Universidade Estadual Paulista

Tese de Doutorado

Morfologia, reprodução e tamanho do genoma dos camarões *Synalpheus* (Decapoda: Caridea): perspectivas evolutivas e história de vida

Isabela Ribeiro Rocha de Moraes

Orientador: Dr. Antonio Leão Castilho
Coorientador: Dr. Alexandre Oliveira Almeida

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MORFOLOGIA, REPRODUÇÃO E TAMANHO DO GENOMA DOS CAMARÕES *SYNALPHEUS* (DECAPODA: CARIDEA): PERSPECTIVAS EVOLUTIVAS E HISTÓRIA DE VIDA

ISABELA RIBEIRO ROCHA DE MORAES

Orientador: Prof. Dr. Antonio Leão Castilho

Co-orientador: Prof. Dr. Alexandre Oliveira Almeida

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Palavras-chave: Camarões carídeos; Dimorfismo sexual; Histologia; Peso DNA; c-value.

Dedicatória

Dedico esta tese a todas as mulheres que se dedicam à educação e ciência, especialmente dona Therezinha, senhora minha avó.

Resumo

Synalpheus não gambarelloides devem receber um esforço em estudos para além de revisões taxonômicas e terem descrições biológicas e ecológicas bem elucidadas para comparações com o grupo dos gambarelloides e a posição evolutiva do gênero dentro de Caridea.

Sobre os aspectos reprodutivos, *Synalpheus* aparentam possuir uma formação prioritariamente simplista, podendo padronizar um caráter primitivo ao grupo quando comparado a outros gêneros e famílias da Infraordem Caridea. Apesar de a Infraordem como um todo ser um grupo bem heterogêneo em morfologias, comportamentos e formações dos aparelhos reprodutores, a ausência de glândulas acessórias, secreções e a morfologia tão distinta neste grupo fazem alusão a caráteres mais primitivos que outros.

O dimorfismo sexual das espécies gonocóricas foi comprovado com base nas morfologias das primeiras pleuras e pleópodos, sendo complementada pela posição dos apêndices internos que, comprovadamente surgem na posição apical do endópodo em fêmeas e fêmeas ovígeras, caracterizando não só o dimorfismo, como também mais uma estratégia acessória ao "breeding dress" para aprimorar a capacidade das fêmeas de incubar ovos.

Por fim, a associação entre os aspectos reprodutivos e biológicos e o padrão do "genome size" ainda permanecem inconclusivos, porém, com as comparações disponíveis na presente tese, pode-se propor que a evolução do tamanho do genoma em camarões carídeos (incluindo *Synalpheus*) seja fortemente relacionada ao padrão biogeográfico com tendência à distribuição polar apresentar os maiores conteúdos de DNA nuclear.

As perspectivas de estudo para o gênero podem ser reformuladas a partir dos presentes resultados, sendo as espécies comprovadamente diferenciadas entre os sexos, os padrões celulares de reprodução esclarecidos, e os primeiros aspectos comparativos do tamanho do genoma elucidados. Os próximos passos para completo entendimento evolutivo do gênero é a descrição de cada vez mais "c-values" para outras espécies de camarões carídeos e a evolução da ciência básica para descrição comportamental e biológica dos *Synalpheus* não gambarelloides que poderão ser comparados aos presentes resultados e corroborar a hipótese do padrão primitivo do gênero.

Abstract

Non-gambarelloides synalpheus should receive an effort in studies beyond taxonomic revisions and have well elucidated biological and ecological descriptions for comparisons with the gambarelloides group and the evolutionary position of the genus within Caridea.

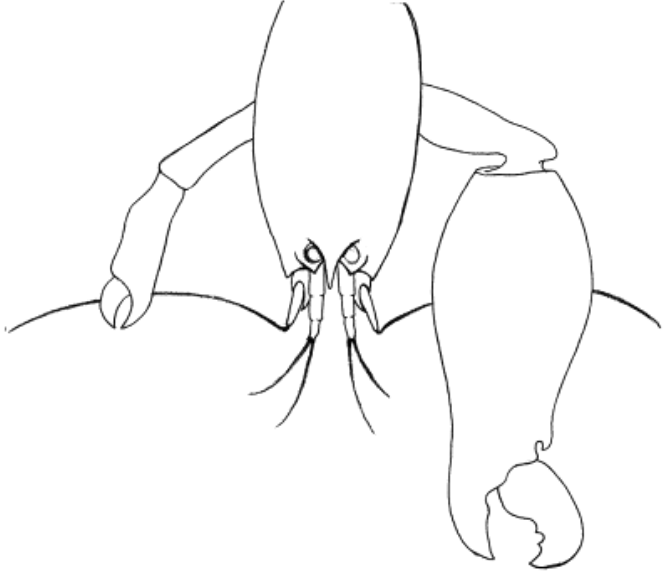
Regarding the reproductive aspects, Synalpheus seem to have a primarily simplistic formation, being able to standardize a primitive character to the group when compared to other genera and families of the Infraorder Caridea. Although the Infraorder as a whole is a very heterogeneous group in terms of morphologies, behaviors and formations of the reproductive systems, the absence of accessory glands, secretions and the morphology so distinct in this group allude to more primitive characters than others.

The sexual dimorphism of the gonochoric species was confirmed based on the morphologies of the first pleura and pleopods, being complemented by the position of the internal appendages that, demonstrably appear in the apical position of the endopod in females and ovigerous females, characterizing not only the dimorphism, but also one more accessory strategy to the "breeding dress" to improve the ability of females to incubate eggs.

Finally, the association between reproductive and biological aspects and the "genome size" pattern still remains inconclusive, however, with the comparisons available in the present thesis, it can be proposed that the evolution of the genome size in carid shrimp (including Synalpheus) is strongly related to the biogeographic pattern with a tendency for the polar distribution to present the highest contents of nuclear DNA.

The study perspectives for the genus can be reformulated from the present results, with the species being proven to be differentiated between the sexes, the cellular patterns of reproduction clarified, and the first comparative aspects of the genome size clarified. The next steps for a complete evolutionary understanding of the genus are the description of increasingly "c-values" for other species of carid shrimp and the evolution of basic science for behavioral and biological description of non-gambarelloid Synalpheus that can be compared to the present results and corroborate the hypothesis of the primitive pattern of the genus.

Sumário



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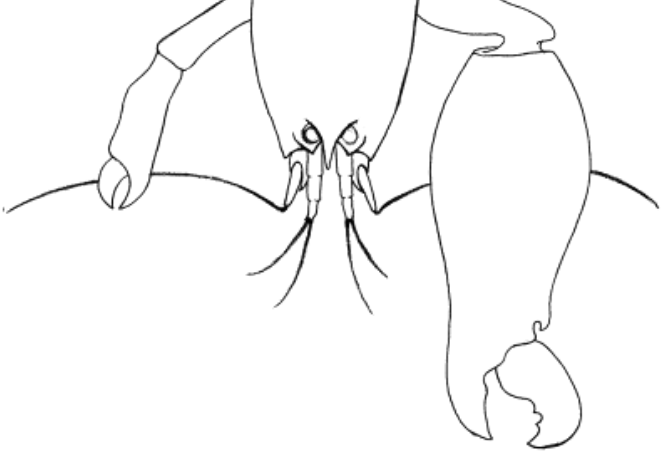

Considerações iniciais

Introdução Geral

Camarões da Infraordem Caridea e contexto histórico do Gênero *Synalpheus*

A Infraordem Caridea Dana 1852 pode ser considerada como um dos principais grupos para compreensão de modelos biológicos e evolutivos inseridos na Ordem Decapoda por serem representados por mais de 3.000 espécies, riqueza menor apenas que os caranguejos da Infraordem Brachyura Latreille, 1802 (De Grave and Fransen, 2011).

Com um formato corpóreo típico de "shrimp like", Caridea é reconhecida por agrupar os indivíduos denominados popularmente como camarões com a carapaça e abdome comprimidos lateralmente (Bauer, 2004), e se distribuie taxonomicamente em mais de 30 famílias em sua divisão, sendo as mais representativas Palaemonidae Rafinesque, 1815 e Alpheidae Rafinesque, 1815 (De Grave and Fransen, 2011).

O gênero *Synalpheus* Spence Bate, 1888, grupo abordado na presente tese, pertence à família Alpheidae e juntamente com o gênero *Alpheus* Fabricius 1798 formam um grupo taxonômico distinto considerando particularidades morfológicas derivadas de seus primeiros pares de pereópodos, especialmente os própodos e dáctilos. São reconhecidamente designados como "Camarões pistola", "Camarões de Estalo" ou "*Snapping shrimps"* devido a essa volumosa quela com um complexo mecanismo de "estalo" utilizados para defesa, comunicação e agressão a ameaças interespecíficas. É capaz de produzir um dos sons mais comuns do ambiente marinho, podendo ser detectado por outras espécies a uma distância de até 1 Km (Au and Banks, 1998; Versulius *et al.*, 2000; Kim *et al.*, 2010). Este "estalo" é causado pelo impacto do rápido fechamento do dedo móvel (dáctilo) no dedo fixo da maior (própodo) do quelípodo.

É descrito em *Alpheus heterochaelis* Say, 1818 que o estalo causa uma implosão de uma bolha de cavitação causada pela água e rapidamente ejetada por um dente especializado localizado no dedo fixo e no dáctilo. O fechamento do dáctilo para produção dessa bolha está entre os movimentos mais rápidos do reino Animalia (Versluis *et al.*, 2000; Schmitz, 2001; Anker *et al.*, 2006).

Synalpheus primariamente foi considerado em sua taxonomia pertencente ao gênero *Alpheus*. Morfologicamente, os grupos são extremamente semelhantes, distinguindo-se especialmente pela presença de epipoditos (processos inseridos nas coxas) em pelo menos os três primeiros pares de pereópodos e no terceiro maxilípede em *Alpheus* e a ausência de qualquer estrutura semelhante em *Synalpheus* (Paulson, 1875; McClure, 2005). A confirmação destas distinções morfológicas, atreladas aos conceitos genéticos, impulsionou a decisão de *Synalpheus* deixar de ser considerado um subgênero para se tornar um táxon independente. A espécie tipo do gênero é *Synalpheus falcatus* Spence Bate 1888 (De Grave, 2008), a qual passou por uma revisão taxonômica, sendo posteriormente considerada como sinonímia de *Synalpheus comatularum* (Coutière 1899).

Henri Coutière (1909) é responsável pela primeira publicação significativa sobre o gênero e propõe a divisão do táxon em 6 grupos informais: *laevimanus*, futuramente considerado como *Synalpheus gambarelloides* (Nardo, 1847); *brevicarpus*; *comatularum*; *neomeris*; *paulsoni*; e *biunculatus*. Desde o princípio das identificações das espécies dos grupos, o autor já considerou a presença de algumas subespécies pela grande semelhança morfológica dos indivíduos em geral, sendo assim, levando em consideração relações biogeográficas e semelhanças morfológicas em status informal, não sendo elevados a subgêneros ou algo semelhante. Ao separar as espécies em grupos, Henri Coutière (1909) ainda registrou pela primeira vez a característica de *Synalpheus*

apresentar em muitos casos simbiose obrigatória com alguns invertebrados, como esponjas e crinoides, servindo como mais um parâmetro para dividi-los em grupos e foi um ponto de partida para a taxonomia moderna no grupo como um todo.

Após as propostas morfológicas e divisão dos grupos dentro do gênero, trabalhos de revisões passaram a ser propostos e discutidos levando em consideração caráters ecológicos, comportamentais e de seleção de hospedeiros pelas espécies de *Synalpheus.* Por exemplo, Banner & Banner (1975) e Chace (1972) analisaram todos os grupos propostos por Coutière (1909) e concluíram que com o avanço nas identificações de novas espécies do gênero, o processo de separá-las em grupos seria cada vez mais infundável. Pelas bases diagnósticas, foi proposto então que apenas três, dos seis grupos primários, poderiam ser válidos para uma caracterização mais geral, isto é, *Comatularum*, *Brevicarpus* e o *Laeviamanus,* agora *Gambarelloides.*

O grupo mais significativo em números de espécies descritas e morfologia particular quase inconfundível "*S. gambarelloides"* pode ser separado pela sequência de cerdas distintas presentes no própodo da primeira menor quela, estruturas discutidas ao longo do tempo como essenciais para o sucesso do grupo em sua simbiose, ou seja, auxiliam na locomoção dos indivíduos pelos canais das esponjas, bem como na alimentação (Banner and Banner, 1975). Ríos & Duffy (2007) propuseram a elevação deste grupo para o *status* do gênero *Zuzalpheus*, utilizando como base principal esta caracterísa completamente particular das cerdas encontrada apenas nos *Gambarelloides*, além do comportamento social simbiótico. Porém, tal proposta foi logo contestada por Anker & De Grave (2008), ao qual argumentam não haver evidências principalmente genéticas que sustentem a separação dos grupos e, portanto, *Zuzalpheus* foi considerado uma sinonímia júnior em *Synalpheus.*

Ferramentas integrativas contemporâneas possibilitaram a discussão moderna sobre a divisão do grupo em contextos genéticos, morfológicos e comportamentais. Hermoso-Salazar *et al.* (2008) propuseram a divisão *Synalpheus* em dois grupos, *Gambarelloides* e *Paulsonii*, sendo todos os outros considerados apenas sinonímias. Apesar de todas as propostas, Anker & De Grave (2008) deixam claro que o número de espécies de *Synalpheus* descritas e registradas ainda é subestimado devido à quantidade de espécies crípticas que o gênero apresenta e pela característica de uma morfologia homogênea, com variação de poucos caracteres de uma maneira geral independente de grupos.

A mais recente análise integrativa e que representa o *status* de classificação atual de *Synalpheus* e seus grupos foi proposta por Hultgren *et al.* (2014), que até o momento registrava cerca de 160 espécies válidas para o gênero. Os autores ressaltam que a grande maioria dos trabalhos comportamentais e taxonômicos na literatura focam apenas em discussões sobre o grupo dos *Gambarelloides* que ultrapassam 70 espécies válidas (representando os maiores dados taxonômicos e biológicos de todo o grupo). O grupo *Brevicarpus* por exemplo representam não mais que uma dúzia de espécies descritas que podem ser simbiontes facultativas em esponjas. *Comatularum* também apresenta cerca de 10 espécies. Segundo os autores, os 6 grupos informais originais propostos para o gênero podem ser levados em consideração para construção de uma filogenia atual, porém confirma-se a relação suportada para apenas 3 grupos já sugeridos na literatura, os citados: *Brevicarpus*, *Gambarelloides* (mais representativo) *e Comatularum*. Dois destes grupos apresentam algum tipo de relação simbiótica obrigatória (*Gambarelloides* com poríferos e *Comatularum* com crinóideos).

Synalpheus é considerado um grupo monofilético e existem espécies potencialmente crípticas que ainda não foram analisadas morfologicamente, o que torna

este processo final ainda mais distante de acontecer. Os estudos com ferramentas integrativas deixam claro que a separação de *Synalpheus* em grupos não pode ser baseada unicamente em caracteres morfológicos, e apesar do respaldo genético, ainda são encontrados obstáculos pela presença de politomias nas construções das árvores filogenéticas. Hultgren *et al.* (2014) ressaltam ainda que estas dificuldades em separações claras dos grupos se devem muito provavelmente ao rápido processo de especiação que ocorre atualmente neste gênero.

A maior parte dos estudos sobre levantamento de espécies, "checklists", biologia populacional e reprodução nem entram no mérito de divisão do gênero em grupo, avaliam *Synalpheus* como um gênero homogêneo evitando discussões. Exceto quando se referem aos "sponge-dwelling shrimps" do grupo *Gambarelloides.* Para a proposta da presente tese, o que deve ser levado em consideração é que comprovadamente nenhuma das espécies a serem analisadas pertencem ao grupo dos *Gambarelloides* ou apresentam algum tipo de simbiose obrigatória. O que torna o trabalho significativo por abordar aspectos aos grupos que necessitam de uma base de informação biológica e taxonômica para aplicações robustas e separações efetivas, caso venham a ocorrer futuramente. Portanto, para facilidade de nomenclatura e compreensão, será abordado ao longo de todo o texto o termo "*Synalpheus não-gambarelloides"* (Morrison *et al.*, 2003) em referência a todas as espécies exploradas na presente tese

Aspectos reprodutivos associados ao gênero *Synalpheus*

Dentro da Infraordem Caridea existem duas categorias para classificação dos caracteres reprodutivos morfológicos e funcionais que determinam dimorfismos sexuais entre os indivíduos: caracteres sexuais primários e secundários, principalmente quando relacionadas às espécies gonocóricas, maioria dos representantes de *Synalpheus.* Os

caracteres sexuais primários referem-se aos testículos em machos e ovários em fêmeas, ocupam em sua maior parte a região do cefalotórax do animal podendo alterar seu tamanho e morfologia conforme o estágio de desenvolvimento, principalmente no caso das fêmeas em que os ovários chegam a atingir a região final do abdome quando em estágio desenvolvido (especialmente nos *Synalpheus*) (Bauer 2004; observação pessoal).

Em fêmeas, o sistema sexual primário é formado pelos ovários e ovidutos, que se abrem em um gonóporo na região do terceiro par de pereópodos e que, geralmente, quando desenvolvidos se estendem desde a carapaça (surgindo do hepatopâncreas) até a região do segundo somito abdominal (Bauer, 2004; Baeza, 2018). *Synalpheus* pode ser considerado como uma exceção a essa regra, uma vez que os ovários desenvolvidos em fêmeas reprodutivas podem atingir até mesmo a região do último somito abdominal, percorrendo todo o corpo do animal, característica pouco reportada entre os camarões carídeos (observação pessoal). Porém, quando as gônadas não estão desenvolvidas, a região interna, com órgãos e musculatura, é basicamente idêntica a dos machos, o que torna muito difícil a designação da distinção sexual entre os espécimes. Em machos, o sistema sexual primário é reconhecido pela composição de um par de testículos, seguidos de vasos deferentes além dos poros genitais que são concentrados na região do quinto par de pereópodos do animal (Bauer, 2004; Espinoza-Fuenzalida *et al.*, 2008; Baeza, 2018).

Além dos aparatos sexuais primários, Bauer (2004) descreve como “aparatos sexuais secundários” todas as estruturas externas envolvidas na reprodução e responsáveis por ditar o dimorfismo sexual em espécies de camarões carídeos. Prioritariamente, estas estruturas são reconhecidas como os “apêndices masculinos” encontrados associados ao segundo par de pleópodos em machos e as aberturas genitais, os “gonóporos” encontrados em machos e fêmeas por onde são liberados os materiais provenientes das gônadas (os gonóporos não são características exclusivas de Caridea).

Synalpheus também se apresenta como exceção à essa "regra" morfológica por ser um dos pouquíssimos grupos que não possui apêndice masculino em machos e a formação dos gonóporos é praticamente imperceptível mesmo sob microscopia de luz. Esta sucessão de particularidades anatômicas e funcionais fazem com que o dimorfismo sexual em *Synalpheus* seja sutil ou praticamente inexistente. Coutière (1909) já observava que a diferenciação dos espécimes em sexos gonocóricos era dificultada com base apenas na morfologia externa, considerando apenas a ausência do apêndice masculino e morfologia das pleuras abdominais dos animais.

As características e morfológicas reprodutivas do gênero *Synalpheus* aparentam refletir um *status* primitivo de evolução das estruturas, comparando a outros gêneros e grupos da Infraordem Caridea, que apresentam características consideravelmente mais complexas e funcionais quando relacionados a comportamentos e estratégias reprodutivas. Analisar os conceitos de formação estruturais e genéticos do gênero é de grande importância para contribuições e interpretações sobre estes parâmetros evolutivos da Infraordem.

"Genome size": Definição e aplicações

É reconhecida a importância e significância do DNA em conter informações hereditárias e avaliações dos parâmetros celulares em massa para os mais diversos organismos, independente de grau taxonômico ou ambiente de distribuição. As avaliações e descrições das informações contidas no DNA podem variar e serem apresentadas de formas qualitativas e quantitativas: Realizando sequenciamento e identificação dos pares de base nas dupla-hélices da molécula, ou ainda medido o "peso" que o genoma pode apresentar (Gregory, 2005; Jeffery, 2015).

Por definição, o termo Genome Size (GS), refere-se à análises quantitativas, avaliando o conteúdo total de DNA contido em um único conjunto de cromossomo. Trata-se de estudos da "biomassa" das informações contidas nos genes e cromossomos dos eucariotos mensurado em picogramas (pg) do DNA por núcleo haploide (Gregory, 2005). Essa biomassa é interpretada a partir da constante denominada como "c-value", derivada da denominação de Swift, 1950 que se refere ao núcleo do DNA nuclear haploide e ao se referir a espécies diploides como os camarões, o c-value e GS são intercambiáveis.

À priori, levando em consideração que esta constante do tamanho do genoma dentro das espécies reflete o papel do DNA como substância hereditária, a primeira implicação poderia indicar que GS provavelmente fosse proporcional ao número de genes dos organismos, portanto, refletindo que a complexidade organizacional dos animais seria diretamente proporcional ao tamanho do genoma, sendo então, organismos mais complexos, apresentando *c-values* maiores (Gregory, 2005)

Na década de 50, os autores Mirsky & Ris (1951), no entanto, foram os pioneiros a observar que o peso do DNA nuclear não está intimamente correlacionado à complexidade do organismo analisado, o que viria a ser considerado adiante como "Paradoxo do c-value". O GS já foi reportado para mais de 10.000 organismos entre plantas e animais, e dentro dos Metazoa, esta variação é bastante significativa, partindo de registros de 0,02 pg e atingindo até 132,83 pg. A tendência das observações e suposições é de que o tamanho do genoma se correlacione fortemente com o tamanho celular, taxa de divisão celular e características intrínsecas a cada organismo como taxa metabólica e taxa de crescimento. Padrões comportamentais e de distribuição geográfica também devem ser levados em consideração (Bainard and Gregory, 2013).

A maioria dos registros sobre GS e c-value de plantas ou animais pode ser acessado (e atualizado por pesquisadores) em uma plataforma online, disponível no

website "https://www.genomesize.com/", além de todos os registros científicos em artigos publicados em periódicos. A maioria dos dados sobre c-value encontrados na plataforma dizem respeito a vertebrados, cerca de pouco mais de 300 registros condizem aos crustáceos, número baixo quando se trata de um subfilo tão especioso. Apesar de pouco representativo, comparando o número de espécies descritas com número de espécies com GS avaliado, a variação do c-value em crustáceos chama muita atenção, com os menores valores registrados como 0.14 pg em copépodos *Cyclops kolensis* Lilljeborg, 1901 até 64.62 pg em anfípodos *Ampelisca macrocephala* Lilljeborg, 1852. Essa grande variação instiga a realização de um esforço cada vez maior para realização de estudos e levantamento de dados neste sentido para interpretações evolutivas.

Os crustáceos Decapoda são dentre os Crustacea os que possuem maiores registros de GS na literatura (atingindo cerca de 200 espécies) e, portanto, é o grupo que mais possibilita interpretações evolutivas sobre a relação do genoma com sua história de vida e adaptações, especialmente condizendo a adaptações aos ambientes que colonizam. Trabalhos indicam que possa existir uma relação entre o tamanho do genoma e ambientes extremos onde os crustáceos podem ser encontrados como, por exemplo, em fontes hidrotermais e regiões polares abrigando espécies com os maiores valores de GS já observados. Entre estes, justamente os camarões carídeos são os que apresentam maiores valores de GS avaliados, atingindo mais de 40 pg (Gregory, 2005; Rees *et al.*, 2008; Hultgren *et al.*, 2018).

Uma das técnicas muito significativas para a avaliação e determinação do GS para espécies diploides é a citometria de fluxo, tecnologia ao qual são medidas as características físico-químicas celulares (como dispersão de luz e fluorescência) de partículas individuais como os núcleos. No citômetro de fluxo, as medições são realizadas conforme as partículas passam uma a uma através de um ponto de luz em alta velocidade

(10^2 – 10^3 partículas/seg.). Pela incidência luminosa o citômetro de fluxo avalia a intensidade relativa de fluorescência de núcleos isolados a partir de células obtidas de tecidos não proliferativos, quantificando assim o "peso" das informações contidas em cada núcleo estimulado (Xavier *et al.*, 2017).

As relações do GS para carídeos são bastante discutidas e devem levar em consideração todos os aspectos como tamanho do animal, comportamento, padrões de reprodução, distribuição geográfica e modelo do cariótipo, que são padrões mutáveis na história evolutiva de um ser vivo (Rees *et al*., 2007, 2008; Bonnivard *et al*., 2009; Dufresne and Jeffery, 2011; Hultgren *et al*., 2018). Sendo assim, todo resultado da ciência básica publicado deve ser levado em consideração, bem como um exaustivo levantamento de dados sobre GS deve ser registrado para descrever a história de vida dos crustáceos, em especial o diverso grupo Caridea e seu enigmático gênero *Synalpheus*.

Perspectivas evolutivas: morfologia, ecologia, e tamanho do genoma em *Synalpheus*

Todas as funções morfológicas, comportamentais e ecológicas envolvendo o gênero *Synalpheus,* como as particularidades quase que exclusivas dos aparatos reprodutores, a ausência do apêndice masculino, o padrão de organização eusocial em *Gambarelloides*, ou mesmo os padrões de simbiose obrigatória e facultativa denotam questões pertinentes sobre o padrão evolutivo do grupo e sua história de vida dentro de Caridea (Duffy, 2003; Chak *et al*., 2015; Chak, 2016).

Em *Synalpheus* já há um esforço significativo para descrição do tamanho do genoma de espécies do grupo *Gambarelloides*, com relações filogenéticas e comportamentais pelo desenvolvimento eusocial e foi avaliada até mesmo variação intraespecífica, podendo o c-value variar de acordo com a distribuição geográfica. Uma

relação biológica esperada da variação do c-value proporcional ao número e tamanho dos cromossomos presentes nos animais também foi proposta (Jeffery *et al*., 2016).

Recentemente, pesquisas visam compreender a relação do tamanho do genoma com aspectos biológicos e taxonômicos de *Synalpheus* gerando importantes discussões registradas na literatura (Chak *et al*., 2021; Hultgren *et al*., 2021). Mesmo que ainda relacionados sempre aos *S. gambarelloides*, pode-se começar a analisar a relação das atividades simbióticas, adicionando o acúmulo de elementos geneticamente transponíveis que espécies deste grupo tendem a apresentar, a relação direta entre GS x DNA não codificante, adicionando ainda os padrões de modelos evolutivos como os diferentes modelos de desenvolvimento (anamórfico regular, anamórfico irregular ou epimórfico), contando ainda o número de estágios larvais que as espécies podem apresentar, que pode ter influência direta do tamanho do genoma (Hultgren *et al*., 2021).

Todos os resultados da presente tese condizem com informações básicas sobre os modelos reprodutivos, morfológicos e do tamanho do genoma das espécies de *Synalpheus não-gambarelloides,* fornecendo novas perspectivas para discussões uma vez que este grupo não apresenta modelo de vida simbiótico obrigatório. Ademais, informações sobre a espermiotaxonomia, padrões do aparato reprodutor masculino e feminino e potenciais reprodutivos das espécies podem servir de base para discussões comparativas e compor ainda mais as informações básicas sobre o grupo para fomentar os padrões evolutivos quando se for comparar o tamanho do genoma dos grupos.

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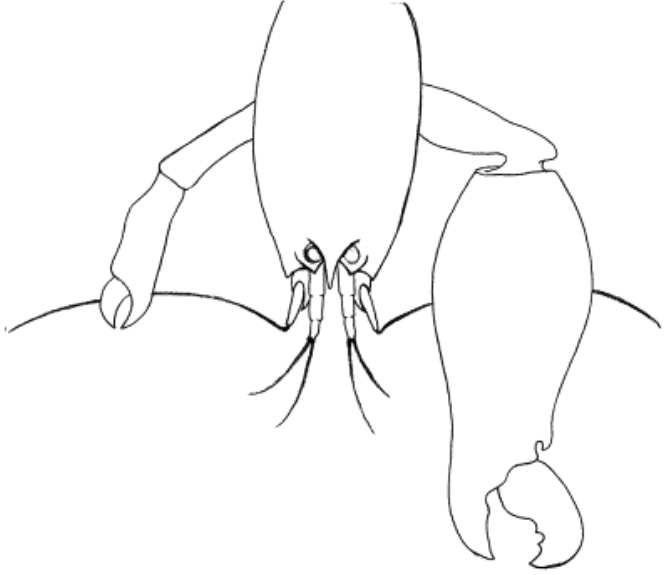


Objetivos e Hipóteses

Objetivo geral da tese

O objetivo da presente tese é descrever os padrões morfológicos, reprodutivos e o tamanho do genoma de camarões carídeos do gênero *Synalpheus* e responder questões enigmáticas e inéditas sobre perspectivas evolutivas e história de vida dos camarões Caridea.

Objetivos específicos

1- Descrever pela primeira vez o aparato reprodutor masculino de *Synalpheus* em uma perspectiva histológica e morfológica, para elucidar questões sobre o desenvolvimento e padrão de composição celular do aspecto reprodutivo.

2- Elucidar o padrão de dimorfismo sexual integrando diferentes ferramentas de descrição entre indivíduos do gênero *Synalpheus* partindo do princípio da impossibilidade atual de diferenciação morfológica de machos e fêmeas, apontando novas perspectivas às descrições morfológicas entre indivíduos.

3- Avaliar o padrão do “genome size”, visto toda a informação biológica recolhida para o grupo de *Synalpheus gambarelloides*, e avaliar uma posição evolutiva do genoma para o gênero em uma perspectiva comparativa dentro de Caridea.

Hipóteses a serem testadas

1- *Synalpheus* apresentam aparelho reprodutor masculino completo, mesmo com a impossibilidade de identificação macroscópica.

2- Machos e fêmeas de *Synalpheus não-gambarelloides* apresentam dimorfismo sexual aparente baseado na primeira e segunda pleura abdominal e morfologia da primeira maior quela.

3- O padrão de "genome size" em carídeos tende a seguir uma especialização biogeográfica e não padrões intrínsecos das espécies como reprodução e comportamento ecológico.

4- Camarões do gênero *Synalpheus* apresentam características primitivas relacionando sua morfologia e tamanho do genoma comparando aos outros grupos da Infraordem Caridea.

Espécies a serem utilizadas como modelo na tese:

Os *Synalpheus não-gambarelloides* utilizados como modelo ao longo de toda tese são espécies amplamente distribuídas no litoral brasileiro, especialmente no litoral do estado de São Paulo, representadas na figura 1.

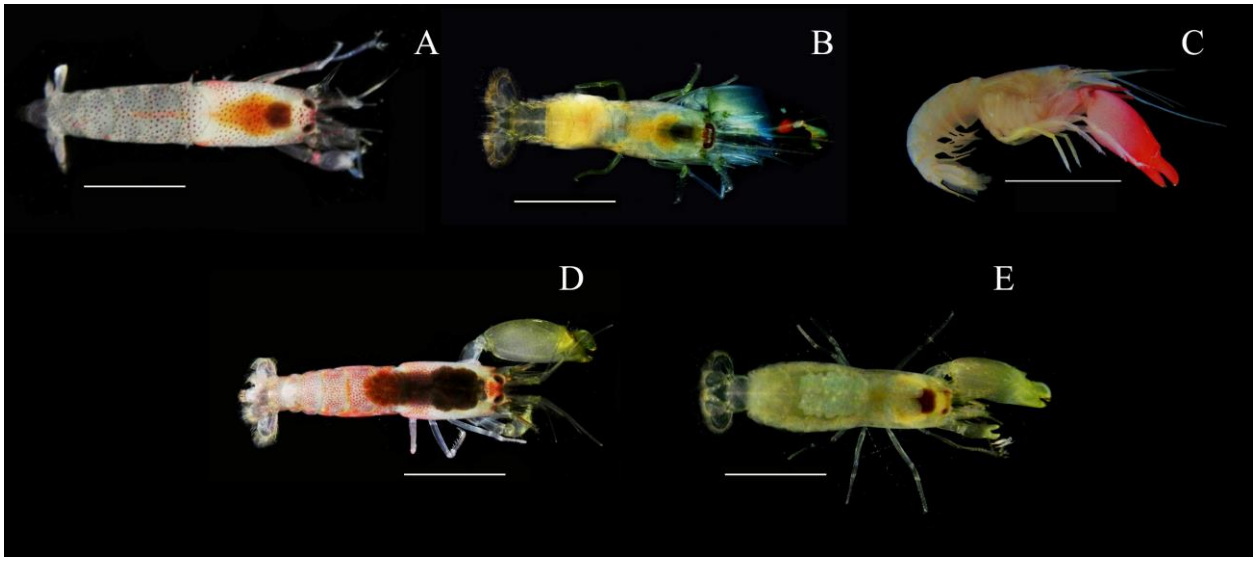
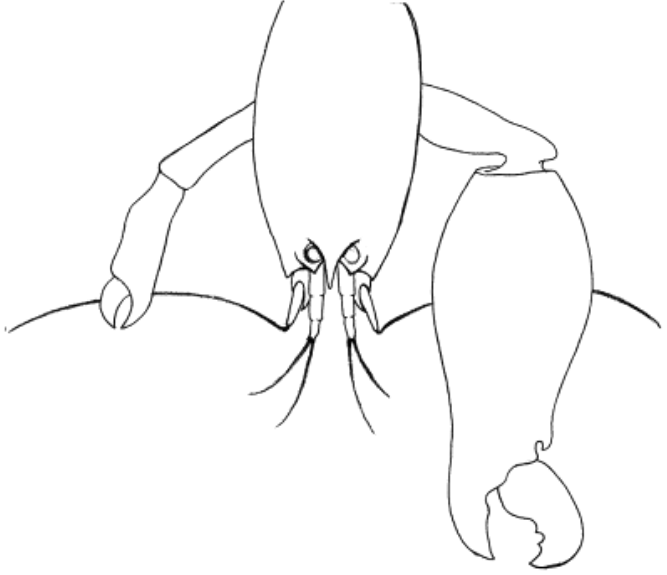


Figura 1: *Synalpheus* apresentados ao longo dos três capítulos da tese: A) *Synalpheus scaphoceris* Coutière, 1910; B) *Synalpheus brevicarpus* (Herrick, 1810)*;* C) *Synalpheus minus* (Say, 1818); D) *Synalpheus fritzmuelleri* Coutière, 1909; E) *Synalpheus apioceros* Coutière, 1909. Barra de escala 2 mm. Fotos: Moraes, IRR.



Capítulo 01

Functional morphology and ultrastructure of the reproductive system of the non-gambarelloides *Synalpheus* Spence Bate, 1888 shrimps (Caridea: Alpheidae)

Abstract

The remarkable non-gambarelloides shrimps of the genus *Synalpheus* is a representative group into Caridea infraorder. The present work aims to provide the first description of the reproductive system of these animals from microscopic and histological perspectives. The reproductive systems, thoracic sterna, and male and female gonopores were observed by scanning electron microscopy. Males has slender and translucid testes and vasa deferentia that are hard to visualize. Because of this condition, sections on histological preparation were made in the entire carapace to secure that all regions of the reproductive system could be demonstrated. We observed that the non-gambarelloides group seems to present a gonochoric condition. The Caridea pattern of reproductive system was observed: the male reproductive system consisted of a pair of lobular testes and vasa deferentia emerging from testes and divided into three regions: proximal, middle, and distal, ending on the opening of the gonopore located at the basis of fifth pereopod coxae. The absence of any spermatophore or androgenic gland to aid the mature spermatozoa was an exception to the Caridea pattern. The spermatogenesis process consists of four stages as spermatogonia, spermatocytes, spermatids, and free mature spermatozoa. Spermatozoa have a classical caridean shrimp shape with a semicircular nucleus region as an “umbrella”, and one single spike. In females, the reproductive system consists of a pair of ovaries, very turgid, occupying the entire abdominal cavity and cephalothorax, extending to the last abdominal somite. The extension of the ovary to the end of the abdome seccion is a very particular situation comparing to other caridean shrimps. Our first panorama of masculine morphology on primary sexual features for *Synalpheus* shrimps distinguished as much simpler formation than other caridean that presents complex male reproductive trait with spermatophores, complex vasa and secretions, androgenic cells and a great number of spermatozoa, and it may be provided a possible primitive aspect of this genus into Infraorder.

Keywords: Decapoda, DIC, reproduction, spermatophore, spermatozoa, vas deferens.

Introduction

The genus *Synalpheus* Spence Bate, 1888 is the second richest of the Family Alpheidae (just behind *Alpheus* shrimps), with more than 150 described species distributed worldwide (Ríos and Duffy, 2007; De Grave and Fransen, 2011; Hultgren and Duffy, 2011; Anker *et al*., 2012). This classification is still in progress, but we can identify two well-separated informal groups. The principal one, gambarelloides group, with almost a half species (= *Zuzalpheus* Ríos and Duffy, 2007, Junior synonym of *Synalpheus* Anker and De Grave, 2008) and non-gambarelloides group, that can be defined at least into two more groups but with no genetic and morphological evidence in separations (Hultgren and Duffy, 2011). Knowing the possibility of the existence of other informal groups, but with all systematic incongruences and gaps for its nomination, at the present discussion we will assume just as non-gambarelloides this shrimp represented by three Atlantic distributed species: *Synalpheus apioceros* Coutière, 1909, *Synalpheus brevicarpus* (Herrick, 1891), and *Synalpheus fritzmuelleri* Coutière 1909. Despite the importance of the genus, most effort and studies published focused on taxonomic, genetic, and phylogenetic parameters, or biology and behavior of the gambarelloides and the eusocial condition proposed for this group (Dardeau, 1984; Duffy and Macdonald, 1999; Hermoso-Salazar *et al.*, 2008; Hultgren *et al.*, 2014; Chak *et al.*, 2015b, 2020).

Caridean shrimps can present a remarkable variety of morphologies, global and ecological distribution, behavior, and biological features, in the same proportion, presents a great variation on models of reproduction that modulates morphologies of the reproductive traits that remains attention to be studied and compared for taxonomic and evolutive studies with the group. We can define many differences in the sexual system in caridean shrimps, since gonochoric condition to many variations in hermaphroditism

patterns (Braga *et al.*, 2009; Nunes *et al.*, 2010; Baeza, 2018). In *Synalpheus* shrimps are not different, hermaphroditism and intersex condition were proposed to many gambarelloides species and they are evaluated in many aspects to identify behaviors on the colony reproduction and defense. (Tóth and Bauer, 2007, 2008; Chak *et al.*, 2015a; Alves *et al.*, 2021).

In the non-gambarelloides, the entire reproductive aspects have never been described before, even not any hermaphroditism condition. Only reproductive behavior and fecundity parameters were analyzed for the group (Felder, 1982; Rebolledo *et al.*, 2014) that seems to present a gonochoric system in the majority by the presence of the heterosexual pair living animals living associated with sponges and corals (Alves *et al.*, 2021). *Synalpheus* until data share the same typical anatomy for the male reproductive system with all caridean shrimps: it is entirely located at the cephalothorax portion of the animal, and consist of two tests at the same region of the hepatopancreas and heart. From each testis emerges a pair of long ducts: the vas deferens that is divided into three distinct regions: Proximal, Middle, and Distal. This vas is responsible for the mature spermatozoa transportation and connects testes to the opening region at the final portion of the cephalothorax, specifically in the coxae of the fifth pereopod (called Gonopore) (Bauer, 2004; Sainte-Marie, 2007; Baeza, 2018).

The anatomy of the female reproductive system, as the pattern of caridean shrimps consist of a paired ovaries, located in the cephalotorax and when present, yolky oocytes, as the mature ovaries extend in the space of the anterior cephalotoracic region and may extend, depending on maturation stages, into few abdominal segments (Bauer, 2004; Cobos *et al.*, 2005). The particularity of *Synalpheus* anatomy is represented by the extension of the ovary that, when mature, can reah almost the fifth abdominal segmentfinal, covering all dorsal portion of the abdomen (personal observation).

Spermatophore production can be observed in many Decapoda crustaceans as well as "free spermatozoa" formation. The structure of the spermatophore is emitted by the male during copulation and contains the sperm involved by an adhesive secretion, produced in the vas deferens. The design and place of the formation of spermatophores are striking, since it can occur on the face of the female body or internally when there is exist a seminal receptacle. The presence or not of this spermathophore structure, as well as its design and chemical composition, is extremely variable among the group. However, its complexity appears to be directly related to the time required from the moment of the sperm transfer until the moment of ovulation (mainly in species with external fertilization). Therefore, in the infraorder Caridea, which the process of copulation and ovulation is usually extremely fast, the spermatophore (when present), remains very simple, without a sheath surrounding spermatozoa and secretion is scarce (Bauer, 1986; Subramoniam, 1991; Lópes-Greco 2012). In *Synalpheus* (at least in the gambarelloides group), there is no evidence of spermatophores formation, or complex structures in spermatozoa formation and its transference (Chak *et al.*, 2015).

The spermatozoa morphology are classified into three groups between Decapoda based on the morphology and quantity of spikes or arms (Holdich, 2002). At first, spermatozoa can be "multistellate" in restrict Pleocyemata groups as Astacidae and Brachyura, presenting many spikes or arms formation around the body and nucleus region as presented in a revision and classification by Poljaroen *et al.* (2010). Some groups do not present any formation like spikes as in some Sergestidae and Aristeidae shrimps (Demestre *et al.*, 1997; Scelzo and Medina, 2004). At last, the "unistellate sperm" formation shows one single spike and is found in most caridean shrimps. Despite that, there is no register about Alpheidae family spermatozoa formation, but it seems to resemble many other species from Palaemonidae, Atyidae, Hippolytidae, Pandalidae, and

Lysmatidae shrimps (Koehler, 1979; Lynn and Clark, 1983; Chow *et al.*, 1989; Cobos *et al.*, 2011; Braga *et al.*, 2016; Tomas *et al.*, 2019). For the Caridea infraorder plus a single spike, is common that the mature spermatozoa be like a "cup" or "umbrella" shape (Felgenhauer and Abele 1991; Bauer 2004), different from many Dendrobranchiata shrimps that can also show one single spike but with a spherical shape (Camargo *et al.*, 2017).

The primary sexual morphology and spermatogenesis description have never been described for the *Synalpheus* shrimps. Chak *et al.* (2015a) used scanning electronic microscopy (SEM) and histological tools to analyze the development stage of male and female gonads in studies with gambarelloides *Synalpheus* to report reproductive skews and competition in colonies of social and communal shrimps, but no description or images were provided to make possible a comparison.

This study aims to evaluate the gonochoric condition in *Synalpheus* non-gambarelloides shrimps, bringing new information for Alpheidae Family. The study also addresses the first overview of the histological characteristics of the reproductive tract in the genus *Synalpheus*, a poorly understandable group within caridean shrimps due to the difficulty in finding primary or secondary characteristics in males.

Material and Methods

Sample sites and methods

The mature males and females of *Synalpheus apioceros* Coutière, 1909, *Synalpheus brevicarpus* (Herrick, 1810) and *Synalpheus fritzmuelleri* Coutière, 1909 used in the morphological analysis were sampled manually by SCUBA diving expeditions during May/2019 and December/2019 in two continental islands: Alcatrazes Archipelago (24°5'43.69"S, 45°51'35.51W) and Couves Island (23°25'S, 44°51'W), and in coastal

areas of the Ubatuba region (Cais do Porto - 23º27'05"S, 45º02'48"W). The sampled shrimps were individualized in plastic tubes, placed into aerated coolers containing water from each sample site, and transported alive to the laboratory at São Paulo State University, *Campus* Botucatu.

Despite we analyzed three species, after observing the same parameters and patterns in all structures, we decided to use one of them (*S. brevicarpus*) to be a "model" for all images provided in the results.

Histological and histochemistry preparation

Due to the very small size of the animal and especially because of the very translucent and slender morphology of the male reproductive system, none of the specimens could be successfully dissected, then, shrimps were anesthetized using clave oil stock, and the entire carapace was dissociated from the abdominal region (without pereopods). Authors tried many times to separate macroscopically the system without success. Therefore, for *Synalpheus* shrimps, the best way to describe the male reproductive system was to look through the entire carapace into resin for microscope preparations, even in those individuals with a big comparative carapace length. The carapace portion was fixed in 4% paraformaldehyde in 0.2 mol L^{-1} sodium phosphate buffer (pH = 7.4) and sampled were transferred to São Paulo State University *Campus* Jaboticabal, to the Invertebrate Morphology Lab (IML), in the same way, proposed by Machado *et al.* (2021).

Despite mature females presenting developed and morphological distinct ovaries into all cephalothorax and abdomen, the same protocol to block the entire animal into resin was applied to guarantee that all portions of cells would be evaluated.

After the fixation period, samples were rinsed in the same buffer, dehydrated in an ascending ethanol series (70–95%), and embedded in historesin in Leica®– HistoResin (Wetzlar, Germany) (glycol methacrylate), with the carapace positioned in a horizontal position. After polymerization, blocks were sectioned with a rotatory microtome. Sections were made in the entire carapace to secure that all regions that containing the reproductive system were sectioned. Slides of the 5 – 7 µm sections were stained with hematoxylin and eosin (HE) (Junqueira and Junqueira, 1983). We used xylidine Ponceau to detect proteins and PAS for neutral polysaccharides (Mello and Vidal, 1980; Pearse, 1985). Also, samples of what we determined as spermatozoa immersed in seminal fluid from the vas deferens were transferred to slides and photographed under differential interference contrast (DIC) microscopy on a Zeiss Axio Imager Z2 microscope (Oberkochen, Germany).

Scanning Electron Microscopy – Gonopore Morphology

Samples of the entire carapace were used, dissecting almost all pereopods maintaining just de coxa region where we inspected for opening gonopore. Samples were transferred to a solution of Karnovsky fixative (2.5% glutaraldehyde and 2% paraformaldehyde) in 0.1 mol L-1 sodium cacodylate buffer (pH 7.4) for at least 24 h, washed three times in the same buffer - 10 minutes wash, dehydrated in 15 minutes ethanol series 3 x (30% 50%, 70%, 80% 90% 95%, 100%). Then hexamethyldisilazane (HMDS) 1:1: 20min and finally HMDS 100%. The HMDS is a powerful solution that became material dry very quickly, so we were putting one drop of the solution with content that emerged on a watchmaker's plate and observing if the carapace was entirely dried to prepare stubs. Samples were attached with adhesive to round stubs of 25 mm in diameter for sputter coating with 5 nm gold in a metallizer Supputering SC 070 Belzer Union before observation in a Scanning

Electron Microscopy (Zeiss® Evo 10), operating with voltages ranging from10-20 kV. We evaluated all pereopods, especially 3rd and 5th, looking into opening gonopore for males and females.

Results

General Anatomy of the male reproductive system

A pair of testes is observed at the anterior carapace region close to the hepatopancreas and behind eye formation. It extends with the vasa deferentia (VD) through all carapace being peripheral when reaching the second to fourth pereopods (close to the gills). At this point, it abruptly changes the direction toward the central region of the body (ventral) reaching the fifth pereopod where the gonopore opening is located (Figure 1).

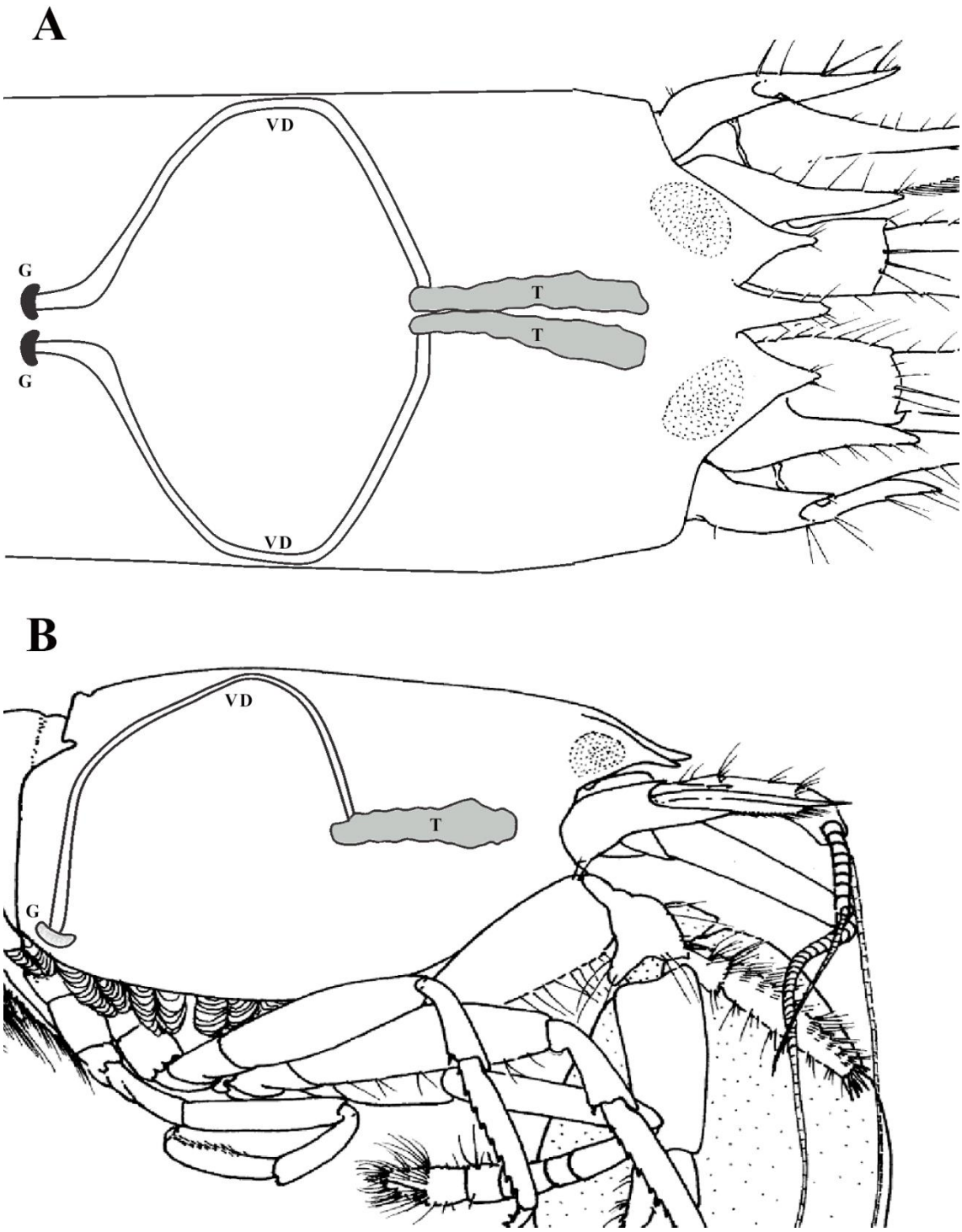


Figure 1: Schematic drawing showing testes and vas deferens of *Synalpheus* non-gambarelloides shrimps (illustration adapted from Didderen *et al.*, 2006). A) Dorsal view; B) lateral view. **T** = Testes; **VD** = Vas Deferens; **G** = Gonopore.

Testes

Testes are concentrated on the dorsal and anterior side of the cephalothorax, just above the hepatopancreas, behind the eyes position, and below the heart, it is lobular morphology, containing conjunctive tissue and a slender musculature in the link between testes and vas deferens (Figure 2A and 2B). Seminiferous tubules were found at the ends of the testicles where sperm formation occurs and it was possible to define four stages of spermatogenesis: spermatogonia, spermatocytes, spermatids, and mature spermatozoa (Figure 2C, 2D and 2E). The spermatogonia is a round cell with scarce cytoplasm and granular nuclei, with very homogeneous chromatin located in the periphery of the seminiferous tubule (Figure 2D). Spermatocytes show large nuclei depicting different stages of the meiotic prophases, with also unconsolidated chromatin (Figure 2C). Spermatid, instead of the two first stages has well-condensed chromatin concentrated in only one pole of the cell, and mature spermatozoa containing nucleus in an “umbrella” shape and spike (entire described bellow) is released into the lumen of the seminiferous tubule (Figure 2E).

Vas Deferens (VD)

The VD is a long tube that initiates at the end of the seminiferous tube. Despite we cannot provide a macroscopic perspective for the entire morphology of testes and VD, it can be defined into different regions, *i.e*, the proximal vas deferens (PVD), the middle vas deferens (MVD) ending in a final portion: the distal vas deferens (DVD).

The PVD, is a narrow tubule in a dorsal position, with a primary layer rounded by well-formed cells very close to the testes with a tiny layer and a concentration of anucleated cells at the margin, inside the vasa we can observe free spermatozoa with the acrosomal vesicle spike and nuclei. (Figures 2F and 2G). MVD is longer than the other

two regions extending from the PVD toward the lateral side of the cephalothorax (from the 2^{sd} pereopod region to the 4/5th) (Figure 3A) with simple cubic epithelium lying on a thin layer of musculature and formed spermatozoa in the lumen (Figure 3 B, 3C, 3D). The DVD is considered here as the portion of the vasa that returns into the most central region in the 5th pereopod (Figure 4A). It is the region of the vasa with cubic epithelium and is wrapped in a thick muscular layer and the two vasa almost touch each other when opening (Figure 4B, 4C, 4D). It does not seem to present an ejaculatory duct, only an enlargement of the musculature on the final portion of the DVD without androgenic glands and a few numbers of free spermatozoa in the lumen (Figure 4D). The entire vasa presented few secretions (discreet on coloration performance) (Figure 4E, 4F) and the presence of typhlosole regions is not observed.

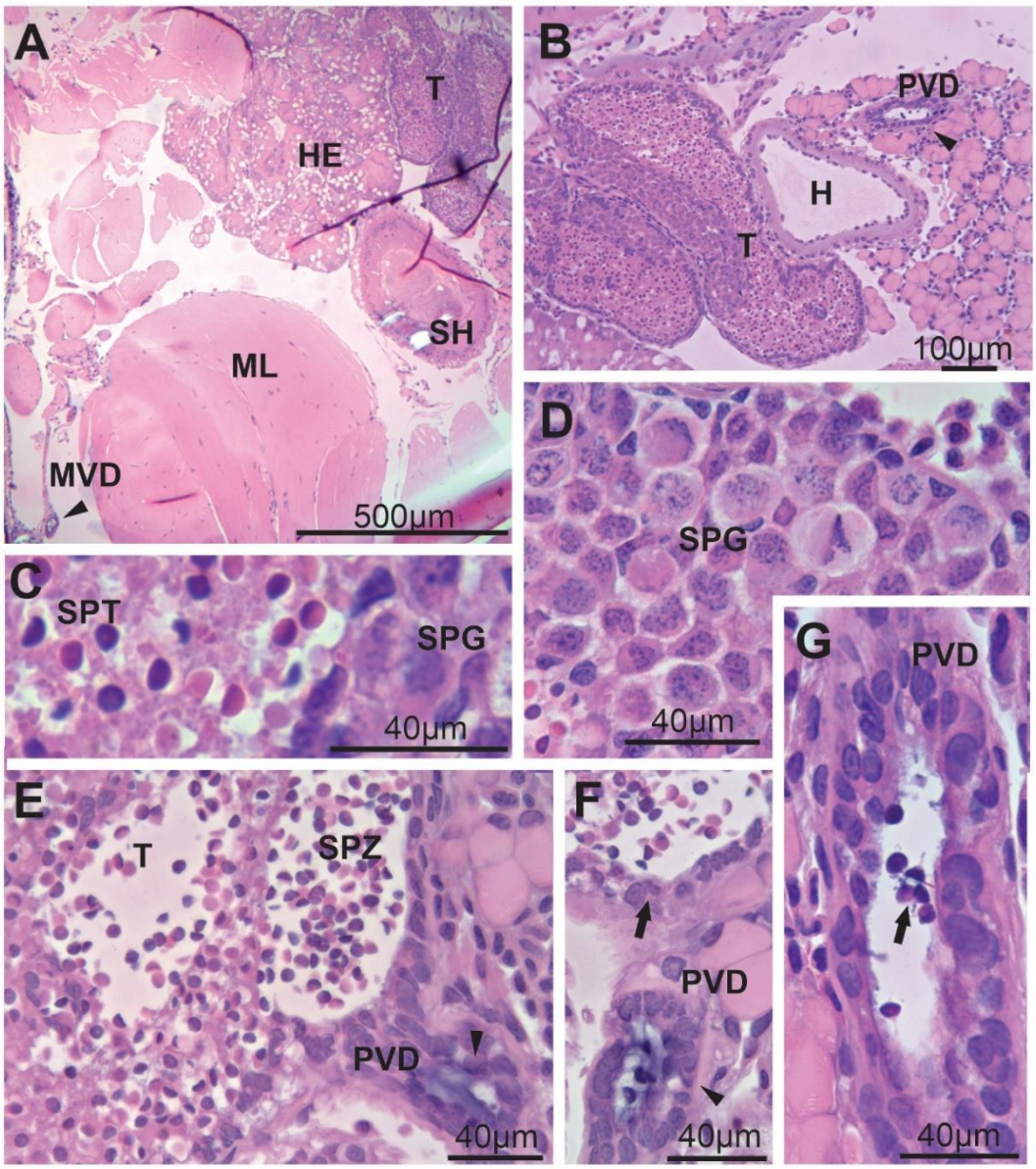


Figure 2: Histology of the male reproductive system of *Synalpheus brevicarpus* stained with hematoxylin and eosin (H&E). Focus on testes and Proximal Vas Deferens: A) Longitudinal view of the carapace containing in the central region: The hepatopancreas **(HE)**; Stomach **(SH)**; Muscular Layer **(ML)** and lobular Testes **(T)**. In the lateral region, a first portion of the Median Vasa Deferens **(MVD)** (black arrowhead). B) Distal view of the testes containing differentiation cells. The heart between testes and Proximal Vas Deferens **(PVD)** (black arrowhead) very proximal to testes formation with free spermatozoa in the lumen. C) Zoom on the proximal view of testes with proliferation cells formed by Spermatogonia cells **(SPG)** and late Spermatids **(SPT)**. D) Spermatogonia cells in the site of different stages of proliferation in one part of the lobular lumen of testes. E) Testes lobe releasing free-formed Spermatozoa **(SPZ)** to the lumen of PVD (black arrowhead); free spermatozoa in the lumen of the final portion of testes. F) Final lobe of testes with free spermatozoa in the lumen (black arrow), very close located to the beginning of the first portion of PVD (black arrowhead). G) Focus on the first proximal opening of PVD with a few spermatozoa in the lumen with primary layer rounding vas. **H** = Heart; **SPC** = Spermatocytes.

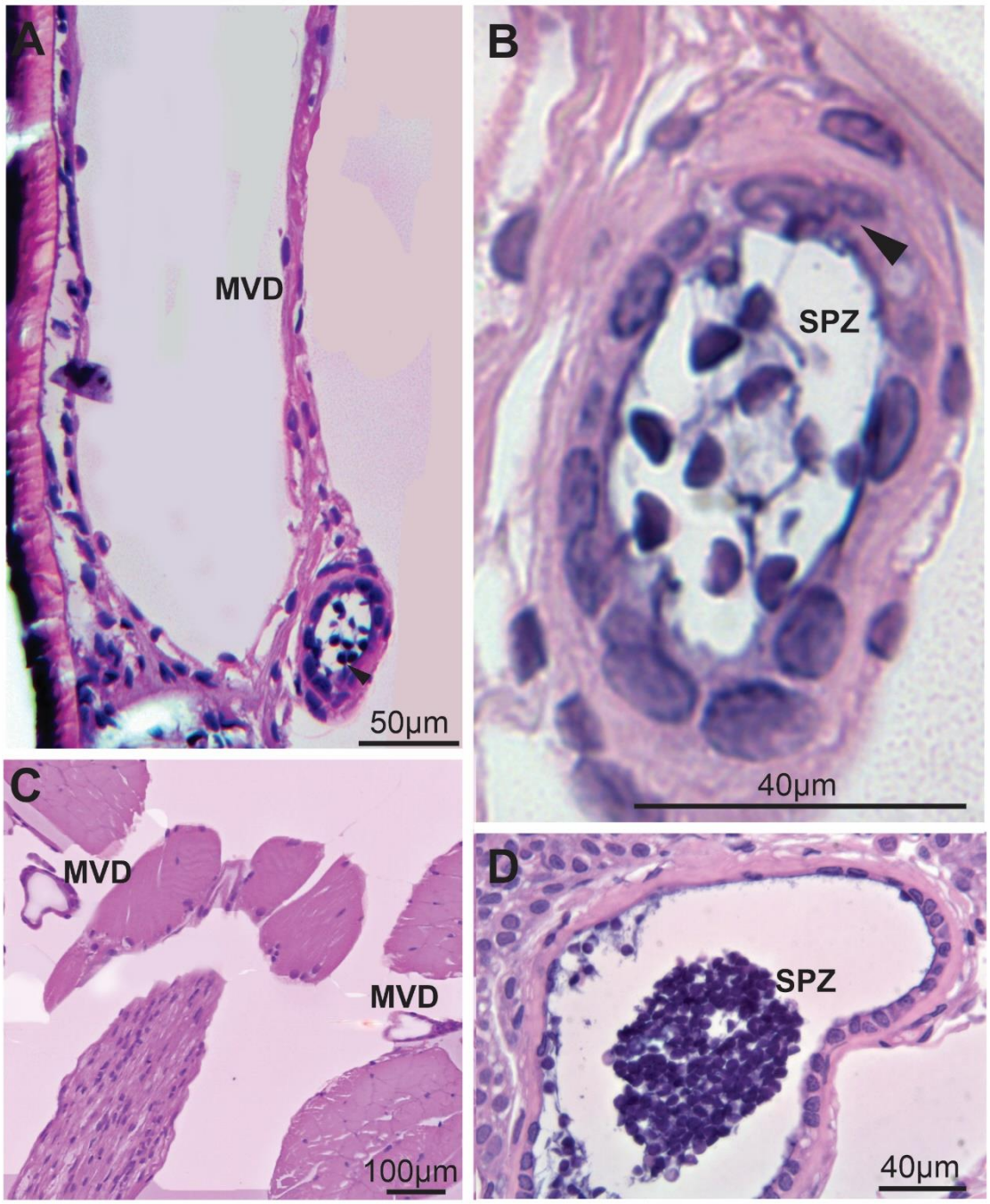


Figure 3: Histology of the male reproductive system of *Synalpheus brevicarpus* stained with hematoxylin and eosin (H&E). Focus on Middle Vasa Deferentia **(MVD)**. A) MVD (black arrow) at the most lateral portion of the cephalothorax with simple cubic epithelium lying on a thin layer of musculature and formed free Spermatozoa **(SPZ)** (black arrowhead) in the lumen. B) Focus view of MVD with a few spermatozoa (black arrowhead points to anuclear cells) in the lumen with primary layer. C) Dorsal view of both MVD changing directions from most lateral portion of the carapace to the most central region. Thin musculature and lumen with few secretions and few spermatozoa. D) Proximal view of the end of MVD with cubic epithelium lying on a thin layer of musculature and spermatozoa clusters to the center of the vas.

Histochemistry

Chemist secretions can be found at most proximal MVD but not in the entire vasa, and none remarkable secretion into all VD. The proximal portion of MVD with spermatozoa and simple cubic epithelium portion was intensely reactive for proteins (Figure 4E). The final portion of MVD with a slender muscular layer and also none remarkable secretion was positive for neutral polysaccharides (Figure 4F). There were also few secretions weakly positive between spermatozoa, with negative nuclei. The acrosomal vesicle is weakly positive for neutral polysaccharides (Figure 4F). The final portion of the DVD presents a remarkable muscular wall, with epithelium intensely reactive for proteins and few glycoprotein granules, with spermatozoa intensely reactive for proteins agglomerates in the center of the vase with just a few secretions.

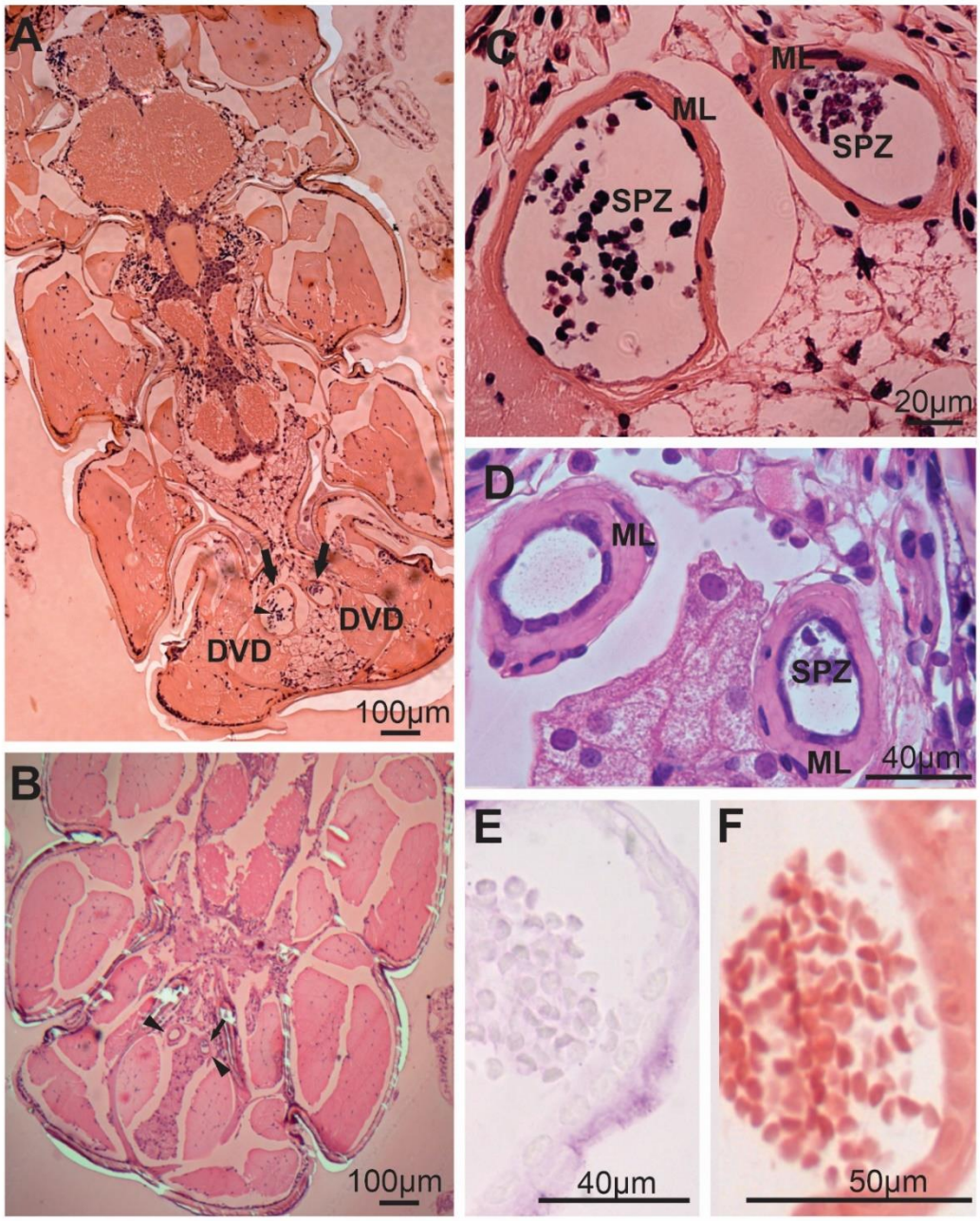


Figure 4: Histology of the male reproductive system of *Synalpheus brevicarpus*. Focus Distal Vasa Deferentia **(DVD)** and histochemistry reactions on vasa. A) View of the entire carapace, musculature very present and distributed into the entire body, DVD (black arrows) located at the most posterior and central part of the carapace, very close to each other. B) Distal view of the final portion of DVD (black arrowheads) with remark for expressive muscular layer rounding the vasa, free Spermatozoa **(SPZ)** (black arrow) into the lumen of the vasa. C) the first portion of DVD with a Muscle Layer **(ML)** thick than in the Middle Vas Deferens **(MVD)** turning more expressive muscle rounding vasa with free spermatozoa in the lumens. D) Final portion of DVD lined with cubic epithelium and wrapped in a thick muscular layer, without androgenic cells and a few numbers of free spermatozoa in the lumen. E) Proximal portion of MVD, intensely proteins with spermatozoa of the lumen with almost nonexistent secretion and the walls of the MVD are a simple cubic epithelium with a thin muscle layer. F) Proximal view of the final portion of MVD with free spermatozoa in the center of the lumen reactive for neutral polysaccharides.

Spermatozoa (SPZ) morphology

Synalpheus seems not to present the process of spermatophores formation and free SPZ is formed since the testes to the final portion of the VD. It is immersed in a discrete secretion (Figure 5A). The mature spermatozoa consist of the main body (nuclear pole) and cap region where we can see the spike typical for caridean shrimps (Figure 5B). The mean size of the spermatozoa was 10.47 ± 0.44 µm in major axis, 6.55 ± 0.44 µm in the minor axis, and 4.12±0.77 µm in the spike length. The spermatozoa are aflagellated with one single and very distinguish rigid spike, with a condensed nucleus (Figure 5C).

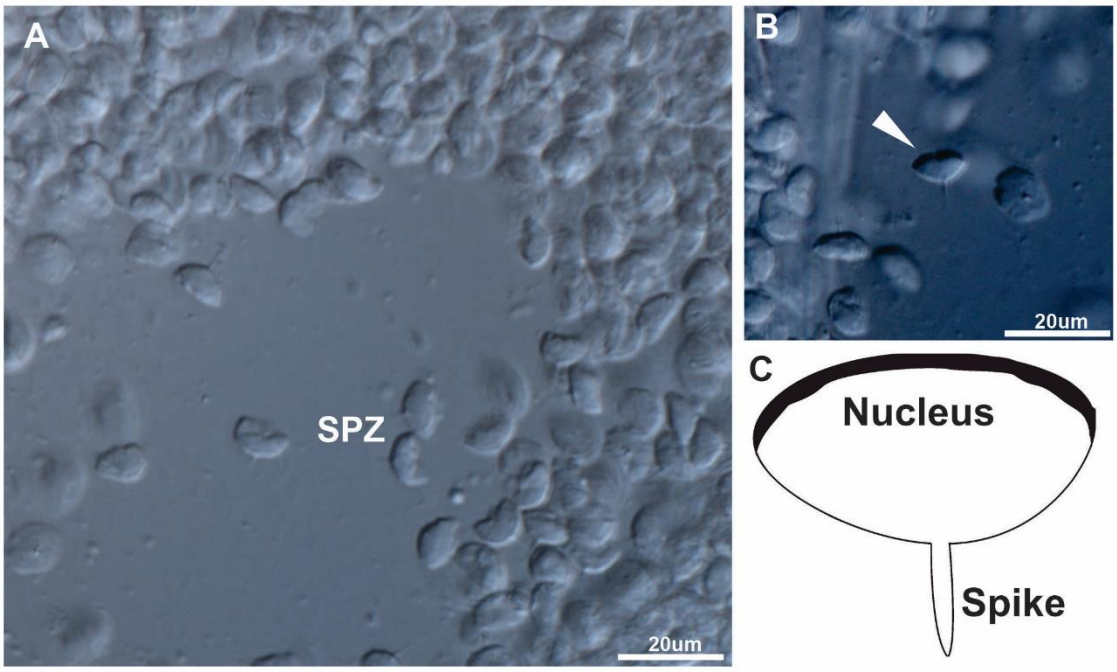


Figure 5: Spermatozoa morphology of *Synalpheus brevicarpus*. A) Spermatozoa agglomerates are shown in Differential Interference Contrast (DIC), especially looking into semicircular as "umbrella" formation on nucleus region, and spike well defined. B) Final portion of Middle Vas Deferens **(MVD)** with free Spermatozoa **(SPZ)** on center in the periphery of the vas under DIC image (white arrowhead points to an "umbrella shape" free spermatozoa). C) Schematic drawing of single SPZ with nucleus and spike region, horizontal distance corresponding on the major axis, and vertical view on the minor axis.

General Anatomy of the female's reproductive system

The female reproductive system consists of a pair of ovaries that is easily visible through the translucid carapace. The ovaries are granular and are very turgid, located mainly in the dorsal portion of the cephalothorax: parallel and dorsal to the digestive tract, anterior lobes reached the anterior region of the carapace, immediately behind the eyestalks; posterior lobes extend into the dorsal region of the abdomen, reaching the third abdominal somite (Figure 6A). The total size of the ovaries, in both length and width dimensions, depended on the stage of ovarian development, but without changes in the color pattern of the oocytes, only increasing the color intensity according to the advancement of development. The mature oocytes were visible, with prominent yolk granules (Figure 6B). Each ovary has two thick lobes with anterior cephalothoracic and a posterior abdominal region. Both lobes are connected and surrounded by a thin layer of connective tissue (Figure 6C). In the anterior ovarian region, the presence of oogonia and some previtellogenic ovarian follicles are observed. Oogonia are small cells with a round, voluminous nucleus in relation to the cytoplasm and are located in the anterior region of the ovary (Figure 6D, 6E). The ovarian follicle, on the other hand, is formed by the oocyte and the follicular cells that surround it, and this previtellogenic ovarian follicle presents an oocyte with a central nucleus and evident nucleolus, the cytoplasm is homogeneous with discrete lipid drops (Figure 6F). In the mature ovary, the oocytes are well-developed and have a larger diameter with the main feature of these oocytes is the cytoplasm filled with acidophilic mature yolk granules and many lipid droplets. In this ovarian stage, the oocytes are surrounded by flattened follicular cells and are well adhered to each other, producing a more compact appearance (Figure 6G).

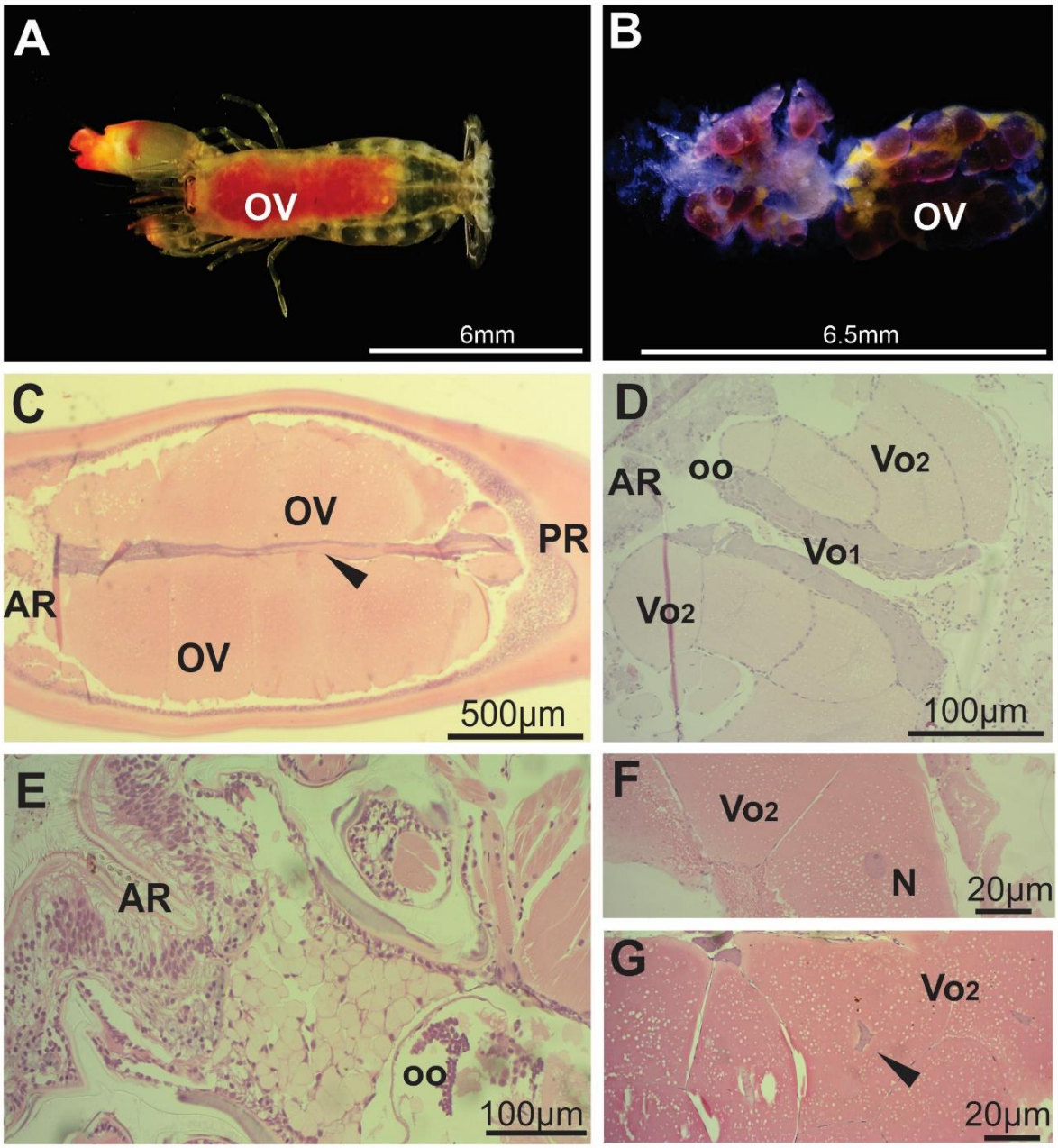


Figura 6. Dorsal view of the mature ovary of *Synalpheus brevicarpus*. A) Ovary **(OV)** in the development stage occupy a large area at cephalothorax and have an intense coloration. B) mature oocytes with prominent yolk granules. C) Ovary with two lobes with anterior cephalothoracic and a posterior abdominal region, connected by a thin layer of connective tissue (black arorwhead). D) The anterior ovarian region with the presence of Oogonia **(OO)**, some previtellogenic ovarian follicles, primary vitellogenic oocyte and secondary vitellogenic oocyte. E) The anterior ovarian region with the presence of oogonia. F) Mature oocytes showing nucleus in the central region surrounded by many acidophilic yolk granules and lipid droplets. G) The ovarian region is completely filled with oocytes mature, which comprise cells with a greater accumulation of lipids and proteins in yolk concentrated around the nucleus (black arorwhead). **N** = nucleus, **Vo1** = primary vitellogenic oocyte, **Vo2** = secondary vitellogenic oocyte, **AR** = anterior region, **PR** = posterior region.

Gonopore opening and morphology

Despite confirming the opening region and corroborating the sexual dimorphism of *Synalpheus* non-gambarelloides spp., and the absence of any intersex or hermaphroditism condiction, we can observe many differences between male and female gonopores morphology. The male-gonopore is presented as just like a simple "pore" with no more than 20 μm width at the mesial region of the coxae of the fifth pereopod (P5) (Figure 7A). It is essential to note the proximity of the coxae on both P5, and gonopores are very close, almost touching each other (Figure 7B). It matches the morphology of the primary sexual system described previously on histological preparation with the very central opening of the vas deferens. Both gonopore opening seems don't present any accessory structure to help the release the spermatozoa mass, it just ends on an opening (Figure 7C, 7D), that will transfer direct the spermatozoa in a quick movement to the female stern.

The female-gonopore is a much more complex opening morphology. It is bigger than the male gonopore and is located at the basis of the coxae on the third pereopod (Figure 8A). We believe that it forms an "open-close" structure that works at the moment that females are releasing their ovulation product (Figure 8B). The more complex structure, when compared with males, is corroborated also by the presence of the "egg guiding setae" surrounding the gonopore, described for other *Synalpheus* shrimps by Tóth and Bauer (2007). Those setae are important structures to direct eggs at the time of the fecundation process and guarantee that all mass will be correctly conducted to the stern to join the sperm mass first released by the male.

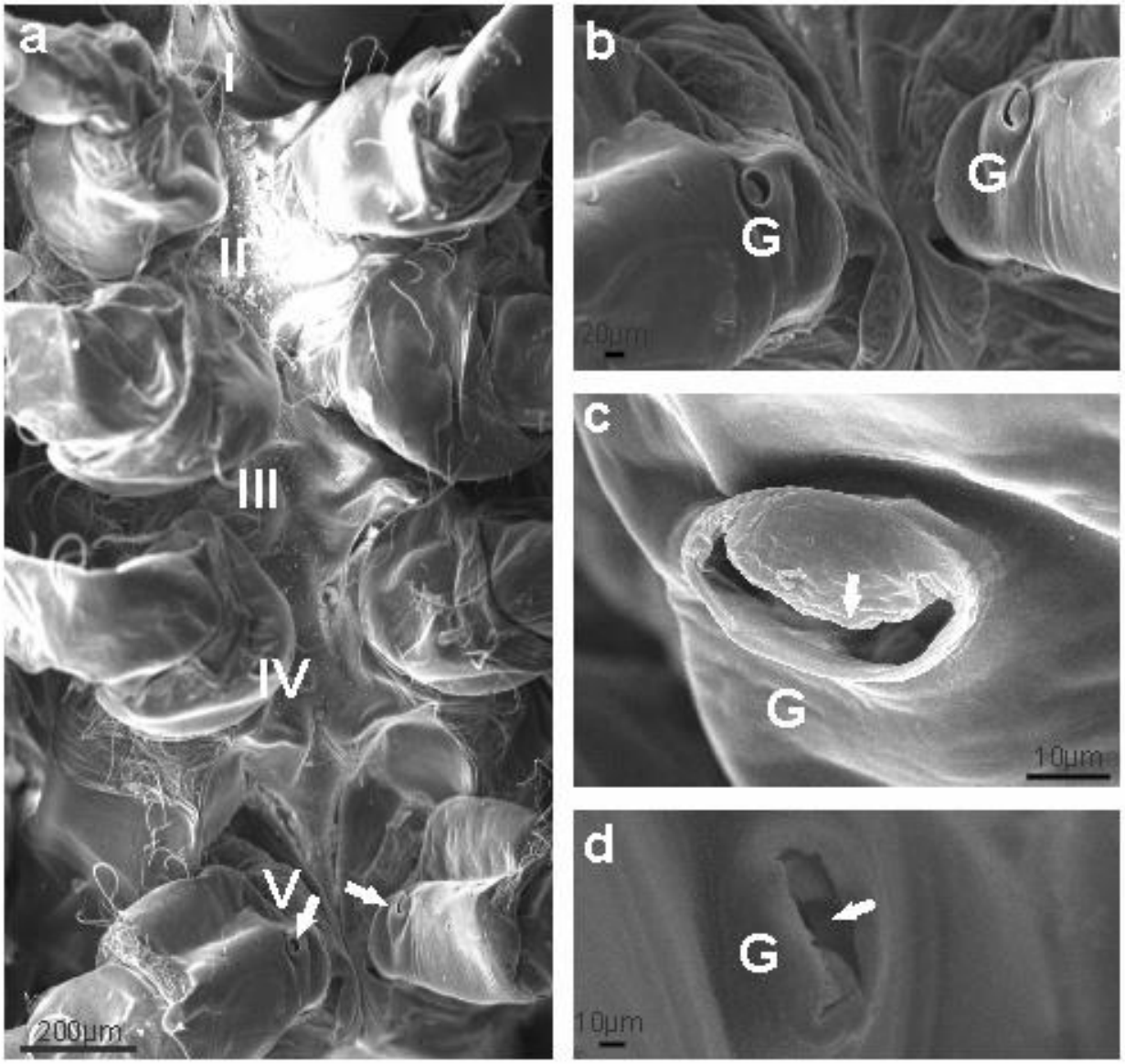


Figure 7: *Synalpheus brevicarpus* Male gonopore morphology under Scanning Electronic Microscopy. A) General view of the male entire sternum and coxa of the five pairs of pereopods **(I, II, III, IV, V)**. Arrow points to a simple gonopore opening located at the basis of the coxa on the fifth pereopods. B) Detail of the two gonopore opening on basis of the coxa on fifth pereopods, very close opening gonopore, almost touching each other, like internal morphology represented on histology images. **G** = Gonopore. C and D) Details on Male form pore to release sperm mass to the female's stern. Very simple form, resembling just a simple pore opening without any accessory structure **(White setae)**.

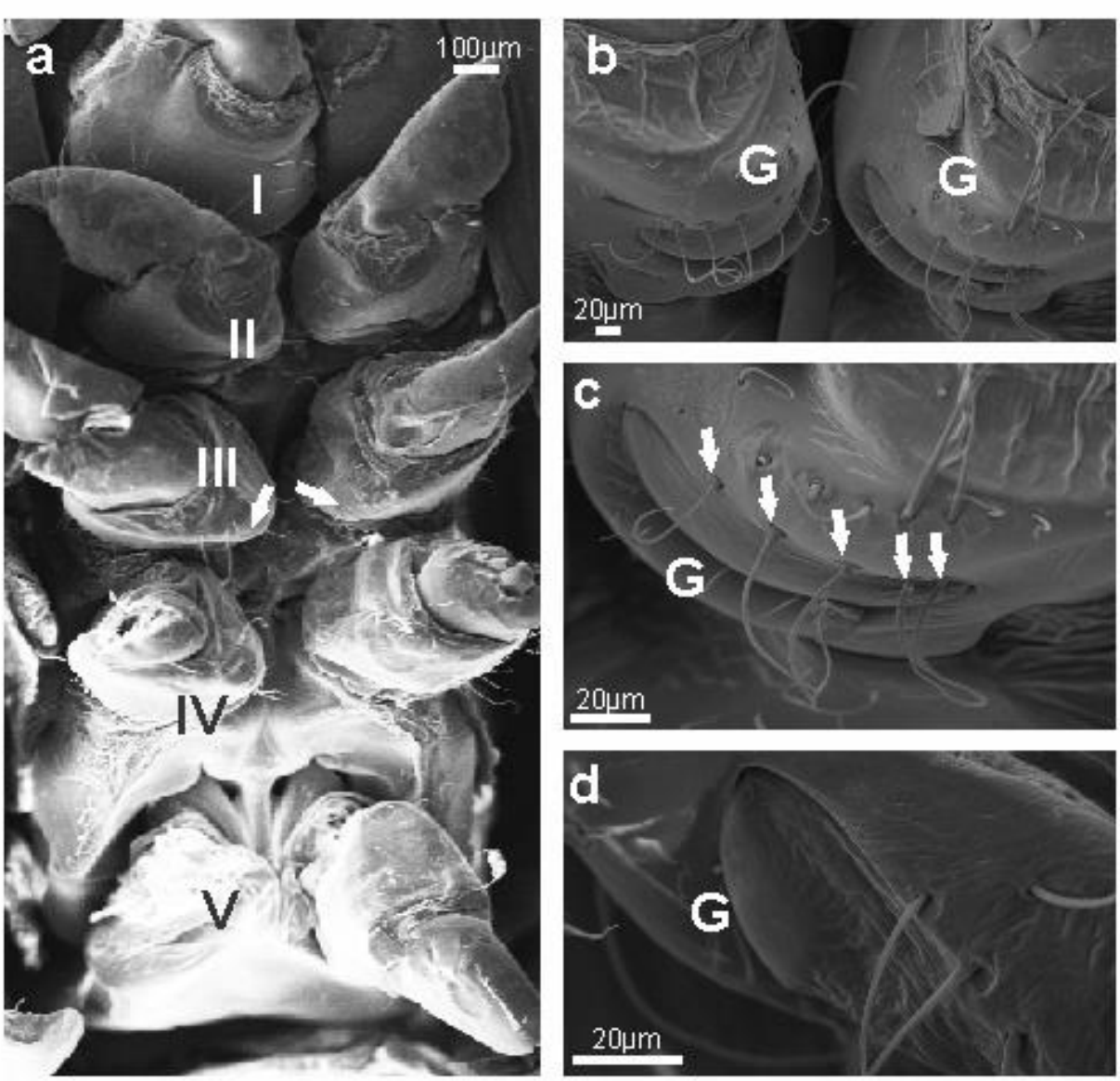


Figure 8: *Synalpheus brevicarpus* Female Gonopore **(G)** morphology under Scanning Electronic Microscopy. A) General view of the female entire sternum and coxa of the five pairs of pereopods **(I, II, III, IV, V)**. Arrow points to elaborated gonopore opening located at the basis of the coxa on the third pereopods. B) Detail of the two gonopore opening on basis of the coxa on third pereopods, "Egg-guiding" setae present surrounding both gonopores and a dorsal view of the "open-close". C and D) Opening morphology of the gonopore at the coxa's basis of the third pereopod. Female form. Without a clear opening, it is maybe opening just at the fecundation time. Egg-Guiding setae present very close to the gonopore opening directing eggs on the fecundation period **(White setae)**.

Despite the presence of the "egg-guiding" setae, the sternum formation in both male and female are very simple. Pereopods are very close to each other, there is no space to form any kind of "chamber" process on the female, even not any elaborated structure to help the mating process in males. Setaes and adhesive structures are also absent, resembling the idea that the copulation process occurs in a very brief period.

Discussion

This is the first panorama for a descriptive study with reproductive aspects in caridean shrimps for the genus *Synalpheus*, in the non-gambarelloides group. Because of the difficulty to access the entire system, the distinct morphology, and the slender aspect of all components in the male reproductive trait, the description was never been successfully made until now.

The strategy of using microscopic analysis, providing histological perspectives, spermatozoa morphology, and internal system structure, like in the present study were never been explored. Other microscopic techniques, like Scanning Electron Microscopy (SEM) to access the sexual system and verify gonochoric, hermaphrodite, or intersex condition in *Synalpheus* species have been used by some authors (Tóth and Bauer, 2007; 2008; Chak, 2016) but always made in a perspective of access information to explain patterns for reproductive behaviors in eusocial groups, linking morphology and mating system.

Caridean shrimps seem to present at least six different types of reproductive strategies into all infraorder, from classic gonochorism, with separated sex specimens, until the different types of hermaphroditism strategies. The gonochoric seems to present the most common pattern (Bauer, 2004; Baeza, 2018) and is recognized for most *Synalpheus* shrimps, especially from the non-gambarelloides group. Gonochorism

implies some morphological differences between males and females in primary (gonads) and secondary (reproductive external traits) characteristics. The *Synalpheus* non-gambarelloides are an exception as the absence of masculina appendix on the endopod of the second pleopod (Bauer, 2004), making it difficult the identification of individuals' sex. Also, gonopore identification are very difficult to access without electronic microscopy (Tóth and Bauer, 2007), turning histological analyzes essential to determine sexual conditions.

The composition of the male sexual system in *Synalpheus* remains the same pattern as in other caridean shrimps but the morphological structures of components, especially in VD, make the genus a particular case, like the slender composition of all vasa and location that it compounds in the cephalothorax region. Most descriptive studies from the sexual system in caridean shrimps are related to a few species of Hyppolytidae, Palaemonidae, and Atyidae families. Those species present one pair of testes on the dorsal region of the cephalothorax, between hepatopancreas and heart. The VD develops into a terminal ejaculatory duct full of spermatophores connecting the gonads trait to the gonopore on basis of the coxae of the fifth pereopod and most of them present the androgenic gland at this final portion of the duct (Espinoza-Fuenzalida *et al.*, 2008; Cobos *et al.*, 2011; Manjón-Cabeza *et al.*, 2011; Paschoal and Zara, 2019).

Despite the components of the reproductive traits be the same that most caridean shrimps, they cannot be found visible in *Synalpheus*, nether under microscopic dissection, so, we must need to imply the morphology by the context seem on slides and histological preparations. Baeza (2018) pointed out that those studies who tested the gonochorism (using histological and morphological features from primary and secondary sexual characteristics) are still missing in caridean groups, revealing the importance of the

present study to be used as a comparison model for Alpheidae and other carideans' families.

The testes of the *Synalpheus* species analyzed in the study exhibit lobular anatomy. The lobular testicular histoarchitecture is present in many species of Decapoda, mainly in Pleocyemata (Tsang *et al.*, 2008). Among caridean shrimps, mostly found in Palaemonidae species of the genus *Macrobrachium* (see Ruiz *et al.*, 2020). The lobular anatomy is characterized by numerous lobes or seminiferous cysts, connected by a central seminiferous duct and each lobe has cells at the same stage of spermatogenesis (Nagao and Munehara, 2003), such structures comprise germ cluster cells, with walls made up of a thin layer of connective tissue and nursing cells (Hobbs *et al.*, 2007).

The difference pattern for studied *Synalpheus* species was the absence of spermatophores and a structure that resembles an androgenic gland. Free spermatozoa are present since the testes with other cells from spermatogenesis, and in all VD. Tomas *et al.* (2019) detailed the male reproductive system for the freshwater Atyidae species *Neocaridina davidi* (Bouvier, 1904) and reported a complex spermatophore formation with a large number of acids glycoconjugates featuring it, very different from the parameter observed here.

Also, in the marine caridean species, like some *Hyppolyte* spp., spermatophores (even the simplest composition) and VD containing secretory substances to spermatophore formation and transport of those compounds are representative and very distinguished into the sexual system (Cobos *et al.*, 2011; Manjón-Cabeza *et al.*, 2011). The opposite seems to occur in *Synalpheus* shrimps, besides not having spermatophores was not observed any remarkable contents of the protein layer surrounding spermatozoa formation, even in the DVD portion. Chak *et al.* (2015a), in a brief description of spermatozoa in *Synalpheus* gambarelloides, detailed that sperm were highly basophilic

with distinct umbrella shape located at testes, vas deferens, and gonopore, but none images or more descriptive character were provided.

Many caridean species, especially *N. davidi*, also present typhlosole formation on a diverse portion of the VD. The secretion of the nutrition of the spermatozoa seems to be provided by those typhlosole formations and the columnar epithelium that form vasa (Tomas *et al.*, 2019), and it is significant for *Macrobrachium* species (Chow *et al.*, 1982; Chow, 1989). Based on analyses of the histochemical results of the present study, *Synalpheus* shrimps do not present any typhlosole formation along the VD, neither present a very representative columnar epithelium to provide a protein supplement for spermatozoa formation. This simple composition remains the hypothesis that a mechanic activity is very important for the reproductive process in *Synalpheus* males, once they may release spermatozoa in just one time for female stern, and the movement of contraction in the vasa is facilitated when it is a continuous and uniform along with all distance, from testes to gonopore.

Synalpheus shrimps analyzed in the present study presented significant differences in the muscular layer that surrounds the entire VD. The layer increases substantially at the distal zone from vasa, forming an expressive wall at the end of the VD, supporting the physical stimulation required to promote spermatozoa movement. In contrast, it is observed in other caridean shrimps that the diameter of the vasa increases while layers decrease from proximal to distal zones, and then, secretion substances and packages of spermatozoa may be required for the successful process of release ejaculatory contents in the female stern (Cobos *et al.*, 2011; Tomas *et al.*, 2019).

The muscular layer pattern from the vasa, the absence of expressive protein productions (without any reactive protein in histochemical results) as very discreet secretion into all VD, absence of an androgenic gland, or ejaculatory duct improves the

hypothesis mentioned before that *Synalpheus* uses a "mechanic" strategy to release male spermatozoa production. Just the muscular movement seems to be enough to transfer spermatozoa mass to the female stern. In addition, *Synalpheus* non-gambarelloides group from invertebrate's symbionts or free-living are mostly found in heterosexual pairs (Vandenspiegel *et al.*, 1998; Alves *et al.*, 2021; personal observation). Therefore, all the process involved on reproduction activity, as the production process of reproductive contents, copulate, fertilization and exteriorization of eggs by females occur in an expressive short-time period, that may explain all simplistic formation of the reproductive trait and sperm product by males. Also, the constant presence of reproductive partners may induce a low output to improve a complex sexual system, once males and females are always available close to each other to reproduce.

The VD in *Exhippolysmata oplophoroides* (Holthuis, 1948) is formed by nuclei cells with dispersing chromatin and a thin fibrous layer of tissue plus layer of muscle formation (Nunes *et al.*, 2010), the same patterns seem to occur at least in PVD of *Synalpheus* shrimps. Along with *Synalpheus*'s MVD, the pattern changes in the same way proposed for penaeoidean shrimps (Subramoniam, 1995) where it is not possible to observe a vas surrounded by more elongated cells and dense nuclei, in which a very smooth muscle layer that completely changes in DVD portion, with almost no tissue layers and expressive musculature compounds.

In *Synalpheus,* the spermiogenesis seems to be presented as almost a simultaneous quick process inside the testes, so, it is possible to identify all type of maturation cells into all part of the testes. It is similar to those of caridean shrimps from the *Hippolyte, Palaemon,* and *Neocaridina* genera despite differences in the number of stages. In the species analyzed in the study, it was observed four stages compared with six in *Hippolyte* (Manjón-cabeza *et al.*, 2011) and ten in *Palaemon* (Papathanassiou and King, 1984b).

In *E. oplophoroides,* the male gametogenesis process increases according to the maturation process of the animal gonads and it is divided into well-reported different stages of gonads with shrimp size in the male phase (Braga *et al.*, 2009; Nunes *et al.*, 2010). This pattern seems to be expected compared with *Synalpheus* shrimps, due to most complex sexual system that the hermaphrodite shrimps *E. oplophoroides* represents. The elongated sexual system of those specimens makes evident a simple and primitive pattern for those Alpheidae gonochoric shrimp of genus *Synalpheus*.

The general morphology of the spermatozoa in caridean shrimps is the same, but it is possible to find some variations among species (better described under Transmission Electronic Microscopy – TEM). The *Synalpheus*' spermatozoa morphology presented resembles those described for *E. oplophoroides*, more like an "umbrella" shape with semicircles than a rounded shape present in Atyidae species as *Neocaridina* (Nunes *et al.*, 2010; Tomas *et al.*, 2019). Besides the corpuscular form variates, the "spike" formation is a constant in spermatozoa for Decapoda, always present in caridean shrimps and it is visible in the *Synalpheus* group analyzed in the present study. The real function of the spike formation in caridean spermatozoa is discussed, but all hypothesis surrounds the premise that it is an important compound on fertilization of the oocytes in female bodies, being that when in contact with oocytes or when in a passive capture and releases of the spermatophores inside female gonads (Burkenroad, 1947). Despite presented in all caridean shrimps, the size of the spike can change into species, and it may be related to the efficiency of the fertilization process. The mean size of the spike in *Synalpheus* shrimps from the present study was 4.12 ± 0.77 µm, smaller than other caridean shrimps as *Neocaridina, Macrobrachium,* and *Palaemonetes* genera that can present a spike from 5 until 15µm length (Tomas *et al.*, 2019).

561 The general morphology of the reproductive system of *Synalpheus* females
562 followed an anatomical pattern common to other groups of Decapoda shrimps, however,
563 the size and extension of mature ovaries was similar to that observed in Penaeidae shrimps
564 (Levi and Vacchi, 1988; Quintero and Gracia, 1998) and in shrimps *Stenopus hispidus*
565 (Olivier 1811) (Gregati *et al.*, 2014), occupying almost all the available space in the
566 cephalothorax and extending to the region of the last abdominal somite. Most of the
567 Caridean shrimps, in contrast, have their ovaries restricted to the cephalothorax, as
568 described in the freshwater species of the genus *Macrobrachium* (see Valenti 1985; Bauer
569 2004; Paschoal and Zara, 2020). In some cases, such as *Hippolyte inermis* Leach 1815,
570 the ovary extends posteriorly at most until the first abdominal somite (Cobos *et al.*, 2005).
571 Caridea is a heterogeneous group both in the morphology of the reproductive
572 system and behavior in the fecundation process. Especially on those eusocial and
573 communal shrimps, the reproductive skews tends to be more specific and adapted to the
574 lifestyle required inside the community structure, and formulating “queen” or “helper”
575 individuals, since one intersex condition may have the potential to become a reproductive
576 male or female specimen and could be useful in its reproductive behavior. In this sense,
577 *Synalpheus* gambarelloides shrimps tend to present both gonochoric and
578 hermaphrodite/intersex conditions (Tóth and Bauer, 2008; Chak *et al.*, 2015a). This
579 pattern does not seem to appear in any species of the free-living and not social non-
580 gambarelloides group analyzed in the present study and can be inferred that intersex is a
581 strategy model for eusocial or communal species, being that free-living or facultative pair
582 living symbiotic performing the separated sex condition.
583 Corroborating the absence of any intersex condiction in the present analyzed
584 species, we can look into the gonopore morphology position. The morphology of the
585 gonopore is the same as gambarelloides males and females but present just in one

pereopod in each specimen. Males showed an opening on a gentle bump on the medial surface of P5 coxae. In the females, it is a semicircular slit with "egg-guiding setae". As previously described by Tóth and Bauer (2007) for another conspecific species, it is a broadly U-shaped slot on the proximal sides of the third pereopod coxae. The morphology of those egg-guiding setae was the same in SEM images provided here and provided by Tóth and Bauer (2007), for *Synalpheus* gambarelloides. Those structures have the function to put embryos during the spawning time in the abdomen direction. Höglund (1943) observed those structures on *Palaemon* shrimps and indicated that in bigger animals, it could be found without using SEM images, which should make it easier to identify females' individuals when dissected specimens. However, in none *Synalpheus* evaluated here, we observed egg-guiding setae on light microscopy, proving that for those animals, the best strategy is keeping using SEM techniques to confirm gonopore morphology and position. The much simplest formation of the structures found in both sterns (male and female), resembles a very quick process to fecundation time. Eggs do not seem to keep much time adhered on female stern because of the lack of structures that resemble any kind of "chamber", that helps to retain most of the spermatozoa or spermatophoric material as described for the Atyidae species *Potimirim potimirim* (Müller, 1881) (see Machado *et al.*, 2021), comparing with this species, another much simplest formation is dealing with setae composition and formation around all the female sternum. Even the "egg-guiding" setae, present in *Synalpheus* females are much smaller and less in number. All those characteristics contribute to the basic and primitive mode of reproduction on those Alpheidae shrimps.

Synalpheus non-gambarelloides shrimps is a remarkable model to analyze the evaluative process for the reproductive pattern in caridean shrimps. This first panorama for a detailed male and female morphology on primary sexual features, being

distinguished as much simpler formation than other caridean shrimps, maybe reinforce a primitive aspect for the genus into the infraorder.

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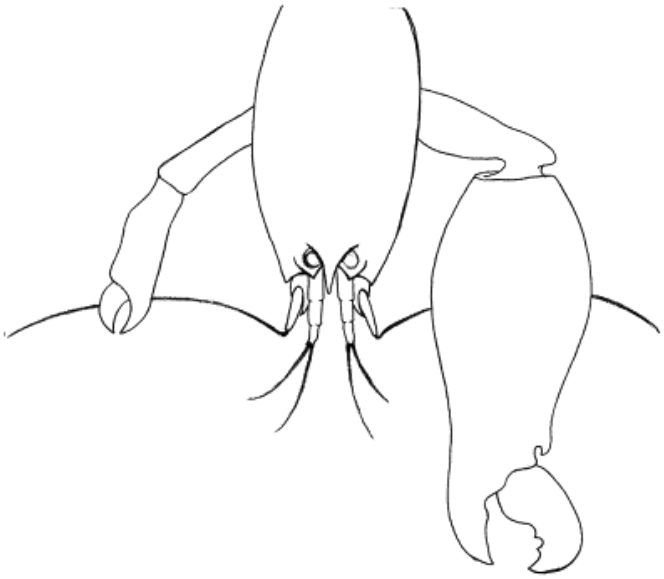

Capítulo 02

Pleopods and Gonopore as morphology tools to assess the sexual dimorphism in *Synalpheus* shrimps

Abstract

The definition of sexual dimorphism is the external morphological differences between sexes. In caridean shrimps, those characteristics are most found in appendix masculina and gonopores opening positions. *Synalpheus* shrimps are an exception to Infraorder Caridea and cannot be sexing by classical morphologies. The present study aims to describe alternatives tools to prove if there is sexual dimorphism in *Synalpheus* spp.. We described reproductive features of non-gambarelloides shrimps *Synalpheus apioceros* and *S. fritzmuelleri* applying: classical and geometric morphometrics tools to compare carapace vs second pleura length and 1st pleura/1st major chela morphologies, respectively; Scanning Electron Microscopy to morphological description of gonopores; and Light Microscopy to describe pleopods and appendix interna. Integrative methods turned possible to evaluate and to propose some new sexual discrimination strategy. Morphologic differences are present at: the pattern of first pleura's posterior projection in males with acute angle and rounded in females and ovigerous female, more robust first chela in males, pleopods with distinct setae in females, and appendix interna position in a more apical portion of females' pleopod than the males' midpoint position. Gonopores have different morphology with much simplest pore formation in males and complex opening-close formation in females. Classical morphometry indicates positive allometry in growing second pleura and carapace length in females. We were able to provide evident sexual dimorphism in *Synalpheus* shrimps, even lacking the masculina appendix and sometimes lacking appendix interna. It also proposed that females invest much more energy in body change to a successful reproduction and breeding process.

Keywords: caridean shrimps, geometric morphometrics, mating behavior, reproductive features.

Introduction

One of the definitions for sexual dimorphism in decapods crustaceans is the external morphological features between sexes (Bildstein *et al.*, 1989; Melo and Masunari, 2017). Bauer (2004) defined it as "secondary sexual characters" present in most caridean and stenopodidean shrimps as the presence of the masculina appendix associated at the endopod of the second pleopod and the gonopore opening at coxae on the third pereopods in females and fifth pereopods in males.

Synalpheus shrimps are a particular group of the Infraorder Caridea since all species do not present masculina appendix associated with pleopods, and the gonopore morphology is very tiny and delicate and almost impossible to visualize under Light Microscopy. Besides that, males and females reach similar sizes making not possible to separate sexes according to the body size, and sexual dimorphism is incompletely understood, and miss described for many species most in not eusocial group (Coutière, 1909; Ríos and Duffy, 2007).

The gonads formation (first sexual characteristics) associated with gonopore morphology are also a technique to identify males and females. However, the ejaculatory ducts are not well developed in males of *Synalpheus'* shrimps as in other caridean shrimps (See results of Chapter 1 and Tóth and Bauer, 2007). When developing a female's gonads, it is much easier to see it into a carapace and abdomen position. Still, when spent gonads, we are almost not able to evaluate for sure the sex of the individual. The different techniques can be applied to sexing animals for the gonopore position and morphology as the much useful Scanning Electron Microcopy (SEM) that is much powerful tool than Light Microscopy (LM) and can provide perfect images to explore the tiniest structure. It is an alternative sexing and describing sexual dimorphism in *Synalpheus* shrimps (Tóth and Bauer, 2007, 2008).

The pleopod morphology is also a structure that is poorly explored on *Synalpheus* descriptions. Besides lacking masculina appendix, most of all *Synalpheus* shrimps present appendix interna (AI) associated on endopod of pleopod from pleopod 2 to pleopod 5 (Dardeau, 1986; Bauer, 2004; Hermoso-Salazar and Hendrickx, 2005), and those structures are proved to be an essential part of the complex changes on the female body to attach embryos on the abdomen. It is part of the "breeding dress" described by Bauer (2004), where females change the body shape to reproduction and breeding period.

The use of characteriscs from pleopods to elucidate sexual dimorphism is completely miss explored in the literature, but it is a potential structure to better understand secondary sexual dimorphism from the *Synalpheus* and other gerera of the Caridea Infraorder.

Synalpheus shrimps are not exclusive into the caridean shrimps with those peculiar characteristics. The Oplophoridae *Janicella* is another genera that species do not present the masculina appendix on pleopods. It is cited that is possible to identify most mature males by the depth of the sinuses in the ventral margin of the pleura of the first and second abdominal somite (Chace, 1986). Some differences into the pleopod morphology in adult specimens were also evidenciated in some species by Cardoso and Young (2005). But those morphologies are just supercially discussed due *Janicella* represent species typicaly found in deep-sea waters, and very little is known and proposed about the biology and morphology of the species. Most works are focused on nothing most than registers and taxonomy feautures.

In other hand, *Synalpheus* spp. are ubiquituous and very well distributed in Atlantic, especially in Brazilian coastal waters, and they can be used as an example to propose morphological features to elucidate question for those particularities in Caridea. In the same way for *Janicella* spp., some works discuss a possible changes on morphology of

the first abdominal pleura in *Synalpheus*, and few studies discuss about the pleopod's designe (Banner and Banner, 1975; Chace, 1986; Dardeau, 1986) .

The present study aims to define a useful and evident character between sex from *Synalpheus* genera. We used *Synalpheus fritzmuelleri* Coutière, 1909, *Synalpheus apioceros* Coutière, 1909, and *Synalpheus brevicarpus* (Herrick, 1891) as parameter. They are non-gambarelloides species widespread and diverse in Brazilian waters and present different morphology patterns inside the same genera, as the absence of the appendix interna in some species.

Material and Methods

Specimens used in the present work were sampled from two continental island of the São Paulo state, Brazil: the Couves island, 23º25'15"S, 44º51'39"W; and the Alcatrazes Archipelago, 24°06′03″S, 45°41′25″W (license number SISBIO 69588-4/2020), using both sample methods: Artificial Refuge Substrate, as described in (Moraes *et al.*, 2021) and by SCUBA diving active search looking for animals associated with the coral *Madracis decactis* (Lyman, 1859).

Pleopods, appendix interna (and the *cincinnulis* structures), as well as gonopore opening, were evaluated from: SEM photographs, light microscopy features, and classical morphometry perspectives.

Pleopod and appendix Morphology

We analyzed specimens from each demographic category (males = M, females = F, ovigerous females = OF) to make the pleopod and appendix descriptions of *S. apioceros*, *S. fritzmuelleri* and *S. brevicarpus*.

In each specimen, we dissected all pleopod (from pleopod 1 = PL l to pleopod 5 = PL 5) from the abdomen region. We disposed then in a slide to photograph using light microscopy (Axioshop 2 fitted with a Zeiss Stemi 2000-C image capture system). Then measured Pleopod Length = PL (from basis to apice of endopod) and Distance to Appendix Interna = AI (from pleopod basis to basis of the AI). We evaluated the position of the AI in relation of the PL, looking for differences in position between males and females. Analyzes were made evaluating the "reason" between PL length and distance to AI, calculated as "AI = a/b", where "a" is the distance to AI and "b" the PL length. When lower values of AI, closest to the basis of endopod the appendix interna is, when hihgher values, more distant from basis and closer to apice they emerge

We also analyzed the seta's morphology associated in each pleopod to evaluate differences between sexes and determine "ovigerous setae" in ovigerous females. Ilustrations of the pleopods and appendix were made using a microscope (Leica DM750) equipped with a camera lucida, and they were vectorized to better understanding of all structures.

Scanning Electron Microscopy – Gonopore Morphology

The entire carapace was used and all the pereopods dissected excepting the coxa region, once it is the known position for the gonopores. Samples were transferred to a solution of Karnovsky fixative (2.5% glutaraldehyde and 2% paraformaldehyde) in 0.1 mol L^{-1} sodium cacodylate buffer (pH 7.4) for at least 24 h, washed three times in the same buffer - 10 minutes wash, dehydrated in 15 minutes ethanol series 3x (30% 50%, 70%, 80% 90% 95%, 100%). Then hexamethyldisilazane HMDS 1:1 20min and finally HMDS 100%. The HMDS is a powerful solution that dries the material very quickly, so one drop of the solution was added with content that emerged on a watchmaker's plate and constant

monitoring were made to see if carapace was entirely dried to prepare stubs. Samples were attached with adhesive to round stubs of 25 mm in diameter for sputter coating with 5 nm gold in a metallizer Supputering SC 070 Belzer Union before observation in a Scanning Electron Microscopy (Zeiss® Evo 10), operating with voltages ranging from10-20 kV.

Results

Morphology of the pleopods

Synalpheus apioceros

Males: The size and setae disposition of the first pleopod is different from the other four pairs. The endopod is 1/3 bigger than the exopod (length = 0.30 ± 0.05 mm). Setae are present in all extensions of the exopod and especially at the distal portion of the endopod. From the second to fifth pairs of pleopods was observed an appendix Interna (AI) associated with the endopod. The morphology of all other pleopods (PL2 to PL5) is similar, little distinguished by the length (Figure 1). The fifth pair of pleopods seems to present as the smallest one, being PL2 to PL4 length of the endopod about 0.82/0.84 mm extension and 0.77 mm in the PL5 (Table 1). The AI of all pleopods present *cincinnulis* at the distal portion in all pleopods (close view of the *cincinnulis*) (Figure 12E, 12F and 12G).

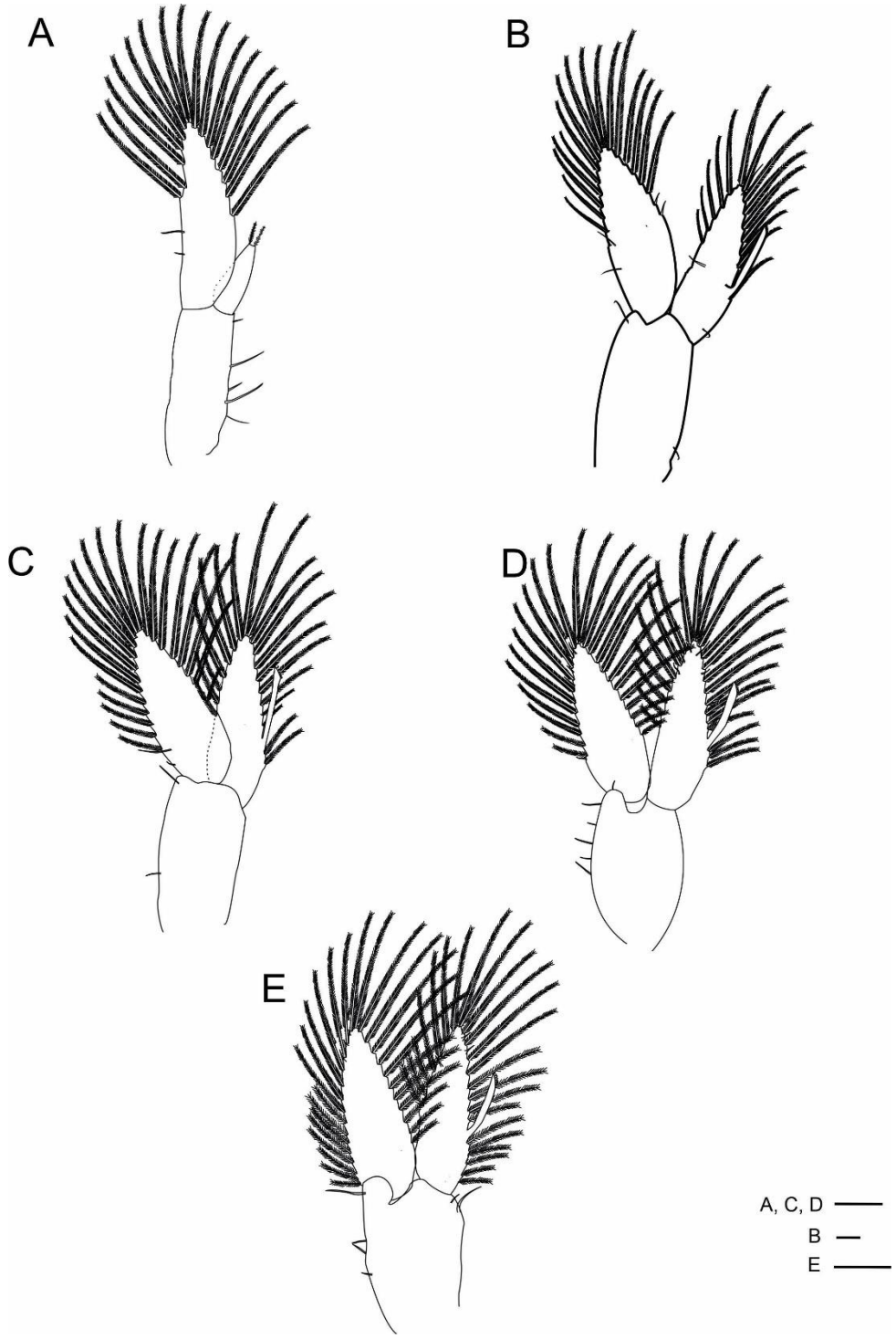

Figure 1: Pleopods from *Synalpheus apioceros* males: A) the first pleopod with exopod bigger than endopod; B) the Second pleopod with an appendix interna on the endopod, setae concentrate on the middle to distal portion of the endopod and exopod; C) the Third pleopod with an appendix interna on the endopod, setae on the middle to distal portion of the endopod and exopod; D) the Fourth pleopod with an appendix interna on the endopod, setae concentrates on the middle to distal portion of the endopod and exopod; E) Fifth pleopod, the smallest one, with an appendix interna on the endopod, setae presented on the entire face of the endopod and exopod. Scale bar 1mm.

Females and Ovigerous Females: The first pleopod, as in males, is different from the other pleopods, but the endopod is bigger compared to the males (0.54 ± 0.20 mm in females and 1.58±0.81 mm). Just as in males, the AI is present from the second to fifth pleopod, associated with the endopod. Pleopods in females are bigger than males, to form an incubatory chamber for embryos. PL2 to PL5 is similar in morphology and in the same way as in males, little different in length. The fifth pleopod remains the smallest one (Table 1). Even if the female is not carrying embryos at the abdomen, we found a clear difference from males' pleopods morphology as the setae is longer and the amount in different being numerous than females in both internal and external margin of the pleopod and the position of the appendix interna, *i.e*, more apical than males. Also, appendix interna is much bigger in this category than in males (Table 1). These features make possible comparisons between sexes (Figures 2 and 3).

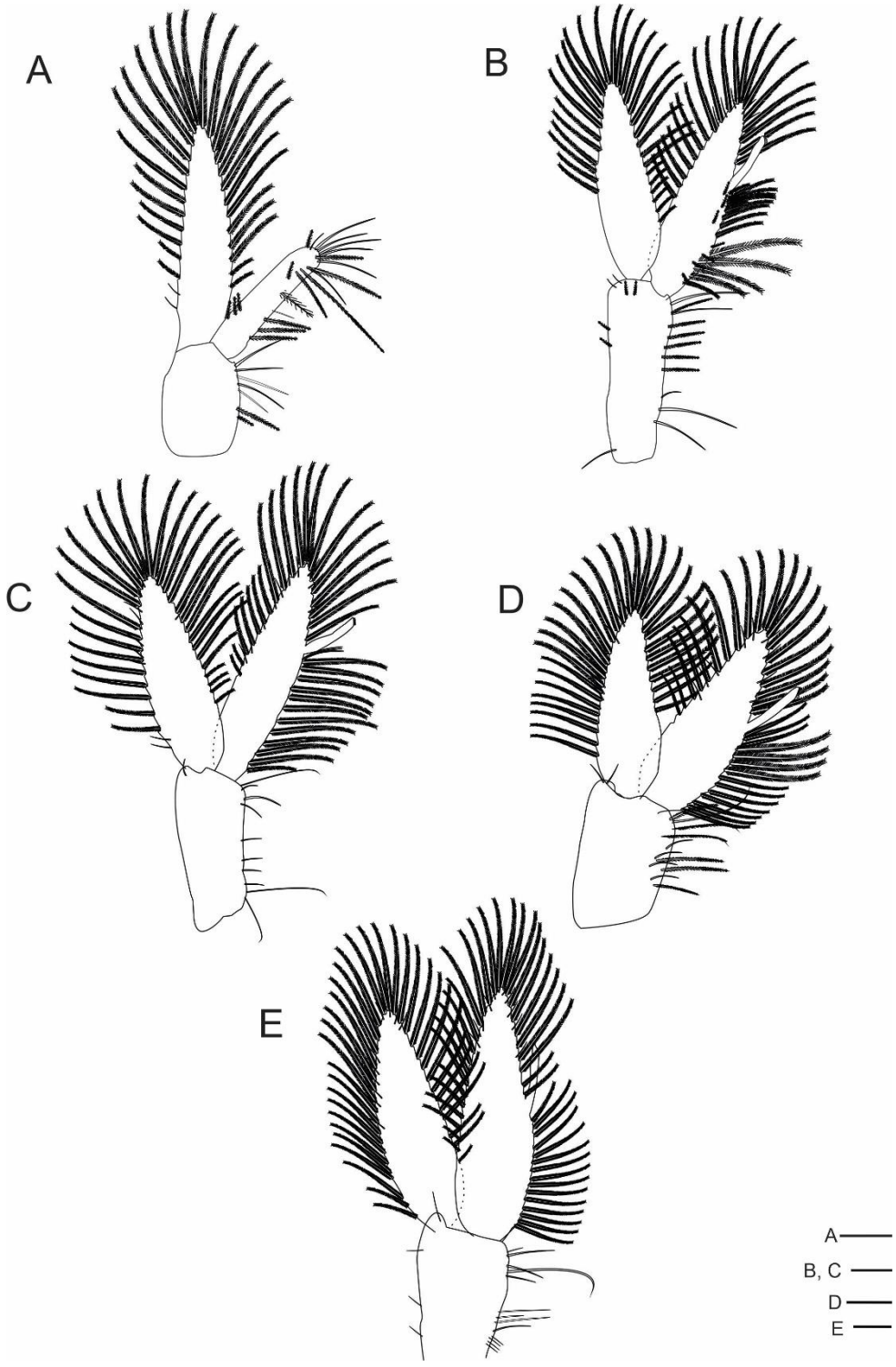

Figure 2: Pleopods from *Synalpheus apioceros* females: A) the first pleopod with exopod bigger than endopod; B) the Second pleopod with an appendix interna on the endopod, C) the Third pleopod with an appendix interna on the endopod; D) the Fourth pleopod with an appendix interna on the endopod; E) Fifth pleopod, the smallest one, with an appendix interna on the endopod. Scale bar 1mm.

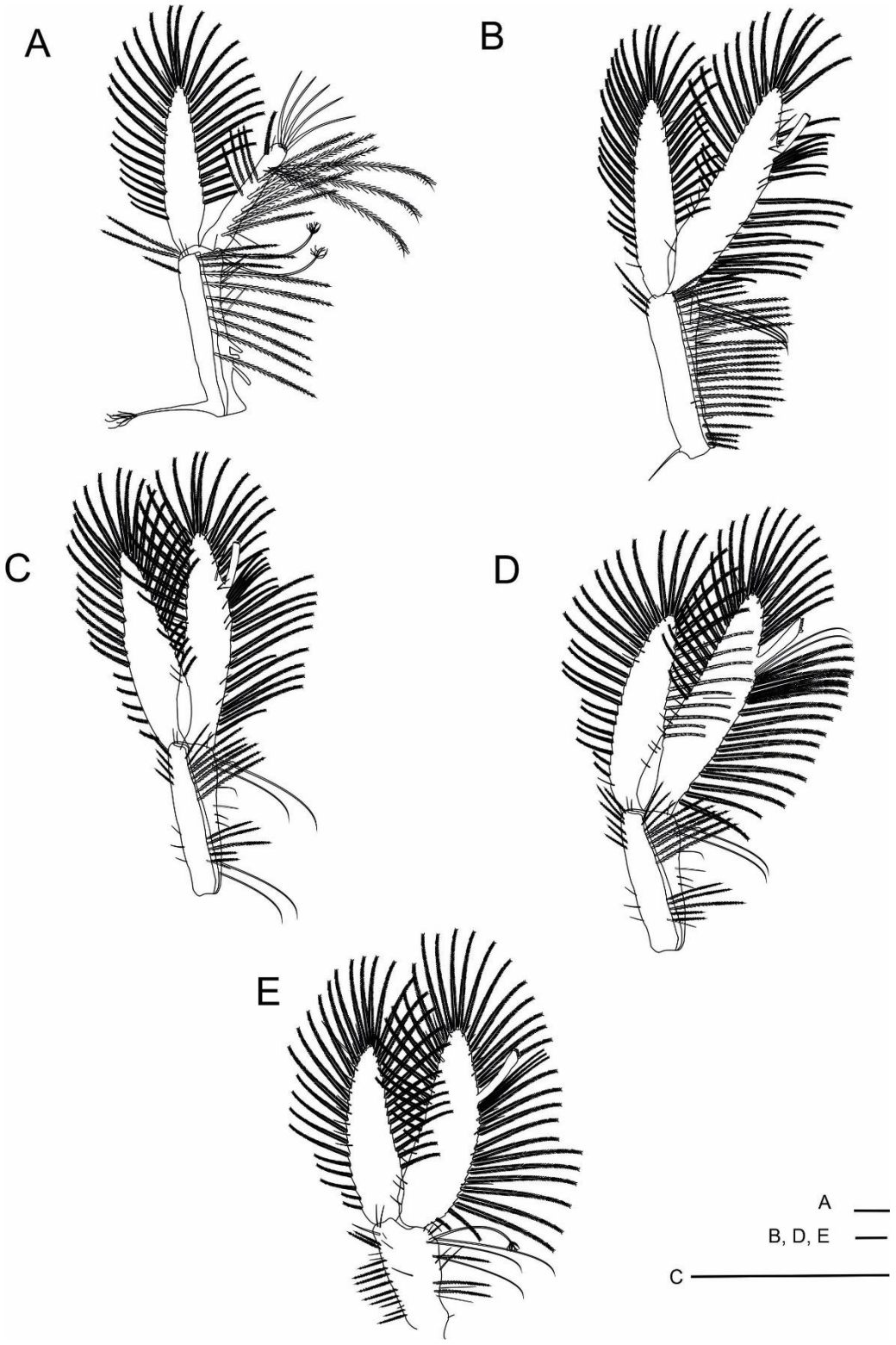

Figure 3: Pleopods from *Synalpheus apioceros* ovigerous females: A) the first pleopod with exopod bigger than endopod; B) the Second pleopod with an appendix interna on the endopod, C) the Third pleopod with an appendix interna on the endopod; D) the Fourth pleopod with an appendix interna on the endopod; E) Fifth pleopod, the smallest one, with an appendix interna on the endopod. Scale bar 1mm.

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200 *Synalpheus fritzmuelleri*

201 Males: Just as in *S. apioceros*, the measure in pleopods are different in males, females,
202 and ovigerous females. However, we cannot find in *S. fritzmuelleri* appendix interna in
203 any endopod from the second until the fifth pleopod. In the same way that the congeneres
204 species described in this study, the first pair of pleopods are different from the other ones
205 (measure mean 0.43±0.15 mm) (Table 1). Once setae are found even in exopod as
206 endopod but are more prominent and numerous at the final portion of the endopod. Setae
207 can be found in both the internal and external margin of the other four pairs, but they are
208 specially presented at PL3 and PL4 (Figure 4).

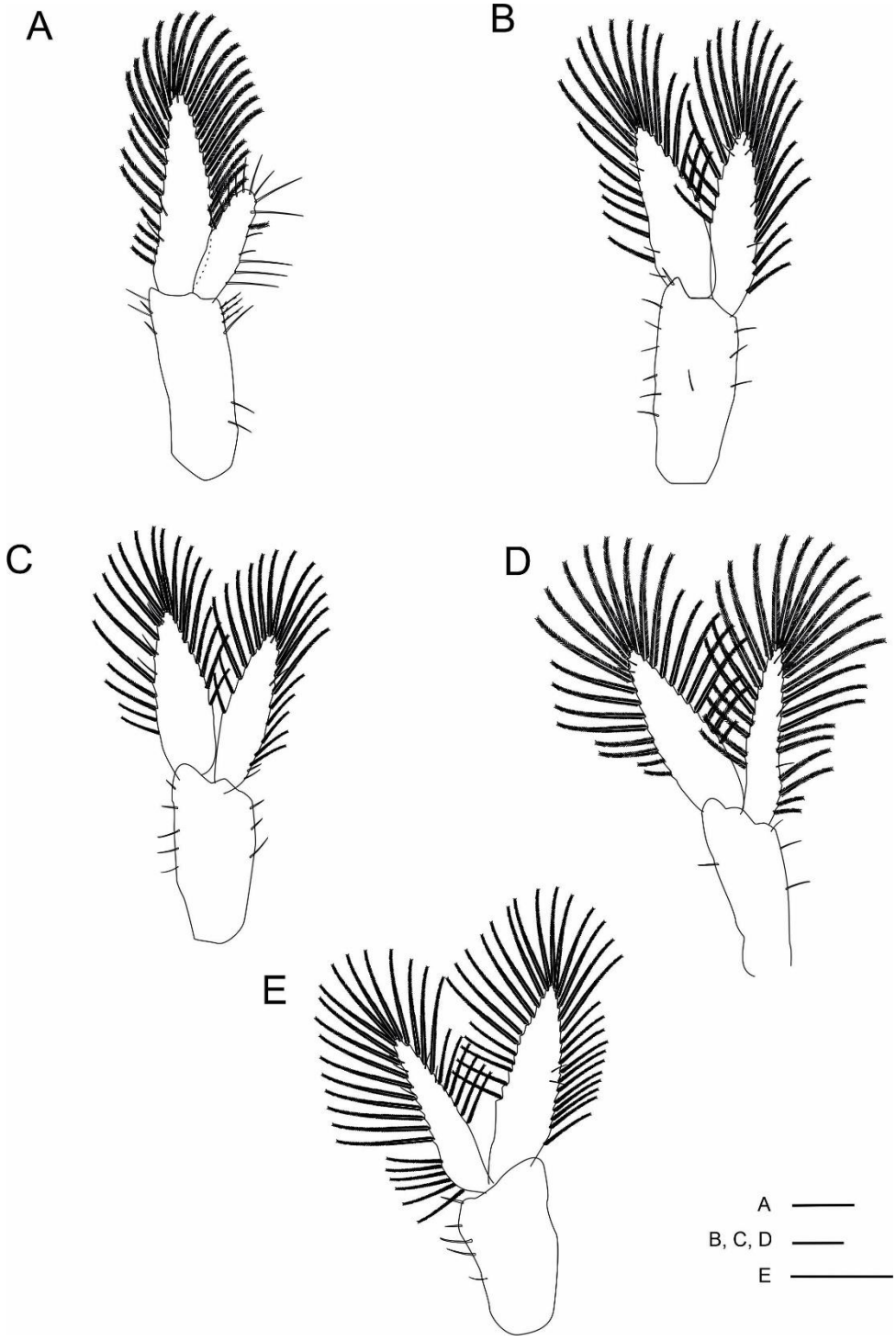

Figure 4: Pleopods from *Synalpheus fritzmuelleri* males: A) the first pleopod with exopod bigger than endopod; B) the Second pleopod without appendix interna on the endopod; C) the Third pleopod without appendix interna on the endopod; D) the Fourth pleopod without appendix interna on the endopod; E) Fifth pleopod, the smallest one, without appendix interna on the endopod. Scale bar 1mm.

Females and Ovigerous female: It is essential to highlight the morphology of females and ovigerous females in *S. fritzmuelleri* specimens, mainly because they present the appendix interna associated with PL2 to PL5. This aspect is a clear evidence for sexual dimorphism helps to identify the sex in the absence of the masculina appendix and due to the difficulty of finding the gonopores using just light microscopy. Beyond the appendix interna, other characteristics are very remarkable on ovigerous female's pleopod morphology, which can help to create a capable incubatory chamber and differ from other males. The first pair of pleopods are different from males, with endopod and exopod almost of the same size (endopod length 1.47±0.22 mm in ovigerous females) changing just in width (Table 1). The presence of an ovigerous seta in protopodite is remarkable. Setae are present in all extensions of pleopods, especially in two of five ones. Appendix interna is present at the 3/4 from the margin of the pleopod. Pleopods are more prominent than males, and the fifth one is also the smallest (Table 1). The incubatory chamber is effective in joining all these different aspects of the pleopods, *e.g.*, bigger exopod and endopod; the presence of appendix interna with *cincinnulis.* First pair of pleopod without an endopod reduced; modified setae presented in almost all pleopods. Despite a tiny species, *S. fritzmuelleri* and other *Synalpheus* shrimps developed great strategies to guarantee the development of their embryos (Figures 5 and 6).

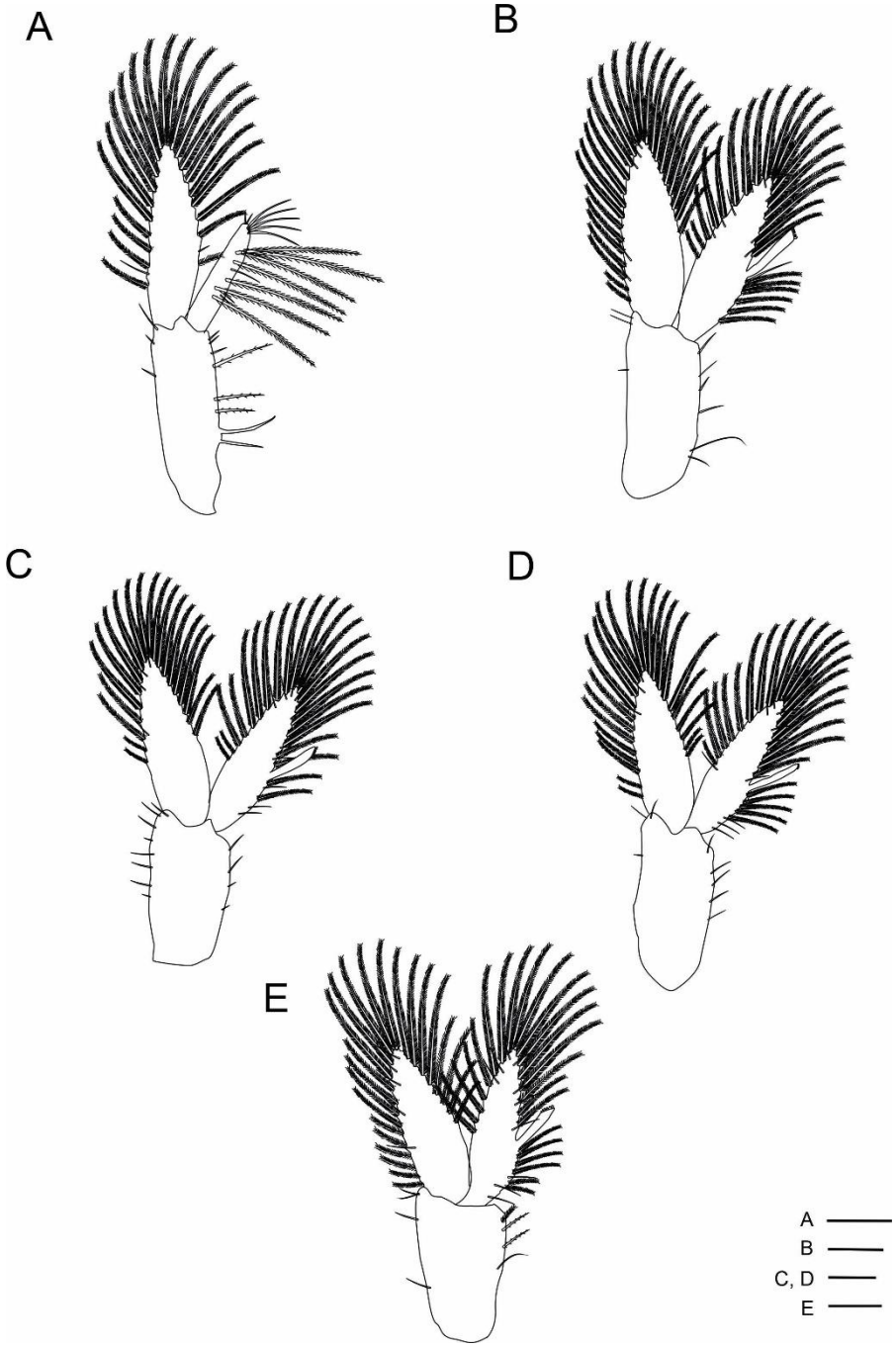

Figure 5: Pleopods from *Synalpheus fritzmuelleri* females: A) the first pleopod with exopod as much bigger as endopod; A) the Second pleopod with an appendix interna on the endopod; C) the Third pleopod with an appendix interna on the endopod; D) the Fourth pleopod with an appendix interna on the endopod; E) Fifth pleopod, the smallest one, with an appendix interna on the endopod. Scale bar 1mm.

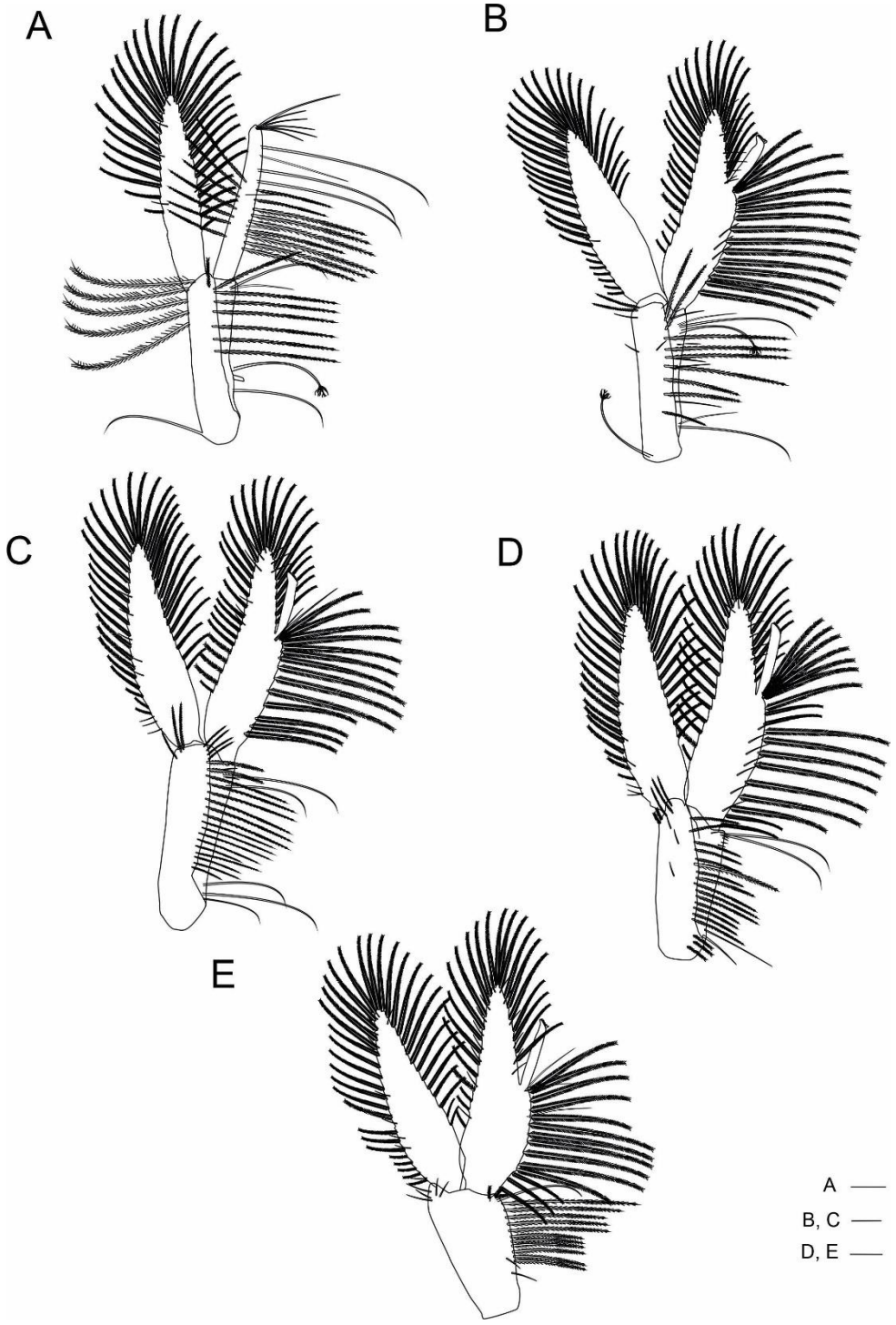

Figure 6: Pleopods from *Synalpheus fritzmuelleri* ovigerous females: A) the first pleopod with exopod as much bigger as endopod; A) the Second pleopod with an appendix interna on the endopod; C) the Third pleopod with an appendix interna on the endopod; D) the Fourth pleopod with an appendix interna on the endopod; E) Fifth pleopod, the smallest one, with an appendix interna on the endopod. Scale bar 1mm.

Synalpheus brevicarpus

Males: Comparing with the other species, *S. brevicarpus* shows the highest mean values in pleopods length, in all categories, but the shape of the structures is basically the same. The first pair of endopod is considering much smaller than others and without any kind of appendix associated. Endopods PL2 to PL5 remain same morphology, with a few simple setaes rounding all structure, and a prominent appendix interna adhered. The endopods 2 to 4 are longer than endopod 5, with mean about 1.01/1.02 mm and 0.9 mm, respectively (Table 1). All appendix interna are provided by *cincinullis* in the same way of other species described (Figure 7).

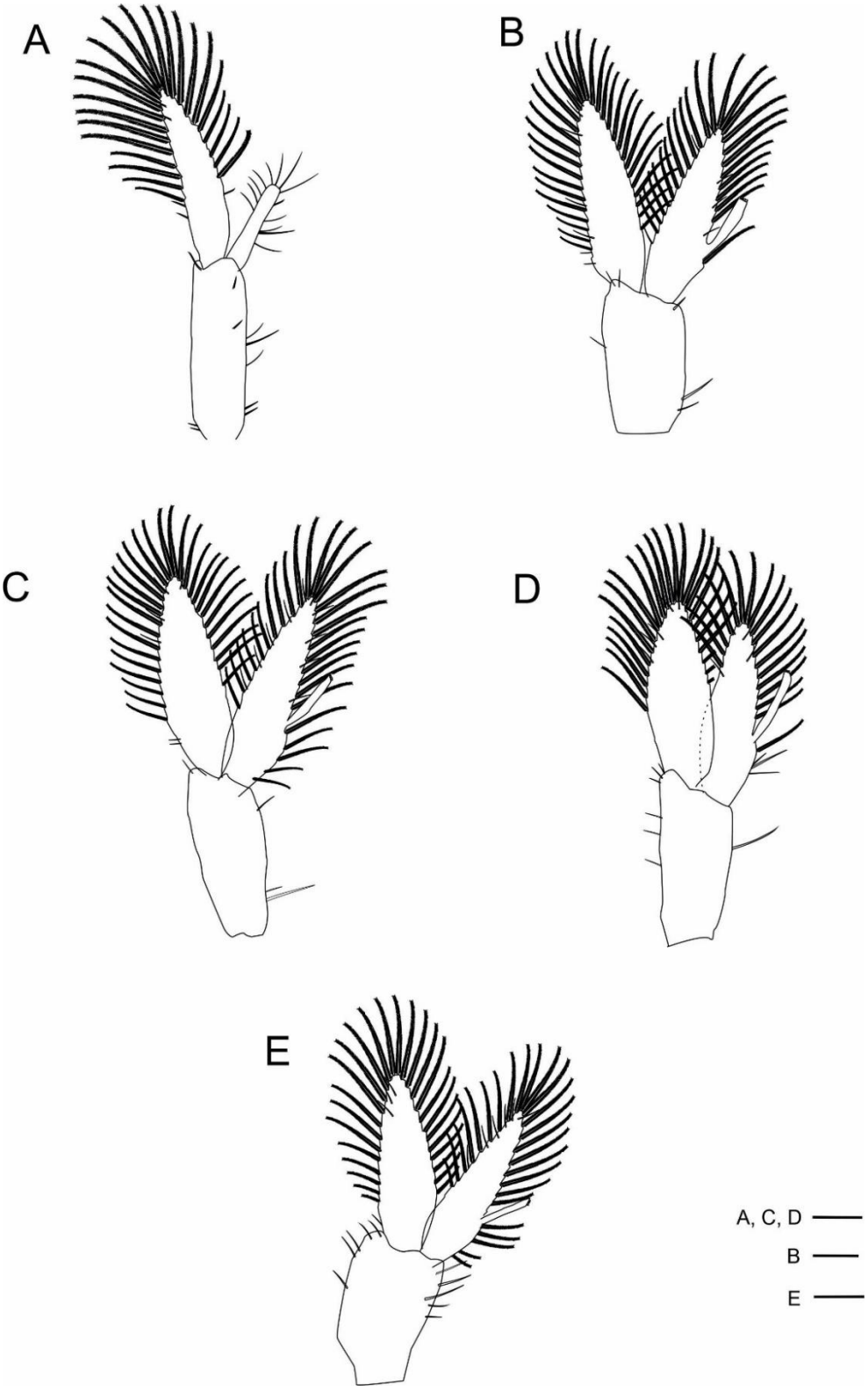

Figure 7: Pleopods from *Synalpheus brevicarpus* males: A) the first pleopod with exopod as much bigger as endopod; A) the Second pleopod with an appendix interna on the endopod; C) the Third pleopod with an appendix interna on the endopod; D) the Fourth pleopod with an appendix interna on the endopod; E) Fifth pleopod, the smallest one, with an appendix interna on the endopod. Scale bar 1mm.

Females and ovigerous females: The endopod of the first pair of pleopods remains as the smaller one as in males, despite it is bigger comparing with males in the females and ovigerous females. The endopd length of the pleopod 2 to 5 are remarkable bigger than in males, reaching more than 1 mm of size (Table 1). The endopod 5 is the smaller one. "Ovigerous setae" are present in all extension of all pleopods, much more prominent and more complex than males. Appendix interna are present and provided *cincinullis*. The length of the appendix interna also increases as the female turns to be able to reproduce and in the spawning period (Table 1, Figure 8).

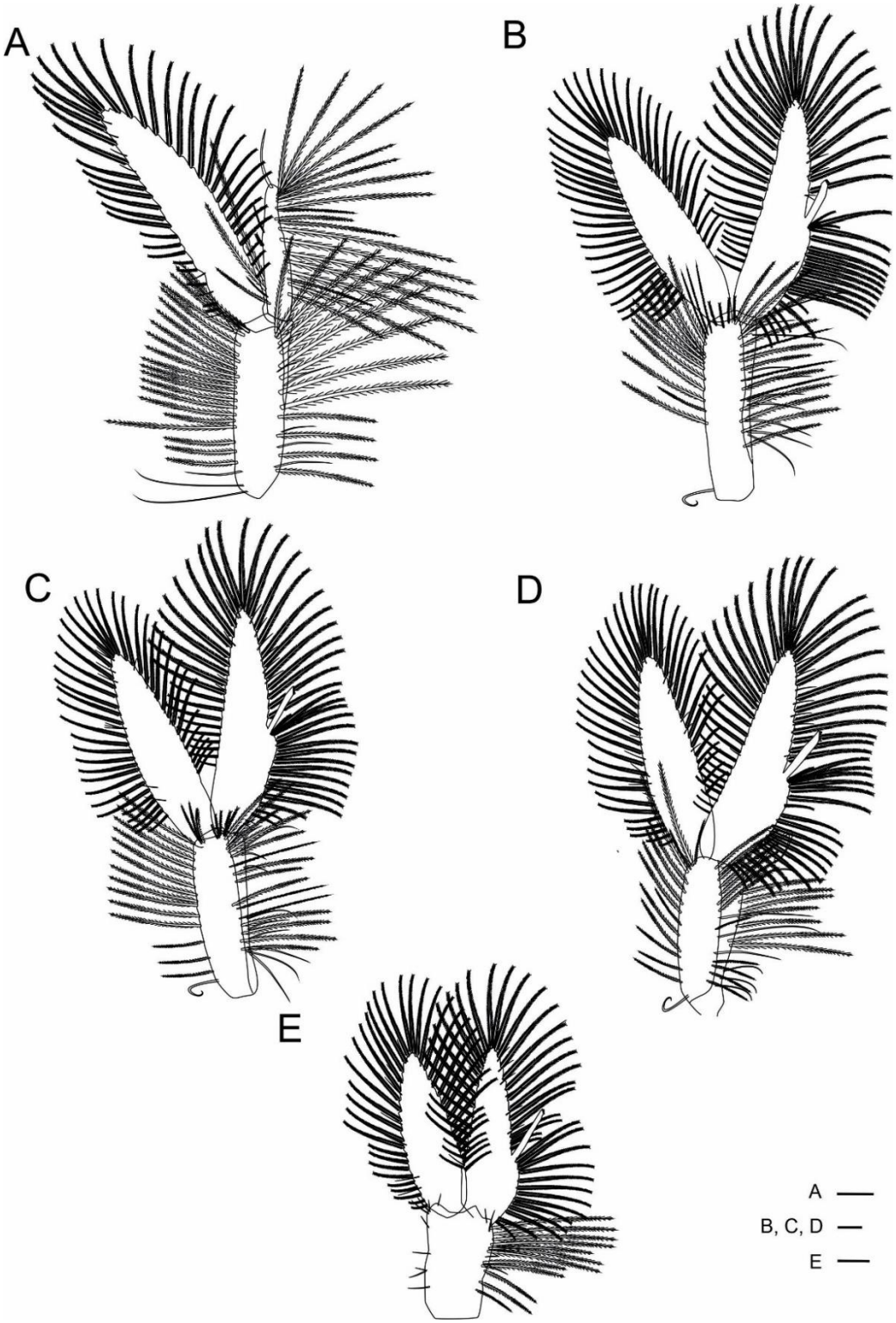

Figure 8: Pleopods from *Synalpheus brevicarpus* ovigerous females: A) the first pleopod with exopod as much bigger as endopod; A) the Second pleopod with an appendix interna on the endopod; C) the Third pleopod with an appendix interna on the endopod; D) the Fourth pleopod with an appendix interna on the endopod; E) Fifth pleopod, the smallest one, with an appendix interna on the endopod. Scale bar 1mm

Table 1: Endopod and Appendix interna length, mean± of the three analyzed species: *Synalpheus apioceros*, *Synalpheus fritzmuelleri* and *Synalpheus brevicarpus*. N= Number of specimens evaluated. Missing values on appendinx interna length of *S. fritzmuelleri* is due the absence of the structure on this species. PL= Pleopod.

Species		**Endopod Length mm (Mean±SD)**				
	N	**PL1**	**PL2**	**PL3**	**PL4**	**PL5**
Synalpheus apioceros						
Male	10	0.30±0.05	0.83±0.19	0.84±0.17	0.82±0.16	0.77±0.15
Female	8	0.57±0.20	1.06±0.29	1.18±0.26	1.20±0.28	1.06±0.23
Ovigerous Female	4	1.58±0.81	2.22±0.94	1.73±0.09	1.80±0.15	1.59±0.20
Synalpheus fritzmuelleri						
Male	14	0.43±0.15	0.81±0.26	0.86±0.28	0.83±0.26	0.74±0.24
Female	5	0.49±0.11	0.80±0.12	0.83±0.14	0.81±0.12	0.71±0.10
Ovigerous Female	11	1.47±0.22	1.71±0.28	1.81±0.34	1.82±0.36	1.49±0.51
Synalpheus brevicarpus						
Male	13	0.46±0.13	1.02±0.24	1.02±0.27	1.01±0.24	0.9±0.20
Female	5	0.61±0.30	1.31±0.87	1.36±0.89	1.3±0.8	1.15±0.78
Ovigerous Female	7	0.86±0.17	2.07±0.64	2.08±0.59	2.10±0.58	1.84±0.53
Species		**Appendix Interna Length mm (Mean±SD)**				
	N		**Pl2**	**Pl3**	**Pl4**	**Pl5**
Synalpheus apioceros						
Male	10		0.30±0.04	0.32±0.04	0.32±0.06	0.28±0.06
Female	8		0.35±0.02	0.38±0.07	0.38±0.06	0.36±0.06
Ovigerous Female	4		0.6±0.24	0.39±0.08	0.46±0.05	0.43±0.06
Synalpheus fritzmuelleri						
Male	14					
Female	5		0.26±0.04	0.28±0.04	0.28±0.03	0.25±0.04
Ovigerous Female	11		0.42±0.04	0.43±0.06	0.46±0.08	0.40±0.08
Synalpheus brevicarpus						
Male	13		0.31±0.07	0.33±0.09	0.34±0.09	0.32±0.07
Female	5		0.36±0.15	0.39±0.16	0.38±0.19	0.36±0.17
Ovigerous Female	7		0.47±0.14	0.52±0.12	0.53±0.15	0.50±0.11

Appendix Interna Position

Looking at morphological differences between males and females pleopods and the position of the appendix interna (when present) at the endopod of pleopod 2 to 5, we were able to define and clarify this difference among sexes.

The position of the appendix interna can be evaluated in two patterns and used in different evidences of sexual dimorphism. In *S. apioceros*, it is clear the diference observed in the reason by the AI equation proposed. Both female and ovigerous female showed higher values of AI than males, demonstrating that interna appendix releases in the most apical region of the endopod, where it is more evident in "ovigerous female" status, and it is clearly a dimorphism proposed for this species (Table 2).

For *Synalpheus fritzmuelleri,* the sexual dimorphism is even more evident using pleopod morphology. None male analyzed presented interna appendix associated at the endopod. All species that was showing this structure had the first pleura rounded and gonopore located at the third pair of pereopods. Comparing non-ovigerous with ovigerous females, the same pattern from *S. apioceros* was observed, with a clear elevation of the local where appendix interna releases. Proving that this is a functional structure to be used at the spawning period (Table 2).

Finally, for the *S. brevicarpus*, this relation between position of the appendix interna is the most subtle to notice when looking for mean size in all pleopods, it is the species that present less differences between those values (Table 2), but still, the same premises can be adopted, despite appendix interna do not heach in the very final portion of the pleopod in ovigerous females. Comparing with the other two species this is the one that presents bigger sizes of pleopods than others and it may be the reason for the lowest diference of the migration of the pleopod (Table 1).

Table 2: Values of the reason calculation for the AI (position of the appendix interna related to the endopod length). Higher and lower values indicate more apical and more basal position rising of the appendix on pleopod respectively.

Species	**AI = a/b**			
Synalpheus apioceros	PL2	PL3	PL4	PL5
Male	0.38±0.13	0.42±0.02	0.39±0.04	0.41±0.04
Female	0.56±0.11	0.64±0.14	0.58±0.11	0.55±0.11
Ovigerous female	0.74±0.03	0.74±0.03	0.72±0.04	0.66±0.07
Synalpheus fritzmuelleri				
Male				
Female	0.49±0.04	0.47±0.06	0.44±0.04	0.43±0.02
Ovigerous female	0.66±0.01	0.65±0.04	0.64±0.03	0.55±0.03
Synalpheus brevicarpus				
Male	0.41±0.01	0.40±0.01	0.39±0.02	0.39±0.04
Female	0.43±0.03	0.42±0.05	0.44±0.04	0.41±0.03
Ovigerous female	0.50±0.03	0.48±0.03	0.47±0.03	0.43±0.02

Comparing both species that present appendix interna in all categories (*S. apioceros* and *S. brevicarpus*), we used the pattern for the second pleopod position to propose a visual graphic comparison. Results of the AI position differences between sexes can be statistically distinguished Kruskall-Wallis (chi-squared=14.36; df=2; p= 0.00076). According to Dunn test, *S. apioceros* showed differences between categories male *versus* female (F=2.77, p=0.002), male *versus* ovigerous females (F=-3.30; p=0.0005), and similarities between females *versus* ovigerous female (F=-0.93; p=0.175) (Figure 9). In *S. brevicarpus* despite mean values are differents, statistical tests pointed well separated groups only in two categories - Dunn test: male *versus* female (F=0.95; p=0.17), male *versus* ovigerous females (F=-3.77;p=0.0001), females *versus* ovigerous females (F=-2.099; p=0.017) - being males and females very close each other. These patterns can be well observed into boxplot graphs (Figure 10).

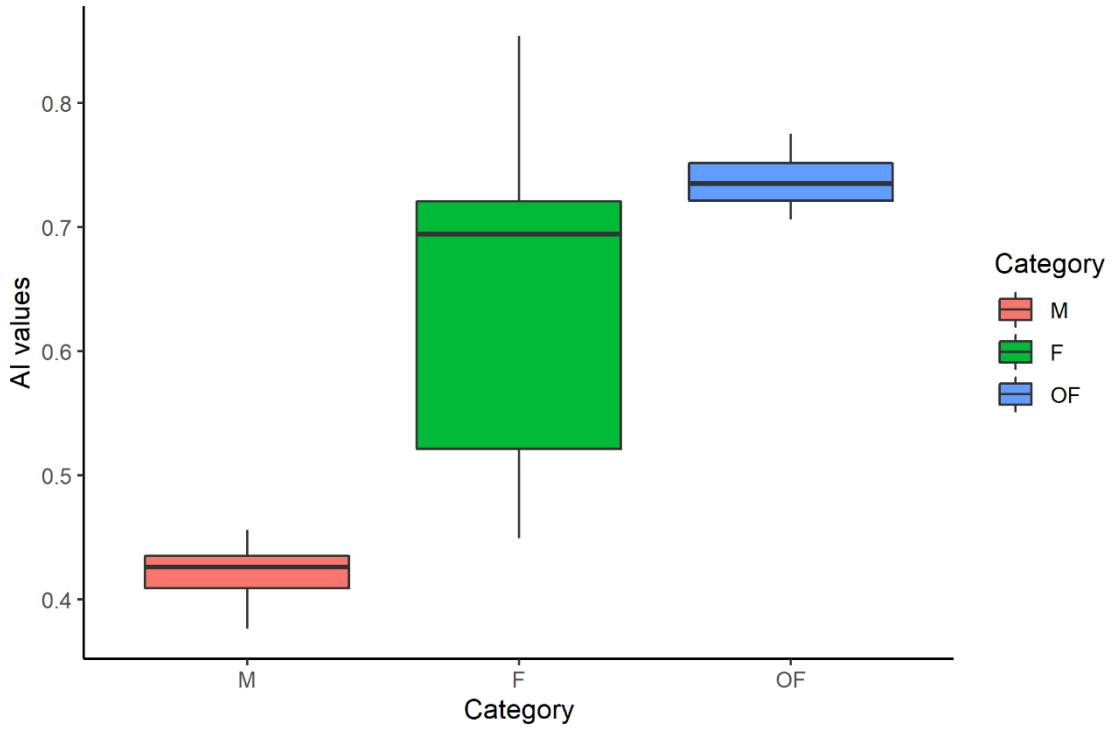

Figure 9: Boxplot representation of the values presented in the reason AI in *Synalpheus apioceros*. Discrepant lower values in males comparing with females confirm hypothesis to uses the appendix interna position as sexual dimorphism in this species.

In *S. brevicarpus* general values are relatively low due the fact that in any categories the appendix reaches the final portion of the pleopod (Figure 10), that is much more evident in *S. apioceros.* In both species it is possible to see differences in mean values that support our premises for uses these morphologies as one of the parameters to confirm sexual dimorphism in those *Synalpheus* species.

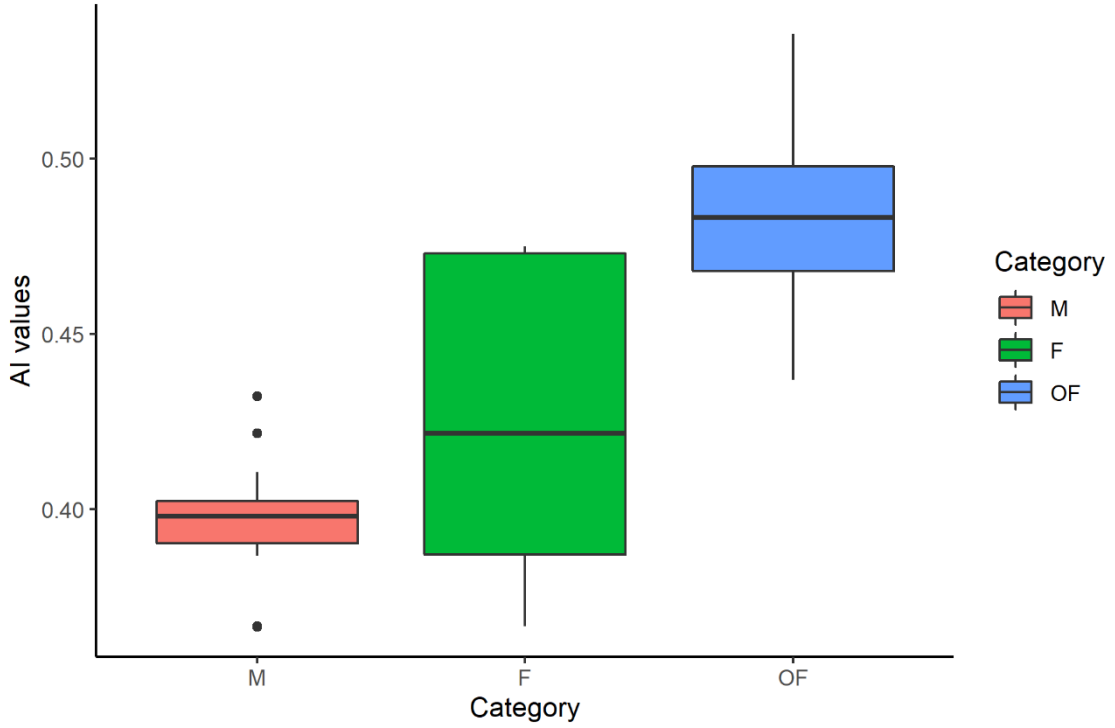

Figure 10: Boxplot representation of the values presented in the reason AI in *Synalpheus brevicarpus*. Mean values on males and females are more discreet than *S. apioceros*, but also indicates an evidence of sexual dimorphism on appendix interna position in this species.

First pleura and gonopores morphology

The morphology of the first pleura was very conclusive, matching with the morphology of the gonopore opening at the basis of the coxae of third or fifth pereopods of the specimens. There are three different patterns of the pleura for *Synalpheus* non-gambarelloides spp. (Figure 11):

Male-form pleura: Consist of the pleura with anterior margin rounded and posterior basal margin forming a prominent projection like an extension of the original morphology. Animals presenting this morphology presented gonopore opening at the basis of the fifth pereopod, as looked at SEM images – confirming the "male" *status* of the animal (Figure 11A).

Female-form pleura: Specific more visible at the ovigerous female, consist at the anterior margin rounded as the male form, but with posterior basal margin exceptionally "rounded" without any projection. Animals presenting this morphology presented gonopore opening at the basis of the third pereopods. Specifically, all females carrying embryos attached to the pleopods (ovigerous females) showed more rounded morphology – confirming the "female" *status* of the animal (Figure 11B).

Intermediate-form pleura: This is the most "confusing" morphology with anterior margin looking at the same as the first two described, but the basis of posterior margin without any projection but also without rounded morphology. It almost formed a straight angle. Animals presenting this morphology were also identified as females looking through SEM images and gonopores showing at the basis of the third pereopods. But none of them were analyzed for ovigerous females, so, the intermediate form is characteristic of "non-ovigerous" female (figure 11B).

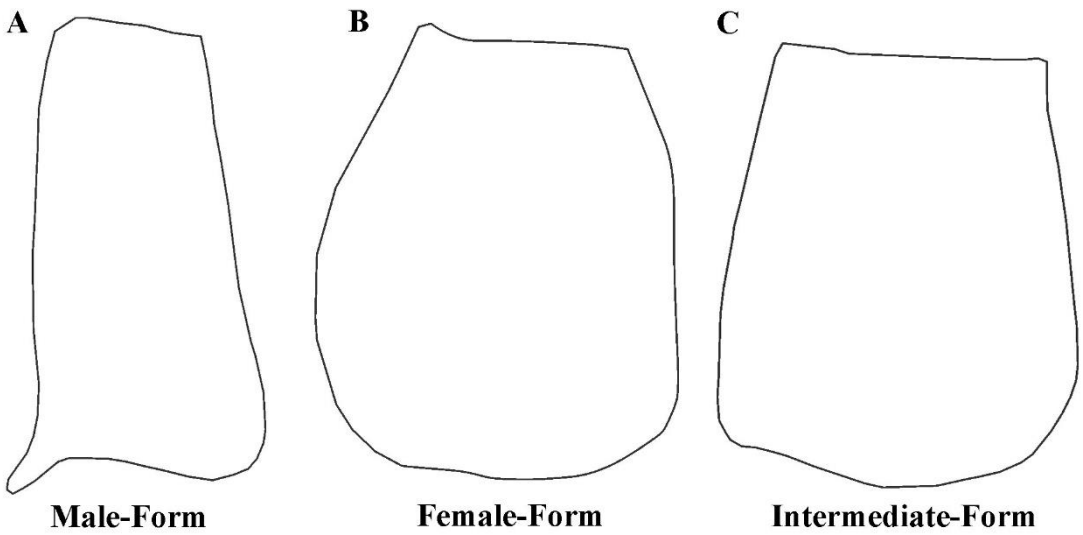

Figure 11: First pleura morphology for all *Synalpheus* shrimps analyzed at the present study. A) Male-Form pleura: anterior region rounded, the posterior part with a visible projection forming a pointed hook extremely different from anterior portion; B) Female-Form pleura: Founded at most in ovigerous or post-spawning females with both anterior and posterior margin extremally rounded; C) Intermediate-Form pleura: Anterior margin rounded and posterior margin forming almost a right angle, without a projection like male-form or turned like female-form. Every specimen with this shape form was considered female.

Despite confirming the opening region and corroborating the sexual dimorphism of *Synalpheus* non-gambarelloides spp., we can observe many differences between male and female gonopores morphology (Figure 12):

Male-gonopore: it is presented as just like a simple "pore" with no more than 20 µm width at the mesial region of the coxae of the fifth pereopod. It is essential to note the proximity of the coxae on both p5, and both gonopores are very close, almost touching each other. It matches the morphology of the primary sexual system described previously with the very central opening of the vas deferens (figure 12A and 12B).

Female-gonopore: it is a much more complex opening morphology. We believe that its forms an "open-close" structure that works at the moment when females are releasing their ovulation product. Both animals with "female-form pleura" or "intermediate-form pleura" presented the same morphology on gonopores (figure 12C and 12D).

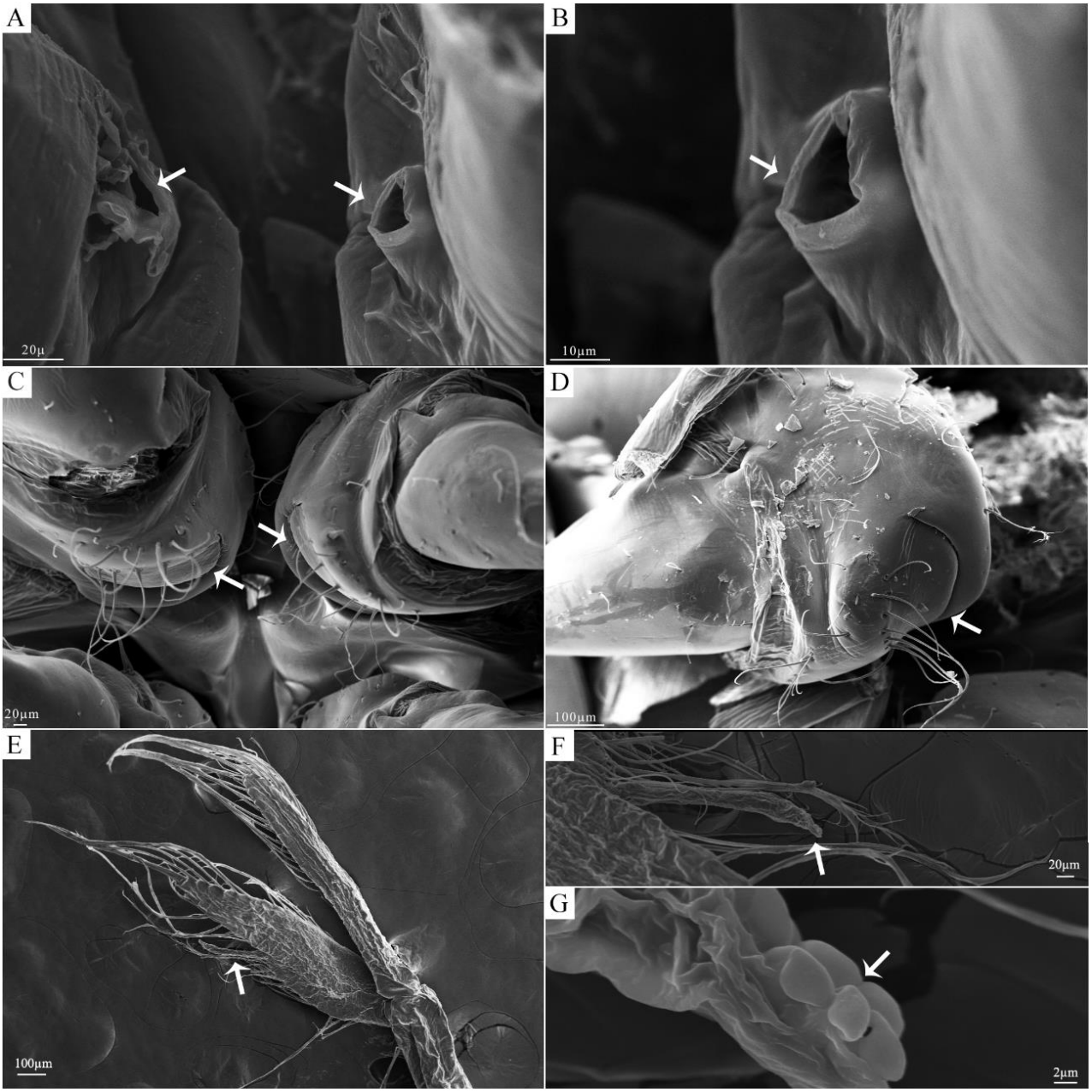

Figure 12: Gonopore and pleopod morphology of *Synalpheus* shrimps under Scanning Electron Microscopy, using *S. fritzmuelleri* as example in representation: A) Gonopore morphology of males opening at the coxae of the fifth pereopods. Arrows points to the simple pore with simple edge. B) Zoom view of the gonopore opening of males and detail of the simple structure wall. C) Gonopore morphology of females opening at the third pereopods. Arrows points to the more elaborated structure of the pore. D) Zoom view of the gonopore opening of females, arrows points to the "open-close" morphology. Egg guides surrounds the pore. E) Second pleopod morphology provided of the appendix interna rising of the endopod. Arrow points to the appendix interna location. F) Detail of the appendix interna structure. Arrow points to the *cincunullils* region. G) Detail of the *Cincinullis* morphology at the apice of the appendix interna. Arrow points to one of the "velcro' sutructures of the region.

Discussion

We provide the first panorama of sexual dimorphism between male and female of *Synalpheus* shrimps, using different techniques. This group is unknown in terms of shapes and structures morphology. The first pleura is one of the remarkable characters to separate males and females with a noticeable change on posterior margin with a projection or rounded morphology, but it is poorly explored on literature. Our results uses combinated characteristics to be used as reference in future works. Male structures are much simpler than females, as pleopods with less and simpler setae and gonopores with just a simple opening. Structures on females and ovigerous females are much more complex since more setae and appendix position to more elaborated gonopore opening morphology. Those results remain biological features because females tend to invest energy in diferent ways as males, like the increase in body changes to guarantee succsesfull reproduction (Bauer, 2004).

For the classical secondary sexual characteristics proposed by Bauer (2004), and almost inexistent or hard to see in *Synalpheus* we can then suggest other morphologies in sexual dimorphism, as the formation of all pleopods pairs and morphology and position of the appendix interna, present at most species of the *Synalpheus* analyzed, so analyzing the combined structures would be the best way for a simple and fast sex discrimination.

The first pair of pleopods in males is much different from the other four pairs. In many caridean shrimps, the first pleopods provide an appendix interna with *cincinnuli* (Bauer, 2004; Baeza, 2018), in which did not occur in *Synalpheus*. The first pleopod is a simple form with exopod much bigger than the endopod. The appendix interna (AI) is also an arbitrary condition that is sometimes present and sometimes absent in many *Synalpheus*, especially in males. Many species just present AI associates from PL2-PL5 in females or ovigerous females, as in *S. fritzmuelleri* in the present study (Dardeau,

1986), and some species of the *S. townsendi* complex (Salazar *et al.*, 2005), but this structures were never been prooved as a sexual dimorphism parameter before.

In males, when present, the insertion of the appendix occurs at almost midpoint of the endopod of PL2-PL5, in lower values at the proposed "AI equation" (lower values calculate more proximity with endopod basis). It had been reported at least two times in other species the appendix interna different position as in *Synalpheus wickstenae* (Hermoso-Salazar and Hendrickx, 2005) that had AI inserted at 0.6 the length of the endopod in ovigerous females *vs.* 0.4 in the males. In *S. williamsi* that had appendix interna inserted at the midpoint in males and apical position on ovigerous female (Ríos and Duffy, 1999).

For the non-ovigerous female, we can see evident differences between the position of the AI in PL2-PL5, as demonstrated in *S. apioceros* in the present study, that all females showed higher values of AI appearing on more apical zone of all pleopods in all females analyzed. Those females presented rounded or intermediate pleura, with or without any embryos attached on pleopod, that corroborates our propose to use the position of AI as a reference to differentiate males from females, when the last do not present embryos attached on pleopods or developed gonads on carapace or even presented an intermediate 1st pleura.

The ovigerous female shows the most particular formation of the pleopods, both in morphology, setae, and appendix interna position, comparing with other groups. Some authors, such as Bauer (2004) and Baeza (2018), defined those specific changes as the "breeding dress" for ovigerous females to be able to spawn and offspring's take care. It consists of the greater space on the abdomen position, changing the first two abdominal pleura especially, with segments becoming wider and an enlarged area in the abdome. Appendix interna morphology and position is one of the strategies to form an efficient

incubation chamber, being able to expand the morphology of the pleopods, associating with more enlarge space between pleural portions, so, the reproduction can be guaranteed. In addition, the basipodite, especially from PL2 – PL5, grew more extensive with a more considerable length of the entire pleopod, just in the same way provided in the present study (Figures 3, 6 and 8). The endopod stays conspicuously long with many "ovigerous seta" very well exemplified by Baeza (2018), and observed here on the ovigerous female of *S. fritzmuelleri.* Those ovigerous setae play a crucial role in attaching embryos and keep them on an abdominal position.

The density and overall length of "normal" setae also increase in ovigerous females. Still, we can see that in non-ovigerous females, setae are bigger and more numerous than in males. On most caridean shrimps, they lost "breeding dress" conditions in the non-ovigerous female. We can propose for *Synalpheus* shrimps that many characteristics became the same, those setae in all extensions in both endopod and exopod and the position of the AI beyond the midpoint of the pleopod. The ovigerous seta on protopodite is lost just like the enlarged shape of the first and second pleura.

As our results, Martínez and Dupré, 2010 analyzed all pleopods of the Rhynchocinetidae *Rhynchocinetes typus* H. Milne Edwards, 1837 and found the 1st pleopods different in length and morphology, but presenting appendix interna. Pleopods of many *Synalpheus* were never genuinely described before, and the function and real importance of many structures are still poorly discussed. For example, on *R. typus* and many other caridean shrimps, the masculina appendix and appendix interna especially on first and second pair of pleopods are supposed to be an important functional structure of the process to transfer sperm mass of the male to the stern of the female (Dupre and Martinez, 2010). On the other hand, in some hermaphrodite species, as *Lysmata* genus, seems to keep a successful transfer of spermatophores without both first and second

pleopod, as showed in ablation experiments of Zhang and Lin (2004), in which put in doubt the real dependence of those structures on reproductive activities. Many other Palaemonidae species also do not present AI associated with the first pair of pleopods and are capable of reproducing as well as those who have (Holthuis, 1952). Even species without any appendix on males as *S. fritzmuelleri* presents a significant success on reproduction. So, it is a very particular detail that should be studied case-by-case, providing results on behavior and images to find a pattern on the mating process.

In the same way, we can discuss the real utility of the *cincinnuli* formation on the top of AI. Bauer (2004) defined that there is a "velcro" with the goal of fastening objects together, like linking pleopods by AI and making more successful the synchronized movements by pleopods as a pair, *i.e.*, natatory movements and reproductive movements, as the "flip" to transfer spermatozoa mass can be even more useful. It is not clear if this is a primitive or derived condition and has never been compared to the reproductive capacity of specimens naturally with AI and *cincinnuli vs.* those without this structure. What we know so far, looking in both *Synalpheus* shrimps analyzed in the present study, are both in the same way capable of reproducing, and the sperm mass formation is the same as shown in chapter 1. Probably making adaptations on behavior conditions during the reproductive cycle.

For the females and ovigerous females, besides, we have registered the position of AI on pleopods, which is remarkably different from males. Those aspects were just mentioned in taxonomic studies and in only a short descriptive way (see Ríos and Duffy, 1999, 2007; Salazar *et al.*, 2005; Hermoso-Salazar and Hendrickx, 2005). We detailed the firstly description of those structures, and we propose here that it is a unique formation, added to "breeding dress," that makes the incubatory chamber more effective. We believe that AI in a more apical zone creates even more space to accommodate

embryos and allow the brushing behavior to avoid embryo loss (Bauer, 2004). Because we are dealing with a small species with limited space into the abdomen, we can consider that any strategy to guarantee that eggs hatch into larva is valid, so we propose that this morphology, accurately described for *Synalpheus* shrimps so far is part of an adaptation process to reproductive success.

The gonopore morphology complements the secondary sexual characteristics between males and females. This feature had only been reported in *Synalpheus* gambarelloides. Males and females both in gambarelloides and non-gambarelloideses groups show a similar body size, and those small features seems to be important to determine the sexual dimorphism. It has already been reported that SEM is an excellent method to evaluate de gonopore morphology. It is a quick method and can analyze many individuals in one time. For the *Synalpheus*, it was applied to differentiate workers, queens, nom-reproductive or reproductive males in colonies of eusocial gambarelloides shrimps (Tóth and Bauer, 2007, 2008).

In the same way, for the non-gambarelloideses free-living shrimps like presented here, the morphology of the gonopore is the same as gambarelloides males and females. Males showed an opening on a gentle bump on the medial surface of coxae Pereopod 5 (P5). In the females, it is a semicircular slit with “egg-guiding setae”. As previous described by Tóth and Bauer (2007) for another congenere species: it is a broadly U-shaped slot on the proximal sides of the third pereopod coxae. Morphology of those egg-guiding setae was the same in SEM images provided here and provided by Tóth and Bauer (2007), for *Synalpheus* gambarelloides. Those structures have the function to put eggs during the spawning time in the abdomen direction. Höglund (1943) observed those structures on *Palaemon* shrimps and indicated that in bigger animals, it could be found without using SEM images, which should make it easier to identify females’ individuals

546 when dissected specimens. However, in none *Synalpheus* evaluated here, we observed
547 egg-guiding setae on light microscopy, proving that for those animals, the best strategy is
548 keeping using SEM techniques to confirm gonopore morphology and position.

549 Gonopore openings are also the best way to identify intersex or hermaphroditism
550 conditions. When they are present in both pereopod 3 and pereopod 5, it is evidence that
551 the animals can reproduce or at least can produce male and female gonads at some point
552 in life. Tóth and Bauer (2008) described the intersex condition on a *Synalpheus*
553 gambarelloides shrimp, looking just on opening gonopore condition. We believe that this
554 feature is mostly found in gambarelloides groups. All species and specimens of the
555 present study were analyzed carefully to confirm the possible intersex or
556 hermaphroditism, but all of them showed gonochorism. Until now, only eusocial and
557 communal species on *Synalpheus* genera got out the pattern of gonochorism with some
558 hermaphroditism or intersex pattern. So, it is a rare occurrence in the genera (Tóth and
559 Bauer, 2008; Chak, Duffy, *et al.*, 2015; Chak, Rubenstein, *et al.*, 2015).

560 The first pleura morphology is not precisely a classic "secondary sexual
561 characteristic" because they do not participate actively in the reproductive process.
562 However, it can be an extremely useful diagnosis morphology to separate males and
563 females in situation that is not possible to access sexual characteristics as gonopore
564 opening and masculina appendix. Many taxonomical studies and diagnoses made for
565 *Synalpheus* shrimps had reported the differences in the 1st pleura morphology, with males
566 showing a lower edge and curved posteroventral corner coming into a hook and females,
567 mainly ovigerous, with a very rounded posteroventral margin. It has been taxonomically
568 reported both for gambarelloides and non-gambarelloides *Synalpheus* (Duffy, 1996;
569 Hermoso-Salazar and Hendrickx, 2005; Salazar *et al.*, 2005; Ríos and Duffy, 2007; Anker
570 *et al.*, 2012). The most challenging part is when we look into the "intermediate" pleura

form, which is not with the hook projection as in males, even rounded as in ovigerous females. In the present study, all "intermediate" pleura specimens, showed in SEM images, presented the gonopore opening on the third pair of pereopods. Hermoso-Salazar and Hendrickx (2005) found the same pattern in *Synalpheus stylopleuron* Hermoso Salazar and Hendrickx (2006) being animals with "intermediate-form" pleura those who were non-ovigerous or were carrying smaller and non-fecundate embryos. In the present study, we did not find any female with non-funded embryos attached to pleopods.

The present results formed an overview of the secondary sexual characteristics of *Synalpheus* non-gambarelloides shrimps, adding morphological information about pleopod formation and differences between sexes, position, and morphology of the appendix interna (when present), morphology, and location of the gonopores. Those sexual characteristics are a functional link between the first sexual characteristics, as shown in chapter one and behavior patterns, to clarify the process of transference of spermatic products from males to females. It is essential to understand all processes and morphology to make possible evaluate the status of the genera into Caridea and Decapoda groups. We can see a representation of evolutionary patterns in those differences into morphology and behavior so that reproduction can be easily distinguished. That information is also relevant when studying genome size patterns in terms of energy allocation where cell size and length of the animals may modulate the genome size, and researches are trying to standard this relation between genome and biology of caridean shrimps, a subject discussed on Chapter IV and V.

We confirm sexual dimorphism for *Synalpheus* shrimps in pleopods' morphology, the position of appendix interna, shape of 1st pleura, and gonopore position and morphology. All those results are essential for future studies with the group to make possible separate males and females and evaluate the *status* of conservations of species

596 in determined area, or event treats with certain biological studies looking for sure the
597 sexes of animals.

598 *Synalpheus* non-gambarelloid groups seem do not present intersex or
599 hermaphroditism conditions. Still, it is an exceptionally poorly known and understood
600 group that needs much attention and deserves the effort to understand and compare
601 biology with gambarelloides congeneres. This first morphological and explained sexual
602 patterns might turn more straightforward future works and make more evident the entire
603 natural history of this remarkable genera.

604

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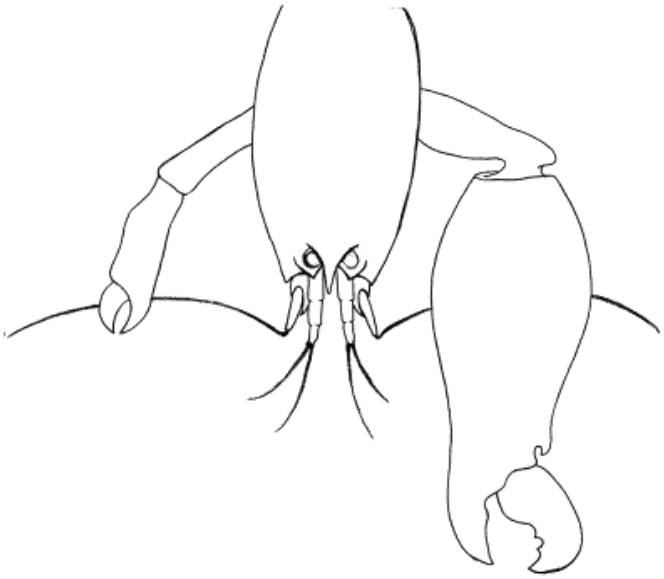

Capítulo 03

Sexual dimorphism and relative growth of the snapping shrimp *Synalpheus fritzmuelleri:* classical and geometric morphometric perspectives

Abstract

The caridean shrimps *Synalpheus fritzmuelleri* is widely distributed into the Atlantic Ocean. It lives associated with invertebrate animals as sponges and corals or as free-living in consolidated substrates. The species evidence poorly kind of sexual dimorphism on secondary sexual characteristics, like other *Synalpheus* spp.. The use of the analyzes of the first pleura and Cheliped morphology to evidence this sexual dimorphism can be a useful strategy since those structures were never been statistically explored before. Here we used geometric and classical morphometry analyses to answer if there are evidences of sexual dimorphism of the first pleura and the cheliped morphology. For the geometric morphometry, we used the first major cheliped with 5 landmarks and 15 semilandmarks, the second pleura with 2 landmarks and 19 semilandmarks, and the second pleura with 2 landmarks and 20 semilandmarks. Classical morphometry was based on carapace length, major cheliped propodus length and width, minor cheliped propodus length and width, and second pleura length. The shape of the first pleura was statistically different into demographic classes with the male-form showing a pointed hooked. The first major cheliped was not evident in morphology to separate sexes. The relative growth of the structures shows that the first major cheliped seems to reach a bigger size, in bigger animals as ovigerous females, but when comparing specimens overlapping the carapace length size, we can observe that males tend to present both larger cheliped in width and length perspectives. So, despite not being evident in morphology, males may use this structure to fight or protect females and territories. We were able to determine sexual dimorphism based on morphology and relative growth of the first pleura and first major cheliped (less evident) for *S. fritzmuelleri* and it can be considered as the first statistical register of those structures on literature. It can be used as tool to apply in other *Synalpheus* shrimps turning much easier future works with this group since the difficult to determine the sex of the specimens nowadays

Keywords: Caridean Morphology, Cheliped, Landmarks, Second Pleura.

Introduction

The Alpheidae shrimp, *Synalpheus fritzmuelleri* Coutière 1909 is one of the most common *Synalpheus* widely distributed in all Atlantic Ocean (Anker *et al.*, 2016). It is commonly found in Brazilian waters, associated with many invertebrates, like sponges and corals, also they can be found free-living in consolidated substrates (Christoffersen, 1982; Anker *et al.*, 2012; Soledade *et al.*, 2015).

Despite very representative, a few efforts have been done to describe biological aspects of this species, being remarkable the studies on presence records (checklists), taxonomy, and a few about reproduction and feeding habits (Coutière, 1909; Goldberg, 1971; Felder, 1982; Dardeau, 1984; Anker *et al.*, 2012).

A fact that might contributes to the lack of populational aspects, morphometry, and relative growth perspectives for this species can be related to the difficulty of correct sex identification of specimens due to the absence of a clear character that separates males and females in the *Synalpheus* spp., once masculina appendix is absent, and the gonopore is very discreet (secondary sexual characteristics used in sexual dimorphism of Caridean shrimps) (Banner and Banner, 1975; Bauer, 2004).

Many taxonomic and morphological descriptions of *Synalpheus* species (*S. fritzmuelleri* included) highlight the morphology of the first abdominal pleura that can be used as alternative evidence of sexual dimorphism, being that in three different shapes: the “male-form”, with an evident hook point at the posteroventral portion of the pleura; the “female-form” with it all rounded shape, and the “intermediate-form” (related to female individuals) with an acute angle in the same region of the “male hook form” (Felder, 1982; Williams, 1984; Ríos and Duffy, 1999). These features are always mentioned systematically, not very clear, or used as sexual dimorphism discussion. It may

work for most *Synalpheus* species, with a few exceptions, like *Synalpheus pectiniger* Coutière (1907) that presents the hook point in all abdominal pleura in males (Coutière, 1909).

Other, but subtle characteristics that can be analyzed on sexual dimorphism is related with the second abdominal pleura and the morphology of the first major Cheliped (the "pistol" Cheliped of the snapping shrimp") that can present distinct growth pattern in male and female specimens (Sganga *et al.*, 2016; Alves *et al.*, 2021), and may be used as an easy way to identify sex in the laboratory or at the field when working with these animals. Despite some discussions around this feature, we can see that these non-usual characteristics (beyond the classical secondary sexual characteristics) as sexual dimorphism were never been entirely explored in the literature in the context to apply statistical tools to evaluate those differences or even clear images to make evident morphological discrepancies. One of the most effective assessments to analyze and compare morphologies with no apparent differences is the strategy to use morphometric analysis, *e.g.*, the Geometric Morphometrics (GM) that evaluates shapes in 2D or 3D sections based on anatomical landmarks and semilandmarks positions with the advantage of describing visual and statistically distortion of some structures, making possible assertive and accurate visualization (Rohlf and Marcus, 1992).

Geometric Morphometrics on caridean shrimps have been applied to analyze morphological shape variation between populations in biogeographical scenarios and to evidence sexual growth patterns based on gender morphological features, *e.g.*, females' carapace and second pleura shape (Torres *et al.*, 2014; Sganga *et al.*, 2016; Silveira Simão *et al.*, 2017).

The use of the analyzes of the first pleura and cheliped morphology to evidence sexual dimorphism can be a useful strategy since those structures were never been

statistically explored before, and then, morphometric geometric can answer visually and numerically if we can keep applying this strategy, at least in *S. fritzmuelleri*.

Another alternative to analyze shape variation is the linear morphometric analysis, which allows detecting morphological differences between sexes based on specifics structures. The relative growth is very applied and used on Decapoda crustaceans because of the particular growth patterns based on molt periods and the metameric disposition of the structures (Hartnoll, 2001; Almeida *et al.*, 2013). It turns possible to visualize the differentiated development of the structures concerning the main body, especially dealing with species presenting heterochely patterns like the *Synalpheus* spp. (Hartnoll, 1978; Pescinelli *et al.*, 2018; Costa-Souza *et al.*, 2019).

Interpretations regarding morphologic structures and growth patterns are important to evaluate sexual dimorphism. Here, those interpretations turn essential when there are no conclusive structures to differentiate sexes between specimens, moreover, the results of those analyzes reflect inferences about biological and behavioral patterns of those animals, like reproductive and predations strategies, for example, as proposed by Alves *et al.* (2021) with one congeners species of the present study.

Those interpretations can be even more elaborated and conclusive as more techniques and tools methods are applied, like in the present thesis that considers from primary sexual characteristics, as gonads development on the firsts chapters to the reflex on external morphological differences as suggested in this chapter. So, evaluating structures is more than just a proposal on taxonomic or systematic differences, it is a big step to interpret the lifestyle and evolutive aspects on behavior and reproduction of a given group.

In the present chapter, we aim to evaluate and statistically describe the morphological secondary characteristics which can be used to identify sexual dimorphism of the snapping shrimp *S. fritzmuelleri* combining classical and morphometric geometric

analyzes to propose the entire "shape" and relative growth of the structures between demographic groups.

The results will improve the knowledge on the structures growth pattern of *Synalpheus* shrimps, and confirm if those new secondary sexual characteristics applied for the group are reliable structures to separate sex, allowing more accurate analyzes in future works with other species of the genera.

Material and Methods

Geometric Morphometrics – Shape Variation

Specimens were sexed based on the appearance of the first abdominal pleura (prominent hook located at the posterior margin in males, rounded shape in females, and acute angle for the intermediate individuals, also considered as females) as proposed by Felder *et al.* (1982); and the presence of embryos attached to pleopods to determine ovigerous females.

Three structures were analyzed in each specimen:

1) The first pleura (66 images analyzed);

2) The second pleura (68 images);

3) The first major cheliped (62 images).

For the first cheliped, five landmarks digitized plus 15 semilandmarks were used to represent homologous curves and surfaces by sets of points. In the same way, two landmarks plus 19 semilandmarks were used on the first pleura and two landmarks plus 20 semilandmarks on the second pleura (Figure 1), using the softwares TpsDig 2.31 and TpsUtil 1.68.

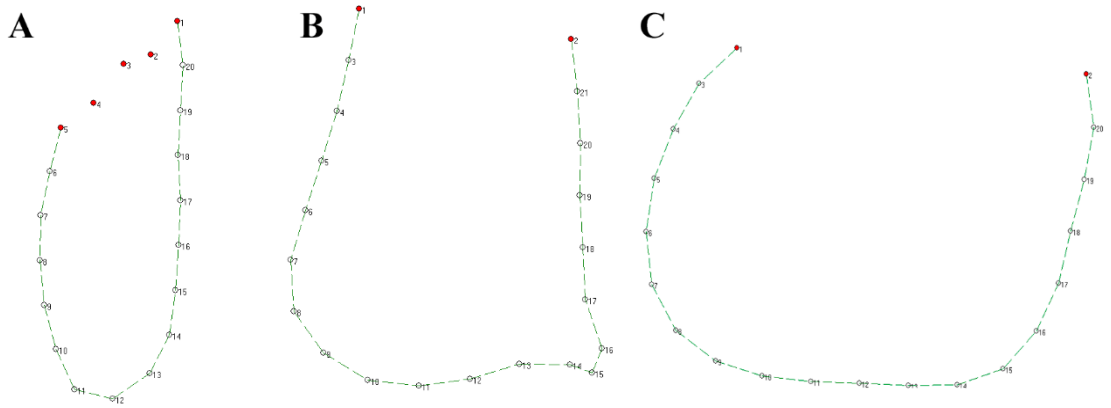

Figure 1: Landmarks disposition in all structures analyzed. Red points represent landmarks, White points represent curves with semilandmarks. A) First major cheliped with five landmarks plus 15 semilandmarks; B) First abdominal pleura with two landmarks plus 19 semilandmarks; C) Second abdominal pleura with two landmarks plus 20 semilandmarks.

All specimens had the structures dissected and photographed underwater in a Petri dish using a stereomicroscope (Zeiss Stemi SV6, fitted with a Zeiss Stemi 2000-C image capture system) equipped with a camera and the Axio Visio software (vers.4.8) with a 1 mm scale bar in each photograph.

The "Thin Plate Spline" (TPS) provided by TPS program deformations tend to visualize the shape from one reference and compare it based on a set of coordinates of homologous points as landmarks. In the present study, semilandmarks were applied to represent the shape of curves and surfaces with a precise equidistance between points as proposed by Gunz and Mitteroecker (2013). Looking at the curved shape of the first cheliped and first and second pleura, the strategy to apply semilandmarks seems applicable.

The coordinates for semilandmarks were aligned with TpsUtil 1.68 and TpsRelW 1.62 softwares (Rohlf, 2004a, 2004b, 2005) using the sliding semilandmark algorithm, on the surface, to remove the effects of arbitrary spacing of semilandmarks.

Statistical analyzes were performed for structures' shape variation assessing discriminant analysis of the uniform components and partial warps scores. Specimens

grouped by sex were used for acquiring the canonical variables that summarize the set of original variables in each grouping (males, females, and ovigerous females) using PAST software (Hammer *et al.,* 2001). We also used a multivariate isometric structures size estimator called the "centroid size", defined as the square root of the sum of the squares of the distances from each anatomic landmark to the center of mass of each configuration (Bookstein, 1991).

According to De Camargo *et al.*, (2015), the resulting variable is used as a single measurement that accounts for the multivariable nature of the size of the structures in subsequent analyzes. This procedure removes only the effects of isometric size. Therefore, the configurations keep both shape variations unrelated to size and allometric variations. We compared all values of centroid sizes through a "t-student*"* test to confirm differences between shapes among sexes, also were employed a PCA to generate a graphical visualization of these differences.

Additionally, discriminant analyzes were applied in both male and female structures of the uniform compounds and partial deformations to compare all of them using the Hotelling T^2 test.

Classical Morphometry

We provided the first panorama for classical morphometry comparing the relative growth of the sexes in the species.

Measurements included the following structures (Figure 2):

- Carapace Length (CL) (Figure 2A);
- Major Cheliped Propodus Length (MaCPL) (Figure 2B);
- Major Cheliped Propodus Width (MaCPW) (Figure 2B);
- Minor Cheliped Propodus Length (MiCPL) (Figure 2C);

- Minor Cheliped Propodus Width (MiCLW) (Figure 2C);
- Second Pleura Length (SPL) (Figure 2D).

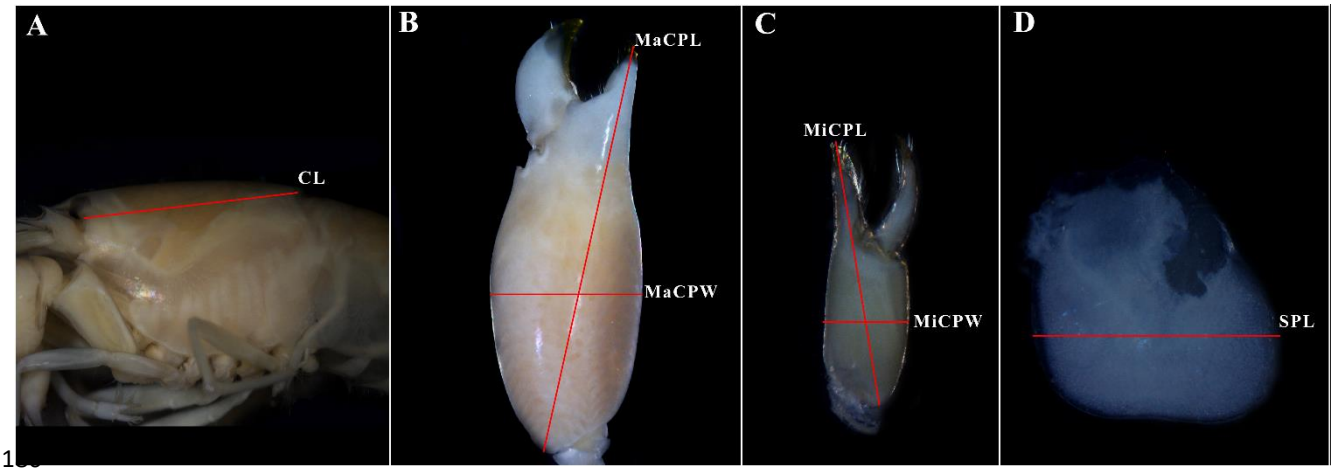

Figure 2: Structure analyzed on linear classical morphometry. A) Carapace Length (CL); B) First Major cheliped length and width (MaCPL; MaCPW); C) First Minor Cheliped Length and Width (MiCPL; MiCPW); D) Second pleura Length (SPL).

The relative growth analysis detects changes in the growth pattern of particular body structures concerning a constantly growing body part, *e.g.*, the carapace length (independent variable). The allometric power equation $y = a\ x^b$ was used in its linearized form ($\log y = \log a + b \log x$) in which "y" is the dependent variable (body structures), "x" is the independent variable (CL), "b" is the allometric coefficient, and "a" is the intercept. The allometric condition "b" was analyzed for each structure ($b = 1$: isometry, $b < 1$: negative allometry, $b > 1$: positive allometry). The allometric growths were tested using the smart (Warton *et al.*, 2012) package in R (R Core Team, 2021). Structures relationship were tested between carapace length (CL, independent variable) against the other structures (MaCPL, MaCPH, MiCPL, MiCPH, SPL, (dependent variables). The allometric relationships were tested using the major axis routine, which is known as "type II linear regression", this analysis decreases the effect of residuals providing a better line of fit for allometric relationships (Warton *et al.*, 2006). Before the analysis, the data were

log-transformed and the allometric coefficients (slopes, b-value) were tested for isometry (b = 1), negative allometry (b < 1), and positive allometry (b > 1) as described above.

Results

A total of 62, 66, and 68 specimens were used to analyze the shape variation of the first major cheliped, the first pleura, and the second pleura morphologies, respectively.

Shape variation – First Major Cheliped and Pleura

The discriminant analyzes did not evidence any statistical differences between the format of the first major cheliped in males and females (Hoteling, T2= 123.4, F=1.428, p= 0.177) (Figure 3A). Most of the landmarks were overlapping with a discreet variation in the shape, that can be observed between landmarks 3 and 4 in the apical region of the propodus (Figure 4B). First pleura was statistically evaluated and discriminant analyzes pointed out a significative shape variation (Hoteling, T2= 984.09, F= 10.526, p= 6.855E-09) (Figure 3B) between males and females, and a very close related morphology between females and Ovigerous female (Figure 5B).

The second pleura was also statistically different between sexes (Hoteling, T2= 278.98, F=3.639, p= 0.0002) (Figure 3C), but in a general view, the format remains the same. These results can be used to explain the female output to expand the second pleura to create the incubator chamber (Figure 6B). However, the first pleura was the best informative structure with the significative difference to represent morphological sexual dimorphism.

At performing Centroid Size (CS) values and PCA with shapes variation, we can visualize differences in all structures. In the same way of discriminant analyzes, the best

230 explanation values for PCA were found in the first pleura shape variation (Wilk's λ =
231 0.0152, df1 = 78, df2 = 52, F = 4.86, p = 7.99E-09) (Figure 5A). The Second pleura was
232 as well separated in centroid size evaluation and well discriminated in PCA evaluation
233 (Wilk's λ = 0.048, d1 = 72, df 2 = 60, F = 2.94, p = 1.448E-05) (Figure 6A). Despite well
234 separated in PCA centroid size analyzes, the major cheliped still the most discreet
235 structure in sexual dimorphism, evaluating just shape in morphometric geometric
236 analyzes (Wilk's λ = 0.076, df1 = 72, df2 = 48, F = 1.74, p = 0.0212) (Figure 4A).

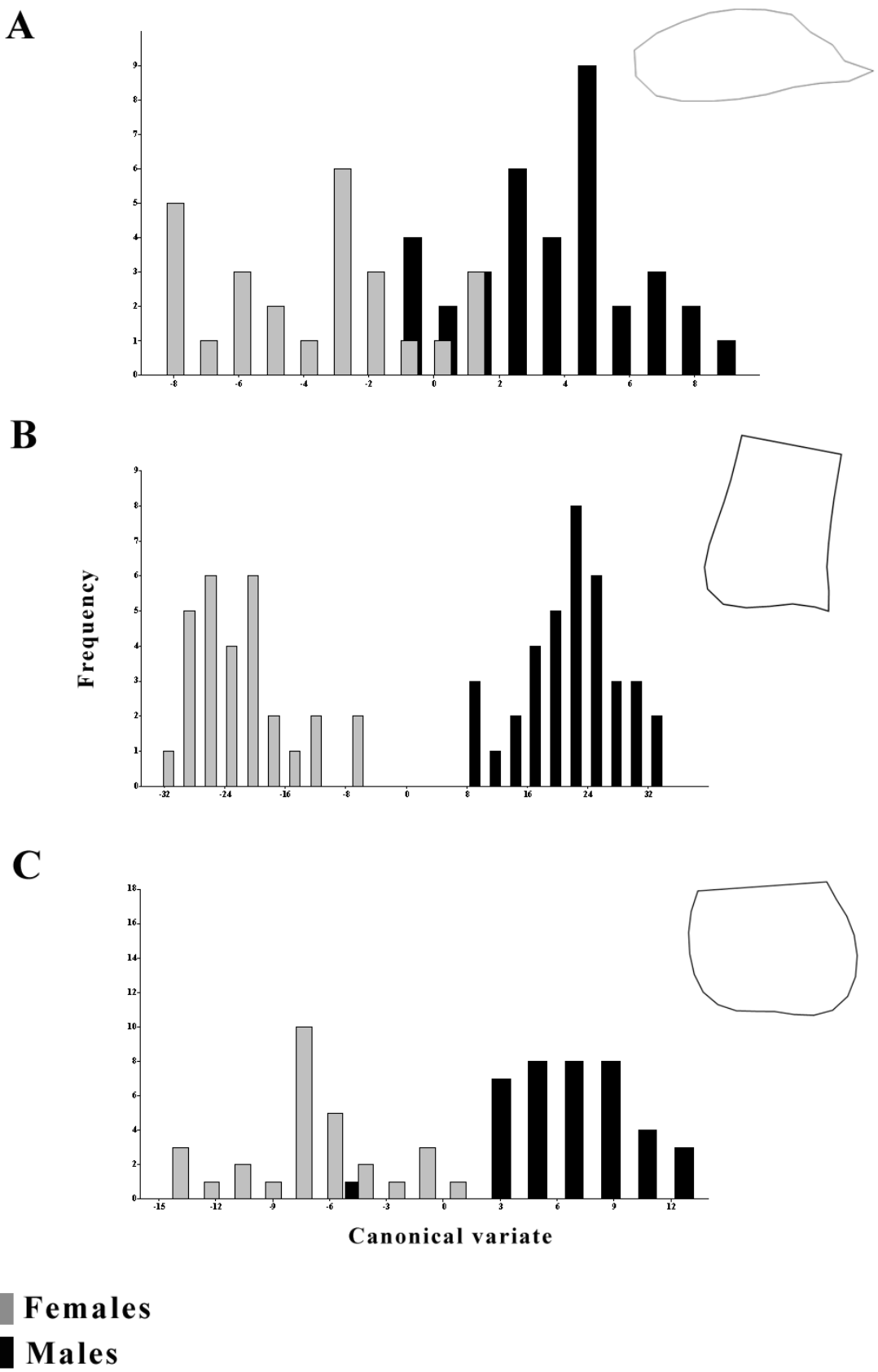

Figure 3: Frequency of canonical variation from discriminant analyzes of the shape variation. A) First Cheliped; B) First Abdominal Pleura; C) Second Abdominal Pleura.

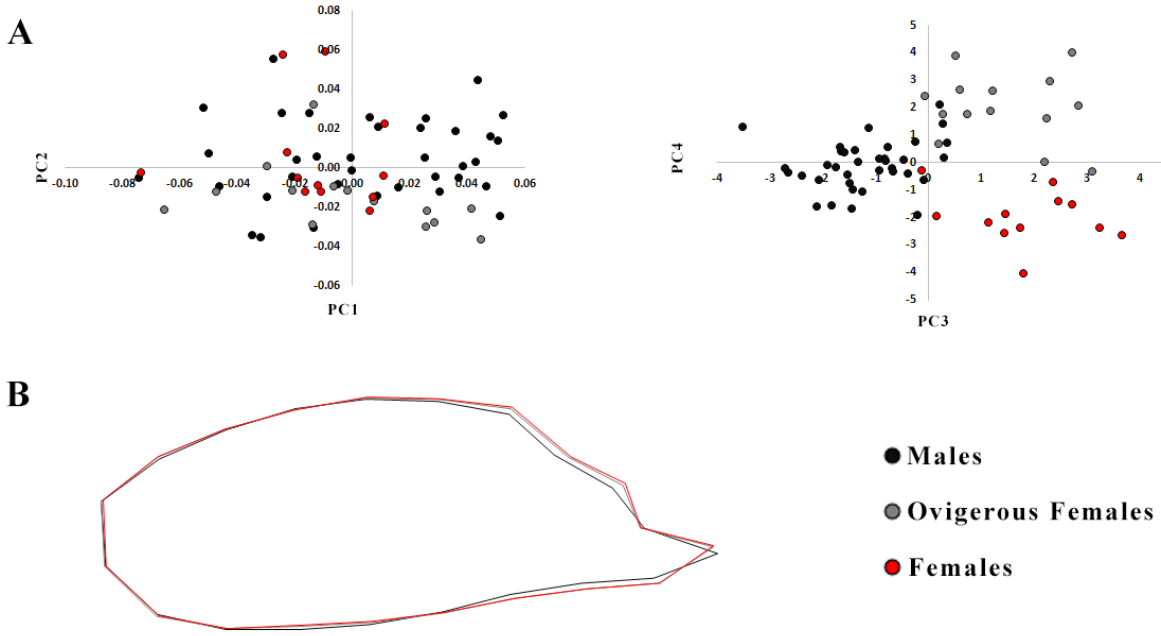

Figure 4: A) PCA representation on variance discrimination of four axes analyzes on shape variation of the structure; B) Shape variation of the first Cheliped morphology – schematic drawn based on landmarks deformations.

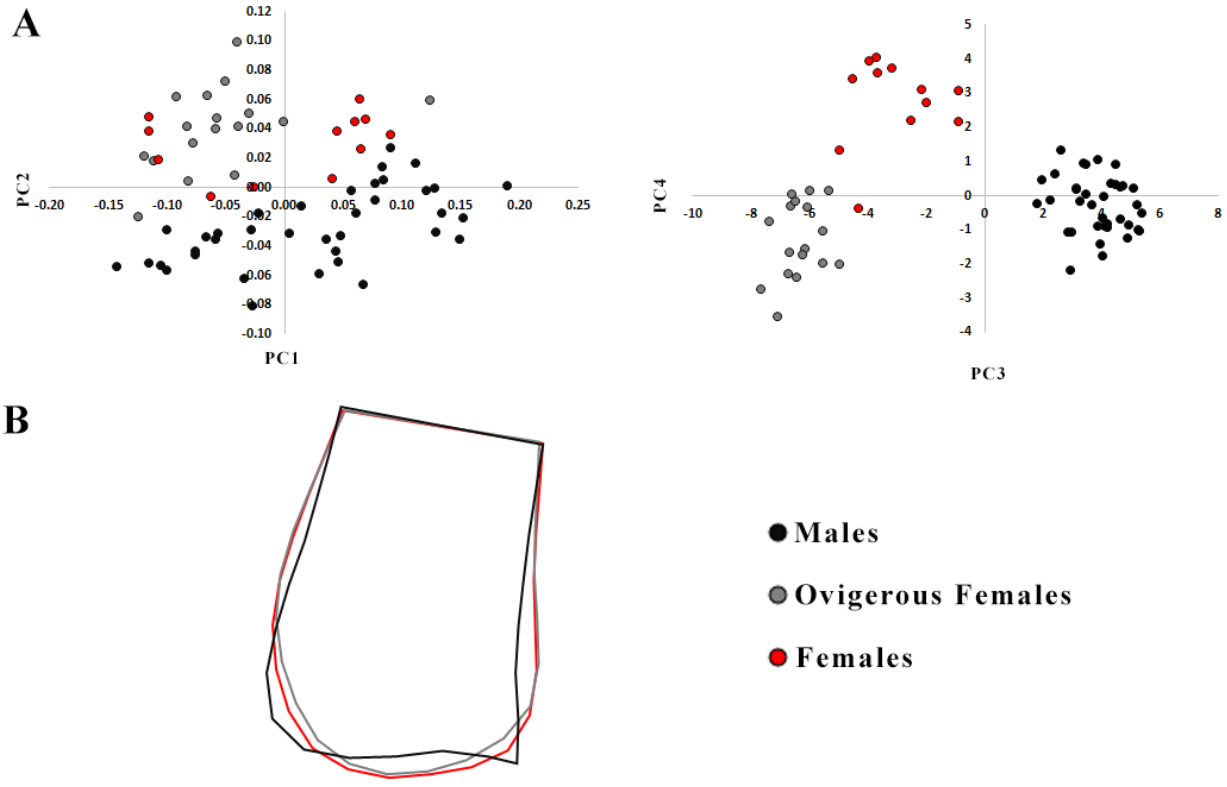

Figure 5: A) PCA representation on variance discrimination of four axes analyzes on shape variation of the structure; B) Shape variation of the First Pleura morphology – schematic drawn based on landmarks deformations.

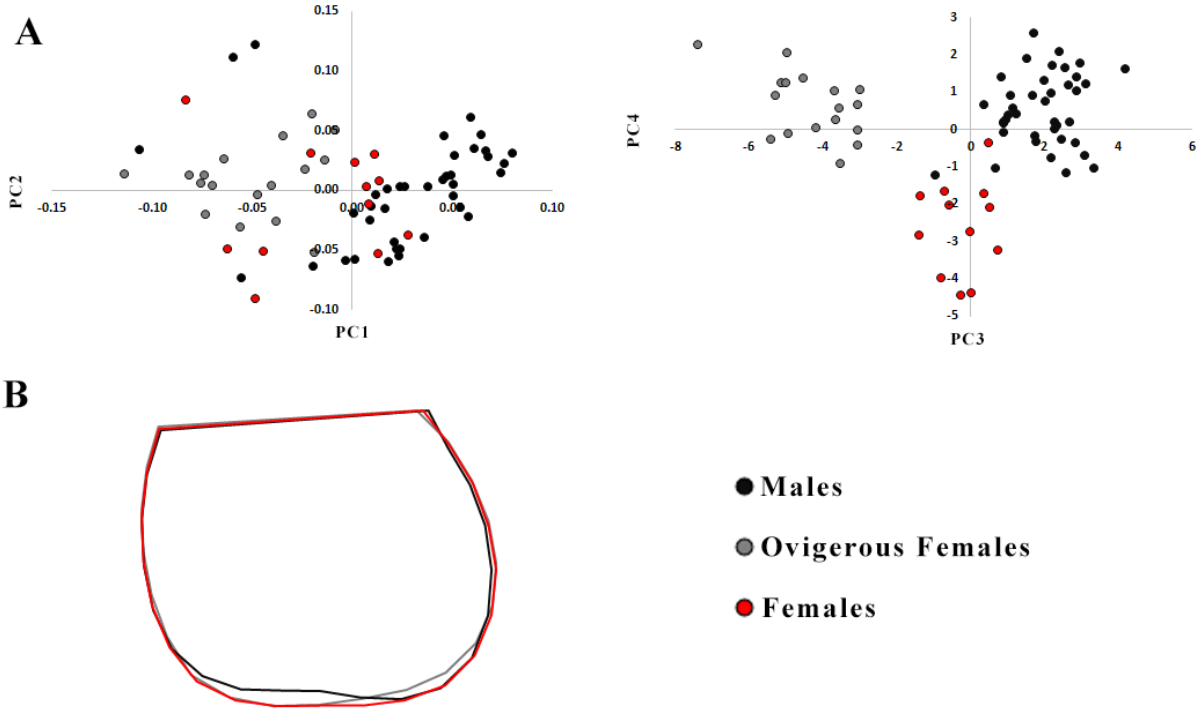

Figure 6: A) PCA representation on variance discrimination of four axes analyzes on shape variation of the structure; B) Shape variation of the Second Pleura morphology – schematic drawn based on landmarks deformations.

Classical Morphometry

A total of 72 specimens were analyzed for relative growth study, being 40 males (M), 14 females (F), and 18 Ovigerous Females (OF). Total Carapace Length varied from 2.72 mm to 7.16 mm. Measurements of the smallest ovigerous female were 4.14 mm. In general, ovigerous female (mean CL size: 5.59 ± 0.85 mm) and female (mean CL size: 4.27 ± 0.61 mm) reached respetitively bigger CL size than males (mean CL size: 4.08 ± 0.84 mm).

Morphometric analysis showed positive allometry for the second pleura from both females and ovigerous females and isometry for males (Table 1). Major cheliped length showed positive allometry for all sexes, and major cheliped width showed positive allometry for males and ovigerous females; however, females presented isometry for this

structure (Table 1). The minor cheliped length showed isometry for all sexes, and minor cheliped width males and ovigerous females showed isometry and females showed positive allometry (Table 1).

Table 1: *Synalpheus fritzmuelleri*. Allometric analyses of transformed morphometric data (log) using carapace length as independent variable. CL=Carapace Length; SPL=Second Pleura Length; MaCPL=Major Cheliped Length; MaCPW=Major Cheliped Width; MiCPL=Minor Cheliped Length; MiCPW=Minor Cheliped Width; *p-value=significance values; "+": positive allometry; "=": isometry.

Relationship	Sexes	Intercept (a)	Slope (b)	p-value	allometry
CL x SPL	Males	-0.46	1.01	0.86	=
	Females	-0.87	1.70	< 0.01*	+
	Ovigerous Females	-0.74	1.56	< 0.01*	+
CL x MaCPL	Males	-0.05	1.40	<0.01	+
	Females	-3.70	7.13	< 0.01	+
	Ovigerous Females	-0.04	1.25	0.04	+
CL x MaCPW	Males	-0.54	1.47	<0.01	+
	Females	-0.40	1.20	0.29	=
	Ovigerous Females	-0.57	1.37	0.04	+
CL x MiCPL	Males	-0.24	1.60	0.24	=
	Females	-0.59	1.58	0.13	=
	Ovigerous Females	-0.17	0.90	0.24	=
CL x MiCPW	Males	-0.75	1.10	0.16	=
	Females	-1.57	2.39	0.04	+
	Ovigerous Females	-0.80	1.12	0.24	=

Looking at the same structures analyzed on morphometric geometric shapes, we evaluated the relative growth according to the carapace length, between the sexes. The first major cheliped seems to reach a bigger size, in bigger animals as ovigerous females. It was observed that males tend to present both larger cheliped in width and length

perspectives (Figure 7A and 7B), when comparing specimens overlapping the CL size (as male and females in the same size-class). Additionally, the growth pattern of the first minor cheliped had the same tendency in lower scale than larger cheliped (Figure 8A and 8B).

The growth patterns of the second abdominal pleura are the most evident structure to compare between sexes, being ovigerous females presenting remarkable larger pleura than females and males, respectively. This corroborates the fact evidenced for the caridean shrimps, that females invest more energy to form an effective incubatory chamber at the time to reproduces and keep embryos attached on pleopods. One of many strategies is the enlargement of the pleural to improve the space in the abdominal region (Figure 9).

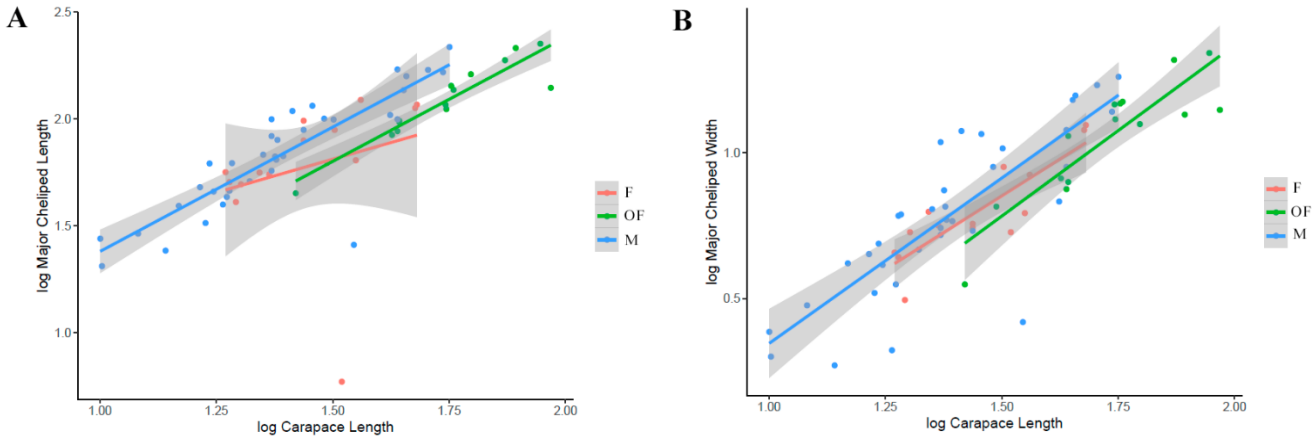

Figure 7: Log of the linear regression of the analyzes on structures growth differences between Males, Females, and Ovigerous Females of the *Synalpheus fritzmuelleri*. A) relative growth of the major cheliped length concerning carapace length; B) relative growth of the major cheliped width in relation with carapace length. F=females; OF=ovigerous females; M=males.

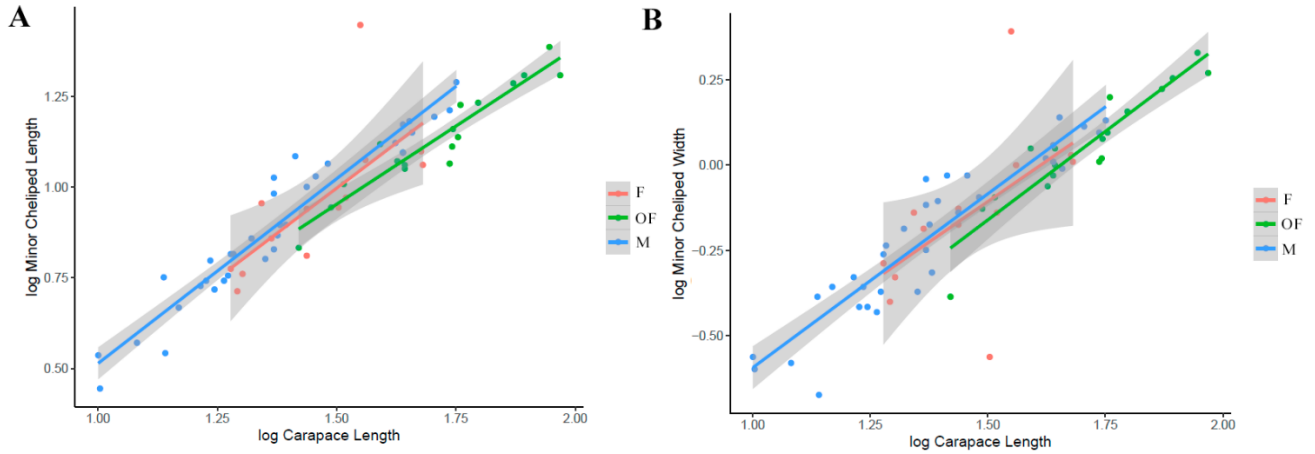

Figure 8: Log of the linear regression of the analyzes on structures growth differences between Males, Females, and Ovigerous Females of the *Synalpheus fritzmuelleri*. A) relative growth of the minor cheliped length concerning carapace length; B) relative growth of the minor cheliped width in relation with carapace length. F = females; OF = ovigerous females; M = males.

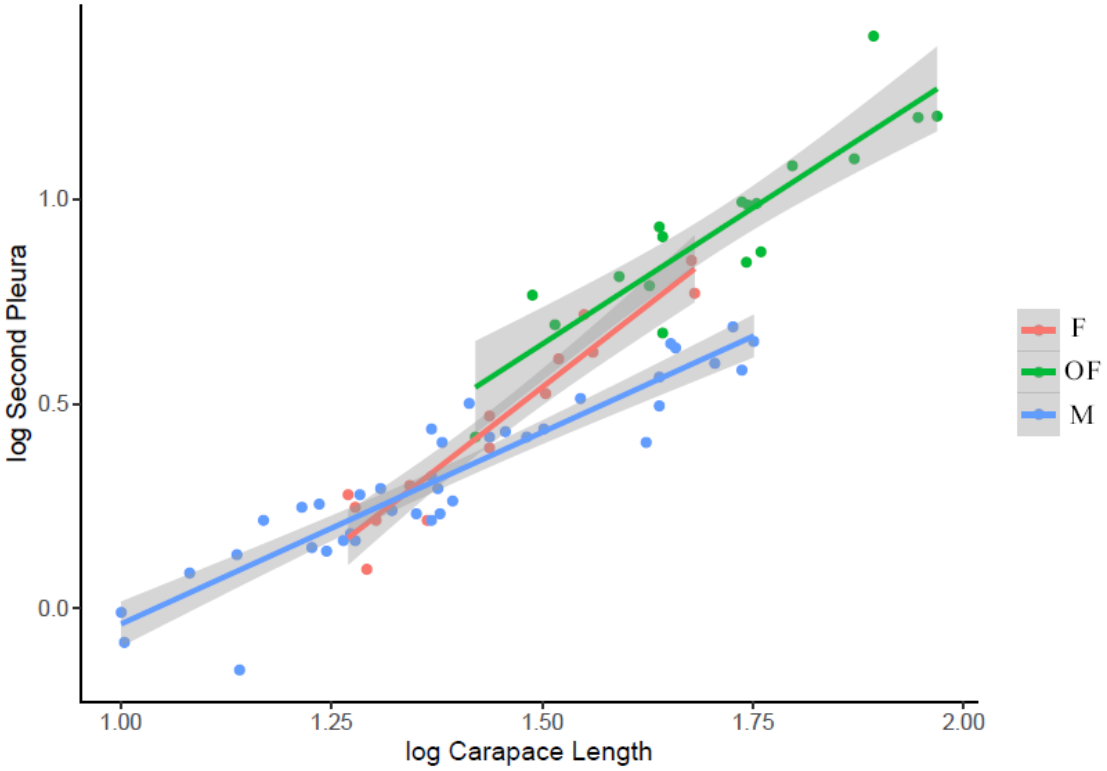

Figure 9: Linear regression of the analyzes on second pleura growth differences between Males (M), Females (F), and Ovigerous Females (OF).

Discussion

Accurate identification of sexes in *Synalpheus* shrimps is very discussed and almost don't confident using macroscopical and classical diagnotic of secondary sexual characteristics for caridean shrimps. These animals lack the presence of the masculina appendix associated with the endopod of the second pleopod, and the observation of the gonopore opening is almost impossible using light microscopy (Bauer, 2004; Tóth and Bauer, 2007, 2008). The purpose of effective and evident characters for this group comes to compound and clarify the literature and morphological studies with species from this genus.

Despite taxonomically reported but short described, the first abdominal pleura can be used as sexual dimorphism in some of the *Synalpheus* shrimps (explored here and in

chapter II). This is the first time that geometric morphometrics was statistically tested and our results for shapes, landmarks, and centroid size analyzes corroborated to find differences, starting a new perspective and panorama for a reliable pattern for sex separation to be applied in more *Synalpheus* species.

Some authors described the character of the first pleura in sexes, especially in description and taxonomic works for other conspecific *Synalpheus* species (Duffy, 1996; Salazar *et al.*, 2005; Ríos and Duffy, 2007), but always just in a brief description of the format, and with a brief discussion around it. Evidencing this structure as an effective sexual character was never been explored before. Therefore, it is one of the morphologies to be taking into account when dealing with sexing *Synalpheus* shrimps (Figure 10). We encourage new studies to apply these numerical references in other species from the genus and formulate a definitive morphological pattern for those animals. Evidencing a clear sexual dimorphism without appendix masculina and gonopore morphologies.

Studying reproductive parameters of the *S. fritzmuelleri*, Felder (1982) proposed that immature juveniles have the morphology of the first pleura similar to the male adults and, eventually, those juveniles can present some non-fecundated eggs attached on pleopods (Coutière, 1909; Banner and Banner, 1975).

We found the "male-form" pleura in some juveniles containing some "minute chalky" eggs (as non-fertilized eggs) on the abdomen. It was observed one case like this in our samples, where one of the specimens analyzed had the first pleura with a discreet projection (almost like an "intermediate" pleura), but having a few, like five non-fecundated eggs attached on pleopods (Figure 11). We assume that cases like this can pose doubt on sex identifications when taking into account only the first pleura morphology. Such as "female-presenting male-form pleura". We believe that the morphology of the first pleura can change from juvenile to adults, especially in females,

that can present an intermediate form at the beginning of life, and than improve the structure during the reproductive period, as one of the process of the "breeding dress" (Bauer, 2004). In males, the hooked posterior point of the pleura become even more prominent.

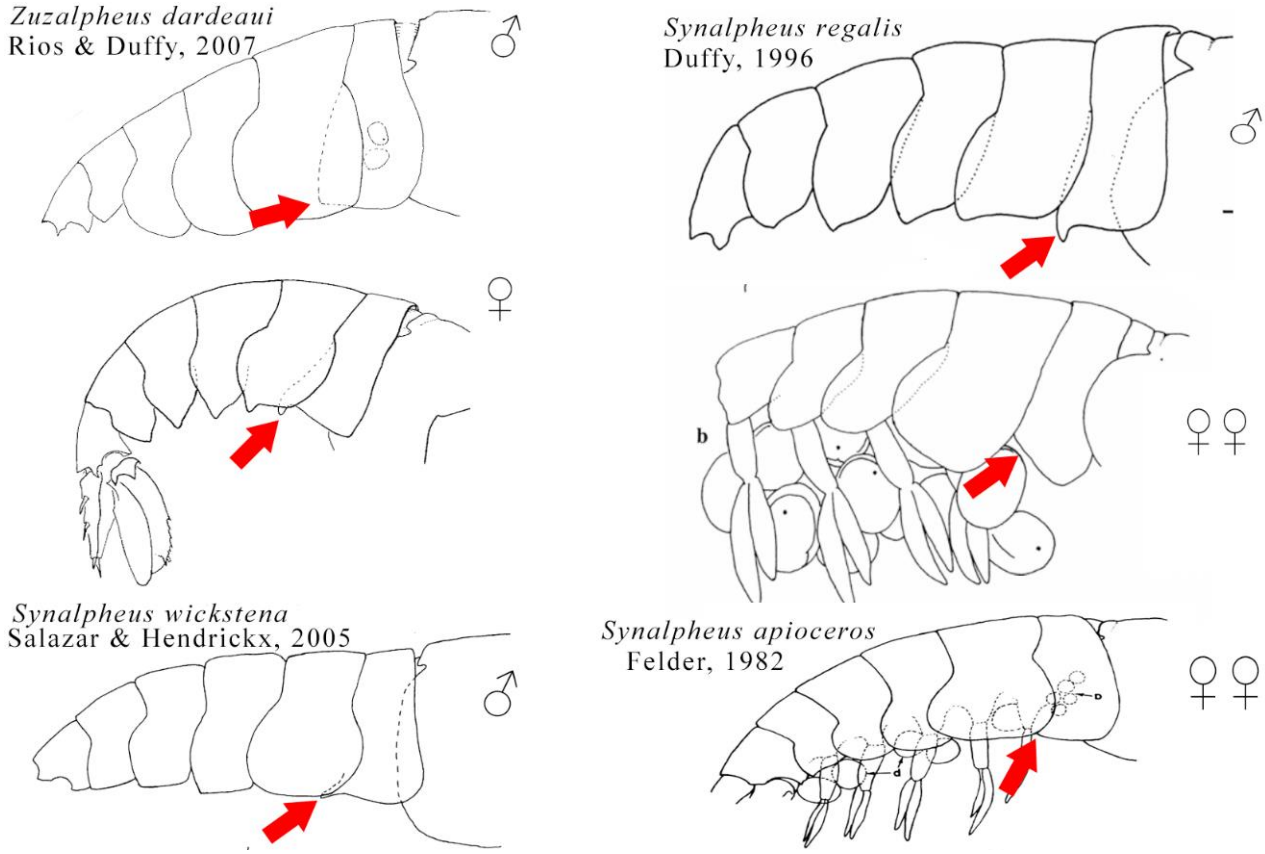

Figure 10: Representation of the taxonomical description of some *Synalpheus* shrimps, mentioning first pleura morphology between sexes. All images provided of publication are mentioned on the image.

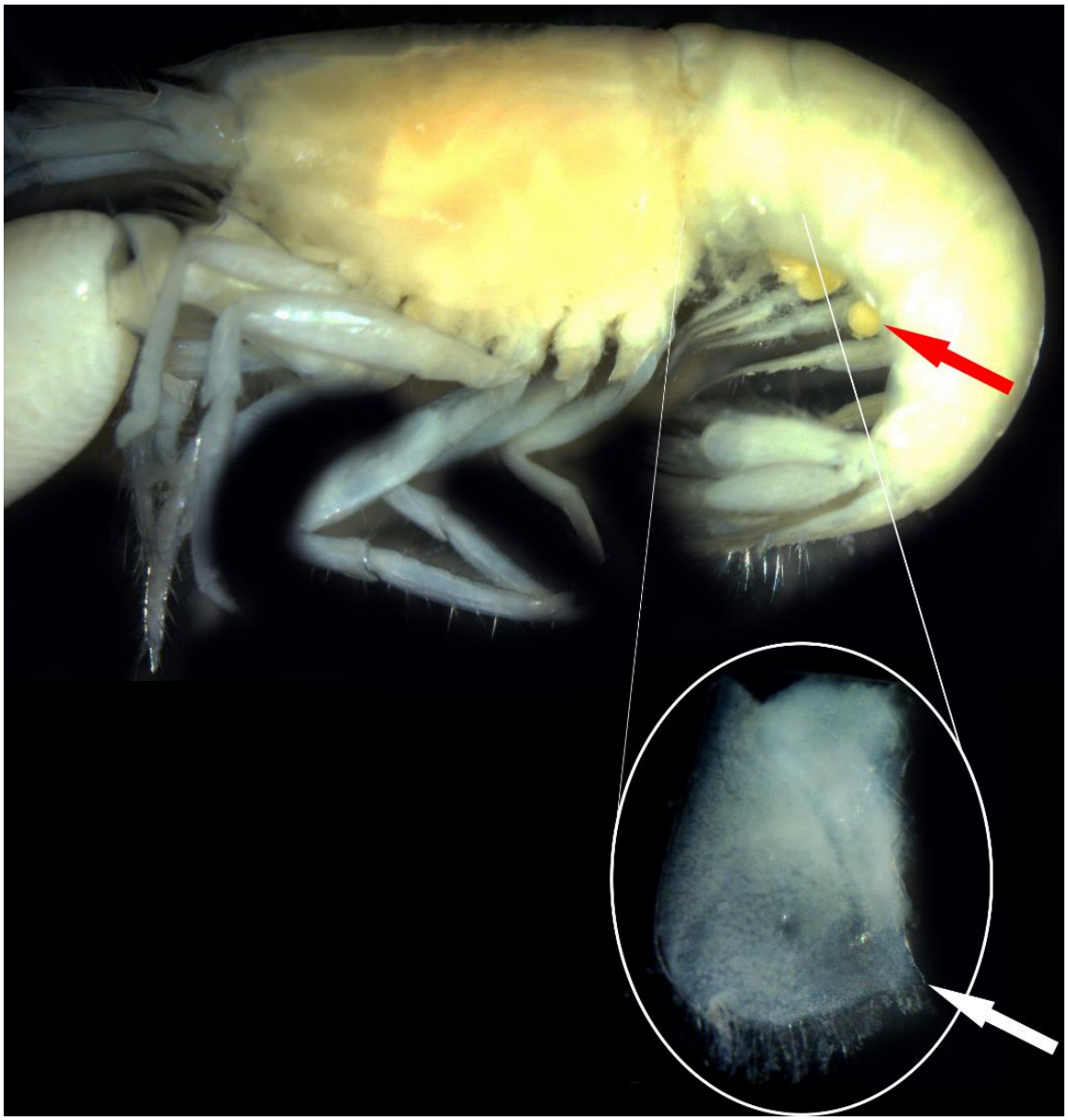

Figure 11: Particular case of one *Synalpheus fritzmuelleri* showing first abdominal pleura as "Male-Form" with a hooked corner on the posterior basis (zoom detail) and a few non-fecundated eggs attached on pleopods. White arrow points to detail on posterior margin of the pleura; Red arrow points to small, yellow, chalky, and few eggs attached on pleopods.

In *S. fritzmuelleri,* the morphology combined with structures seems to be the best way to confirm sexual dimorphism. In most cases, the abdominal pleura morphology could answer this question, but when dealing with particular cases, as Figure 11, we propose to look for the presence of the appendix interna associated on endopod of the pleopods (as demonstrated in chapter II) since males do not present any kind of appendix. So, the combination of the structures Pleura + Plepods can be an usefull way to determine sex in those animals.

Geometric morphometric tools have been used in shrimps more recently to compare different populations of some species and evaluate their specific changes among geographic areas (Bissaro *et al.*, 2013). In Caridea, it was applied to analyze sexual dimorphism in some species. Silveira Simão *et al.* (2017) used the protopodite of pleopod shape to evidence morphological features in *Pasiphaea* species. Some other studies used the carapace shape to prove sexual dimorphism as *Macrobrachium* caridean shrimps, which answered well on those analyzes (Torres *et al.*, 2014; Melo and Masunari, 2017). The second abdominal pleura is also one of the structures analyzed on sexual dimorphism as the pattern described by Sganga *et al.* (2016) for *Neocaridina* species, with the second pleura more prominent in adult females than males.

Despite the second abdominal pleura being evidence for sexual dimorphism, it turns more evident when females are already carrying embryos on pleopods or just in the period after the spawning, as we can see in our results in geometrical and classical morphometries. So, it is not a constant and evident morphological structure like the first pleura and appendix interna, being considered more an accessory structure than a confident diagnostic for sexual dimorphism.

Most of the works that use geometrical morphometries differences in caridean shrimps look for differences in the carapace shapes (Torres *et al.*, 2014; Sganga *et al.*,

2016; Melo and Masunari, 2017). None of those species presented the first weapon developed cheliped like snapping shrimp from *Synalpheus* genera. It is known that for those snapping and pistol shrimps, the carapace morphology is not the best structure to evidence sexual dimorphism (Costa-Souza *et al.*, 2019; Pescinelli *et al.*, 2020; Alves *et al.*, 2021). Here, instead of the carapace shape, we referenced the first major cheliped as a parameter for sexual dimorphism.

Some analyzes of those structures were evaluated by other authors using classical morphometry, Alves *et al.* (2021) proposed sexual dimorphism relating first major cheliped for the non-gambarelloide *Synalpheus brevicarpus* (Herrick, 1891), as suggested in the present study, being very close results with females reaching discreet, but bigger sizes in carapace length and males presenting robust and bigger first major cheliped than specimens of the same size. Looking at and comparing those results, we proposed that the first major cheliped also can be used at the time of differentiating sexes when looking for specimens at the same size class. Despite the absence of the masculina appendix and the discreet gonopore with difficult visualization, our results showed at least two "non-conventional" sexual characteristics for sexing species of *Synalpheus* genera.

This relative growth parameter of the first major cheliped can change in *Synalpheus* shrimps when analyzing species with communal or eusocial behaviors (Chak *et al.*, 2015), where the reproductive skews can modulate the sexual dimorphism in gambarelloides species (mandatory sponge symbionts).

Caridean shrimps that present pair-living behaviors tend to have not pronounced differences in carapace growth, being males and females reaching similar size-trend. This fact makes easier the mating system process, once they will copulate with the same partner most of the time, and will live under the same conditions, especially when associated with other invertebrate animals (Jormalainen, 1998; Thiel and Baeza, 2001).

In general, Alpheidae shrimps from “Pistol” and “Snapping” groups (*Alpheus* and *Synalpheus* genera) present a remarkable heterochely characterized for using the first major cheliped for aggressiveness, courtship displays, and grooming, while the first minor cheliped is used to capture and manipulating food (Mariappan *et al.*, 2000). The discreet dimorphism from the carapace is also reflected in the somatic growth on the first cheliped, where males and females tend to present some similar growth patterns of this structure, with a discreet, but visible prominent growth in males. This can be explained by the aggressive and territorialistic behavior reflecting physiological patterns (Pescinelli *et al.*, 2018, 2020; Costa-Souza *et al.*, 2019). So, males presenting a robust major cheliped, maybe can use this as strategy at the time to protect females and the possible host from predators. Despite looking into the morphology of the minor cheliped, it did not bring a confident results on behavior and sexual dimorphism, being possible to discuss that the most important and used structure remains in the first major Cheliped, responsible for the “pistol” movement in this group.

. The positive allometry of the second pleura growth corroborates the very evident growth of this structure relating to the female’s body size, that maybe can be related to the fact of the extensive investment by females in ovarian development and secondary sexual characteristics to form an effective process to guarantee successful embryo production. So, it is clear that females tend to invest energy in all reproductive processes, from histological development to somatic growth of secondary sexual characteristics, while males seem to tend to invest in somatic growth of the chelipeds to protect the host and territory, and also make easy the mating system process.

In summary, the three structures can be used at the time to differentiate sexes of *S. fritzmuelleri* species, *i.e.*, the first major cheliped (even in a discreet way), first pleura, and second pleura morphology, being the first pleura the clearest and representative one.

Also, *S. frizmuelleri* represents an example of behavior and physiological patterns reflected on morphological features, since pair-bond animals tend to present growth patterns in the same way as seen in our results. Those morphological characteristics studied here will be usefull in future works with this and other *Synalpheus* species, being confident in the application of the "non-usual" secondary sexual appendages morphology.

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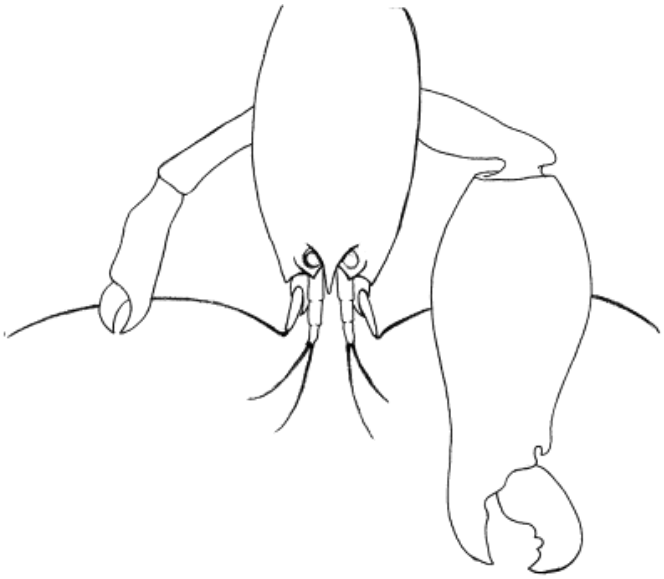

Capítulo 04

Patterns of genome size variation in caridean shrimps: a new data for nongambarelloides *Synalpheus* genus

Abstract

Genome size (GS) or DNA nuclear content is considered a useful index for making inferences about evolutionary models and life history in animals, including taxonomic, biogeographical, and ecological scenarios. However, patterns of GS variation and their causes in crustaceans are still poorly understood. This study aimed to describe the GS of five Neotropical *Synalpheus* nongambarelloides shrimps (*S. apioceros, S. minus, S. brevicarpus, S. fritzmueller, and S. scaphoceris*) and compare the C-values of all Caridea Infraorder in terms of geography and phylogenetics. All animals were sampled in the coast of São Paulo State, Brazil and GS was assessed by flow cytometry analysis (FCA). The C-values ranged from 7.89 pg in *S. apioceros* to 12.24 pg in *S. scaphoceris*. Caridean shrimps had higher GS than other Decapoda crustaceans. The results reveal a tendency of obtaining larger genomes in species with direct development in *Synalpheus* shrimps. In addition, a tendency of positive biogeographical correlation with Caridea Infraorder was also observed. This study provides remarkable and new protocol for FCA, which led to the discovery of new information regarding GS of caridean shrimps, especially for Neotropical *Synalpheus, which* represents the second-largest but poorly studied group in the Caridea Infraorder.

Keywords: C-value, caridean shrimps, Decapoda, DNA content

Introduction

Decapoda crustaceans and genome size

The Order Decapoda constitutes a large representative group in the extensive and specious Phylum Crustacea. It is extremely diverse due to described species, ecological and geographical distribution, and sexual system and behaviors, which instigates the need for constant evaluation and study of its general biology and evolution. Special attention has been drawn by the evaluation of species of Decapoda by Chace (1951), Martin and Davis (2001), and De Grave *et al.* (2009) in which more than 15,000 Decapoda species distributed into about 3,000 genera (with at least other 3,000 fossil species), were registered.

The evolutionary relationships, adaptations process, and life history (considered here as reproduction, life cycle models, and evolutional patterns) among species belonging to the Order are constantly discussed and reformulated, as new information regarding morphology, behavior, and/or genetics features are described (Dixon *et al.*, 2003; Porter *et al.*, 2005). To make inferences about the life history and biological aspects of this diverse group, a more robust and confident analysis is needed. Nowadays, with the advent of new technologies and science descriptions, discussions on the topic can become even more complex and elaborated by combining classical genetic analyses with the new genome size (GS) parameters (Jeffery *et al.*, 2016).

Among the genetic studies, GS appears to be a new feature for population, biogeographical, and evolutionary studies (Blommaert 2020; Panzera *et al.*, 2007). The genome of plants and animals can be described qualitatively and quantitatively, since GS describes the “mass” of the genome or nuclear DNA in terms of “C-value,” which is expressed as picograms (pg), where 1 pg = 10^{-12} g (Gregory, 2005).

Genome size variation in animals

It is established that GS varies among organisms, which represents essential information for genomic studies from an evolutionary perspective (Gregory, 2001). However, GS variation is not necessarily related to organizational complexity of animals, which is described as the "C-value paradox" (Bainard & Gregory, 2013; Gregory, 2005) or more recently as "C-value enigma" (due to the highly complex interpretations of the values and biological aspects of the organisms) (Elliot & Gregory, 2015). Theories about GS evolution patterns of eukaryotes are constantly formulated and discussed in literature from the perspective of genetics (such as noncoding DNA influences and transcriptome-wide selection efficacy) to phenotypic and adaptive process in determined species and locations (Chak *et al.*, 2021; Hultgren *et al.*, 2018; Lefébure *et al.*, 2017). Decapod crustaceans form an expressive group that is used as a model of these theories and enigma, since they exhibit extensive species diversity and a nuclear GS that vary (>40-fold) among species (1.07–40.89 pg) (Rees *et al.*, 2008; Gregory's Database: www.genomesize.com).

Genome size of caridean shrimps

The Infraorder Caridea forms also an expressive group in terms of richness of species and diversity of environments, with well-established findings on their biology, morphology, and behavior. Therefore, they represent an excellent biological model to study taxonomic, biogeographical, and ecological process that may have kind of influences on the GS patterns. The group is more consistent on its methods of measuring GS and presents more robust information regarding variation in species distribution and

habitats in comparison with other Decapoda's Infraorder. Brachyura, for instance, presents the largest register of taxonomically described specimens; however, it has been poorly explored in terms of GS (Jeffery *et al.*, 2016; Rees *et al.*, 2008).

Caridean shrimps seem to form a more interesting group because their GS was estimated to be within the range of 4.10–40 pg (Bachmann & Rheinsmith, 1973; Jeffery *et al.*, 2016; Rees *et al.*, 2008). The relationship between the number of described species on caridean shrimps and the number of GS described remains controversial, regardless of the methodology used. From more than 3,000 taxonomic valid species described, less than 100 species had their GS evaluated (De Grave & Fransen, 2011; Gregory's Database: www.genomesize.com), which makes the interpretation and formulation of a robust hypothesis about the biology and distribution of these animals difficult in terms of DNA content (Rees *et al.*, 2008).

Considering the current information available in literature, there exists a tendency of species having a higher quantity of GS in more polar distribution than in tropical areas (Hultgren *et al.*, 2018). This study aimed to describe certain characteristics of some snapping shrimps of the genus *Synalpheus* Bate (1888) (Decapoda, Alpheidae) that are found in Neotropical waters to determine if this new data improves the pattern estimation between the GS and habitat or geographical variables.

This genus can be divided into two informal groups: gambarelloides (mandatory symbionts of sponges) and nongambarelloides (free-living or facultative symbionts in coral and sponges) (Anker & De Grave, 2008; Ríos & Duffy, 2007). All data available in literature regarding GS and Gregory's Database of the *Synalpheus* are registered for specimens of gambarelloides group (Jeffery *et al.*, 2016)

This is the first register of GS for nongambarelloides group of *Synalpheus* shrimps. This work focused on five species that are widely distributed throughout the

Neotropical ecosystem and found in the Brazilian coast: *S. apioceros* (Coutière, 1909); *S. minus* (Say, 1818); *S. brevicarpus* (Herrick, 1891); *S. fritzmuelleri* (Coutière, 1909); and *S. scaphoceris* (Coutière, 1910). Characterization of the GS will add new information on *Synalpheus*'s GS database and provide answers to questions, such as "How caridean shrimps show patterns and adaptations in the environment?" and "How GS can be used to understand the biogeographical and local caridean's species distribution, especially *Synalpheus* shrimps?"

Answers to these questions would be assential for the futures description of the evolutionary process underlying the diversity of Decapoda worldwide.

Materials and methods

Sampling methods and species analyses

Synalpheus apioceros, *S. minus*, *S. brevicarpus*, *S. fritzmuelleri*, and *S. scaphoceris* were sampled from the "Couves" island (27°25′17″ S–44°51′36″ O) and "Itaguá beach" (23°27′32″ S–35° 02′ 96″) (Table 1), Ubatuba/SP, Southeastern Brazil, using SCUBA diving for active searching and Artificial Substrates of Refuge to capture as much species as possible (the sampling methods were described in the study by Moraes *et al.*, 2021). The specimens were taken alive to the laboratory to initiate the process of the GS description by flow cytometry analysis (FCA) according to the protocol adapted from Xavier *et al.* (2017).

Flow cytometry analysis

Animals were previously anesthetized using clove oil stock solution and saltwater (300 mg/ml). Then, a small portion (~2 mm^2) of muscle tissue was extracted carefully

from the animal's abdominal region. The subsequent FCA was conducted according to the procedures described by Xavier *et al.* (2017) for *Astyanax lacustris* (Characiformes: Characidae) (Lütken, 1875) with some modifications. First, the tissue was treated with 100 µL of lysing solution (9.53 mM $MgCl_2.7H_20$, 47.67 mM KCl, 15 mM Tris, 74 mM sucrose, 0.6% Triton X-100, and pH 8.0) to extract the cell nuclei.

In addition, the RNA was quantified using a Qubit RNA HS Assay Kit along with a Qubit® 2.0 fluorometer. This procedure was employed to obtain the best RNAs concentration, since the assay kit provides an accurate and selective method of quantitation of low-abundance RNA in samples; however, this method cannot be employed to quantify DNA, protein, or free nucleotides (datasheet ThermoFisher). Therefore, following this procedure, a control test tube and three test tubes with different RNAse concentrations were quantified: (1) control without RNAse, (2) 1 mg/mL RNAse, (3) 2 mg/mL RNAse, and (4) 3 mg/mL RNAse. After addition of RNAse, the samples were incubated at 37°C and vortexed for 10 seconds at 5 min intervals. The digestion was carried out in the test tube containing 44.6 ng/ml RNA and in other test tubes. Afterward, the media concentration of 2 mg/mL RNAse was used as a standard for FCA.

Finally, the material was filtered with a 30 µm mesh filter (Celltrics, Partec, Munster, Germany) and the nuclei suspensions were stained with 1 mg/mL PI that binds double-stranded DNA. Cell cycle analysis of at least 20,000 nuclei for each sample was done by FCA using a FACSCanto II flow cytometer with FACSDiva software. Data analysis and histogram construction were done with FlowJo, vX.10.6 version (Tree Stars Inc) and expressed as median fluorescence intensity (Figure 1). Figure 1 shows a representative histogram outlining the gating strategy for the analysis. Gates were used to generate a SSC vs. PI plot and DNA histogram.

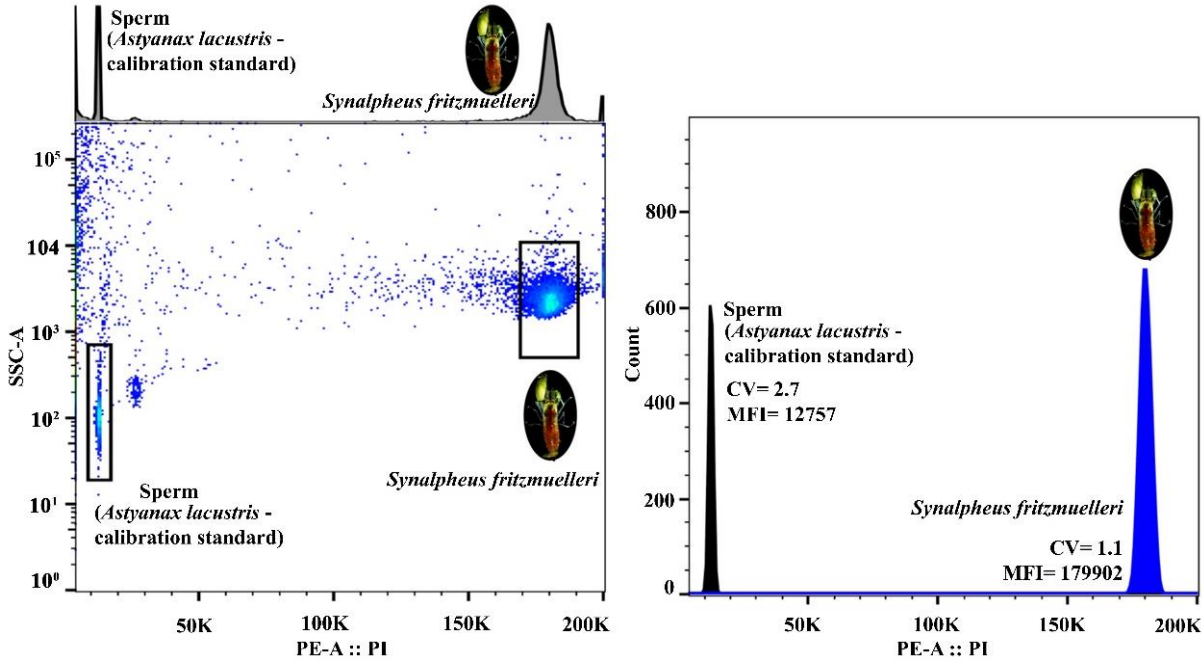

Figure 1: Sample Flow Cytometry analysis showing gating strategy. Propidium Iodide (PE-A:: PI) versus Side Scatter parameter (SSC-A: parameter that measure the amount of the laser beam that bounces off of particulates inside of the cell), showing the gates of calibration standard (*Astyanax bimaculatus*) and the caridean shrimp, *e.g.*, *Synalpheus fritzmuelleri*. CV: coefficient of variance, MFI: median fluorescence intensity, Count: nuclei number.

To estimate the DNA content, all analysis was conducted with a sperm sample of *A. lacustris* and then used as a calibration standard, since the species presents a DNA content of 1.47 pg (Carvalho *et al.*, 2002). The sperm samples (~2 µL) were processed as previously mentioned for caridean tissue. After staining, the sperm sample was added to each shrimp sample (~10 µL per sample). The coefficient of variance was controlled and maintained below 3% in order to generate accurate analysis (Figure 1).

Most studies reporting GS in caridean shrimps use mainly two methodologies to obtain information on nuclear cells: Feulgen Image Analysis Densitometry (FIAD) (Bachmann & Rheinsmith, 1973; Jeffery *et al.*, 2016; Jeffery & Gregory, 2014; Jimenez *et al.*, 2010) and FCA (Bonnivard *et al.*, 2009; Rees *et al.*, 2008). Both techniques produce the same results and can be used to compare the final data without any setback or errors on the C-value (Jeffery *et al.*, 2016). Therefore, FCA was adopted in the present study;

175 however, in addition, we compared our results with the information in literature obtained
176 by FIAD.
177
178 ***Taxonomic relationship and phylogenetic reconstruction of Synalpheus***
179
180 We compared the C-values among the groups, obtaining a general panorama from
181 the crustacean decapod's GS and detail of Caridea Infraorder and *Synalpheus* genus
182 (gambarelloides with direct development or abbreviated larval stage *versus*
183 nongambarelloides with at least two larval stages). All specimens analyzed in literature
184 and in this study in terms of biological distribution and C-value are listed in Table 1 and
185 supplementary material. Differences among the groups were determined by analysis of
186 variance, followed by Tukey *posthoc* test and illustrated with box-plots. The statistical
187 analyses described below were performed using R v.3.5.3 software.
188 To contrast the shrimp traits with GS, phylogenetically controlled analysis of the
189 Bayesian phylogeny from a genetic data (mitochondrial locus COI from Genbank) was
190 performed. We adapted the methodology of Valdes *et al.* (2020) to obtain the Bayesian
191 inference. A secondary calibration of divergence times were used to obtain an ultrametric
192 *Synalpheus* phylogeny tree. A birth-death process (Stadler, 2009) with an initial random
193 tree was conducted. The substitution model of selection was implemented with AC = AT,
194 AG = CT, and CG = GT with empirical base frequencies and four gamma categories. As
195 a secondary calibration point, a prior was set for the age of the common ancestor of the
196 genus *Synalpheus*, with a mean of 4 and standard deviation and 0.5 (Hurt *et al.*, 2021).
197 Two independent runs, consisting of 100×10^6 Markov Chain Monte Carlo iterations and
198 sampling change every 2000 generations, were used. Convergence was checked using
199 Tracer v. 1.7.1 (Rambaut *et al.*, 2018), obtaining an effective sample size greater than 200
200 for all parameters. Tree and log files were combined using LogCombiner included in

BEAST2 (Bouckaert *et al.*, 2014). The caridean shrimps *Macrobrachium lohena* (Banner & Banner, 1960) and *Alpheus heterochaelis* (Say, 1818) were used as out-group.

Phylogenetically controlled analyses

Statistical analyses to evaluate the effect of mean carapace length (CL), Latitude Midpoint, and Hemisphere on GS were according to our data and those of Gregory's Database and Jeffery *et al*. (2016) (and its supplementary material) regarding the caridean Infraorder, specifically the *Synalpheus* genus. A Generalized least square analysis was performed to incorporate these variables (without interactions) and a model selection procedure was performed based on corrected Akaike Information Criteria (AICc) (Burnham & Anderson, 2002). For this purpose, all combinations between these variables were generated and evaluated using the *dredge* function of the MuMIn package (Bartoń, 2020). From the list, models with an AICc delta <4 were selected and this subset of variables was evaluated using the *model.sel* function of the same package. For these analyses, Pagel's λ (pgls) was previously estimated from a full model (that includes all variables) with Restricted Maximum Likelihood (ML). Thus, in the models described above, the value of lambda (λ) was equal to 1, thus indicating that the distribution of the residuals of the full model fit as expected by Brownian Motion. The same is true if we only consider the GS of Pagel (where the value of λ is 0.99). Finally, for significant variables, we performed correlations using both raw species means and phylogenetic independent contrasts (PICs). For all analysis, in order to avoid spurious correlations forced by these nonobjective species, the out-group was not included.

Results

Caridean diversity and genome size

A total of 30 *Synalpheus* individuals of the five species were analyzed (6 specimens for each species sampled) and it was found that the C-values (mean ± SD) ranged from 7.89 ± 0.05 pg to 12.24 ± 0.54 pg (Table 1 and Figure 2). The difference between the GS among species was significant ($p < 0.001$) and pairwise multiple comparison (Tukey *posthoc* test) revealed that all pairwise species comparison were significant ($p < 0.05$).

We found similar C-values between the groups of Decapoda crustaceans when the results of the present study are combined with data from Gregory's database and other GS values in literature (available at supplementary data), except for caridean shrimps in which the C-value ranged higher that of other groups (Figure 3 and Table 2). Furthermore, Caridea presented higher amplitude of C-value and a large range of GS among Decapoda (Figure 3).

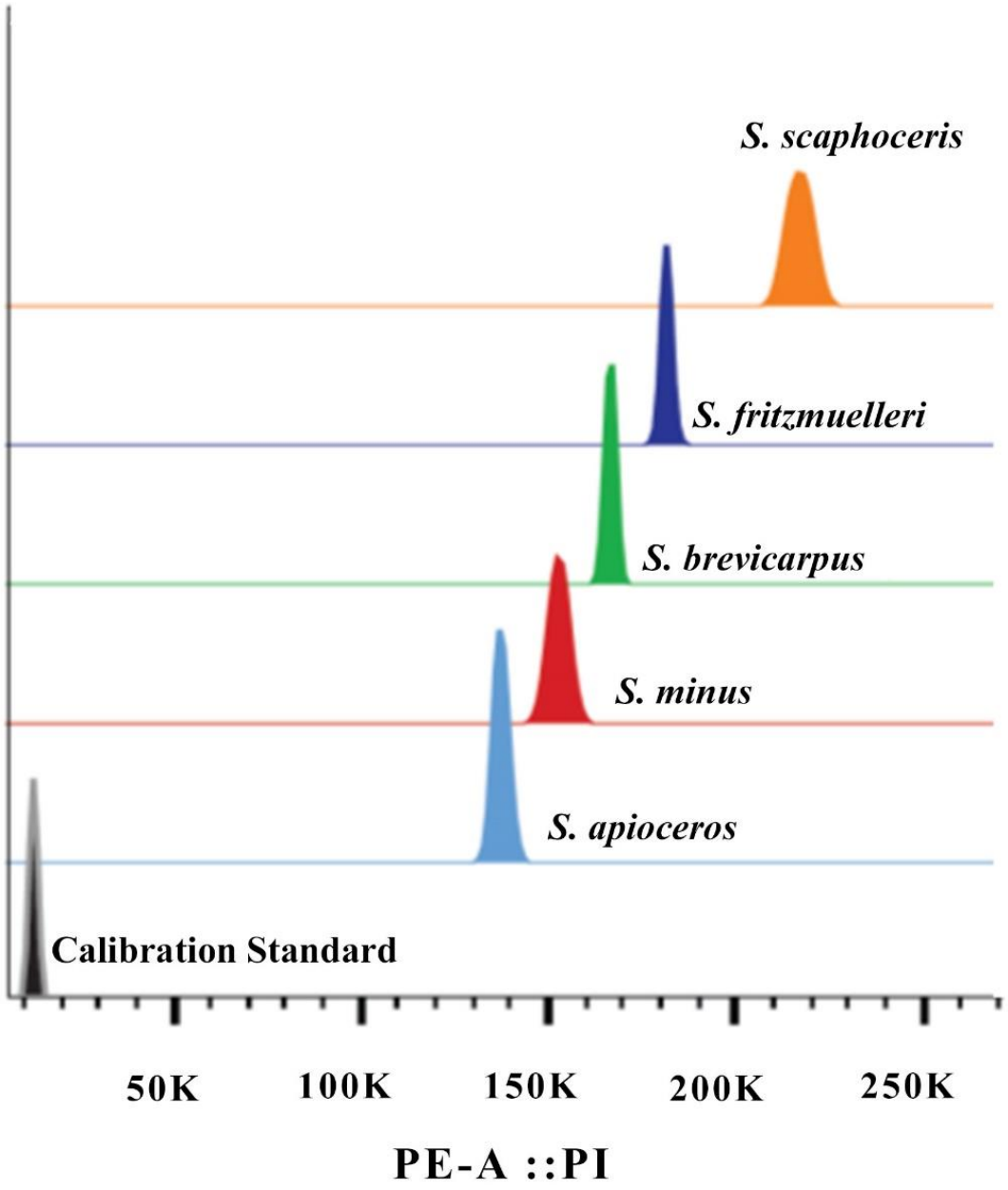

Figure 2: Histogram design (median fluorescence intensity - MFI) for values found on *Synalpheus* and calibration standard species. All genome size measured per species was different as *Astyanax lacustris* (black histogram = calibration standard), *Synalpheus apioceros* (light blue histogram), *Synalpheus minus* (red histogram), *Synalpheus brevicarpus* (green histogram), *Synalpheus fritzmuelleri* (dark blue histogram), and *Synalpheus scaphoceris* (orange histogram).

291 **Table 1:** List of *Synalpheus* spp. analyzed in the present study with Mean and Standard
292 Deviation in the Genome Size values (pg), Geographical coordinates (GC) from the
293 present study and geographical distribution of species in the world.

294

Species	C-value (pg)	CL (mm)	GC	Distribution
Synalpheus apioceros	7.89 ± 0.05	3.95 ± 0.90	23°26′S 45°02′W	Atlantic Ocean, United States to Brazil (Almeida *et al.*, 2016)
Synalpheus minus	8.99 ± 0.22	4.10 ± 0.99	23°26′S 45°02′W	Atlantic Ocean: the United States to Brazil (Cunha *et al.*, 2015; Christoffersen, 1998; Cunha *et al.*, 2015; Almeida *et al.*, 2016)
Synalpheus brevicarpus	9.77 ± 0.37	4.28 ± 1.13	23°26′S 45°02′W	West Atlantic (Anker *et al.*, 2016a)
Synalpheus fritzmuelleri	10.46 ± 0.09	3.96 ± 0.83	23°26′S 45°02′W	Western Pacific, Caribbran Sea and Brazil. (Coutière, 1909; Anker *et al.*, 2012, 2016b; Coutière, 1909; Christtoffersen, 1998; Anker *et al.*, 2012, 2016)
Synalpheus scaphoceris	12.24 ± 0.54	3.99 ± 0.86	23°26′S 45°02′W	Atlantic Ocean: the Gulf of Mexico to Brazil (Dardeau, 1986; Anker *et al.*, 2012; Christtofersen, 1979; 1998; Dardeau, 1986; Anker *et al.*, 2012)

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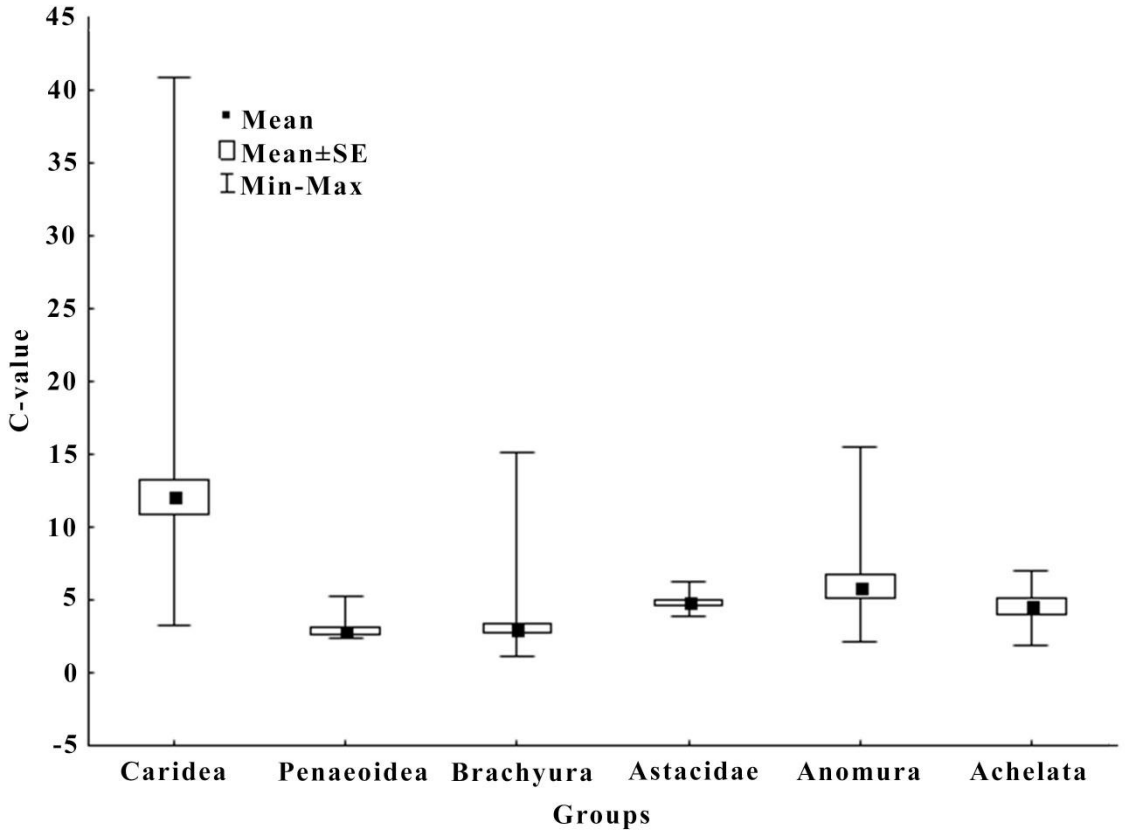

Figure 3: BoxPlot analyzes to compare the Genome Size into different groups in Decapoda Crustaceans: Caridea, Penaeoidea, Brachyura, Astacidae, Anomura, and Achelata were used as a data basis.

Table 2: List of Decapoda Crustaceans big groups analyzed from database - Genomesize.com - with number of specimens analyzed from the present study and literature registers, and C-value picograms (mean ± SD) contents.

Group	Number of Species	C-value (pg)
Caridea	47	12.07 ± 8.24
Anomura	17	5.91 ± 3.32
Astacidae	11	4.84 ± 0.74
Achelata	10	4.57 ± 1.77
Brachyura	53	3.33 ± 2.25
Penaeoidea	12	2.85 ± 0.78

No differences were detected (Kruskal-Wallis test, $p = 0.17$) in GS comparison among three categories of *Synalpheus*, considering the life-history trends and development types (Figure 4). The mean GS for the three distinct groups was: (1) 8.73 ± 3.76 pg for Gambarelloides *Synalpheus* with indirect development, which presents at least one larval phase in development; (2) 10.63 ± 2.12 pg for gambarelloides *Synalpheus* with direct development or at least postlarvae abbreviated development; and (3) 9.87 ± 1.63 pg for nongambarelloides *Synalpheus* (not sponge obligatory symbiont), which was used in the present study, without any evidence of direct development.

The selection model analysis included the CL, CL + hemisphere, and CL + Latitude as the best models for explaining the variation in GS in *Synalpheus.* However, only CL showed significant coefficients (Table 3). Pagel test showed a high phylogenetic signal in GS ($\lambda = 0.99$) (Figure 5). The PICs regression between GS and CL showed a negative relationship ($R^2 = 0.654$, $p < 0.0001$), with a slope of −0.305 (Figure 6). Notwithstanding, the results of pgls and PICs regression have shown a hard polytomy between *S. longicarpus* (Herrick, 1891) and *S. dardeaui* (Ríos & Duffy, 2007). When both analyses are repeated after excluding these species, the relationship between GS and CL disappeared (Figure 6).

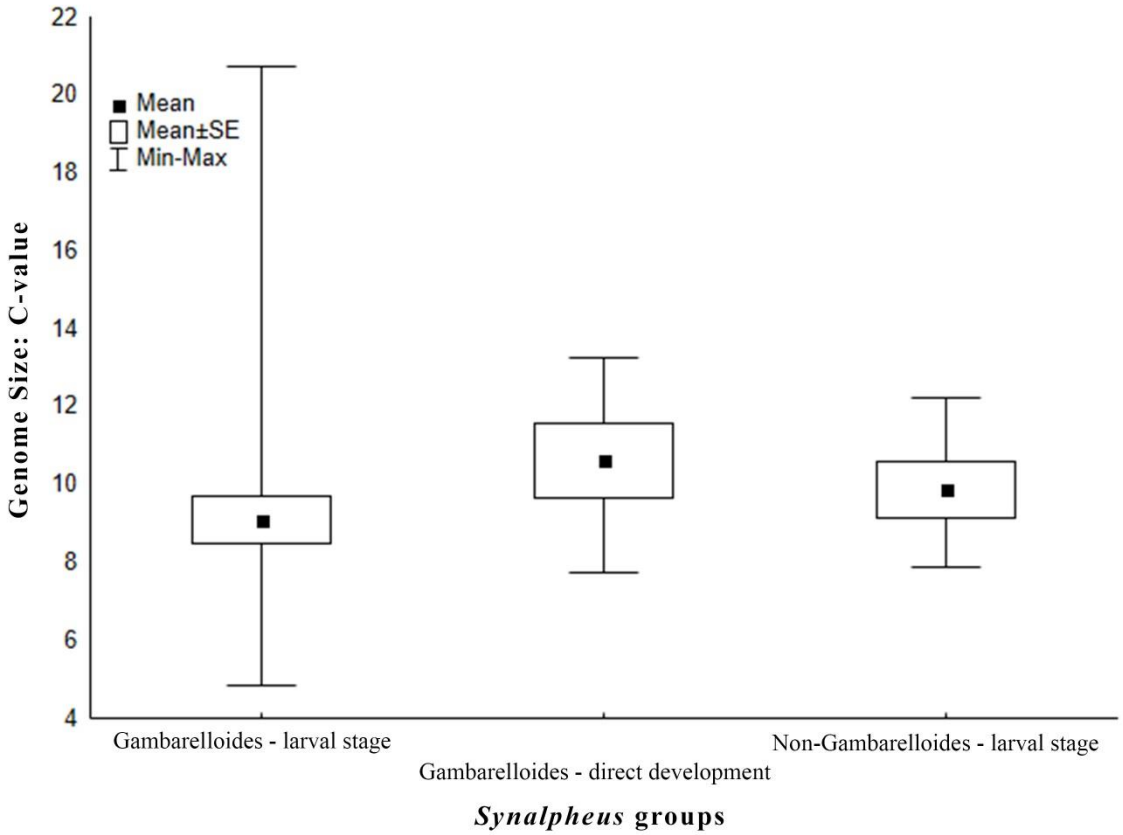

Figure 4: BoxPlot analyzes to compare the Genome Size into different groups of ecology and development patterns: categories are: *Synalpheus* gambarelloides eusociais with indirect development; *Synalpheus* gambarelloides eusociais with direct development and *Synalpheus* non-gambarelloides free pair-living with indirect development.

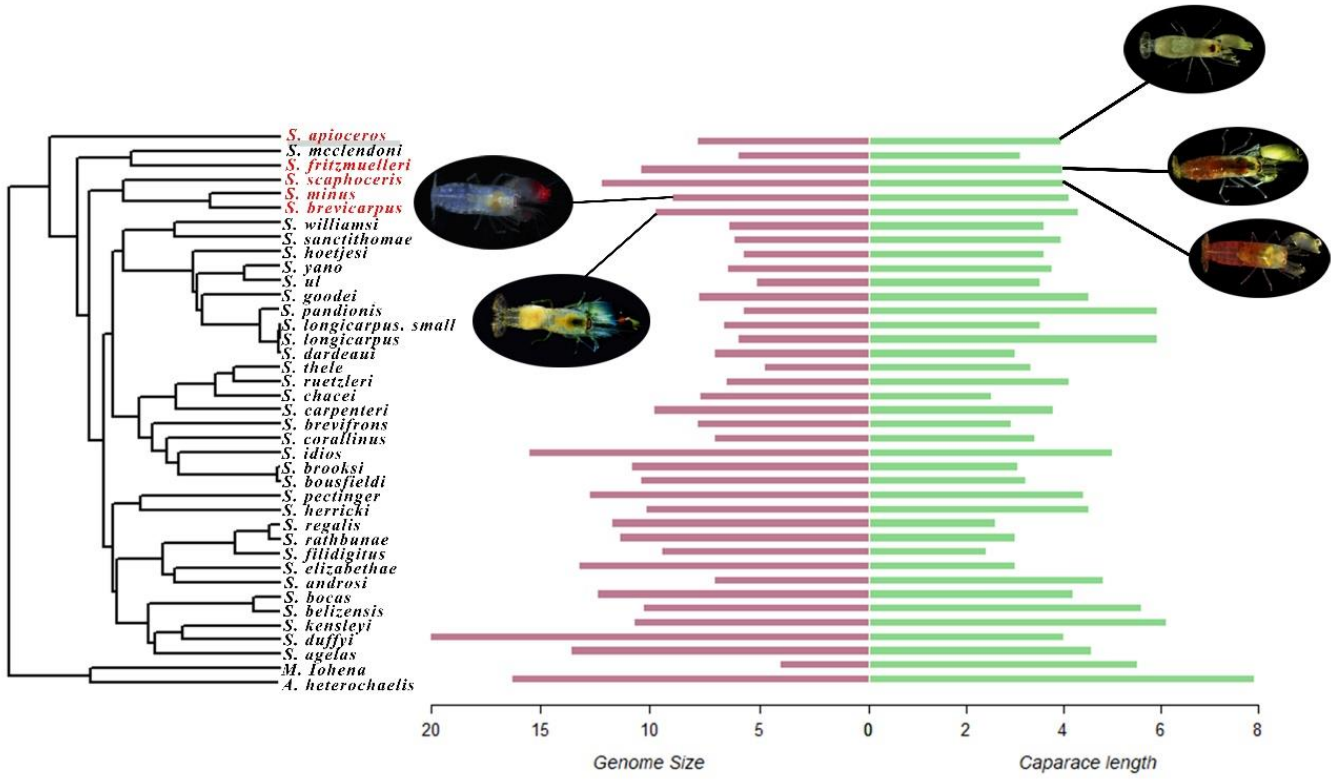

Figure 5. Phylogenetic controlled comparison of genome size and carapace lenght in shrimps from *Synalpheus* genus.

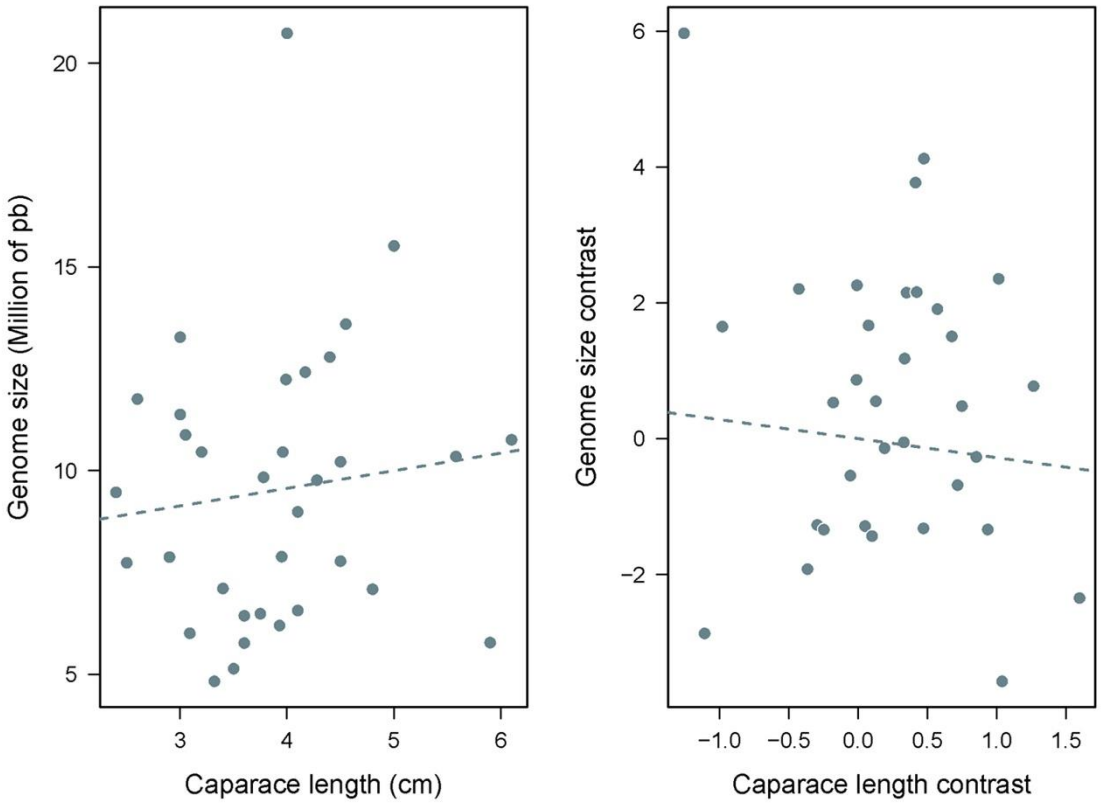

Figure 6: Correlations using both raw data and phylogenetic independent contrasts (PIC) between mean carapace length of *Synalpheus* species and genome size. Outgroup and polytomy between *S. longicarpus* and *S. dardeaui* was excluded.

Discussion

Genome size and reproductive aspects

The life history and reproductive strategies of species, as a modulatory content to the GS in many animals, seems to have a trend involving lower C-values for those R-selected species (that tends to invest in a numerous offspring with great dispersion capacity), thus favoring small cells, rapid growth, and lower DNA contents, in contrast with K-selected species (that tends to invest on the quality of the offspring with lower fecundity and more parental care to ensure the survival of specimens to perpetuate and spread the genes) with larger cells and lower growth pattern (Rees *et al.*, 2007, 2008).

In the present study, the R-selected decapods were represented by the Penaeoidea group (Dendrobhanchiata). The K-selected ones were represented by the Pleocyemata, *i.e*, Caridea, Brachyura, Astacidae, and Achelata (Fenwick, 1984; Parry, 1981). The mean GS values of all groups within Dendrobrachiata and Pleocyemata were closely related, despite a little lower value for Dendrobrachiata, and exceeded the caridean shrimps that present a mean GS larger than that of the other groups (Figure 3). There is scanty data available on the GS description of Decapoda, especially for R-selected groups, to conclude a confident panorama in this regard. However, there exist a tendency in correlation between GS and reproductive strategy.

In addition, a pattern in the *Synalpheus* genus, in which the animals with abbreviated or direct development tend (with some variations) to present higher GS values was observed. These animals have mean C-values up to 10 pg while species with many larval stages tend to show lower C-values. Although, more robust data is needed to present a robust conclusion, it can be considered in this genus.

Body size and biogeographical perspectives

The relationship between CL and geographical distribution can be determined as proposed by Hultgren *et al.* (2018), but it is still under evaluations. Moreover, the PIC analyses seem to be very useful in making those patterns clearer when dealing with larger data, especially for the *Synalpheus* group.

Given the variability in the GS patterns within the crustacean groups, *e.g.* decapod infraorders (Figure 3), the relationship between GS and carapace size in monophyletic small groups (excluding out-groups species and hard polytomies on two species) may be very useful to validate the general patterns. In this study, using a phylogenetically controlled analysis, the relationship between GS and size disappears when PIC was

404 performed for *Synalpheus* genus, which was similar to the significance of size in the pgls
405 analysis.

406 Based on the factors presented above, GS seems to be linked with evolution and
407 adaptive patterns. For the caridean shrimps, this fact is clear based on the phylogeny and
408 biogeographical/ecological distribution of the species (Hultgreen, *et al.*, 2018). In this
409 regard, the C-value can be modulated in relation to the environmental and ecological
410 implication. The evolutionary variation can be intrinsic to speciation, for instance, the
411 biogeographical latitudinal distribution can be considered for the general worldwide
412 caridean shrimps and development pattern can be considered for the Neotropical's
413 distributed *Synalpheus*.

414 Hultgren *et al.* (2018) tested several variables related to GS in crustaceans. For
415 Decapoda, only the caridean shrimps presented the tendency of a positive relationship
416 between latitudinal distribution and GS. However, the authors also noted negative
417 relationships between GS and a number of larval stages, such that the larger genomes
418 were observed in species with abbreviated or direct development. For instance, species
419 from Caridea Infraorder with the largest C-value, such as *Sclerocrangon ferox* (Sars,
420 1877) (Decapoda, Crangonidae), usually presents direct development and polar
421 distribution (Figure 7 and supplementary data). We corroborated and reaffirmed the
422 latitudinal trend proposed by Hultgren *et al.* (2018) by adding more data on the caridean
423 shrimps from tropical distribution and comparing them with those described in the
424 literature. Therefore, the tendency of a latitudinal factor was maintained with the idea of
425 positive correlation between both parameters (GS vs. Latitude).

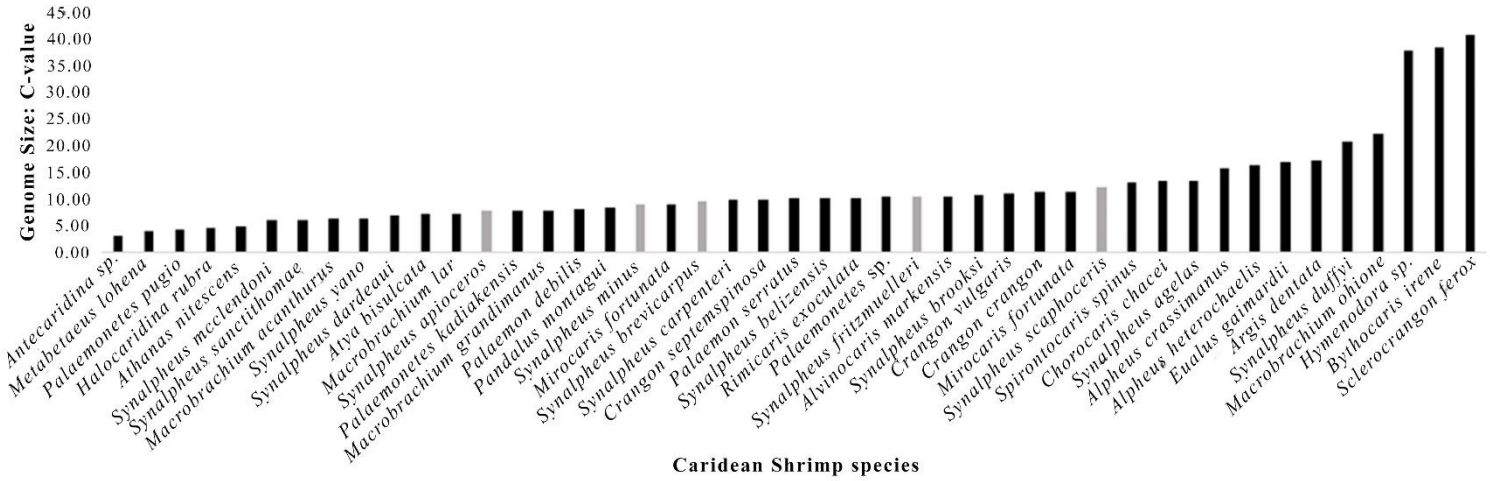

Figure 7. Genome Size of most of the caridean shrimps analyzed on literature and database from Genomesize.com (excluding values found on supplementary data from Jeffery *et al.* 2016): Gray bars indicate species values analyzed in the present study

Development pattern in Synalpheus spp.

In this study, some species of the snapping shrimp *Synalpheus* with abbreviated or direct development (Duffy & Macdonald, 2009) were found. All the species with proven direct development already had the GS patterns available in the supplementary material from Jeffery *et al.* (2016), *i.e*, *S. elizabethae* (Ríos & Duffy, 2007) (C-value = 13.28 pg), *S. regalis* (Duffy, 1996) (C-value = 11.76 pg), *S. filidigitus* (Armstrong, 1949) (C-value = 9.47 pg), *S. chacei* (Duffy, 1998) (C-value = 7.74 pg), and *S. brooksi* (Coutière, 1909) (C-value = 10.88 pg). Our comparative group showed the same tendency proposed by Hultgren *et al.* (2018) with higher GS values for *Synalpheus* gambarelloides with direct or abbreviated development (Figure 5). We suggest the incorporation of more samples and species with larval description to this pattern in order to corroborate or validate the hypothesis mentioned above, but emphasizing once again that the evolutionary events must be independently assessed in the groups, since ontogenetic

characteristics of development can also be modulated by epigenetic factors, more than just biogeographical distribution (Gibny & Nolan, 2010).

For the snapping shrimps, considering all the species that present an obligatory symbiosis with the Porifera in the gambarelloides group, we found species with a particular behavior similar to eusociality, where just one female (the queen) is the reproductive female in the colony (Duffy, 1996; Duffy, *et al.*, 2000; Duffy, 2003) and this condition can be considered in the reproduction and evolution aspects of the genus.

Future perspectives in genome size science with Synalpheus shrimps

From the foregoing, as a conclusion to the hypothesis made for this genus, it can be stated that GS pattern is strongly related with life history and reproduction. We suggest that the next steps should involve the analysis of many *Synalpheus* species and evaluation of the following: (1) the fecundity and reproductive output of species from "the queens" and not the eusocial species; (2) the number of larval stages; and 3) the reproductive biology and population sizes of species.

Rubenstein *et al.* (2008) conducted microsatellite studies on multiple species of *Synalpheus* and suggested a duplication event in the genome of these caridean shrimps. However, Jeffery *et al.* (2016) evaluated the GS and karyotype of four *Synalpheus* species and found no evidence of polyploidy. Since the authors correlated only the size of the chromosomes with the size of the genome, they suggested that segmental duplications or paleopolyploidy (*i.e*, ancient polyploidy in the lineage of the shrimps that may be difficult to detect due to duplicate gene deletion over evolutionary time (Garsmeur *et al.*, 2014) may explain the microsatellite data and the large GS in many species of *Synalpheus*.

Chromosomal studies could be useful in elucidating several issues that may influence the GS. For example, the karyotype could aid the knowledge of possible

euploidy and aneuploidy events (Jeffery *et al.*, 2016); the pattern of heterochromatin can assist in the quantification of repetitive (inactive) regions of DNA and transposable elements (Sumner, 2003; Vitturi *et al.*, 1997), and studies of molecular cytogenetic with epigenetic markers aid the understanding of adaptive genomic evolution (Barzotti *et al.*, 2006; Jablonka, 2017). For the *Synalpheus*, analyses of this same perspective were recently started by Chak *et al.* (2021) with the gambarelloides group. There is a need to improve the parameters for nonsymbiotic species as done in this present result.

In conclusion, this work described, for the first time important information regarding the DNA content obtained by FCA among five *Synalpheus* nongambarelloides species. We hypothesize that the environmental and cytogenic adaptations are fundamental to explain the modulation of GS and patterns of this genetic features among caridean shrimps. Also, for the *Synalpheus* genera, specific adaptation and reproductive patterns seem to modulate the GS behavior in subtropical species, especially the development process, since exist a tendency of the species with direct development to present the largest GS content, thus reinforcing the importance of the knowledge of the basic biology of species in making inference regarding their evolution history and patterns.

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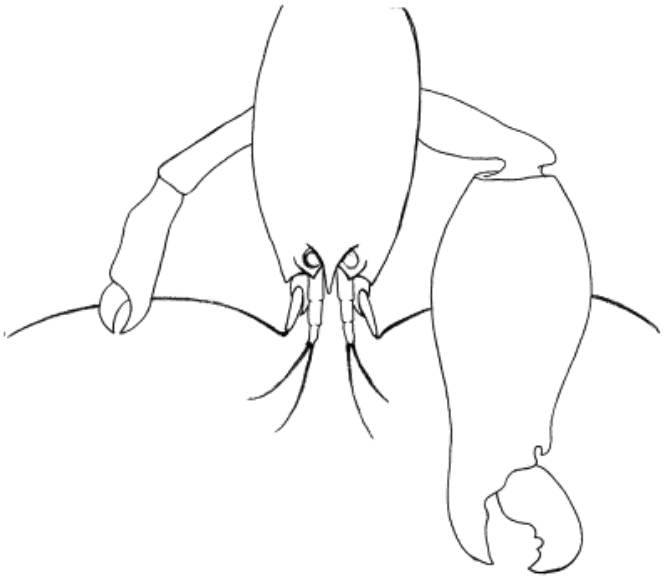

Capítulo 05

Perspectives of the genome size in the Caridea Infraorder using latitudinal variation as evaluation model

Abstract

The genome size, considered as the "biomass" of the information content at genes and chromosomes in eukaryotes can be related to the cellular size, rate of cellular division, cytogenetics variations, and intrinsic characteristics and metabolic perspectives in each organism. But also, those values can variate into biogeographical perspectives into Metazoa, as proposed in Bergmann's theory that habitats in higher latitudes and depths (correlating lower temperatures) imply a higher cellular size, life cycle, and consequently in greater body size of animals. So, it is available to propose a direct correlation into genome size (GS) x biogeographical distribution. In the present study we evaluated neotropical Alpheidae shrimps and compared the genome size with other literature data available as: environment where the caridean shrimps are found, and latitudinal gradient and caridean sizes (carapace length) classes. We were able to observe different patterns of GS comparing species that inhabit the deep sea (beyond 100m deep) with other environments. The same can be found when observing the latitudinal trend in GS from caridean shrimps, in which species that inhabit higher latitudes present significantly higher GS. Here, Bergmann's theory seems to be applied even when we evaluate more neotropical species. The next step in future works will have the basis on information acquired in results presented in works like the present from Nuclear DNA weight joining the basic biological sciences from distribution to behaviors to confirm and turn even more robust the theory proposed here.

Keywords: Bergmanns' Theory, C-value, Flow Citometry, latitudinal paradigm.

Introduction

The Nuclear DNA content is presented as one of many tools used to investigate evolutionary perspectives and lifehistory of animals alive. Is considered as the “biomass” of the information content at genes and chromossomes in eukaryots, and measured in Picogrames (pg) of the DNA per haploid nuclei, compared and visualized as a constant determined as “C-value” (Gregory, 2005).

The genome size (GS) has been already reported for more than 10.000 organisms between plants and animals, and is considered as a very new and promissore technique, that can be related with the celular size, rate of celular division, cytogenetics variations and intrinsic characteristics and metabolic perspectives in each organisms (Bainard and Gregory, 2013; Gregory *et al.*, 2013).

One of those variables that can answer, or at least help on interpretations of those values is the biogeographical position of animals. The animals with higher values on “c-value” content registered between Crustacea are from the Amphipoda and Caridea group sampled in polar regions (Rees *et al.*, 2008; Hultgren *et al.*, 2018). The classical Bergmann’s theory (Bergmann, 1948) proposes that habitats in higher latitudes and depths (correlating lower temperatures) implies in a higher celular size, life cycle and consequently in a greater body size of animals.

This principle was applied in a diverse group, joining positives and negatives relationships. One of the most sucessful examples of this theory is related just with of Amphipoda and Copepods (Campbell *et al.*, 2021), in wich generes species distributed on Atlantic Ocean and circumpolar Arctic present distincs longevity and body sizes, being the polar animals notable with elevated values in both perspectives. Because of the great longevity and longer life spectancy, the polar species are capable to growth during more

time, reaching, biger body sizes, even tending to the gigantism. It can be tested in intra or interspecific levels (Timofeev, 2001). We can evaluate those perspectives also in the genome size expression, as proposed in the present study using, Decapoda as model to evaluate.

The theoretical foundation supporting Bergmann's principle is directly related to latitudinal variation, decrease in ambient temperature, longevity attributed to cell and body size. In species with Polar distribution, it is expected to find these patterns and associate the proposed principle with the assumptions of GS (the larger the body size, the greater the weight of nuclear DNA).

The aim of the present work is to provide new data about Alpheidae species from tropical Brazilian region and look at the panorama of the genome size using all available data of the literature about the caridean shrimps and their distribution worldwide, reforcing the first evaluation proposed by Hultgren *et al.* (2018) that latidunal distribution may have one of the strongest correlation with the genome size in the Caridea Infraorder.

Material e Métodos

Sample methods

The Alpheidae shrimps *Alpheus formosus* Gibbes, 1850 and *Alpheus thomasi* Hendrix and Gore, 1973 were sampled in the Couves island (23°25'S; 44°51'W), Ubatuba, São Paulo state, Brazil, associated with corals fragments of the *Madracis decatis* (Lyman, 1859) species. The pieces of the corals were individualized in plastic bags to posterior inspection looking for the associated fauna. Every specimen found was separated and kept alive in an aerated thermic box to be transported to the laboratory at São Paulo State University, Botucatu region.

The animals were identified using taxonomical Keys (Soledade and Almeida, 2013), and then submitted to the genome size measurement using flow cytometry analyzes. All genetic procedures were conducted in collaboration with the Laboratory of immunology from São Paulo State University.

Other caridean shrimps used in this analyzes were the same proposed on Chapter 4 of the present thesis.

Flow cytometry analyzes

Animals were anesthetized using a clove oil stock solution and saltwater (300mg/ Ml^{-1}). A small portion of the muscle tissue was extracted carefully from the animal's abdominal region. The subsequent Flow Cytometric Analysis (FCA) was conducted according to the procedures of Xavier *et al.* (2017), for *Astyanax lacustris* (Lütken, 1875) (Characiformes: Characidae) with some modifications. Firstly, the tissue was treated with 100 µL of lysing solution (9.53 mM $MgCl_2.7H_20$; 47.67 mM KCl; 15 mM Tris; 74 mM sucrose, 0.6% Triton X-100, pH 8.0) in order to extract the cell nuclei. Additionally, the RNA was quantified using a Qubit RNA HS Assay Kit followed by estimation by Qubit® 2.0 fluorometer. Such a procedure was used to obtain the best RNAs concentration in which all the RNA was digested since this assay kit provides an accurate and selective method for the quantitation of low-abundance RNA samples and not quantitates DNA, protein, or free nucleotides (datasheet ThermoFisher). Finally, the material was filtrated in a 30-µm mesh filter (Celltrics, Partec, Munster, Germany) and the nuclei suspensions were stained with 1 mg/mL of Propidium Iodide (PI) that binds to double-stranded DNA. Cell cycle analysis by flow cytometry was carried out using a FACSCanto II flow cytometer with FACSDiva software, which analyzed at least 20 000 nuclei for each

sample. Data analysis and histogram construction were carried out with the software FlowJo, vX.10.6 version (Tree Stars Inc), and was expressed by the median fluorescence intensity (MFI).

To estimate the DNA content, all analyses were conducted with a sperm sample of *A. lacustris* and were then used as a calibration standard since the species presents a DNA content of 1.47 pg (Carvalho *et al.*, 2002). The sperm samples (~2 µL) were processed as mentioned previously for caridean tissue. The sperm sample was added to each shrimp sample (~10 µL per sample). The coefficient of variance was controlled and maintained below 3% to generate accurate analysis.

Statistical evaluations

Statistical analyzes were performed to test differences in the GS into differents categories according to our data, plus Gregory's Database and Jeffery *et al.* (2016) (and its supplementary material) regarding the infraorder Caridea. Two Way Analysis ANOVA and post-hoc Tukey analyses and box-plot comparison graphs were made to show differences between the parameters:

(1) Environment where the caridean shrimps are found: freshwater, estuarine sites, shallow-water marine system, and deep-sea marine system;

(2) Latitudinal gradient that the caridean shrimps are found: 3 – 23°S, 3 – 23°N, 23 – 43°N, 43 – 63°N, 63 – 53°N;

(3) Caridean sizes (carapace length) classes: 1 – 2.99 mm, 3 – 5.99 mm, 6 – 8.99 mm, 9 – 11.99mm, 12 – 14.99mm, 15 – 17.99mm, 18 – 20.99mm, >21mm.

All analyzes were conducted with programs Rstudio and Statistica version 12.0.

We sorted the C-values by groups to obtain a general panorama from the crustacean decapod's GS and detailed for Caridea infraorder.

Results

Five abdominal samples were taken from the *Alpheus formosus* varying from 1.73mm to 2.71mm of Carapace Length and used to demonstrate the genome size. The "*c-value"* obtained were mean 8.87±0.62 pg and can be well observed at histogram and fluorescence graphs (Figure 1).

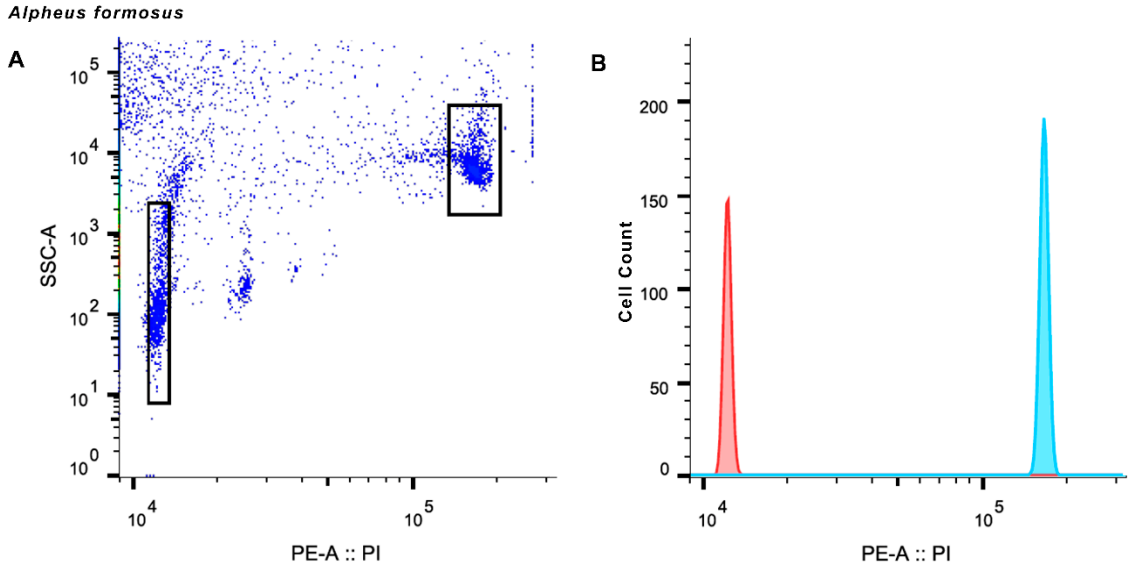

Figure 1: *Alpheus formosus* graphs of stained cells and evaluations of the genome size: A) Fluorescence intensity of the cell's dispersion under the flow cytometry tool. B) Histogram design for all values found in the present study. The red peak represents the control species *Astyanax lacustris.* The blue peak indicates the 2n cells from abdominal region of the *Alpheus formosus*. PE-A::PI=The fluorescence intensity stained by nuclei; Cells count= The number of nuclei counted on the flow cytometry.

The *Alpheus thomasi* were evaluated for one sample from abdominal region of the animal measuring 4.75mm of CL size. The *c-value* was 7.69 pg and results are also well represented at fluorescence and histogram graphs (Figure 2).

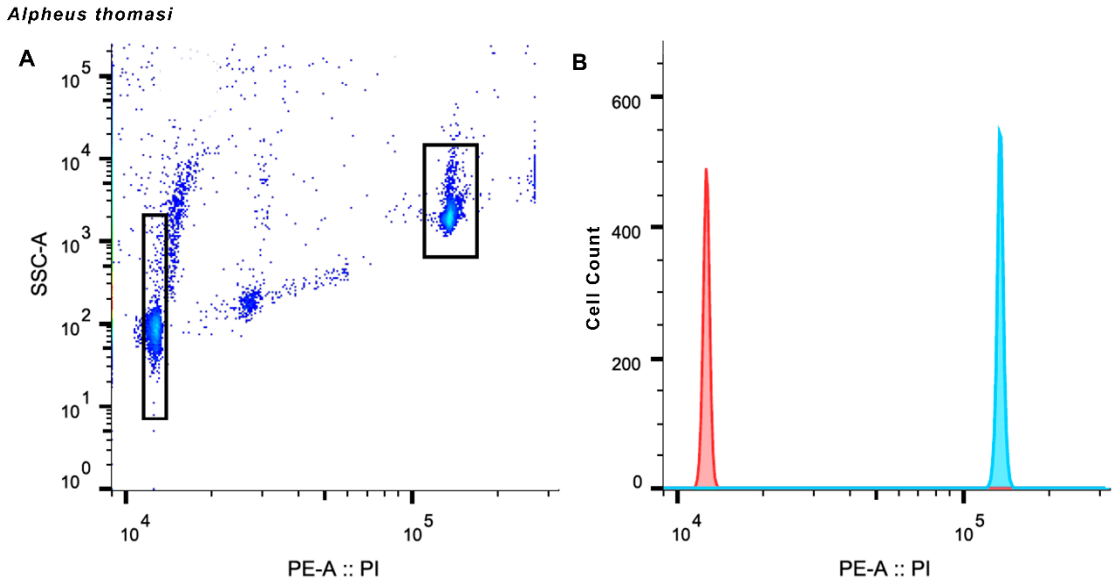

Figure 2: *Alpheus thomasi* graphs of stained cells and evaluations of the genome size: A) Fluorescence intensity of the cell's dispersion under the flow cytometry tool. B) Histogram design for all values found in the present study. The red peak represents the control species *Astyanax lacustris.* The blue peak indicates the 2n cells from abdominal region of the *Alpheus thomasi;*. PE-A::PI=The fluorescence intensity stained by nuclei; Cells count= The number of nuclei counted on the flow cytometry.

Results of *Synalpheus* shrimps are presented on **chapter 4**.

ANOVA tests only shows significative statistical differences into the "latitudinal distribution" among the Caridean shrimps: $F=10.798$, $P<0.01$. Observing the box-plot graphs, comparing all the variables, beyond the numeric results, it is possible to observe some particularities and make a few inferences on GS and life-history patterns of caridean shrimps.

In a general scenario, taking into account the different habitats in which caridean shrimps inhabit, we can observe different patterns of GS comparing species that inhabit deep sea (beyond 100m deep) with another environment (Figure 3).

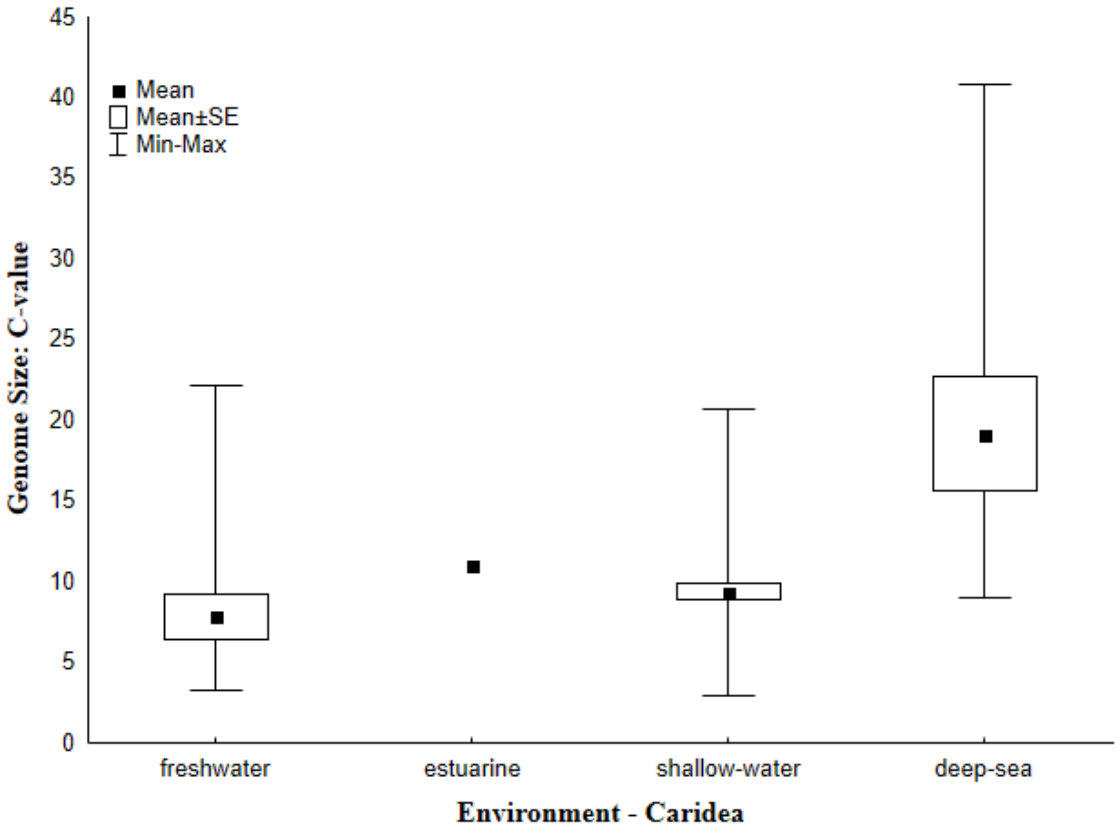

Figure 3: BoxPlot analyzes to compare the Genome Size into different environments that caridean shrimps can be sampled: Freshwater; Estuarine sites; Shallow-Water Marine System; Deep-sea marine system.

The same can be found when observing the latitudinal trend in GS from caridean shrimps, in which species that inhabit higher latitudes present significant higher GS (Figure 4). Therefore, it can be interpreted that genome size could be linked with local adaptations, once in deep sea environments and temperate/polar regions the animals would need further adaptations due the limits imposed by these extreme environments, which can be reflected through the discrepant GS and its genetics specifications (Hultgreen *et al.*, 2018).

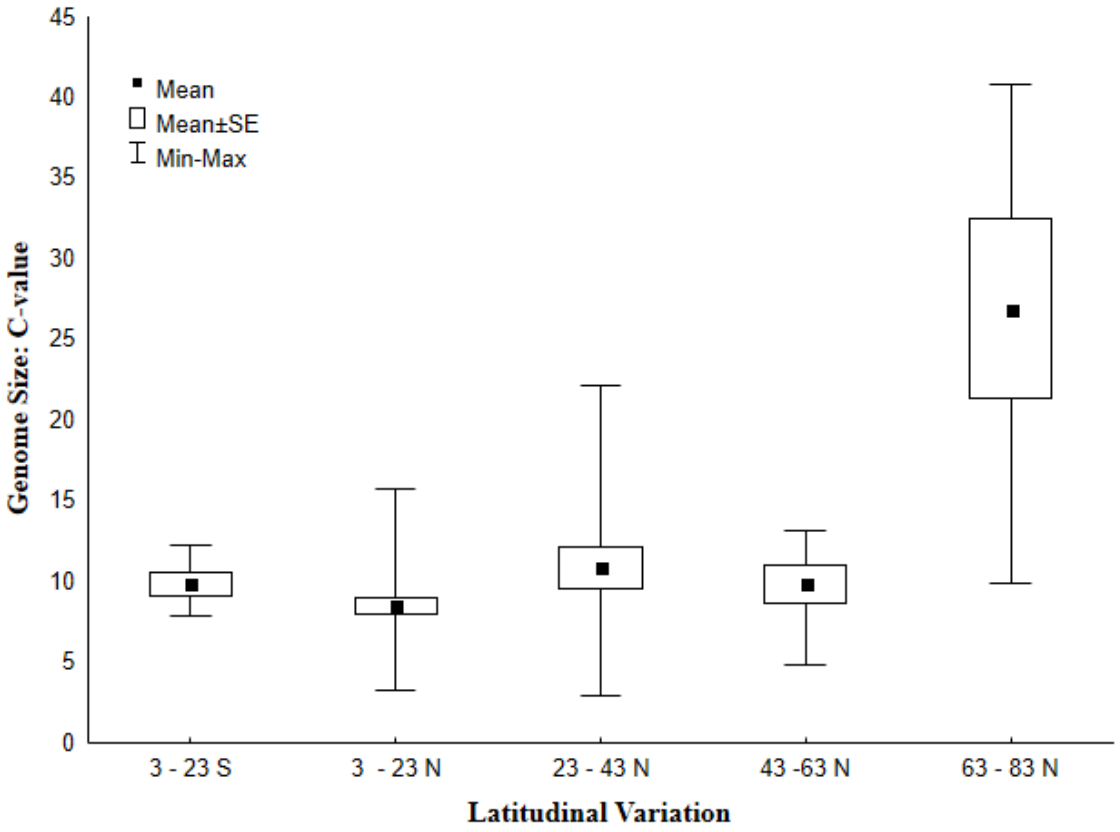

Figure 4: BoxPlot analyzes to compare the Genome Size into the different latitudinal distribution of caridean shrimps. Latitudinal registers were grouped into five classes: 3 – 23°S; 3 – 23°N; 23 – 43°N; 43 – 63°N; 63 – 53°N.

Another interpretation of GS, which has already been approached in different scenarios with distinct crustaceans' groups is the relationship between GS and body size length. Among the different Caridea groups, this relationship does not seem significant, neither presents any relationship pattern looking at box-plot and simple linear regression graph results (Figure 5).

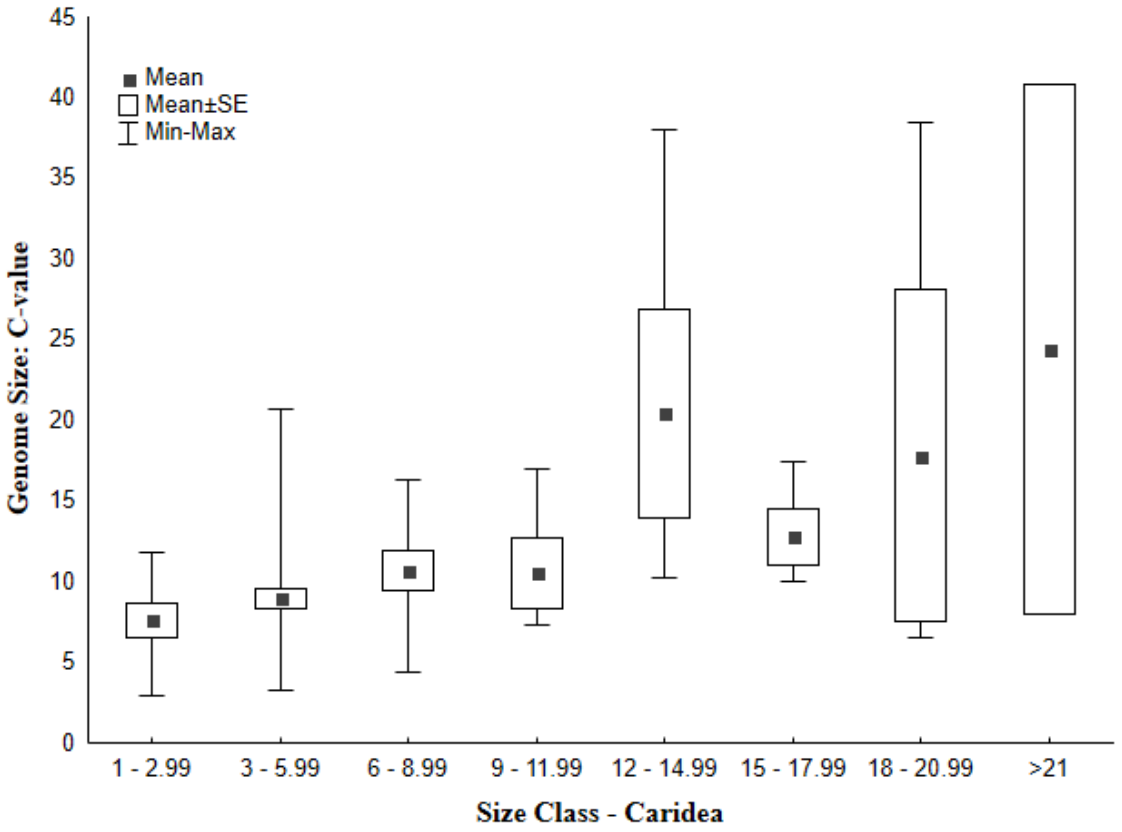

Figure 5: BoxPlot analyzes to compare the Genome Size into different size classes of caridean shrimps. Carapace Lenght were grouped into 8 classes: 1 – 2.99mm; 3 – 5.99mm; 6 – 8.99mm; 9 – 11.99mm; 12 – 14.99mm; 15 – 17.99mm; 18 – 20.99mm; >21mm

Discussion

Among Crustaceans, the higher values on the GS are present on the Amphipods that show a first panorama of how geographical distribution, reproductive patterns, and life history can modulate the patterns of GS with a considerable higher value found into species with more polar distribution (Rees *et al.*, 2007). Into the caridean shrimps, the same pattern seems to occur: specimens with more polar distribution presents 40 pg while tropical species ranges from 2 to 15 pg, in which shows a very strong relationship between GS and species with polar distribution. This pattern remains the same looking into those more results presented here in the two tropical species analyzed. Also, the higher values

found in caridean shrimp are also related with “deep-sea” species, which remains the fact that the environment has a great activity on GS variation. These large genomes described in polar and “deep sea” environments may be related with large amount of heterochromatin in the genomic composition (providing protection and maintenance of DNA stability), essential event for the survival of animals under stress conditions (Yang *et al.*, 2011; Jansen *et al.*, 2018).

The Order Decapoda is a very specious and diverse group, then, comparing the entire group, as it is proposed for other crustaceans as copepods and amphipods remains more difficult. The division in Infraorders seems to be essential, for example, looking into the Phylogenetic independent contrast (PIC) analyzes proposed by Hultgren *et al.*, 2018, just the Caridea and Anomura groups seems to present any correlation with genome size and latitudinal variations.

The PIC analyzes correlates many variables with the GS gaining phylogenetics relations as co-variables. And, despiting we devided Decapod into groups, when this analyzes is applied, we can see some tendencies but many of them are still not very clear, that makes evident the great need to dedicate a effort to register the much data as possible to improve as possible the species’ number. Those variables can be related to: the number of the larval stage on species, timing of development and reproduction (Knowlton, 1973; Jeffrey, 2015), and in larger scales, the accumulation of the transposons and non-coding DNA (Chak *et al.*, 2021; Ennis *et al.*, 2021).

The phenotypic relation, like the body size and geographical distribution *versus* the GS can be also related and explained by the slower metabolism founded in the environments with lower temperatures (Hessen *et al.*, 2008; Hessen and Persson, 2009), *i.e.*, metabolism activity maybe is inversely proportional than GS. This pattern was proved by Vinogradov (1995) in mammals and by Gregory (2002) in birds.

Those proposed patterns in lower temperatures, as polar regions and deep-sea shares even more similar characteristics proposed by Martens (1997) in which polars and deep-sea species present an evolutive convergence in a series of characteristics like, *e.g.*, gigantism's tendency. This proposoal runs in the same sence of the Bergman's theory, that had been tested for some marine crustaceans (Campbell *et al.*, 2021) and need further analyzes from freshwaters animals that are very poorly explored and need more data to compare with marine representants.

If considered just for species into tropical shallow waters this latitudinal cline is not so clear. We analyzed the values found into all caridean shrimps and difference into species in Arctic distribution, like *Sclerocrangon* G. O. Sars, 1883, *Bythocaris* G. O. Sars, 1870 and *Hymenodora* G. O. Sars, 1887, that were remarkable with all of them with more than 35 pg each (Rees *et al.*, 2008). However, considering only *Synalpheus* and *Alpheus* shrimps, the higher GS (*S. duffyi* with 20.74 pg) is shown in specimens into the Caribbean Sea (Jeffery *et al.*, 2016), demonstrating that the evolutionary and adaptative events should be evaluated independently in the natural groups (monophyletic).

The genome size seems to be presented as a link between the evolution and adaptative patterns to the caridean shrimps, it tends to be a little clear when we look for phylogenetics situations and the geographical/ecological (like the highest values founded on Antarctic species) (Rees *et al.*, 2008; Hultgren *et al.*, 2018). In this sense, the c-value can be modulated on the function of the need in the particular environmental region and variation intrinsic to the speciation process. And that is why the basic science, as checklists species, population biology, fecundity, and all information about the species on the literature are now so important in these more applicable studies and evolutive perspectives (Gregory *et al.*, 2000; Gregory, 2001; Hessen and Persson, 2009).

The next step in future works will have the basis on information acquired in results presented in works like the present from Nuclear DNA weight joining the basic biological sciences from distribution to behaviors that will be a useful link to the understanding of evolution from the most variety of species in higher taxonomical levels.

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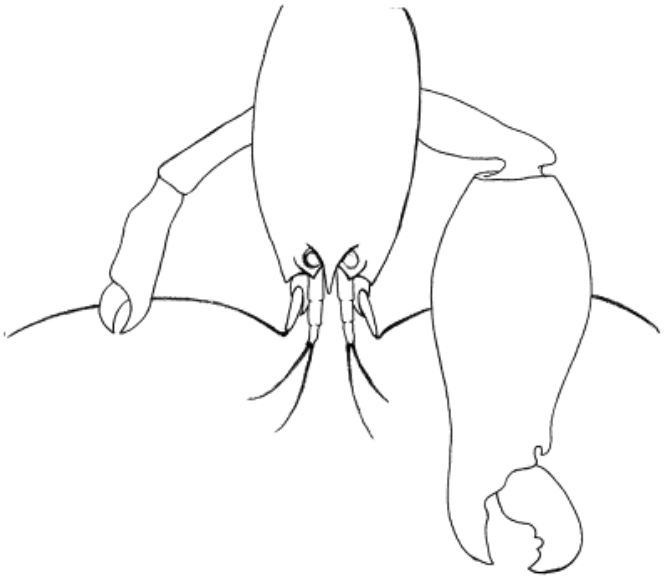


Considerações finais

Conclusões e considerações para futuras pesquisas

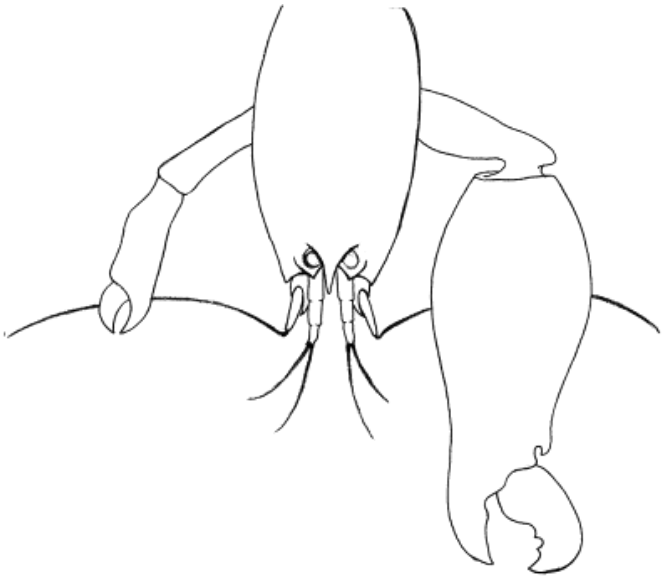
Synalpheus não gambarelloides devem receber um esforço em estudos para além de revisões taxonômicas e terem descrições biológicas e ecológicas bem elucidadas para comparações com o grupo dos *Gambarelloides* e a posição evolutiva do gênero dentro de Caridea.

Sobre os aspectos reprodutivos, *Synalpheus* aparentam possuir uma formação prioritariamente simplista, podendo padronizar um caráter primitivo ao grupo quando comparado a outros gêneros e famílias da Infraordem Caridea. Apesar de a Infraordem como um todo ser um grupo bem heterogêneo em morfologias, comportamentos e formações dos aparelhos reprodutores, a ausência de glândulas acessórias, secreções e a morfologia tão distinta neste grupo fazem alusão a caráteres mais primitivos que outros.

O dimorfismo sexual das espécies gonocóricas foi comprovado com base nas morfologias das primeiras pleuras e pleópodos, sendo complementada pela posição dos apêndices internos que, comprovadamente surgem na posição apical do endópodo em fêmeas e fêmeas ovígeras, caracterizando não só o dimorfismo, como também mais uma estratégia acessória ao "breeding dress" para aprimorar a capacidade das fêmeas de incubar ovos.

Por fim, a associação entre os aspectos reprodutivos e biológicos e o padrão do "genome size" ainda permanecem inconclusivos, porém, com as comparações disponíveis na presente tese, pode-se propor que a evolução do tamanho do genoma em camarões carídeos (incluindo *Synalpheus*) seja fortemente relacionada ao padrão biogeográfico com tendência à distribuição polar apresentar os maiores conteúdos de DNA nuclear.

As perspectivas de estudo para o gênero podem ser reformuladas a partir dos presentes resultados, sendo as espécies comprovadamente diferenciadas entre os sexos, os padrões celulares de reprodução esclarecidos, e os primeiros aspectos comparativos do tamanho do genoma elucidados. Os próximos passos para completo entendimento evolutivo do gênero é a descrição de cada vez mais "c-values" para outras espécies de camarões carídeos e a evolução da ciência básica para descrição comportamental e biológica dos *Synalpheus* não gambarelloides, que poderão ser comparados aos presentes resultados e corroborar a hipótese do padrão primitivo do gênero.



Material suplementar

Material suplementar tamanho do genoma.

Tabela referencial aplicada nos capítulos 04 e 05 com todos os dados utilizados para comparações do tamanho do genoma de camarões carídeos. Incluindo: Espécies, O grupo informal dos representantes do gênero *Synalpheus*, Ditribuição latitudinal das espécies avaliadas, Tipo de ambiente que a espécie é encontrada, Tamanho, "C-value" e referência.

Species	*Synalpheus* group	Latitudinal distribution	Environment distribution	Size Class	C-value	References
Synalpheus "longicarpus small"	Gambarelloides	23 - 43 N	shallow-water	3 - 5.99	6.69	Jeffery *et al.* 2016 supplementary material
Synalpheus agelas	Gambarelloides	3 - 23 N	shallow-water	3 - 5.99	13.60	Jeffery *et al.* 2016
Synalpheus androsi	Gambarelloides	3 - 23 N	shallow-water	3 - 5.99	7.09	Jeffery *et al.* 2016 supplementary material
Synalpheus ankeri	Gambarelloides	3 - 23 N	shallow-water	3 - 5.99	13.3	Jeffery *et al.* 2016 supplementary material
Synalpheus belizensis	Gambarelloides	3 - 23 N	shallow-water	3 - 5.99	10.35	Jeffery *et al.* 2016
Synalpheus bocas	Gambarelloides	3 - 23 N	shallow-water	3 - 5.99	12.42	Jeffery *et al.* 2016 supplementary material
Synalpheus bousfieldi	Gambarelloides	3 - 23 N	shallow-water	3 - 5.99	10.46	Jeffery *et al.* 2016 supplementary material
Synalpheus brevifrons	Gambarelloides	3 - 23 N	shallow-water	1 - 2.99	7.88	Jeffery *et al.* 2016 supplementary material
Synalpheus carpenteri	Gambarelloides	3 - 23 N	shallow-water	3 - 5.99	9.84	Jeffery *et al.* 2016
*Synalpheus cayoneptunus**	Gambarelloides	23 - 43 N	shallow-water	1 - 2.99	2.88	Jeffery *et al.* 2016 supplementary material
Synalpheus corallinus	Gambarelloides	3 - 23 N	shallow-water	3 - 5.99	7.11	Jeffery *et al.* 2016 supplementary material
Synalpheus dardeaui	Gambarelloides	3 - 23 N	shallow-water	3 - 5.99	7.09	Jeffery *et al.* 2016
*Synalpheus dominicensis**	Gambarelloides	3 - 23 N	shallow-water	1 - 2.99	5.79	Jeffery *et al.* 2016 supplementary material
Synalpheus duffyi	Gambarelloides	23 - 43 N	shallow-water	3 - 5.99	20.74	Jeffery *et al.* 2016
Synalpheus goodei	Gambarelloides	3 - 23 N	shallow-water	3 - 5.99	7.78	Jeffery *et al.* 2016 supplementary material
Synalpheus herricki	Gambarelloides	3 - 23 N	shallow-water	3 - 5.99	10.22	Jeffery *et al.* 2016 supplementary material
Synalpheus hoetjesi	Gambarelloides	3 - 23 N	shallow-water	3 - 5.99	5.77	Jeffery *et al.* 2016 supplementary material
Synalpheus idios	Gambarelloides	3 - 23 N	shallow-water	3 - 5.99	15.52	Jeffery *et al.* 2016 supplementary material
Synalpheus kensleyi	Gambarelloides	3 - 23 N	shallow-water	6 - 8.99	10.76	Jeffery *et al.* 2016 supplementary material
*Synalpheus kuadramanus**	Gambarelloides	3 - 23 N	shallow-water	1 - 2.99	7.74	Jeffery *et al.* 2016 supplementary material
Synalpheus longicarpus	Gambarelloides	3 - 23 N	shallow-water	3 - 5.99	5.99	Jeffery *et al.* 2016 supplementary material

Synalpheus mcclendoni	Gambarelloides	3 - 23 N	shallow-water	3 - 5.99	6.01	Jeffery *et al.* 2016
Synalpheus pandionis	Gambarelloides	3 - 23 N	shallow-water	3 - 5.99	5.78	Jeffery *et al.* 2016 supplementary material
Synalpheus pectiniger	Gambarelloides	3 - 23 N	shallow-water	3 - 5.99	12.79	Jeffery *et al.* 2016 supplementary material
Synalpheus rathbunae	Gambarelloides	23 - 43 N	shallow-water	3 - 5.99	11.38	Jeffery *et al.* 2016 supplementary material
Synalpheus ruetzleri	Gambarelloides	3 - 23 N	shallow-water	3 - 5.99	6.57	Jeffery *et al.* 2016 supplementary material
Synalpheus sanctithomae	Gambarelloides	3 - 23 N	shallow-water	3 - 5.99	6.20	Jeffery *et al.* 2016
Synalpheus thele	Gambarelloides	3 - 23 N	shallow-water	3 - 5.99	4.83	Jeffery *et al.* 2016 supplementary material
Synalpheus ul	Gambarelloides	3 - 23 N	shallow-water	3 - 5.99	5.14	Jeffery *et al.* 2016 supplementary material
Synalpheus williamsi	Gambarelloides	3 - 23 N	shallow-water	3 - 5.99	6.44	Jeffery *et al.* 2016 supplementary material
Synalpheus yano	Gambarelloides	3 - 23 N	shallow-water	3 - 5.99	6.49	Jeffery *et al.* 2016
Synalpheus brooksi	Gambarelloides - direc development	3 - 23 N	shallow-water	3 - 5.99	10.88	Jeffery *et al.* 2016
Synalpheus chacei	Gambarelloides - direc development	3 - 23 N	shallow-water	1 - 2.99	7.74	Jeffery *et al.* 2016 supplementary material
Synalpheus elizabethae	Gambarelloides - direc development	3 - 23 N	shallow-water	3 - 5.99	13.28	Jeffery *et al.* 2016 supplementary material
Synalpheus filidigitus	Gambarelloides - direc development	3 - 23 N	shallow-water	1 - 2.99	9.47	Jeffery *et al.* 2016 supplementary material
Synalpheus regalis	Gambarelloides - direc development	3 - 23 N	shallow-water	1 - 2.99	11.76	Jeffery *et al.* 2016 supplementary material
Synalpheus apioceros	Non-Gambarelloides	3 - 23 S	shallow-water	3 - 5.99	7.89	Present work
Synalpheus brevicarpus	Non-Gambarelloides	3 - 23 S	shallow-water	3 - 5.99	9.77	Present work
Synalpheus fritzmuelleri	Non-Gambarelloides	3 - 23 S	shallow-water	3 - 5.99	10.46	Present work
Synalpheus minus	Non-Gambarelloides	3 - 23 S	shallow-water	3 - 5.99	8.99	Present work
Synalpheus scaphoceris	Non-Gambarelloides	3 - 23 S	shallow-water	3 - 5.99	12.24	Present work
Metabetaeus lohena		3 - 23 N	freshwater	3 - 5.99	4.10	Bachmann & Rheinsmith (1973)

Athanas nitescens	43 -63 N	shallow-water	3 - 5.99	4.84	Bonnivard *et al.* (2009)
Alpheus crassimanus	3 - 23 N	shallow-water	6 - 8.99	15.80	Bachmann & Rheinsmith (1973)
Alpheus heterochaelis	23 - 43 N	shallow-water	6 - 8.99	16.31	Rheinsmith *et al.* 1974
Mirocaris fortunata	23 - 43 N	deep-sea	6 - 8.99	9.00	Bonnivard *et al.* (2009)
Rimicaris exoculata	23 - 43 N	deep-sea	15 - 17.99	10.39	Bonnivard *et al.* (2009)
Alvinocaris markensis	23 - 43 N	deep-sea	3 - 5.99	10.58	Bonnivard *et al.* (2009)
Mirocaris fortunata	23 - 43 N	deep-sea	6 - 8.99	11.50	Bonnivard *et al.* (2009)
Chorocaris chacei	23 - 43 N	deep-sea	15 - 17.99	13.35	Bonnivard *et al.* (2009)
Antecaridina sp.	3 - 23 N	freshwater	3 - 5.99	3.30	Bachmann & Rheinsmith (1973)
Halocaridina rubra	3 - 23 N	freshwater	3 - 5.99	4.70	Bachmann & Rheinsmith (1973)
Atya bisulcata	3 - 23 N	freshwater	6 - 8.99	7.20	Bachmann & Rheinsmith (1973)
Crangon septemspinosa	63 - 83 N	shallow-water	9 - 11.99	9.92	Rees *et al.* 2008
Crangon vulgaris	43 -63 N	estuarine	6 - 8.99	11.00	Dixon *et al.* (2001)
Crangon crangon	43 -63 N	shallow-water	12 - 14.99	11.35	Bonnivard *et al.* (2009)
Argis dentata	63 - 83 N	deep-sea	15 - 17.99	17.42	Rees *et al.* 2008
Sclerocrangon ferox	63 - 83 N	deep-sea	<	40.89	Rees *et al.* 2008
Spirontocaris spinus	43 -63 N	deep-sea	3 - 5.99	13.22	Rees *et al.* 2008
Eualus gaimardii	63 - 83 N	deep-sea	9 - 11.99	16.97	Rees *et al.* 2008
Bythocaris irene	63 - 83 N	deep-sea	18 - 20.99	38.47	Rees *et al.* 2008
Hymenodora sp.	63 - 83 N	deep-sea	12 - 14.99	38.00	Dixon *et al.* (2001)
Palaemonetes pugio	23 - 43 N	freshwater	6 - 8.99	4.35	Jimenez *et al.* (2010)
Macrobrachium acanthurus	23 - 43 N	freshwater	18 - 20.99	6.48	Rheinsmith *et al.* 1974
Macrobrachium lar	3 - 23 N	freshwater	9 - 11.99	7.30	Bachmann & Rheinsmith (1973)
Palaemonetes kadiakensis	23 - 43 N	freshwater	9 - 11.99	7.91	Rheinsmith *et al.* 1974
Macrobrachium grandimanus	3 - 23 N	freshwater	>21	8.00	Bachmann & Rheinsmith (1973)
Palaemon debilis	3 - 23 N	freshwater	3 - 5.99	8.20	Bachmann & Rheinsmith (1973)
Palaemon serratus	43 -63 N	shallow-water	12 - 14.99	10.21	Bonnivard *et al.* (2009)
Palaemonetes sp.	23 - 43 N	freshwater	6 - 8.99	10.42	Gregory database
Macrobrachium ohione	23 - 43 N	freshwater	12 - 14.99	22.16	Rheinsmith *et al.* 1974
Pandalus montagui	43 -63 N	shallow-water	18 - 20.99	8.53	Rees *et al.* 2008

Cabeça fria,
coração quente.