported by the ships (Dineen et al., 2001). The successful establishment of *C. hellerii* along the Brazilian coast is also facilitated by its fast growth and short time to reach sexual maturity,

ability to store sperm and produce multiple broods, and the carnivorous generalist diet that enables the exploration of different habitats (Dineen et al., 2001; Mantelatto and Garcia, 2001; Sant'Anna et al., 2015). Invasive species may impact the biology and survival of native species, thus, the invasion of *C. hellerii* is suggested to be responsible for the population decline of *Cronius ruber* (Lamarck 1818) in the rocky shores of Santos-São Vicente Bay, in the coast of São Paulo (Mantelatto et al., 2009; Sant'Anna et al., 2012).

There is a considerable knowledge about the ecology of *C. hellerii* (Dineen et al., 2001; Mantelatto and Garcia, 2001; Bolaños et al., 2012; Sant'Anna et al., 2012; Watanabe et al., 2015), but the morphology of the reproductive system has not been described yet. Improving the knowledge on the biology of invasive species possibly helps to minimize their negative effects on the native species (Balasubramanian and Suseelan, 1998; Bolaños et al., 2012). Reproductive features of Brachyurans can help to explain the invasion success of exotic species. Brachyurans have a bilateral, H-shaped male reproductive system located in the cephalothorax, comprised by a pair of testes, vasa deferentia, and ejaculatory ducts. The testes are tubular organs classified into two types, lobular or tubular, and extend from the periphery of the anterior edge of the cephalothorax to the central region of the cephalothorax (Nagao and Munehara, 2003; Simeó et al., 2009). The testes of portunids, at least to *Callinectes*, are classified as lobular type according to the histology (Zara et al., 2012; Nascimento and Zara, 2013).

The vas deferens is connected to the testis and is divided into three regions: anterior (AVD), middle (MVD), posterior (PVD) (Krol et al., 1992). The sperm is transported to the AVD where the spermatophores are produced (Zara et al., 2012). Most of the granular seminal fluid is produced in the MVD by many lateral outpocketings (Cronin, 1947; Johnson, 1980; Zara et al., 2012; Nascimento and Zara, 2013). The mature spermatophores of portunoids have a

spermatophore wall (pellicle) and, in *Carcinus maenas* Linnaeus 1758 and *Arenaeus cribrarius* (Lamarck 1818), spermatophore dehiscence has not been detected in seawater (Spalding, 1942; Zara et al., 2014). The PVD has lateral outpocketings and nearly no spermatophores. Although it participates in the seminal fluid production, this fluid is homogeneous rather than granular as in the MVD (Ryan, 1967; Johnson, 1980; Zara et al., 2012; Nascimento and Zara, 2013; Ravi et al., 2014; Waiho et al., 2017). In *Charybdis japonica* (A. Milne-Edwards, 1861) a peculiar feature was described: the lateral outpocketings are restricted to the PVD (Wong and Sewell, 2015).

The general morphology of the vas deferens described above was based on studies on Portunoidea species from the genera *Callinectes*, *Carcinus*, and *Portunus*, and is related to the production of sperm plugs (Spalding, 1942; Ryan, 1967; Johnson, 1980; Bawab and El-Sherief, 1988; Zara et al., 2012; Nascimento and Zara, 2013). The sperm plug seals off the female seminal receptacle and prevents subsequent copulations (Spalding, 1942; Hartnoll, 1969; Diesel, 1991; Jensen et al., 1996; Hines et al., 2003; Wolcott et al., 2005; Guinot et al., 2013), and most species of the family Portunidae are known to produce sperm-plugs (Hartnoll, 1969; McLay and López-Greco, 2011).

In crabs, the reproductive investment has been commonly evaluated using the gonadosomatic (GSI) and the hepatosomatic (HSI) indexes, which also help to detect reproductive peaks and seasonality of reproductive events (López-Greco et al., 1999). These indices were mostly used to evaluate the reproduction strategy of Portunid species that produce sperm plugs such as *Callinectes* spp. (Cronin, 1947; Zara et al., 2012; Nascimento and Zara, 2013), *Carcinus aestuarii* Nardo, 1847 (Baklouti et al., 2013), and *S. serrata* (Quinitio et al., 2007). These species often have a high GSI; however, it was demonstrated that *C. japonica* has low GSI (Wong and Sewell, 2015). This is an unusual feature, given that species that produce

sperm plugs usually produce large quantities of seminal secretion (Hartnoll 1969; Hines et al., 2003; Zara et al., 2012; Nascimento and Zara 2013, Zara et al., 2014).

Since the invasion success of *C. hellerii* seems to be at least in part attributed to its reproductive features, in this study we investigate the morphology of the male reproductive system of *C. hellerii* and also the production of seminal fluid, through the combination of histochemistry and ultrastructure. The seasonal seminal fluid production, gonadosomatic index, spermatophore production and dehiscence were evaluated to fill the gaps in the biological knowledge of this species. Thus, we expect that this invasive species do not follow the seminal fluid production predicted to all other sperm plug Portunidae species. This information can answer important questions regarding the reproductive success of this invasive species.

MATERIAL AND METHODS

Field sampling and gross morphology

The crabs were monthly collected from May 2009 to April 2010 in three beaches of São Vicente Bay, São Paulo, Brazil: Itararé (Porchat Island), Milionários, and Gonzaguinha. The sampling methods and cephalothoracic width (CW) measurements followed Sant'Anna et al. (2012). The crabs were identified and classified by sex and sexual maturity based on the abdomen morphology and adherence to the thoracic sternite. The abdominal adherence is not visible in very small animals and in these cases the male reproductive system was checked macroscopically. The presence of spermatophores was confirmed by squashes of the MVD (Sant'Anna et al., 2012; Zara et al., 2012). The animals were anesthetized by chilling (5 min at -20ºC) (López-Greco et al., 1999). For the macroscopic study of the reproductive system the

dorsal carapace and heart were removed, and the animals were immersed in the same light microscopy fixative to be analyzed under a Leica stereomicroscope.

Hepatosomatic and gonadosomatic indices

The reproductive system and hepatopancreas of males were separately weighed using an analytical scale (0.001g). The gonadosomatic (GSI) (vasa deferens and testes) and hepatosomatic (HSI) indices were obtained by the ratio of the wet organ weight (WW) to the total animal body wet weight (AW) and transformed into percentage (WW/AW x 100) (Kyomo, 1988; Zara et al., 2012; Nascimento and Zara, 2013). Due to the range of 30 to 35 mm of carapace width comprises both juvenile and mature adult individuals (with spermatophores) and there were no juveniles above this size class; the animals were classified into size classes of 5 mm range. The Kolmogorov-Smirnov test was applied to check the normality of the data. The post-hoc Dunn test ($P \leq 0.01$) was performed when the Kruskal-Wallis test indicated a significant difference between GSI and HSI per the seasons and size classes (Sokal and Rohlf, 1995).

Light microscopy, and transmission and scanning electron microscopy (TEM and SEM)

Tissue samples of nine males (40–65 mm CW) were fixed for 24 h in 4% paraformaldehyde prepared with seawater from the sampling site. After fixation, the samples were kept overnight in sodium phosphate buffer 0.2 M (pH 7.2). Then they were dehydrated in an ascending ethanol series (70−95%) and embedded in glycol methacrylate resin (Leica® historesin kit) for 72 h at 4ºC. After polymerization, the blocks were sectioned (4–7 µm) using a rotating Leica RM2245 microtome. The histological description was based on the hematoxylin and eosin technique (HE) (Junqueira and Junqueira, 1983). Proteins were detected by staining with Bromophenol blue (Pearse, 1985) and xylidine ponceau (Mello and Vidal, 1980). Acidic

and neutral polysaccharides were detected by staining with Alcian blue (pH 2.5 and pH 1.0) (Junqueira and Junqueira, 1983) and periodic acid of Schiff (PAS), respectively (Junqueira and Junqueira, 1983). The simultaneous use of Alcian Blue/PAS (Junqueira and Junqueira, 1983) was used to describe the distribution of acidic and neutral polysaccharides in the tissue.

The preparation of tissue samples for the transmission electron microscopy (TEM) was done by the following procedures. Samples were fixed in 2.5% glutaraldehyde with 0.2% picric acid (Mancini and Dolder, 2001; Zana et al., 2001) and 0.1 M sodium cacodylate in buffered seawater (pH 7.6) for 4 h. They were washed twice for 15 min in the same buffer and post-fixed in 1% buffered osmium tetroxide for 1 hour. Then, they were "en bloc" stained in 2% uranyl acetate, dehydrated in an ascending acetone series (70–100%), and embedded in Epon-Araldite resin. After polymerization, the blocks were sectioned in a Leica UC7 ultramicrotome. Ultrathin sections were collected on a copper grid and contrasted in 2% uranyl acetate and 2% lead citrate (Zana et al., 2001). The samples were photographed with a Jeol J1010 at 80 kV electron beam.

For the scanning electron microscopy (SEM), the first gonopod pair of males, previously fixed in paraformaldehyde 4%, was removed and washed in phosphate buffer 0.2 M (pH 7.6), dehydrated in an ethanol series (30–100%), and dried in critical-point dryer (EMS 850). The sample was attached to stubs and sputtering-coated with gold (5 nm) in a sputtering Denton Vacuum Desk II. The samples were photographed with a Zeiss Evo 10 using a 10 kV electron beam.

Spermatophore dehiscence

The protocol described by Beninger et al. (1993) was adapted to evaluate the spermatophore dehiscence in seawater. The main change was the use of a microscope instead of a stereomicroscope due to the spermatophores' small size. Spermatophores from the distal AVD (AVDd) and MVD of three *C. hellerii* mature males were mixed and diluted in 3 ml of seawater. After a quick and gentle mixing of the reproductive system contents, a 100 µL aliquot was placed on an excavated slide and covered with a coverslip. The site with the highest number of spermatophores (over 30) was located using a 10X objective lens and photographed for 90 min at 5-min intervals, using a Zeiss AxioVision differential interference phase contrast microscope (DIC).

The seminal fluid was observed after going through the gonopod I. The seminal fluid of the AVDd and MVD were mixed in 1 ml of seawater. After a quick and gentle mix, a 300 µm aliquot was taken with a 1 ml syringe and the seminal fluid was slowly pulled through the gonopod I. After this procedure, the fluid that went through the gonopod I was placed on an excavated slide, covered with a coverslip, and observed under the same microscope.

RESULTS

Gross morphology and GSI and HSI indices

The bilateral H-shaped male reproductive system of *C. hellerii* has a pair of testes extending from the anterior margin of the cephalothorax to the hepatopancreas (Fig. 1A and B). The reproductive organs are small and the testes are as long as the vasa deferentia (Fig. 1B). The testes are connected to each other by a transverse commissure below the stomach, near the pair

of vasa deferentia, beneath the heart (Fig. 1A and C). The testis seems like a whitish tangled tube filled with many seminiferous lobules beneath the connective tissue (Fig. 1D) and ends in the vas deferens (Fig 1.A–C). The transverse commissure has seminiferous lobules and connects laterally to each testis that extends to the vas deferens (Fig. 1C, E and F). The vas deferens is divided into three anatomical regions. In fresh dissections, the AVD is a milky convoluted tube of smooth surface that has two anatomical portions. The proximal AVD (AVDp) is a thin and very convoluted tube connected to the testis. The distal AVD (AVDd) is a wide and convoluted duct that leads to an even wider duct (Fig. 1C and E). The AVDd is filled with a large amount of seminal fluid (pale yellow in fixed animals), extends deeply into the ventral cephalothoracic region, and opens in the MVD. The MVD is very thin and filled with the same milky secretion observed in the AVD (Fig. 1C and E). The MVD has many small rounded folds, the outpocketings (Fig. 1E and G). The MVD ends in the PDV. This transition is marked by an abrupt change in the luminal fluid color from milky (or pale yellow) to translucent (Fig. 1C, E and G). The PVD is located ventrally in the cephalothoracic cavity. Its diameter increases toward the posterior margin of the cephalothorax where the PVD anteriorly bents toward the gonopore opening (Fig. 1A–C and H). The PVD outpocketings increase in number and diameter from the proximal to the distal portion (Fig. 1B and C). The PVD ends in the ejaculatory duct, which is a cuticle-lined epithelium with smooth surface, without outpocketings, that opens in the gonopore at the base of the gonopod I (Fig. 1H). Interestingly, the male reproductive system of very small (30.2 mm CW) and large (72.7 mm CW) *C. helerii* individuals had the same features, and no diferent development stages were detected upon the macroscopic evaluation.

In total, we dissected and evaluated 164 males through the sampling year. The mean GSI was 0.42 ± 0.25 (0.10–1.62) and the mean HSI was 5.12±1.57 (2.09–11.09). Neither the GSI (H

= 10.85, p = 0.012) nor the HSI (H = 0.2983, p = 0.96) varied seasonally (Fig. 2A). The smallest physiologically and morphologically mature male had 30.2 mm CW, although juveniles were also present in the 30–35 mm size class. The GSI did not differ between the size classes (H=20.05, p=0.011) (Fig. 2B). On the other hand, the HSI differed between a few classes (H=36.74; p<0.01): the 40−45mm differed from the 65−70 mm class (Dunn test, p<0.01), and the 45−50mm class differed from the 60−65 mm and 65−70 mm classes (Dunn test, p<0.01) (Fig. 2B).

Spermatophore and seminal fluid formation

The spermatozoa coming from the seminiferous duct are released in the vas deferens, where they are packed and stored. The AVDp and AVDd have different functions and characteristics. The convoluted AVDp has a simple columnar epithelium and an irregular basophilic nucleus at the cell base, and an evident nucleolus (Fig. 3A, B). The epithelium lies on a thin connective and muscular layers (Fig. 3B). The AVDp lumen is full of free spermatozoa immersed in a basophilic type I secretion (Fig. 3B). This secretion was negative for proteins, whereas the acrosomal vesicle of the spermatozoon was positive (Fig. 3C, D). The type I secretion is rich in carbohydrates, positive for neutral polysaccharides and strongly positive for acidic polysaccharides in Alcian blue at pH 2.5 and 1.0. The acrosomal vesicle was only reactive to PAS when stained with PAS/Alcian blue (Fig. 3E, F). In the AVDd, the type I secretion will form the spermatophore. Under TEM, the cytoplasm of this region has a well-developed rough endoplasmic reticulum (RER), irregular nucleus with dispersed heterochromatin blocks, and sometimes more than one large nucleolus (Fig. 3G, H). The basal cytoplasm has RER and many

mitochondria. The epithelium underneath the basal lamina lays on a connective tissue and muscular fibers (Fig. 3H, I). In the cell apex there are many RER cisternae, Golgi complex, and electron-dense vesicles, which are related to the secretion rich in acidic polysaccharides (Fig. 3J, K). The electron-dense vesicles are released by exocytosis among the short microvilli (Fig. 3L). Two types of secretion are identified in the lumen of the AVDp: the type I secretion is a homogeneous slightly electron-dense secretion that surrounds the spermatozoa and the type II secretion is less electron-dense and present in small areas among the sperm masses (Fig. 3L, M).

The AVDd is a less convoluted and very long duct with a lumen wider than in the AVDp (Fig. 4A) and it is the main site for the spermatophore storage. The epithelium is flat and simple with a flat- to round-shaped nucleus immersed in a very basophilic cytoplasm (Fig. 4B). Below the epithelium there is a thick acidophilic muscle layer positive for proteins (Fig. 4B, C). The type II secretion is produced in the AVD and separates the spermatozoon masses into mucous-type spermatophores (Fig. 4B–F). This secretion produces coenospermic spermatophores containing many or few spermatozoa, but cleistospermic spermatophores (one spermatozoon per spermatophore) are also found in the AVD (Fig 4C–E). The acidophilic luminal type II secretion is intensively positive for proteins, positive for neutral polysaccharides and negative for acidic polysaccharides (Fig 4C–F). The type I secretion has the same characteristics as in the AVDp and the mature spermatophore is immersed in a basophilic compound negative for proteins, positive for neutral polysaccharides, and strongly positive for acidic polysaccharides (pH 2.5). The acidic polysaccharides were preferentially stained by the conjugated PAS/Alcian blue staining (Fig. 4C–F).

Under TEM, the cells of the AVDp, and AVDd, have a basal nucleus and the cytoplasm is filled with RER, Golgi complex, and apical secretion vesicles (Fig. 4G–I). The basal region has

basal membrane folds and mitochondria. The epithelial basal lamina lays on a thin connective tissue over a muscle layer (Fig. 4H). In the apical cytoplasm, the Golgi complex shows a typical morphology and small electron-dense secretion vesicles are found in the *trans* face (Fig. 4I). These vesicles are also found next to the microvilli (Fig. 4J-L). The luminal type II secretion occurs among the spermatophores and becomes more electron-dense than that in the AVDp (Fig 4G, J-L). This secretion corresponds to the acidophilic glycoprotein under light microscopy (Fig. 4B-F). The mature spermatophores formed by the less electron-dense type I secretion have the same electron-density as in the ADVp (Fig. 4L). The spermatophore wall is absent, characterizing the spermatophore of the mucous-type. They vary from a rounded to an irregular spermatozoa mass, and it seems that larger spermatophores are formed by the fusion of smaller ones (Fig 4G, J-L).

The MVD is a short and narrow region containing a homogeneous acidophilic secretion, without any granules, surrounding a smaller amount of spermatophores than the AVDd. The epithelium is simple cubic, with irregular basophilic cytoplasm and nucleus (Fig. 5A, B). In this region, the vas deferens has discrete small outpocketings (Fig. 5A). The MVD luminal secretion has the same features as in the AVDd; it is intensely stained by Xylidine ponceau, positive for PAS, and negative for Alcian blue. These features characterize a glycoprotein with neutral polysaccharides of the type II secretion (Fig. 5C−E). Inside the spermatophores, the type I secretion is basophilic and negative for basic polysaccharides and total proteins (Fig. 5B−E). However, the secretion is intensely reactive for acidic polysaccharides in Alcian blue pH 2.5 but not in pH 1.0 (Fig. 5E, F). Under TEM, the MVD cells have an irregular nucleus with one or more large nucleolus, and the cytoplasm is also filled with a well-developed RER and many Golgi complex (Fig. 5G, H). The basal region is very irregular, with basal membrane folds and a

muscle layer juxtaposed to the epithelium (Fig. 5H). The apical cytoplasm has several secretion vesicles less electron-dense than those in the AVD, which are also released by exocytosis in the MVD (Fig. 5G, I, J). The luminal secretion is slightly heterogeneous, and it is possible to note different electron-densities in its components, making the secretion less electron-dense than in the AVDp (Fig. 5J). The spermatophores in the MVD are still composed of a wall-less sperm mass immersed in the type I secretion that is more electron-dense than the luminal matrix (Fig. 5K).

The PVD is a long tube, wider than the MVD, with outpocketings that become larger along the tube toward the distal end. The PVD also lays on a muscle tissue organized in layers of parallel fiber bundles and is thicker than the AVD or MVD (Fig. 6A–C). The cells of the simple PVD epithelium vary from cubic to elongate and have a large, central and basophilic nucleus (Fig. 6A. B). The PVD lacks spermatophores. A large amount of secretion with acidophilic granules surrounded by a basophilic homogeneous matrix fills the lumen (Fig. 6A, B). The matrix is weakly stained for proteins but the granules are positive (Fig 6C, D), and, unlike the unstained granules (Fig 6E, F), the matrix is strongly reactive for neutral and acidic polysaccharides (Fig. 6E, F). Under TEM, the epithelial cells are polarized and have a basal RER and a large amount of electron-dense secretion vesicles occupying the median to apical cytoplasm region (Fig. 6G). The basal region shows shallow basal membrane folds with many mitochondria (Fig. 6H). The nucleus is lobulated and contains many heterochromatin blocks. Secretion vesicles occur in the perinuclear cytoplasm (Fig. 6I). Next to the nuclear envelope there are well-developed Golgi with typical morphology (Fig. 6J, K). The apical region of these cells is filled with electron-dense vesicles (Fig. 6L, M) and the apical edge is marked by irregular

microvilli, clearly identified by the cytoskeleton elements (Fig. 6L, N). The luminal secretion has an electron-dense and homogeneous matrix and few less electron-dense materials (Fig. 6O).

Spermatophore dehiscence and ultrastructure of the Gonopod I

The spermatophores collected from the AVDd and MVD of adult males were partially dehiscent in seawater. The process of dehiscence occurs exponentially in the first few minutes. After 10 min, nearly 40% of spermatophores ruptured in contact with seawater (mostly small spermatophores), and, after 90 min, the percent of rupture was above 50% (Fig 7A). Intact spermatophores swelled by 12.42% (± 5.46) after 90 min; their size varied from 14671 ± 7766 μm^2 (at the start) to 16275 ± 8258 μm^2 (Fig 7B and C). The spermatophores in the seminal fluid going through the gonopod I ruptured just after being collected from the gonopod I aperture. Comparing the same microscopic area, the amount of free spermatozoa was higher than the number of spermatophores placed in seawater. Many fragmented rounded spermatophores (and a few non-ruptured) were detected in the first minutes (Fig. 7D). The first gonopod is a slender and long structure; the basal 1/3 is enlarged and the apical 2/3 is slender (Fig. 7E). Two rows of mesiodorsal sensilla are noticed in the apical 1/3. The transversal-sectioned 1/3 apical shows a narrow gonopod channel with 33 ± 7 µm of diameter (Fig. 7E and F). Next to the end of the basal 2/3 of the first gonopod, just before the mesioventral bents toward the sternum, the fractured gonopod still has a slender channel with 61 ± 4 µm (Fig. 7G). Both fractured areas have a smooth inner channel surface (Fig. 7H). In mesioventral view, the slender apex has another row of sclerotized sensilla, which runs toward the mesioventral opening. These apical sensilla lay on a carena where the seminal fluid goes through (Fig. 7I and J). Inside the gonopod opening there

are some materials on the inner surface (Fig. 7K). At a high magnification, the material corresponds to small tooth-like protuberances opposite to the seminal fluid stream, and many bacteria are attached to the surface (Fig 7L).

FIGURES

Figure 1. Internal anatomy and gross morphology of the male reproductive system in *Charybdis hellerii*. **(A)** Testes and vas deferens lying over the hepatopancreas of an adult *C. hellerii* (CW=55.6mm); **(B)** Complete reproductive system showing the "H-shaped" pair of testes and vas deferentia; **(C)** General view of the reproductive system; the posterior vas deferens bents (arrow) before the ejaculatory duct; **(D)** Detailed view of the testes depicting several seminiferous lobes; **(E)** Convoluted anterior vas deferens with a well-developed distal portion and a very short median portion containing spermatophores (arrow); **(F)** Commissure that joins the testes; **(G)** Transition between the median and posterior portions of the vas deferens, where spermatophores are no longer observed (arrow); **(H)** Transition between the posterior vas deferens and ejaculatory duct. AVDp, Proximal portion of anterior vas deferens; AVDd, distal portion of anterior vas deferens; C, commissure; ED, ejaculatory duct; Gl, gills; H, hepatopancreas; MVD, median van deferens; OT, outpocketings; PVD, posterior vas deferens; SL, seminiferous lobes; ST, stomach; T, testes; VD, vas deferens.

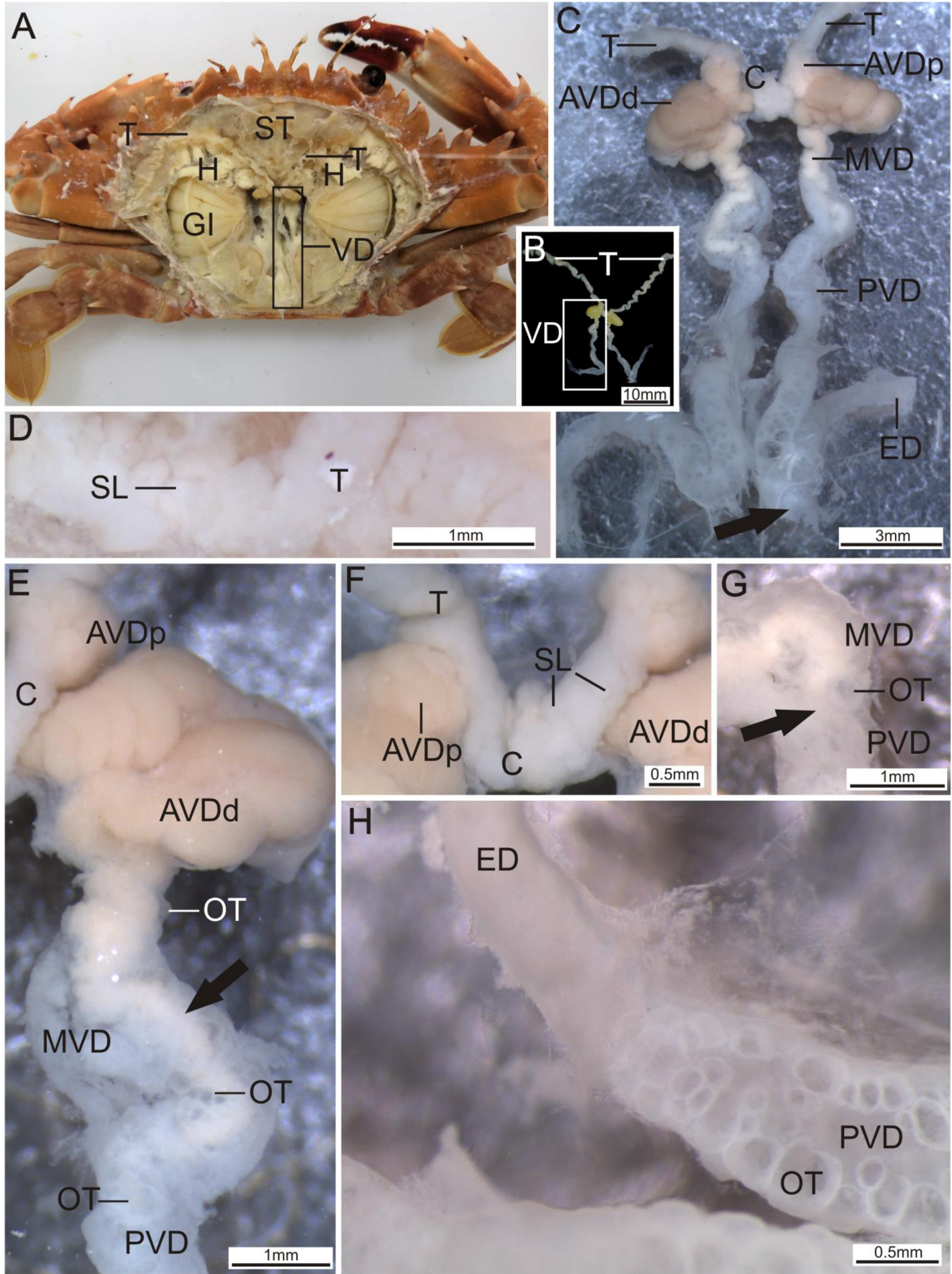
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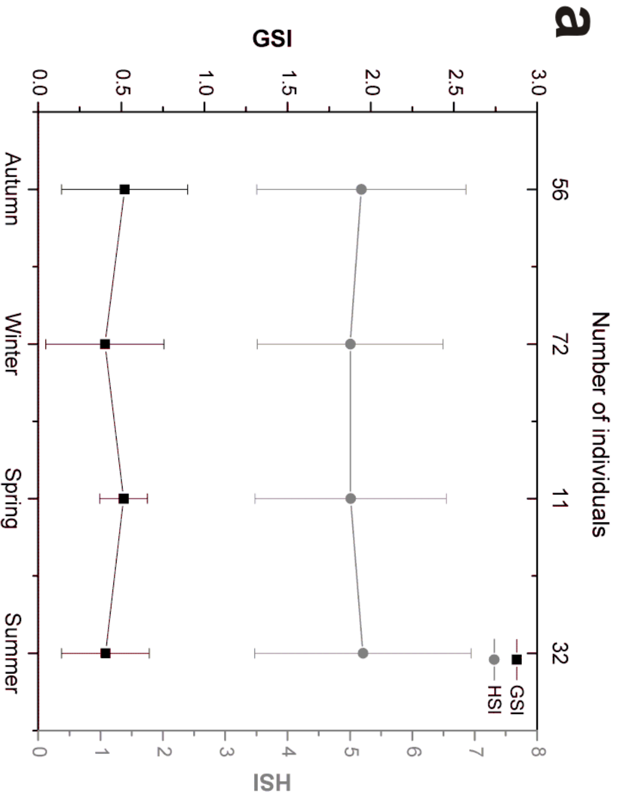
Figure 2. Gonadosomatic (GSI) and Hepatosomatic (HSI) indices. Average variation of GSI and HSI indices of males of *Charybdis hellerii* analyzed by season (A) and per size class (B). Equal letters indicates statistical differences.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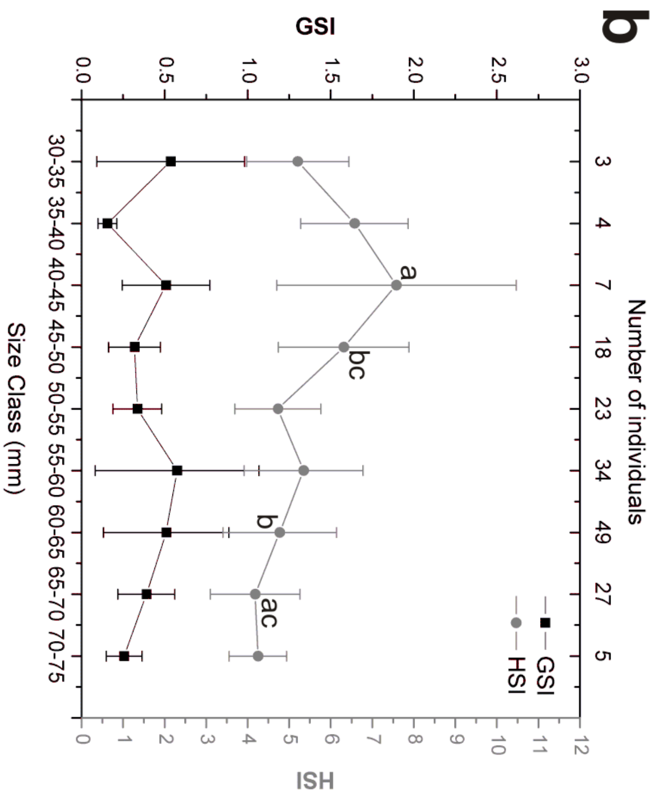
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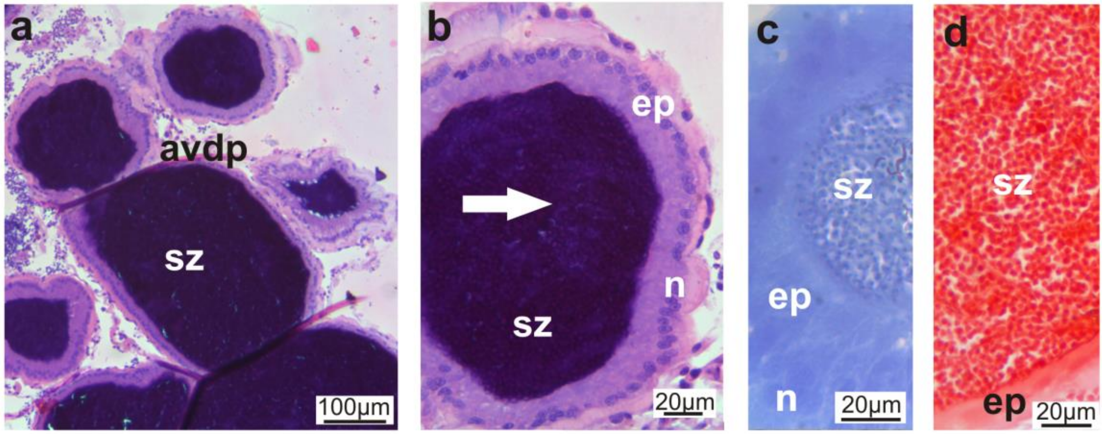
Size Class (mm)

Figure 3. Proximal portion of the anterior region of vas deferens (AVDp). **(A)** Overview of AVDp. HE staining. **(B)** Detail of the AVDp with columnar epithelium, in a mature male. Free spermatozoa in acidophilic secretion (arrow). HE staining. **(C)** AVDp in bromophenol blue, showing that the epithelium is positive for basic proteins, but the matrix is negative. **(D)** Luminal secretion of the AVDp, non-reactive to Xylidine Ponceau. **(E)** AVDp secretion, strongly positive upon the simultaneous staining by the periodic acid of Schiff (PAS) and Alcian blue (AA) pH 2.5. The black arrow shows the secretion reactive for acid polysaccharides; the white arrow shows the presence of neutral polysaccharides in the spermatozoa. **(F)** Secretion strongly positive for AA pH 1.0 (arrow). **(G)** Basal region of the AVDp epithelium with large quantities of rough endoplasmic reticulum. **(H)** Detail of the basal lamina, showing muscular tissue. **I** Detail of the muscular fibers. **(J)** Detail of the large quantity of rough endoplasmic reticulum and electron-dense vesicles. **(K)** *Trans* face of Golgi complex with electron-dense vesicles. **(L)** Apical region of the epithelium with presence of rough endoplasmic reticulum and electron-dense vesicles being released to the lumen. Presence of two secretions: one more electron-dense (black arrow) and other less electron-dense (white arrow). **(M)** Free spermatozoa enclosed by two secretions: one more electron-dense (white arrow) and other less electron-dense (black arrow). bl, basal lamina; ct, conjunctive tissue; ep, epithelium; gc, Golgi complex; mc, muscular fibers; n, nucleus; nu, nucleolus; rer, rough endoplasmic reticulum; sz, spermatozoa; v, vesicles.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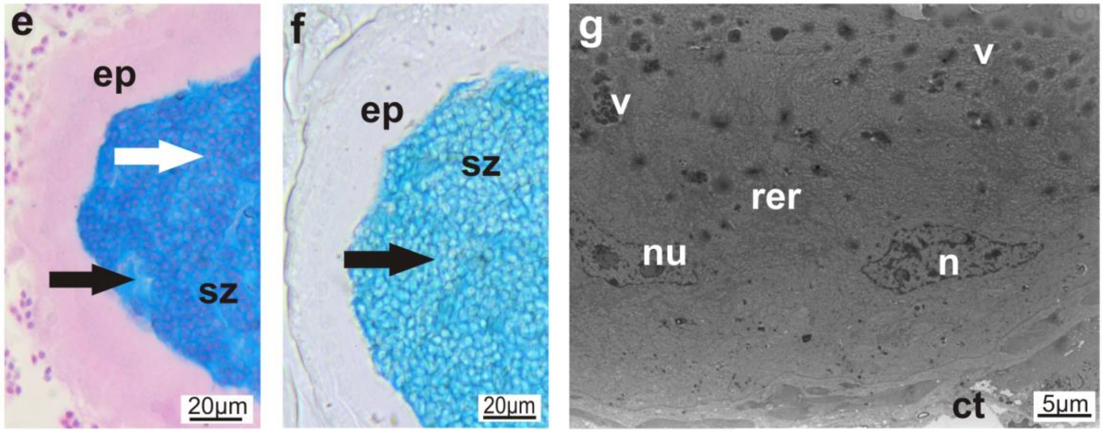
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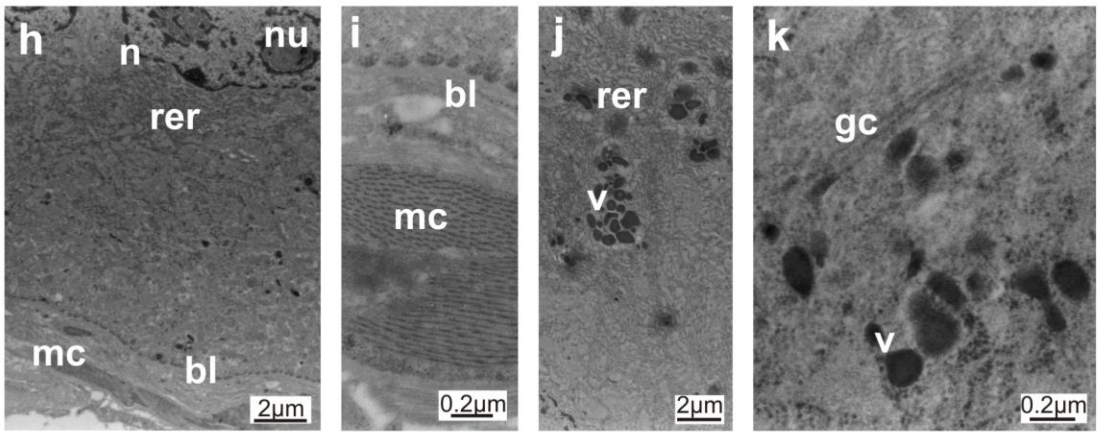
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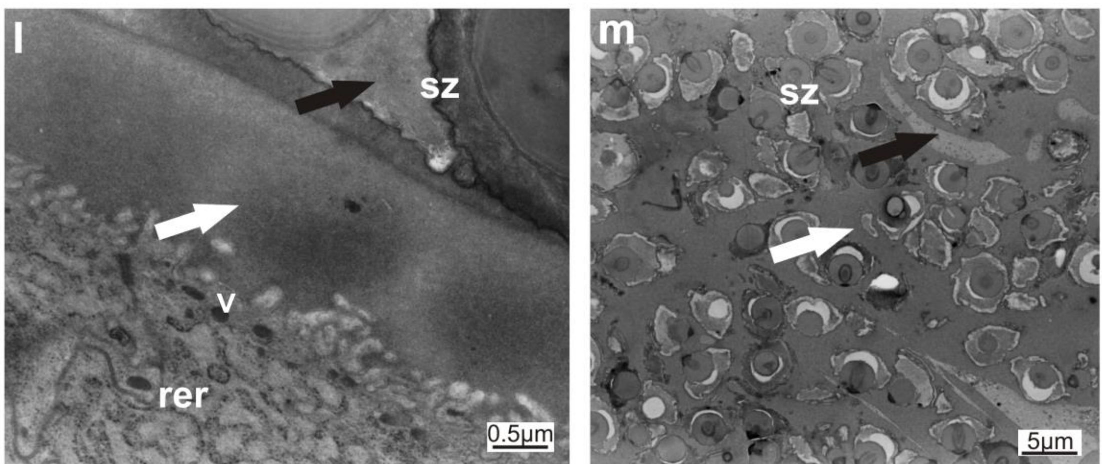
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Figure 4. Distal portion of the anterior vas deferens (AVDd). **(A)** Overview of AVDd. HE staining. **(B)** Detail of AVDd with mature spermatophores surrounded by acidophilic secretion (arrows). Notice the epithelium and muscular fibers. HE staining. **(C)** Luminal secretion matrix of the AVDd positive (black arrow) for total proteins. Inside the spermatophore there was no reaction for proteins (white arrow). Xylidine ponceau staining. **(D)** Simultaneous staining by the periodic acid of Schiff (PAS)/Alcian blue (AA) pH 2.5 in AVDd, strongly positive. The secretion that encloses the spermatophore is positive for neutral polysaccharides (black arrow), unlike the secretion inside the spermatophore (white arrow). **(E)** Secretion weakly positive for neutral polysaccharides (black arrow), and positive reaction inside the spermatophore (white arrow). Detail of a cleitospermic spermatophore (arrowhead). **(F)** Secretion of AVDd, negative for acid polysaccharides (black arrow) but positive inside the spermatophores (white arrow). **(G)** AVDd epithelium. Presence of less electron-dense secretion inside the spermatophore (white arrow), and a more electron-dense secretion being released (black arrow). **(H)** Basal region with muscle fibers, mitochondria and rough endoplasmic reticulum. **(I)** Median region of epithelium with Golgi complex and a large quantity of rough endoplasmic reticulum. **(J)** Difference between the secretion enclosing the spermatophore (black arrow) and the secretion inside the spermatophore (white arrow). **(K)** Cell apex with Golgi complex and vesicles with a more electron-dense secretion in the lumen and less electron-dense vesicles inside the spermatophore (white arrow). **(L)** Detail of the cell apex showing the electon-dense vesicles and both electron-dense (black arrow) and a less electron-dense (white arrow) luminal secretions. ep, epithelium; gc, Golgi complex; m, mitochondria; mc, muscle fiber; n, nucleus; nu, nucleolus; rer, rough endoplasmic reticulum; sp, spermatophore; sz, spermatozoa; v, vesicles.

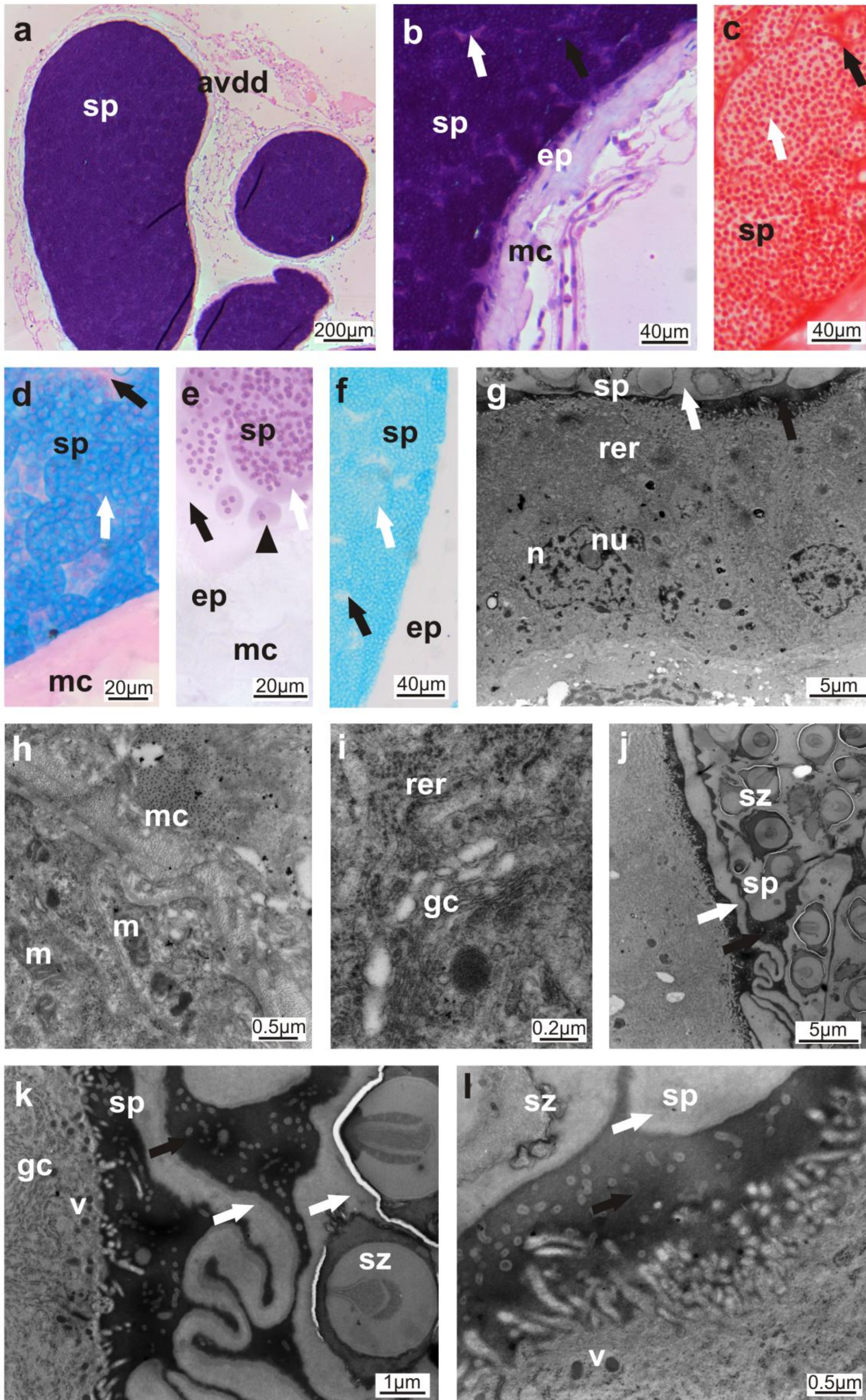
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Figure 5. Middle region of vas deferens (MVD). **(A)** Overview of MVD characterized by the large quantity of acidophilic secretion, completely formed spermatophore and small outpocketings. HE staining. **(B)** Detail of MVD showing acidophilic luminal secretion (black arrow) and basophilic secretion of the spermatophore (white arrow). **(C)** Luminal secretion (black arrow) of the MVD in bromophenol blue, strongly positive for basic proteins, as well the spermatophore secretion (white arrow) also positive but less intense. **(D)** MVD in Xylidine ponceau with luminal secretion (black arrow) strongly positive for total proteins. The spermatophore secretion shows a less intense reaction (white arrow). **(E)** MVD lumen with a secretion strongly positive for neutral polysaccharides (black arrow), and acid polysaccharides in the spermatophore (white arrow). Notice the presence of cleistospermic spermatophore (arrowhead). Staining by the periodic acid of Schiff (PAS)/Alcian blue (AA) pH 2.5. **(F)** MVD in AA pH 1.0 showing a non-reactive secretion in the lumen (black arrow) but reactive in the spermatophore (white arrow). Notice the presence of cleistospermic spermatophore (arrowheads). **(G)** Overview of MVD epithelium with a large quantity of rough endoplasmic reticulum and Golgi complex (GC) and secretory electron-dense vesicles in the cell apex (black arrow), in the lumen there is a less electron-dense secretion (white arrow). **(H)** MVD basal region with a large quantity of rough endoplasmic reticulum. **(I)** Detail of the apex showing a large quantity of RER and the release of vesicles in the lumen (arrowhead). **(J)** Presence of microvilli in the cell apex. **(K)** Spermatophore with a more electron-dense secretion (white arrow) than the lumen (black arrow). bl, basal lamina; ep, epithelium; gc, Golgi complex; l, lumen; mv, microvilli; mc, muscle fibers; n, nucleus; nu, nucleolus; rer, rough endoplasmic reticulum; ot, outpocketings; sp, spermatophore; sz, spermatozoa; v, vesicles.

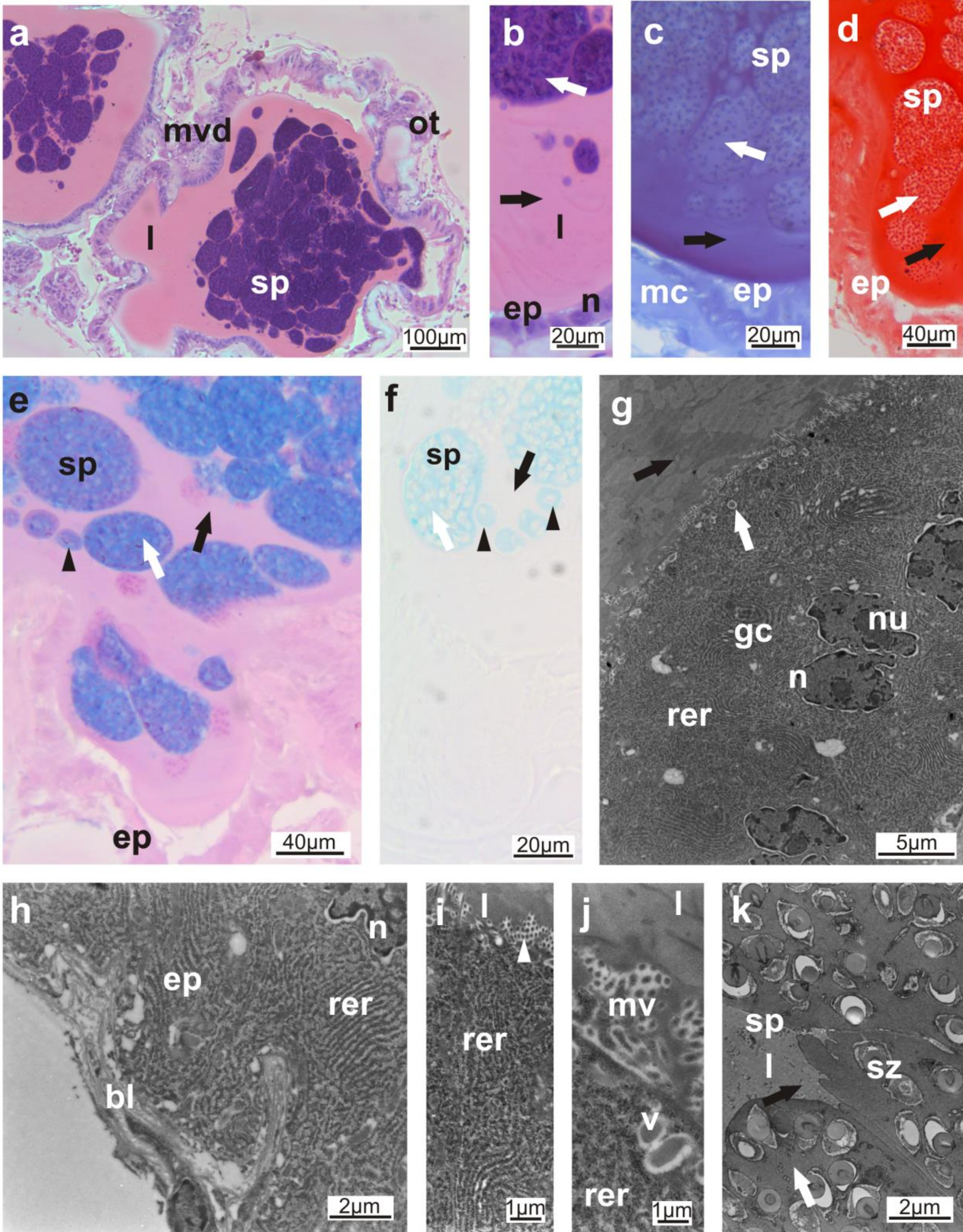
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Figure 6. Posterior region of vas deferens (PVD). **(A)** Overview of PVD. The luminal secretion shows presence of basophilic secretion (white arrow) and acidophilic (black arrow) granules. Also show columnar epithelium and muscular tissue. HE staining. **(B)** Detail of PVD showing basophilic secretion (white arrow) and basophilic granules (black arrow). HE staining. **(C)** PVD in xylidin ponceau with secretion granules (white arrow) weakly positive for total proteins and small outpocketings. **(D)** Detail of the PVD secretion, weakly positive for proteins **(E)** Luminal secretion (white arrow) positive for neutral polysaccharides; granules without reaction (black arrow). **(F)** Secretion (white arrow) positive for AA pH 2.5 and granules without reaction (black arrow). **(G)** Epithelial tissue of PVD with many vesicles. **(H)** Detail of the basal region with muscular tissue and mitochondria. **(I)** Detail of central nucleus and the beginning of the formation of the vesicles. **(J)** Base-median cytoplasm with Golgi complex rough endoplasmic reticulum. **(K)** Detail of developed Golgi complex and the rough endoplasmic reticulum. **(L)** PVD apex showing many vesicles. **(M)** Detail of the vesicles with less electron-dense regions and its envelope (arrowhead). **(N)** Detail of the PVD apex with many vesicles and evaginations to the lumen. **(O)** Detail of the electron-dense luminal secretion with some less electron-dense material. Ep, Epithelium; GC, Golgi complex; L, lumen; M, mitochondria; MC, muscle fiber; MV, Microvilli; N, nucleus; Nu, nucleolus; OT, outpocketings; RER, rough endoplasmic reticulum; S, secretion; V, vesicles.

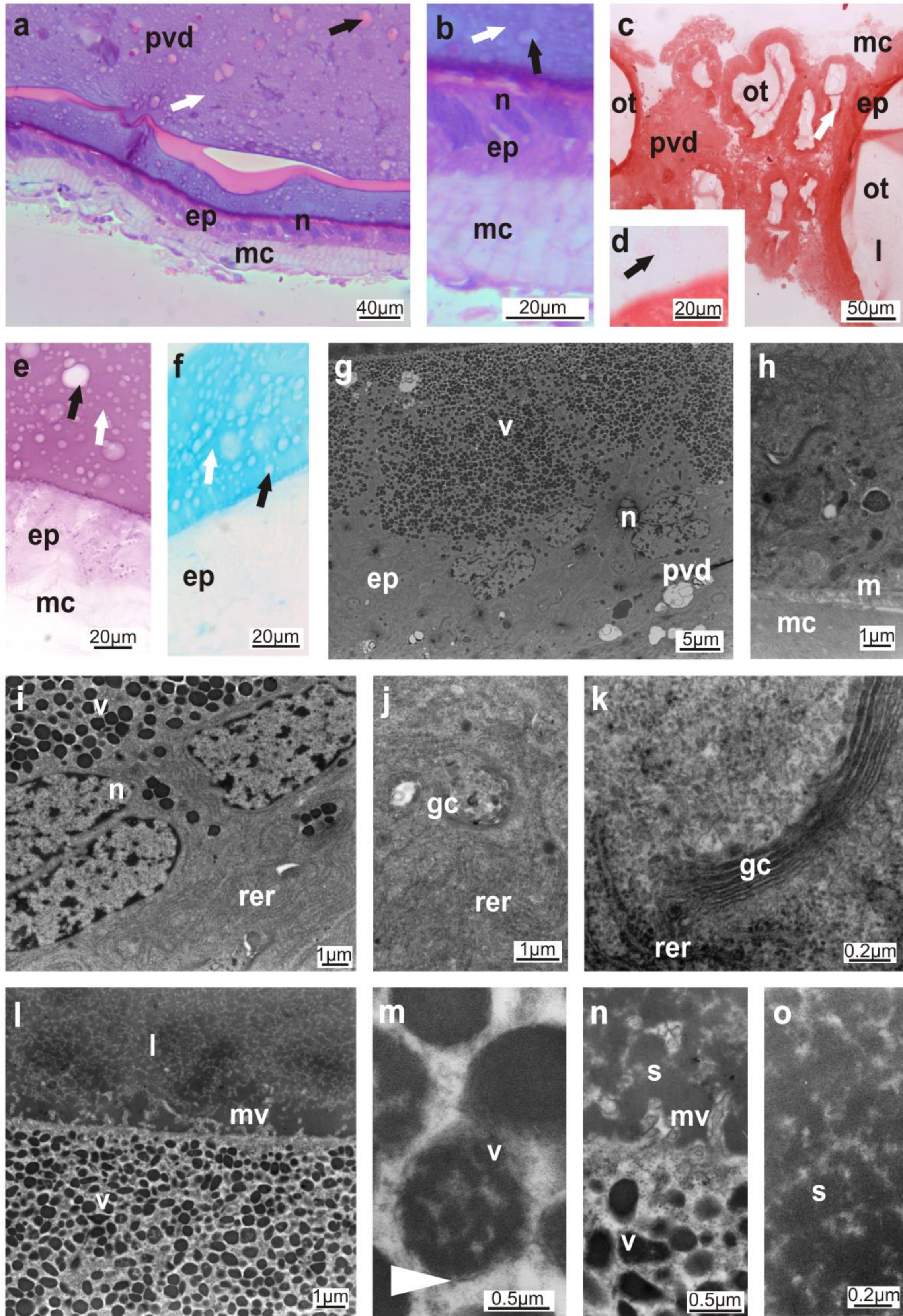
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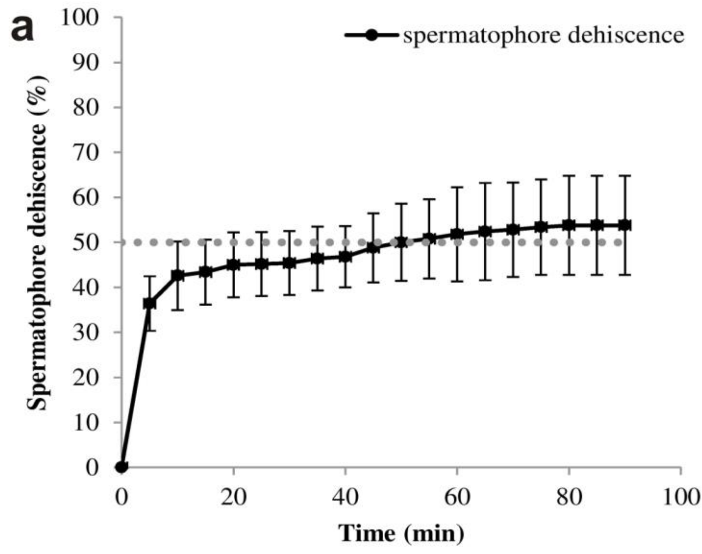
Figure 7. Dehiscence and first gonopod ultrastructure. **(A)** Percentage of dehiscence after 90 min after contact with seawater of spermatophores from posterior region of anterior vas deferens. **(B and C)**. Differential interference phase contrast microscopy showing the mucous-type spermatophores in time 5 min and 90 min of exposition to seawater, respectively. Notice the cone-like projections containing spermatozoa (arrow), and free spermatozoa (arrowhead). The bars depict the swelled spermatophore during the experiment. **(D)** Mechanically squeezed seminal fluid after going through the gonopod I. Most of the spermatozoa are free in the sample and some spermatophores have an irregular morphology (arrow), while only a few seem intact. **(E)**. Mesiodorsal view of the slender gonopod I, under scanning electron microscopy. Two rows of sensilla (arrow) are notice and the numbers indicate the fractured points. **(F and G)** Fractured points 1 and 2 from the previous picture showing the slender gonopod channel apex (arrows). **(H)** Detail of smooth surface (arrow) of the gonopod channel. **(I)** Mesioventral view of the slender apical 2/3 of gonopod I and one row of sensilla (arrow). **(J)** Detail of the mesioventral gonopore opening with a carena and an additional row of sensilla in front of the aperture (arrow). **(K)** Material (arrow) inside the gonopod opening. **(L)** Detailed view of the gonopod opening with the tooth-like protuberances in opposite streaming position (arrow) and many bacteria attached on the cuticle (arrowhead). CH = gonopod channel; GI = first gonopod; O = gonopod opening; SN = sensilla; SP = spermatophores; SZ = spermatozoa, SZ.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spermatophore dehiscence
Spermatophore dehiscence (%)
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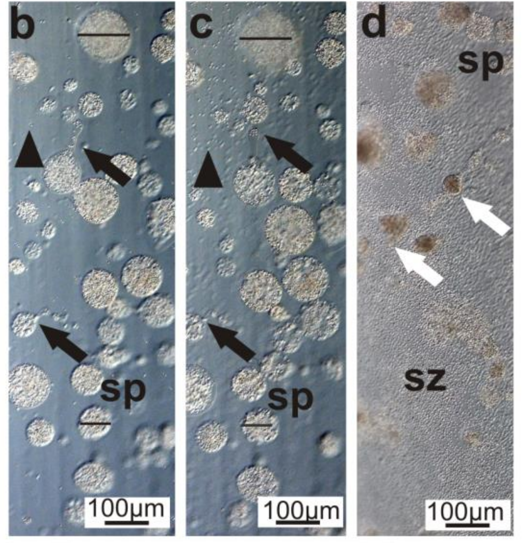
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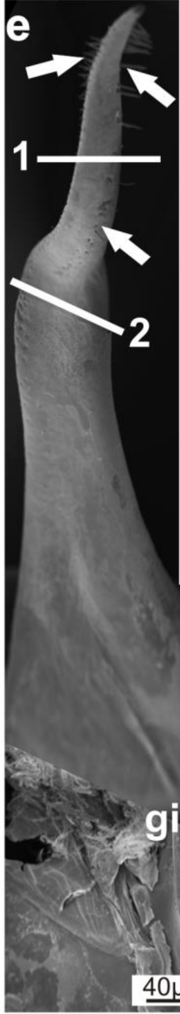
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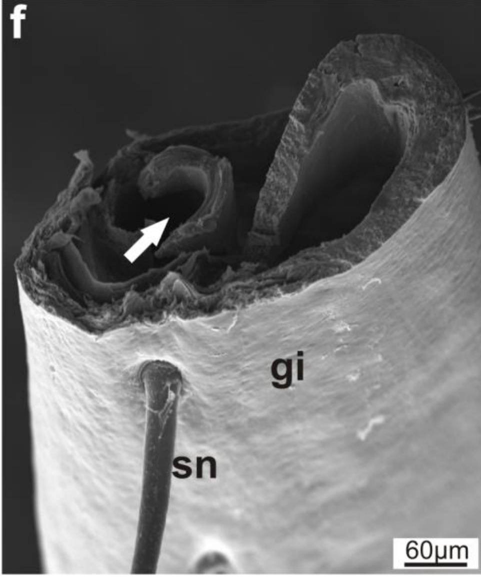
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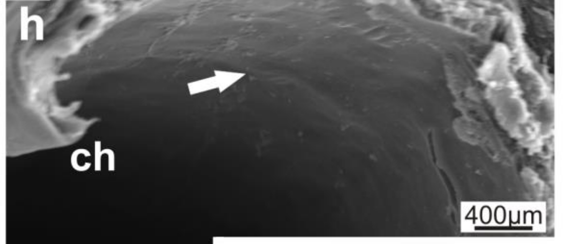
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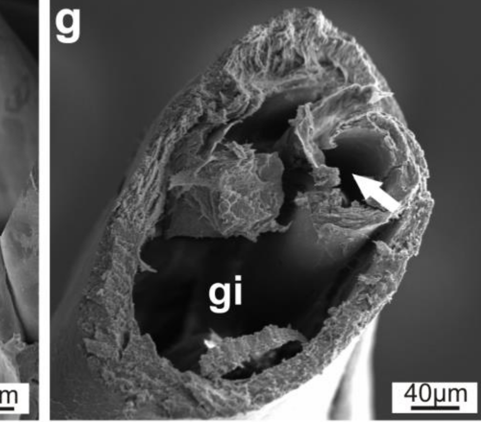
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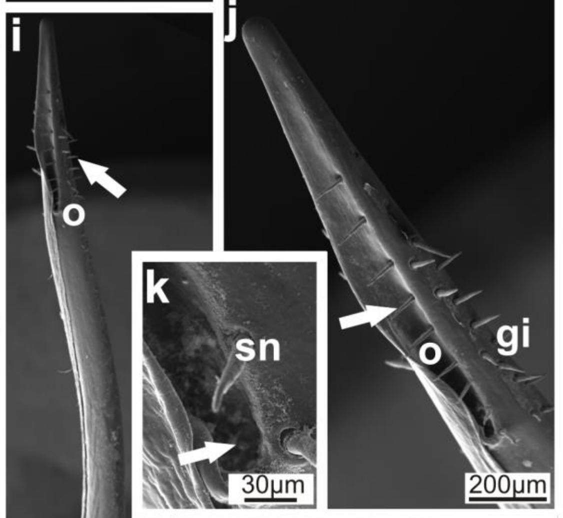
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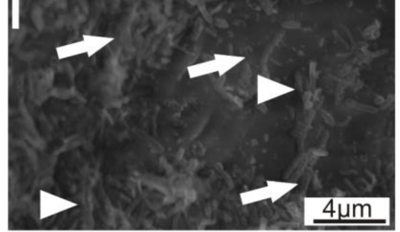
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DISCUSSION

Somatic indices and macroscopic development stages of the reproductive system

This study revealed that *C. hellerii* differs from the other Portunidae regarding the seminal fluid and spermatophore production. The patterns described here for *C. helleri* may be a character of the Thalamitinae. The first dissonant trait is the much lower GSI (0.4%) of *C. helleri*, irrespective of size class or season, which interestingly, the male GSI of *C. japonica* —a congeneric species— was also 0.4% and, like in *C. hellerii*, did not vary seasonally (Wong and Sewell, 2015). However, this is very different when compared to those of other Portunidae species that produce sperm plugs (Bawab and El-Sherief, 1988; Costa and Negreiros-Fransoso, 1998; Mantelatto and Fransozo, et al., 1999). For example, in *Callinectes danae* Smith 1869, *Callinectes ornatus* Ordway 1863 and *Portunus pelagicus* Linnaeus 1758, the amount of fluid in the vas deferens led to GSI values of 3.5%, 4.0% and 4.2%, respectively (Bawab and El-Sherief, 1988; Zara et al., 2012; Nascimento and Zara, 2013; Efrizal et al., 2015). The low GSI of *C. hellerii* points to a different pattern of seminal fluid production and storage that could play a key role during the transference to the female seminal receptacle.

The second character is the absence of different macroscopic development stages in *C. hellerii.* In most portunids, the relative volume of the male reproductive system changes along the maturation, thus, the HSI decreases during the passage from juvenile/developing male phase to the mature phase, while the GSI reaches its peak. These relative differences lead to the diferent macroscopic development stages (rudimentary, under-development, mature) (Zara et al., 2012; Nascimento and Zara, 2013) that have been observed in *Achelous spinimanus* (Latreille, 1819) as *Portunus spinimanus* in Santos and Negreiros-Fransozo (1999), *A. cribrarius*, *Po.*

pelagicus, *Ca. danae*, *Ca. ornatus*, as well as in the congeneric *C. japonica* (Pinheiro and Fransozo, 1998; de Lestang et al., 2003; Zara et al. 2012; Nascimento and Zara 2013; Wong and Sewell, 2015, respectively). In *Ca. danae* and *Ca. ornatus* (which produce sperm plugs), part of the nutrients stored in the hepatopancreas is mobilized to the rapid development of the male reproductive system and explains the low number of adult males classified as developing males (Zara et al., 2012; Nascimento and Zara, 2013).

The macroscopic classification of the reproductive system has been an important tool in many studies on the population biology of Portunidae crabs (Pinheiro and Fransozo, 1998; Costa and Negreiros-Fransozo, 1998; Mantelatto and Fransozo, 1999; Santos and Negreiros-Fransoszo, 1999; de Lestang et al., 2003; Efrizal et al., 2015). However, in this study we did not observed the stages seen in *C. japonica* (Wong and Sewell 2015) since the testes and vas deferens of *C. hellerii* were always visible and never became empty of spermatophores (irrespectively of size or season). Moreover, very small and large males had very similar GSI and HSI, and these indices did not vary seasonally. Overall, the lack of macroscopic stages in *C. hellerii* indicates that even the smaller males are morphologically and physiologically mature and able to mate, which could be very interesting trait to the invasion sucess. A similar feature occurs in the close relative species *Thalamita picta* Stimpson, 1858, *Thalamita prynma* (Herbst, 1803) and *Thalamita sima* H. Milne Edwards, 1834, small males can mate with large females, which increases the competition for females to mate (Norman, 1996; Norman et al., 1997; Norman et al., 1999). The size of the adult females ranges from 35.5 to 63.1 mm of CW and the adult males ranged from 30.2 to 72.7 mm of CW. In our study area, the sex ratio was 3:1 (male:female) (Sant'Anna et al., 2012). Thus, we propose that the smaller and larger males can reproduce once they can mate with the smaller and larger adult females, respectively. An early start of reproductive activity

could be advantageous in species that lack the mate guarding behavior, and/or in the cases in which the females copulate while hard-shelled (i. e., during the intermolt) and can be an advantage to the success of invasion.

Vas deferens and seminal fluid production

The third peculiar trait refers to the vas deferens of *C. hellerii.* Despite being divided in three regions, as in other Brachyura (Johnson 1980; Krol et al., 1992; Castilho et al., 2008; Zara et al., 2012; Nascimento and Zara, 2013; Ravi et al., 2014; Tiseo et al., 2014; Wong and Sewell, 2015), this somatic duct is quite different from those of other Portunidea species that produce sperm plugs (Spalding, 1942; Johnson; 1980; Zara et al., 2012; Nascimento and Zara, 2013). In *C. hellerii*, the AVD has also two portions, AVDp and AVDd, with different functions and histochemical characteristics. Although being chemically distinct, the epithelium cells of the AVDp and AVDd have the same ultrastructural characteristics. The cytoplasm of these cells is filled by RER and there are several secretion vesicles next to the apical edge. Thus, the epithelium is a typical secretor of proteins, as described in other ultrastructural studies carried out with the portunid *P. pelagicus* and other Majoidea (Hinsch and Walker, 1974; Simeó et al., 2009; Ravi et al., 2014). However, the AVD luminal secretion is chemically distinct. The AVDp has a narrow and convolute lumen filled with proteins and free spermatozoa masses immersed in basophilic secretion type I, positive for acidic polysaccharides and negative for neutral polysaccharides. On the contrary, in *Ca. danae* and *Ca. ornatus* (which produce sperm plugs) the secretion type I was very reactive for neutral polysaccharides (Zara et al*.,* 2012; Nascimento and Zara, 2013).

The AVDd is formed by wider and less convoluted ducts and is the main site of storage of mature spermatophores. This is unique to Portunidae and other swimming crabs like Carcinidae and Ovalpidae species (Spalding, 1942; Johnson, 1980; Uma and Subramonian, 1984; Stewart et al., 2010; Zara et al., 2012; Nascimento and Zara, 2013; Vallina et al., 2014; Ravi et al., 2014; Wong and Sewell, 2015; Waiho et al., 2017). The same pattern can be seen in published photographs of *C. japonica*, despite the fact that the narrow MVD was described as the site of spermatophore storage (Wong and Sewell, 2015). Moreover, in *Charybdis smithii* (McLeay, 1838) the authors described the AVD as the main site of spermatophore storage, but they mention that the storage is similar as in other portunids (where the MVD is the main site of storage), despite the absence of photographs depicting the MVD or PVD (Balasubramanian and Suseelan, 2000).

The luminal secretions play a central role in the spermatophore formation. The luminal secretion type II is an acidophilic glycoprotein without acidic polysaccharides, unlike the secretion type I inside the sperm masses. The composition of the secretion type II is similar in *C. hellerii*, *Ca. danae* and *Ca. ornatus* (Zara et al., 2012; Nascimento and Zara, 2013). However, these secretions play a different role in *C. hellerii* since the secretion type II separates the spermatozoa immersed in type I secretion, forming the spermatophores. In contrast, in the AVDd of *Ca. danae* and *Ca. ornatus* the glycoprotein secretion type II infiltrates among the type I secretion (rich in acidic polysaccharides at this portion) to produce the spermatophore wall or pellicle, which is absent in *C. hellerii* (Zara et al., 2012; Nascimento and Zara, 2013). Thus, in *C. hellerii* the spermatophores are just masses of spermatozoa trapped in the secretion type I, characterizing a mucous type spermatophore (*sensu* Klaus et al., 2009; 2013). The mucous spermatophores are characterized by a variable morphology and number of spermatozoa per

spermatophore. In *C. hellerii* it is possible that some spermatophores fuse to each other forming large coenospermic spermatophores. Although it was not observed here, Klaus et al. (2013) reported this phenomenon in the freshwater crab *Sundathelphusa phillippina* (von Martens, 1868), which also has the mucous spermatophore. In *C. hellerii* the mucous type spermatophore could be advantageous to a fast dehiscence releasing the spermatozoa to become ready to fertilization.

The MVD of *C. hellerii* has a narrow lumen forming a short duct filled with homogeneous acidophilic, glycoprotein secretion type II, like in the AVDd. Thus, the MVD produces the same seminal fluid component as the AVDd. However, the amount of seminal fluid increases in the MVD due to the outpocketings (*sensu* Johnson, 1980). As the seminal fluid increases in the MDV, the spermatophores seem looser. The MVD lateral outpocketings are smaller than those in the PVD, in *C. hellerii* and in *C. japonica* (see the photographs in Wong and Sewell, 2015). The MVD outpocketings occur in all Portunidae species, however they are more numerous and larger in all species that produce sperm plugs like *Callinectes* and *Portunus*, and in the carcinid *Car. maenas* (Spalding, 1942; Ryan, 1967; Johnson, 1980; Zara et al., 2012; Nascimento and Zara, 2013; Ravi et al., 2014). Exceptionally, MVD outpocketings are absent in *S. olivacea* and in *S. serrata* (Uma and Subramonian, 1984; Waiho et al., 2017), and *S. serrata* produces sperm plugs (Jayasancar and Subramonian, 1997). The MVD of *C. hellerii* cannot be considered the main site of spermatophore storage and its luminal secretion is a homogeneous glycoprotein without granular compounds. This granular seminal fluid is found in all Portunidae and close related Gerionidae, Carcinidae and Ovalipidae species that produce sperm plugs (Spalding, 1942; Ryan, 1967; Johnson, 1980; Uma and Subramonian, 1984; Hinsch, 1986, 1988; Stewart et al., 2010; Zara et al., 2012; Nascimento and Zara, 2013; Ravi et al., 2014; Vallina et

al., 2014; Waiho et al., 2017). Thus, this is another evidence that the vas deferens of *C. hellerii* is not involved in the sperm plug formation. In the MVD of *C. hellerii* is possible to notice a morphological variation: there are coenospermic spermatophores containing few or many spermatozoa, and some cleistospermic spermatophores, containing only one spermatozoon per spermatophore. The occurrence of cleistospermy is more common in freshwater crabs that have fewer large lecithotrophic eggs, and is considered as a strategy to avoid polyspermy (Guinot et al., 1997; Klaus et al., 2009; 2013). Tiseo et al. (2014) proposed that cleistospermy is related to a differential dehiscence. Dehiscence is quicker in cleistospermic than in coenospermic spermatophores, at least in *Pachygrapsus gracilis* (Saussure, 1858) and *Pachygrapsus transversus* (Gibbes, 1850). Like in *C. hellerii*, these two species have acidic polysaccharides inside the spermatophores. Thus, the mucous-type spermatophore of *C. hellerii* seems morphologically variable and simpler, which could be associated to the dehiscence process during the sperm transference.

The PVD of *C. hellerii* has three main characteristics: 1) absence of spermatophores; 2) increase of outpocketings and seminal fluid and absence of accessory glands or caeca; and 3) a fluid-like secretion rich in acidic polysaccharides. The spermatophores are also lacking in the PVD of *C. japonica* and all portunid, carcinid and ovalipid species that produce sperm plugs, or that are likely to produce (Spalding, 1942, Ryan, 1967; Uma and Subramonian, 1984; Nascimento and Zara, 2013; Ravi et al., 2014; Vallina et al., 2014; Waiho et al., 2017). Species that produce sperm packets also lack spermatophores in the PVD (Beninger et al., 1988; Diesel, 1989, 1990; Benhalima and Moriyasu, 2000; Sal Moyano et al., 2010; Antunes et al., 2016; 2018; Oliveira and Zara, 2018), but unlike in *C. hellerii,* these species have accessory glands whose secretions mixes with the PVD secretion, creating a gel that groups the sperm into packets

inside the seminal receptacle (Antunes et al., 2016; 2018). Usually, in species that lack sperm plugs and sperm packets, the spermatophores are found along the entire vas deferens, including the PVD (Cuartas and Souza, 2007; Castilho et al., 2008; Sant'Anna et al., 2007).

The GSI values of *C. hellerii* and *C. japonica* are very low, compared to species that produce sperm plugs, and more similar to species that lack them, such as *C. smithii* and other Thalamininae as *T. picta*, *T. prynma* and *T. sima* (Norman, 1996; Norman et al., 1997; Balasubramanian and Suseelan 1998).

The increase in size and number of PVD outpocketings filled only with luminal secretion is a characteristic of all Portunoidea (Spalding, 1942; Cronin, 1947; Ryan, 1967; Johanson, 1980; Zara et al., 2012; Ravi et al., 2014; Wong and Sewell, 2015; Waiho et al., 2017), also noted in the gross anatomy of the gerionid *Chaceon fenneri* (Manning and Holthuis, 1984) as *Geryon fenneri* in Hinsch (1988), which produces sperm plugs. The presence of outpocketings and absence of spermatophores in the PVD seem to be an apomorphy shared by all Portunoidea, recently raised to families (Spiridonov et al., 2014), irrespectively of whether they produce sperm plugs or not. On the other hand, the cells of the PVD outpocketings have several electron-dense secretory vesicles that produce a basophilic seminal fluid. This fluid is positive for neutral and acidic polysaccharides and has small protein granules, and differs from the acidophilic fluid of the Portunoidea that produce sperm plugs (Ryan, 1967; Uma and Subramonian, 1984; Ravi et al., 2014; Waiho et al., 2017). In *Ca. danae* and *Ca. ornatus,* the PVD fluid is a glycoprotein without anionic polysaccharides (Zara et al., 2012; Nascimento and Zara, 2013). The presence of a basophilic secretion is described in the PVD and accessory glands of the sperm packet species *Ch. opilio*, including neutral and acidic polysaccharides (Benhalima and Moriyasu, 2000). In addition, Antunes et al. (2016, 2018) demonstrated that the distinct secretions from the accessory

glands and PVD are mixed to produce the gel that separates the sperm packets in the seminal receptacle of *Stenorhynchus seticornis* (Herbst, 1788). This PVD gel has the same histochemical composition found in *C. hellerii*. Thus, we suggest that the PVD secretion of *C. hellerii* acts as seminal gel that pushes the old ejaculates toward the dorsal region of the seminal receptacle, as described in Majoids (Diesel, 1980; 1991; Sal Moyano et al., 2010; Antunes et al., 2016).

The occurrence of multiple mating with hard-shelled females —which is a common behavior of species that produce sperm packets— has been recorded in *Charybdis longicollis* Leene, 1938, *C. japonica*, *T. picta*, *T. prynma* and *T. sima* (Innocenti et al., 1998; Norman, 1996; Norman et al., 1997; 1999; Baker et al., 2018). Nonetheless, only free sperm, without sperm packets, have been observed in the seminal receptacle of *C. helleri* and *C. japonica* (Sant'Anna et al., 2012; Wong and Sewell, 2015). This and other features argue against the presence of sperm packets in *C. hellerii*. The seminal receptacle in Portunidae is classified as the dorsal-type, in which the oviduct opening is opposite to the vagina. However, in species that produces sperm plugs, like *A. cribrarius* and *Car. maenas*, the oviduct changes to a more ventral position, closer to the vagina (but always opposite to the vagina), while the plug disappears (Spalding, 1942; Zara et al., 2014). Since this process moves the sperm mass toward a more ventral position, if *C. hellerii* had sperm packets these movements would mix the sperm and the sperm packets would lose their function, which is to avoid polyspermy.

Considering that multiple and hard-shelled matings occur in *C. hellerii*, as in *C. longicollis* and *C. japonica* (Innocenti et al., 1998; Baker et al., 2018), and that apparently there are no sperm packets in their seminal receptacle, the PVD seminal fluid and the acidic polysaccharide secretion inside the spermatophores may have simpler functions. One of the functions of the PVD seminal fluid could be protection of the sperm released from the spermatophores. Several

authors proposed that male secretions with acidic polysaccharides act in the seminal receptacle as a defense against harmful agents and bacteria (Johnson 1980; Sasikala and Subramonian, 1987; Beninger et al. 1993; Jensen *et al.* 1996; Benhalima and Moriyasu, 2000). In *C. hellerii*, there are many bacteria attached to the small teeth near the opening of gonopod I, along the passage of the seminal fluid. The acidic polysaccharides may trap these bacteria just before the transfer and release of spermatozoa from the spermatophores, since they are stored in the AVDd.

The PVD seminal fluid can also be involved in spermatophore dehiscence. The spermatozoa of *C. hellerii* are immersed in a neutral and acidic polysaccharide spermatophore, which increases the protection against pathogens. However, the absence of a wall (pellicle) makes the spermatophore more susceptible to different environmental agents. The spermatophore hydration promoted by the acidic polysaccharides (i. e., attraction of water dipole) causes a quick swelling and the formation of a cone-like projection around it. This cone-like projection produces a streaming that releases the spermatozoa, as seen in *S. serrata* (Uma and Subramonian, 1979). In *C. hellerii*, the swelling occurs in the first 15 min and suddenly the dehiscence stabilizes, which indicates that the hydration itself is not enough to promote the dehiscence (Beninger et al., 1993). On the other hand, when the seminal fluid is pumped through the gonopod I, sperm is released among many ruptured, irregular spermatophores, and only a few are still intact. The crossing tooth-like protuberances and a few sclerotized sensilla near the apex of the gonopod I aperture, along the passage of the seminal fluid, could help rupturing the mucous spermatophore (Rorandelli et al., 2008; Antunes et al., 2018). However, the hydration and cuticular projections do not seem to be strong enough to cause the full dehiscence, and this process may be completed by a mechanical process. The gonopod I channel of *C. hellerii* is as smooth as in *S. seticornis* (Antunes et al., 2018) and becomes very narrow from the middle

toward the apex. Thus, during the passage along these portions, the spermatophore receives a significant mechanical pressure that triggers the dehiscence. Thus, the mechanical force could be the step stone in the spermatophore disruption, adding to the effect of the other structures (Spalding, 1942; Beninger et al., 1993; Antunes et al., 2018). The mechanical force is associated to the presence of muscle layers surrounding the PVD, AVD and MVD indicating that they act during the transfer of seminal fluid, in addition to the corporeal muscle contraction which is known to occur in other Portunidae (Ryan, 1967; Hartnoll, 1969; Jivoff et al., 2007).

Lastly, another important function of the PVD seminal fluid could be to facilitate the mixing of spermatozoa from multiple previous matings, which is well-known in other Thalamitinae (Innocenti et al., 1998; Norman, 1996; Norman et al., 1997; 1999; Baker et al., 2018). If it does occur in *C. hellerii* as well, the mixing of sperm due to the lack of sperm plugs or sperm packets could be a very important feature contributing to the invasion success of this species. The multiple paternities increase the genetic variability of each brood and could explain the high intraspecific variability that has been observed in *C. hellerii* through the application of different molecular markers (Negri et al., 2018).

In conclusion, the male reproductive system of *C. hellerii* differs from all the other native Brazilian Portunidae species that produce sperm plugs studied so far, and is more similar to species that do not produce sperm plugs or sperm packets. Our results highlight the importance of this different pattern of seminal fluid production and spermatophore dehiscence and predict that this is the pattern of all Thalamitinae. This model of seminal fluid production can be an important factor increasing the invasion success of *C. hellerii* (and probably of *C. japonica*) for allowing more than one male to transfer its seminal fluid to the female. However, further studies addressing the histology and ultrastructure of the female seminal receptacle, mating behavior,

and paternity are needed confirm that these traits are important, or even decisive, factors explaining the success of *C. hellerii* worldwide.

ACKNOWLEDGMENTS

The authors thank the São Paulo Research Foundation FAPESP (JP # 2005/04707-5 and Biota Intercrusta #2018/13685-5 to FJZ) and Coordenação de Aperfeiçoamento de Nível Superior – CAPES, program Ciências do Mar II (#1989/2014 – 23038.004309/2014-51) granted to FJZ and TTW PhD fellowship associated to CAPES CIMAR II #1989/2014, CAPES financial code 001. FJZ acknowledge the receipt of research grant from CNPq (PQ2# 303314/2017-6). Additional thanks are due to Márcia F. Mataqueiro, Dra. Claudia Fiorillo and Monika Iamonte for technical support; to the Invertebrate Morphology Laboratory of UNESP- FCAV and the Electron Microscopy Laboratory of UNESP – FCAV, Jaboticabal and Rio Claro. This study was conducted in accordance with the Brazilian laws (FJZ - MMA SisBio permanent license #34587).

REFERENCES

Antunes, M., Zara F.J., López-Greco, L.S., Negreiros-Fransozo, M.L. (2016). Morphological analysis of the female reproductive system of *Stenorhynchus seticornis* (Brachyura: Inachoididae) and comparisons with other Majoidea. *Invertebrate Biology,* 135: 75–86.

Antunes, M., Zara, F.J., López-Greco, L.S., Negreiros-Fransozo, M.L. (2018). Male reproductive system of the arrow crab *Stenorhynchus seticornis* (Inachoididae). *Invertebrate Biology,* 137: 171–184.

Baker, C.F., Gublin, Y., Reeve, K.A. (2018). Observations of hard-shell mating in the portunid crab *Charybdis japonica* (A. Milne-Edwards, 1861) (Decapoda: Brachyura). *Journal of Crustacean Biology*, 38: 504-508.

Baklouti, S., Derbali, A., Dhieb, K., Jarboui, O. (2013). Reproductive biology of the Mediterranean green crab, *Carcinus aestuarii* Nardo, 1847 (Crustacea: portunidae), in the Gulf of Gabes (Tunisia, Central Mediterranean). *Cahiers de Biologie Marine*, 54: 411-417.

Balasubramanian, C.P., Suseelan, C. (1998). Reproductive biology of the female deepwater crab *Charybdis smithii* (Brachyura: Portunidae) from the India Seas. *Asian Fisheries Science*, 10: 211-222.

Balasubramanian, C.P., Suseelan, C. (2000). Male reproductive system and spermatogenesis in the deep water crab *Charyhdis smithii* McLeay (Brachyura: Portunidae). *Indian Journal of Fishery*, 47: 275-282.

Bawab, F.M., El-Sherief, S.S. (1988). Stages of the reproductive cycle of the female crab *Portunus pelagicus* (L., 1758) based on the anatomical changes of the spermatheca (Decapoda, Brachyura, Portunidae). *Crustaceana*, 54: 139–148.

Beninger, P.G., Elner, R.W., Foyle, T.P., Odense, P.H. (1988). Functional anatomy of the male reproductive system and the female spermathecae in the snow crab *Chionoecetes* opilio (O. Fabricius) (Decapoda: Majidae) and a hypothesis for fertilization. *Journal of Crustacean Biology*, 8: 322-332.

Beninger, P.G., Lanteigne, C., Elner, R.W. (1993). Reproductive processes revealed by spermatophore dehiscence experiments and by histology, ultrastructure, and histochemistry of the female reproductive system in the snow crab *Chionoecetes opilio* (O. Fabricius). *Journal of Crustacean Biology*, 1: 1-6.

Benhalima, K., Moriyasu, M. (2000). Structure and function of the posterior vas deferens of the snow crab, *Chionoecetes opilio* (Brachyura, Majidae). *Department of Fisheries and Oceans, Maritimes Region, Science Branch, Invertebrate Fisheries Division,* 37: 11–23.

Bolaños, A.J, Baeza J.A., Hernandez, J.E., Lira, C., López, R. (2012). Population dynamics and reproductive output of the non-indigenous crab *Charybdis hellerii* in the south-eastern Caribbean Sea. *Journal of the Marine Biological Association of the United Kingdom*, 92: 469-474.

Carqueija, C.R.G., Gouvêa, E.P. (1996). A ocorrência, na costa brasileira, de um Portunidae (Crustacea, Decapoda), originário do Indo-Pacífico e Mediterrâneo. *Nauplius,* 4:165- 168.

Castilho, G.G., Ostrensky, A., Pie, M.R., Boeger, W.A. (2008). Morphology and histology of the male reprodutive system of the mangrove land crab *Ucides cordatus* (L.) (Crustacea, Brachyura, Ocypodidae). *Acta Zoological,* 89: 157-161.

Cronin, L.E. (1947). Anatomy and histology of male reproductive system of *Callinecetes sapidus*

Rathbun. *Journal of Morphology*, 81: 209-239.

Costa, T.M., Negreiros-Fransozo, M.L. (1998). The reproductive cycle of *Callinectes danae* Smith, 1869 (Decapoda, Portunidae) in the Ubatuba region, Brazil. *Crustaceana*, 71: 615-627.

Cuartas, E.I., Sousa, L.G. (2007). Ultrastructural study of the spermatophores and spermatozoa in *Uca uruguayensis* (Decapoda, Brachyura, Ocypodidae). *Biociências*, 15: 21-28.

de Lestang, S., Hall, N.G., Potter, I.C. (2003). Reproductive biology of the blue swimmer crab (*Portunus pelagicus*, Decapoda: Portunidae) in five bodies of water on the west coast of Australia. *Fishery Bulletin*, 101: 745-757.

Diesel, R. (1989). Structure and function of the reproductive system of the symbiotic spider crab *Inachus phalangium* (Decapoda: Majidae): observations on sperm transfer, sperm storage and spawning. *Journal of Crustacean Biology*, 9: 266-277.

Diesel, R. (1991). Sperm competition and mating behavior in Brachyura. In: Bauer, R.T. e Martin, J.W. (eds), *Crustacean Sexual Biology.* Columbia University Press, New York, pp. 145–163.

D'incao, F., Martins, S.T.S. (1998). Occurrence of *Rhithropanopeus harrisii* (Gould, 1841) in the southern coast of Brazil (Decapoda, Xanthidae). *Nauplius*, 6: 191-194.

Dineen, J.F., Clark, P.F., Hines, A.H., Reed, S.A., Walton, H.P. (2001). Life history, larval description, and natural history of *Charybdis hellerii* (Decapoda, Brachyura, Portunidae), an invasive crab in the western atlantic. *Journal of Crustacean Biology*, 21: 774-805.

Efrizal, A., Arshad, M.S. Kamarudin, C.R. Saad, Amin, S.M.N. (2015). Some Aspects of Reproductive Biology of Blue Swimming Crab (*Portunus pelagicus* (Linnaeus, 1758)) Under Laboratory Conditions. *Journal of Fisheries and Aquatic Science,* 10: 77-91.

El-Sherief, S.S. (1991). Fine structure of the sperm and spermatophores of *Portunus pelagicus* (L.) (Decapoda, Brachiura). *Crustaceana,* 61, 271-279.

Erkan, M., Tunali, Y., Balkis, H., Oliveira, E. (2009). Morphology of testis and vas deferens in the Xanthoid crab, *Eriphia verrucosa* (Forskal, 1775) (Decapoda: Brachyura). *Journal of Crustacean Biology*, 29: 458-465.

Geiger, W., Alcorlo, P., Baltanas, A., Montes, C. (2005). Impact of an introduced Crustacean on the trophic webs of Mediterranean wetlands. In: *Issues in Bioinvasion Science 2005* (pp. 49-73). Springer Netherlands.

Gregatti, R.A., Fransozo, V, López-Greco, L.S., Negreiros-Fransozo, M.A. (2010). Reproductive cycle and ovarian development of the marine ornamental shrimp *Stenopus hispidus* in captivity. *Aquaculture,* 306: 185-190

Guinot, D., Jamieson, B.G.M., Tudge, C.C. (1997). Ultrastructure and relationships of spermatozoa of the freshwater crabs *Potamon fluviatile* and *Potamon ibericum* (Crustacea, Brachyura, Potamidae). *Journal of Zoology,* 241: 229-244.

Guinot, D., Tavares, M., Castro, P. (2013). Significance of the sexual openings and supplementary structures on the phylogeny of brachyuran crabs (Crustacea, Decapoda, Brachyura), with new nomina for higher-ranked podotreme taxa. *Zootaxa*, 3665: 1-414.

Hartnoll, R.G. (1969). Mating in the Brachyura. *Crustaceana,* 16: 161-181.

Hines, A.H., Jivoff, P.R., Bushmann, P.J., Montfrans, J.V., Reed, S.A., Wolcott, D.L., Wolcott, T.G. (2003),. Evidence for sperm limitation in the blue crab, *Callinectes sapidus*. *Bulletin of Marine Science*, 72: 287-310.

Hinsch, G.W. (1986). A comparison of sperm morphologies, transfer and sperm mass storage between two species of crab, *Ovalipes ocellatus* and *Libinia emarginata*. *International Journal of Invertebrate Reproduction e Development*, 10: 79–87.

Hinsch, G.W. (1988). Ultrastructure of the sperm and spermatophores of the goldencrab *Geryon fenneri* and a closely related species, the red crab *G. quinquedens*, from the eastern gulf of Mexico. *Journal of Crustacean Biology*, 8: 340-345.

Hinsch, G.W., Walker, M.H., (1974). The vas deferens of the spider crab, *Libinia emarginata*. *Journal of Morphology*, 143: 1-20.

Innocenti, G., Vannini, M., Galil, B.S. (1998). Notes on the behavior of the portunid crab *Charybdis longicollis* Leene parasitized by rhizocephalan *Heterosaccus dollfusi* Boschma. *Journal of Natural History*, 32: 1577-1585.

Jamieson, B.G.M. (1994). Phylogeny of the Brachyura with particular reference to the Podotremata: evidence from a review of spermatozoa ultrastructure (Crustacea: Decapoda). *Philosophical Transections of the Royal Society of London,* 345: 373-393.

Jayasankar, V., Subramoniam, T. (1997). Proteolytic activity in the seminal plasma of the mud crab, *Scylla serrata* (Forskal). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 116: 347-352.

Jensen, P.C., Orensanz, J.M., Armstrong, D.A. (1996). Structure of the female reproductive tract in the Dungeness crab (*Cancer magister*) and implications for the mating system. *The Biological Bulletin*, 190: 336-349.

Jivoff, P., Hines, A.H., Quackenbush, L.C. (2007). Reproductive Biology and Embryonic Development. p. 255-286. In: L.E. Cronin and V.S. Kennedy (eds), The Blue Crab *Callinectes sapidus*. Maryland Sea Grant College.

Johnson, P.T. (1980). *Histology of the blue crab Callinectes sapidus: a model for the Decapoda*. Praeger, New York. 440p.

Junqueira, L.C.U., Junqueira, L.M.M.S. (1983). *Técnicas Básicas de Citologia e Histologia.* Santos, São Paulo. 123p.

Katsanevakis, S., Wallentinus, I., Zenetos, A., Leppäkoski, E., Çinar, M.E., Oztürk, B., Grabowski, M., Golani, D., Cardoso, A.C. (2014). Impacts of invasive alien marine species on ecosystem services and biodiversity: a pan-European review. *Aquatic Invasions*, 9: 391-423.

Kerckhof, F.; Haelters, J.; Gollasch, S. (2007). Alien species in the marine and brackish ecosystem: the situation in Belgian waters. *Aquatic Invasions,* 2: 243-257.

Klaus, S., Schubart, C.D., Brandis, D., (2009). Ultrastructure of spermatozoa and spermatophores of old world freshwater crabs (Brachyura: Potamoidea: Gecarcinucidae, Potamidae and Potamonautidae). *Journal of Morphology*, 270: 175-193.

Klaus, S., Münzner, S., Modenbach, A., Streit, B., Tudge, C.C. (2013). Spermatophore formation and sperm ultrastructure of *Sundathelphusa philippina* (Crustacea: Brachyura: Gecarcinucidae). *Acta Zoologica (Stockholm)*, 94: 267-272.

Krol, R.M., Hawkins, W.E., Overstreet, R.M. (1992). Reproductive components, pp. 295–343. In, Harrison, F. W., Humes, A. G. (eds.), *Microscopic Anatomy of Invertebrates*. Vol. 10. Decapoda Crustacea. Wiley-Liss Inc., New York.

Kurtz, K., Martínez, Soler, F., Ausio, J., Chiva, M. (2008). Histones and nucleosomes in Cancer sperm (Decapod: Crustacea) previously described as lacking basic DNA aassociated proteins: A new model of sperm chromatin. *Journal of Cellular Biochemistry*, 10: 574-584.

Kurtz, K., Ausió, J., Chiva, M. (2009). Preliminary study of sperm chromatin characteristics of the brachyuran crab *Maja brachydactyla*. Histones and nucleosome-like structures in decapod crustacean sperm nuclei previously described without SNBPs. *Tissue and Cell*, 41: 334-44.

Kyomo, J. (1988). Analysis of the relationship between gonads and hepatopancreas in males and females of the crab *Sesarma intermedia*, with reference to resource use and reproduction. *Marine Biology,* 97: 87-93.

Langreth, S.G. (1969). Spermiogenesis in *Cancer* crabs. *Journal of Cell Biology*, 43: 575–603.

Lemaitre, R. (1995). *Charybdis hellerii* (Milne Edwards, 1867), a nonindigenous portunid crab (Crustacea: Decapoda: Brachyura) discovered in the Indian River lagoon system of Florida. *Proceedings of the Biological Society of Washington*, 108: 643-648.

López-Greco, L.S., Lopez, G.C., Rodriguez, E.M. (1999). Morphology of spermatheca in the estuarine crab *Chasmagnathus granulata* Dana 1851 (Graspidae, Sesarminae). *Journal of Zoology*, 249: 490-493.

Mancini, K., Dolder, H. (2001). Ultrastructure of apyrene and eupyrene spermatozoa from the seminal vesicle of *Euptoieta hegesia* (Lepidoptera: Nymphalidae). *Tissue and Cell,* 33: 301-308.

Mantelatto, F.L., Fransozo, A. (1999). Reproductive biology and moulting cycle of the crab *Callinectes ornatus* Ordway, 1863 (Decapoda – Brachyura – Portunidae) from the Ubatuba region, São Paulo, Brazil. *Crustaceana*, 72: 63-76.

Mantelatto, F.L.M., Garcia, R.B. (2001). Biological aspects of the of nonindigenous portunid crab *Charybdis hellerii* in the western tropical south Atlantic. *Bulletin of Marine Science,* 68: 469-477.

Mantelatto, F.L., Robles, R., Schubart, C.D., Felder, D.L. (2009) Molecular Phylogeny of the Genus *Cronius* Stimpson, 1860, with Reassignment of *C. tumidulus* and Several American Species of *Portunus* to Genus *Achelous* De Haan, 1833 (Brachyura: Portunidae). In: Martin, J.W., Crandall, K.A., Felder, DL (eds), *Crustacean Issues: Decapod Crustacean Phylogenetics*. CRC Press, pp 567-579.

McLay, C.L., López Greco, L.S. (2011). A hypothesis about the origin of sperm storage in the Eubrachyura, the effects of seminal receptacle structure on mating strategies and the evolution of crab diversity: how did a race to be first become a race to be last? *Zoologischer Anzeiger*, 250: 378–406.

Melo, G.A.S., Bertini, G., Fransozo, A. (2000). Occurrence of the eastern pacific species *Pilumnoides perlatus* (Poeppig, 1836) in the southeastern Brazilian coast. *Nauplius*, 8: 89-91.

Melo, G.A.S.D., Crivelaro, T.B. (2002). Primeira ocorrência de *Polybius navigator* (Herbst) (Decapoda, Brachyura, Portunidae) no Atlântico ocidental. *Revista brasileira de Zoologia*, 19: 233-238.

Mello, M.S.L, Vidal, B.C. (1980). *Práticas de Biologia Celular*. São Paulo: Edgar Blucher-FUNCAMP. 69p.

Medina, A., Rodríguez, A. (1992). Spermiogenesis and sperm structure in the crab *Uca tangeri* (Crustacea, Brachyura), with special reference to the acrosome differentiation. *Zoomorphology,* 111: 161-165.

Nagao J., Munehara, H. (2003). Annual cycle of testicular maturation in the helmet crab *Telmessus cheiragonus*. *Fisheries Science*, 69: 1200-1208.

Nascimento, F.A., Zara, F.J. (2013). Development of the male reproductive system in *Callinectes ornatus* Ordway, 1863 (Brachyura: Portunidae). *Nauplius*. 21: 161-177.

Negreiros-Fransozo, M.L. (1996). The zoea I of *Charybdis hellerii* (A. Milne-Edwards, 1867) (Decapoda, Portunidae) obtained in laboratory. *Nauplius*, 4: 165-168.

Negri, M., Schubart, C.D., Mantelatto, F.L. (2018). Tracing the introduction history of the invasive swimming crab *Charybdis hellerii* (A. Milne-Edwards, 1867) in the Western Atlantic: evidences of high genetic diversity and multiple introductions. *Biological invasions*, 20: 1771-1798.

Norman, C.P. (1996). Reproductive biology and evidence for hard-female mating in the brachyuran crab *Thalamita sima* (Portunidae). *Journal of Crustacean Biology*, 16: 656-662.

Norman, C.P., Hirano, Y.J., Miyazaki, T. (1997). Hard-female mating in the brachyuran crab *Thalamita prymna* (Portunidae). *Crustacean Research*, 26: 62-69.

Norman, C.P., Nuka, T., Miyazaki, T. (1999). Mating behavior in *Thalamita picta* (Brahyura: Portunidae) and comparisons with congeneric species. *Crustacean Research*, 28: 16-23.

Oliveira, L.J.F., Zara, F.J. (2018). An investigation into the male reproductive system of two freshwater crabs from the Amazon: is there a sperm plug or packet formation? *Marine and Freshwater Behaviour and Physiology*, 51: 227-249.

Pearse, A.G.E. (1985). *Histochemistry: Theorical and Applied*. JeA Churchill, London. 998p.

Pearson, P.J., Walker, M.H. (1975). Alteration of cytochrome-C oxidase activity during spermatogenesis in *Carcinus maenas*. *Cell and Tissue Research*, 164: 401–410.

Pinheiro, M.A.A., Fransozo, A. (1998). Sexual maturity of the speckled swimming crab *Arenaeus cribrarius* (Lamarck, 1818) (Decapoda, Brachyura, Portunidae), in the Ubatuba littoral, São Paulo State, Brazil. *Crustaceana*, 71: 434-452.

Pochon-Masson, J. (1983). Artropoda – Crustacea. In: Adiyodi, R.G, Adiyodi, K.G. (Eds.) *Reprodutive Biology of Invertebrates. Vol II Spermatogenesis and Sperm Function*. New York: Wiley e Sons.

Quinitio, E.T., de Pedro, J., Parado-Estepa, F.D. (2007). Ovarian maturation stages of the mud crab *Scylla serrata*. *Aquaculture Research,* 38: 1434-1441.

Ravi, R., Manisseri, M.K., Sanil, N.K. (2014). Structure of the male reproductive system of the blue swimmer crab *Portunus pelagicus* (Decapoda: Portunidae). *Acta Zoologica*, 95: 176-185.

Ricciardi, A., Steiner, W.W., Mack, R.N., Simberloff, D. (2000). Toward a global information system for invasive species. *BioScience*, 50: 239-244.

Rorandelli, R., Paoli, F., Cannicci, S., Mercati, D., Giusti, F. (2008). Characteristics and fate of the spermatozoa of *Inachus phalangium* (Decapoda, Majidae): description of novel sperm structures and evidence for an additional mechanism of sperm competition in Brachyura. *Journal of Morphology*, 269: 259-271.

Ryan, E.P. (1967). Structure and function of the reproductive system of the Portunus sanguinolentus (Herbst) (Brachyura: Portunidae). I. The male system. In: *Proceedings of the Symposium on Crustácea at Erkulan, Índia, 1965. Part II. Symposium Series* 2: 506-521 + III pl.

Sal Moyano, M.S., Gavio, M.A., Cuartas, E.I. (2010). Morphology and function of the reproductive tract of the spider crab *Libinia spinosa* (Crustacea, Brachyura, Majoidea): pattern of sperm storage. *Helgoland Marine Research*, 64: 213-221.

Sant'Anna, B.S., Pinheiro, M.A.A., Mataqueiro, M., Zara, F.J. (2007). Spermathecae of the mangrove crab *Ucides cordatus*: a histological and histochemical view. *Journal of the Marine Biological Association of the United Kingdom*, 87: 903-911.

Sant'Anna, B.S., Watanabe, T.T., Turra, A., Zara, F.J. (2012). Relative abundance and population biology of the non-indigenous crab *Charybdis hellerii* (Crustacea: Brachyura: Portunidae) in a southwestern Atlantic estuary-bay complex. *Aquatic Invasions*, 1: 347-56.

Sant'Anna, B.S., Branco, J.O., Oliveira, M.M., Boos, H., Turra, A. (2015). Diet and population biology of the invasive crab *Charybdis hellerii* in southwestern Atlantic waters. *Marine Biology Research*, 11: 814-823.

Santos, S., Negreiros-Fransozo. M.A. 1999. Reproductive cycle of the swimming crab *Portunus spinimanus* Latreille (Crustacea, Decapoda, Brachyura) from Ubatuba, São Paulo, Brazil. *Revista Brasileira de Zoologia* 16: 1183-1193.

Sasikala, S.L., Subramoniam, T. (1987). On the occurrence of acid mucopolysaccharides in the spermatophores of two marine prawns, *Penaues indicus* (Milne-Edwards) and *Metapenaeus monoceros* (Fabricius) (Crustacea: Macrura). *Journal of Experimental Marine Biology and Ecology*, 113: 145-153.

Simeó, C.G., Ribes, E., Rotllant, G. (2009). Internal anatomy and ultrastructure of the male reproductive system of the spider crab *Maja brachydactyla* (Decapoda: Brachyura). *Tissue and Cell,* 41: 345-361.

Simeó, C.G., urtz, K., Chiva, M., Ribes, E., Rotllant, G. (2010). Spermatogenesis of the Spider Crab *Maja brachydactyla* (Decapoda: Brachyura). *Journal of Morphology*, 271: 394-406.

Sokal, R.R., Rohlf, F.J. (1995). *Biometry.* New York, W.H. Freeman and Company. 887p.

Spalding, J.F. (1942). The nature and formation of the spermatophores and sperm plug *Carcinus maenas*. *Quarterly Journal of Microscopical Science*, 83: 399-422.

Spiridonov, V.A., Neretina, T.V., Schepetov, D. (2014). Morphological characterization and molecular phylogeny of Portunoidea Rafinesque, 1815 (Crustacea Brachyura): Implications for understanding evolution of swimming capacity and revision of the family-level classification. *Zoologischer Anzeiger*, 253: 404-429.

Stewart, M.J., Steward, P., Soonklang, N., Linthong, V., Hanna, J.P., Duan, W., Prasert, S. (2010). Spermatogenesis in the blue swimming crab, *Portunus pelagicus*, and evidence for histones in mature sperm nuclei. *Tissue and Cell*, 42: 137-150.

Taboga, S.R., Dolder, M.A.H. (1991). Análise histológica da espermatogênese de *Hyla ranki* (Amphibia, Anura, Hyllidae). *Brazilian Journal of Morphological Sciences*, 8: 66-71.

Tavares, M., Mendonça Jr, J.B. (1996). *Charybdis hellerii* (A. Milne Edwards, 1867) (Brachyura: Portunidae), eighth nonindigenous marine decapod recorded from Brazil. *Crustacean Research*, 25: 151-157.

Tavares, M., Mendonca Jr, J.B. (2011). The occurrence of the Indo-Pacific swimming crab *Scylla serrata* (Forskål, 1775) in the Southwestern Atlantic (Crustacea: Brachyura: Portunidae). *Aquatic Invasions*. 6: 49-51.

Tiseo, G.R., Mantelatto, F.L., Zara, F.J. (2014). Is cleistospermy and coenospermy related to sperm transfer? A comparative study of the male reproductive system of *Pachygrapsus transversus* and *Pachygrapsus gracilis* (Brachyura: Grapsidae). *Journal of Crustacean Biology*, 34: 704-716.

Tudge, C.C. (2009). Spermatozoal morphology and its bearing on decapod phylogeny. In: Martin, J.W., Crandall, K.A., Felder, D.L. (eds). *Crustacean Issues 18: Decapod Crustacean Phylogenetics*. Boca Raton, FL: CRC Press, Taylor e Francis Group. pp 101–119.

Uma, K., Subramoniam, T. (1979). Histochemical characteristics of spermatophore layers of Scylla serrata (Forskal) (Decapoda: Portunidae). *International Journal of Invertebrate Reproduction*, 1: 31-40.

Uma. K., Subramoniam, T. (1984). A comparative study on the spermatophore formation in *Scylla serrata* (Forskal) (Decapoda: Brachyura) and *Clibanarius longitarsus* (De Haan) (Decapoda: Anomura). *Journal of the Marine Biological Association of India*, 26: 103-108.

Vallina, M., Sal Moyano, M.P., Cuartas, E.I., Gavio, M.A. (2014). Reproductive system and size maturity of the paddle crab *Ovalipes trimaculatus* (Brachyura: Portunidae) along the Argentine coast. *Journal of Crustacean Biology*, 34: 357-366.

Vaughn, J.C, Locy, R.D. (1969). Changing nuclear histone patterns during development. III. The deoxyribonucleic acid content of spermatogenic cells in the crab *Emerita analoga*. *Journal of Histochemistry Cytochemistry*, 17: 591-600.

Vaughn, J.C., Hinsch, G.W. (1972). Isolation and characterization of chromatin and DNA from the sperm of the spider crab, *Libinia emarginata*. *Journal of Cell Science*, 11: 131-152.

Vaughn, J.C, Thomson, L.A. (1972). A kinetic study of DNA and basic protein metabolism during spermatogenesis in the sand crab, *Emerita analoga*. *Journal of Cell Biology*, 52: 322–337.

Waiho, K., Fazhan, H., Jasmani, S., Ikhwanuddin, M. (2017). Gonadal development in males of the orange mud crab *Scylla olivacea* (Herbest, 1796) (Decapoda, Brachyura, Portunidae). *Crustaceana* 90: 1-19.

Wang, Y.L., Zhang, Z.P., Li, S.J. (1997). Ultrastructure of spermatogenesis in the crab *Scylla serrata*. *Acta Zoologica Sinica,* 43: 249-254.

Watanabe, T.T., Zara, F.J., Hattori, G.Y., Turra, A., Sant'Anna, B.S. (2015). Biological associations of color variation in the Indo-Pacific swimming crab *Charybdis hellerii*. *Anais da Academia Brasileira de Ciências*, 87: 219-232.

Wolcott, D.L., Hopkins, C.W.B., Wolcott, T.G. (2005). Early events in seminal fluid and sperm storage in the female blue crab *Callinectes sapidus* Rathbun: effects of male mating history, male size and season. *Journal of Experimental Marine Biology and Ecology*, 319: 43-55.

Wong, N.A., Sewell, M.A. (2015). The reproductive ecology of the invasive Asian paddle crab, *Charybdis japonica* (Brachyura: Portunidae), in northeastern New Zealand. *Invertebrate Biology*, 134: 303-317.

Yu, K., Hou, L., Zhu, J.Q., Ying, X.P., Yang W.X. (2009). KIFC1 participates in acrosomal biogenesis, with discussion of its importance for the perforatorium in the Chinese mitten crab *Eriocheir sinensis*. *Cell Tissue Research*, 337: 113-123.

Zana, U., Lino-Neto, J., Dolder, H. (2001). Ultrastructure of spermatozoa in *Plebeia droryana* Friese (Hymenoptera: Apidae: Meliponina). *Journal of Hymenoptera Research,* 10: 261-270.

Zara, F.J., Toyama, M.H., Caetano, F.H., Lopéz-Greco, L.S. (2012). Spermatogenesis, spermatophore and seminal fluid production in the adult blue crab *Callinectes danae* (Portunidae). *Journal of Crustacean Biology*, 32: 249-62.

Zara, F.J., Pereira, G.R., Sant'Anna, B.S. (2014). Morphological changes in the seminal receptacle during ovarian development in the speckled swimming crab *Arenaeus cribrarius*. *The Biological Bulletin*, 227: 19-32.

Capítulo 3

New insights into sperm storage and mating behavior in Portunidae based on the non native crab *Charybdis hellerii*

Prólogo – Capítulo 3

Assim como o capítulo anterior, este capítulo teve origem inicial nos dados do projeto Jovem Pesquisador FAPESP do meu orientador, porém houve diversos desdobramentos até chegar aos resultados aqui apresentados.

Durante coleta de animais para meu trabalho publicado em 2015 nos Anais da Academia Brasileira de Ciências, foi encontrado em campo um casal de *Charybdis hellerii* em cópula com carapaça rígida. Essa observação de campo originou toda a introdução de mais estudos tanto na questão de comportamento reprodutivo, como na análise da paternidade da espécie.

Capítulo 3

New insights into sperm storage, paternity and mating behavior in Portunidae Rafinesque, 1815 based on the non-native crab *Charybdis hellerii* (A. Milne-Edwards, 1867)

ABSTRACT

Charybdis hellerii is a non native Portunidae crab that is originally native to the Indo-Pacific Ocean and is now found around the world. In this work, we studied the sperm storage pattern in the seminal receptacle (SR) during the conciliated ovarian cycle to understand the mating behavior of this invasive species. The SR underwent histology and transmission electron microscopy examinations. The characteristic sperm plug found in portunid species is absent in *C. hellerii*, and the SR maintains a constant volume throughout the ovarian cycle. Spermatophores or sperm packets were not found at any stage of the ovarian cycle, and only free spermatozoa were found in the SR lumen. The free spermatozoa in the SR were immersed in a glycoprotein secretion consisting of neutral and acidic polysaccharides, with the latter compound only found after mating. The dorsal region of the SR produces glycoprotein secretions in a stratified epithelium that forms a dense layer. These secretions are released primarily via holocrine mechanism. Mating in *C. hellerii* occurs with females in the intermolt stage; each female mates with more than one male, and each mating lasts for a short duration. This is reflected in the paternity of the offspring, as the analysis showed that polyandry occurs in *C. hellerii*. In conclusion, the absence of a sperm plug and sperm packets in the SR, which maintains it morphology during ovarian development conciliated to more than one male mating with hard-shelled female *C. hellerii*, differs from most Portunidae and has a similar pattern as that of Thalamitinae. This provides an advantage for increasing the genetic variability as a key role to establishing the species in new areas.

Keywords: Portunoidea, ultrastructure, poliandy, bioinvasion, Thalamitinae

INTRODUCTION

Charybdis hellerii (A. Milne-Edwards, 1867) is a portunid crab native to the Indo-Pacific (Lemaitre, 1995) that is now widespread in the Western Atlantic and is found from Florida to the south coast of Brazil (Mantelatto and Dias, 1999; Dineen et al., 2001; Negri et al., 2018). Adults, juveniles and ovigerous females have been frequently collected along the Brazilian coast (Tavares and Mendonça 1996; Mantelatto and Dias, 1999; Sant'Anna et al., 2012; Watanabe et al. 2015), which confirms that the species is well established (Mantelatto and Garcia, 2001; Ferreira et al., 2008). *Charybdis hellerii* has many traits that favor the colonization of new areas, such as long periods of larval development (44 days), rapid growth and maturation (Dineen et al., 2001), omnivorous and varied diet (Sant'Anna et al., 2015), ability to store sperm and ability to produce multiple clutches with a high fecundity (Dinnen et al., 2001; Sant'Anna et al., 2012).

In Brachyura, the organ that stores mating products is called the spermatheca (spermathecae) (Spalding, 1942; Hartnoll, 1969; Beninger et al., 1988; Sainte-Marie and Sainte-Marie, 1998; Lopez-Greco et al., 1999; Rotllant et al., 2007; Sant´Anna et al., 2007) or the seminal receptacle (SR) (Johnson, 1980; Diesel, 1989, 1990, 1991; Guinot and Quenette, 2005; López-Greco et al., 2009). In Eubrachyura, this organ has an ecto-mesodermal origin (Diesel, 1991; Guinot and Quenette, 2005), and according to Guinot and Quenette (2005), the most appropriate nomenclature is the seminal receptacle due to this double origin. Spermatheca is used for Podotremata crabs with an ectodermal origin of the sperm storage chamber without any connection to the ovary (Guinot and Quenette, 2005; Guinot et al., 2013). McLay and López Greco (2011) provide details about this discussion and the evolutionary relationship of the histological and anatomical structure of the SR among the brachyurans.

In Portunidae, as well as in other Brachyura, the SR is a pair of organs connected to the ovaries by oviducts; it is classified as dorsal, intermediary or ventral type according to the insertion of the oviducts in the SR; the vagina lies ventrally (Diesel 1991; McLay and Lopez-Greco, 2011). However, some new findings have increased this classification since the oviduct can also open into the vagina of dorippid crabs (Hayer et al., 2016; Vehof et al., 2017). Although the traditional classification is insufficient for Eubrachyura (Hayer et al., 2016; Vehof et al., 2017), we still use this classification in this study because it fits our goals in portunid crabs. The SR are positioned on both sides of the cephalothoracic cavity, below the heart (Pyle and Cronin, 1950), and are ventrally connected to the vagina, which opens on the sternite of the sixth thoracic segment and forms the genital opening, the vulva or female gonopore (Pyle and Cronin, 1950; Johnson, 1980; Beninger et al., 1988; Diesel, 1990; Moriyasu and Benhalima, 1998; Lopez Greco et al., 1999; 2009).

In some majoid species, the sperm and secretions from males may participate in the sperm competition process (Diesel, 1989; 1991). In this case, the seminal fluid and spermatophores are stored in the ventral type SR, and the sperm packets from the last male have priority to fertilize the oocytes (Diesel, 1988, 1989, 1991; Beninger et al., 1993; Sainte-Marie et al., 2000; Sal Moyano et al., 2009; Antunes et al., 2016, 2018). In other Brachyura, such as cancrids, portunids, ovalipids and carcinids, the secretion from the vas deferens forms a sperm plug inside the SR or in the vagina and vulva (Spalding, 1942; Hartnoll, 1969; 1988; Hinsch, 1988, Jensen et al., 1996; Pardo et al., 2013). In Portunidae, the sperm plug seals the SR to prevent competitor males or future mates from transferring additional sperm (Hartnoll, 1969, Johnson, 1980; Jivoff 1997a and b, Jivoff et al., 2007; Wolcott et al., 2005; Zara et al., 2014). Thus, the sperm plug can diminish or avoid sperm competition (Parker, 1970; Jivoff 1997). However, this plug disappears after

some time, and the SR becomes flaccid, as observed in *Carcinus maenas* (Linnaeus, 1758) in Spalding (1942); *Callinectes sapidus* Rathbun, 1896 in Johnson (1980), Hines et al., (2003) and Wolcott et al., (2005); *Portunus hawaiiensis* (Herbst, 1783) as *P. sanguinolentus* in Ryan (1967), *Portunus pelagicus* (Linnaeus, 1758) in Bawab and El-Sherief (1988); *Ovalipes ocellatus* (Herbst, 1799) in Hinsch (1986); and *Arenaeus cribrarius* (Lamarck, 1818) in Pinheiro and Fransozo (2002) and Zara et al. (2014). The plug dissolution occurs at the same time as the ovarian development to open space in the cephalothoracic coelom as a result of the huge area that the waxy-like plug occupies in the body (Hines et al., 2003; Wolcott et al., 2005; Zara et al., 2014). This reduction can trigger ovarian development (Zara et al., 2014).

Although sperm plug formation and soft-shelled mating are common traits in Portunidae, in the subfamily Thalamitinae, this trait is variable because there are species of *Charybdis* which mate in soft-shelled (Sumpton, 1990; Soundarapandian et al., 2013) and hard-shelled condition (Innocenti et al., 1998; Baker et al., 2018). Moreover, at least three species of *Thalamita* also mate in the hard-shelled condition (Norman, 1996; Norman et al., 1997; 1999). This characteristic is usually found in sperm packet species of Inachoididae, Oregoniidae, and Epialtidae (Diesel, 1989; 1991; Beninger et al., 1993; Sainte-Marie et al., 2000; Sal Moyano et al., 2009; Antunes et al., 2016, 2018) and in semiterrestrial and terrestrial crabs without evidence of packets or plugs as described for Ucididae (Sant'Anna et al., 2007), Ocypodidae (Nakasone and Murai, 1998), Gecarcinidae (López-Victoria and Werding, 2008) and Grapsidae (Brockerhoff and McLay, 2005). *Charybdis hellerii* is a successful invasive species, similar to its congener, *Charybdis japonica* (A. Milne-Edwards, 1861), which is becoming established outside its native range (Wong and Sewell, 2015, Baker et al., 2018), and it can be a possible competitor or aggressive predator influencing native species (Mantelatto and Garcia, 2001; Mantelatto et al.,

2009; Sant'Anna et al., 2012; Negri et al., 2018). Therefore, knowing the basic reproductive biology, specifically the pattern of sperm storage and mating behavior of *C. helleri*, is important to increase knowledge to develop management strategies.

These facts open the question whether the polyandry occurs in *C. hellerii* or not. Molecular analyssis reveals that polyandry is highly diffused in the nature, including several species of birds and mammals which were classically considered monogamous (Waser et al., 2006; Soulsbury, 2010; Chambers et al., 2014). In invertebrates, other researchs also shows that the multiple paternity is also a very common event (Yue and Chang, 2010; Jossart et al., 2014). Recent research also points that females of several crustacean has polyandrous behavior, corroborated through short tandem repeats (STR) analysis, which shows multiple paternity in fertilysed eggs (Baggio et al., 2011; Jensen and Bentzen 2012; Ma et al., 2013; Rojas-Hernandez et al., 2014; Dennenmoser and Thiel, 2015).

In this work, we studied the functional morphology of sperm storage in the SR of the non native crab *C. hellerii* during the ovarian cycle. We focused on whether this crab showed different patterns of storage compared to other sperm plug Portunidae or sperm packet species. We also studied the mating behavior in the laboratory to identify some advantageous traits and the paternity of the offspring that could add to others already known to explain the success of its invasion worldwide.

MATERIALS AND METHODS

Animal samples

Non native *C. hellerii* crabs were collected monthly from April 2009 to April 2010. They were collected during low tide on the rocky shores of the Milionários (Porchat Island, 23°58’34.10’’S; 46°22’18.48’’W), Itararé (23º58’39.95’’S; 46º22’08.79’’W) and Gonzaguinha Beaches (23º58’21.49’’S; 46º23’04.48’’W) at São Vicente, São Paulo state, Brazil. The animals were collected using ring traps (the traps consisted of a metal ring 30 cm in diameter, with nylon net and baited with fish) placed every 10 meters and visited every 10 min over a 2-h period. Simultaneously, two people manually collected the crabs found below the water line (Sant’Anna et al., 2012). In the laboratory, the live crabs were identified according to Lemaitre (1995) and maintained in identified plastic boxes with constant aeration until analysis.

Determination of ovarian development

In the laboratory, the females were classified as juveniles or adults based on the shape of the abdomen and whether it was attached to the sternite (Van Engel, 1990; Watanabe et al. 2015). The cephalothoracic width (CW) of the crabs was measured (excluding lateral spines) using a caliper (0.05 mm).

To assess the morphological changes of the SR during adult ovarian development, the animals were anesthetized by chilling (5 min at -20°C) and were then dissected. The ovaries of adult individuals were classified macroscopically based on the maturation stage, according to their color and size with respect to the hepatopancreas. Five adult ovarian development stages were macroscopically defined: rudimentary (RUD), developing (DEV), intermediary (INT), mature or ripe (MAT) and ovigereous (OV) (Costa and Negreiros-Fransozo, 1998; Sant’Anna et

al., 2012; Zara et al., 2013). After these procedures, the SRs from each ovarian stage were dissected and rapidly weighed on an analytic scale (0.001 g).

The normality of the SR weight data was tested by the Shapiro-Wilk test for each ovarian stage, and the weight of the SR in the ovarian stages was compared by the Kruskal-Wallis test complemented by the Dunn test, with a significance level of $P<0.05$ (Sokal and Rohlf, 1995).

Light and transmission electron microscopies

The seminal receptacles of juvenile (virgin) and adult female crabs (al least three individuals) at each ovarian stage were fixed in 4% paraformaldehyde prepared with water from the environment in 0.2 M sodium phosphate buffer (pH 7.2) for 24 h. After fixation, the samples were washed in the same buffer; during the last bath, the gross anatomy was examined under stereomicroscopy. The buffered materials were dehydrated in alcohol (70-95%) and embedded in Leica® methacrylate resin following the histological routine. Serial sections 4 to 7 µm in size were obtained using a rotary microtome. The hematoxylin and eosin (HE) technique was used for the traditional histological description according to Junqueira and Junqueira (1983). The histochemistry procedure to identify proteins was conducted using the Xylidine ponceau (Mello and Vidal, 1980) and mercuric-bromophenol blue (Pearse, 1985) stains. The techniques PAS and Alcian Blue pH 2.5 were used to identify neutral polysaccharides with 1-2 glycol groups (neutral) and acid polysaccharides (Junqueira and Junqueira, 1983). The basophilic characteristics of the compounds were also tested using toluidine blue pH 4.0 (Junqueira and Junqueira, 1983). The images were obtained in a Zeiss Axio Imager Z2 light microscope.

For transmission electron microscopy (TEM), small fragments from the dorsal and ventral regions of the SR from juveniles and adults individuals, with RUD, DEV, MAT, and OV ovaries (N=3 each) were fixed in cold 3% glutaraldehyde prepared with seawater in 0.1 M cacodylate buffer (pH 7.3), added to 0.2% picric acid (Mancini and Dolder, 2001), washed in the same buffer and postfixed with 1% buffered osmium tetroxide (pH 7.3). The samples were washed in the same buffer and stained "en bloc" using aqueous 1% uranyl acetate; they were then dehydrated in an ascending acetone series and embedded in Epon-Araldite resin. Ultrathin sections were obtained using a Leica® UC7 ultramicrotome, and the copper grids were contrasted with 2% uranyl acetate and 2% lead citrate. Images were obtained using a TEM Philips CM100 and Jeol J1010 with 80KV electron beam emission. The ovarian stages INT was not used to TEM since we did not detect any weight or morphological difference among RUD, DEV, MAT or OV seminal receptacles.

Laboratory and field mating observations

In July 2011 at Itararé Beach, an intermolt mating pair of *C. hellerii* with a cephalothoracic width (CW) of 44.4 mm in the male and 43.7 in the female was manually collected among the rocks at low tide. Additional animals were also collected from Itararé Beach in July 2017 to conduct laboratorial mating experiments. The animals were acclimated during five days of feeding with penaoidean shrimp. Nine hard-shelled intermolting mating pairs with a CW ranging from 45.7 to 61.2 mm in males and 41.2 to 47.6 mm in females were maintained in a water-recirculation system of eight tanks under controlled salinity (35‰), aeration, room temperature of 23±0.5°C and photoperiod (12L:12D) adapted from Gregati et al. (2010). The mating arenas

were three tanks of 80x30x30 cm provided with living rocks, beach sand and mollusk shells. The recirculating system among the tanks was never interrupted to test whether the mating occurred independently of a probable pheromone communication. To test whether females might have multiple mating encounters, one female was released into the tank arena with the first male. Just after the mating, the male was removed, and 24 h later we introduced a second male and then a third one, always with an interval of 24 h. All mating behaviors were recorded using a digital camera. In trials in which the copulation did not start within one h, the attempt was considered unsuccessful.

Paternity test

For the paternity test, five ovigerous females were collected from the field with carapace width varying from 41.5 to 48.2 mm. The eggs were frozen at -20ºC in seawater to keep the DNA viable. The DNA from the mother tissue and an aliquot of 200 eggs were used to amplify Cfe01, Cfe05, CfeMIH2 and CfeMIH3 which were microsatellite loci of *Charybdis feriata* (Ma et al., 2013) that had similarity for *Charybdis hellerii.*

The PCR conditions for amplification were (94ºC for 5 min.) x1, (94ºC for 40 s., 48ºC (for Cfe01), 50ºC (for Cfe05), 49ºC (for CfeMIH2) and 48ºC (for CfeMIH3) for 30 s., 72ºC for 40 s.) x 35. The PCR products were screened on 8% non-denaturing acrylamide gel after electrophoresis at 70V for 2 h.

Specifict microsattelite characterization

To develop specific primers for *C. helleri,* additional amplifications from Cfe01, Cfe05, CfeMIH2, CfeMIH3 were size fractioned on agarose gel (0.8%) and the DNA bands of interest were recovered using Qiaquick gel extraction columns and concentrated using QiaQuick PCR

purification columns (Qiagen, Valencia, Calif.) according to the manufacturer's instructions. The purified DNA were ligated on plasmid vector (pCR 4-TOPO) then dialised. The ligated plasmids were used to transform electrocompetent DH5α MAX Efficiency *Escherichia coli* (T. Escherich, 1885) cells. The cells were plated on X-Gal/IPTG Luria-Bertani (LB) kanamycin agar and left to grow for 16 h at 37ºC. The positive colonies were inoculated on sterile test tube with 5µl of liquid LB kanamycin medium and left to grow for another 16 h at 37º in a temperature controlled shaker. The cells were concentrated on centriguge then lisated to extract the cloned plasmids. The concentrated plasmids then were eluted and added DNAse-free RNAse A. then rested for 15 min at 37ºC. The DNA then were fractioned on agarose gel (0.8%) and the ligated DNA was amplified with Big Dye Terminator Sequencing via PCR using the plasmid primers M13-Forward (-20) (5´-GTAAAACGACGGCCAG-3´) and M13-Reverse (5´-CAGGAAACAGCTATGAC-3´). The clones were sequenced on ABI 3730 DNA Analyser (Life Technologies – Applied Biosystems). The primers for *C. hellerii* were selected comparing similarities from the primers developed for *C. feriata* (Ma et al., 2013).

RESULTS

The SR during ovarian development and gross morphology

The weight of the SR of RUD and MAT adult females was slightly higher than that of OV females. However, no difference (KW, H=1.62; *P*=0.81) in the SR weight of the invasive crab was noted throughout ovarian development (Fig. 1).

The SR of juvenile *C. hellerii* is elliptical and slender (Fig. 2A). The RUD ovary, i.e., previtellogenesis, is whitish in color and is also attached to the dorsal region surface of the SR (Fig. 2B). The oviduct is connected ventrally in the SR (Fig. 2A and C). The vagina is long and is anchored in several bundles of muscular tissue (Fig. 2D). The SR of adult RUD inseminated ovigerous *C. hellerii* is round-auricular shaped, and the sperm mass is concentrated in the ventral region of the SR; however, there are streams of whitish material going to the dorsal region (Fig. 2E). It has the same general aspect as a juvenile female. Despite being filled with secretions, the oviduct maintains the ventral connection with the SR, which occurs close to the ventral region lined with the cuticle (Fig. 2F). The gonopore of the female is round-elliptical shaped, and there is no external evidence of a sperm plug (Fig. 2G). In the DEV female that copulated three consecutive times under laboratorial conditions, the SR was still auricular shaped but was swollen and the SR have a soft consistency. The whitish sperm is concentrated in the ventral region. However, many streams of whitish material are found among the translucent secretion from the ventral to dorsal areas of the SR. There is no evidence of a sperm plug or sperm packets (Fig. 2H).

Histology, histochemistry and ultrastructure of the seminal receptacle

During the JUV stage, the SR is elliptoid to ovoid shaped, and in the longitudinal section, the dorsal region displays a few small folds in the lumen while the main fold is found close to the transition to the ventral region (Fig 3A). In some JUV females, the fold occupies a large luminal area (Fig 3B). The dorsal region is composed of three layers: 1) the outermost connective layer and 2) a collagenous layer in contact to 3) the dense layer, which is the inner most layer and consists of stratified epithelium (Fig 3C). The lumen shows small quantities of basophilic secretion, usually in the dense layer or in the ventral region of the SR (Fig 3A-C). This secretion is intensely stained to indicate proteins and is also positive for neutral polysaccharides (Fig 3D-F). Anionic polysaccharides are absent in the basophilic secretion (Fig 3G). The same secretion is observed in the ventral region (Fig. 3A). The basal cells of the dense layer lie next to the connective layer which are displayed as well-organized collagen bundles and fibroblasts (Fig 3H). The basal cells in the stratified epithelium contain many mitochondria, rough endoplasmic reticulum (RER) and round nuclei (Fig 3H). The cells of the apical part of the dense layer are filled with a basophilic substance in large vesicles while the basal layer has many cells undergoing mitotic division (Fig 3C and I). Under a view of the ultrastructure, the last strata of the dense layer show cells with apical large and less electron-dense vesicles and many small electron-dense vesicles (Fig. 3J). These cells are undergoing holocrine secretion, and the nuclei have condensed chromatin or pyknosis (Fig 3I). The SR ventral is deeply folded where it comes in contact with the dorsal mesodermic-origin region. In JUV females, the ventral region has a small luminal volume and is continuous with the vagina. The ventral epithelium is monostratified, covered by cuticle and lies on a collagenous layer where many muscular fibers

are attached (Fig. 3A, K and L). The primary characteristic of the ventral epithelium is the cytoplasm filled with mitochondria (Fig. 3M).

In adult females from the RUD to OV stages, and in the DEV female that copulated three times under laboratorial conditions, the SR remained a ventral type with the oviduct opening in the dorsal region close to the transition to the ventral regions (Fig 4A-C). The more central section shows masses of spermatozoa grouped in figures that remember strata. However, the peripheral sections reveal that all spermatozoa material are mixed and connected by flows of sperm widespread in SR regions (Fig 4B). Only free spermatozoa immersed in basophilic secretions and surrounded by small amounts of acidophilic secretion were observed; no spermatophore was found (Fig. 4A-C). The ventral region is distended due to the secretion and spermatozoa (Fig. 4A and C). Sperm plugs or packets were absent in the SR of all *C. hellerii* RUD and DEV females analyzed (n=17). The ovary along the ventral region, and the oviduct opens in the dense layer close to the ventral region, which is continuous with the vagina (Fig. 4C). The dorsal dense layer appears more compacted because of the seminal fluid in the SR lumen (Fig 4C and D). This mesoderm origin region has the same characteristics found in JUV females, with an external and thin connective layer; a collagenous layer; and a fibrous, thicker, dense inner layer (Fig. 4D). Under a view of the ultrastructure, the connective layer has many fibroblasts that include cytoplasm filled with well-developed rough endoplasmic reticulum (RER) and mitochondria. Well-organized collagen fibers were observed among the fibroblasts. The stratified epithelium of the dense layer shows the basal layer of cells with RER and mitochondria (Fig 4E). The epithelium undergoes continuous desquamation, and the apical scaling cells release their contents through a specific holocrine mechanism. The cytoplasm accumulates into vesicles, and the large apical vesicles typical of JUV females were absent (Fig

4F). The cells close to the lumen have pyknotic nuclei and a disrupted cell membrane; the presence of significant cell debris surrounded by membrane is noted, so these cells resemble apoptotic bodies, which is the final fragmentation associated with this type of holocrine secretion (Fig. 4G and H). The apoptotic-like bodies form a layer of distinct secretion types named type I, which differs from the secretion among the spermatozoa (type II) (Fig. 4H and I). Secretion type I is strongly reactive to proteins and positive for neutral polysaccharides (Fig. 4J–L), whereas it is negative for acidic polysaccharides with pH values of 1 and 2.5 (Fig. 4M). Conversely, the secretion type II, which is spread among the free spermatozoa, was weakly positive for proteins but reacted intensively to neutral and acid polysaccharides (Fig. 4J–M).

Sperm transfer behavior

Under laboratorial conditions, of the nine females of *C. hellerii* used, four mated in the intermolt stage (hard-shelled condition). The female mating behavior could be divided into three repertoires: 1) hidden/immobile, where the female remained immobile while trying to hide (sometimes half-buried in the substrate); 2) escape, where the female swam away from the male individual; and 3) acceptance, where the female accepted the advance of the male or was captured even when trying to flee. For *C. hellerii* males, the repertoires were divided as follows: 1) walking/swimming, where the male dislocated himself in the aquarium; 2) immobile, where the male remained motionless in the aquarium; 3) advance, where the male tried to capture the female when perceived; 4) female manipulation, which is the act of the male putting the female into a copulatory position after the female is captured; 5) gonopod insertion, in which the male inserts the gonopods and transfers the seminal fluid after the female is in the copulatory position; and 6) released, when the male swims upward.

Most of the time, *C. hellerii* females were immobile (Table 1) next to stones or were partially burrowed in the substratum. When not receptive, *C. hellerii* females tried to flee (Table 1), but if captured, there were no longer any signs of struggle. The male tried to approach the female when he had visual contact, but there no precopulatory behavior was observed; the male simply tried to capture the female. Some *C. hellerii* males did not search for the females and remained motionless after some time (Table 1). As the male captures the female, it begins to manipulate the female with the chelipeds to put her in an upside-down copulatory embrace (Fig. 5A and B). During the copulatory embrace, the female always opens her abdomen so the male can insert gonopod I to begin performing the sperm transfer (Fig. 5C and D), in our observations, the male never opened the female abdomen. During the sperm transfer, the male might move to a different location with the female underneath, and the female would clasp the male with all her pereopods (Fig. 5E). *Charybdis hellerii* has a long sperm transfer period; the mating duration ranged from 60 min to five hours (177.5±105.5 min). After the sperm transfer, the female unclasps the embrace and the male goes upward (Fig. 5F). There is no postcopulatory behavior; the individuals seclude themselves individually after the mating. We had only one successful multiple mating with a female (CW 44.7 mm) who accepted three different males (CW of 54.0, 60.4, and 60.5 mm) with a 24 h interval between each copula. The male copula lasted 94, 143 and 166 min each following mating average (177.5±105.5 min).

Paternity test and microsattelite charcterization

The paternity test showed that *C. hellerii* is polyandrous. In this case, the loci Cfe01, Cfe05, CfeMIH2 and CfeMIH3 were polymorphic (Table 2). Specifically Cfe01, the DNA from the eggs had multiple bandings (Fig. 6A) showing that the heritage was from at least two

different males. In the loci Cfe05 and CfeMIH3, the offspring had different genotype from the mothers, but it was not possible to clearly see the polyandry (Fig. 6B and C). The loci CfeMIH2 also had polymorphy, and it it was also possible to see polyandry (Fig 6D). The sequenced PCR products from the amplifications using the *C. feriata* primers were searched for similar sequences. Overall the sequences for the primers were very similar to the used for *C. feriata*. The four specific primers that can be used for *C. hellerii* are listed on Table 3

FIGURES and TABLES

Figure 1. Mean weight (g) and standard deviation (thicks) of the seminal receptacle during the ovarian cycle. The number of crabs analyzed per development stage is shown in parentheses. Rudimentary (RUD), developing (DEV), intermediate (INT), mature (MAT) and ovigerous (OV).

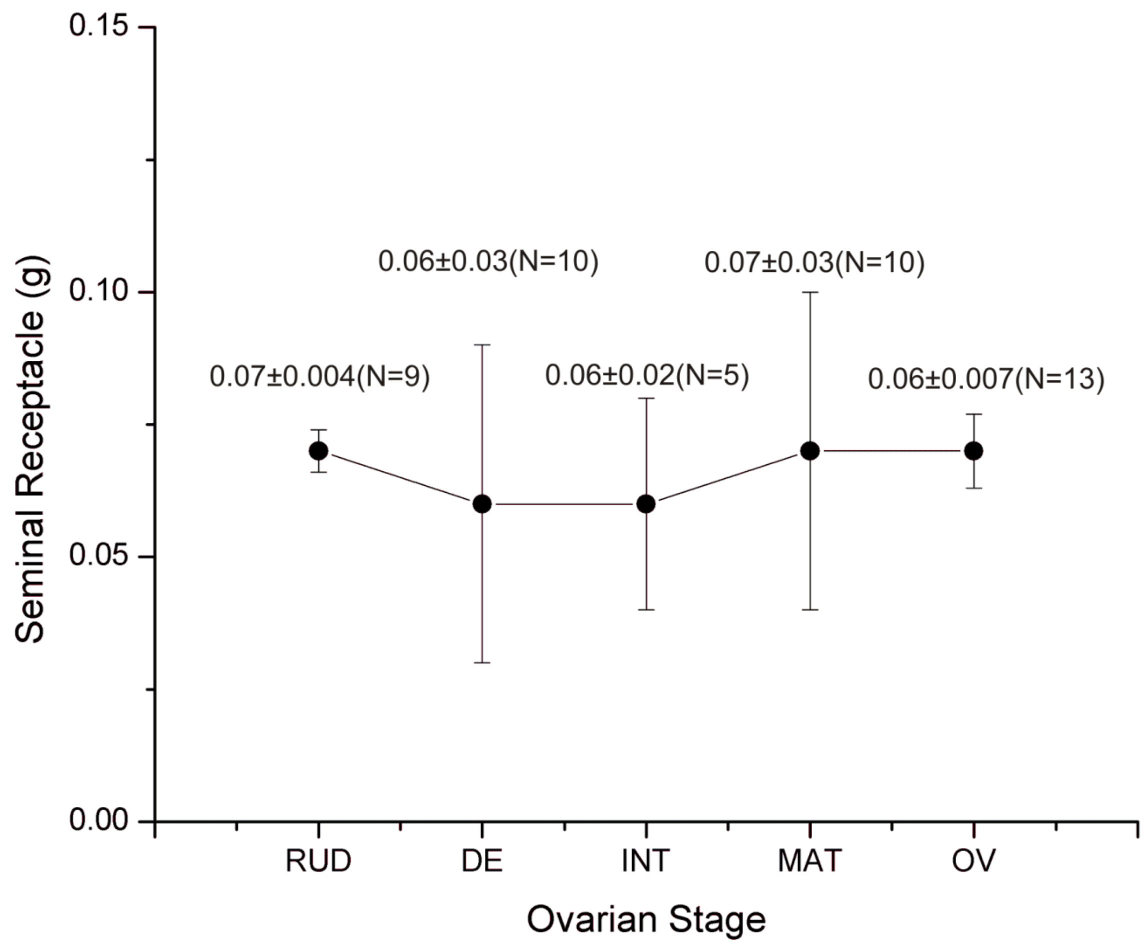
0.15
0.10
0.05
0.00
Seminal Receptacle (g)
0.07±0.004(N=9)
0.06±0.03(N=10)
0.06±0.02(N=5)
0.07±0.03(N=10)
0.06±0.007(N=13)
RUD
DE
INT
MAT
OV
Ovarian Stage

Figure 2. Gross morphology of the seminal receptacle of *Charybdis hellerii* – **A.** General view of the juvenile seminal receptacle. Black arrow depicts the position of the oviduct. **B.** Detailed view of the juvenile ovary, with translucent oocytes (arrow). **C.** Detailed view of the ventral region of the juvenile seminal receptacle with discrete oviduct. **D.** Detailed view of the vagina with several bundles of muscular tissue. **E.** General view of adult ovigerous female with developing ovaries of *C. hellerii,* with spermatozoa in the ventral region, and the strains of whitish secretion going to the dorsal region (arrowheads). Arrow depicts the gonopore aperture. **F.** Detailed view of the seminal receptacle ventral region, with evident oviduct and the presence of spermatozoa (arrow). **G.** Detailed view of *C. hellerii* gonopore. **H.** Seminal receptacle of a female inseminated three times with developing ovary, with the presence of spermatozoa mixed in the translucent secretion in the dorsal region (arrowheads). d, dorsal region; m, muscle tissue; od, oviduct ov, ovary; sr, seminal receptacle; sz, spermatozoa; v, ventral region; vg, vagina.

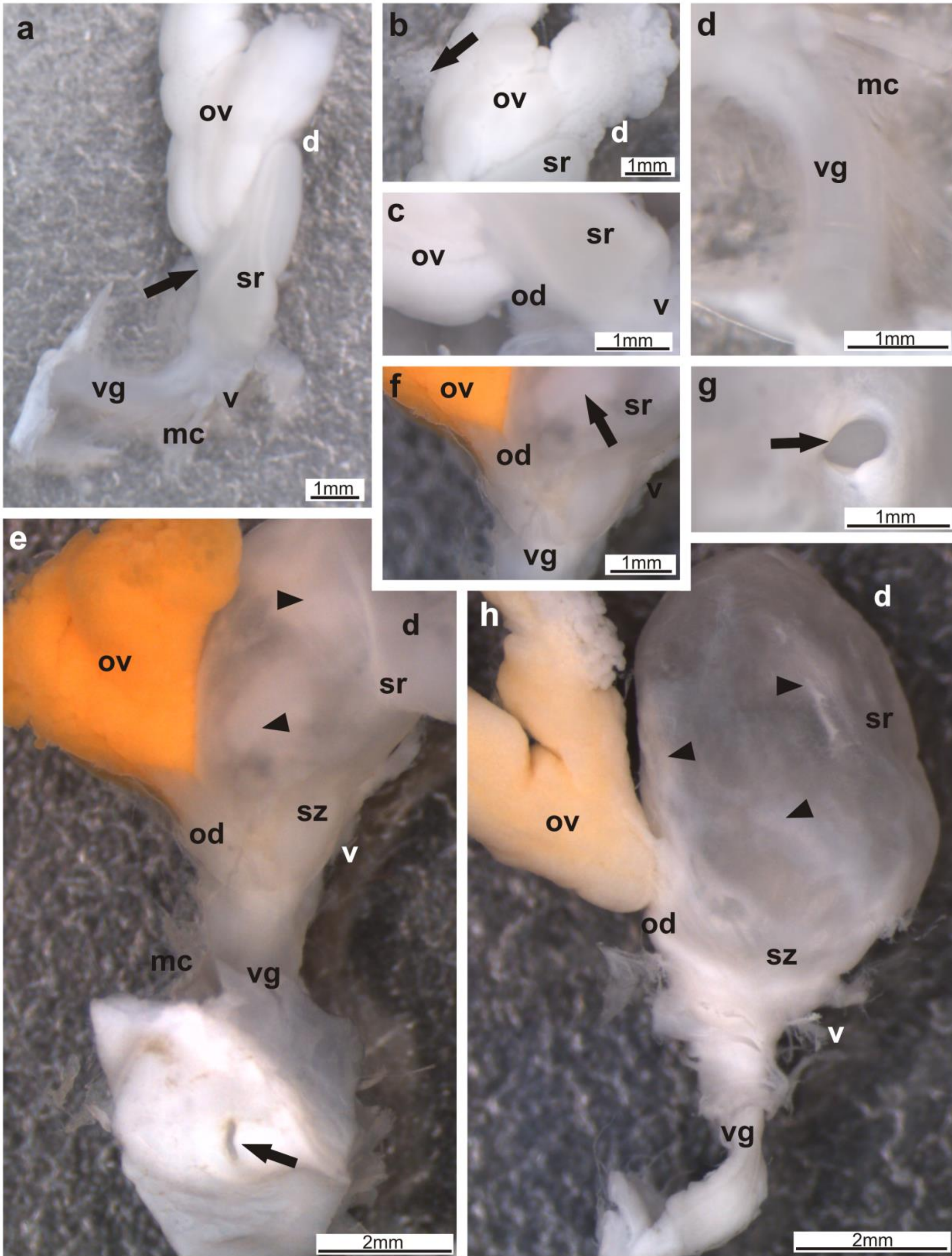
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Figure 3. Juvenile female seminal receptacle of *Charybdis hellerii.* **A.** Micrographs assembled sequentially showing an empty seminal receptacle with small basophilic secretion. The oviduct is inserted in the ventral region through modification in the seminal receptacle epithelium (arrow). **B.** Details of the juvenile seminal receptacle with several folds in the epithelium (arrowheads). **C.** Details of the epithelium of the dorsal region of the dense layer with cells undergoing mitosis (arrow). **D and E.** Secretions being liberated by holocrine mechanism (arrow) rich for proteins. **F.** Luminal secretion reactive to neutral polysaccharides. **G.** Luminal secretion without reaction for acid polysaccharides (arrow). **H.** Ultrastructural aspect of the dorsal region of the seminal receptacle. **I**. Apical region of the dense layer showing flaking of cells containing a large vacuole being released by exocytosis (black arrow). Compact nuclei being released (white arrow). **J.** Fusion process of electron-dense vesicles (black arrow) to a large heterogeneous vesicle (white arrow). **K.** General aspect of the receptacle. The oviduct connects in the ventral region of the seminal receptacle (arrow). **L.** Ultrastructure of the ventral region with the cuticle above the epithelium. **M.** Detailed view of the apical region of the ventral region with several mitochondria (arrow). c, cuticle; cl, collagen; ct, connective tissue; d, dorsal; dl, dense layer; l, lumen; mc, musculature; n, nucleus; ov, ovary; od, oviduct; s, secretion; v, ventral.

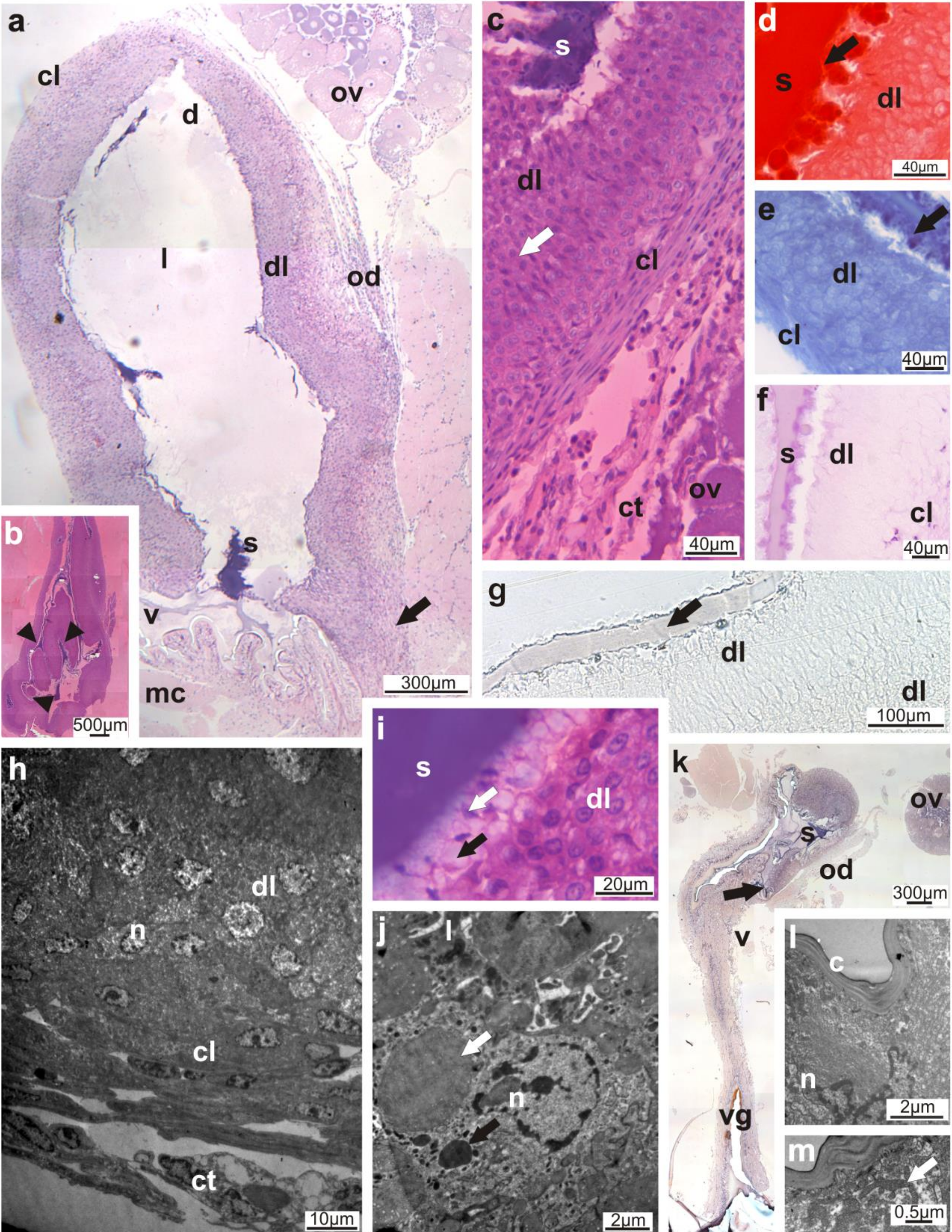
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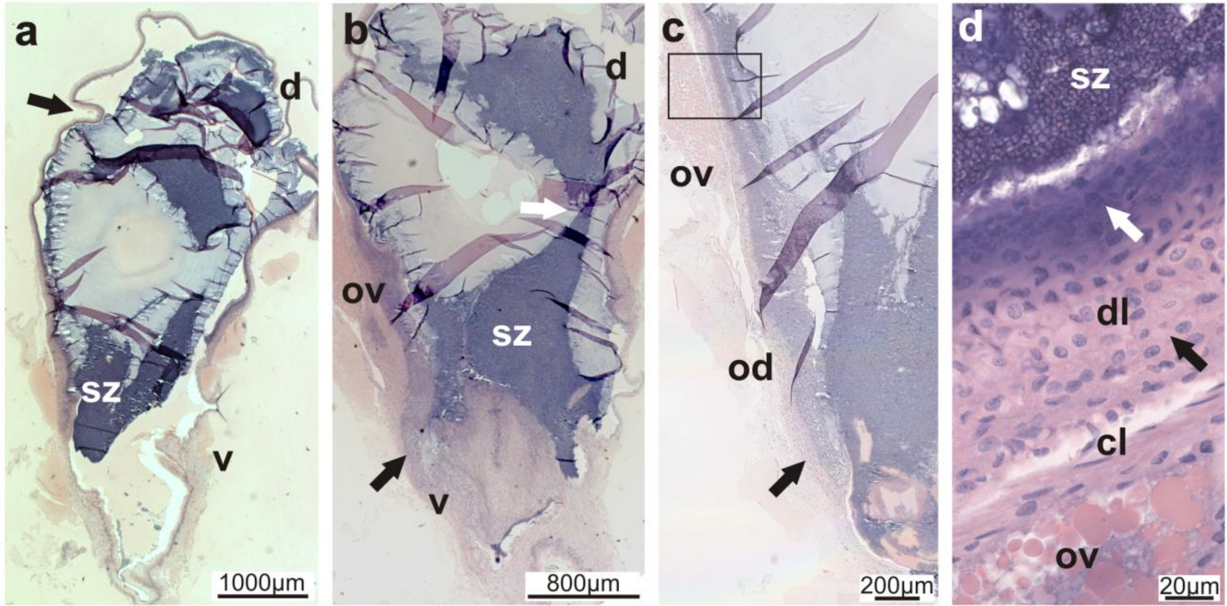
Figure 4. Developing *C. hellerii* female after three consecutive mating attempts. **A.** MosaiX photos assembled showing RUD receptacle with accumulated sperm on the ventral region. The absence of secretions or sperm plugs should be noted. The modified epithelium in the transition between the dorsal and ventral types forms a fold in the lumen (arrow) **B.** Peripheral section showing that the ventral and dorsal sperm masses are connected (white arrow). Black arrow shows the oviduct insertion. **C.** Detailed view of the ventral region of the seminal receptacle with the oviduct connection (arrow). **D.** Detailed view of the seminal receptacle epithelium (marked in the rectangle in **C**), with a more developed dense layer with acidophilic cytoplasm in the basal region (black arrow) and basophilic cytoplasm in the apical region (white arrow). **E.** Ultrastructural aspect of the connective layer depicting well-organized collagen fibers (arrow) and cytoplasm filled with rough endoplasmic reticulum and mitochondria. **F.** Apex of the dense layer depicting cells producing secretions (arrow) distinct from that where the spermatozoa are found. **G.** Apical cells dislodge to the lumen showing compact electron-dense nuclei and the cytoplasm of the dense layer filled with less electron-dense vesicles (arrow). **H.** Spermatozoa immersed in secretions less electron-dense than the secretions liberated by the dense layer. **I.** Spermatozoa immersed in secretions. **J and K.** Weak reaction for xylidine and bromophenol blue among free spermatozoa (black arrow). The secretion next to the dense layer is positive for this compound (white arrow). **L.** Secretions strongly reactive to PAS close to the sperm (black arrow). The secretion next to the dense layer was also reactive to Schiff (white arrow). **M.** Secretions among free spermatozoa that is intensely positive for acid polysaccharides (black arrow). The secretion next to the dense layer was not reactive (white arrow), Alcian blue pH 2.5. cl, collagenous layer; d, dorsal; dl, dense layer; l, lumen; m, mitochondria; n, nucleus; od, oviduct; ov, ovary; rer, rough endoplasmic reticulum; s, secretion; sz, spermatozoa; v, ventral.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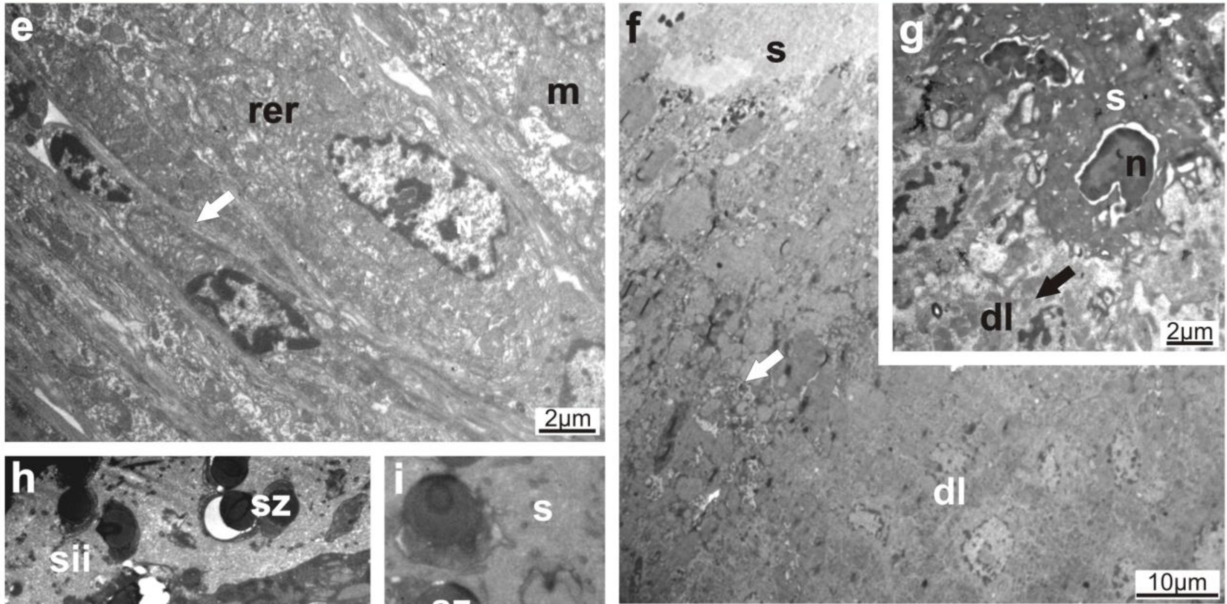
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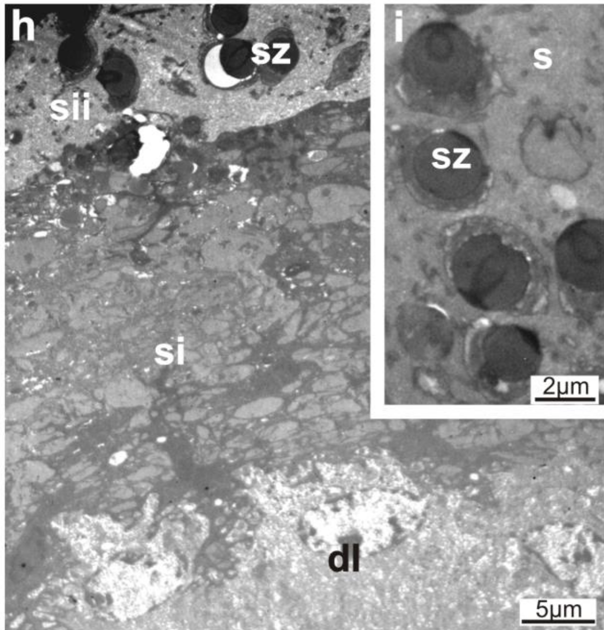
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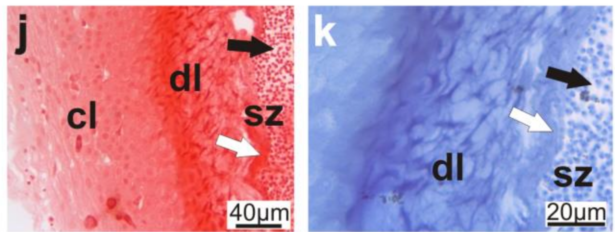
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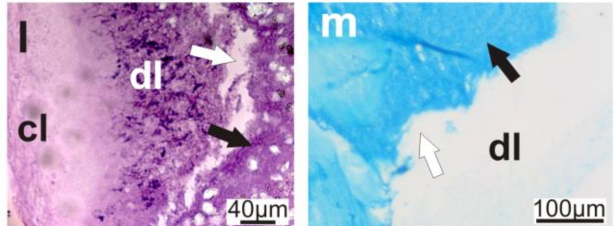
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Figure 5. Mating behavior of *Charybdis hellerii* – **(A)** Capture of the female by the male. **(B)** Male manipulating the female into the upside-down position. **(C)** Female opening the abdomen (arrow) to allow male gonopod insertion. **(D)** Male inserting the gonopod (arrow) into the female to begin the sperm transference. **(E)** Detailed view of the gonopod I of the male inserted into the female (arrow) and the grasping of the female with the pereopods. **(F)** Male releasing the female and swimming upward.

a
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♀

b
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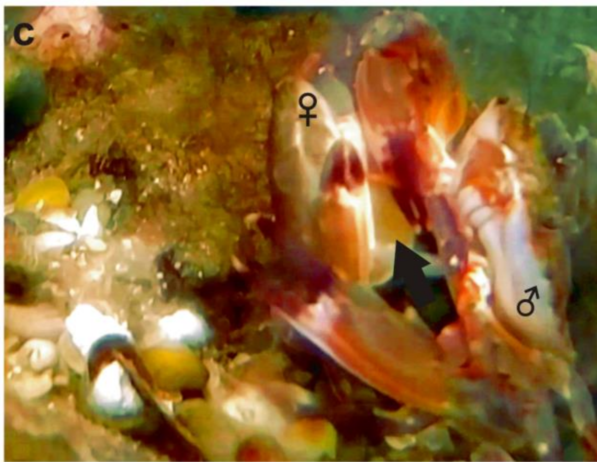
♂


c
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e
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f
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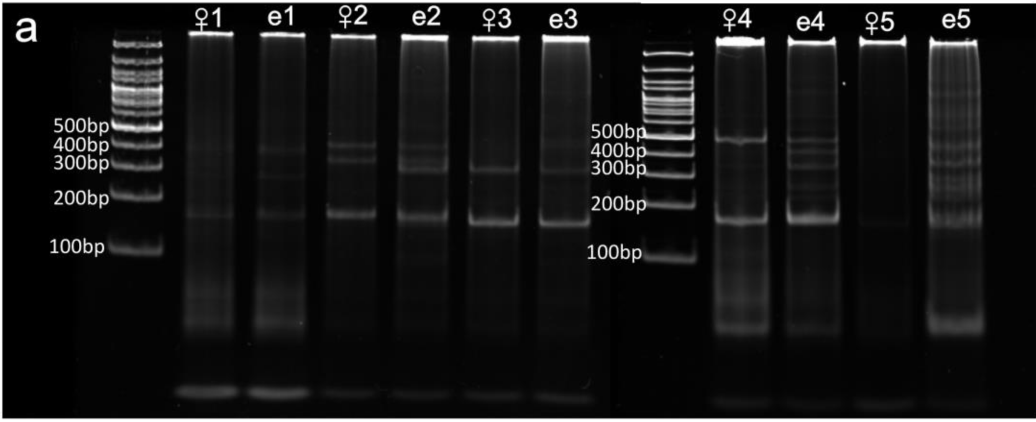
Figure 6. Screening of acrylamide gel (8%) of the PCR products from the DNA of the 5 *Charybdis hellerii* ovigerous females and its offsprings. **A.** Loci Cfe01; **B.** Loci Cfe05; **C.** Loci CfeMIH2; **D.** Loci CfeMIH3. Carapace width of the females: ♀1, 41.5 mm; ♀2, 48.2 mm; ♀3, 42.4 mm; ♀4, 44.8 mm; ♀5, 46.6 mm. ♀, ovigerous females; e, eggs.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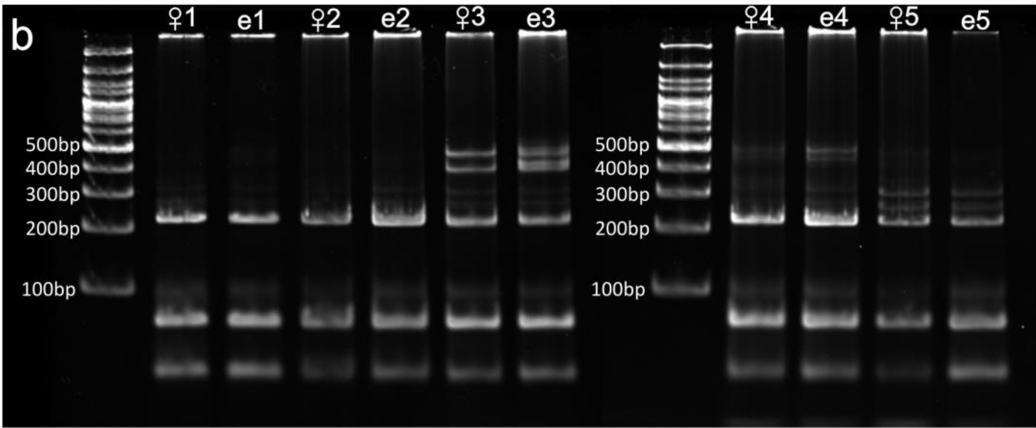
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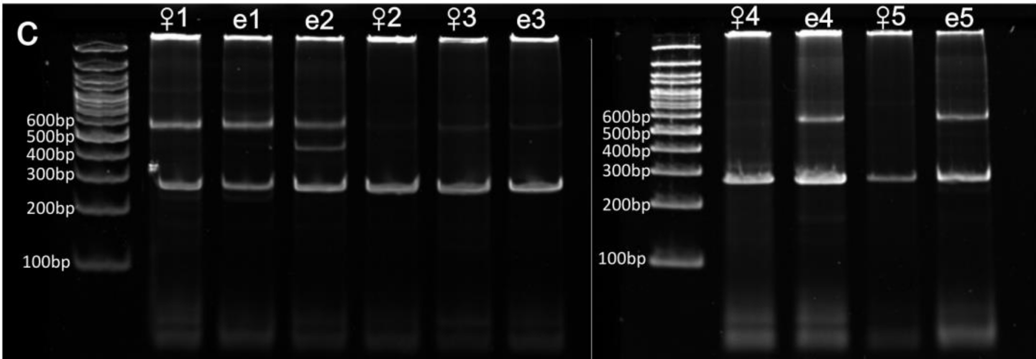
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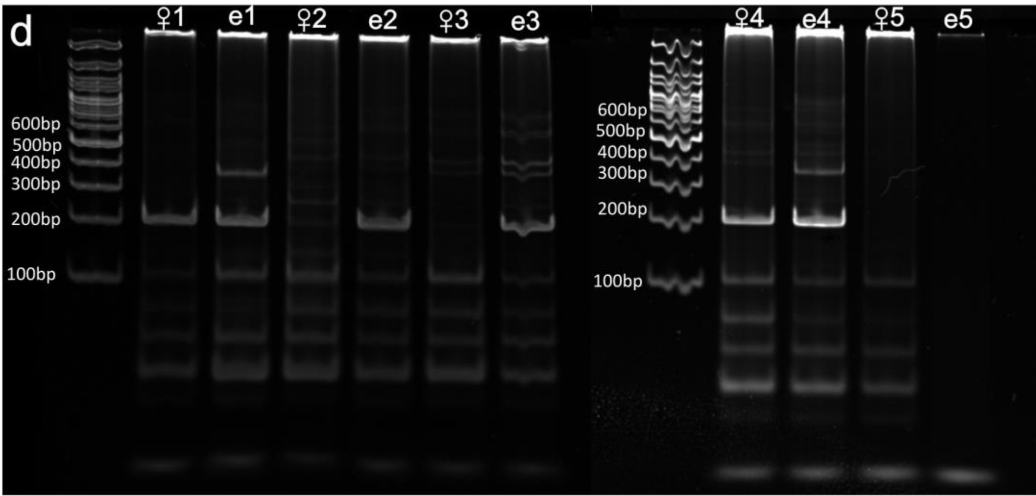
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Table 1. Catalogue of behavioral repertoire of *Charybdis hellerii.* Time of behavioral acts (minutes), with the number of acts in parentheses.

	Behavioral category	Time behavioral act (min)	
		Succesful mating	Unsuccesful mating
Females	Immobility	8.98±4.46	58.83±0.79
	Escape	0.69±0.52 (1.5 times)	1.17±0.78 (8.0 times)
	Male acceptance	0.2±0.12	0
Males	Walking/Swimming	2.27±0.62	6.94±2.29
	Immobile	4.58±2.55	52.09±2.46
	Advance	3.04±2.74 (2.75 times)	0.96±0.64 (7.2 times)
	Female manipulation	10.01±1.45	0
	Abdomen aperture	0.11±0.02	0
	Gonopod insertion	0.41±0.05	0

Table 2. Genotype of *Charybdis hellerii* females and alleles of the offspring to four *loci* (Cfe01, Cfe05, CfeMIH2 and CfeMIH3). Alleles were designated based on the length of the base pair. Bold numbers in the egg alleles indicates maternal alleles.

Female	Female genotype	Egg alleles
1		
Cfe01	159; 371	**159**; 265; **371**
Cfe05	84; 210	**84**; 105; **210**; 252; 462
CfeMIH2	252; 576	**252**; **576**
CfeMIH3	56; 112; 196	**56; 112**; **196**
2		
Cfe01	159; 318	**159**; 265; **318**; 371
Cfe05	84; 105; 210; 294	**84**; **210**; **294**
CfeMIH2	252	**252**; 432; 576
CfeMIH3	56; 112; 140; 196; 252	**56; 112; 196**
3		
Cfe01	159; 265	**159; 265**; 424
Cfe05	84; 105; 210; 378; 435	**84; 105; 210; 378**; 420
CfeMIH2	252; 576	**252; 576**
CfeMIH3	56; 112; 392	**56; 112;** 196; 336; **392**; 560; 672
4		
Cfe01	159; 477	**159**; 212; 265; 424; **477**
Cfe05	84; 105; 231; 525	**84; 105; 231**; 545
CfeMIH2	288; 720	180; **288**; 576; 1116
CfeMIH3	56; 112; 224; 476; 532; 784	**56; 112; 224**; 420; **532**; 764
5		
Cfe01	159	**159**; 265; 848
Cfe05	84; 231; 273; 315	**84**; 105; **231; 273**; **315**
CfeMIH2	288; 720	**288**; 612; 1152
CfeMIH3	56; 112	-

Table 3. Locus and PCR primer sequence for four microsattelite *loci* developed for *C. hellerii.* F, forward sequence; R, reverse sequence.

Locus		Sequence 5'-3'
Che01	F	TCACTCACTCGAAAGCCGCGG
	R	CTGTGTGAAATTGTTATCCGCTC
Che02	F	CTACCCGACGGAGCGAGCTC
	R	AGCTAACTCACATTAATTGCGTT
Che03	F	CTAAATTCAATTCGCCCTATA
	R	TTCGCGGCCGCTAAATTCAA
Che04	F	GAAGATCGGAAAGGAAGAGC
	R	GCTTCTCGTACCTTCGCTAC

DISCUSSION

The main findings of our work are as follows: 1) the absence of weight variation in the SR during ovarian development, 2) the absence of sperm plugs in the SR, 3) females can copulate in the hard-shelled condition including with more than one male without forming sperm packets and spermatophores, leading to a seminal fluid mix in the SR, 4) the ventral type of SR, first described to Portunidae, maintains the same anatomical, histological and ultrastructural organization during ovarian development and 5) The offpring of *C. hellerii* has multiple paternity. All these findings will be discussed below.

The weight of the seminal receptacle of the non native crab *C. hellerii* did not change significantly during ovarian development; thus, our findings are quite different from that reported for most of Portunidae species studied (Spalding 1942; Ryan, 1967; Johnson, 1980; Hines et al., 2003; Wollcott et al., 2005; Zara et al., 2014). According to Hines (1982) and Wolcott et al. (2005), allometric constraints in the inner space of the cephalothoracic carapace influence and determine the amount of eggs produced in each ovarian cycle; the sperm plug dissolution is also necessary to this process. In the following species, the SR decreases in volume after mating and creates more space in the body cavity for ovary development: *Chasmagnatus granulatus* (Dana, 1851), as *Neohelice granulata* in López-Greco et al. (1999), *Metacarcinus magister* (Dana, 1852), as *Cancer magister* in Jensen et al., (1996) and Shauna and Hankin (2004) and portunids (Ryan, 1967; Hinsch, 1986, Bawab and El-Sherief, 1988; Pinheiro and Fransozo, 2002; Wolcott et al., 2005; Zara et al., 2014). Therefore, the allometric constraints proposed by Hines (1982) do not apply for *C. hellerii,* which indicates that the females do not have these constraints and can mate while their ovary is in any developmental stage. These characteristics could explain the large number of females and ovigerous females with mature

ovaries found in the same study area population (Sant'Anna et al., 2012) and demonstrate that female *C. hellerii* have multiple spawnings with sperm from more than one male.

The sperm plug is absent in the invasive crab *C. hellerii* and could be an advantage to an invasive species that could receive seminal fluid from different males. The absence of a sperm plug is also reported in the close relative species of Thalamininae, *Thalamita picta* Stimpson, 1858, *Thalamita prynma* (Herbst, 1803), *Thalamita sima* H. Milne Edwards, 1834 and *Charybdis smithii* (McLeay 1838), although it was only observed in this last species (Norman, 1996; Norman et al., 1997; Balasubramanian and Suseelan, 1998). The sperm plug is a characteristic of Portunoidae crabs found in different genera such as *Carcinus*, *Ovalipes*, *Scylla*, *Callinectes*, *Portunus* and *Areneaus* (Spalding, 1942; Ryan, 1967; Hartnoll, 1969; Johnson, 1980; Hinsch, 1986; Bawab and El-Sherief, 1988; Jayasankar and Subramoniam, 1997; Zara et al., 2014). The sperm plug is rigid with the consistency of paraffin wax (Ryan, 1967; Johnson, 1980; Jivoff et al., 2007); it produces a barrier in the seminal receptacle that limits or prevents the transfer of genetic material from other males in subsequent mating (Hartnoll, 1969; Johnson, 1980; Wolcott et al., 2005). This plug disappears shortly after mating in the Portunidae *Ca. sapidus* (Hines et al. 2003; Wolcott et al., 2005), *P. hawaiiensis* (as *P. sanguinolentus* in Ryan, 1967), *P. pelagicus* (Bawab and El-Sherief, 1988), *O. occelatus* (Hinsch, 1986), *A. cribrarius* (Pinheiro and Fransozo, 2002; Zara et al. 2014), in the varunid *Ch. granulatus* (Lopéz-Greco et al., 1999) and in some Carcinidae and Cancridae, despite the last species having an internal plug (Spalding, 1942; Jensen et al., 1996). Hartnoll (1969) proposed that the sperm plug might be a vestigial feature of Brachyura ancestors that adheres the spermatophores/spermatozoa to shallow depressions of the female sternum. The sperm plug has possibly been lost in Thalamitinae, and its absence in *C. hellerii* may be considered homoplasy which in this case favors the invasion of

new environments because the female once mature, could mate any time possible. Thus, the absence of a sperm plug in *C. hellerii* is a feature which is shared with many different families of Majoidea (Hartnoll, 1969; Beninger et al., 1988, 1993; Diesel, 1989, 1991; Sainte-Marie and Sainte-Marie, 1998; Sal Moyano et al., 2009, Antunes et al., 2016), Dorippidae (Hayer et al., 2016; Vehof et al., 2017), Leucosiidae (Hayer et al., 2015), Ocypodidae (Lopez-Greco et al., 2009), Ucididae (Sant'Anna et al., 2007) and Pinnotheridae (Becker et al., 2011).

In Portunidae, secretions produced for the absorption or elimination of the sperm plug seem to contribute to the gradual dissolution of the spermatophore wall (Wolcott et al., 2005; Jivoff et al., 2007); they are displaced to the ventral region of the SR (Zara et al., 2014). In addition, the weight of the SR decreased, and free spermatozoa were only observed in females with mature ovaries (Wolcott et al., 2005; Zara et al., 2014). Spalding (1942), Ryan (1967), Hartnoll (1969) and Wolcott et al. (2005) suggest that the sperm plug has no nutritional role in Portunidae. Following the same line of thought, the natural absence of the plug in *C. hellerii* shows that it is not essential for nutrition, long storage or maintenance of sperm viability (Jeyalectumie and Subramoniam, 1991) and argues against the nuptial gift proposed for Portunidae (Hines et al., 2003; Wolcott et al., 2005). Jivoff (1997a and b) and Jivoff et al. (2007) consider the sperm plug function of preventing further mating as the leading adaptive advantage in Portunidae. Thus, unlike other members of this family, the absence of the sperm plug in *C. hellerii* allows the female to mate with several males, which is similar to what was reported in three species of *Thalamita* (Norman, 1996; Norman et al., 1997, 1999), *Charybdis longicollis* Leene, 1938 in Innocenti et al. (1998) and *C. japonica* (Baker et al., 2018); this activity allows increased genetic variability of the offspring. This agrees with the results reported by Sant'Anna et al. (2012), who suggested that female crabs spawn multiple times and that the sex ratio is 3:1

in favor of males, indicating intense competition for females in the population in São Vicente, Brazil, which could explain the success of *C. hellerii* in this region.

Another important result of this study is the absence of sperm packets even in females that mated three times with different males. The presence of sperm packets is a common occurrence in species that undergo hard-shelled mating, as described for Majidae (Benhalima and Moriyasu, 2000; Sainte-Marie et al., 2000; Sal Moyano et al., 2010), Inachoididae (Diesel, 1989, and 1990; Antunes et al., 2016, 2018) and Trichodactylidae (Oliveira and Zara, 2018). However, in those species, the vas deferens has accessory glands that seem to contribute to the formation of the sperm packets (Diesel, 1989, 1990; Benhalima and Moriyasu, 2000; Sainte-Marie et al., 2000; Antunes et al., 2016, 2018; Oliveira and Zara, 2018). In Inachoididae, the mixture of the secretions from the accessory glands with PVD secretions creates a gel that separates the sperm masses (Antunes et al., 2016, 2018). In *C. japonica*, the PVD depicts outpocketings, but there is no accessory gland (Wong and Sewell, 2015), which is also the case in *C. hellerii* and explains the absence of sperm packets in the SR. Additionally, because Sesarmidae (Zimmerman and Felder 1991), Varunidae (López-Greco et al., 1999) and Ocypodidae (López-Greco et al., 2009) have no evident sperm packets and can mate with several males before ovulating, it was proposed that the sperm stored could represent a mixture of sperm from several different males. This could be the case of *C. hellerii* and possibly of *C. japonica* because they have similar characteristics regarding their mating system and sperm storage (Wong and Sewell, 2015; Baker et al., 2018).

In *C. hellerii,* the ability to mate in the hard-shelled intermolt condition is in concordance with the absence of the sperm plug. In the species that have an internal sperm plug, i.e., a plug that fills the SR (Hartnoll, 1969), the females mate in the soft-shelled molt condition, as reported

for Portunidae (to review, Hartnoll, 1969; McLay and Lopes-Greco, 2011). Although it was reported that females of *Charybdis feriata* (Linnaeus, 1758) and *Charybdis natator* (Herbst, 1794) mate in the soft-shelled state (Soundarapandian et al., 2013; Sumpton, 1990), our results are the third report that the genus undergoes hard-shelled mating, as reported for *C. longicollis* and *C. japonica* (Innocenti et al., 1998; Baker et al., 2018). In addition, three more species of *Thalamita* open the question of whether the ability of females to mate in the hard-shelled condition could be traits of Thalamitinae, which could reinforce the validity of this subfamily proposed by Evans (2018). Soft-shelled mating seems to be the primary characteristic of Portunidae; moreover, the genus *Charybdis* might exist in an intermediate position between the ancestral condition (soft-shelled) and the most derived (hard-shelled) state of the mating system in Brachyura (McLay and Lopez-Greco, 2011). In this study, the males that copulated were larger than the females; however, in one field observation during samplings made by Watanabe et al. (2015), an intermolt couple with the same CW size was captured (Sant'Anna and Watanabe, personal communication). This was also similarly reported in *Thalamita* spp. where the couplings occur with males that are smaller than females, and in many cases, were the same size as females (Norman, 1996; Norman et al., 1997, 1999). Interestingly, males with a mature reproductive system may exist in the small size classes in *C. hellerii* (Watanabe et al., in preparation). Thus, further mating experiments using small males are required to consider another trait in favor of increasing the genetic variability of larvae by small males participating as functional males in the population.

Unlike what was observed in *C. japonica* (Baker et al., 2018), the *C. hellerii* males did not seem to chemically perceive the females most of the time. Thus, if the female individuals are about to molt, the male individuals might be able to chemically perceive the soft-shelled molting

females. In *C. helleri*, there was no pre or postcopulatory guarding, and the copula was short, similar to *T. sima* (Norman, 1996), *T. prymna* (Norman et al., 1997), *T. picta* (Norman et al., 1999) and *C. japonica* (Baker et al., 2018); however, it was dissimilar from soft-shelled Portunidae females (for review, Pinheiro and Fransozo, 1999; Jivoff et al., 2007), and this trait is characteristic of sperm plug species (Hartnoll, 1969, Sastry, 1983; Norman et al., 1999). Thus, those behaviors are also in favor of allowing the females to mate with other males after copulation, as found here in *C. hellerii* under laboratory conditions. Conversely, the fact that *C. hellerii* females barely explored the tanks suggests a cryptic female behavior, supporting the suggestion originated in population biology (Sant`Anna et al., 2012). Thus, the possibility of only one male mate that fertilizes the oocyte cannot be ruled out.

The morphology and ultrastructure of the SR do not change during ovarian development. The seminal receptacle of *C. hellerii* is covered with a thin layer of connective tissue and is called the connective layer. In the dorsal region beneath the connective layer, there is an intermediary layer rich in collagen fibers and fibroblasts; this is called the collagen layer; the innermost layer is formed by cells arranged in a stratified-like epithelium, without basal lamina, is called the dense layer. This common arrangement of the SR in layers is quite similar to that observed in origoiniids (Beninger et al., 1988, 1993; Sainte-Marie and Sainte-Marie, 1998), inachids (Diesel, 1989), varunids (López-Greco et al., 1999), ucidids (Sant´Anna et al., 2007) and other portunids (Ryan, 1967; Johnson 1980; Zara et al,. 2014).

The dense layer in *C. hellerii* shows glandular activity and is characterized by cells that become progressively filled with numerous active Golgi complexes and electron-dense vesicles as they reach apical layers close to the lumen, similar to other crabs *Chionoecetes opilio* (Fabricius, 1780) in Beninger et al. (1993). In JUV females, the secretory activity appears less

intense, and the apical layer cells form a large vesicle among the small vesicles. These cells release their contents at the same time as the cells lose their junctions. The holocrine-type secretions found in adult SR cells of *C. hellerii* show a similar pattern; however, this is a peculiar type of holocrine secretion without a large vesicle, as in JUV. The cells become filled with many small cytoplasm vesicles that we call apoptotic-like vesicles; these vesicles do not became enlarged, and the cell disrupts just before the desquamation to display a pyknotic nucleus. This holocrine secretion pattern is different from the sperm plug species *A. cribrarius* (Zara et al., 2014). In this species, the disintegration of the secretory cells occur as they complete their terminal differentiation; consequently, they became enlarged and filled with secretory products, losing their cell junctions, leading to desquamation and then cell disruption. This follows the usual pattern of mammalian holocrine secretion (Mescher, 2018), which is likely associated with enzyme production to sperm plug dissolution (Zara et al., 2014). In another portunid, *Scylla serrata* (Forskal 1775), sperm plug dissolution occurs by means of enzymes (Jayasankar and Subramoniam, 1997). Thus, the difference in the holocrine mechanism found here could be associated with different secretory components and functions, probably one acting in spermatozoa protection or maintenance in *C. hellerii*. The increase in ultrastructure knowledge, including apoptotic techniques, is an interesting cell biology model to use for understanding different patterns of holocrine secretion and how it could be related to key roles of spermatozoa maintenance, sperm plug dissolution or its importance to sperm packet species. Independent of the cell mechanism, the glandular layer cells (dense layer) appear to follow the predictable pattern of increasing the decomposition and degradation into the lumen, where they disintegrate and discharge their secretions (Diesel, 1989). The holocrine secretory activity of the dorsal epithelium was described by several authors in sperm plug portunoids species (Spalding, 1942;

Ryan, 1967; Johnson, 1980; Bawab and El-Sherief, 1989; Jayasankar and Subramoniam, 1997; Zara et al., 2014), sperm packet majoid species (Beninger et al., 1993, González-Pisani and López Greco, 2007; Sal Moyano et al., 2009; Antunes et al., 2016) or species with only free sperm in the SR, such as Leucosiidae and Ucididae (Sant'Anna et al., 2007; Hayer et al., 2015). The ultrastructural results reported in this work conclude that *C. hellerii* cells are involved in the secretory activity, although the reasons for cell scaling and desquamation require further investigation.

Between the dorsal and ventral regions, a modified epithelium that folds into the SR lumen is observed in *C. hellerii* but does not display an important specific function. In *Ca. sapidus,* this epithelium is referred to as the dorsal epithelium (Johnson, 1980), epithelial layer or tiphosolar projection, as in *P. hawaiiensis* (Ryan, 1967), and a possible movement function has already been proposed for this modified epithelium (Hartnoll, 1968; Johnson, 1980). In *A. cribrarius*, this epithelium covers the area where the spermatozoa are stored and appears as a columnar epithelium with long microvilli (Zara et al., 2014). This modified epithelium in the transition to the dorsal and ventral regions differs from the *velum* found in *I. phalagium*, which delimits the fertilization chamber and is formed by columnar cells and muscle fibers (Diesel, 1989) or the velum of *Libinia spinosa* Milne Edwards 1834, which is modified according to the SR condition (González-Pisani et al., 2012) and does not seem to be present in *C. hellerii*. The ventral region is lined by the cuticle over a true monostratified epithelium connected to the vagina, both of which are of ectodermal origin. The ventral region and the vagina show muscle insertions that are likely related to muscular contractions to open the vagina and vulva during egg extrusion. Moreover, they can move to aid oocytes in finding nonmotile sperm, as proposed to majoids (Diesel, 1989; Beninger et al., 1993). In Portunidae, the oviduct connection usually is dorsal

(Ryan, 1967; McLay and López-Greco 2011; Zara et al., 2014); however, in *Car. maenas* and *A. cribrarius*, the SR changes its morphology as the sperm plugs disappear, and the oviduct becomes close to the ventral side. The oviduct is maintained in opposition to the vagina, maintaining the dorsal type arrangement (Spalding, 1942; Zara et al., 2014). This was not observed in *C. hellerii* because the SR maintains the same morphological ventral type opening of the oviduct, independent of the gonadal stage.

Another interesting event is the presence of free spermatozoa throughout the entire ovarian development, even after mating. This result agrees with the dehiscence model associated with the acid polysaccharides mucous type spermatophores of the coenospermia and the cleistorpemia found in *C. hellerii* (Watanabe et al. in preparation). According to Tiseo et al. (2014), species that have spermatophores in cleistospermia with acid polysaccharides can suffer fast dehiscence, which makes the spermatozoa available for fertilization. In addition, the acid polysaccharides found in the SR of *C. hellerii* are received exclusively from the males. Thus, the male provides a nuptial product that could act as a bactericidal and bacteriostatic agent (Sazikala and Subramonian, 1987; Beninger et al., 1993; Benhalima and Moriyasu, 2001). In *Ch. opilio*, the seminal fluid secretions from the PVD form a mucous acid substance that acts as catalytic and antimicrobial agent (Benhalima and Moriyasu, 2001). In Portunidae, the seminal plasma of *Scylla serrata* acts as an antimicrobial agent, and the SR secretions likely reduce bacterial infections during sperm storage (Jayasankar and Subramoniam, 1999). The sperm stored in the interior of the SR appear to depend on both the seminal fluid and the secretions from the epithelium to survive (Adiyodi and Anilkumar, 1988). Thus, in *C. hellerii*, beyond acting on spermatozoa maintenance, the SR also seem important to prevent microorganism proliferation; each mating can also act as a new dose of this component.

The multiple paternities demonstrated to *C. hellerii* are a trait that gives to invasive species higher genetic variability of the offspring (Facon et al., 2006). This characteristic could be important to invasive species to the success of an invasion, but there are other factors that assist in the invasion (Facon et al. 2006). There are other reasons that a species could be considered a good invader. In carps, they are highly adaptable, have broad diet, quick larval development and produce a high number of offspring (Koehn 2004). In seastar *Asteria amurensis* Lutken, 1871 the main reasons accounted for the invasion success are that are voracious predators and has a high fecundity, multiple spawning during the year and a key characteristic of be capable of asexual reproduction (Byrne et al., 1997; Ross et al., 2004). Other invader crabs other than *Charybdis*, such as the Varunidae *Hemigrapsus sanguineus* (De Haan, 1835), Carcinidae *Car. maenas* and Portunidae *Ca. sapidus* have successfully stablished themselves outside their native range (Epifanio, 2013; Grosholz and Ruiz, 1996; Nehring, 2011, respectively). In these crabs, only *H. sanguineus* is reported to mate in intermolt stage (Anderson and Epifanio, 2010), in both *Car. maenas* and *Ca. sapidus* it is found the sperm plug in the female reproductive system (Spalding, 1942; Wolcott et al., 2005). The previously findings for the species such as generalist diet, high fecundity, quick larval development, and spread through ballast water (Dineen et al., 2001; Sant'Anna et al., 2015; Negri et al., 2018), have great importance in bioinvasion of marine species, and it also occurs in the other invader crabs (Grosholz and Ruiz, 1996; Nehring, 2011; Epifanio 2013). Thus, the polyandry of *C. hellerii* is another important trait which could be a key to the success of the establishment of new places.

In conclusion, the seminal receptacle of *C. hellerii* does not change morphologically during the ovarian cycle in adult females, unlike which has been observed for the Portunidae family studied thus far (Ryan, 1967; Johnson, 1980; Bawab e El-Sherif, 1988; Zara et al., 2014). The

invasive crab *C. hellerii* shows a new genetic material storage pattern in Portunidae. In this pattern, there is no sperm plug or sperm packet, and the spermatozoa are not stored in spermatophores. This pattern of storage, together with the ability of the female to mate in the intermolt stage (hard-shelled) and with more than one male (poliandy), have direct implications on increasing the genetic variability of the offspring and may be crucial key to be adding to other factors previously studied to explain the success of the invasion of this species around the world.

ACKNOWLEDGMENTS

The authors are grateful to the São Paulo Research Foundation (FAPESP grants JP #05/04707-5, Biota Intercrusta #2018/13685-5 to FJZ and IC #2009/18383-8 to GRRP), CNPq (FJZ PQ2 #303314/2017-6), PROPe-UNESP primeiros projetos and the Coordenação de Aperfeiçoamento de Nível Superior - CAPES - Ciências do Mar II #1989/2014 - 23038.004309/2014-51 granted to FJZ and TTW PhD fellowship as part of CIMAR II CAPES financial code 001 for the financial support. The authors also thank the Electron Microscopy Laboratories of UNESP Rio Claro, particularly Monika Iamonte, for the technical support and also Dra. Claudia Fiorillo from the UNESP Jaboticabal facilities. This research was conducted according the Brazilian laws (FJZ, MMA – Sisbio permanent license #34587).

REFERENCES

Adiyodi, K.G., Anilkumar, G., (1988). Reproductive biology of invertebrates – Arthropoda - Crustacea.Vol. 3, Accessory sex glands. John Wiley e Sons, Chichester, England. pp. 261-318.

Anderson, J.A., Epifanio, C.E. (2010). Mating and sperm storage of the Asian shore crab *Hemigrapsus sanguineus*. *Journal of Shellfish Research*, 29: 497-502.

Antunes, M., Zara F.J., López-Greco, L.S, Negreiros-Fransozo, M.L. (2016). Morphological analysis of the female reproductive system of *Stenorhynchus seticornis* (Brachyura: Inachoididae) and comparisons with other Majoidea. *Invertebrate Biology,* 135: 75-86.

Antunes, M., Zara, F.J., López-Greco, L.S., Negreiros-Fransozo, M.L. (2018). Male reproductive system of the arrow crab *Stenorhynchus seticornis* (Inachoididae). *Invertebrate Biology,* 137: 171-184.

Baker, C.F., Gublin, Y., Reeve, K.A. (2018) Observations of hard-shell mating in the portunid crab *Charybdis japonica* (A. Milne-Edwards, 1861) (Decapoda: Brachyura). *Journal of Crustacean Biology*, 38: 504-508.

Bawab, F.M., El-Sherief, S.S. (1988). Stages of the reproductive cycle of the female crab *Portunus pelagicus* (L., 1758) based on the anatomical changes of the spermatheca (Decapoda, Brachyura, Portunidae). *Crustaceana*, 54: 139-148.

Bawab, F.M., El-Sherief, S.S. (1989). Contributions to the study of the origin, nature and formation of the plug in the spermatheca of the female crab *Portunus pelagicus* (Linnaeus, 1766) (Decapoda, Brachyura). *Crustaceana,* 57: 9-24.

Becker, C., Brandis, D., Storch, V. (2011). Morphology of the female reproductive system of European Pea crabs (Crustacea, Decapoda, Brachyura, Pinnotheridae). *Journal of morphology,* 272: 12-26.

Benhalima, K., Moriyasu, M. (2000). Structure and function of the posterior vas deferens of the snow crab, *chionoecetes opilio* (Brachyra, Majidae). *Invertebrate Reproduction e Development,* 37: 11-23.

Benhalima, K., Moriyasu, M. (2001). Prevalence of bacteria in the spermathecae of female snow crab, *Chionoecetes opilio* (Brachyura: Majidae). *Hydrobiologia*, 449: 261-266.

Beninger, P.G, Elner, R.W., Foyle, T.P., Odense, P.H. (1988). Functional anatomy of the male reproductive system and female spermatheca in the snow crab *Chionoecetes opilio* (O. Fabricius) (Decapoda: Majidae) and the hypothesis for fertilization. *Journal of Crustacean Biology*, 8: 322-332.

Beninger, P.G., Lanteigne, C., Elner, N.W. (1993). Reproductive processes revealed by spermatophore dehiscence experiments and by histology, ultrastructure and histochemistry of

the female reproductive system in the snow crab *Chionoecetes opilio* (O. Fabricius). *Journal of Crustacean Biology*, 13: 1-16.

Byrne, M., Morrice, M.G., Wolf, B. (1997). Introduction of the northern Pacific asteroid *Asterias amurensis* to Tasmania: reproduction and current distribution. *Marine Biology*, 127: 673-685.

Brockerhoff, A.M., McLay, C.L. (2005). Mating behavior, female receptivity and male-male competition in the intertidal crab *Hemigrapsus sexdentatus* (Brachyura: Grapsidae). *Marine Ecology Progress Series*, 290: 179-191.

Brown, G.G. (1966). Ultrastructure studies of sperm morphology and sperm egg interaction in the decapod *Callinectes sapidus. Journal of Ultrastructure Research,* 14: 425-441.

Crandall, K.A., Ported, M.L., Perez-Losada. (2009). Crabs, shrimps, and lobsters (Decapoda). 36: 293-297. In: The Timetree of Life, SB Hedges; S. Kumar (Eds). Oxford on line.

Costa, T.M, Negreiros-Fransozo, M.L. (1998). The reproductive cycle of *Callinectes danae* Smith, 1869 (Decapoda, Portunidae) in the Ubatuba region, Brazil. *Crustaceana*, 71: 615-627.

Diesel, R. (1988). Discrete storage of multiple-mating sperm in the spider crab *Inachus phalangium. Naturwissenchaften,* 75: 148-149.

Diesel, R. (1989). Structure and function of the reproductive system of the symbiotic spider crab *Inachus phalangium* (Decapoda: Majidae): observations on sperm transfer, sperm storage and spawning. *Journal of Crustacean Biology*, 9: 266-277.

Diesel, R. (1990). Sperm competition and reproductive success in a decapod *Inachus phalangium* (Majidae): A male ghost spider crab that seals off rivals sperm. *Journal of Zoology*, 220: 213-223.

Diesel, R. (1991). Sperm competition and mating behavior in Brachyura. In: In: Bauer, R.T. e Martin, J.W. (eds), *Crustacean Sexual Biology.* Columbia University Press, New York, pp. 145-163.

Dineen, J.F., Clark, P.F., Hines, A.H., Reed, S.A., Walton, H.P. (2001). Life history, larval description, and natural history of *Charybdis hellerii* (Decapoda, Brachyura, Portunidae), an invasive crab in the western Atlantic. *Journal of Crustacean Biolo*gy, 21: 774-805.

Edwards, E. (1966). Mating behavior in the European edible crab (*Cancer pagurus L.) Crustaceana,* 10: 23-30.

Epifanio, C.E. (2013). Invasion biology of the Asian shore crab *Hemigrapsus sanguineus*: a review. *Journal of Experimental Marine Biology and Ecology*, 441: 33-49.

Facon, B., Genton, B.J., Shykoff, J., Jarne, P., Estoup, A., David, P. (2006). A general eco-evolutionary framework for understanding bioinvasions. *Trends in ecology & evolution*, 21: 130-135.

Ferreira, C.E.L., Junqueira, A.O.R., Villac, M.C., Lopes, R.M. (2008). Marine bioinvasions in the Brazilian coast: Brief report on history of events, vectors, ecology, impacts and management of non-indigenous species. In: Biological Invasions in Marine Ecosystems. Ecological studies 204, Chapter 27.

González-Pisani, X., López Greco, L.S. (2007). Estructura de la espermateca del cangrejo arana *Leurocyclus tuberculosus* (Majoidea). In: *Proceedings of the XII Congreso Latinoamericano de Ciencias del Mar*, Florianópolis, Brasil, April (p. 14-17).

González-Pisani, X., Barón, P., López-Greco, L.S. (2012). Functional anatomy of the female reproductive systems of two spider crabs (Decapoda, Majoidea). *Invertebrate. Biology,* 131: 61-74.

Gregati, R.A., Fransozo, V., López-Greco, L.S., Negreiros-Fransozo, M.L. (2010). Reproductive cycle and ovarian development of the marine ornamental shrimp *Stenopus hispidus* in captivity. *Aquaculture*, 306: 185-190.

Grosholz, E.D., Ruiz, G.M. (1996). Predicting the impact of introduced marine species: lessons from the multiple invasions of the European green crab *Carcinus maenas*. *Biological Conservation*, 78: 59-66.

Guinot, D., Quenette, G. (2005). The spermatheca in podotreme crabs (Crustacea, Decapoda, Brachyura, Podotremata)and its phylogenetic implications. *Zoosystema*, 2: 267-342.

Guinot, D., Tavares, M., Castro, P. (2013). Significance of the sexual openings and supplementary structures on the phylogeny of brachyuran crabs (Crustacea, Decapoda, Brachyura), with new nomina for higher-ranked podotreme taxa. *Zootaxa*, 3665: 1-414.

Hartnoll, R.G. (1969). Mating in Brachyura. *Crustaceana*, 9: 161-181.

Hartnoll, R.G. (1988). Growth and molting. *Biology of the land crabs*, p. 186-210.

Hayer, S., Schubart, C.D., Brandis, D. (2015). Morphology and function of the female reproductive system of *Ebalia tumefacta* (Decapoda, Brachyura, Leucosiidae). *Journal of morphology*, 276: 517-525.

Hayer, S., Köhnk, S., Boretius, S., Brandis, D. (2016). Ever more complex: a new type of organization of reproductive organs in female *Dorippe sinica* Chen, 1980 (Decapoda: Brachyura: Dorippidae). *Zoology*, 119: 455-463.

Hines, A.H. (1982). Allometric constraints and variables of reproductive effort in brachyuran crabs. *Marine Biology,* 69: 309-320.

Hines, A.H., Jivoff, P.R., Bushmann, P.J., Montfrans, J.V., Reed, S.A., Wolcott, D.L., Wolcott, T.G. (2003). Evidence for sperm limitation in the blue crab, *Callinectes sapidus. Bulletin of Marine Science*, 72: 287-310.

Hinsch, G.W. (1986). A comparison of sperm morphologies, transfer and sperm mass storage between two species of crab, *Ovalipes ocellatus* and *Libinia emarginata. International Journal of Invertebrate Reproduction and Devevelopment,* 10: 79-87

Hinsch, G.W. (1988). Morphology of the reproductive tract and seasonality of reproduction in the golden crab, *Geryon fenneri* from the eastern Gulf of Mexico. *Journal of Crustacean Biology*, 8: 254-261.

Innocenti, G., Vannini, M., Galil, B.S. (1998). Notes on the behavior of the portunid crab *Charybdis longicollis* Leene parasitized by rhizocephalan *Heterosaccus dollfusi* Boschma. *Journal of Natural History*, 32: 1577-1585.

Jayasankar, V., Subramoniam, T. (1997). Proteolytic activity in the seminal plasma of the mud crab, *Scylla serrata* (Forskal). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 116: 347-352.

Jayasankar, V., Subramoniam, T. (1999). Antibacterial activity of seminal plasma of the mud crab *Scylla serrata* (Forskal). *Journal of Experimental Marine Biology and Ecology,* 236: 253-259.

Jensen, P.C., Orensanz, J.M., Armstrong, D.A. (1996). Structure of the female reproductive tract in the Dungeness crab (*Cancer magister*) and implications for the mating system. *Biological Bulletin,* 190, 336-349.

Jeyalectumie, C., Subramoniam, T. (1991). Biochemistry of seminal secretions of the crab *Scylla serrata* with reference to sperm metabolism and storage in the female. *Molecular Reproduction and Development*, 30: 44-55.

Jivoff, P. (1997a). The relative roles of predation and sperm competition on the duration of the post-copulatory association between the sexes in the blue crab, *Callinectes sapidus. Behavior Ecology and Sociobiology,* 40: 175-185.

Jivoff, P. (1997b). Sexual competition among male blue crab, *Callinectes sapidus*. *The Biological Bulletin*, 193: 368-380.

Jivoff, P., Hines, A.H., Quackenbush, L.S. (2007). Reproductive biology and embryonic development. In: Kennedy, V.S. and Cronin, L.E., editors. The Blue Crab, *Callinectes sapidus*. Maryland Sea Grant, College Park, Maryland, p. 255-298.

Johnson, P.T. (1980). *Histology of the blue crab Callinectes sapidus: a model for the Decapoda*. Praeger, New York. 440p.

Junqueira, L.C.U, Junqueira, L.M.M.S. (1983). *Técnicas Básicas de Citologia e Histologia.* Santos, São Paulo. 123p.

Koehn, J.D. (2004). Carp (*Cyprinus carpio*) as a powerful invader in Australian waterways. *Freshwater biology*, 49: 882-894.

Lemaitre, R. (1995). *Charybdis hellerii* (Milne Edwards, 1867), a nonindigenous portunid crab (Crustacea: Decapoda, Brachyura), dicovered in the Indian River lagoon system of Florida. *Proceeding of Biological Society of Washington,* 108: 643-648.

López Greco, L.S., Lopez, G.C., Rodriguez, E.M. (1999). Morphology of spermatheca in the estuarine crab *Chasmagnathus granulata* Dana 1851 (Graspidae, Sesarminae). *Journal of. Zoology*, 249: 490-493.

López-Greco, L.S., Fransozo, V., Negreiros-Fransozo, M.L., Santos, D.C. (2009). Comparative morphology of the seminal receptacles of *Ocypode quadrata* (Fabricius, 1787) (Brachyura, Ocypodoidea). *Zootaxa*, 210: 41-50.

López-Victoria, M., Werding, B. (2008). Ecology of the Endemic Land Crab *Johngarthia malpilensis* (Decapoda: Brachyura: Gecarcinidae), a Poorly Known Species from the Tropical Eastern Pacific. *Pacific Science,* 62: 483-493.

Mancini, K., Dolder, H. (2001). Ultrastructure of apyrene and eupyrene spermatozoa from the seminal vesicle of *Euptoieta hegesia* (Lepidoptera: Nymphalidae). *Tissue and Cell,* 33: 301-308.

Mantelatto, F.L.M., Dias, L.L. (1999). Extension of the know distribution of *Charybdis hellerii* (A. Milne Edwards, 1967) (Decapoda, Portunidae) along the western tropical South Atlantic. *Crustaceana*, 72: 617-620.

Mantelatto, F.L.M., Garcia, R.B. (2001). Biological aspects of the of non-indigenous portunid crab *Charybdis hellerii* in the western tropical south Atlantic. *Bulletin of Marine Science,* 68: 469-477.

Mantelatto, F.L., Robles, R., Schubart, C.D., Felder, D.L. (2009) Molecular Phylogeny of the Genus *Cronius* Stimpson, 1860, with Reassignment of *C. tumidulus* and Several American Species of *Portunus* to Genus *Achelous* De Haan, 1833 (Brachyura: Portunidae). In: Martin, J.W., Crandall, K.A., Felder, DL (eds), *Crustacean Issues: Decapod Crustacean Phylogenetics*. CRC Press, p. 567-579.

McLay, C.L., López Greco, L.S. (2011). A hypothesis about the origin of sperm storage in the Eubrachyura, the effects of seminal receptacle structure on mating strategies and the evolution of crab diversity: how did a race to be first become a race to be last? *Zoologischer Anzeiger*, 250: 378-406.

Mello, M.S.L, Vidal, B.C. (1980). *Práticas de Biologia Celular*. São Paulo: Edgar Blucher-FUNCAMP. 69p.

Mescher, A.L. (2018) *Junqueira's Basic Histology Text and Atlas*. 15th ed, McGraw-Hill, New York. 1136p.

Moriyasu, M., Benhalima, K. (1998). Snow crabs, *Chionoecetes opilio* (O. Fabricius, 1788) (Crustacea: Majidae) have two types of spermatophore: hypotheses on mechanism of fertilization and population reproductive dynamics in the southern Gulf of St. Lawrence, Canada. *Journal of Natural History*, 32: 1651-1665.

Nakasone, Y., Murai, M. (1998). Mating behavior of *Uca lactea perplexa* (Decapoda: Ocypodidae). *Journal of Crustacean Biology*, 18: 70-77.

Negri, M., Schubart, C.D., Mantelatto, F.L. (2018). Tracing the introduction history of the invasive swimming crab *Charybdis hellerii* (A. Milne-Edwards, 1867) in the Western Atlantic: evidences of high genetic diversity and multiple introductions. *Biological invasions*, 20: 1771-1798.

Nehring, S. (2011). Invasion history and success of the American blue crab *Callinectes sapidus* in European and adjacent waters. In: *In the wrong place-alien marine crustaceans: distribution, biology and impacts* (pp. 607-624). Springer, Dordrecht.

Norman, C.P. (1996). Reproductive biology and evidence for hard-female mating in the brachyuran crab *Thalamita sima* (Portunidae). *Journal of Crustacean Biology*, 16: 656-662.

Norman, C.P., Hirano, Y.J., Miyazaki, T. (1997). Hard-female mating in the brachyuran crab *Thalamita prymna* (Portunidae). *Crustacean Research*, 26: 62-69.

Norman, C.P., Nuka, T., Miyazaki, T. (1999). Mating behavior in *Thalamita picta* (Brahyura: Portunidae) and comparisons with congeneric species. *Crustacean Research,* 28: 16-23.

Oliveira, L.J.F., Zara, F.J. (2018). An investigation into the male reproductive system of two freshwater crabs from the Amazon: is there a sperm plug or packet formation? *Marine and Freshwater Behaviour and Physiology*, 51: 227-249

Pardo, L.M., Riveros, M., Fuentes, J.P., López-Greco, L. (2013). Functional morphology of the seminal receptacle in the crab *Metacarcinus edwardsii*. *Invertebrate Biology*, 132: 386-393.

Parker, G.A. (1970). Sperm competition and its evolutionary effect on copula duration in the fly *Scatophaga stercoraria*. *Journal of Insect Physiology,* 16: 1301-1328.

Pearse, A.G.E. (1985). *Histochemistry: Theoretical and Applied*. JeA Churchill, London. 998p.

Pinheiro, M.A.A., Fransozo, A. (1999). Reproductive behavior of the swimming crab *Arenaeus cribrarius* (Lamarck, 1818) (Crustacea, Brachyura, Portunidae) in captivity. *Bulletin of Marine Science*, 64: 243-253.

Pinheiro, M.A.A., Fransozo, A. (2002). Reproduction of the speckled swimming crab *Arenaeus cribrarius* (Brachyura: Portunidae) on the Brazilian coast near 23º30'S. *Journal of*

Crustacean Biology, 23: 416-428.

Pochon-Masson, J. (1968). L'ultrastructure des spermatozoïdes vésiculaires chez les Crustacés Décapodes avantet au cours de leur dévagination expérimentale. I. Brachyoures et Anomoures. *Annales des Sciences Naturelles (Zoologie et Biologie Animale),* 10: 1-98.

Pochon-Masson, J. (1983). Artropoda – Crustacea. In: Adiyodi, K.G., Adiyodi (Eds.) Reprodutive Biology of Invertebrates. Vol II Spermatogenesis and Sperm Function. New York: Wiley e Sons.

Pyle, R., Cronin, E. (1950) The general anatomy of the blue crab: *Callinectes sapidus* Rathbun. Chesapeake Biological Laboratory. Solomons Island, Maryland. p. 24-29.

Ryan, E.P. (1967). Structure and function of reproductive system of the crab *Portunus sanguinolentus* (Herbst) (Brachyura: Portunidae). I – The Male system. Proceedings of the Symposium on Crustacean, Ernakulam. Part III, pp. 506-521. Marine Biological Association of India. Bangalore, India: Bangalore Press

Ross, D.J., Johnson, C.R., Hewitt, C.L., Ruiz, G.M. (2004). Interaction and impacts of two introduced species on a soft-sediment marine assemblage in SE Tasmania. *Marine Biology*, 144: 747-756.

Rotllant, G., Gonzáles-Gurriarán, L.F., Benhalima, K., Ribes, E. (2007). Ovarian maturation of the multi-spawning spider crab *Maja brachydactyla* (Decapoda: Majidae) with special reference to yolk formation. *Marine Biology,* 152: 383-394.

Sal Moyano, M.P., Gavio, M.A., Cuartas, E.I. (2009). Morphology and function of the reproductive tract of the spider crab *Libinia spinosa* (Crustacea, Brachyura, Majoidea): pattern of sperm storage. *Helgoland Journal of Marine Research,* 64: 213-221.

Saint-Marie, G., Saint-Marie, B. (1998). Morphology of the spermathecae, oviduct, intermediate chamber and vagina the adult snow crab (*Chionoecetes opilio). Canadian Journal of Zoology*, 76: 1589-1604.

Sainte-Marie, G., Sainte-Marie, B. (1999). Reproductive products in the adult snow crab (*Chionoecetes opilio).* II. Multiple types of sperm cells and of spermatophores in the spermathecae of mated females. *Canadian Journal of Zoology*, 77: 451-462.

Sainte-Marie, G., Sainte-Marie, B., Sévigny, J.M. (2000). Ejaculate storage patterns and the site of fertilization in female snow crabs (*Chionoecetes opilio*; Brachyura, Majidae). *Canadian Journal of Zoology*, 78: 1902-1917.

Sant`Anna, B.S., Pinheiro, M.A.A., Mataqueiro, M., Zara, F.J. (2007). Spermathecae of the mangrove crab *Ucides cordatus:* a histological and histochemical view. *Journal of Marine Biology Association of United Kingdom,* 87: 903-911.

Sant`Anna, B.S., Watanabe, T.T., Turra, A., Zara, F.J. (2012). Relative abundance and population biology of the non-indigenous crab *Charybdis hellerii* (Crutacea: Brachyura: Portunidae) in a southwestern Atlantic estuary-bay complex. *Aquatic Invasions,* 7: 347-356.

Sastry, A.N. (1983). Ecological aspects of reproduction. *Environmental adaptations*, 8: 170-179.

Sazikala, S.L., Subramonian, T. (1987). On the occurrence of acid mucopolysaccharides in the spermatophores of two marine praws, *Penaeus indicus* (Milne-Edwards) and *Metapenaeus monoceros* (Fabricius) (Crustacea: Macrura). *Journal of Experimental Marine Biology and Ecology,* 113: 145-153.

Shauna J.O., Hankin, D. (2004). The sperm plug is a reliable indicator of mating success in female dungeness crabs, *Cancer magister*. *Journal of Crustacean Biology,* 24: 314–326.

Sokal, R.R., Rohlf, F.J. 1995. *Biometry.* New York, W.H. Freeman and Company. 887p.

Soundarapandian, P., Varadharajan, D., Ilavarasan, N., Kumar, J., Kumar, A. (2013). Mating Behaviour of Flower Crab, *Charybdis feriata* (Linnaeus). *Journal of Marine Science: Research e Development*, 3: 127.

Spalding, J.F. (1942). The nature and formation of the spermatophores and sperm plug *Carcinus maenas. Quarterly Journal of Microscopical Science*, 83: 399-422.

Sumpton, W. (1990). Biology of the rock crab *Charybdis natator* (Herbst) (Brachyura: Portunidae). *Bulletin of Marine Science*, 46: 425-431.

Tavares, M., Mendonça Jr., J.B. (1996). *Charibdis hellerii* (A. Milne Edwards, 1867) (Brachyura: Portunidae), eighth nonindigenous marine decapod recorded from Brazil. *Crustacean Research*, 25: 151-157.

Van Engel, W.A. (1990)**.** Development of the reproductively functional form in the male blue crab, *Callinectes sapidus. Bulletin of Marine.Science,* 46: 13-22.

Vehof, J., Scholtz, G., Becker, C. (2017). Morphology of the female reproductive system of three dorippid crabs (Crustacea: Decapoda: Brachyura: Dorippidae) and the role of accessory cuticle structures associated with seminal receptacles. *Invertebrate Biology*, 136: 271-289.

Watanabe, T.T., Zara, F.J., Hattori, G.Y., Turra, A., Sant'Anna, B.S. (2015). Biological associations of color variation in the Indo-Pacific swimming crab *Charybdis hellerii*. *Anais da Academia Brasileira de Ciências*, 87: 219-232.

Wolcott, D.L., Hopkins, C.W.B., Wolcott, T. G. (2005). Early events in seminal fluid and sperm storage in the female blue crab *Callinectes sapidus* Rathbun: effects of male mating history, male size and season. *Journal of Experimental Marine Biology and Ecology,* 319: 43-55.

Wong, N.A., Sewell, M.A. (2015). The reproductive ecology of the invasive Asian paddle crab, *Charybdis japonica* (Brachyura: Portunidae), in northeastern New Zealand. *Invertebrate Biology*, 134: 303-317.

Zara, F.J., Toyama, M.H., Caetano, F.H., López-Greco, L.S. (2012). Spermatogenesis, spermatophore and seminal fluid production in the adult blue crab *Callinectes danae* (Portunidae). *Journal of Crustacean Biology,* 32: 249-262.

Zara, F.J., Gaeta, H.H., Costa, T.M., Toyama, M.H., Caetano, F.H. (2013). The ovarian cycle histochemistry and its relationship with hepatopancreas weight in the blue crab *Callinectes danae* (Crustacea: Portunidae). *Acta Zoologica,* 94, 134-146.

Zara, F.J., Pereira, G.R.R., Sant'Anna, B.S. (2014). Morphological Changes in the Seminal Receptacle During Ovarian Development in the Speckled Swimming Crab *Arenaeus cribrarius*. *Biological Bulletin,* 227: 19-32.

Zimmerman, T.L., Felder, D.L. (1991) Reproductive ecology of an intertidal brachyuran crab, *Sesarma sp*. (nr.*reticulatum*) from the Gulf of Mexico. *Biological Bulletin*, 181: 387–401.

Considerações finais

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1- Morfologia do sistema reprodutor

Por meio dos resultados deste estudo, podemos observar as diferenças entre o sistema reprodutor masculino de Portunidae nativo tipicamente produtor de "plug" espermático do gênero *Callinectes* e do siri invasor *Charybdis hellerii*. A diferença do volume dos *vasa deferentia*, além de uma composição química diferente entre as espécies, mostram indiretamente estratégias reprodutivas distintas. Desta maneira, em *Callinectes,* temos a produção de um grande volume de secreção, que vão formar o "plug" espermático no receptáculo seminal das fêmeas e espermatóforos com presença de parede. Já no caso de *C. hellerii*, temos um baixo volume de secreção, estocagem de espermatóforos em uma região distinta da maioria dos outros Portunidae, espermatóforos sem a presença de parede, o que confere deiscência por ação mecânica e osmótica.

As observações no sistema reprodutor masculino refletem nos resultados obtidos para o sistema reprodutor feminino, no qual observamos a presença de espermatozoides livres dentro do receptáculo seminal, juntamente com polissacarídeos ácidos, o que não é encontrado no receptáculo seminal vazio. Além disso, não observamos em nenhum momento a presença de "plug" espermático ou pacotes espermáticos o que juntamente com a secreção holócrina do receptáculo seminal, misture o material genético dentro dele.

2 – Comportamento reprodutivo e análise genética da prole

As investigações morfológicas do sistema reprodutor de *C. hellerii* deram evidências indiretas para a forma que esta espécie se reproduz. Em condições laboratoriais, juntamente com

o que foi observado em campo, vimos que *C. hellerii* se reproduz sem depender que a fêmeas esteja em estágio de muda. Estas observações, juntamente com os resultados morfológicos se refletiram no estudo genético da prole da espécie. *Charybdis hellerii* é comprovadamente uma espécie poliândrica. A hipótese de que o comportamento reprodutivo e morfologia do sistema reprodutor de *C. hellerii* segue o padrão mais comum em Portunidae foi refutada. Estas características aqui descritas são adicionais a outras já descritas por estudos anteriores, as quais em conjunto explicam como esta espécie é bem sucedida na invasão de novos habitats. Assim, com está aberta a possibilidade de que a cópula em estágio intermuda, ausência de "plug" espermático e poliandria descritos para *C. hellerii* serem um padrão para a subfamília Thalamitinae o que seria uma característica relacionada à filogenia deste grupo, devendo ser estudada na região de dispersão deste gênero, o Indo-pacífico.