UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
Câmpus de Botucatu
INSTITUTO DE BIOCIÊNCIAS
Pós-Graduação em Ciências Biológicas – Zoologia

Tese de Doutorado

A análise interdisciplinar comparativa entre os camarões *Macrobrachium amazonicum* (Heller, 1862) e *Macrobrachium pantanalense* Dos Santos, Hayd & Anger, 2013 suporta a diferenciação interespecífica?

Caio dos Santos Nogueira

Orientador: Prof. Dr. Rogerio Caetano da Costa
Coorientador: Prof. Dr. João Alberto Farinelli Pantaleão

Botucatu – SP
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Caio dos Santos Nogueira

Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas – Zoologia do Instituto de Biociências de Botucatu, Universidade Estadual Paulista “Júlio de Mesquita Filho” – UNESP – Câmpus de Botucatu, como parte dos requisitos para a obtenção do título de Doutor em Ciências Biológicas – Zoologia.

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Botucatu – SP

2023

FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: MARIA CAROLINA ANDRADE CRUZ E SANTOS-CRB 8/10188

Nogueira, Caio dos Santos.
A análise interdisciplinar comparativa entre os camarões *Macrobrachium amazonicum* (Heller, 1862) e *Macrobrachium pantanalense* (Dos Santos, Hayd & Anger, 2013) suporta a diferenciação interespecífica? / Caio dos Santos Nogueira. - Botucatu, 2023

Tese (doutorado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Instituto de Biociências de Botucatu
Orientador: Rogerio Caetano da Costa
Coorientador: João Alberto Farinelli Pantaleão
Capes: 20402007

1. Hibridização. 2. Morfometria. 3. Reprodução animal. 4. Larvas - Anfíbios (Crustáceos, peixes, invertebrados, etc.) 5. Zoologia - Classificação.

Palavras-chave: Desenvolvimento larval; Hibridização; Morfometria geométrica; Sistema reprodutor; Taxonomia integrativa.

Agradecimentos

Gostaria de expressar minha gratidão ao Prof. Dr. Rogerio Caetano da Costa por confiar em mim e ter me concedido a oportunidade de desenvolver esse trabalho. Além disso, agradeço sinceramente pelos seus valiosos conselhos e orientações ao longo dos últimos seis anos. Estou confiante de que essa experiência será fundamental para o meu desenvolvimento profissional no futuro.

Gostaria de agradecer ao meu coorientador, Prof. Dr. João Alberto Farinelli Pantaleão, por sua ajuda desde a época do mestrado e por ter me ensinado muito sobre o mundo dos crustáceos e do mundo científico em geral. Obrigado por ter aceitado coorientar minha Tese e por ter concordado com a ideia da temática. Sem sua ajuda, este trabalho não teria sido concluído.

Gostaria de expressar minha gratidão ao Prof. Dr. Fernando José Zara, que mais uma vez aceitou colaborar comigo em outro projeto. Muito obrigado pelos conselhos e dicas, não só no capítulo do qual teve participação direta, mas também por todas as discussões que tivemos sobre minha pesquisa. Sua contribuição foi essencial para a conclusão deste trabalho.

A Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), a qual financiou a execução minha Tese diretamente pelo processo 2019/00661-3, e também pelo Projeto Temático Biota INTERCRUSTA, processo 2018/13685-5.

Ao Ministério do Meio Ambiente e ao Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA) por conceder a licença para coleta de material nas áreas de estudo.

Ao Instituto de Biociências – IB e à Pós-Graduação em Ciências Biológicas Zoologia da UNESP de Botucatu e ao Departamento de Biodiversidade e Bioestatística.

Ao Departamento de Ciências Biológicas, da Faculdade de Ciências da UNESP de Bauru, o qual o Laboratório de Biologia de Camarões (LABCAM) é vinculado.

A toda minha família, e em especial à minha mãe e meu irmão, por todo o apoio e suporte nas minhas decisões desde que decidi trilhar esse caminho.

A minha namorada Natalia, por toda paciência, apoio e conselhos que me concedeu ao longo desses longos anos de pós-graduação, muito obrigado por tudo. Sem você nada seria possível.

Aos meus irmãos de vida, Isabela e Rafael. Muito obrigado por sempre estarem ao meu lado nesses últimos anos, vocês tiveram um papel fundamental no desenvolvimento desse trabalho. Espero que nossa amizade perdure e que todos nós consigamos atingir nossos objetivos de vida.

Ao meu grande amigo e parceiro de pesquisa, Alexandre (Dino). Muito obrigado por toda a ajuda nesses últimos quatro anos. Espero que continuemos animados, e trabalhando juntos com nossa empolgação pelas “armas”.

A segunda família que ganhei nesses últimos anos graças a Rep. do Amor. Abner, Bruno (Sushi), Gabriel, Milena, Rafael e Victor, vocês são muito especiais para mim.

Aos amigos que ganhei durante esses anos de pós-graduação, Alexandre (Seu Jorge), Bruno Tayar, Gabriel, Giovana, Júlia, Lizandra, Lucas (Marginal), Mariana (Magrela) e Régis, por todos os momentos que passamos juntos, fazendo com que meus dias fossem mais alegres.

A todos os integrantes do Laboratório de Biologia de Camarões (LABCAM - UNESP/FC), desde os antigos aos mais novos, vocês foram fundamentais durante todos esses anos.

A todos os integrantes do Laboratório de Morfologia de Invertebrados (IML - UNESP/FCAV), Barbara, Camila, Fernanda, Lucas, Marcia e Maria Alice, por me acolherem tão bem e pela grande ajuda no desenvolvimento do meu mestrado e doutorado.

Aos integrantes do Laboratório de Bioecologia e Sistemática de Crustáceos (LBSC – USP/FFCLRP), por me recepcionarem tão bem nas minhas visitas ao laboratório durante o meu doutorado.

Mais uma vez, muito obrigado a todos.

Sumário

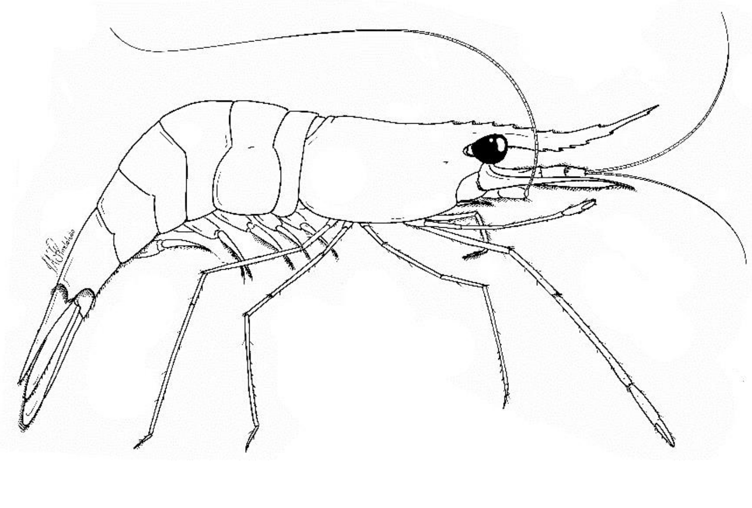
CAPÍTULO IV: Morphology and morphometrics of larvae of *Macrobrachium amazonicum* (Heller, 1862) and *M. pantanalense* Dos Santos, Hayd & Anger, 2013: a comparative approach among different lineages and phenotypes

Resumo

Macrobrachium amazonicum e *M. pantanalense* são duas espécies de camarões dulcícolas proximamente relacionadas que ocorrem no Brasil. *Macrobrachium pantanalense* é uma espécie endêmica das regiões pantaneiras, e constituía um clado presente dentro do complexo de *M. amazonicum*. A descrição dessa linhagem como espécie gerou controvérsias quanto a sua validade, principalmente pela alta semelhança morfológica com *M. amazonicum* e por conta do baixo distanciamento genético entre as espécies. Desse modo, o presente estudo teve como objetivo avaliar se ferramentas comumente utilizadas na taxonomia integrativa suportam a existência de *M. pantanalense*. Foram utilizadas quatro ferramentas; experimentos de hibridização, morfometria geométrica, descrição da anatomia do sistema reprodutor masculino (SRM) e ultraestrutura do espermatozoide, e morfologia e morfometria larval. Em todas essas abordagens integrativas foram observadas diferenças interespecíficas. Nos experimentos de hibridização, foi constatado um isolamento reprodutivo entre esses organismos, portanto, essas espécies não copulam naturalmente. A morfometria geométrica evidenciou diferenças no formato em cinco estruturas taxonomicamente relevantes. Não houve diferença na morfologia do SRM, entretanto, ocorreram variações histoquímicas nas secreções que compõem o espermatóforo. O formato do espermatozoide apresentou variações entre as duas espécies. As larvas apresentaram variações heterocrônicas e morfométricas ao longo do seu desenvolvimento. Desse modo, essas análises integrativas demonstram que ocorrem variações entre traços biológicos de *M. amazonicum* e *M. pantanalense*, sustentando a separação de ambas as linhagens. Evidentemente, algumas dessas diferenças são sutis, o que pode estar correlacionado ao fato de que o processo de especiação entre esses grupos seja recente.

Palavras-chave: Hibridização; Morfometria geométrica; Sistema reprodutor; Desenvolvimento larval; Taxonomia integrativa

Considerações iniciais



Considerações iniciais

Complexos de espécies são grupos constituídos por diferentes linhagens de organismos que possuem uma variabilidade genética relativamente elevada, mas que em muitos casos são morfologicamente similares (Mayr, 1999; De Queiroz, 2007). Quando não existem caracteres morfológicos que auxiliam na discriminação desses grupos, essas diferentes linhagens são consideradas como espécies crípticas (Bickford et al., 2007). Ao longo dos últimos anos, o número de estudos que abordam a variabilidade morfológica de linhagens que constituem complexos de espécies aumentou consideravelmente, com vários estudos discriminando essas espécies ou então agregando esses organismos como espécies crípticas, nos mais variados táxons animais (Bagley et al., 2015; Alama-Bermejo et al., 2016; Johnson et al., 2018; Calixto-Cunha et al., 2021).

Em crustáceos decápodes podemos observar muitos exemplos nos quais complexos de espécies foram discriminados morfologicamente ou então agregados como espécies crípticas (Carvalho et al., 2013; Almeida et al., 2014; Moraes et al., 2016; Crivellaro et al., 2018; Cunha et al., 2021; Siriwut et al., 2021). Nem sempre a análise da morfologia externa fornece caracteres determinantes para discriminar complexos de espécies, desse modo, alguns estudos mais recentes têm utilizado outras ferramentas para buscar diferenças interespecíficas entre essas linhagens. Em alguns casos, por exemplo, são realizados experimentos de hibridização, descrição da ultraestrutura espermática e da morfologia larval, análises da variação geométrica de estruturas taxonomicamente relevantes ou então análises citogenéticas (Marques & Pohle, 1995; Baylac et al., 2003; Introini et al., 2013; Alevi et al., 2014; Mendonça et al., 2016).

O camarão dulcícola *Macrobrachium amazonicum* (Heller, 1862) possui ampla distribuição geográfica ao longo do território brasileiro, ocorrendo do Norte ao Sudeste do Brasil (Magalhães et al., 2005). Essa espécie foi investigada quanto à variabilidade genética entre diversas populações distribuídas ao longo do país, e foi possível detectar a existência de três diferentes linhagens que possuem divergências genéticas (Vergamini et al., 2011). Portanto, esses organismos foram classificados da seguinte forma: Clado I – populações oriundas do interior da região hidrográfica amazônica; Clado II – populações oriundas da região hidrográfica do pantanal; Clado III – populações oriundas da região da costa Norte do Brasil, e que habitam ambientes estuarinos (Vergamini et al., 2011).

Apesar dessas linhagens variarem geneticamente, a distância que as separa não extrapola os limites interespecíficos delimitados para o gênero *Macrobrachium* por Liu et al. (2007) (5,5% e 15% para o gene 16S e COI, respectivamente). Mesmo assim, alguns anos após a realização do estudo de Vergamini et al. (2011), um grupo de pesquisadores analisou as populações que constituem o Clado II e descreveram uma nova espécie, *Macrobrachium pantanalense* Dos Santos, Hayd & Anger, 2013. Os autores relataram que apesar dessa distância genética ser relativamente baixa considerando o gênero, ainda assim ocorrem divergências entre alguns caracteres morfológicos, fisiológicos e reprodutivos que justificariam a descrição desse novo táxon (Dos Santos et al., 2013).

Posteriormente, foi realizado um estudo de caso sobre a situação dessas duas espécies, levando em consideração casos similares que ocorreram com outras espécies proximamente relacionadas de crustáceos decápodes (Weiss et al., 2015). Esses mesmos autores discutem sobre até que ponto apenas a distância genética entre duas linhagens deve ser utilizada para a delimitação de espécies, e reforçam a importância da utilização de mais ferramentas que podem suportar, ou não, a existência de novos. Seguindo essa tendência, um recente estudo evidenciou algumas diferenças morfológicas entre esses dois táxons, principalmente relacionada ao número de dentes presentes no rostro de *M. amazonicum* e *M. pantanalense*, dando suporte a separação dessas linhagens (Calixto-Cunha et al., 2021).

Nesse sentido, o presente estudo teve como objetivo analisar *M. amazonicum* e *M. pantanalense* em diferentes perspectivas biológicas com o uso de quatro ferramentas que são amplamente utilizadas na delimitação de espécies nos mais variados táxons animais. Dentre essas ferramentas, foram realizados experimentos de hibridização, análises de morfometria geométrica, a descrição do sistema reprodutor masculino e da ultraestrutura do espermatozoide, e a comparação da morfologia e morfometria larval. Todas essas ferramentas são comprovadamente eficazes na separação de espécies, e já foram utilizadas na discriminação de espécies de crustáceos (Marques & Pohle, 1995; Graziani et al., 2003; Camargo et al., 2016; Siriwut et al., 2021). Nossa hipótese principal é de que *M. amazonicum* e *M. pantanalense* são espécies diferentes, portanto, predizemos que esses grupos apresentem diferenças biológicas que serão evidenciadas pelas ferramentas mencionadas. Possivelmente, algumas das diferenças encontradas podem ser sutis, uma vez que a distância genética entre essas linhagens ainda é pequena, o que evidencia que o processo de especiação entre esses grupos seja recente.

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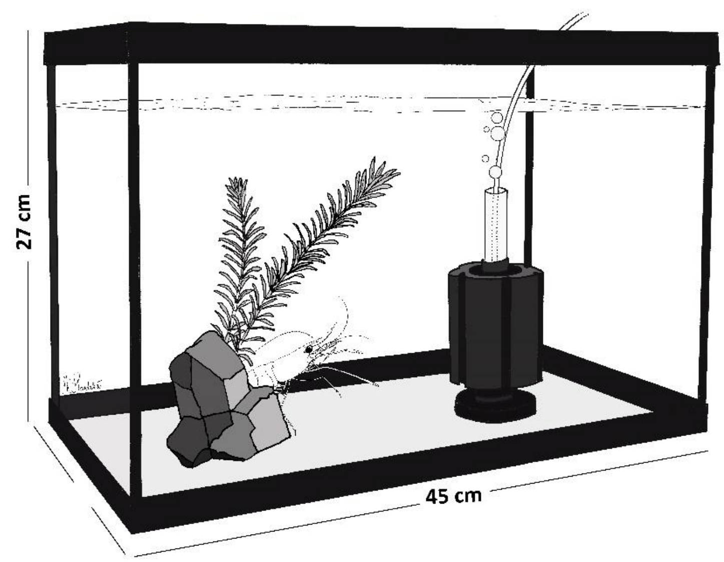
Capítulo I

Hybridization experiments between the freshwater prawns *Macrobrachium amazonicum* (Heller, 1862) and *M. pantanalense* Dos Santos, Hayd & Anger, 2013 (Decapoda: Palaemonidae)



Abstract

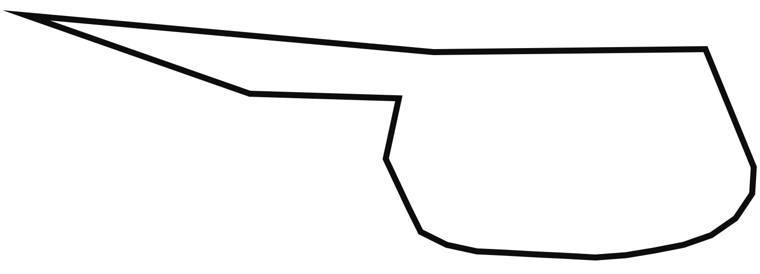
The freshwater prawns *Macrobrachium amazonicum* and *M. pantanalense* are phylogenetically closely related. The present study analysed the occurrence of copulation between these two species. Specimens of *M. amazonicum* were collected from the Tietê River in Cambaratiba (SP), whereas *M. pantanalense* specimens were collected from the Lagoa Baíazinha, in Miranda (MS). Six different experimental groups were used, MA♂ × MP♀, MA♀ × MP♂ (interspecifc), MA♂ × MA♀, MP♂ × MP♀ (intraspecific), MA♀ and MP♀ (individualised; MA = *M. amazonicum* and MP = *M. pantanalense*). The incubation times of the ovigerous females were monitored in all experiments. Some females from all experimental groups were randomly selected and had their oocytes photographed to verify the occurrence of embryonic development. No larvae hatched in the interspecific experimental groups, whereas, in intraspecific groups, almost all experiments (95%) presented larval hatching. The incubation period differed significantly ($P < 0.001$) between the intraspecific groups and all the other experimental groups, but not between each other ($P > 0.05$). The interspecific and individualised groups showed no significant ($P > 0.05$) difference between them. In addition, oocytes from the interspecific experimental groups did not present characteristics of embryonic development. Our results suggest that *M. pantanalense* and *M. amazonicum* populations do not copulate, which corroborates the proposal of recent speciation.

Keywords: Genetic distance; Intraspecific recognition; Pantanal; Reproductive barrier; Sexual behavior

Link de acesso ao artigo: https://doi.org/10.1071/MF20226

Capítulo II

Variation in body structure shape as a tool for discriminating between the freshwater prawns *Macrobrachium amazonicum* (Heller, 1862) and *M. pantanalense* Dos Santos, Hayd & Anger, 2013 (Caridea: Palaemonidae)



Abstract

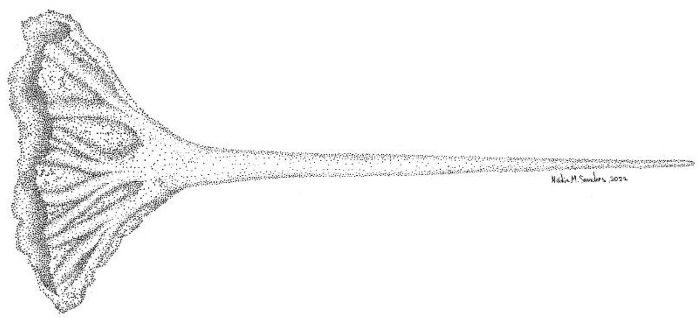
Macrobrachium amazonicum and *M. pantanalense* are two closely related freshwater prawn species, the latter having been described from populations that were previously considered *M. amazonicum*. Distinguishing these two lineages from the available taxonomic description can be a complex task due to the morphological similarity of these organisms. In these cases, the investigation of morphological characters that help in the differentiation of these groups is of great value. Thus, the present study aimed to analyze taxonomically informative structures through geometric morphometrics analyses, evaluating the informative potential of these structures in the separation of these lineages. The selected structures were carapace, dactyl, propodus, scaphocerite and telson. The analyzed dactyl and propodus are from the second pair of pereiopods (chelipeds). Three populations of each species were analyzed. All structures were photographed and marked with landmarks and semilandmarks for the acquisition of shape variables. The shape and size of all structures differed statistically between the two species, regardless of sex. Among the observed morphometric variations, the most evident were in the carapace and telson. In the carapace, the position of the first ventral tooth of the rostrum is different, while the posterior region of the telson is proportionally thinner in *M. amazonicum* and wider in *M. pantanalense*. Our results indicate that the carapace and the telson are informative structures that can help distinguish these two groups. Furthermore, our results support the proposal presented in other studies that *M. amazonicum* and *M. pantanalense* may be in a relatively recent process of speciation.

Keywords: Integrative taxonomy; *Macrobrachium amazonicum*; *Macrobrachium pantanalense*; Morphometry; Speciation

Link de acesso ao artigo: https://doi.org/10.1016/j.jcz.2023.03.003

Capítulo III

Male reproductive system of *Macrobrachium amazonicum* (Heller, 1862) and *M. pantanalense* Dos Santos, Hayd & Anger, 2013: new insights into the analysis of sperm morphology in crustaceans



Abstract

Studies on the male reproductive system (MRS) and sperm ultrastructure can provide important information about reproductive biology and also about relationships between crustacean species. Therefore, the present study aimed to describe the functional anatomy of the MRS, and the ultrastructure and morphometry of sperm of two closely related freshwater prawn species, *Macrobrachium amazonicum* (MA) and *Macrobrachium pantanalense* (MP), in order to detect possible interspecific differences. Prawns were anesthetized and their MRS fixed and processed following histological routines and for scanning electron (SEM) and transmission (TEM) microscopy. Histochemical analysis was performed to detect neutral and acidic polysaccharides and proteins. The anatomy of the MRS consists of a pair of testes that are interconnected to the vas deferens (VD). No differences in the anatomy of the MRS were observed between the two species. The main differences between the two species are related to the histochemistry of the secretions present in the medial and distal region of the VD, and the shape and size of spermatozoa. SEM and TEM analyses showed variations in the shape of spermatozoa of both species, and this variation was found to be significant by geometric morphometrics. We found that the use of these tools was important to highlight differences between caridean prawns, mainly in cases of closely related species. Thus, with the differences found mainly in the spermatozoa, it becomes clear that MA and MP are two different taxa and reinforce that these groups underwent a speciation process.

Keywords: Integrative approach; Morphometry; Pantanal; Reproduction; Spermiotaxonomy.

Introduction

The morphology of the male reproductive system (MRS) in decapod crustaceans follows a general anatomical pattern, composed of a pair of testes that present a tubular anatomy, varying in their morphological complexity (very or slightly convoluted) (López-Greco, 2007; Buranelli et al., 2014; Fransozo et al., 2016; Antunes et al., 2018; Tomas et al., 2019; Watanabe et al., 2020). From the testes, a pair of *vasa deferentia* (VD) is found, which can also present morphological variations. The VD can be subdivided into distinct regions due to remarkable morphological characteristics (*e.g.*, increased vas diameter, presence of accessory glands), or they may not present any type of differentiation, being only an elongated tube that connects the testes to the male gonopores located on the coxae of the fifth pair of pereiopods (Chow et al., 1982; Buranelli et al., 2014; Tiseo et al., 2014: Antunes et al., 2018; Watanabe et al., 2020).

In addition to the morphology of the MRS, the ultrastructure of spermatozoa also presents unique morphological characteristics among crustaceans (Braga et al., 2013; Tan et al., 2020). In most cases, these spermatozoa are immobile, as they lack flagella, a structure associated with sperm motility (Jamieson, 1991; Tan et al., 2020). Among decapod crustaceans, we can observe a wide variety of spermatozoa shapes (Tudge, 1992; Braga et al., 2013; Niksirat et al., 2013; Camargo et al., 2016; 2017; 2020). In prawns, the spermatozoa have a tack or inverted umbrella shape, divided into a main body and an acrosomal vesicle. The acrosomal vesicle is subdivided into two other portions, the acrosomal cap on the main body (anterior portion) and the spike (posterior portion) (Chow et al., 1989; Braga et al., 2013; Camargo et al., 2016; 2017).

The number of studies addressing morphological variations of the MRS and the ultrastructure of spermatozoa of decapod crustaceans has been growing over the last few years. In general, these studies describe the morphology of these structures and make comparisons between different taxonomic levels (*e.g.*, family, genus, or species; Chow et al., 1989; Camargo et al., 2016; Fransozo et al., 2016; Oliveira & Zara, 2018; Oliveira et al., 2021). These comparisons demonstrate efficacy in discriminating these groups, therefore, they serve as excellent tools that support the separation of taxa and their relationships, regardless of the taxonomic level (Camargo et al., 2016; 2017; Fransozo et al., 2016; Oliveira et al., 2021).

There are species complexes in many genera of decapod crustaceans, which are composed of different animal lineages that present a certain degree of genetic variability, while these organisms are morphologically similar (Mathews & Anker, 2009; Lai et al., 2010; Almón et al., 2022). In these cases, the application of integrative morphological analyses for the discrimination of closely related species can be very useful, as well as describing the MRS and the ultrastructure of the spermatozoa, precisely to highlight differences or similarities between these organisms (Introini et al., 2013; Camargo et al., 2016; 2017).

In the genus *Macrobrachium* Spence Bate, 1868, one of the major genera of freshwater prawns, there are some examples of complexes that were unified as a single species, or complexes where it was evidenced that they were actually constituted by different species (Murphy et al., 2004; Rossi & Mantelatto, 2013; Siriwut et al., 2020). In Brazil, in past fewyears, a new species of this genus was described, *Macrobrachium pantanalense* Dos Santos, Hayd & Anger, 2013, and this species was one of the lineages that constituted the clades of the *Macrobrachium amazonicum* complex (Heller, 1862) (Clade II; Vergamini et al., 2011). However, due to the low genetic distance between these lineages, few differences are observed that support the separation of these taxa (Nogueira et al., 2020; Calixto-Cunha et al., 2021). Overall, the morphological variation described between these two groups is still inconsistent, mainly because *M. amazonicum* presents high phenotypic variability, with populations presenting different ecological and morphological characteristics, very similar to *M. pantanalense* (Dos Santos et al., 2013; Paschoal & Zara, 2020; Calixto-Cunha et al., 2021). On the other hand, there are some reproductive and coloration characteristics that diverge between *M. amazonicum* and *M. pantanalense*, and therefore support the separation of these species (Dos Santos et al., 2013; Nogueira et al., 2020).

Thus, the present study aims to analyze the MRS, ultrastructure, and spermatozoa morphometrics of *M. amazonicum* and *M. pantanalense*, searching for further biological features that support the separation of these two groups. We predict that some traits of the male reproductive system, as well as sperm ultrastructure, may show variations, even with the low genetic distance that exists between these two lineages.

Material and methods

The specimens of *M. amazonicum* were collected from the Tietê River, at the Ibitinga hydroelectric power plant, located in the municipality of Cambaratiba, São Paulo state, Brazil (24°44'29" S; 49°01'27" W). The specimens of *M. pantanalense* were collected at the type-locality in Baiazinha Lagoon, a permanent lagoon that has connectivity with the Miranda River during the rainy season, located in the municipality of Miranda, Mato Grosso do Sul state, Brazil (20°15'49" S; 56°23'15" W). The prawns were collected using a sieve that was handled in the marginal vegetation of these water bodies. After collection, the animals were transported alive in thermal boxes with proper aeration to the laboratory. In this location, the prawns were kept alive in aquariums until the moment of dissection.

To perform the spermatozoa ultrastructure analysis and description of the male reproductive system, some specimens (n = 5 for each analysis) were anesthetized by chilling for 10 minutes (-20°C). Then, the carapace and gills were removed to improve visualization of the male reproductive system, and a small amount of fixative coherent with the microscopy technique to be employed was dripped along the dissection. Glutaraldehyde 2.5% in 0.08M sodium cacodylate buffer (pH 7.2) was used for scanning and transmission electron microscopy (Paschoal and Zara, 2019), and 4% paraformaldehyde in 0.2M phosphate buffer (pH 7.3) was used for the anatomical description of the male reproductive system and histology.

Anatomy and histology of the male reproductive system

The dissected material was fixed for 24 hours. Then, the material was washed twice in 0.2 M phosphate buffer (pH 7.2) for 15 minutes. During this process, the MRS anatomy was photographed under a Leica MZ15 stereo microscope, attached to a base with transmitted illumination equipped for Hoffman contrast. Subsequently, the samples were dehydrated in an increasing ethanol series (70 to 95%) and embedded in Leica® historesin glycol-methacrylate. The blocks were cut with 4-7 µm thickness on a rotary microtome. The slides were stained with hematoxylin and eosin (H&E) for general histological description of the testis and vas deferens (Junqueira & Junqueira, 1983), and with toluidine blue pH 4.0 for visualization of the nucleus and the different phases during cell division. For histochemical analysis, Alcian blue (pH 2.5) and periodic acid-Schiff (PAS) techniques were used to identify the acid and neutral polysaccharides, respectively (Pearse, 1960; Junqueira & Junqueira, 1983). The anionic Xylidine ponceau staining was used for total protein identification (Mello & Vidal, 1980). The slides were analyzed and photographed under a Leica DM 2000 microscope and Leica LAS software.

Ultrastructure of the spermatozoa

For transmission electron microscopy (TEM), only small portions (1 mm^3) of the distal region of the vas deferens (DVD) were fixed. The samples were then washed three times in the same buffer, each for 10 minutes. Post-fixation was carried out with 1% buffered osmium tetroxide for 2 hours. After washing in buffer, the samples were “En Bloc” contrasted with 1% aqueous uranyl acetate (overnight, 4°C). Dehydration was performed with a progressive sequence of acetones (50 to 95%) and the samples were embedded with Epon-Araldite® resin. Semi-thin and ultra-thin sections were obtained using a Leica UC7 ultramicrotome with a diamond knife. The grids with ultra-thin sections (50-60nm) were contrasted with 2% aqueous uranyl acetate and 0.4% lead citrate (Reynolds, 1963; Paschoal & Zara, 2019).

For scanning electron microscopy (SEM), the male reproductive system, sternum, and the second pair of pleopods from individuals were subjected to the same fixation and post-fixation procedure described for TEM. Additionally, the VD of the MRS was squashed between a slide and coverslip. This squash was suspended in fixative and dripped onto coverslips treated with poly-L-lysine to promote spermatozoa adhesion. Subsequently, all samples were dehydrated in ethanol series (70-100%) and completely dried at the EMS 850 critical point. Next, the samples were attached to supports and coated by cathodic spraying with gold in Denton vacum desk II sputtering (Paschoal & Zara, 2019). The samples were observed and photographed with a Zeiss EVO10 SEM, at 10-20 kV.

Linear and geometric morphometrics of spermatozoa

For the preparation of this material, the distal region of the vas deferens (DVD) fixed according to the histology protocol was used. The vas deferens was sectioned at the curvature that delimits the end of the medial region and the beginning of the distal region. Subsequently, the DVD was placed on a slide, where two drops of methylene blue were dripped (Paschoal & Zara, 2018). Then, a coverslip was inserted, performing a crushing movement to disperse the seminal fluid on the slide. The spermatozoa were photographed under differential interference contrast microscopy (DIC or Nomarski) using a Zeiss Axio Imager Z2 microscope (Oberkochen, Germany).

The size of *M. amazonicum* and *M. pantanalense* spermatozoa was compared using two dimensions: spermatozoa length and main body width (Figure 1). A total of 20 spermatozoa from 20 individuals of each species were measured. Additionally, comparisons were made between males of the same species to verify if there is variation in spermatozoa size within each species. The spermatozoa position was standardized to avoid any bias in the analysis due to erroneous measurement of this structure. The standard position of the spermatozoa was exemplified in Figure 1A.

Spermatozoa length and main body width were compared between the two species using the Mann-Whitney test. The Kruskal-Wallis test and Dunn's post hoc test were used to perform pairwise comparisons of spermatozoa length and main body width among males of each species. These analyses were performed after conducting a Shapiro-Wilk test, which revealed a non-normal distribution pattern of the data (Shapiro-Wilk; Sperm length – $W = 0.983$ and $p < 0.001$; Main body width – $W = 0.986$ and $p < 0.001$). All analyses were performed with the Past software v.4.05 (Hammer et al., 2001; Zar et al., 2010).

The spermatozoa shape of *M. amazonicum* and *M. pantanalense* was compared using 20 individuals of each species through geometric morphometric methods. One spermatozoon from each individual was photographed, and the standard position in which the spermatozoa were photographed is the same as exemplified in Figure 1A.

As for linear morphometrics, the spermatozoa were individually photographed under differential interference contrast (DIC) microscopy using a Zeiss Axio Imager Z2 microscope (Oberkochen, Germany) coupled with an imaging system using the Zeiss AxioVision software. All photos were taken by the same person, and the magnification (400x) and spermatozoa position were standardized in all photos (Viscosi & Cardini, 2011).

A total of five landmarks were digitized in the spermatozoa photographs to characterize the shape of this structure (Figure 1B). All landmarks were digitized using the tpsDig 2.04 and tpsUtil 1.26 software (Rohlf, 2004; 2005). Subsequently, the variables describing the spermatozoa shape were obtained using the tpsRelw 1.49 software (Rohlf, 2010) after performing a Generalized Procrustes Analysis (GPA).

To investigate whether the spermatozoa shape of *M. amazonicum* and *M. pantanalense* presents statistical differences, a Hotelling *t*-test (T^2) test was performed, followed by a discriminant analysis. The tpsRegr 1.31 software (Rohlf, 2009) was used to represent the spermatozoa shape associated with the discriminant analysis axes.

Results

The male reproductive system of *M. amazonicum* and *M. pantanalense* showed differences, mainly related to the histochemical patterns of the secretions present in the regions of the *vasa deferentia* (mainly MVD and DVD). Additionally, differences in the size and shape of the spermatozoa of both species were also observed.

Anatomy of the male reproductive system

The male reproductive system in *M. pantanalense* and *M. amazonicum* is comprised of a pair of elongated testes that run anteroposteriorly, with the anterior lobes being longer and thicker than the posterior lobes (Figure 2A). At the mid-region, the testes open into the vas deferens (VD) (Figure 2A-C), which extends lateroventrally in the cephalothorax up to the fifth pair of pereiopods. The VD is divided into three regions: proximal (PVD), medial (MVD), and distal (DVD). The PVD is connected to the testis and is morphologically characterized by its concentric coiling, which gradually decreases as the VD progresses and becomes straight (Figure 2B and C). The MVD begins as a straight tube from the end of the coiled region of the PVD, with the diameter slightly increasing until the last region of the VD (Figure 2D and E). The DVD corresponds to the area of the VD with the largest diameter, and at the end of this region, there is a dilation of the vessel called the ejaculatory duct (ED). Additionally, there is a group of cells on the muscle of the ED, facing the anterior region of the cephalothorax, constituting the androgenic gland (AG; Figure 2F-G).

Under SEM, the male gonopores of *M. pantanalense* and *M. amazonicum* are located in the artrodial membrane between the coxopodite of the fifth pair of pereiopods and the abdominal sternum (Figure 3A). The gonopore is covered with extensions of the coxopodite and its opening is oriented horizontally towards the opposite opening (Figure 3B-D). The second pair of pleopods is composed of the exopod, endopod, appendix masculina, and appendix interna (Figure 3E-G). In the appendix masculina, it is possible to observe the presence of long bristles (mainly in the anterior region) and short simple bristles (mainly in the posterior region) distributed from the anterior to the posterior region (Figure 3E-G). At the apex of the appendix interna, it is possible to observe hook-shaped structures known as cincinulli (Figure 3H and I). The spermatozoa ultrastructure under SEM shows two regions, namely, the main body and the acrosomal vesicle (Figure 3J-K). The latter region is subdivided into two other regions, which are the acrosomal cap (standardized as the anterior region of the acrossomal vesicle) and the spike (posterior region of the acrossomal vesicle). The acrosomal cap occupies most of the main body of the spermatozoa and has its surface composed of longitudinal stripes from the base of the spike to the margin of the cap. The free posterior margin of the cap becomes concave producing a funnel-like aspect of the posterior margin of the main body (Figure 3L and M). The spermatozoa of *M. amazonicum* has a more concave margin of the main body in relation to the margin of the main body of the spermatozoa of *M. pantanalense* (Figure 3L and M).

Spermatozoa ultrastructure under TEM

In both species, the spermatozoa are dispersed in the type I secretion present inside the vas deferens (Figures 4A and 5A). In longitudinal sections, the two regions of the spermatozoa can be observed: the main body and the acrosomal vesicle, with the latter region presenting the spike and the acrosomal cap. The spermatozoon has a tack-like shape due to the tapered shape of the main body and its association with the elongated acrosomal vesicle, which has a projection of the spike (Figures 4A and 5A). The acrosomal cap covers the main body of the spermatozoa through thin fibers that unite at a broad base that gives rise to the region of the spike (Figures 4C and 5B). The spike is elongated and is composed of bundles of longitudinally striated fibers, which resemble collagen fibrils (Figures 4D, E, and 5C). At the end opposite the base of the spike, the acrosomal cap is filled with electron-dense material and does not completely cover the main body, leaving an area of free plasma membrane up to the concave face of the main body. The membrane of the acrosomal cap is continuous with the plasma membrane (Figure 4F and G). Below the acrosomal cap, the main body is marked by the presence of a voluminous nucleus that is mixed with the cytoplasm, and the nuclear region is filled with granular chromatin. Membranous vesicles with two membrane units and membranes that resemble cristae are observed immersed in this nucleus-cytoplasmic material (Figures 4F-H and 5D-F).

Regarding the ultrastructural differences between the two species, it was observed that the region of the acrosomal cap of the *M. pantanalense* spermatozoa is wider, presenting the free margin of the main body as more angular and shallower (Figure 4C). On the other hand, in *M. amazonicum*, the same region of the acrosomal cap is thin, with the free margin of the main body more rounded and deeper, presenting a concave edge (Figure 5B).

Histological and histochemical analyses

Testes

The testes were histologically characterized as lobular or acinar type. The testicular lobules are surrounded by accessory cells, which in turn are surrounded by connective tissue (Figure 6A and B). Spermatogonia, primary and secondary spermatocytes can be observed at the periphery of the testicular lobules (Figure 6A). In addition, inside the lobules, it is possible to observe free spermatozoa in attached channels that form the seminiferous ducts (Figure 6C and D). Each lobule or acinus is connected to the seminiferous duct by a small collecting duct. This duct receives mature spermatozoa, which are enveloped by secretions (Figure 6D). The lumen of the seminiferous duct is composed of a basophilic secretion, which is weakly reactive to proteins, and toluidine blue, very weakly to acid polysaccharides, and strongly reactive to neutral polysaccharides (Figure 6A-F). The spermatozoa are weakly reactive to acid polysaccharides and strongly reactive to proteins, toluidine blue, and neutral polysaccharides (Figure 6C-F). The histological and histochemical analyses of the testes conducted in the present study did not reveal any differences between *M. amazonicum* and *M. pantanalense*. Thus, only the histological and histochemical patterns of *M. pantanalense* testes were depicted in Figure 6.

Vas deferens

Proximal region (PVD)

The epithelium in the PVD is characterized by the occurrence of a typhlosole on one of the duct faces, while the other face has a simple epithelium composed of cubic to squamous cells. The typhlosole is composed of a group of compacted columnar cells, forming a protrusion directed towards the lumen. The nucleus of the epithelial cells is irregular, and the duct wall is lined by a thin muscular layer (Figure 7A and B).

In the lumen, spermatic masses are present, occupying most of the central region, and the spermatozoa are immersed in a slightly basophilic secretion called type I. In addition, type I secretion is weakly reactive to proteins, slightly basophilic and reactive to toluidine blue and acidic polysaccharides, and strongly reactive to neutral polysaccharides (Figure 7C-F). Along the PVD, specifically on the typhlosole face, the presence of a type II secretion can be observed (Figure 7B). This secretion is also slightly eosinophilic, non-reactive to acidic polysaccharides, weakly reactive to neutral polysaccharides, and strongly reactive to proteins and toluidine blue (Figure 7B-E). The acrosomal vesicle of the spermatozoa was moderately reactive to neutral polysaccharides and strongly reactive to proteins, toluidine blue (β-metachromasia), and acidic polysaccharides (Figure 7C-F). The histological and histochemical analyses of the PVD region conducted in the present study did not reveal any differences between *M. amazonicum* and *M. pantanalense*. Thus, only the histological and histochemical patterns of *M. pantanalense* testes were depicted in Figure 7.

Medial region (MVD)

The epithelium in the MVD is still characterized by two different types of cells. On one face of the duct, it is possible to visualize simple columnar cells that constituted the former typhlosole. Thus, this epithelium, which varies from cubic to columnar, is composed of secretory cells, which are no longer prominent towards the lumen, as occurs in the PVD, but rather form a specialized face of the MVD (Figure 8A and B). The other face of the duct remains with cells forming a simple squamous epithelium. In this region, the epithelium of the VD is seated on a thicker muscular layer than in relation to the PVD (Figure 8A and B).

Spermatozoa are immersed in a weakly basophilic type I secretion, which is surrounded by an acidophilic type II secretion (Figure 8B). In this region, it is possible to note the presence of a type III secretion that surrounds the other two secretions and that has a eosinophilic character (Figure 8B).

In the MVD, some of the compounds forming the future spermatophore (i.e., secretions I, II, and III) showed histochemical differences between the two species, the main differences being related to the reactivity of toluidine blue and neutral polysaccharides.

In *M. pantanalense*, the type I secretion was weakly reactive to toluidine blue and acid polysaccharides, moderately reactive to proteins, and strongly reactive to neutral polysaccharides. The type II secretion was non-reactive to acid polysaccharides, weakly reactive to toluidine blue, and moderately reactive to proteins and neutral polysaccharides (Figure 8C-H). On the other hand, the type III secretion was non-reactive to acid polysaccharides and toluidine blue, moderately reactive to neutral polysaccharides, and strongly reactive to proteins (Figure 8C-F). In *M. amazonicum*, the type I secretion was weakly reactive to toluidine blue and acid polysaccharides, moderately reactive to proteins, and strongly reactive to neutral polysaccharides. The type II secretion was non-reactive to acid polysaccharides, moderately reactive to proteins and toluidine blue, and strongly reactive to neutral polysaccharides (Figure 8C-H). Meanwhile, the type III secretion was non-reactive to acid polysaccharides and toluidine blue, moderately reactive to neutral polysaccharides, and strongly reactive to proteins (Figure 8C-H). The acrosomal vesicle of the spermatozoa was strongly reactive to proteins, acid polysaccharides, and neutral polysaccharides in both species (Figure 8C-H).

Distal region (DVD)

In the DVD, the lumen is notably wider than in the two anterior regions (i.e., PVD and MVD), forming the ejaculatory duct at the end of the VD. Unlike in the PVD and MVD, only one type of cell, consisting of simple cuboidal epithelium, is observed in the DVD. The volume of secretion in this region may have caused compression of the columnar cells that originated the typhlosole region. Additionally, a thick muscular layer can be observed around the epithelium of the DVD (Figure 9A-C).

Apparently, in the matrix of this vessel region, type I and II secretions are mixed, surrounding the spermatozoa (Figure 9A and B). Type III secretion is characterized as basophilic and surrounds the entire eosinophilic matrix of type II secretion (Figure 9B and C). In the DVD, some of the compounds that form the future spermatophore (i.e., secretion I, II, and III) presented histochemical differences between the two species. The main differences were related to the reactivity to toluidine blue and neutral and acidic polysaccharides.

In *M. pantanalense,* type I secretion was weakly reactive to proteins and toluidine blue, moderately reactive to acidic polysaccharides, and strongly reactive to neutral polysaccharides (Figure 9D-I). Type II secretion was non-reactive to acidic polysaccharides, weakly reactive to toluidine blue, moderately reactive to neutral polysaccharides, and strongly reactive to proteins (Figure 9D-I). Type III secretion was non-reactive to acidic polysaccharides and toluidine blue, and moderately reactive to proteins and neutral polysaccharides (Figure 9D-I). In *M. amazonicum*, type I secretion was weakly reactive to proteins and toluidine blue, moderately reactive to acidic polysaccharides, and strongly reactive to neutral polysaccharides (Figure 9D-J). Type II secretion was non-reactive to neutral and acidic polysaccharides, moderately reactive to toluidine blue, and strongly reactive to proteins (Figure 9D-J). Type III secretion was non-reactive to acidic polysaccharides and toluidine blue, moderately reactive to proteins, and strongly reactive to neutral polysaccharides (Figure 9D-J). The acrosomal vesicle of the spermatozoa was strongly reactive to proteins, acidic polysaccharides, and neutral polysaccharides in both species (Figure 9D-J).

Spermatozoa morphometrics

Significant differences were observed in spermatozoa length (Mann-Whitney; Z = 16.47 and $p < 0.001$) and main body width (Mann-Whitney; Z = 21.1 and $p < 0.001$) between the species. The spermatozoon of *M. amazonicum* is longer, while the main body of the spermatozoon of *M. pantanalense* is wider. Spermatozoa length varied from 19.0 to 24.4 µm (21.7 ± 1.1 µm) and 15.2 to 22.8 µm (20.3 ± 0.8 µm) in *M. amazonicum* and *M. pantanalense*, respectively. In addition, the main body width varied from 10.3 to 16.0 µm (13.6 ± 1.3 µm) and 13.2 to 21.3 µm (16.0 ± 1.0 µm) in *M. amazonicum* and *M. pantanalense*, respectively.

Among males of both species, there was 46.2% and 65.2% significant variation in spermatozoa length (Kruskal-Wallis; H = 361.27 and $p < 0.001$) and main body width

(Kruskal-Wallis; H = 555.94 and $p < 0.001$), respectively. Among males of *M. amazonicum*, there was 5.78% and 25.26% significant variation in spermatozoa length (Kruskal-Wallis; H = 70.16 and $p < 0.001$) and main body width (Kruskal-Wallis; H = 168.46 and $p < 0.001$), respectively. Among males of *M. pantanalense*, there was 6.84% and 7.89% significant variation in spermatozoa length (Kruskal-Wallis; H = 70.03 and $p < 0.001$) and main body width (Kruskal-Wallis; H = 83.02 and $p < 0.001$), respectively.

Discriminant analysis showed that the spermatozoa morphology of *M. amazonicum* and *M. pantanalense* is statistically different (Hotelling $T^2 = 46.25$; F = 6.69; $p < 0.001$). The analysis separated the two species with 90% effectiveness (Figure 10A). The spermatozoa morphology of both species varied mainly in the region of the main body and spike. In *M. amazonicum*, the spike of the spermatozoon is longer than that of *M. pantanalense*. On the other hand, the main body of the spermatozoon of *M. pantanalense* is more robust than that of *M. amazonicum* (Figure 10B).

Micrographic documentation

Figure 1. Spermatozoa of *Macrobrachium pantanalense* Dos Santos, Hayd & Anger, 2013. A. Field of view showing how spermatozoa were analyzed, marked spermatozoa are in the standard measurement position (Magnification 400x). B. Landmarks (in red) that were inserted for characterization of the shape of the spermatozoa of *M. amazonicum* and *M. pantanalense*. C. Illustration of a spermatozoon of *M. pantanalense* and morphological dimensions that were measured for the execution of linear morphometry analyses. MBW = Main body width and SL = Spermatozoa length.

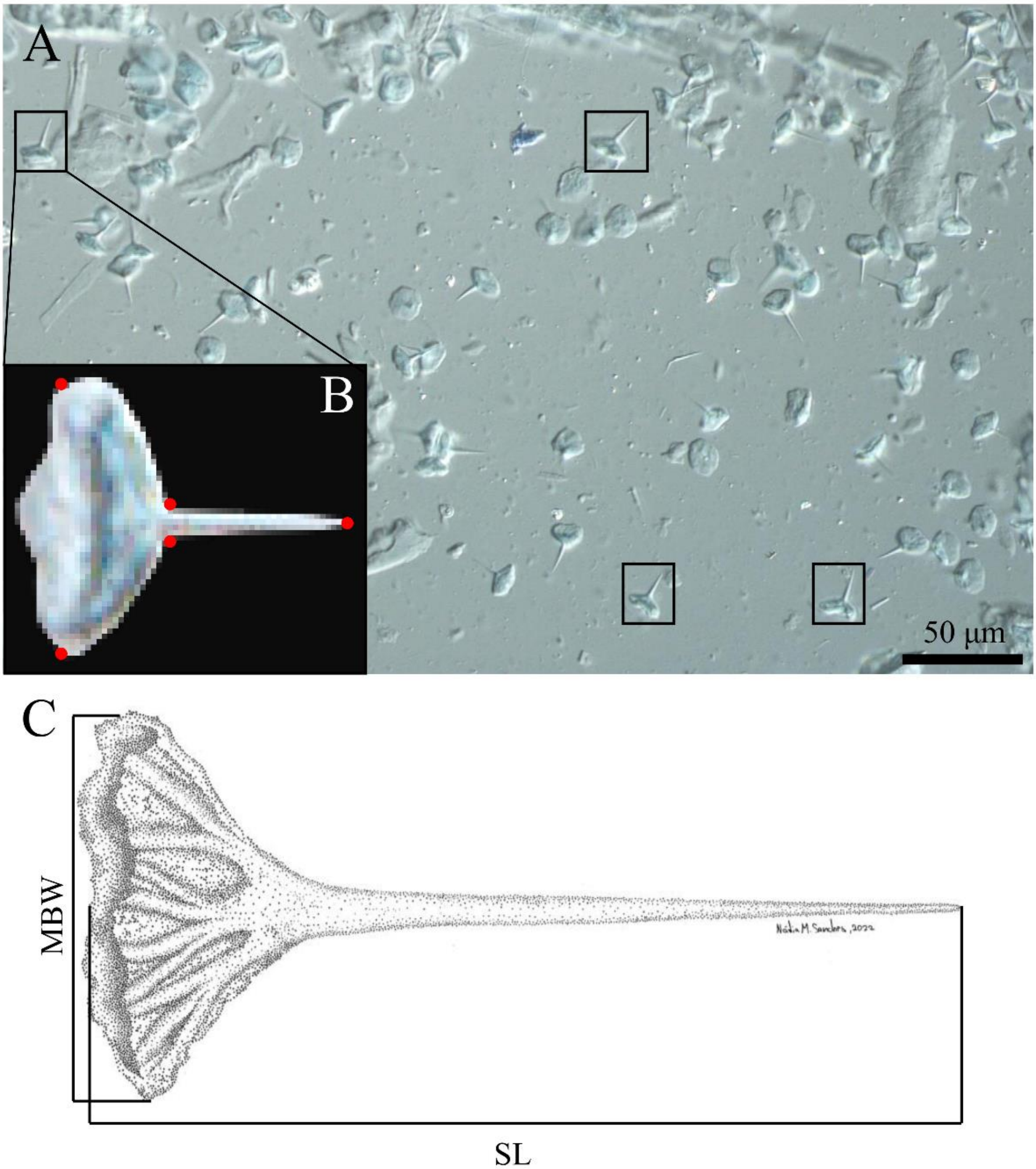
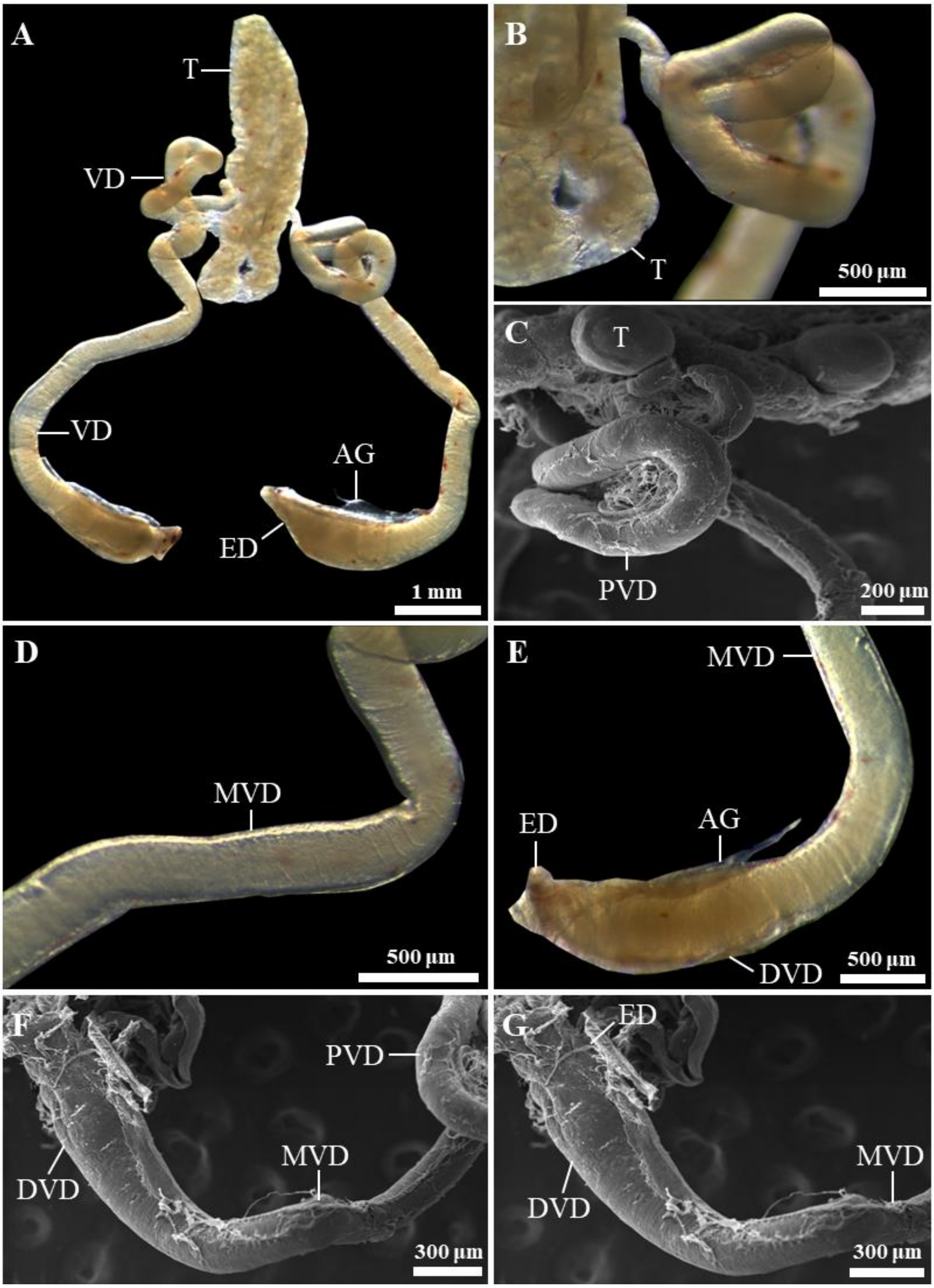

A
B
50 µm
C
MBW
SL

Figure 2. Male reproductive system of *Macrobrachium pantanalense* (Dos Santos, Hayd & Anger, 2013). A. Overview of the male reproductive system, showing the testes associated with the convoluted region of the vas deferens (PVD) and the subsequent regions (MVD and DVD). B and C. View of the connection between the testis and PVD. D and E. Overview of the MVD and DVD, respectively. F. View of the MVD highlighting the connection between PVD, MVD, and DVD. G. Detail of the DVD highlighting the separation between MVD and DVD and the ejaculatory duct. AG = Androgenic gland, ED = Ejaculatory duct, T = Testes, VD = Vas deferens, PVD = Proximal vas deferens, MVD = Medial vas deferens, and DVD = Distal vas deferens.


A
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300 µm

Figure 3. Morphology of the sternum, second pair of pleopods, and spermatozoa of *Macrobrachium pantanalense* (Dos Santos, Hayd & Anger, 2013). A. Ventral view of the male sternum, showing the five pairs of pereiopods and male gonopores located on the fifth pair of pereiopods (white arrows). B. Detail of the fifth pair of pereiopods, with the opening of the male gonopore indicated by white arrowheads. C and D. Individual views of the coxae of each pereiopod (fifth pair), with the opening of the gonopores indicated by white arrowheads. E. General morphology of a pleopod (from the second pair), showing the exopod, endopod, male appendix, and internal appendix. F. Detail of a pleopod, showing the endopod, appendix masculina, and appendix interna. G. Detail of the appendix masculina. H. Detail of the appendix interna (asterisk), with the white arrow showing the cincinulli. I. Detail of the cincinulli (white arrow) present in the appendix interna. J and K. General view of a spermatozoon of *M. pantanalense* and *M. amazonicum*, respectively. L and M. Detail of the region of the acrosomal cap and main body of a spermatozoon of *M. pantanalense* and *M. amazonicum*, respectively. AI = Appendix interna, AM = Appendix masculina, EN = Endopod, EX = Exopod, AC = Acrosomal cap, AV = Acrosomal vesicle, and MB = Main body.

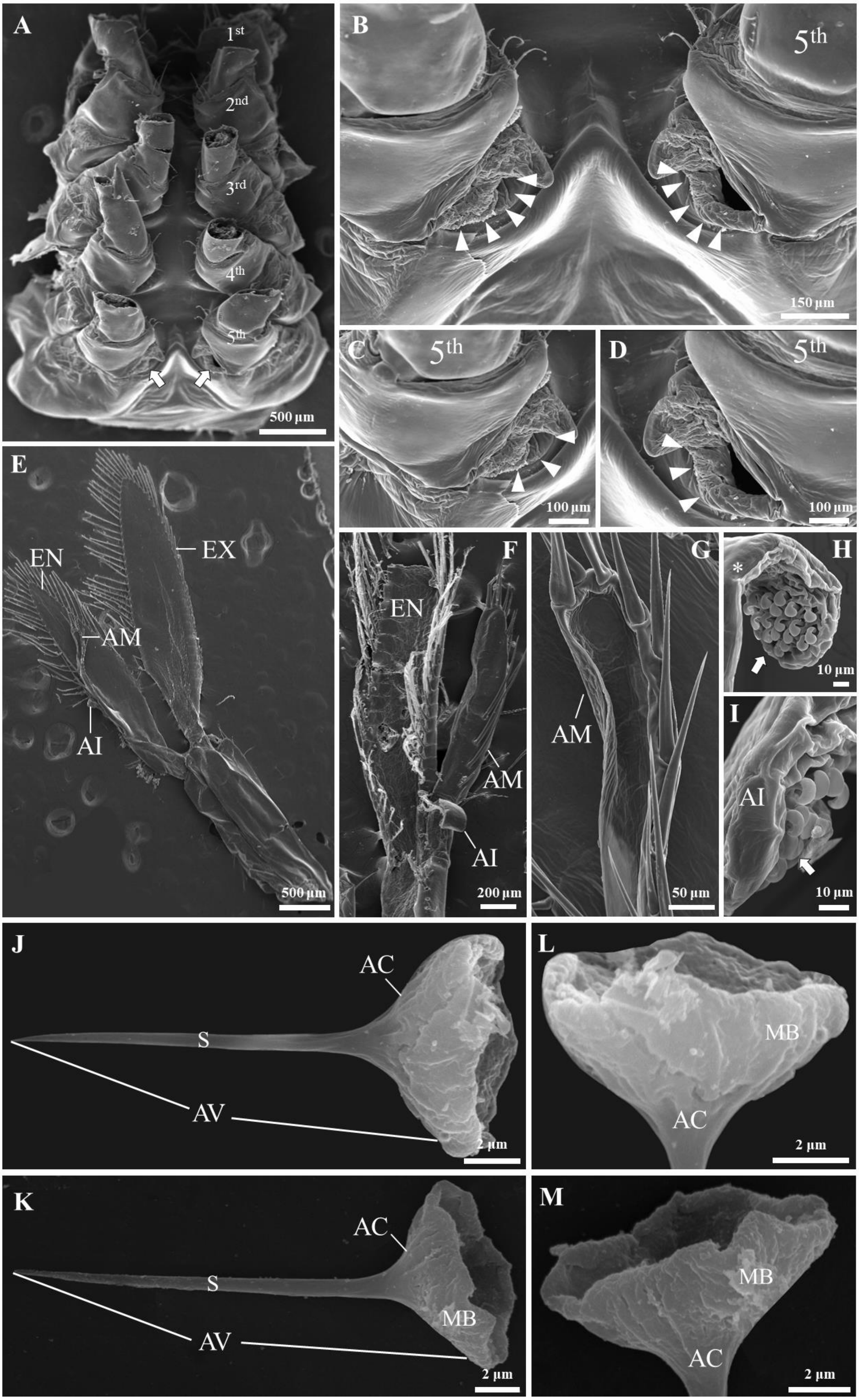

A
1st
2nd
3rd
4th
5th
500 µm
B
5th
150 µm
C
5th
100 µm
D
5th
100 µm
E
EN
EX
AM
AI
500 µm
F
EN
AM
AI
200 µm
G
AM
50 µm
H
*
10 µm
I
AI
10 µm
J
AC
S
AV
2 µm
L
MB
AC
2 µm
K
AC
S
MB
AV
2 µm
M
MB
AC
2 µm

Figure 4. Ultrastructure of the spermatozoa of *Macrobrachium pantanalense* Dos Santos, Hayd & Anger, 2013. A. Distal region of vas deferens showing free and immersed spermatozoa among secretions. B. Longitudinal section of the spermatozoon showing the acrosomal vesicle, which is composed of the acrosomal cap and spike, located above the main body and nucleus. Black arrows point to the free margin of the main body, which is angular and shallow. C. Detail of the main body and acrosomal cap region, showing the presence of organelles similar to degenerated mitochondria inside the nucleus, and a double membrane that delimits the entire nucleus-cytoplasmic region. D and E. Longitudinal and transverse section of the spike region, respectively. This region is composed of bundles of longitudinal fibers that resemble collagen fibers. F. Margin of the acrosomal cap on the main body. The main body contains the nucleus mixed with cytoplasm and many vesicles. G. Detail of the end of the acrosomal cap filled with electron-dense material, marking the limit of the acrosomal vesicle. The cap is delimited by a membrane unit (black arrows) and does not completely cover the main body. The vesicles present in the nucleus-cytoplasmic material have two membrane units (white arrows) and internal membranes (black arrowheads). H. Detail of the nucleus-cytoplasmic material with fibrillar chromatin and vesicles with two membrane units (white arrows) and internal membranes (black arrowheads). AC = Acrosomal cap; AV = Acrosomal vesicle; N = Nucleus; MB = Main body; MP = Plasma membrane; S = Spike; SPZ = Spermatozoa; SE = Secretion.

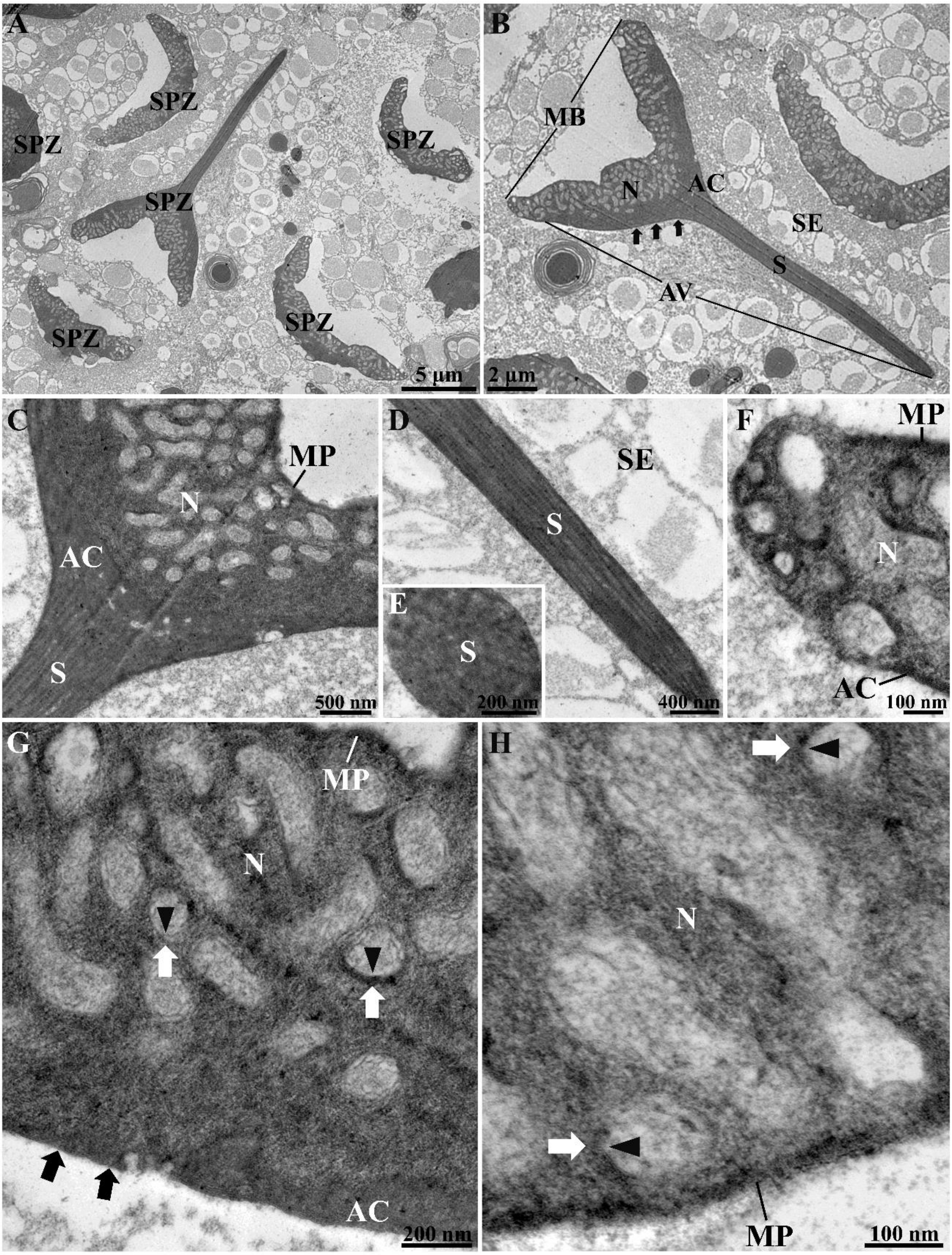

A
SPZ
SPZ
SPZ
SPZ
SPZ
SPZ
5 µm
B
MB
N
AC
SE
S
AV
2 µm
C
MP
N
AC
S
500 nm
D
SE
S
400 nm
E
S
200 nm
F
MP
N
AC
100 nm
G
MP
N
AC
200 nm
H
N
MP
100 nm

Figure 5. Ultrastructure of the spermatozoa of *Macrobrachium amazonicum* (Heller, 1862). A. Longitudinal section of the spermatozoa showing the acrosomal vesicle, which is composed of the acrosomal cap and spike, located above the main body and nucleus. B. Detail of the main body and acrosomal cap region showing the presence of organelles similar to degenerated mitochondria within the nucleus. In addition, there is a double membrane that delimits the entire nucleus-cytoplasmic region. White arrows point to the free margin of the main body, which is concave, rounded, and deep. C. Longitudinal section of the spike region. It can be observed that this region is composed of bundles of longitudinal fibers that resemble collagen fibers. D and E. Details of the main body region, highlighting the acrosomal cap located above the nucleus and the double membrane that delimits the entire nucleus-cytoplasmic region. The vesicles present in the nucleus-cytoplasmic material have two membrane units (white arrows) and internal membranes (black arrowheads). F. Detail of the nucleus-cytoplasmic material with fibrillar chromatin and vesicles with two membrane units (white arrows) and internal membranes (black arrowheads). AC = Acrosomal cap; AV = Acrosomal vesicle; N = Nucleus; MB = Main body; MP = Plasma membrane; S = Spike; SPZ = Spermatozoon; SE = Secretion.

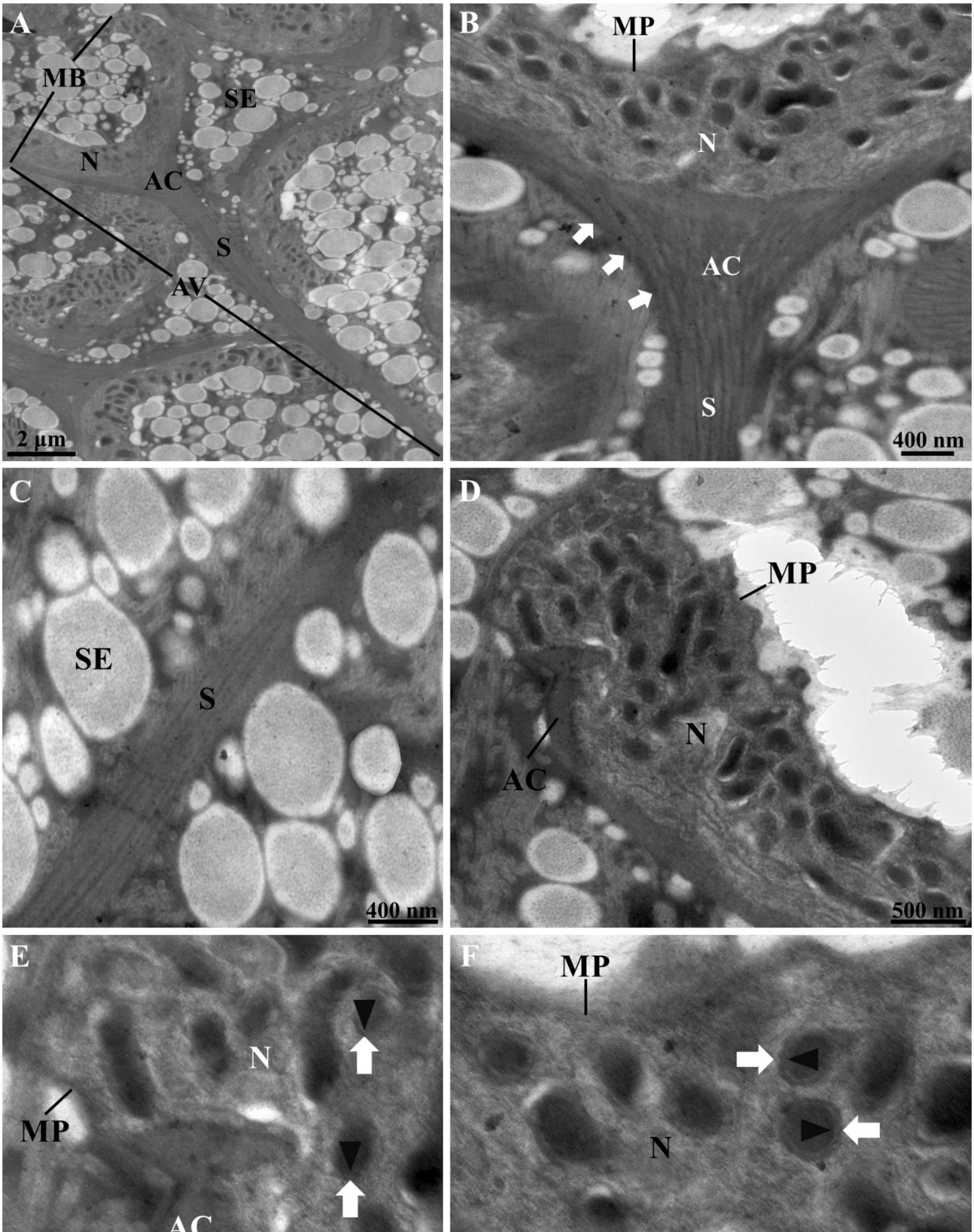

A
MB
SE
N
AC
S
AV
2 µm
B
MP
N
AC
S
400 nm
C
SE
S
400 nm
D
MP
N
AC
500 nm
E
N
MP
AC
200 nm
F
MP
N
200 nm

Figure 6. Morphological and histochemical description of the testes of *Macrobrachium pantanalense* Dos Santos, Hayd & Anger, 2013. A. General morphology of the testes, showing the spermatozoa immersed in secretion in the lumen of the seminiferous tubules. B. Detail of a lobule, showing the spermatozoa and the spermatogenic zone. C. Details of the seminiferous tubule lumen, where the spermatozoa are located in a matrix weakly reactive to proteins, while the germ cells are strongly reactive. D. Details of the seminiferous tubule lumen, where the spermatozoa are located in a matrix strongly reactive to neutral polysaccharides, and the germ cells (spermatocytes and spermatozoa) were also strongly reactive to neutral polysaccharides. E. Details of the seminiferous tubule lumen, where the spermatozoa are located in a matrix formed by a secretion weakly reactive to acidic polysaccharides, while the germ cells (spermatocytes and spermatozoa) were strongly reactive to acidic polysaccharides. F. Detail of the seminiferous tubule, showing germ cells present in the central portion of the tubule. [Toluidine blue (pH 4.0)]. CT = Connective tissue, SGZ = Spermatogenic zone, SPZ = Spermatozoon, SCI = Spermatocyte I.

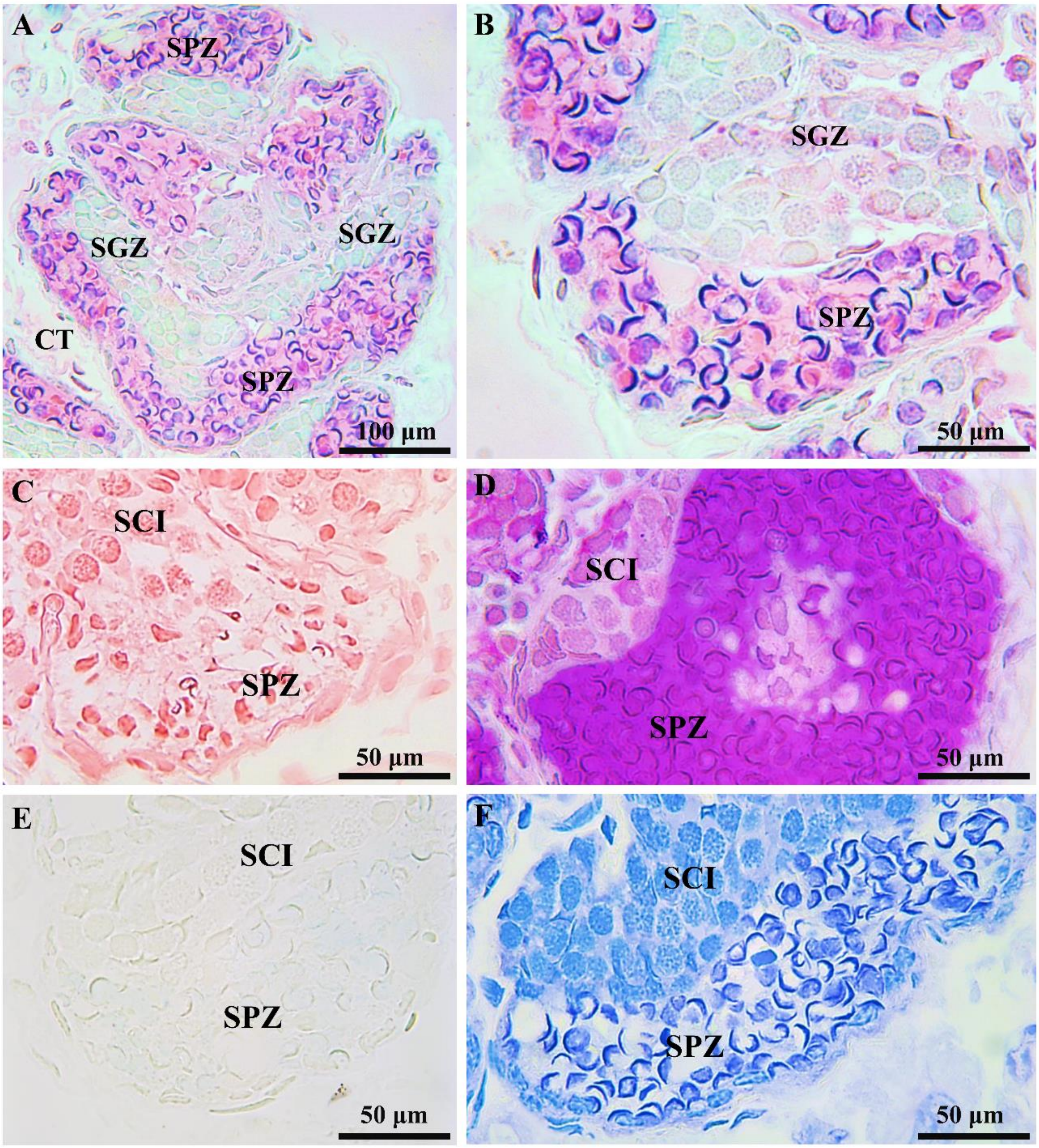

A
SPZ
SGZ
SGZ
CT
SPZ
100 µm
B
SGZ
SPZ
50 µm
C
SCI
SPZ
50 µm
D
SCI
SPZ
50 µm
E
SCI
SPZ
50 µm
F
SCI
SPZ
50 µm

Figure 7. Morphological and histochemical description of the proximal region of the vas deferens of *Macrobrachium pantanalense* Dos Santos, Hayd & Anger, 2013. A. Cross section of the proximal region of the vas deferens (PVD), showing spermatozoa immersed in type I secretion. The epithelium was characterized by the presence of a typhlosole in one of the walls, while the other wall was characterized by the formation of cuboidal cells, with irregular nuclei and covered by a thin muscular layer. B. Detailed cross section of the proximal region of the vas deferens, in addition, in this region it is possible to observe the presence of a slightly eosinophilic type II secretion. C. Detail of the cross section of the PVD, type I secretion was weakly reactive to proteins, while type II secretion and the acrosomal vesicle of the spermatozoa were strongly reactive. D. Detail of the cross section of the PVD, type I secretion was weakly reactive to neutral polysaccharides, while type II secretion and the acrosomal vesicle of the spermatozoa were strongly and moderately reactive, respectively. E. Detail of the cross section of the PVD, type I secretion was weakly reactive to acidic polysaccharides, while type II secretion was non-reactive. The acrosomal vesicle of the spermatozoa was strongly reactive to acidic polysaccharides. F. Detail of the cross section of the PVD, showing the spermatic matrix surrounded by type I secretion, and this surrounded by type II secretion. [Toluidine blue (pH 4.0)]. EP = epithelium, MU = Muscle, SI and II = Secretion type I and II, SPZ = Spermatozoa, TY = Typhlosole.

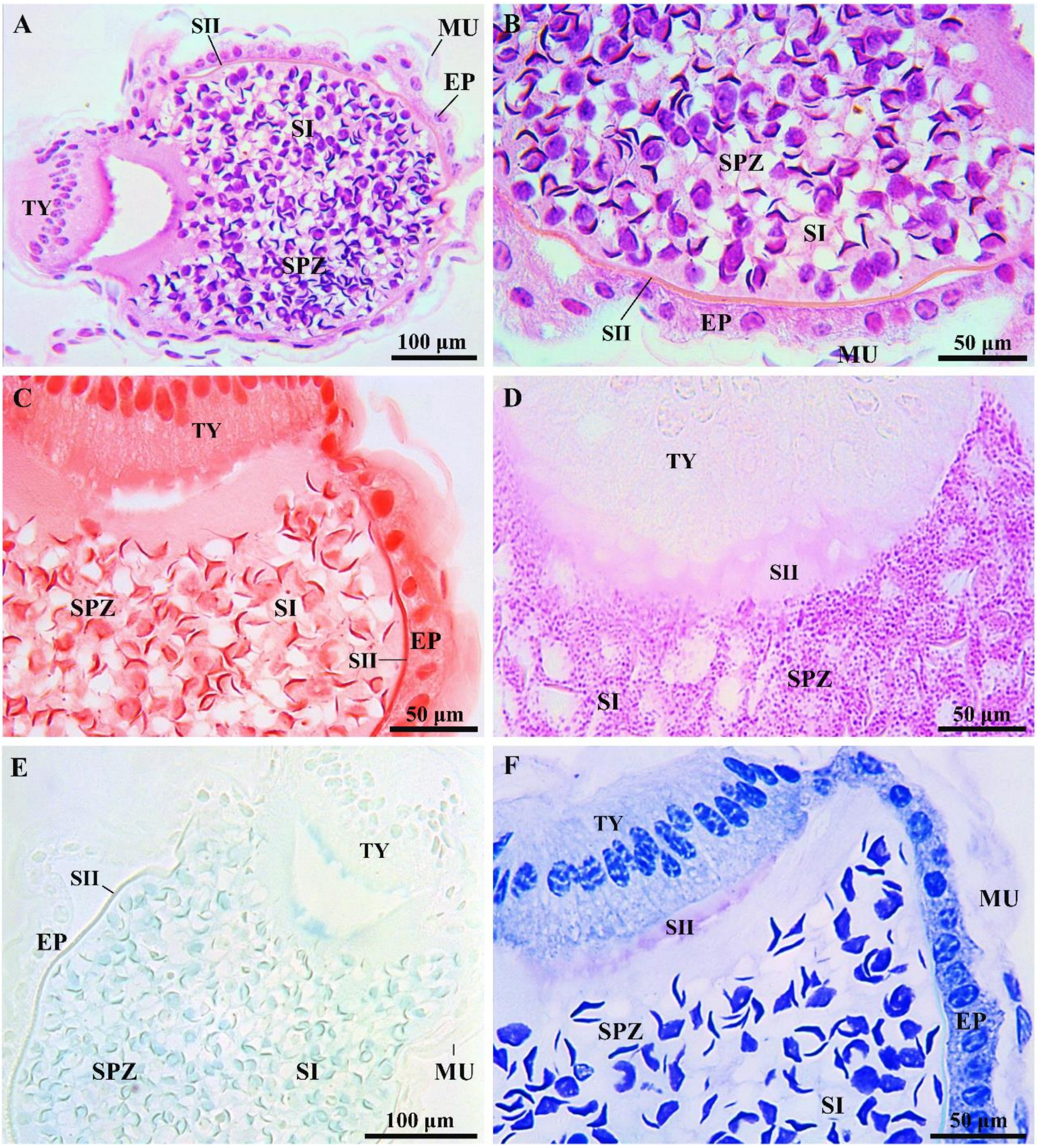

A
SII
MU
EP
SI
TY
SPZ
100 µm
B
SPZ
SI
SII
EP
MU
50 µm
C
TY
SPZ
SI
EP
SII
50 µm
D
TY
SII
SPZ
SI
50 µm
E
TY
SII
EP
SPZ
SI
MU
100 µm
F
TY
SII
MU
SPZ
EP
SI
50 µm

Figure 8. Morphological and histochemical description of the medial region of the vas deferens of *Macrobrachium pantanalense* Dos Santos, Hayd & Anger, 2013. A. Longitudinal section of the medial region of the vas deferens (MVD), showing spermatozoa immersed in type I secretion (basophilic), surrounded by type II (acidophilic) and type III (acidophilic) secretions. The epithelium of this region was characterized by two types of cells, one of the walls is composed of columnar cells, and the other epithelial wall was characterized by the formation of simple cuboidal cells. The muscular layer in this region is thicker than in the proximal region (PVD). B. Detailed longitudinal section of the medial region of the vas deferens (MVD). C. Detail of the longitudinal section, type I and II secretions were moderately reactive to proteins, while type III secretion was strongly reactive. D and E. Detail of the longitudinal section of MVD in *M. pantanalense* and *M. amazonicum*, respectively. The main difference between the two species was type II secretion, which was moderately and strongly reactive to neutral polysaccharides in *M. pantanalense* and *M. amazonicum*, respectively. F. Detail of the longitudinal section, type I secretion was weakly reactive to acidic polysaccharides, while type II and III secretions were non-reactive. G and H. Detail of the longitudinal section of MVD in *M. pantanalense* and *M. amazonicum*, respectively. The main difference between the two species was type II secretion, which was weakly and moderately reactive to toluidine blue in *M. pantanalense* and *M. amazonicum*, respectively [Toluidine blue (pH 4.0)]. MU = Muscle, SI, II and III = Secretion type I, II and III, SPZ = Spermatozoa, EP = Epithelium.

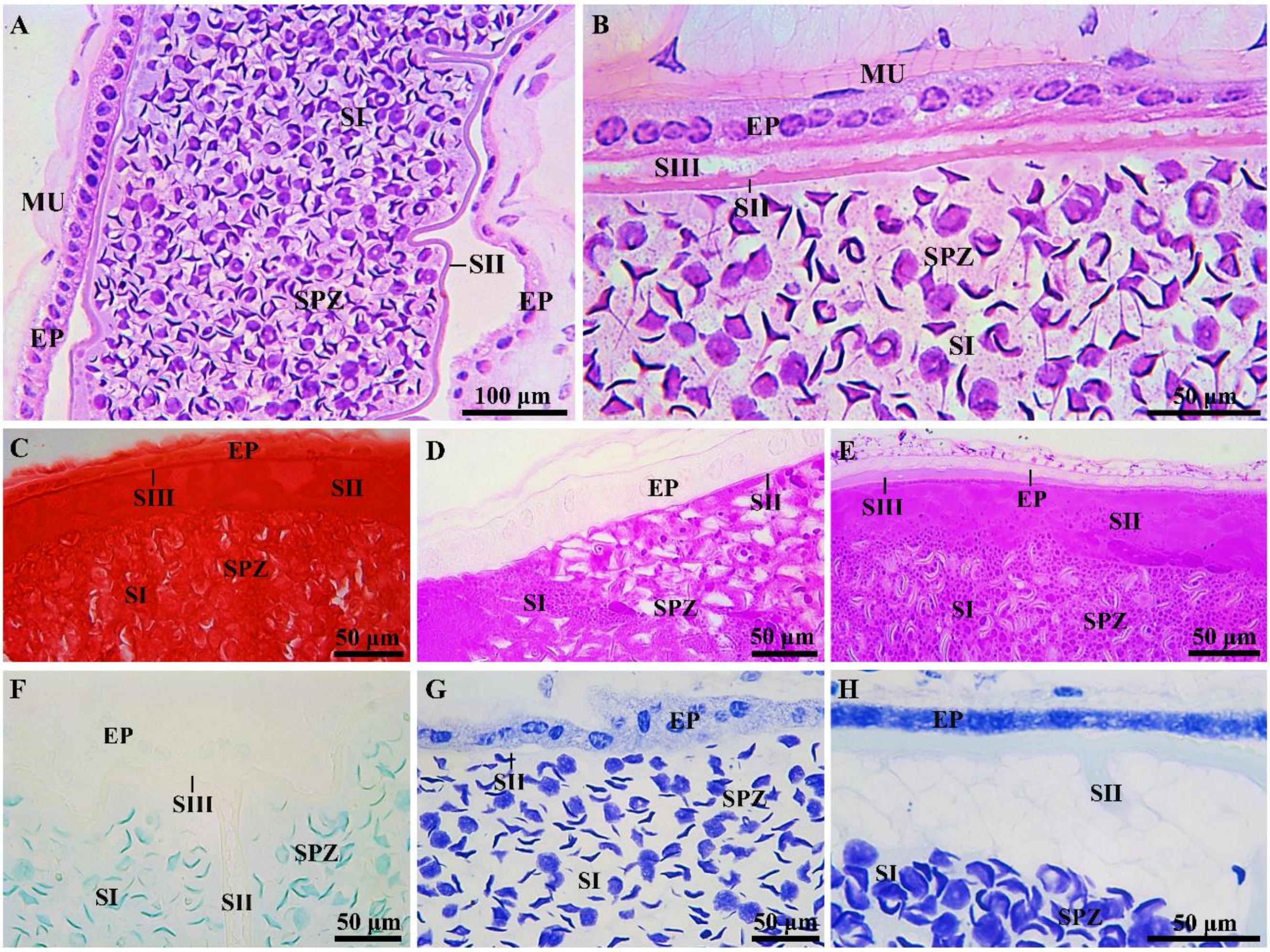

A
MU
EP
SI
SPZ
SII
EP
100 µm
B
MU
EP
SIII
SII
SPZ
SI
50 µm
C
EP
SIII
SII
SPZ
SI
50 µm
D
EP
SII
SI
SPZ
50 µm
E
SIII
EP
SII
SI
SPZ
50 µm
F
EP
SIII
SPZ
SI
SII
50 µm
G
EP
SII
SPZ
SI
50 µm
H
EP
SII
SI
SPZ
50 µm

Figure 9. Morphological and histochemical description of the distal region of the vas deferens of *Macrobrachium pantanalense* Dos Santos, Hayd & Anger, 2013. A. Longitudinal section of the distal region of the vas deferens (DVD), showing spermatozoa immersed in secretion. Apparently, type I and II secretions are mixed and surrounded by type III secretion, which is acidophilic. The epithelium of this region is characterized by only one type of cell, which is simple cuboidal. Note the thick muscular layer in this region. B and C. Detailed longitudinal section of the distal region of the vas deferens (DVD), showing spermatozoa immersed in secretion, as well as the characteristic epithelium and musculature of the region. D. Detail of the longitudinal section of the DVD, type I secretion was weakly reactive to proteins, while type II and III secretions were strongly and moderately reactive to proteins, respectively. E and F. Detail of the longitudinal section of the DVD in *M. pantanalense* and *M. amazonicum*, respectively. The main difference between the two species was the reaction of type II secretions (moderately reactive in *M. pantanalense* and non-reactive in *M. amazonicum*) and type III secretions (moderately reactive in *M. pantanalense* and strongly reactive in *M. amazonicum*) to neutral polysaccharides. G and H. Detail of the longitudinal section of the DVD in *M. pantanalense* and *M. amazonicum*, respectively. The main difference between the two species was the reaction of type I secretion, which was moderately and weakly reactive to acidic polysaccharides in *M. pantanalense* and *M. amazonicum*, respectively. I and J. Detail of the longitudinal section of the DVD in *M. pantanalense* and *M. amazonicum*, respectively. The main difference between the two species was the reaction of type II secretion, which was weakly and moderately reactive to toluidine blue in *M. pantanalense* and *M. amazonicum*, respectively. MU = Muscle, SE = Secretion, SPZ = Spermatozoa, EP = Epithelium.

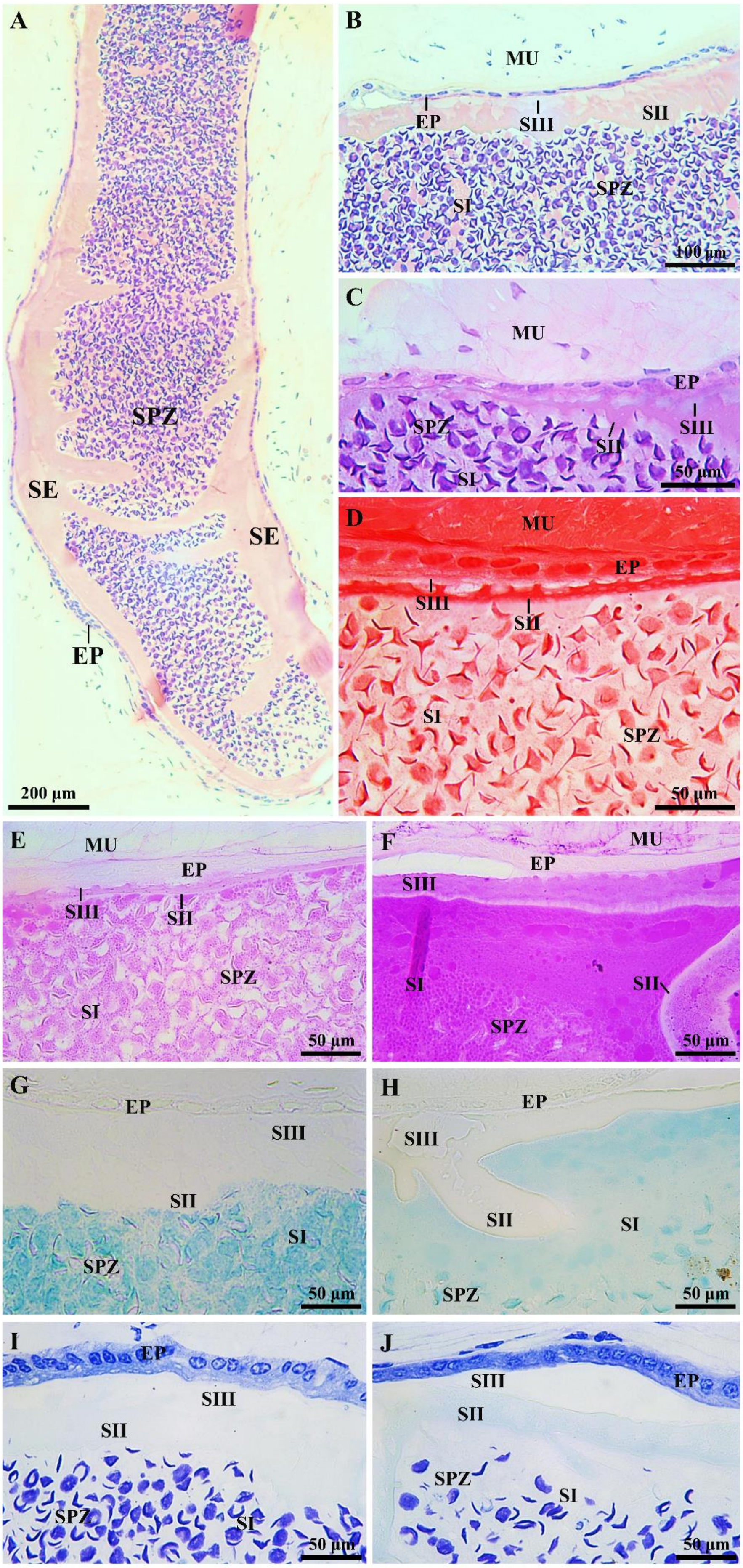
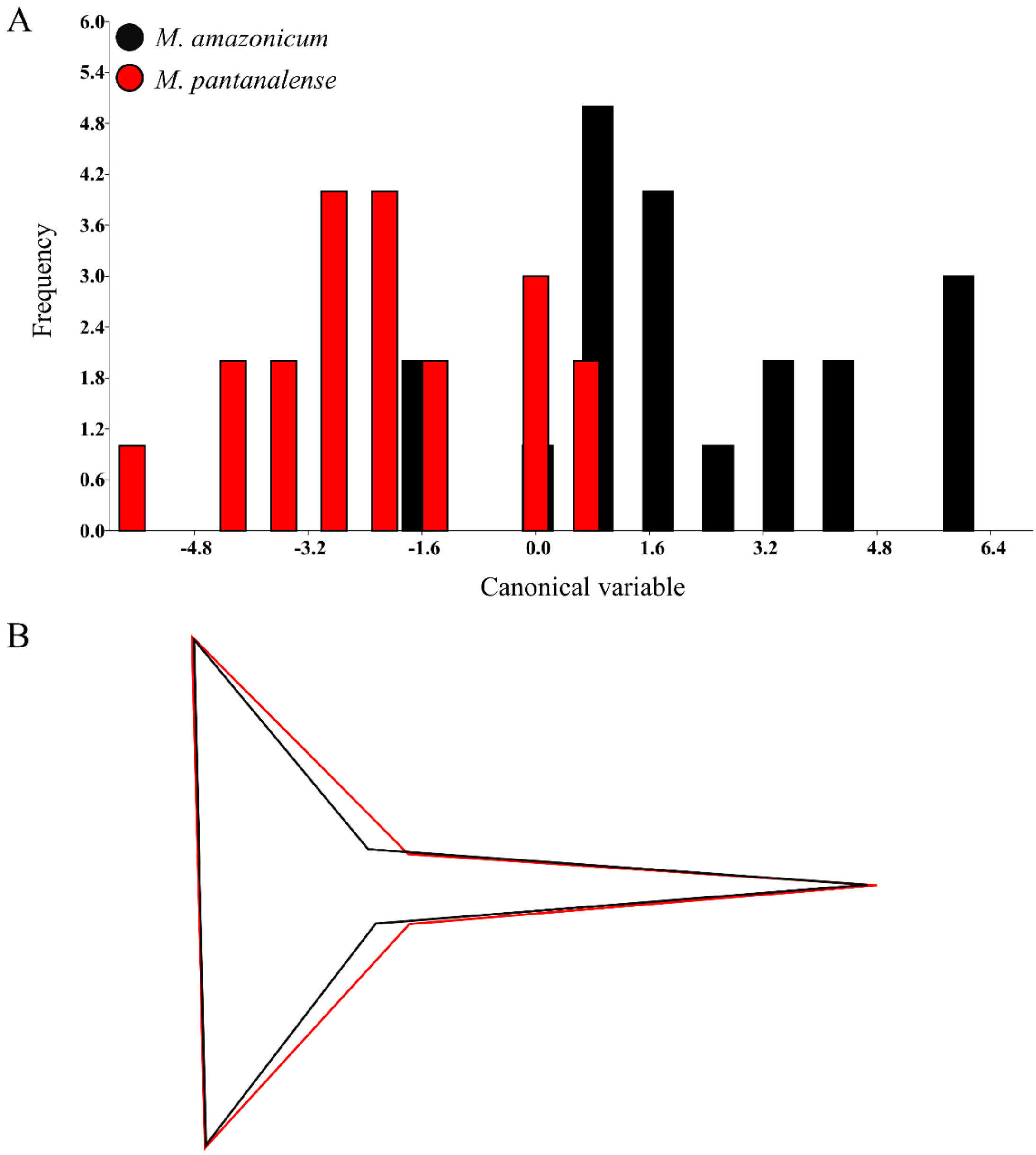

A
SPZ
SE
SE
EP
200 µm
B
MU
EP
SIII
SII
SI
SPZ
100 µm
C
MU
EP
SIII
SPZ
SII
SI
50 µm
D
MU
EP
SIII
SII
SI
SPZ
50 µm
E
MU
EP
SIII
SII
SPZ
SI
50 µm
F
MU
EP
SIII
SI
SII
SPZ
50 µm
G
EP
SIII
SII
SI
SPZ
50 µm
H
EP
SIII
SII
SI
SPZ
50 µm
I
EP
SIII
SII
SPZ
SI
50 µm
J
SIII
EP
SII
SPZ
SI
50 µm

Figure 10. Variation in the shape of spermatozoa of *Macrobrachium amazonicum* (Heller, 1862) and *M. pantanalense* Dos Santos, Hayd & Anger, 2013. A. Histogram originated from discriminant analysis showing the separation of the two species according to spermatozoa shape. Discrimination achieved more than 90% effectiveness. B. Graphical representation of sperm shape of *M. amazonicum* (in black) and *M. pantanalense* (in red). The spike region is longer in *M. amazonicum*, while the main body region is more robust in *M. pantanalense*.


A
M. amazonicum
M. pantanalense
6.0
5.4
4.8
4.2
3.6
3.0
2.4
1.8
1.2
0.6
0.0
Frequency
-4.8
-3.2
-1.6
0.0
1.6
3.2
4.8
6.4
Canonical variable
B

Discussion

According to the results, differences were found in the histochemical patterns of secretions in some regions of the *vasa deferentia*, as well as in the morphology of the spermatozoa between *M. amazonicum* and *M. pantanalense*. These differences, particularly in histochemical patterns, may have a direct relationship with the origin location of each species, since the chemical composition of secretions is related to the physicochemical characteristics of the water where these animals inhabit. Additionally, this study provides a novel description of the ultrastructure of the spermatozoa of two species of the *Macrobrachium* genus, as well as new insights into the morphology and histochemistry of the male reproductive system of *M. pantanalense*.

Anatomy and histochemistry of the male reproductive system

The external morphology of the male reproductive system of *M. pantanalense* is similar to the general pattern observed in other species of *Macrobrachium* (Chow, 1982; Paschoal & Zara, 2020, 2022; Nogueira et al., 2023), where the *vasa deferentia* opens from the lobes in the medial region of the testes. In addition, at the end of the *vasa deferentia*, the androgenic gland (AG) can be observed, a structure responsible for the development of secondary sexual characteristics. The development of this gland accompanies the growth of the animal; therefore, a larger animal also has a more extensive AG (Paschoal & Zara, 2019). This trait may influence the exaggerated development of some secondary sexual characteristics, as observed in males of some *Macrobrachium* species (Karplus & Barki, 2019).

The musculature surrounding the regions of the *vasa deferentia* corroborates what has been observed in other species of decapod crustaceans, *i.e.*, the proximal region is surrounded by a thin layer that increases in caliber and becomes thicker towards the distal region of the *vasa deferentia* (Tomas et al., 2019; Paschoal & Zara, 2020; 2022; Nogueira et al., 2023). The larger muscle volume in the distal region is related to ejaculation, which requires force to extrude the spermatophore during copulation (Chow et al., 1982; Simeó et al., 2009; Paschoal & Zara, 2022; Nogueira et al., 2023), and this is the role of the more developed musculature located in the distal region of the vas deferens, mainly surrounding the ejaculatory duct (DVD).

Sperm production begins in the lumen of the testes, where the presence of spermatozoa can be observed, similar to what occurs in other species of caridean prawns and brachyuran crabs (Paschoal & Zara, 2020; Tomas et al., 2019; Watanabe et al., 2020; Nogueira et al., 2023). Examining the histochemical patterns of this region, it can be said that among the compounds analyzed, neutral polysaccharides were the most evident and the only strongly reactive secretion. Some studies suggest that this compound may be produced by Sertoli cells found in the lobes of the testes, and the function of neutral polysaccharides would be to assist in the transport and nutrition of the sperm matrix from the testicular region to the vas deferens (López-Greco et al., 2007; Nunes et al., 2010).

In the PVD, the occurrence of a typhlosole was observed on one of the vessel walls, which is projected towards the lumen and increases the secretion production in the sperm duct. The typhlosole is constituted by a modification that occurs in the epithelial cells, and its occurrence is recorded in some species of caridean and penaeid prawns (Chow et al., 1989; Bauer & Cash, 1991; Chow et al., 1991; Tomas et al., 2019; Paschoal & Zara, 2022; Nogueira et al., 2023), and can be considered common in the genus *Macrobrachium*. Although many studies do not discuss the presence of the typhlosole, it is possible to observe that this structure is present in the reproductive system of some species already studied (Rao et al., 1987; Chow et al., 1989; Butcher & Fielder, 1994; Tripathi et al., 2014). As mentioned earlier, the main function of the typhlosole is to increase the secretion production within the *vasa deferentia*, which will consequently influence the constitution of the spermatophore. Reports suggest that the morphology of the spermatophore differs between groups that possess a typhlosole and those that do not (Chow et al., 1989), due to the volume of adhesive secretion produced only on one side of the seminal duct. This causes the sperm mass to be asymmetric throughout the regions of the *vasa deferentia*, which remains so when the spermatophore is attached to the female's sternum (Chow et al., 1989; Paschoal & Zara, 2022; Nogueira et al., 2023).

Considering that histochemical analyses have shown differences between species related to the secretions of the VD and their possible correlation with the environment, understanding the distribution of species becomes essential. Despite *M. amazonicum* having a wide distribution throughout Brazilian territory and possessing great adaptive potential (*i.e.*, being able to inhabit freshwater and brackish environments), this group is still considered native to estuarine environments in northern Brazil (Vergamini et al., 2011; Weiss et al., 2015). Meanwhile, *M. pantanalense* is native to the Paraná River basin, occurring exclusively in freshwater environments (Vergamini et al., 2011; Weiss et al., 2015). It is known that the spermatophore in caridean prawns has morphophysiological adaptations that help protect this structure from the moment it is attached to a female's sternum (Chow et al., 1982; Sasikala & Subramoniam, 1987; Bauer & Cash, 1991; Subramoniam, 1993). Therefore, due to the different physicochemical characteristics of the natural environments of *M. amazonicum* and *M. pantanalense*, the composition of the protective and adhesive matrices (which constitute the spermatophore) must be suitable for the water characteristics so that no damage occurs to this structure, which would make the process of fertilizing oocytes unfeasible and explains the histochemical variation found here.

Morphology, morphometry, and ultrastructure of spermatozoa

There is limited information available on spermatozoa length for few species of penaeid and caridean prawns (Kim et al., 2003; Camargo et al., 2017). According to these data, it can be observed that in most cases, caridean prawn spermatozoa are larger compared to penaeid prawn spermatozoa (Braga et al., 2013; Camargo et al., 2017). Among caridean prawns, *M. amazonicum* and *M. pantanalense* have smaller spermatozoa than those observed for *Pandalopsis japonica* Balss, 1914 (Pandalidae family) and *Rhynchocinetes typus* H. Milne Edwards, 1837 (Rhynchocinetidae family) (55 µm and 67 µm in length, respectively) and larger than those observed for *Hippolyte obliquimanus* Dana, 1852 (Hippolytidae family) (6.2 µm in length). Therefore, it can be inferred that caridean prawns present a wide variation in sperm size across different families.

Several factors can influence the variation that occurs in the length or width of the spermatozoa between species of the same group, regardless of taxa. The main factors are sperm competition, female choice, male age, cryptic species, and genetic influences (Pitnick et al., 2008; Smith et al., 2016b). The variation observed in sperm size in this study may be related to the speciation process that involves the two analyzed groups (Weiss et al., 2015; Calixto-Cunha et al., 2021), as the mean variation in size is still apparently low (although statistically different). Furthermore, it is known that variation in sperm length can result in reproductive isolation between groups of cryptic species (Bode et al., 2010), a factor that was observed between *M. pantanalense* and *M. amazonicum* through behavioral experiments (Nogueira et al., 2020). These findings support the argument that this variation is a result of the recent speciation process of these two groups, in accordance with the premises proposed by Pitnick et al. (2008) for interspecific and intraspecific variations related to sperm features.

The main difference in the external morphology of spermatozoa between the two species analyzed here is located in the concavity of the main body, where the spermatozoa of *M. pantanalense* have a more angular main body compared to that observed in *M. amazonicum*. Furthermore, this difference in the shape of the main body's concavity was confirmed as significant by geometric morphometric analyses. Prawn spermatozoa can show a wide external morphological variation. For example, penaeid prawns have spermatozoa that possess a main body with a spherical, bulging or elongated shape, while the majority of caridean prawns have spermatozoa with a conical main body (Braga et al., 2013). Even among other caridean prawn species, the spermatozoa can present wide morphological variability, mainly related to the projections of the acrosomal vesicle located near the spermatozoa's main body, as observed in *H. obliquimanus*, *P. potimirim*, and *R. typus* (Dupré & Barros, 1983; Terossi et al., 2012; Machado et al., 2021). The main body of the spermatozoon is extremely important in the moments preceding oocyte fertilization since it is the spermatozoon region that makes the first contact with the female gamete, initiating the fertilization process (Chow & Sandifer, 2001; Braga et al., 2013). Therefore, morphological differences located in this region may be related to the recognition of the spermatozoon receptors by the oocyte ligands, which may be distinct between *M. pantanalense* and *M. amazonicum*.

The application of geometric morphometrics to test minute differences in the shape of decapod crustacean sperm is unprecedented. Until now, this tool has only been applied to analyze morphological variation in sperm from some groups of vertebrates, *e.g.*, fish and rodents (Sánchez et al. 2013, Pavlov & Emel'yanova, 2018). Due to the high effectiveness presented in this study, we highlight that this tool has the potential to be applied in identifying variations in spermatozoa shape among congeneric species, as differences in the external morphology of spermatozoa are expected to be less evident among congeneric species than the differences observed among species from different genera or families (Chow & Sandifer, 2001; Braga et al., 2013). We emphasize that this method should be performed using fresh samples to avoid potential deformations that may occur to the material after fixation.

The ultrastructural analyses confirmed the patterns observed by SEM and morphometry, where it was observed that the margin of the main body of the spermatozoa of *M. pantanalense* is more angular than the margin of the main body of the spermatozoa of *M. amazonicum*, which is concave and rounded, making the base of the spike narrower. In addition, TEM showed that the cause of this greater concavity is possibly the width of the acrosomal cap, which is wider in *M. pantanalense*. Additionally, comparing the ultrastructure of the spermatozoa of *M. amazonicum* and *M. pantanalense* with other congeners, variations in morphology were observed (Butcher & Fielder, 1984; Dougherty et al., 1986; Poljaroen et al., 2010). Like *Macrobrachium australiense* Holthuis, 1950 (Butcher & Fielder, 1984), the spermatozoa of *M. amazonicum* and *M. pantanalense* also lack the structures called "acrosomal sacs", differing from what was observed in *Macrobrachium rosenbergii* (De Man, 1879) (Poljaroen et al., 2010). The function of these acrosomal sacs is still uncertain, the authors who described this structure report that these vesicles may be part of the acrosome and contain acrosomal enzymes (Poljaroen et al., 2010). However, such an aspect has never been proven, and there are other studies that also analyzed the spermatozoa of *M. rosenbergii* and did not find these structures (Lynn & Clark, 1983), which makes the real function of the acrosomal sacs even more uncertain.

As mentioned earlier, the first interaction between the sperm and the oocyte occurs through the main body that will make contact with the oocyte membrane. However, fertilization of the oocyte is carried out by the other end of the sperm, the spike (Brown, 1966; Chow & Sandifer, 2001). Therefore, the hypothesis raised by Poljaroen et al. (2010), that the enzymes present in the acrosomal sacs are related to oocyte fertilization, is not supported, as the fertilization process is carried out by another region of the spermatozoon. These acrosomal sacs may actually be degenerated mitochondria, due to their morphological appearance (Medina et al., 1994a; 1994b; Braga et al., 2013). These features are reinforced by the present study, since the vesicles found in the nucleus-cytoplasmic region have a double membrane unit and membranous remnants that resemble cristae, which are morphological characteristics presented by mitochondria.

When analyzing studies that describe the external morphology or ultrastructure of caridean prawns spermatozoa, we note a high degree of morphological similarity, especially with the inverted cup or tack-shaped shape being a recurrent pattern among genera of this infraorder (Dupré & Barros, 1983; Lynn & Clark, 1983; Terossi et al., 2012; Tomas et al., 2019). This morphological similarity is even greater when comparing species within the same genus (Lynn & Clark, 1983; Butcher & Fielder, 1984). However, in this study, we demonstrate the power of integrating three different tools (DIC, SEM, and TEM) in the area of spermiotaxonomy, effectively highlighting the variation in size, shape, and morphology of the spermatozoa of *M. amazonicum* and *M. pantanalense*, a result that would not have been possible without the integration of these tools. Therefore, we recommend that future studies addressing the ultrastructure of caridean prawns spermatozoa use the same method employed here, as the use of only some of these tools may lead to incorrect interpretations regarding the morphology or ultrastructure of these organisms' spermatozoa.

We have shown the occurrence of marked differences in several traits of the male reproductive system of *M. amazonicum* and *M. pantanalense*, specifically the composition of secretions from some regions of the *vasa deferentia* and also in the size and shape of the spermatozoa of both species. These results complement the findings on reproductive behavior and geometric morphometrics of taxonomically important structures, reinforcing that *M. amazonicum* and *M. pantanalense* are two distinct groups that have undergone a process of speciation.

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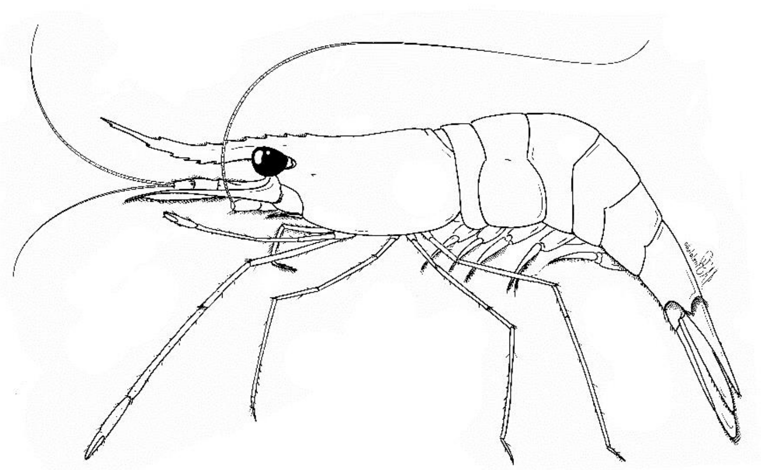
Capítulo IV

Morphology and morphometrics of larvae of *Macrobrachium amazonicum* (Heller, 1862) and *M. pantanalense* Dos Santos, Hayd & Anger, 2013: a comparative approach among different lineages and phenotypes



Abstract

Larval morphology can provide important information about the life history of decapod crustaceans. Many studies have been dedicated to describing larval morphology and comparing morphological characteristics between congeneric species, which has proven useful in delimiting species, since it usually corroborates taxonomic rearrangements initially carried out by molecular or adult morphology analyses. Thus, the present chapter aimed to compare some larval traits between *Macrobrachium amazonicum* and *M. pantanalense*, such as development time, morphology, and larval size. Larvae from two different phenotypes of *M. amazonicum* were analyzed, from estuarine and hololimnetic populations. Parental females were separated into tanks, and subsequently, the larvae were cultured in the laboratory. The larvae were measured and subsequently dissected. In sequence, the first five zoea stages of each phenotype and species were analyzed for their morphology. Differences in larval development time, morphology, and size were observed. Development through the early larval stages of *M. amazonicum* occurs at a faster rate than observed for *M. pantanalense* larvae. The main morphological difference between the two species was the stage at which pereiopod 5 (P5) appears, *i.e.*, zoea IV and III in *M. amazonicum* and *M. pantanalense*, respectively. In addition, size variation was observed, with *M. pantanalense* larvae being larger in the early stages, and in the more advanced stages, this size difference decreases. The differences found between the two species corroborate that *M. amazonicum* and *M. pantanalense* are distinct lineages. Even though these groups are separated by a low genetic distance, the existing differences are conclusive, and therefore, these organisms can be considered as two distinct taxonomic entities.

Keywords: Amazon River Prawn; Freshwater; Heterochrony; Pantanal; Zoea

Introduction

The first studies that analyzed aspects of larval morphology in decapod crustaceans date back to the 17th and 18th centuries (Leeuwenhoek, 1699 [published only in 1807]; Linné, 1767; Bosc, 1802). At that time, the decapod larvae were confused and considered as adult organisms (Bosc, 1802; Leach, 1815), therefore, in many cases, these individuals were described as new species, or even allocated to new genera, e.g., *Zoea* Bosc, 1801, and *Megalopa* Leach, 1814 (Anger, 2001). Later, Thompson (1828) proposed that these two genera were not composed of adult individuals but rather by organisms in the larval life stage with pelagic habits. The evidence that supported these claims was raised from observations of these animals in the laboratory. From that moment on, larval morphology began to be considered and used as an important source of data in taxonomic studies (Milne-Edwards, 1834; 1837; 1840). In addition, some studies from this period also used larval forms to explain traits of natural history and evolutionary trends in crustaceans (Milne-Edwards, 1834; Müller, 1864).

Since then, especially between the 20th and 21st centuries, there has been an increase in the number of studies on larvae of decapod crustaceans (Anger, 2001; 2006). Therefore, some biological aspects of these organisms have been intensely studied, such as morphology, ecology, resistance, and behavior (Morgan, 1987; Queiroga & Blanton, 2005; Torres et al., 2011; Clark, 2016). Generally, studies describing the morphology of zoeal stages of congeneric species seek for morphological variations that support species separation, which are initially discriminated according to adult morphology or molecular analyses (Fincham & Figueras, 1986; Clark, 2016). Some studies make evolutionary inferences and correlate larval morphological adaptations with the conquest of new types of environments (Vogt, 2013; Ralabais & Gore, 2017).

The colonization of new habitats such as freshwater environments, or adaptation to inhospitable environments such as the deep waters of the ocean by some species of decapod crustaceans has led to a series of changes in biological traits (Jalihal et al., 1993; Bauer, 2004; Wowor et al., 2009; Smith et al., 2013; Vogt, 2013; Olesen, 2018). Among these traits, embryonic and post-embryonic development have undergone some adaptations, such as their extreme abbreviation (Van Dover, 1985; Magalhães, 1988; Bauer, 2004; Vogt, 2013). In these cases, most of the development occurs within the egg, and the organisms that hatch can be larvae in an advanced morphological state or juveniles. This is a strategy that has evolved in these organisms and can significantly increase their chances of survival (Jalihal et al., 1993; Bauer, 2004; Vogt, 2013; 2016).

The caridean prawn species that exhibit extreme abbreviation in post-embryonic development (ALD) inhabit environments with limited food availability, such as small rivers or arctic and deep ocean regions (Anger, 1995; Thatje et al., 2003; Bauer, 2004). On the other hand, extended larval development (ELD) is a more common pattern among most marine and amphidromous species, which typically inhabit environments with higher food or nutrients availability (Anger, 2001). Thus, organisms that possess ALD have adapted and developed a strategy where their offspring do not need to feed until reaching the juvenile phase, minimizing mortality due to the scarce food source in their habitat (Anger, 2001).

Interestingly, there is a limited group of caridean prawn species that exhibit ELD and inhabit freshwater environments, *i.e.*, environments with low food availability (Anger, 2001). Currently, nine species are known to display this type of larval development in hololimnetic environments, and among them, two species occur in Brazil, *Macrobrachium amazonicum* (Heller, 1862) and *M. pantanalense* Dos Santos, Hayd & Anger, 2013 (Marco-Herrero et al., 2019; Calixto-Cunha et al., 2021). *Macrobrachium* *amazonicum* is a phenotypically plastic species found in estuarine and freshwater environments, with populations exhibiting high morphological variation along its distribution (Paschoal & Zara, 2020). Meanwhile, *M. pantanalense* is closely related to this first species, being described from populations that were recognized as *M. amazonicum* and are restricted to the Pantanal region of Brazil (Dos Santos et al., 2013).

The larval morphology of both species is known, Marco-Herrero et al. (2019) described the morphology of all zoeal stages of *M. pantanalense* and compared it to the larvae of amphidromous populations of *M. amazonicum*, described by Magalhães (1985). This comparison was made to address the taxonomic problem involving these two lineages. *Macrobrachium amazonicum* and *M. pantanalense* are separated by a low genetic distance (Weiss et al., 2015), and this characteristic stimulated a series of studies that addressed different aspects of the biology of these two organisms, seeking for differences that could help to understand the speciation process involving these two lineages (Weiss et al., 2015; Marco-Herrero et al., 2019; Nogueira et al., 2020; Calixto-Cunha et al., 2021; Nogueira et al., 2023). The larval morphology provided support for the separation of these two groups (Marco-Herrero et al., 2019), however, there is still a gap to be filled, since there are no studies that have addressed the morphology of larvae from hololimnetic populations of *M. amazonicum*.

Given the scenario described above, the present study aimed to compare the morphology of the zoeal stages of hololimnetic populations of *M. amazonicum* with amphidromous populations of the same species, and additionally with the larvae of *M. pantanalense*. The comparison of these two populations with the larvae of *M. pantanalense* will be of utmost importance in understanding the speciation process between these lineages. In addition, we compared other larval aspects, such as development time and size. We predict that morphological variations may occur between the two species, however, these variations may be subtle due to the low genetic distance that separates these lineages.

Material and methods

Sampling of ovigerous females and cultivation of larvae

Ovigerous females from hololimnetic populations of *M. amazonicum* were collected from the Tietê River, at the Ibitinga hydroelectric power plant, located in the municipality of Cambaratiba, state of São Paulo, Brazil (24°44’29” S; 49°01’27” W). This population is characterized by large-sized individuals that exhibit distinct morphotypes. Ovigerous females of *M. pantanalense* were collected at Baiazinha lagoon, type locality of this taxon, located in the municipality of Miranda, state of Mato Grosso do Sul, Brazil (20°15’49” S; 56°23’15” W). Ovigerous females from amphidromous populations of *M. amazonicum* were obtained at the Shrimp Farming Sector of the Aquaculture Center (CAUNESP) of São Paulo State University "Júlio de Mesquita Filho" (UNESP), in the municipality of Jaboticabal, state of São Paulo, Brazil. These farmed animals come from the municipality of Santa Bárbara do Pará, Pará state (northern Brazil), where they originally inhabit estuarine areas connected to the ocean (Meireles et al., 2013). Five ovigerous females of each population type were used.

The collections in the Tietê River and Baiazinha lagoon were performed using a sieve that was handled in the submerged vegetation present in the margin of these environments. The ovigerous females found were transferred to plastic bags containing water from the site and stored in thermal boxes with constant aeration. Subsequently, the shrimps were transported to the laboratory.

The ovigerous females were kept individually in aquariums (10L), with temperature controlled and similar to the natural environment. The females were fed daily with industrial food (TetraColor TETRA®) until the moment of larval hatching. After hatching, the parental females were removed from the aquariums and preserved in 70% ethanol. The larvae were cultivated in the same aquarium where they hatched from the eggs, maintaining parameters similar to the natural environment of each species (Photoperiod 12:12h; temperature 25–26°C; constant aeration). Daily, the larvae were fed ad libitum with newly hatched *Artemia* sp. nauplii, and from the emergence of the first zoeas V, a ground shrimp-specific feed (Natural Shrimp® Reproduction) was introduced as additional food. The water from each aquarium was siphoned daily to remove organic debris present in the bottom.

The cultivation of larval *M. amazonicum* of an amphidromous origin was carried out at the shrimp farming sector of the Aquaculture Center of São Paulo State University "Júlio de Mesquita Filho" (CAUNESP), following all protocols proposed in Valenti et al. (1998; 2009).

Description and comparison of larval morphology

For larval morphological description, ten larvae were collected on alternate days (24-hour intervals) and transferred to a solution of 70% ethanol and glycerin (1:1). The larval stages were identified by performing a comparative analysis of morphology based on previous descriptions of *M. amazonicum* and *M. pantanalense* (Guest, 1979; Magalhães, 1985; Marco-Herrero et al., 2019), and taking into account the progression of days when they were collected. For morphological observations and comparisons, larvae from at least five different ovigerous females of each species (*M. amazonicum* and *M. pantanalense*) or phenotype (*M. amazonicum* amphidromous and hololimnetic) were used. The larval culture aimed to acquire all zoeal stages and the decapodite.

The comparison of larval morphology was carried out following all the data compiled by Marco-Herrero et al. (2019), who investigated differences between larvae of *M. pantanalense* and amphidromous *M. amazonicum* larvae (Magalhães, 1985). Initially, we added comparative information about larvae of hololimnetic *M. amazonicum* and reviewed all the information compiled by Marco-Herrero et al. (2019), according to the larvae cultured in the present study. A comparison of the morphology of all appendages was performed among the larvae of the species and phenotypes up to the zoea V stage. In subsequent stages (*i.e.*, zoea VI, VII, VIII, and IX), we noted a large morphological variability (*e.g.*, the amount of setae present on an article or appendage) within the same larval stage among larvae of the same species or phenotype, which could make inter-specific comparison unfeasible.

In figures of larval morphological comparison, photographs of larval appendages were used as they allowed for the visualization of morphological differences between the larvae of each species, obviating the need for illustrations. Thus, we initially provide an overview of the anatomy of the structure where the differences between the two species were observed. Subsequently, these differences are demonstrated in detail using photographs of each species or phenotype.

Larval morphometrics

A comparison of carapace length (CL, distance between the tip of the rostrum and the posterior margin of the carapace) was performed among larvae of different phenotypes of *M. amazonicum* and *M. pantanalense*. The larvae were photographed using a Zeiss Stemi 2000C stereo microscope coupled to a camera with an imaging system. The larvae were measured using the measurement tool of Zeiss AxioVision software.

In morphometric analyses related to the CL of each zoeal stage, larvae from all stages were used for which a minimum sample was obtained that followed the premises for statistical comparisons (Zar, 2010). The normality of the data was assessed using the Shapiro-Wilk test, and then the CL of larvae from each group was compared using a Kruskal-Wallis test followed by Dunn's post-hoc test (Zar, 2010).

Results

The five parental females of each *M. amazonicum* population type, amphidromous and hololimnetic, had an average CL of 15.26 ± 0.93 and 13.9 ± 1.71 mm, respectively. The five parental females of *M. pantanalense* had an average CL of 9.2 ± 1.01 mm. The development between the zoea I stage and the decapodite lasted 21 days for *M. amazonicum* of hololimnetic origin and *M. pantanalense*, while for *M. amazonicum* of amphidromous origin, the entire larval development lasted 17 days (Table I).

The larval development time of *M. amazonicum* varied in relation to that observed in *M. pantanalense*, regardless of the phenotype. In *M. amazonicum*, the transition between zoea II and III occurred by the fifth day of culture, and the transition between zoea III and IV occurred between the fifth and ninth day of culture. In *M. pantanalense*, the transition between zoea II and III occurred by the seventh day of culture, and the transition between zoea III and IV occurred between the ninth and thirteenth day of culture (Table I). The diet of larvae from different phenotypes and species was standardized until the emergence of zoea V in the culture, so comparisons between the time of progression of more advanced larval stages (ZV to ZIX) were not performed due to the possible influence of differential feeding on larval development time.

Table I. Developmental time between larval stages throughout the culture days. MAA = *Macrobrachium amazonicum* amphidromous phenotype; MAH = *Macrobrachium amazonicum* hololimnetic phenotype; MP = *Macrobrachium pantanalense*; PL = Post-larvae; Z = Zoea.

Phenotypes/Days	Days of culture										
	1	3	5	7	9	11	13	15	17	19	21
MAA	ZI	ZII	ZIII/ZIV	ZIV/ZV/ZVI	ZVI	ZVI/ZVII	ZVII	ZVII/ZVIII	ZIX/PL	-	-
MAH	ZI	ZII	ZIII/ZIV	ZIV	ZIV	ZV	ZV	ZV/ZVI/ZVII	ZVI/ZVII	ZVI/ZVII	ZVIII/ZIX/PL
MP	ZI	ZII	ZII	ZIII	ZIV	ZIV	ZIV/ZV	ZVI	ZVI	ZVII	ZVIII/ZIX/PL

Larval morphology

The morphology of all body appendages in the first five larval stages (ZI to ZV) of both *M. amazonicum* phenotypes and *M. pantanalense* was analyzed. Below, we describe only the morphological characters that showed variations between the two species and/or *M. amazonicum* phenotypes.

In zoea I, the only difference found between the species was the number of setae in the coxal endite of the maxillule (Figure 1). Five setae were found in *M. amazonicum* (in both phenotypes), while six setae were observed in *M. pantanalense* (Figure 1B and C). In this larval stage, the morphological variation observed here differed from the existing larval descriptions for both species (see Table 2 and specific references).

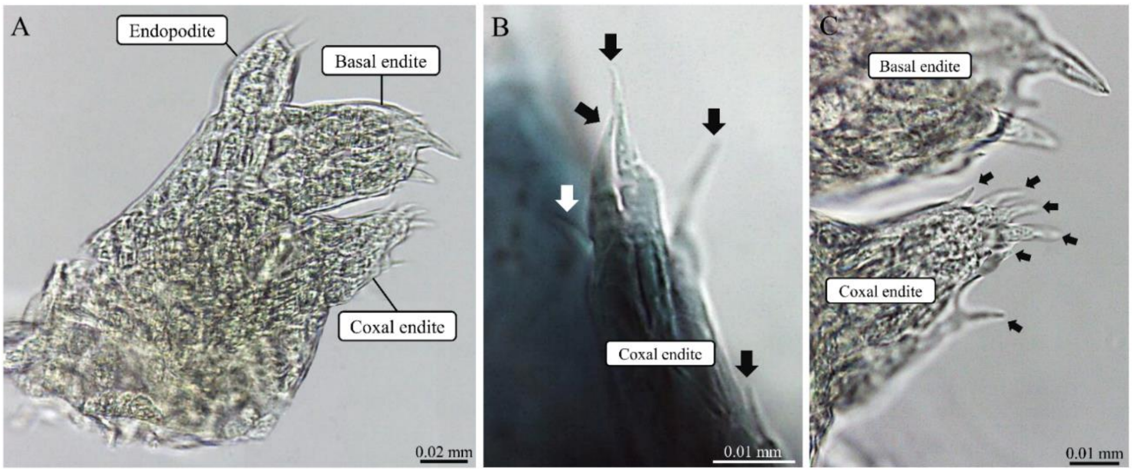


Figure 1. Variation in maxillule morphology between *Macrobrachium amazonicum* and *M. pantanalense* (zoea I). A. General anatomy of the maxillule, composed of the endopodite and basal and coxal endites. B. Detail of the ventral view of the coxal endite of *M. amazonicum*. Arrows indicate the five setae present in this structure (observed in both populations). C. Detail of the coxal endite region of the maxillule of *M. pantanalense*. Black arrows indicate the six setae present in this structure.

In zoea II, a morphological difference was found in the basis of maxilliped 1 (Figure 2A). The basis of maxilliped 1 in *M. amazonicum* has 5-6 setae, while in *M. pantanalense* it has 4 setae (Figure 2B and C). At this larval stage, this morphological variation differed from the larval description that exists for *M. pantanalense* (Table 2 and specific references).

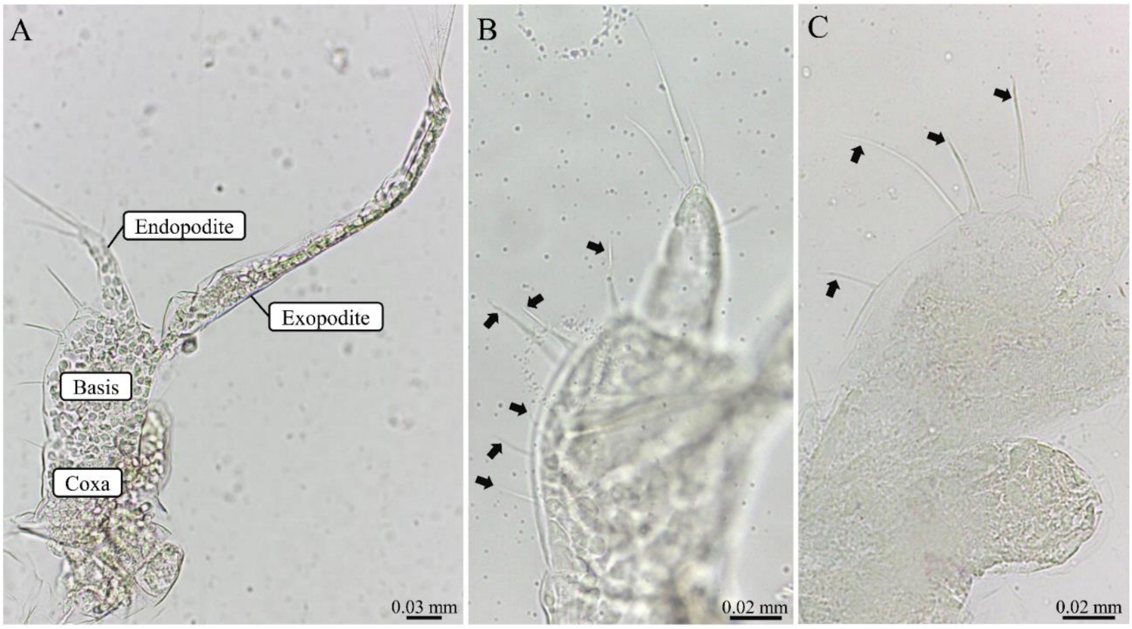


Figure 2. Morphological variation of the maxilliped 1 between *Macrobrachium amazonicum* and *M. pantanalense* (zoea II). A. General anatomy of the maxilliped 1, which is composed of coxa, basis, endopodite, and exopodite. B. Detail of the basis and endopodite of the maxilliped 1 of *M. amazonicum*, the black arrows point to the six setae present on the basis of this appendage. C. Detail of the basis of the maxilliped 1 of *M. pantanalense*, the black arrows point to the four setae present on the basis of this appendage.

In zoea III, the only morphological difference observed between the species was in the characteristics of the pereiopod 5 (P5; Figure 3A). In *M. pantanalense*, this structure is composed of five articles, indicating its functional nature and featuring a pair of setae on each of the last two articles (Figure 3A and B). Conversely, in both phenotypes of *M. amazonicum*, this structure exhibited the morphology of a non-functional uniramous bud (Figure 3C). This morphological variation corroborated the larval descriptions that exist for both species (Table 2 and specific references).

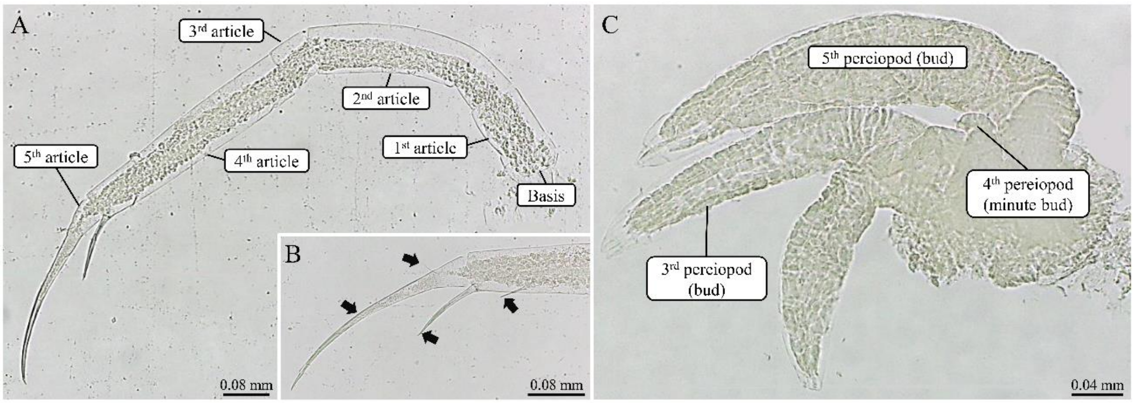


Figure 3. Morphological variation of the fifth pereiopod between *Macrobrachium amazonicum* and *M. pantanalense* (zoea III). A. General anatomy of the functional fifth pereiopod of *M. pantanalense*, which is composed of coxa (not shown in the photo), basis, and five other articles (ischium, merus, carpus, propodus, and dactyl, respectively). B. Detail of the posterior region of the fourth article and the entire extension of the fifth article. The black arrows point to the two setae present in each of the mentioned articles. C. Overall view of the buds of the third, fourth, and fifth pereiopods of *M. amazonicum*. The fourth pereiopod is the smallest bud, being named "minute bud" in the literature.

In zoea IV, the main morphological differences between the species were observed in the endopodite of pereiopod 3 and pereiopod 5 (Figures 4 and 5). From this stage, morphological variation was observed among the phenotypes of *M. amazonicum* (Table 2). In pereiopod 3, a variation in the number of setae on the second article of the endopodite was observed, with one seta present in *M. amazonicum* of hololimnetic origin, while *M. pantanalense* does not have setae on this same article. Additionally, *M. amazonicum* of amphidromous origin also lacks setae on the second article of the endopodite of pereiopod 3 (Figures 4A-C). In pereiopod 5, which is now functional in both species, the main difference is related to the number of setae present on the articles of this appendage. In *M. amazonicum*, at least one seta is observed on each of the five articles of pereiopod 5 (Figures A, C, and D), while in *M. pantanalense*, setae are only observed on the last two articles, which have three and two setae, respectively (Figure 5B). At this larval stage, this morphological variation differed from the larval description that exists for *M. amazonicum* (Table 2 and specific references).

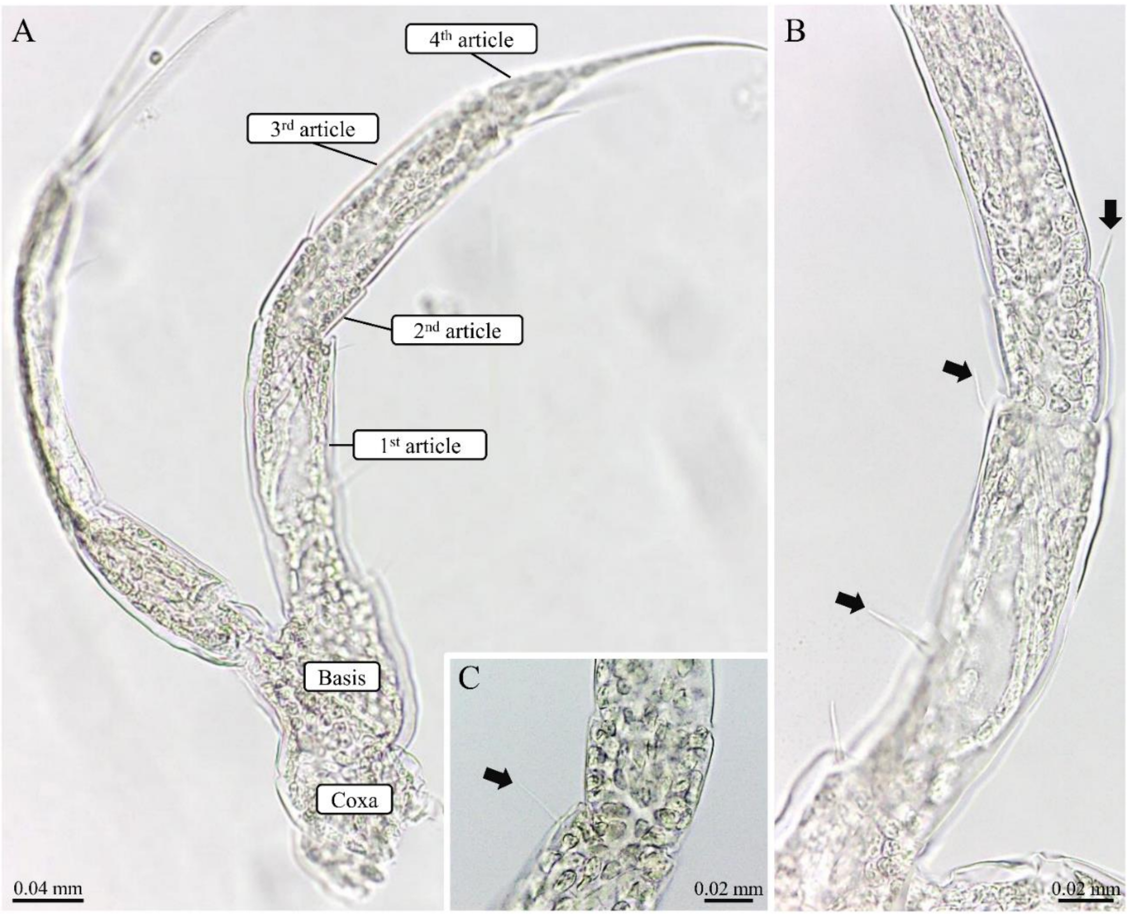


Figure 4. Morphological variation of the pereiopod 3 between *Macrobrachium amazonicum* and *M. pantanalense* (zoea IV). A. General anatomy of the pereiopod 3 of *M. amazonicum*, which is composed of coxa, basis, and four other articles. B. Detail of the first two articles and the anterior region of the third article of the pereiopod 3 of *M. amazonicum* from hololimnetic origin. The black arrows point to the two setae present in the first article and to one seta present in the second article. C. Detail of the posterior region of the first article, second article, and anterior region of the third article of the pereiopod 3 of *M. pantanalense*. The black arrow points to the seta located in the posterior region of the first article. There are no setae present in the second article of the pereiopod 3 of the zoea IV of this species.

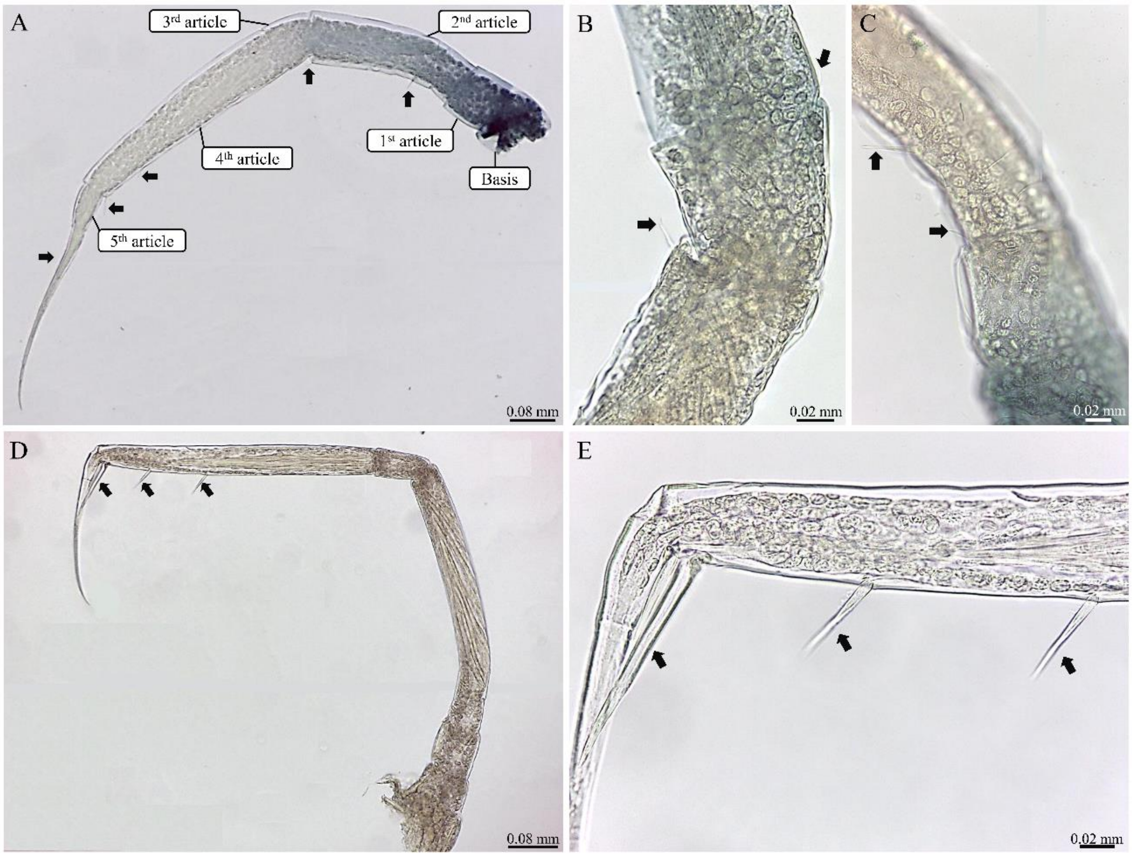


Figure 5. Variation in the morphology of pereiopod 5 between *Macrobrachium amazonicum* and *M. pantanalense* (zoea IV). A. General anatomy of pereiopod 5 in *M. amazonicum*. This appendage is composed of coxa (not shown in the photo), basis, and five articles (ischium, merus, carpus, propodus, and dactylus, respectively). The black arrows indicate the setae present on the articles of this structure. *M. amazonicum* exhibits setae on all articles of this appendage. B. Detail of the posterior region of the second article, third article, and anterior region of the fourth article. The black arrows point to the setae present on the second and third articles. C. Detail of the first article and anterior region of the second article. The black arrows point to the setae present on these articles. D. Pereiopod 5 of *M. pantanalense*. In this species, setae are observed only on the last two articles of this appendage (black arrows). E. Detail of the posterior region of pereiopod 5 in *M. pantanalense*, highlighting the setae present on the fourth article (black arrows).

In zoea V, the greatest morphological variation between the two species was observed. These differences are related to the number of setae on the endopodite of the antenna, caridean lobe of maxilliped 1, basis and endopodite of pereiopod 4, and on pereiopod 5 (Table 2). In the first and third articles of the endopodite of the antenna of *M. amazonicum*, setae are observed, while in *M. pantanalense* setae are not observed in the first articles (Figure 6A-D). In addition, in the last article of the endopodite of the antenna, between five and six setae can be found in *M. amazonicum* and four setae in *M. pantanalense* (Figure 6E-G). In the caridean lobe of maxilliped 1 of *M. amazonicum*, three plumose setae are found, while in *M. pantanalense*, only one plumose seta was observed (Figure 7A-C).

In the phenotypes of *M. amazonicum*, one to two setae can be observed on the basis of pereiopod 4, while no setae are observed in *M. pantanalense* (Figure 8A–C). In the third article of the endopodite of pereiopod 4 of *M. amazonicum*, three setae are observed, while in *M. pantanalense* only two setae are observed in this same article (Figure 8D and E). Furthermore, we highlight that the setae of *M. pantanalense* are twice as long as those of *M. amazonicum* (pereiopod 4). In the pereiopod 5, a morphological variation occurs similar to what was described in zoea IV of both species (Figure 9). In *M. amazonicum*, at least one seta is observed in each of the five articles of pereiopod 5, while in *M. pantanalense*, setae are only observed in the last two articles, each with three setae (Figure 9A–F). Additionally, in the fourth article of pereiopod 5 of *M. amazonicum*, more setae (four to six) are observed compared to those observed in *M. pantanalense* (always three setae) (Figure 9D–F). At this larval stage, some of the morphological variations observed here differed from the larval descriptions that exist for both species (Table 2).

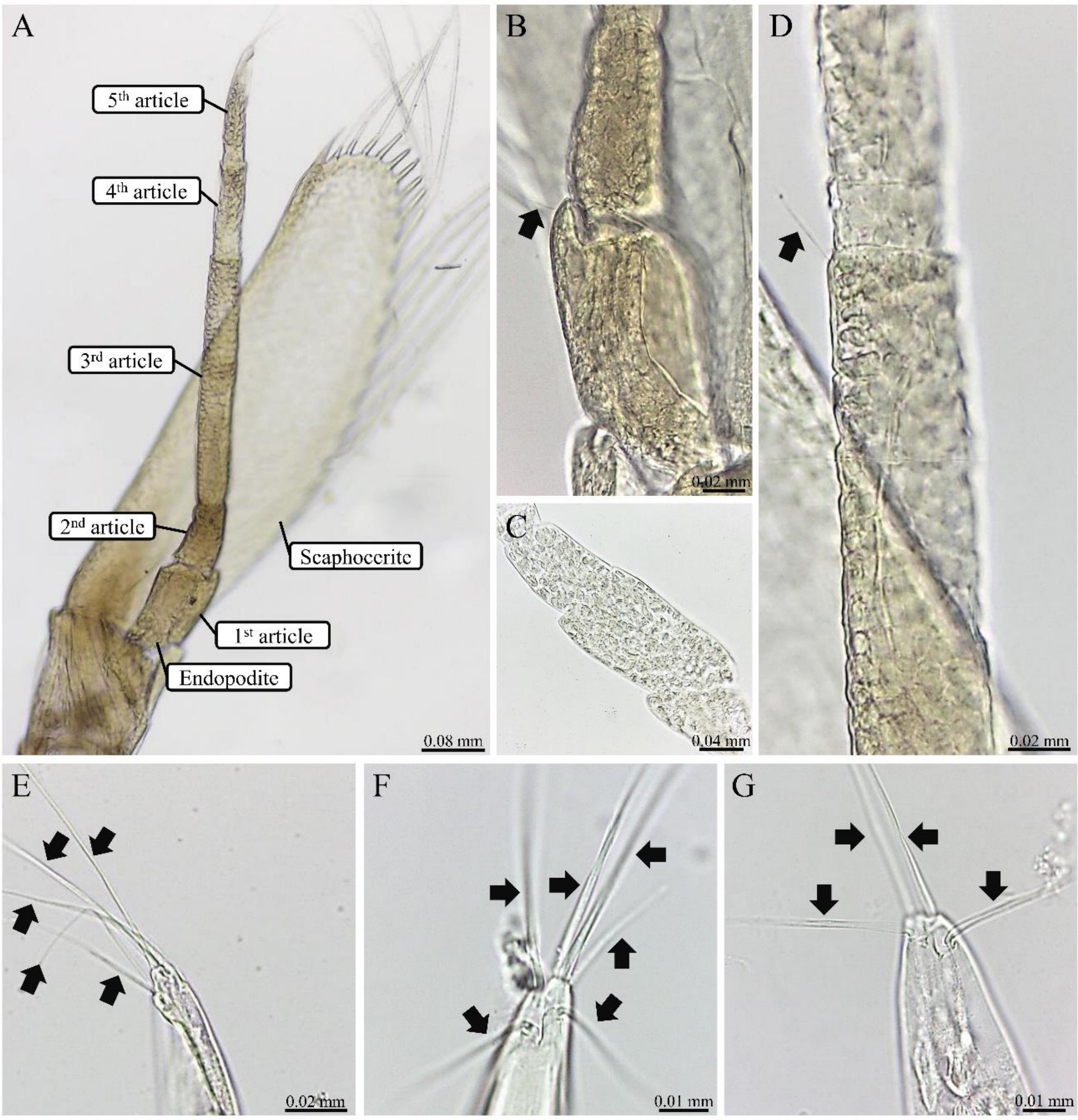


Figure 6. Variation of the morphology of the antenna endopodite between *Macrobrachium amazonicum* and *M. pantanalense* (zoea V). A. General anatomy of the antenna of *M. amazonicum*. This appendage is composed of the protopodite, the scaphocerite (exopodite), and the endopodite, the latter structure being divided into five articles. B and C. Detail of the first two articles of the endopodite of the antenna of *M. amazonicum* and *M. pantanalense,* respectively. The black arrow highlights the presence of a seta in the first article of the endopodite of the antenna of *M. amazonicum*, while no setae are observed in *M. pantanalense*. D. Detail of the posterior region of the third article and the anterior region of the fourth article of the endopodite of the antenna of *M.*

amazonicum. The black arrow highlights the presence of a seta in the third article of the endopodite of the antenna of *M. amazonicum* (this seta was observed only in the amphidromous phenotype.). No setae are observed in *M. pantanalense* in this article. E, F, and G. Detail of the posterior region of the last article of the endopodite of the antenna of *M. amazonicum* from an amphidromous and hololimnetic origin and *M. pantanalense*, respectively. In *M. amazonicum*, between five and six setae can be found in this article, while four setae are observed in *M. pantanalense*.

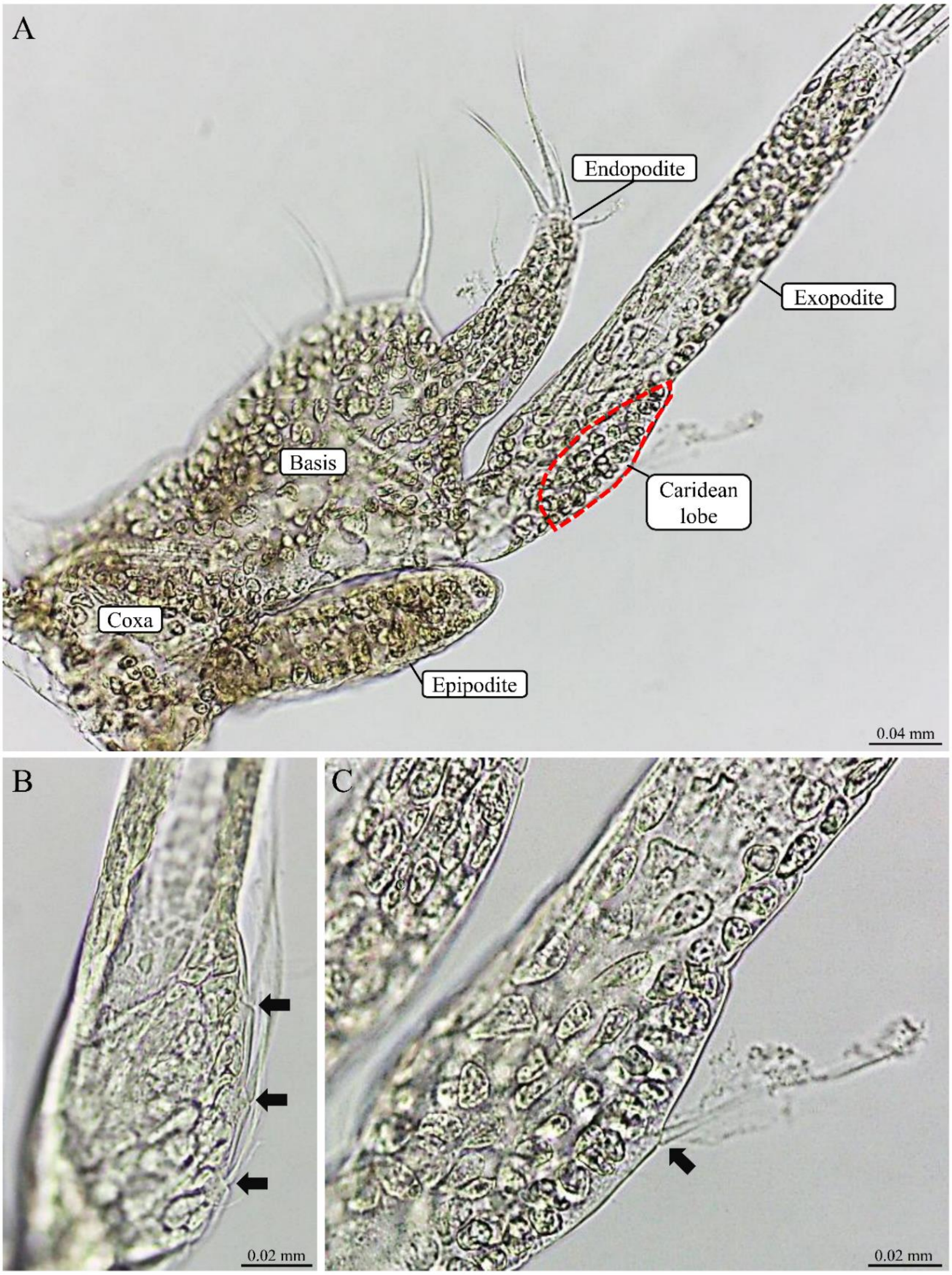


Figure 7. Variation in the morphology of maxilliped 1 between *Macrobrachium amazonicum* and *M. pantanalense* (zoea V). A. General anatomy of maxilliped 1 of *M. pantanalense*. This appendage is composed of coxa + epipodite, basis, exopodite, and endopodite. In the exopodite, a projection is formed in the proximal region of this

structure, which is known as the caridean lobe (marked in red). B and C. Detail of the caridean lobes of *M. amazonicum* and *M. pantanalense*, respectively. The black arrows point to the plumose setae present in this projection. In *M. amazonicum,* three setae are observed, while in *M. pantanalense,* only one seta is observed.

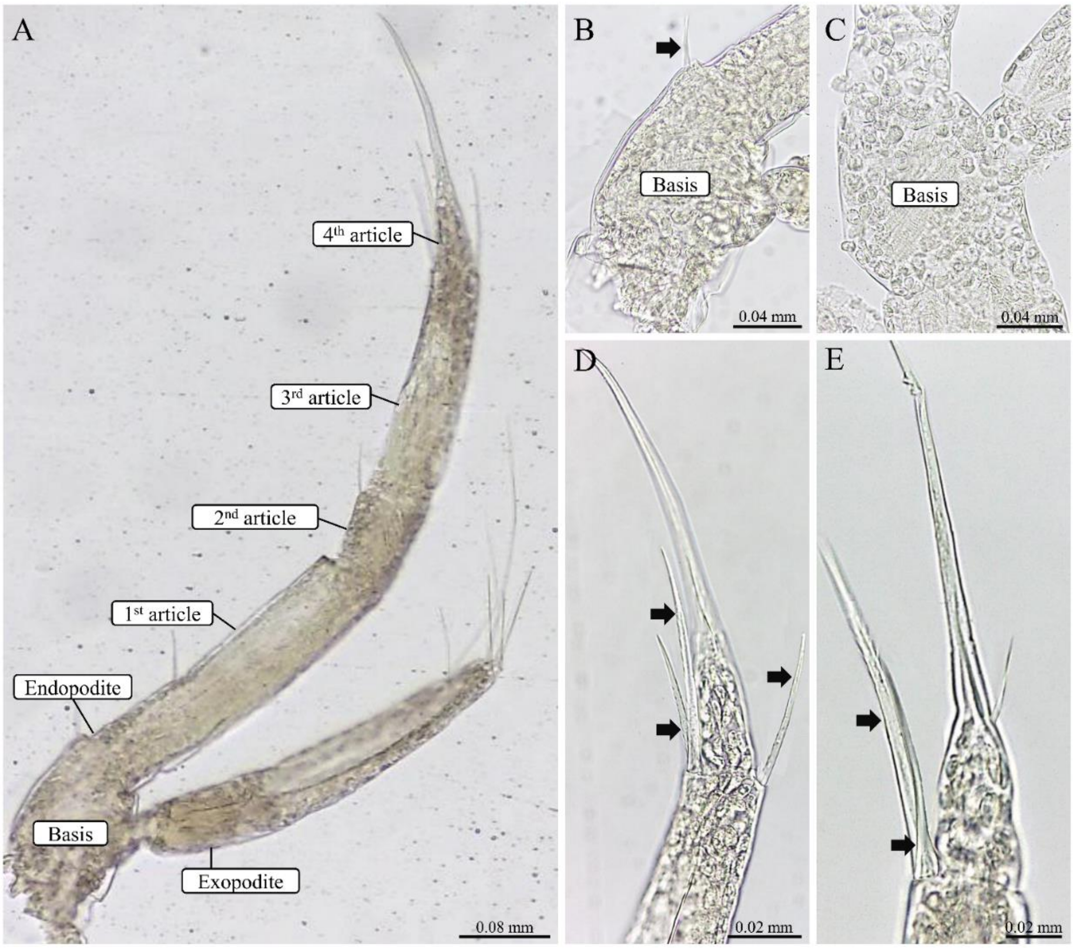


Figure 8. Morphological variation of the pereiopod 4 between *Macrobrachium amazonicum* and *M. pantanalense* (zoea V). A. General anatomy of a fourth pereiopod of *M. amazonicum*. This appendage is composed of coxa (not shown in the figure), basis, exopodite, and endopodite, with the latter structure divided into four articles. B and C. Detail of the basis of the fourth pereiopod of *M. amazonicum* and *M. pantanalense*, respectively. The black arrow points to the seta present in the basis of the fourth pereiopod of *M. amazonicum*, while no setae are observed in *M. pantanalense*. D and E. Detail of the posterior region of the third and fourth article of the endopodite of the fourth pereiopod of *M. amazonicum* and *M. pantanalense*, respectively. The black arrows point to the three setae present in the third article of the endopodite of the fourth pereiopod of *M. amazonicum*, while in *M. pantanalense* only two setae are observed in this same article.

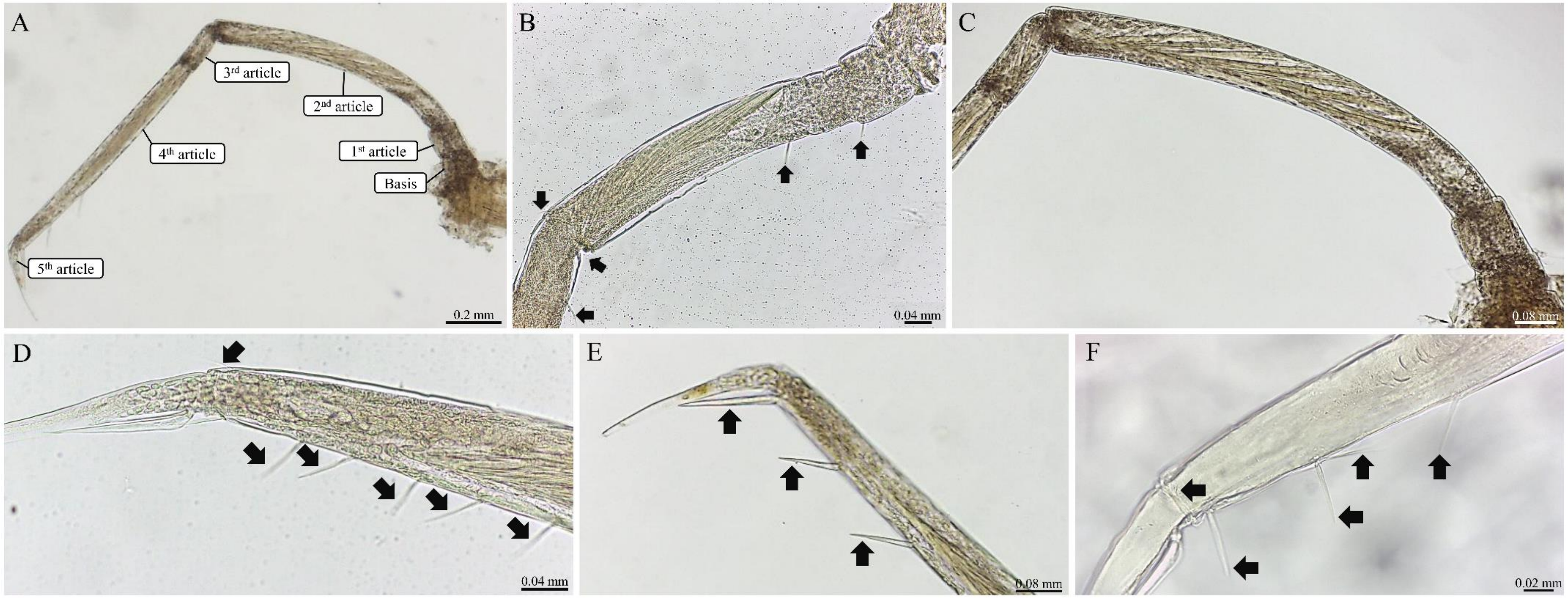


Figure 9. Variation in morphology of pereiopod 5 between *Macrobrachium amazonicum* and *M. pantanalense* (zoea V). A. General anatomy of pereiopod 5 of *M. pantanalense*. This appendage is composed of coxa (not shown in the figure), basis, and five articles (ischium, merus, carpus, propodus and dactylus, respectively). B and C. Detail of the first, second and third article of pereiopod 5 of *M. amazonicum* and *M. pantanalense*, respectively. Black arrows highlight the presence of setae since the first article of pereiopod 5 in *M. amazonicum*, which are not observed in *M. pantanalense*. D, E and F. Detail of the posterior region of the fourth and fifth articles of the pereiopod 5 of amphidromous *M. amazonicum*, *M.*

pantanalense and hololimnetic *M. amazonicum*, respectively. Black arrows highlight the presence of setae in article 4, which are notably more abundant in *M. amazonicum*, regardless of phenotype.

Table 2. Morphological differences between the first larval stages (ZI to ZV) of *Macrobrachium amazonicum* from amphidromous origin (MAA), hololimnetic origin (MAH), and *Macrobrachium pantanalense* (MP). [1]Magalhães (1985); [2]Present study; [3]Marco-Herrero et al. (2019). Numbers in parentheses correspond to cases in which the number of setae was observed in a single specimen.

Zoea	Structure	Region	Species/Phenotypes				
			MAA[1]	**MAA[2]**	**MAH[2]**	MP[3]	**MP[2]**
I	Maxillule	Coxal endite	4	5	5	5	6
II	Maxiliped 1	Basis	6	5–6	5–6	5	4
III	Pereiopod 5	-	Bud	Bud	Bud	0,0,0,2,2	0,0,0,2,2
IV	Pereiopod 3	Endopodite	2,1,2,1	2,0,2,2	2,1,2,2	2,0,2,2	2,0,2,2
	Pereiopod 5	-	4–art 2,2,3,1	5–art 1,2,1,3,2	5–art 1,2,1,3,2	5–art 0,0,0,3,2	5–art 0,0,0,3,2
V	Antena	Endopodite	4–art 0,0,0,5	5–art 1,0,1,1(0),5	5–art 1,0,0,0(1),6(5)	6–art 0,0,0,1,0,5	5–art 0,0,0,1(0),4(5)
	Maxiliped 1	Caridean lobe	2	3(2)	3(4)	2-3	1
	Pereiopod 4	Basis	1	1(2)	2	0	0
		Endopodite	2,0,2,1	2,1–2,3,2	2,1,3,2	2,1,2,2	2,1,2,2
	Pereiopod 5	Endopodite	3,2,5,2	1(2),3,2,6(7),3	1,2–3,2,4–6,3	0,0,0,3,3	0,0,0,3,3

Larval morphometrics

Statistical differences were observed in CL among almost all larval stages analyzed between *M. amazonicum* and *M. pantanalense* (Kruskal-Wallis; $p < 0.05$; Table 3). *Macrobrachium pantanalense* larvae were larger than those of *M. amazonicum*, especially in the early zoeal stages (Zoea I and II; Table 4). As of the zoea III stage, the difference between species decreased, although it remained significant (only between *M. amazonicum* hololimnetic and *M. pantanalense*; Table 3 and 4). Moreover, the size of larvae from both *M. amazonicum* phenotypes differed in the zoea III and IV stages. No statistical difference in larval size was observed in zoea V (Table 3).

Table 3. Kruskal-Wallis and Dunn's post hoc test results. Analyses showed statistical differences ($p < 0.05$) in the CL of some of the first larval stages (ZI to ZV) of *M. amazonicum* of amphidromous origin (MAA), *M. amazonicum* of hololimnetic origin (MAH), and *M. pantanalense* (MP). *Statistical difference

Stages	Comparison	Kruskal-Wallis (H)	Kruskal-Wallis (*p*)	Dunn (*p*)
ZI	MAA × MAH	9.72	< 0.01*	0.6
	MAA × MP			0.04*
	MAH × MP			< 0.01*
ZII	MAA × MAH	19.82	< 0.001*	0.16
	MAA × MP			< 0.01*
	MAH × MP			< 0.001*
ZIII	MAA × MAH	13.6	< 0.01*	< 0.001*
	MAA × MP			0.08
	MAH × MP			< 0.01*
ZIV	MAA × MAH	12.41	< 0.01*	< 0.01*
	MAA × MP			< 0.01*

	MAH × MP			0.79
	MAA × MAH			-
ZV	MAA × MP	1.12	0.56	-
	MAH × MP			-

Table 4. Size variation (mean ± standard deviation; mm CL) among the first larval stages (ZI to ZV) of *M. amazonicum* of amphidromous origin (MAA), *M. amazonicum* of hololimnetic origin (MAH), and *M. pantanalense* (MP). The two species differ statistically in size in almost all larval stages (Mann-Whitney, $p < 0.05$), except for the zoea V stage.

Larval stage/Phenotype	MAA	MAH	MP
ZI	0.83 ± 0.04	0.82 ± 0.07	0.87 ± 0.03
ZII	0.88 ± 0.04	0.91 ± 0.06	0.97 ± 0.07
ZIII	1.03 ± 0.04	0.91 ± 0.07	0.99 ± 0.06
ZIV	1.23 ± 0.06	1.07 ± 0.09	1.09 ± 0.11
ZV	1.33 ± 0.09	1.31 ± 0.11	1.33 ± 0.09

Discussion

Differences in development, morphology, and size were observed between the first five zoeal stages of *M. amazonicum* (MA) and *M. pantanalense* (MP). Interestingly, there were also morphological and morphometric differences between the *M. amazonicum* phenotypes; however, the differences between the species (MA *vs.* MP) are more conspicuous than those between the phenotypes (MAA *vs.* MAH). Some morphological differences were recorded between the zoeal stages analyzed here and the larvae of *M. amazonicum* and *M. pantanalense* initially described by Magalhães (1985) and Marco-Herrero et al. (2019), respectively. These variations deserve special attention in future studies, as they may be related to the occurrence of cryptic lineages not yet known for these organisms. Nonetheless, the morphological differences between the two species are robust, confirming the predictions of the present study.

On the fifth day of culture, a desynchronization in the development of zoeal stages between the two species was observed. While *M. amazonicum* larvae (both phenotypes) molted to stage ZIII, *M. pantanalense* larvae remained at stage ZII, molting to ZIII only on the seventh day of culture. Upon hatching, these prawn larvae have a yolk reserve that is the main source of food and provides the energy needed for these organisms to carry out the first molting processes (Magalhães & Walker, 1988). Most of this reserve is consumed in the first days of life since the larvae do not feed during the first larval stage (ZI; Magalhães, 1985; Magalhães & Walker, 1988), but in some cases, yolk droplets are still found in ZII and ZIII (Magalhães & Walker, 1988; Anger & Hayd, 2010). It is known that *M. amazonicum* larvae have a larger yolk reserve when compared to *M. pantanalense* larvae (Anger & Hayd, 2010). Thus, even if these organisms have standardized feeding during culture, *M. amazonicum* larvae would still have a greater energy reserve than *M. pantanalense*, which may have influenced the observed developmental rate between the two species in the first days of culture.

The morphological differences observed among the larvae may be related to the speciation process that involves both species (Weiss et al., 2015), since the variation in P5 is evident, and was also observed by Marco-Herrero et al. (2019). The main difference in this structure is heterochronic, meaning that the chronology of P5 development varies between species (McNamara, 2012). In the zoea III stage, we observed that the P5 of *M. amazonicum* has a uniramous bud-like shape, while in *M. pantanalense*, the same structure is functional and articulated. In the zoea IV stage, the P5 is functional in both species, and a variation in the configuration of the setae present between the articles was observed, with *M. amazonicum* having more setae between the articles of P5, and this difference becomes even greater in zoea V. Heterochronic differences in post-embryonic development are common among decapod crustacean species, especially when comparing organisms with ALD and ELD (Clark, 2005; Møller et al., 2020). However, it is also possible to observe heterochronic differences among organisms that have the same type of post-embryonic development, but in these cases, the differences are less marked (Clark, 2005; Batel et al., 2014). In the present study, the early development of P5 in *M. pantanalense* is a notable variation and may be related to the diversification process that occurred between these two lineages.

Since *Macrobrachium acanthurus* (Wiegmann, 1836) represents the sister group of *M. amazonicum* (Mantelatto et al., 2021) and consequently also of *M. pantanalense*, and *M. acanthurus* presents the functional P5 only in ZIV (Choudhury, 1970), we can consider that this character (functional P5 in ZIV) represents a plesiomorphic condition, while the presence of the functional P5 in the ZIII of *M. pantanalense* represents an apomorphic condition. Using the principle of parsimony and adopting Clade III (Vergamini et al., 2011) as the sister group of *M. pantanalense*, we can consider that this character (functional P5 in ZIII) must have a unique origin in *M. pantanalense*. Therefore, we hypothesize that the anticipation of the appearance of the functional P5 in ZIII is an autapomorphy of *M. pantanalense*.

There were also morphological variations in other structures, as shown in Table I. In the case of these structures, the observed variation between the two species is mainly related to the number of setae that are present on the articles of some appendages. Although these differences are not as evident as the observed heterochronic variation, they should not be disregarded. Some recent studies have compared larval morphology among several species of caridean prawns (Pescinelli et al., 2017; Pantaleão et al., 2020; Santos et al., 2020; Almeida et al., 2021), and often, the main differences found among these species are related to the number or type of setae present on the articles of the same appendage. In addition, we emphasize that some of the species compared in these studies are separated by larger genetic distances than the distance between *M. amazonicum* and *M. pantanalense* (Almeida et al., 2014; Cunha et al., 2020). For this reason, we emphasize that even though these differences may seem less conspicuous than those observed in the P5 (between ZIII and ZV), they are extremely important and once again demonstrate how *M. amazonicum* and *M. pantanalense* diversified.

The larvae of both species showed size variations, regardless of phenotype. Differences were observed between the first four zoeal stages, with greater discrepancies in the first two zoeas. Larvae of *M. pantanalense* were larger than those of *M. amazonicum*. This result was expected since there is a correlation between egg size and zoea I size (Sastry, 1983; Hines, 1986; Hancock, 1998), and the eggs of *M. pantanalense* are larger than those of *M. amazonicum*, especially in the final stage of embryonic development (Hayd & Anger, 2013; Pantaleão et al., 2018; Silva et al., 2017). Thus, these results corroborate what was initially pointed out by Dos Santos et al. (2013) and Hayd & Anger (2013), when variations were suggested between some reproductive traits of these organisms, including the variation between the egg size of *M. amazonicum* and *M. pantanalense*.

We found differences in some post-embryonic development characteristics of *M. amazonicum* and *M. pantanalense*, related to development time, morphology, and larval size. Despite the occurrence of differences in several appendages, we emphasize that the morphological variation that occurs in P5 is noteworthy and can be used to identify these two species even at the larval stage. Therefore, the differences observed here add to other biological characteristics that vary between these two species (*e.g.*, reproductive isolation, reproductive aspects, and body shape) and corroborate that *M. amazonicum* and *M. pantanalense* are two lineages separated by low genetic distance but with differences that support the separation of these organisms as two taxonomic entities.

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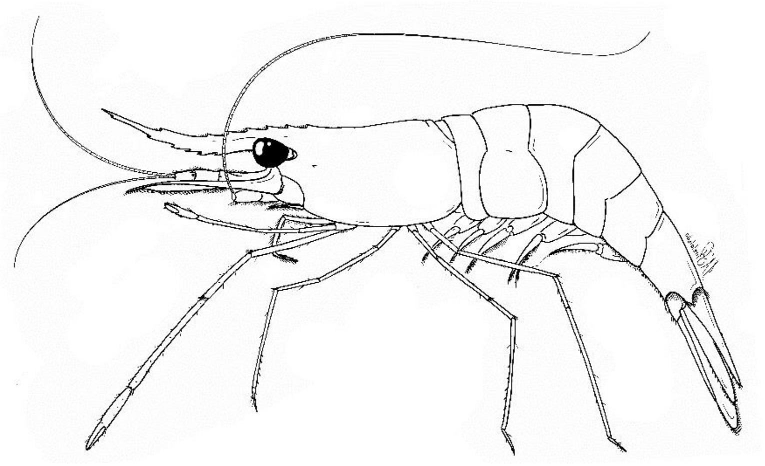
Considerações finais



Considerações finais

Com base nos resultados expostos em todos os capítulos, evidenciamos que existem diferenças entre diversas características biológicas de *M. amazonicum* e *M. pantanalense*, o que confirma a hipótese central desse estudo. Em um primeiro momento (Capítulo 1), foi observado que existe um isolamento reprodutivo entre esses organismos, ou seja, ambas as linhagens não se reconhecem sexualmente e, portanto, não copulam. Possivelmente, essa falta de reconhecimento pode estar relacionada com os feromônios sexuais, os quais tendem a ser específicos para cada espécie.

Foram observadas diferenças estatísticas no formato de todas as estruturas analisadas com a ferramenta de morfometria geométrica. Duas delas foram sugeridas como potenciais estruturas para serem utilizadas na identificação dos grupos, *i.e.*, a carapaça e o telson. Porém, como foi retratado no capítulo em questão (Capítulo 2), o presente estudo não possui escopo taxonômico. Portanto, recomendamos que sejam realizadas análises adicionais com mais populações para verificar se o padrão observado nas seis populações aqui analisadas se repete em outras que estão distribuídas ao longo do Brasil.

As características do sistema reprodutor masculino e a ultraestrutura do espermatozoide também suportam a separação de *M. amazonicum* e *M. pantanalense* (Capítulo 3), sendo evidenciadas diferenças nas secreções que compõem o espermatóforo e no formato do espermatozoide. As diferenças nesses traços reprodutivos foram inesperadas, uma vez que esses caracteres são altamente conservativos. Ainda assim, foi possível elencar algumas características reprodutivas que divergem entre esses organismos, e essas somam-se as características que já foram reportadas na descrição e em outros estudos sobre *M. pantanalense*.

A morfologia e a morfometria larval (Capítulo IV) forneceram informações que seguem a mesma tendência exposta nos três primeiros capítulos, ou seja, que *M. amazonicum* e *M. pantanalense* são linhagens distintas, com várias diferenças em seus aspectos biológicos. Dentre as diferenças apontadas nesse capítulo, chamamos a atenção para a variação heterocrônica do pereópodo 5, tempo de desenvolvimento e tamanho das larvas das duas espécies. Essas diferenças somam-se aos achados descritos por Marco-Herrero et al. (2019), consolidando que esses dois grupos apresentam variações interespecíficas desde a fase de vida larval.

Podemos concluir que essas duas linhagens são duas entidades taxonômicas que apresentam diferenças em sua biologia, todas as ferramentas aqui utilizadas foram eficazes na discriminação desses dois grupos, dando suporte à sua separação. Entretanto, entendemos que a descrição original de *M. pantanalense* apresenta algumas inconsistências pelo fato de que a maioria das informações descritivas sejam baseadas em proporções de tamanho em relação a *M. amazonicum*, o que pode gerar problemas na identificação, uma vez que *M. amazonicum* é uma espécie fenotipicamente plástica, com populações de pequeno e grande porte ocorrendo ao longo do Brasil. Desse modo, algumas das características aqui levantadas podem ser muito úteis em futuros estudos que almejem redescrever *M. pantanalense*. Finalmente, reforçamos a importância da utilização de ferramentas de taxonomia integrativa em estudos que abordem a discriminação de espécies que são proximamente relacionadas.