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Ecologia e Reprodução de *Macrobrachium amazonicum* (Crustacea, Palaemonidae) em diferentes sistemas aquáticos

Gicelle Maria Farias da Silva

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Tese apresentada ao Programa de Pós-Graduação em Ecologia, Universidade Federal do Pará e Embrapa Amazônia Oriental, como requisito para a obtenção do título de Doutora em Ecologia.
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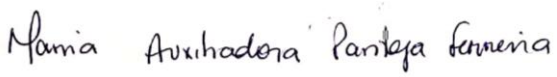

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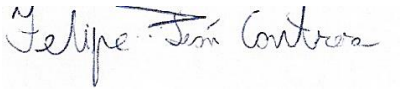

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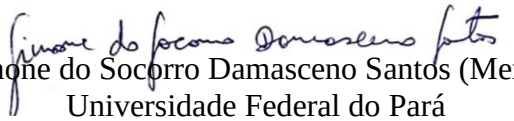

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Epígrafe

"Talvez não tenha conseguido fazer o melhor, mas lutei para que o melhor fosse feito. Não sou o que deveria ser, mas Graças a Deus, não sou o que era antes".

Marthin Luther King

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RESUMO

Macrobrachium amazonicum é uma espécie de camarão endêmico da América do Sul com ampla distribuição geográfica e de importância biológica e econômica para a Amazônia. Essas populações apresentam variações quanto às características ecológicas, comportamentais e de histórias de vida. Contudo, grandes lacunas sobre adaptação do *M. amazonicum* ao ambiente, relacionados aos fatores que subsidiam a sobrevivência/reprodução do animal ainda constituem entraves para o entendimento de seu ciclo de vida. O objetivo desta tese foi investigar os efeitos dos ecossistemas aquáticos, de estuário e rio, nos traços da história de vida das populações de *M. amazonicum*. Desse modo, esta tese foi organizada em três capítulos. O primeiro descreve e compara o sistema reprodutor dos morfotipos de machos adultos através da morfometria dos túbulos seminíferos, células germinativas, e frequência dessas células nos morfotipos TC (*Tranlucent claw)*, CC (*Cinamon claw)* e GC (*Green claw*) de *M. amazonicum* e estabelecer uma nova descrição da espermiogênese para a espécie. O segundo capítulo descreve se o ciclo hidrológico, no estuário e no rio, influenciam nas condições ambientais e nas populações de *M. amazonicum*. Os dados descritos neste capítulo mostraram que o ciclo hidrológico, em cada ambiente, apresenta fatores abióticos específicos que interferem na dinâmica de tolerância para a sobrevivência, podendo ser fatores limitantes para as alterações nos traços da história de vida de populações de *M. amazonicum.* No terceiro capítulo, foi investigado a ocorrência de trade-off entre crescimento e reprodução em populações de *M. amazonicum,* de diferentes sistemas aquáticos. Nesse estudo, estabelecemos que em ambientes de rio, as fêmeas não apresentam estágio de repouso e há predomínio de machos morfotipo TC, determinando a existência de trade-off entre crescimento e a reprodução como estratégia para se manter no ambiente. Nossos dados demonstraram que *M. amazonicum* representa um modelo para estudos sobre os processos em nível populacional, comportamental, taxonômico e reprodutivo em diferentes ecossistemas.

Palavras-chave: camarão da Amazônia, ambiente, estratégia reprodutiva, gônada

ABSTRACT

Macrobrachium amazonicum is endemic of South America with a large geographical distribution and both biological and economical importance to Amazon. It shows variations in the ecological characteristics, behavior and life-history traits between populations. However, large gaps in the adaptation of *M. amazonicum* to the environment, related to factors that support the survival / reproduction of the animal, are still obstacles to the understanding of its life cycle. The objective of this thesis was to investigate the effects of aquatic, estuary and river ecosystems in life history traits of M. amazonicum populations. Thus, this thesis was organized into three chapters. The first chapter described and compared the reproductive system of adult male morphotypes using the morphometry of the seminiferous tubules, germinative cells and the frequency of these cells in the TC (*Tranlucent claw)*, CC (*Cinamon claw)* and GC (*Green claw*) morphotypes of *M. amazonicum.* In this work we have established a new description of spermyogenesis for the species. The second chapter aimed to describe if the hydrological cycle, in the estuary and in the river, influence the environmental conditions and the populations of *M. amazonicum*. The data showed that the hydrological cycle, in each environment, presents specific abiotic factors that interfere with the survival tolerance dynamics, and may be limiting factors for changes in the life history traits of the populations. In the third chapter, the objective was to investigate the occurrence of trade-offs between growth and reproduction in populations of *M. amazonicum* from different aquatic systems. In this study, we established that in female environments, *M. amazonicum* females do not present resting stage and there is a predominance of male CT morphotype, determining the existence of trade-off between growth and reproduction as a strategy to stay in the environment. Our data demonstrated that *M. amazonicum* represents a model for studies on the understanding of processes at the population, behavioral, taxonomic and reproductive levels in different ecosystems.

Keywords: Amazon river prawn, environment, reproductive strategy, gonad.

ESTRUTURA DA TESE

Levando em consideração as diretrizes, a Tese se encontra na seguinte estrutura:

1. Introdução geral, onde o leitor irá encontrar uma breve apresentação sobre a espécie *M. amazonicum,* incluindo a taxonomia, morfologia, distribuição geográfica, aspectos ecológicos, reprodutivos e sua importância econômica.

Objetivos da Tese

Com a presente tese, investigamos os efeitos dos ecossistemas aquáticos, estuário e rio, em traços da história de vida de populações de *Macrobrachium amazonicum.*

Assim, a tese está organizada em três capítulos:

Capítulo I- Morphometry, frequency and ultrastructure of male germ cells in morphotypes of the freshwater prawn *Macrobrachium amazonicum* (Decapoda: Palaemonidae)

Este capítulo descreve e compara a morfometria dos túbulos seminíferos e das células germinativas e estabelecer a frequência dessas células nos diferentes morfotipos de *M. amazonicum*. Ainda propõe uma nova descrição da espermiogênese nessa espécie.

Manuscrito publicado em **Zoologischer Anzeiger** – 2019, Qualis A2 -Área Biodiversidade-CAPES.

Capítulo II- Is the hydrological cycle a limiting factor for the life-history traits of a freshwater prawn?

Este capítulo relata traços da história de vida das populações de *Macrobrachium amazonicum* em dois ecossistemas distintos durante um ciclo hidrológico. Baseado em estudos que características ambientais de um ecossistema podem interferir no crescimento, desenvolvimento e reprodução dos camarões.

O manuscrito foi submetido à *Ecology and Evolution* – 08/2019, Qualis A1 - CAPES.

Capítulo III- Energy allocation trade-off in *Macrobrachium amazonicum*, with no resting stage

Este capítulo relata um novo padrão de perfil reprodutivo em *Macrobrachium amazonicum*. Baseado nos dados de que, em fêmeas, o estadiamento ovariano ocorre simultaneamente ao desenvolvimento embrionário, e, em machos, a ausência ou a diminuição da ocorrência de morfotipos dominantes não comprometem a reprodução.

O manuscrito será submetido à *Plos One* – 2019, Qualis A1- CAPES.

INTRODUÇÃO GERAL

1-Aspectos e distribuição da espécie *Macrobrachium amazonicum.*

Entre os crustáceos, a ordem Decapoda Latreille, 1802 é uma das mais bem sucedidas apresentando aproximadamente 10.000 espécies, essenciais em ambientes aquáticos. Esses organismos são importantes no aspecto ecológico, por realizarem o processamento da matéria orgânica e a manutenção do fluxo de energia (Bond-Buckup & Buckup, 1989; Müller et al., 1999; Magalhães, 2003; Gonçalves & Aranha, 2004), atuando na cadeia como predadores, detritívoros e presas (Porto, 1998).

A maioria das espécies conhecidas de camarão pertence à infraordem Caridea Dana, 1852 com aproximadamente 2.400 espécies agrupadas em 270 gêneros e 27 famílias. Dentre estas, se destaca a família Palaemonidae Rafinesque, 1815 composta por 980 espécies (De Grave & Fransen, 2011) distribuídas em todos os continentes, vivendo principalmente em ambientes de água salobra e doce (Holthuis, 1952; Christoffersen, 1989; Fransen, 2015), possuindo a segunda maior diversidade na ordem Decapoda (Anger, 2013).

O gênero *Macrobrachium* Spence Bate, 1868 é um dos mais importantes da família Palaemonidae, com 243 espécies, destas muitas são de interesse comercial e distribuídas em regiões tropicais e subtropicais (Da Silva et al. 2004; Silva et al. 200 7; De Grave & Fransen, 2011). No Brasil são registradas 17 espécies da família Palaemonidae (Pileggi & Mantelatto, 2010). Dentre estas espécies destaca-se *Macrobrachium amazonicum* (Heller, 1862) que representa cerca dos 85% de camarões de água doce pescados no Brasil (New, 2000).

Esta espécie é endêmica da América do Sul com ampla distribuição geográfica, sendo encontrada nas bacias dos rios Orinoco, Amazonas e Paraguai (Holthuis, 1952). No estado do Pará essa espécie é conhecida como camarão-regional (Moraes-Riodades, et al., 1999), nas demais regiões recebe o nome de camarão-da-Amazônia (Moraes-Valenti & Valenti, 2002), camarão-

canela e camarão-sossego (Valenti, 1985). Habita ambientes lacustres, inundáveis e lóticos e é muito abundante nas águas ricas em sedimentos da bacia do rio Amazonas, reservatórios, em rios e lagos do Pantanal (Odinetz-Collart, 1999; Bialetzki et al., 1997; Magalhães et al., 2005). No entanto, é raro em riachos de água preta onde o teor de nutrientes é baixo (Odinetz-Collart & Rabelo, 1996) e não ocorre em águas frias de elevadas latitudes (Bauer, 2004; Murphy & Austin, 2005; Wowor et al., 2009). A presença dessa espécie em ambientes dulcícolas está relacionada de forma positiva às características ambientais como a temperatura da água, a velocidade da corrente, o tipo de substrato dominante, a profundidade do corpo d'água e a estabilidade do ambiente, além de uma relação destes com a disponibilidade de recursos existentes no meio (Teixeira & Sá, 1998; Müller et al., 1999).

O camarão-da-Amazônia (Figura 1) é caracterizado por apresentar um rostro longo que ultrapassa distintamente o escafocerito, com dentes na porção superior e inferior. O telson apresenta uma extremidade pontiaguda. Apresenta exoesqueleto que oferece proteção contra predadores, e para crescer o animal realiza ecdises, e, neste momento, ele se refugia em tocas porque se encontra vulnerável a predadores. Geralmente os machos adultos são maiores que as fêmeas e apresentam os quelípodos proporcionalmente mais desenvolvidos. Apresentam coloração transparente e quase incolor (Sawaya, 1946; Cervigón et al., 1992). Os machos adultos são classificados em morfotipos de acordo com características macroscópicas como morfometria, morfologia, coloração dos quelípodos e com base na organização das células germinativas (Moraes-Riodades & Valenti, 2004; Silva et al., 2009). Os morfotipos masculinos são uma característica da espécie, mas o desenvolvimento, ou a falta de desenvolvimento, da estrutura completa da população masculina pode ser dependente das características ambientais de cada local (Maciel et al., 2009; Vergamini, 2009).

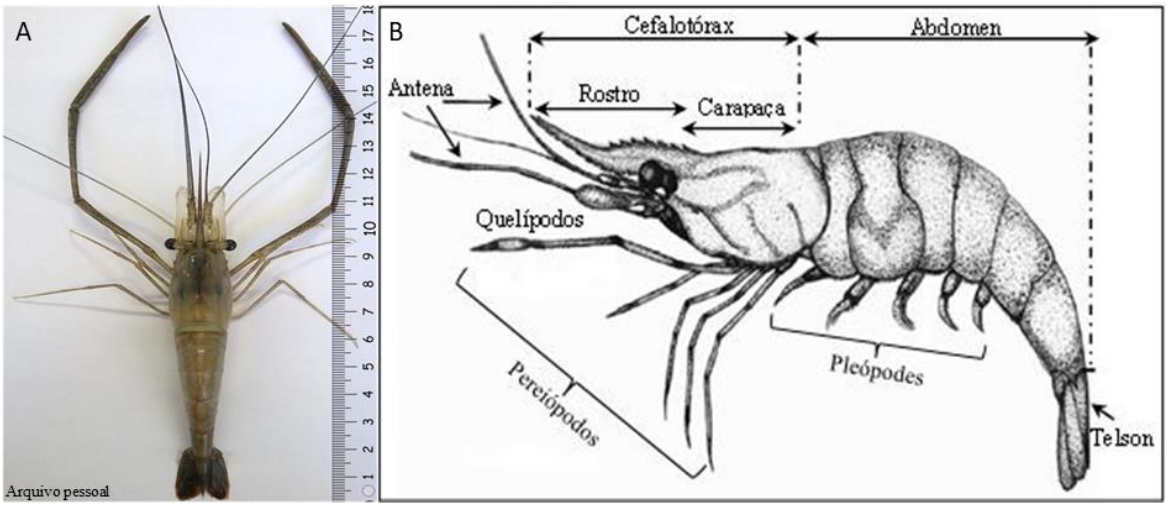

Figura 1. *Macrobrachium amazonicum.* (A) Vista dorsal (B) Desenho esquemático com dimensões corporais. Adaptado de Pantaleão et al. (2012).

2. Características reprodutivas de *M. amazonicum*

Estudos demonstram que existem variações entre populações de *M. amazonicum*, no que diz respeito as características ecológicas, comportamentais e de histórias de vida (Hayd & Anger, 2013). Apresentando ainda variações intraespecíficas quanto às características morfológicas e genética (Vergamini et al., 2011).

A biologia reprodutiva no gênero *Macrobrachium* tem sido bem investigada, para a determinação do período reprodutivo, maturidade sexual, fecundidade, tamanho dos ovos, volume da massa de ovos e suas relações com outros fatores ambientais e temperatura (Müller & Carpes 1991; Souza & Fontoura 1996; Lima & Oshiro 2000; Nazari et al., 2003). No entanto as informações são ainda limitadas no que se refere à relação das diferenças na biologia do animal com os fatores do ambiente.

O ciclo de vida de *M. amazonicum* é compreendido pelas fases de ovo, larva, juvenil e adulto, a maioria dos camarões apresenta fertilização externa. Os machos apresentam uma estrutura chamada espermatóforo que armazena os espermatozóides. A fêmea é submetida a uma muda de pré-acasalamento e, em seguida, o macho deposita o espermatóforo no gonóporo, localizado na

superfície torácica e ventral da fêmea. Os ovos recém-liberados são verde-escuros e mudam de cor até se tornarem translúcidos, pouco antes da eclosão (Romero, 1982; Dougherty & Sandifer, 1984; Rego et al., 2004). O desenvolvimento embrionário oscila de 12 a 18 dias e o desenvolvimento larval de 20 a 23 dias (Guest, 1979; Gamba, 1984; Magalhães, 1985). Após a metamorfose, os juvenis nadam rapidamente na coluna d'água, e assumem um hábito bentônico.

Os machos adultos de *M. amazonicum* (Figura 2) são classificados em quatro morfotipos baseados no comprimento, coloração e espinação dos quelípodos: TC- *Translucent Claw*, CC- *Cinnamon claw*, GC1- *Green Claw* 1 e GC2- *Green Claw* 2 (Moraes-Riodades & Valenti, 2004). No entanto, com base na organização das células germinativas nos testículos Silva et al. (2009) estabeleceu apenas três morfotipos: TC, CC e GC. Enquanto, para fêmeas adultas (Figura 3), de acordo com a observação macroscópica e histológica foi descritos cinco estágios de maturação ovariana, imaturo, em maturação, maduro, ovado e reorganizado (Chaves & Magalhães, 1993; Ribeiro, 2006; Ferreira et al., 2012).

Estudos demonstram que *M. amazonicum* em estuários medem cerca de 10-16 cm, apresentam alta fecundidade e precisam da água salgada ou salobra para completar o desenvolvimento larval. Enquanto, camarões que vivem em ambientes isentos de influência salina, como em rios, lagos e outros corpos de água, são 5 cm menores que do ambiente salino, apresentam baixa fecundidade e sua metamorfose é completa em água doce (Odinetz-Collart & Rabelo, 1996; Moraes-Valenti & Valenti, 2010). Desse modo as estratégias reprodutivas e a estrutura populacional de *M. amazonicum* são fortemente dependentes do local, influenciadas por particularidades hidrológicas e geográficas do meio como as chuvas e inundações, o fluxo de água, a temperatura e a distância da água salobra (Maciel & Valenti, 2009; Meireles et al., 2013). Esta estratégia permite a reprodução contínua do animal, com picos reprodutivos nos períodos chuvosos (Odinetz-Collart, 1993; Bialetzki et al., 1997; Sampaio et al., 2007; Bentes et al., 2011). Porém, ainda são limitadas

as informações quanto aos fatores ambientais que subsidiam a sobrevivência/reprodução contínua de *M. amazonicum.*

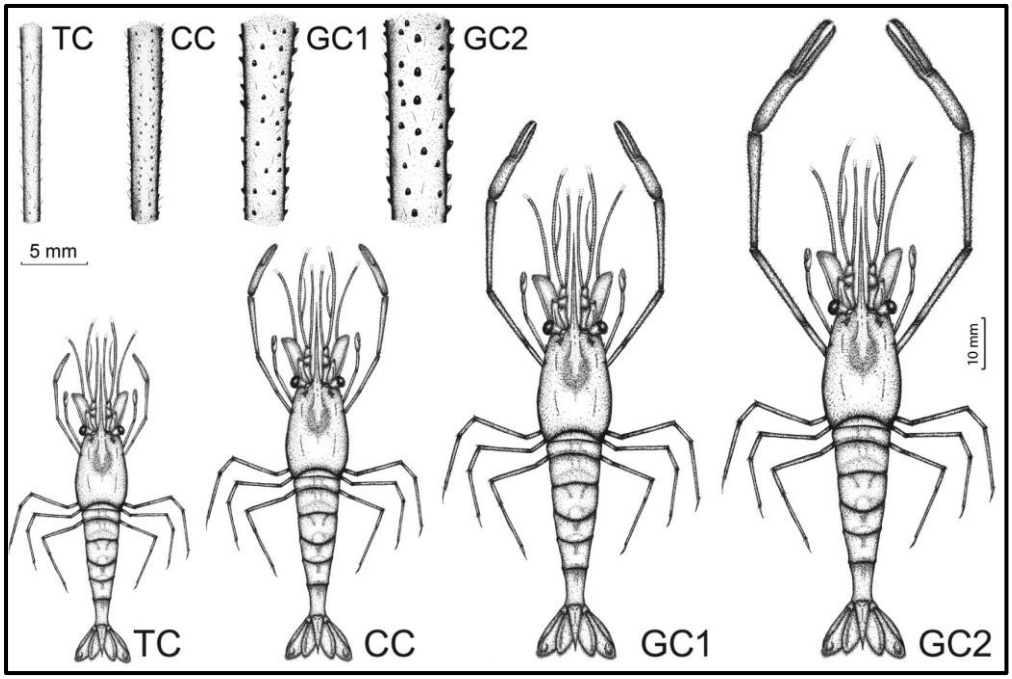

Figure 2. Esquema dos quatro morfotipos de machos de *M. amazonicum* classificados de acordo com coloração e espinação dos quelípodos. TC: Quela translúcida; CC: Quela canela; GC1: Quela verde 1; GC2: Quela verde 2. (Pantaleão et al.2014).

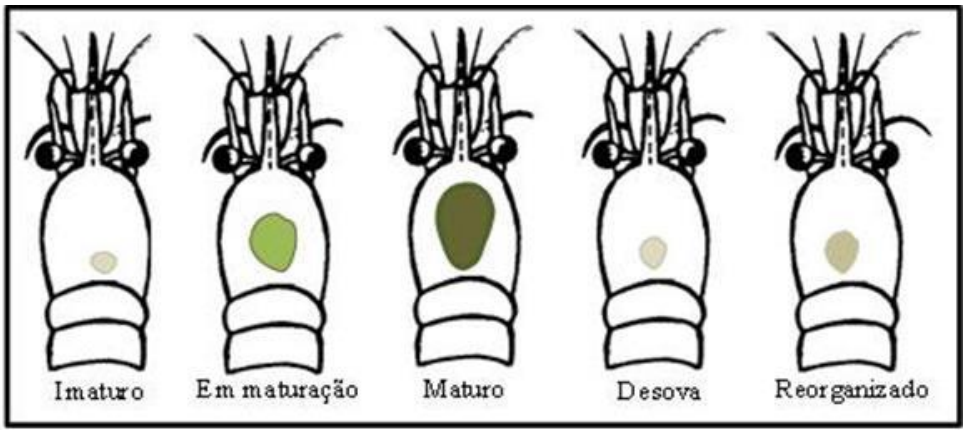

Figure 3. Esquema dos estágios de desenvolvimento ovariano de *M. amazonicum*.

3. Importância econômica de *M. amazonicum*

A carcinicultura de água doce tem sido reconhecida como uma forma de produzir crustáceos com baixo impacto ambiental (New, 2010). Esse sistema adapta-se muito bem aos sistemas familiares de produção e atende aos preceitos de uma aquicultura sustentável (Moraes-Valenti & Valenti, 2010).

No Brasil *M. amazonicum* que apresenta potencial para o cultivo comercial (Moraes-Valenti & Valenti, 2010), é uma espécie que possui rápido crescimento, rusticidade, fácil manutenção em cativeiro (Guest, 1979; Barreto & Soares, 1982; Valenti, 1985). Favorece o cultivo sustentável em empresas que usam mão-de-obra familiar (Valenti, 1993; New et al., 2000) e condições econômico e social às comunidades ribeirinhas que utilizam como fonte de renda e alimentação (Valenti et al., 1989; Albertoni et al., 2003a,b).

Essa espécie é largamente explorada pela pesca artesanal na região Nordeste (Gurgel & Matos, 1984) e nos Estados do Pará e Amapá (Odinetz-Collart, 1987; Odinetz-Collart & Moreira, 1993). Segundo os dados da FAO (2010), foram produzidas, aproximadamente, 413.000 t de camarões de água doce no ano de 2008.

A pesca tradicional do *M. amazonicum* na Amazônia é baseada principalmente no uso de armadilhas artesanais, confeccionadas em madeira, chamadas de matapis, (Figura 4) (Odinetz - Collart, 1993; Vieira, 2003).

Figure 4. Imagens de "Matapis", armadilhas tradicionais usadas para capturar *Macrobrachium amazonicum* na Amazônia.

A pesca do *M. amazonicum* é uma das principais atividades econômicas, segundo o Instituto de Pesquisa em Ciências e Tecnologia do Estado do Amapá cerca de 50 toneladas de camarão foram desembarcadas por ano no principal porto local, entre 1998 e 2000. Nas ilhas do Marajó, norte do Brasil estima-se que 750 kg de camarões são capturados por mês (Vieira & Araújo- Neto, 2006). *M. amazonicum* constitui uma das espécies mais exploradas do gênero, embora a biologia básica seja conhecida, ainda são escassas informações sobre os atributos de sua população e sua exploração. Atualmente, a principal atenção está relacionada à sua captura para consumo (Bauer, 2011a, 2011b)**.**

CHAPTER I

Morphometry, frequency and ultrastructure of male germ cells in morphotypes of the freshwater prawn *Macrobrachium amazonicum* (Decapoda: Palaemonidae)

Gicelle M. F. Silva, Yanne A. Mendes, Ivana K. S. Viana, Liziane A. B. Gonçalves, Renata S. Oliveira, Rossineide M. Rocha & Maria A. P. Ferreira.

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Abstract

Males of the freshwater prawn species, *Macrobrachium amazonicum*, have been staged histologically into morphotypes, *Translucent claw* (TC), *Cinnamon claw* (CC) and *Green claw* (GC). However, information on reproductive system anatomy and spermatogenesis among morphotypes is scarce. Our aim was to describe the frequency of spermatogenic cells, morphometry of seminiferous tubules and spermatogenic cells, and spermiogenesis to establish any differences among morphotypes. Specimens were captured and sexed. Testes were dissected, processed, and analyzed using light, transmission, and scanning electron microscopy. Macroscopically, testes were symmetrical, elongated, and translucid, with long vasa deferentia ending in ampoules. Histologically, testes exhibited seminiferous tubules with the germinal epithelium containing male germ cells in different stages of development and supporting cells. The diameter of seminiferous tubules was largest (P<0.05) in the GC morphotype. Germ cell types were evaluated based upon size, as well as their cytoplasm and nuclear chromatin organization. Spermatozoa were the only

germ cells displaying no differences in size among morphotypes. According to shape and localization, we classified spermatids into three subtypes: St_1 located in the germinal epithelium with scant cytoplasm, a concave nucleus, and condensed chromatin; St_2 sickle-shaped, with a concave, thin nucleus; and, St_3 cup-shaped, with a developing acrosomal vesicle in the convex portion of the cell. Notably, St_2 and St_3 were found in the lumen of the seminiferous tubules and vasa deferentia. Spermatids and spermatozoa were floating in a large amount of amorphous glycoprotein-rich material. Spermatozoa were cup-shaped and displayed an acrosome fashioning a long spike with regular and compact cross-striations. In summary, we established differences in size and frequency of male germ cells among morphotypes and described spermatid characteristics not yet observed in other prawn species. Altogether, this information contributes to expanding our knowledge on taxonomy, phylogenetic relationships, and reproduction among Palaemonidae species.

Keywords: Amazon river prawn; Spermatogenesis; Spermiogenesis; Sperm; Palaemonidae

1. Introduction

Macrobrachium amazonicum (Heller, 1862), a freshwater prawn belonging to the order Decapoda and family Palaemonidae with year-round reproductive activity, is of interest for commercial farming (Odinetz Collart, 1991; New, 2000) due to its rapid growth, resistance to diseases, and easy management (Guest, 1979; Valenti, 1985; New, 2005). An in-depth knowledge of gonadal structure and spermatogenesis would therefore be crucial for aquaculture (Diamond et al., 2008; Ceballos-Vázquez et al., 2010; Alfaro-Montoya and Hernández, 2012). This knowledge would also be important for establishing morphological and phylogenetic relations among arthropods (Brown, 1970; Bauer and Holt, 1998; Tudge et al., 2001; Tudge, 2009; Terossi et al., 2012; Fransozo et al., 2016).

Many studies have described spermatogenesis and sperm structure in several species of palaemonids (Burkenroad, 1947; Pochon-Masson, 1969; Papathanasiou and King, 1984a, 1984b; Barros et al., 1986), especially in the genus *Macrobrachium* (Chow et al., 1982; Lynn and Clark, 1983a, 1983b; Dougherty and Sandifer, 1984; O'Donovan et al., 1984; Butcher and Felder, 1994; Yang et al., 1998; Poljaroen et al., 2010). Microscopically, prawn testes present seminiferous tubules containing the spermatogenic lineage cells. Some studies have described spermatogenesis as a rapid process with a high rate of cell division (King, 1948; Bell and Lightner, 1988). Spermatogonia undergo a finite number of mitotic divisions giving rise to primary spermatocytes, which then undergo a first round of meiosis, and become secondary spermatocytes. Completion of meiosis yields spermatids that differentiate into spermatozoa in a process termed spermiogenesis (Carvalho, 1980; Bell and Lightner, 1988; Butcher and Felder, 1994, Yang et al., 1998; Poljaroen et al., 2010; Tripathi and Pandey, 2014). However, little is known in regards to the germ cell stages comprising the spermatogenic process and sperm formation.

The sperm morphology of decapod crustaceans differs from that in most other animals since they are aflagellate and immotile (Tudge, 2009). In these species, sperm consist of a main body with

appendages termed spikes (Shigekawa and Clark, 1986; Bell and Lightner, 1988; Tudge et al., 2001). Based upon the number of spikes, they can be classified as unistellate, typical of natantians (shrimp), or multistellate, found in reptantians (crabs, crayfish, and lobsters) (Wilson, 1928; Lu, 1976; Talbot and Summers, 1978). Therefore, these differences are based upon the presence or absence of a spike and/or acrosome (Brown, 1970; Koehler, 1979). Natantian spermatozoa have a spherical or cup-shaped head and a small, fixed spike consisting of microtubules and contractile proteins (Clark Jr. et al., 1973; Koehler, 1979). Conversely, reptantian sperm display several spikes or appendages of nuclear or cytoplasmic origin (Krol et al., 1992; Holdish, 2002). Within the decapod order, there are also additional differences that are important regarding the fertilization process; for instance, natantian spermatozoa may (Cummings, 1961) or may not (Papathanassiou and King, 1983b) display an acrosome. In the absence of the acrosome, the spike is formed by filament polymerization and plays a role in sperm-egg recognition and binding at fertilization (Papathanassiou and King, 1983b; Butcher and Felder, 1994). Conversely, in some species, namely of the Peneidean decapod family, a spike arises from the acrosome altogether forming what has been termed the acrosomal complex (Medina et al., 2006; Braga et al., 2013; Hou et al., 2013; Camargo et al., 2015; Fransozo et al., 2016). This diversity in spermatozoal morphology is an important premise used in taxonomic and phylogenetic studies (Jamieson, 1991; Jamieson and Tudge, 2000; Martin and Davis, 2001).

Information regarding testis and gamete morphology is limited for *Macrobrachium* species, as reported in *M. acanthurus* (Carvalho, 1980), *M. rosenbergii* (Chow and Taki, 1982), *M. australiensis* (Butcher et al., 1994) and *M. amazonicum* (Silva et al., 2009; Paschoal and Zara, 2018). In these species, spermatozoa are cup-shaped with the presence of a pointed spike of undefined ultrastructure. In regards to *M. amazonicum,* cultivated adult males were classified according to their macroscopic characteristics, color and spination, as well as the morphology and morphometry of the cheliped, into four morphotypes: *Translucent claw* (TC), cheliped length

ranging from 20.0 to 54.3 mm, translucent colorless, and devoid of spines; *Cinnamon claw* (CC), cheliped length ranging from 24.7 to 54.8 mm, translucent greenish with brown pigments, and some slender spines; *Green claw 1* (GC1), cheliped length ranging from 56.2 to 98.8 mm, and either opaque greenish or whitish with small tubercles, or translucent greenish with brown pigments, and some spines; and, *Green claw 2* (GC2), cheliped length ranging from 71.9 to 175.6 mm, with color and spine morphology similar to GC1 (Moraes-Riodades and Valenti, 2004). However, based upon germ cell organization in the testis, only three morphotypes were identified (TC, CC, and GC) (Silva et al., 2009). Moreover, there is no descriptive information regarding spermiogenesis for this prawn. Therefore, the aim of this study was to detail testis morphometry and spermiogenesis comparing the *M. amazonicum* morphotypes in order to establish a study model for a better understanding of the fertilization process in this genus and to support the application of future techniques that allow for its preservation.

2. Materials and Methods

2.1. Samples

Specimens of *M. amazonicum* were collected bimonthly from January to December 2016 in Mosqueiro Island, Pará, Brazil (1°13'25" S, 48°17'40" W) using artisan traps. Animals were transported alive in an isothermal box filled with local water. Once in the laboratory, 165 adult males were identified based on the presence of the male sexual appendage in the second pair of pleiopods. The identification of male morphotypes followed the classification proposed by Moraes-Riodades and Valenti (2004) and Silva et al. (2009). Subsequently, males were anesthetized on ice and the gonads were dissected, fixed, and submitted to microscopic analysis.

2.2. Light Microscopy

Whole testes from the different morphotypes were dissected, fixed in Bouin's solution for 24 h, and paraffin embedded (Prophet et al., 1995). Then, 5 µm thick sections were placed on glass

slides and stained with hematoxylin and eosin for histological examination. Subsequently, the replicated sections were stained with Periodic Acid-Schiff (PAS) (Prophet et al., 1995) to detect the mucopolysaccharide in prawn testis. All images were recorded using a Nikon (NIKON Eclipse Ci-E) photomicroscope connected to a DS-Ri1 (NIKON, Japan) digital camera.

2.3. Transmission Electron Microscopy (TEM)

For TEM, testis fragments were fixed in Karnosky's solution (4% of parafolmaldehyde, 2% of glutaraldehyde in a buffer solution of sodium cacodylate 0.1 M, pH 7.3) for 24h, post-fixed in 1% osmium tetroxide solution buffered with sodium cacodylate (0.1 M, pH 7.3) for 2 h and contrasted *en bloc* with 1% of uranyl acetate. Tissues were dehydrated in a graded acetone series, and then infiltrated and embedded in Epon (EMS 14120 Embed 812). One µm and 60 nm thick sections were cut using an ultramicrotome (Leica EMUC6). The 1 µm sections were stained with toluidine blue for light microscopy examination. The ultrathin 60 nm sections were stained with uranyl acetate and lead citrate and examined under a TEM LEO 906E electron microscope (Carl Zeiss, Oberkoshen, Germany).

2.4. Scanning Electron Microscopy (SEM)

For SEM, testis fragments were fixed in Karnovsky's solution for 3 h at 4 °C. Samples were post-fixed with 1% osmium tetroxide solution buffered with sodium cacodilate (0.1 M, pH 7.3) for 2 h at room temperature. The fragments were then dehydrated in a graded ethanol series, and critical point dried using CO_2 (CPD 030 Baltec). Specimens were mounted on stubs, coated with gold and analyzed using a LEO 1430 SEM (LEO-ZEISS, Cambridge, England).

2.5. Cell frequency, Morphometry and Statistical analysis

Specimens from each morphotype were considered in all analyses (Silva et al., 2009). For quantification and morphometry measurements, histological sections were photographed on a

Nikon Photomicroscope (Nikon Eclipse CI) coupled to a digital camera (Nikon DS-RI1) and analyzed using NIS-elements software (BR4.00.07, Nikon). To measure spermatogenic cell distribution frequency, we analyzed 90 seminiferous tubules per morphotype. In each tubule, the number of spermatogonia, spermatocytes, spermatids, and spermatozoa was counted. For morphometric analysis, the mean diameter of seminiferous tubules and germ cells was calculated and compared among morphotypes. Means were checked for normality using the Shapiro-Wilk test and analyzed by the Kruskal-Wallis test ($P<0.05$) (Zar, 1999). When significance was detected a multiple comparison test was applied. All analyses were performed using the R Development Core Team Program (2016).

3. Results

3.1. Male reproductive system

A total of 165 male *M. amazonicum* specimens were collected and classified into each of three morphotypes as follows: TC (n=39), CC (n=81) and GC (n=45). Anatomically, the male reproductive system consisted of two symmetrical, elongated and translucent testes located in the cephalothorax (Fig. 1A). Two long vasa deferentia, one for each testis, ended in the fifth pair of pereiopods (Fig. 1B). In its final portion, each vas deferens displayed an ampoule (Fig. 1B and C). Histologically, the testes consisted of irregular seminiferous tubules, each tubule surrounded by a simple epithelium with the corresponding germinal epithelium and a lumen with spermatozoa floating in an amorphous PAS-positive substance (Fig. 1F and G). Vasa deferentia and ampoules were lined by a simple cuboid epithelium with eccentrically located tall epithelial cells, and a lumen filled with spermatozoa (Fig. 1E and G). The mean diameter of the seminiferous tubules differed *($P<0.05$)* among the TC (140.60 µm), CC (142.24 µm) and GC (164.20 µm) morphotypes, with the latter displaying the largest (Table 1).

3.2. Germinal epithelium

The germinal epithelium (Fig. 2A) comprised spermatogenic cells in different stages that included spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids and spermatozoa (Figs. 2, 4, 5, 6 and 7). Based on cell size and location, as well as chromatin organization, primary spermatocytes were classified into 4 stages including prophase I (leptotene, zygotene, pachytene, diplotene, and diakinesis), metaphase I, anaphase I, and telophase I. Interestingly, three types of spermatids were identified in the seminiferous tubules of *M. amazonicum*.

The size of primary spermatocytes and spermatids differed ($P<0.05$) between the TC and CC morphotypes. For all germ cells except spermatozoa, diameter differed ($P<0.05$) between the GC and each of the two other morphotypes. The frequency of spermatogenic cells in the germinal epithelium was different ($P<0.05$) among the three morphotypes, with the lower sperm frequency observed in the CC morphotype (Fig. 3).

3.2.1. Spermatogonia (Sg)

Spermatogonia were found only in the basal portion of the germinal epithelium. These cells presented a round to slightly oblong shape, with scant cytoplasm and evenly distributed nuclear chromatin, and one or two nucleoli (Fig. 2B, C and D). Mean diameter ranged from 6.95 to 7.19 μm (Table 1). The frequency of spermatogonia in the seminiferous tubules of the TC, CC, and GC morphotypes was 7%, 5%, and 3%, respectively (Fig. 3). While this frequency was not significantly different between the TC and CC morphotypes, it did differ between each of these and the GC morphotype ($P<0.05$).

3.2.2. Primary spermatocytes (Sc1)

For all three morphotypes, primary spermatocytes were the largest cells in the germinal epithelium, ranging from 7.50 to 10.78 μm in diameter (Table 1). In the leptotene stage, these cells presented scant cytoplasm containing mitochondria and vesicles, and a nucleus with heterochromatin (Figs. 2E and F; 4A and B). In zygotene, the nucleus was ovoid and contained irregularly condensed chromatin; the formation of the synaptonemal complex adjacent to the nuclear membrane began at this stage (Figs. 2E; 4B and C). In pachytene, the nucleus was rounded and synaptonemal complexes were clearly visible (Fig. 4E, F, H and I). In diplotene, condensed chromatin aggregated near the nuclear envelope (Fig. 4D and E). In diakynesis, the nuclear envelope disappeared and several irregular chunks of condensed, thick chromatin were observed within the cytoplasm (Fig. 4F and J). In metaphase I all chromatin became condensed into a single chunk located in the center of the cell in the absence of a nuclear membrane (Fig. 4G). Whereas cells in anaphase were not viewed, spermatogonia in telophase I were characterized by the formation of two new nuclei and chromatin decondensation (Fig. 4E).

3.2.3 Secondary spermatocytes (Sc2)

Secondary spermatocytes were smaller, ranging from 5.98 to 7.22 μm in diameter, with significant differences among morphotypes (Table 1). These cells also presented a scant cytoplasm containing mitochondria, and a compact nucleus with irregularly distributed heterochromatin (Fig. 4H).

3.2.4. Spermatids (St)

Spermatid diameter ranged from 3.91 to 5.07 μm (Table 1). According to their shape and localization, the spermatids were subdivided into three subtypes: spermatid 1 (St_1), spermatid 2 (St_2), and spermatid 3 (St_3). The St_1, which were found within the germinal epithelium, presented

cytoplasm with vesicles, a concave nucleus, and condensed chromatin (Figs. 5A-D; 7). The St_2 were sickle-shaped, and also presented vesicles within their cytoplasm, as well as a concave, thin nucleus; this stage was characterized by a budding acrosomal vesicle located in the convex portion of the cell (Figs. 5B, C and E, F). The most advanced St_3 were cup-shaped, and during this stage the acrosomal vesicle underwent significant changes, giving rise to a condensed structure (Fig. 5G, H and I). Altogether, the acrosomal vesicle and condensed structure formed the acrosomal complex. Subtypes St_2 and St_3 were found in the lumen of the seminiferous tubules and vasa deferentia, surrounded by PAS-positive material (Figs. 1F, G; 5). Only St_1 and St_3 displayed significant differences in the mean diameter among morphotypes. Seminiferous tubules from the CC morphotype contained the highest frequency of spermatids (Fig. 3).

3.2.5. Spermatozoa (Sz)

Spermatozoa were smaller, ranging from 3.49 to 3.56 μm in diameter. While located within the lumen of the seminiferous tubules and surrounded by a large amount of amorphous PAS-positive material, immature sperm were cup-shaped and displayed a relatively short single spike (Fig. 5I; 6A). The spike originated from the acrosomal vesicle and showed regular and compact cross-striations (Fig. 6B-F). Spermatozoa had very scant cytoplasm and a nucleus with condensed chromatin (Fig 6B-E). Once in the vas deferens, spermatozoa were also cup-shaped but displayed a longer spike (Figs. 6A-H; 7). Differences in the frequency of spermatozoa among morphotypes were detected only within the seminiferous tubules (i.e. 30% for TC, 68% for GC vs. 2% for CC; Fig. 3).

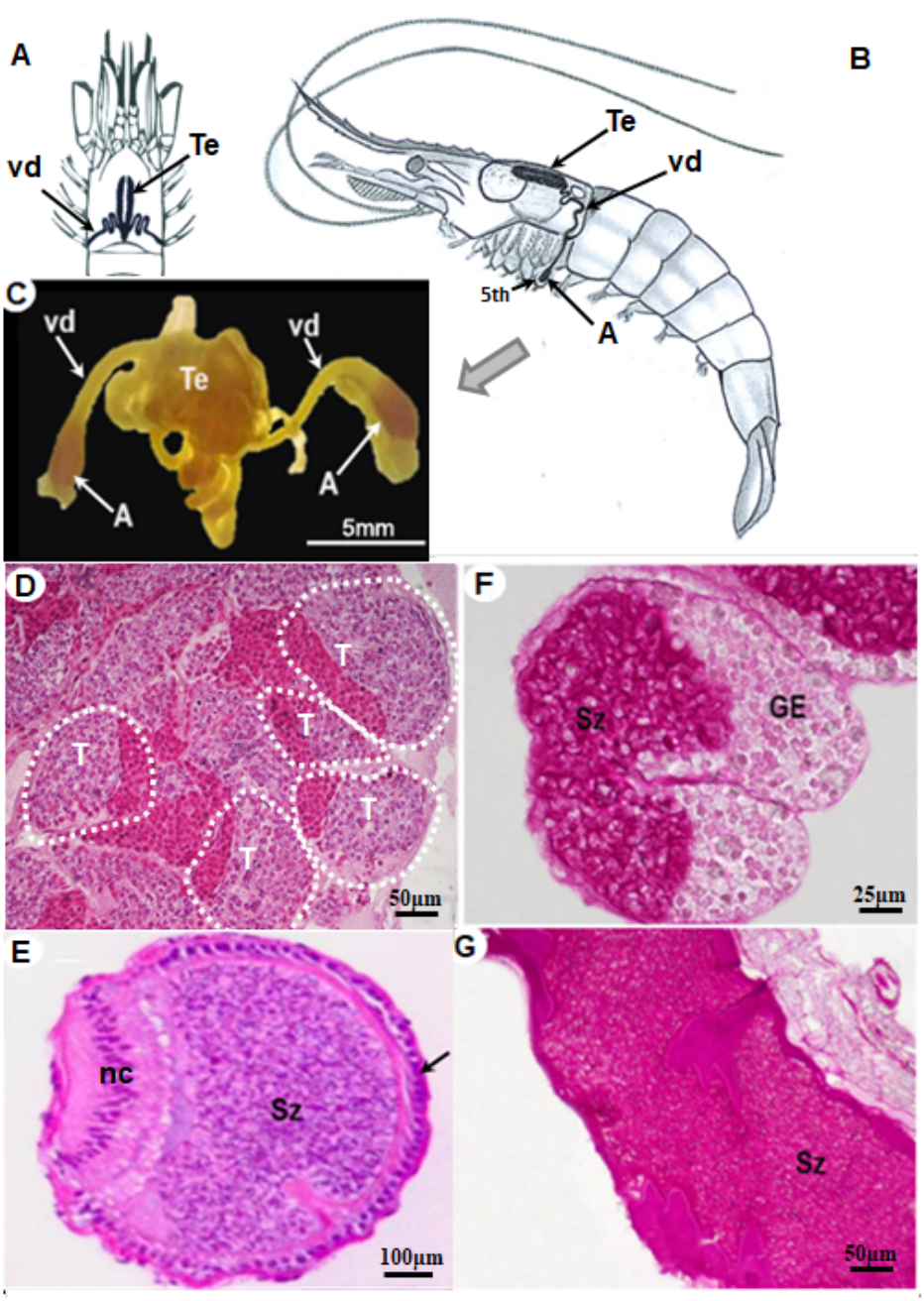

Fig. 1. Morphology of the male reproductive system in *M. amazonicum*. Illustration showing (A) dorsal view and (B) lateral view of testes and vasa differentia located on the cephalothorax. (B) The vasa deferentia display a terminal ampoule ending at the level of the fifth pair of pereiopods. (C) Dissected reproductive tract. (D, E) Photomicrographs of sections stained with H&E. (D) Testis section showing seminiferous tubules surrounded by connective tissue (100X). (E) Cross-section of the vas deferens surrounded by a simple cuboidal epithelium (arrow) with eccentrically located nurturing cells and spermatozoa (100X). (F, G) Sections displaying PAS- positive reaction. (F) Detail of seminiferous tubule showing the germinal epithelium and spermatozoa surrounded by

glycoprotein (400X). (G) Longitudinal section of the vas deferens with luminal spermatozoa surrounded by glycoprotein (200X). A: terminal ampoule, vd: vas deferens, GE: germinal epithelium, nc: nurturing cells, Sz: spermatozoa, T: seminiferous tubules, Te: testes. Scale bars = (C) 5 mm, (D, G) 50 µm, (F) 25 µm, and (E) 100 µm.

Table 1

Mean±SD of diameter of seminiferous tubules and spermatogenic cells in the different morphotypes of *Macrobrachium amazonicum.*

Diameter (µm)	**Morphotypes of *M. amazonicum***			**CV(%)**	***p-value***
	TC n=3	**CC n=3**	**GC n=3**		
Seminiferous tubules (n=90)	140.60 ± 25.20[b]	142.24 ±9.07[c]	164.20± 38.09[a]	22.69	**0.001**
Spermatogonia (n=100)	6.95 ± 0.52[b]	7.19 ± 0.85[b]	7.04± 0.72[a]	9.90	**0.001**
Spermatocyte 1 (n=100)	7.50 ± 0.75[c]	10.78± 0.72[a]	8.00± 0.71[b]	8.55	**0.001**
Spermatocyte 2 (n=100)	5.98 ± 0.47[b]	6.04 ± 0.52[b]	7.22± 0.70[a]	8.75	**0.001**
Spermatid 1 (n=100)	4.55 ± 0.39[b]	4.24 ± 0.53[c]	5.07± 0.50[a]	10.36	**0.001**
Spermatid 3 (n=100)	4.02 ± 0.49[b]	3.91 ± 0.40[b]	4.28± 0.45[a]	11.00	**0.001**
Spermatozoa (n=100)	3.49 ± 0.34	3.53 ± 0.30	3.56 ± 0.34	9.37	0.318

[a,b,c] Different superscripts within row denote significant differences between morphotypes. TC: *Translucent claw*; CC: *Cinnamon claw*; GC: *Green claw.*

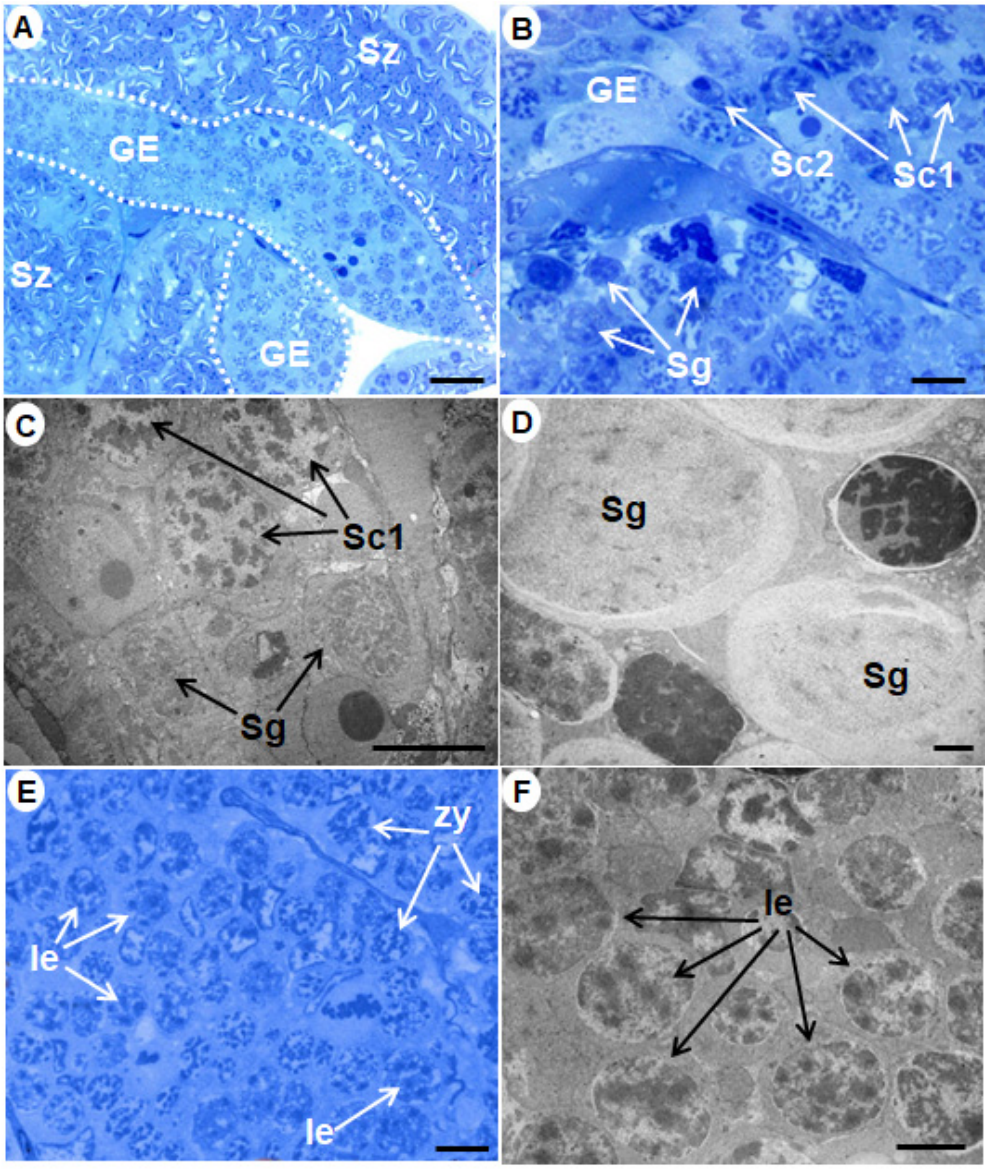

Fig. 2. Light and TEM photomicrographs of the germinal epithelium in the male of *M. amazonicum.* (A) Germinal epithelium and spermatozoa. (B) Germinal epithelium: spermatogonia (Sg), primary spermatocyte (Sc1) and secondary spermatocyte (Sc2). (C-D) Spermatogonia (Sg) with rounded nucleus and uniform chromatin. (E and F) Primary spermatocytes: Leptotene (le), reduced cytoplasm and heterochromatin; Zygotene (zy), beginning of the formation of the synaptonemal complex near the nuclear membrane. Scale bars = (A, B, E) 10 µm, (C, F) 10 µm, (D) 2 µm. GE: Germinal epithelium, Sz: spermatozoa.

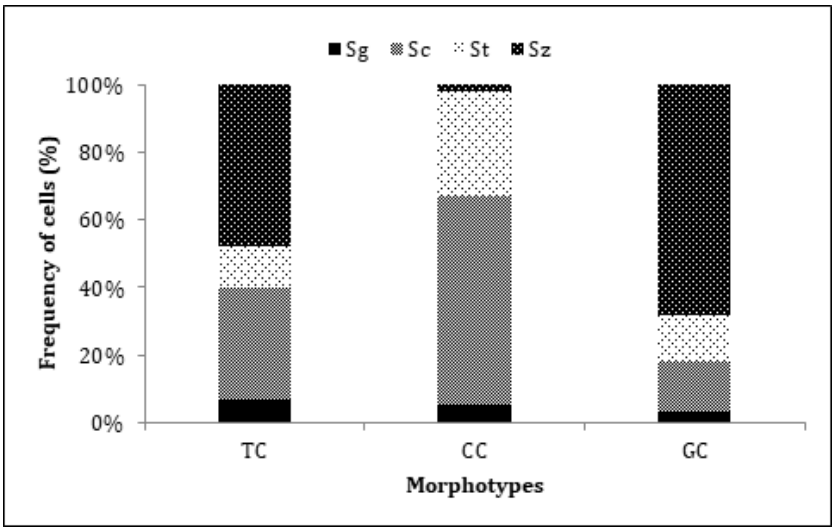

Fig. 3. Frequency of spermatogenic cells in the different morphotypes of *M. amazonicum*. TC: *Translucent claw*; CC: *Cinnamon claw*; GC: *Green claw*. Sg: Spermatogonia; Sc: Spermatocyte; St: Spermatid; Sz: Spermatozoa.

3.2.6. Somatic Cells

Irregularly-shaped cells were also observed adhered to the basal lamina of the germinal epithelium surrounding germ cell lineage cells. These cells displayed elongated nuclei and cytoplasm containing numerous vesicles, mitochondria, and endoplasmic reticulum (Figs. 4K and L).

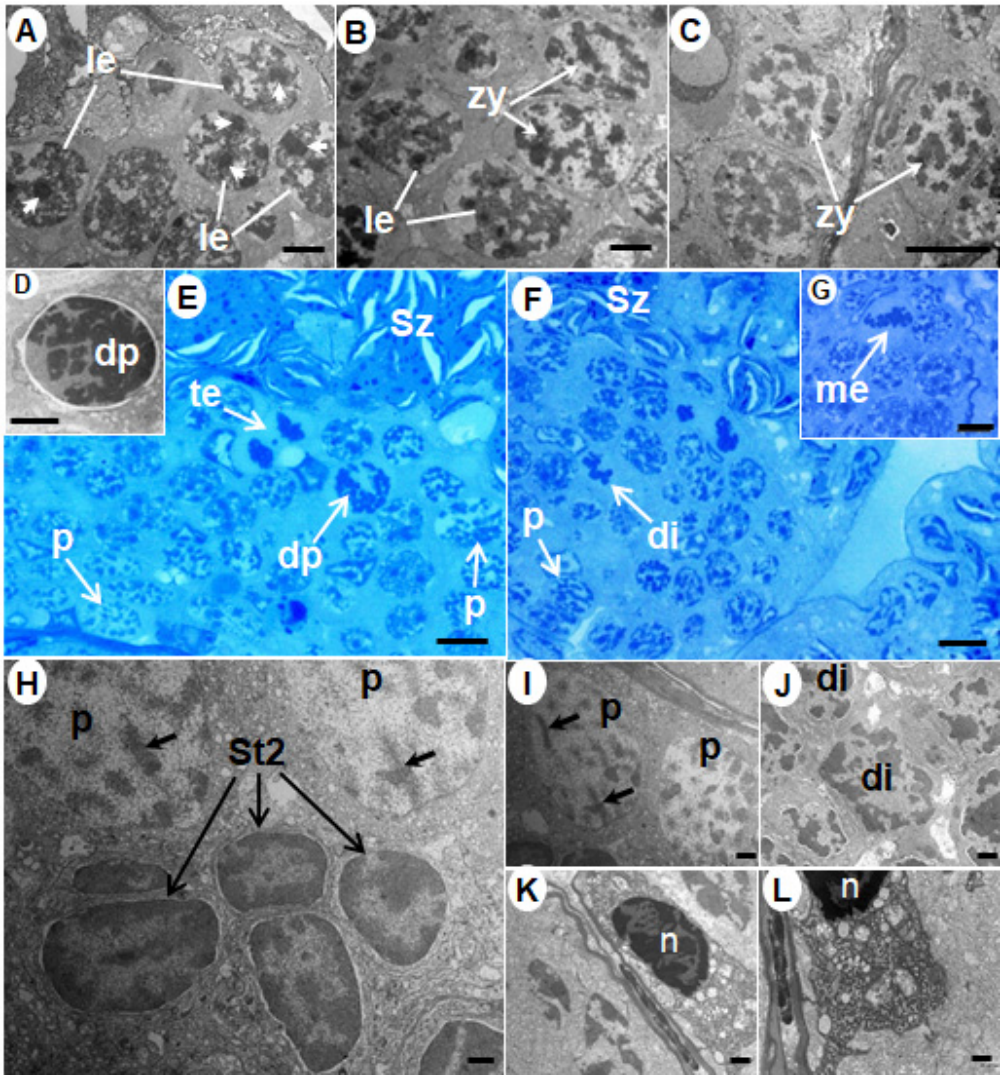

Fig. 4. Light and TEM photomicrographs of the germinal epithelium of the male of *M. amazonicum* showing spermatocytes and somatic cells. (A-J) Primary spermatocytes: (A, C) Leptotene (le), reduction of cytoplasm and heterochromatin; Zygotene (zy), beginning of the formation of the synaptonemal complex near the nuclear membrane. (D) Diplotene (dp), organization of chromatin aggregation near the nuclear membrane; (E) Pachytene (p), Telophase (te) formation of two new nuclei and chromatin decondensation; (F) Pachytene (p), and Diakinesis (di); (G) Metaphase (me), chromatin located in the middle portion of the spermatocyte; (H-I). Secondary spermatocyte; Pachytene (p), synaptonemal complexes (arrow) were clearly visible; (J) Diakinesis (di), loss of nuclear membrane and thick chromatin. (K-L) Somatic cell. Sz: spermatozoa, n: nucleus, c: cytoplasm. Scale bars = (A, B, H, I, J, K , L) 2 µm, (C, E, F, G) 10 µm, (D) 25 µm..

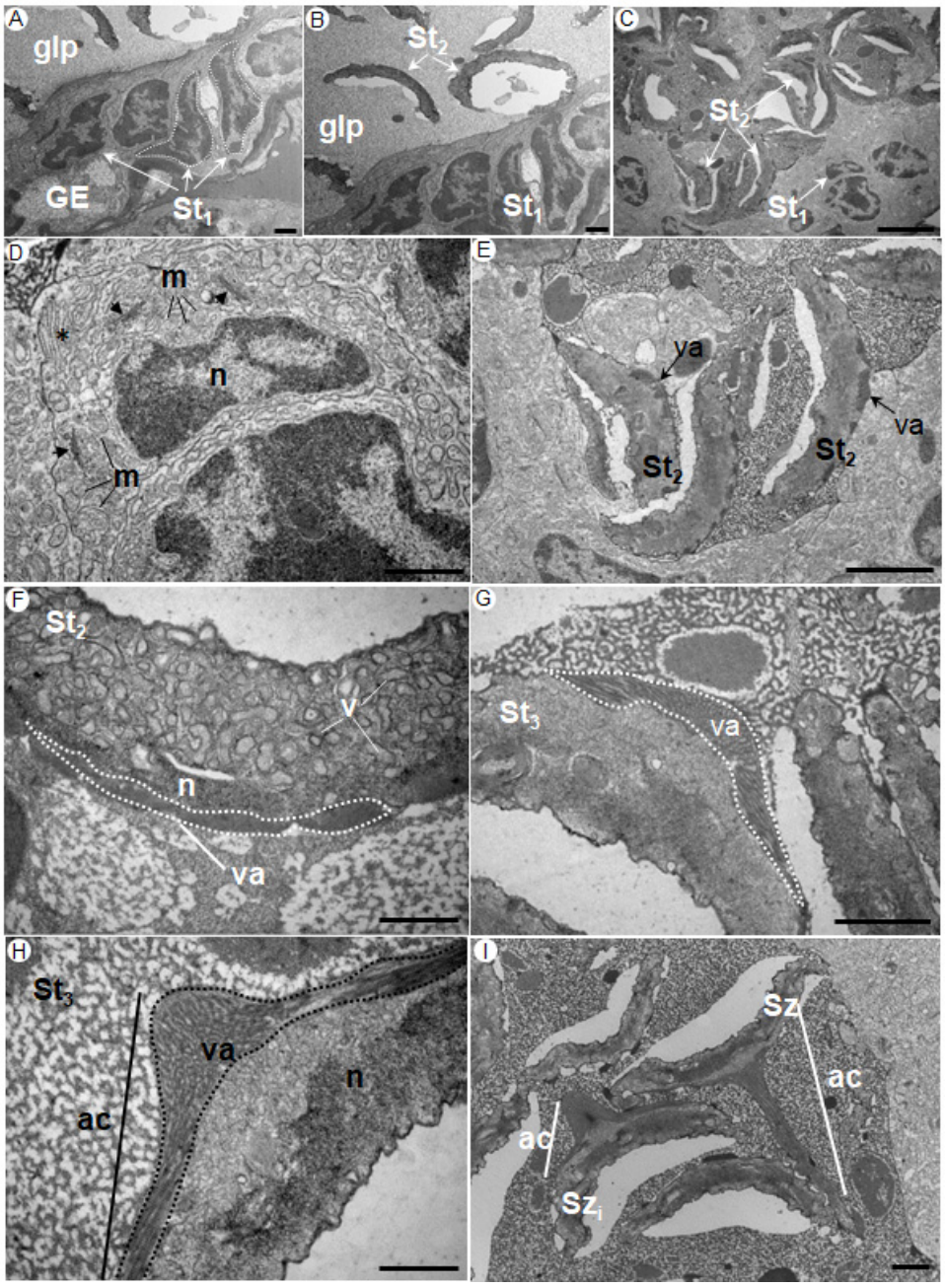

Fig. 5. TEM photomicrographs of the germinal epithelium and seminiferous tubules of *M. amazonicum.* (A-D) Spermatids 1 (St_1): cytoplasm with mitochondria, endoplasmic reticulum (*), Golgi apparatus (arrows) and vesicles; concave nucleus. (E-F) Spermatids 2 (St_2), sickle-shaped with scant cytoplasm and a concave, thin nucleus. (G and H) Spermatid 3 (St_3), cup-shaped; acrosomal vesicle undergoes significant changes giving rise to spike. (I) Immature spermatozoa (Sz_i) and mature Spermatozoa (Sz). av: acrosomal vesicle, ac: acrosomal complex, glp: glycoprotein, m: mitochondria, (n: nucleus, S: spike, Sz: spermatozoa. Scale bars = (F, G, H) 1 µm, (A, B, D, I) 2 µm, (E) 5 µm, (C) 10 µm.

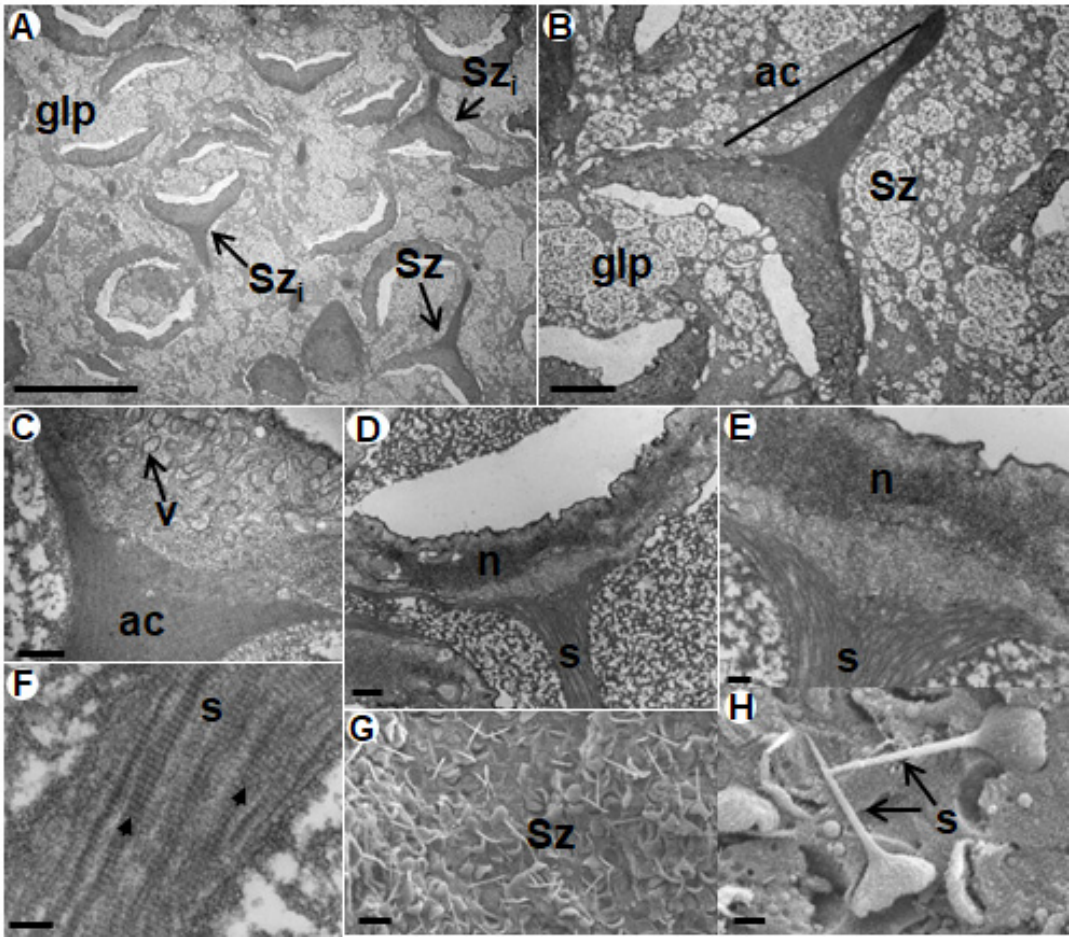

Fig. 6. TEM and SEM photomicrographs of the vasa deferentia of *M. amazonicum.* (A-H) Spermatozoa floating in glycoprotein in the vas deferens. (A) Immature spermatozoa (Sz_i) and mature spermatozoa (Sz). (B) Cup-shaped spermatozoa, nucleus with decondensed chromatin, (C-F) Details of spermatozoa showing nucleus with decondensed chromatin and presence of striations on the spike (thin arrows). (G-H) Cup-shaped spermatozoa with prominent spikes floating in glycoprotein in the vas deferens. av: acrosomal vesicle, ac: acrosomal complex, c: cytoplasm, glp: glycoprotein, n: nucleus, s: spike, Sz_i: immature spermatozoa, Sz: mature spermatozoa, v: vesicles. Scale bars = (A, G) 10 µm, (B, H) 2 µm, (C, D) 500 nm, (E, F) 200 nm, (G, H) 200 nm.

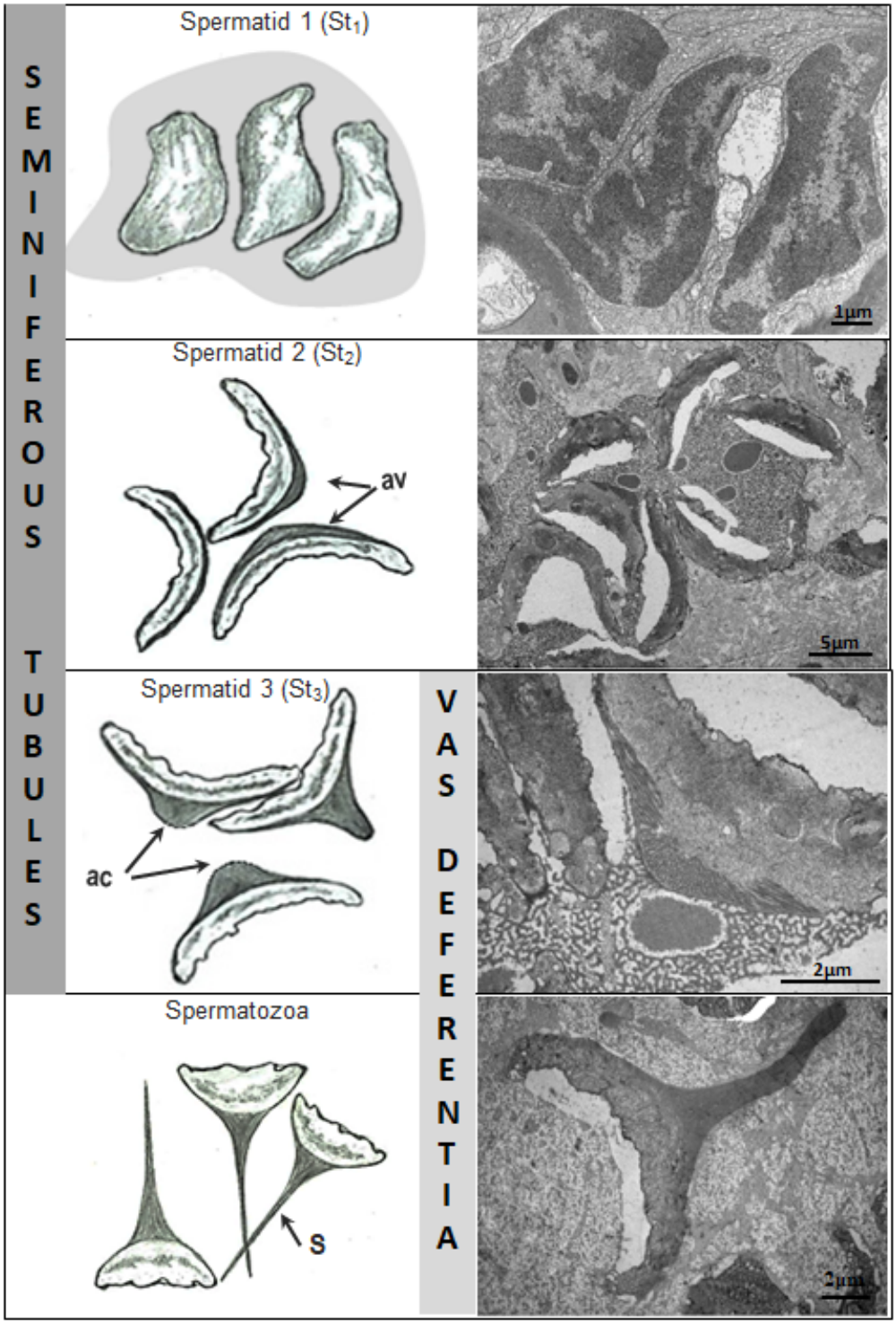

Fig.7. Illustration depicting stages of spermiogenesis in *M. amazonicum.* Spermatids 1 (St_1): with concave nucleus, condensed chromatin and scant cytoplasm located in the germinal epithelium. Spermatids 2 (St_2): individualized sickle-shaped with thin nucleus. Spermatids 3 (St_3): the most advanced stage, cup-shaped, with acrosomal vesicle and developing spike in the convex portion of the cell. Altogether, the acrosomal vesicle and spike formed the acrosomal complex. Subtypes St_2

and St_3 are located in the lumen of the seminiferous tubules and vasa deferentia, surrounded by PAS-positive material. Mature spermatozoa were cup-shaped with a long, single spike; located in the lumen of the vas deferens. av: acrosomal vesicle, ac: acrosomal complex, S: spike.

4. Discussion

The goal of this study was to detail and contrast differences in reproductive anatomy and sperm morphology among morphotypes of the freshwater prawn *M. amazonicum.* Macroscopically, the testes were similar to those in other prawns of the Palaemonidae family, such as *M. rosenbergii* (Chow et al., 1982) and *M. acanthurus* (Carvalho, 1980), as well as other peneideans (King, 1948; Eldred, 1958; Bell and Lightner, 1988). In *M. amazonicum*, differences in the size of the seminiferous tubules and of spermatogenic cells and in the frequency of cell types among morphotypes reflected specific characteristics related to sperm production efficiency. Previous studies suggested that the CC morphotype accumulated more nutrients than the others, likely in preparation for the reproductive period (Papa et al. 2004), facilitating energy mobilization for gonadal development (Sureshkumar and Kurup. 1999). This was supported here by the observation of a high frequency of germ cells in meiosis together with a relatively low number of spermatozoa in the CC morphotype. Conversely, a low frequency of spermatogonia and spermatocytes and large numbers of spermatozoa were observed in the GC morphotype, which is the dominant morphotype in this species and displays high reproductive activity. A similar pattern of germ cell frequency was also observed in the dominant morphotype (blue claw, BC) in adult males of *M. rosenbergii* (Okumura and Hara, 2004). Interestingly, the work by Moares-Riodades and Valenti (2004) showed a developmental progression among morphotypes from TC, to CC, to GC1/2 as the more mature individuals, which is in agreement with our findings in regards to sexual maturation and spermatogenesis. Previous studies also suggested that morphotypes represented individuals of similar age and that removal of the most mature morphotype within a population drove morphotype

progression through rapid growth (Kuris et al., 1987; Moares-Riodades and Valenti, 2004; Wortham and Van Maurik, 2012). Altogether, this leads us to hypothesize that more mature individuals may display behavioral mechanisms to suppress sexual maturation of less mature morphotypes, thus limiting competition for mating or other resources (Barki et al., 1992). Conversely, less mature morphotypes would be reproductively arrested and remain as a pool for future population replenishment. Further studies are needed to test these assumptions.

Based upon the histologic and ultrastructural analysis of the *M. amazonicum* gonad we have now a better understanding of its structure and cellular organization. In this regard, following examination of many testis sections in this study, we observed interconnections among seminiferous tubules that may suggest a syncytial structure. Herein, we did observe an intimate relationship among spermatogenic cells of *M. amazonicum,* supporting the presence of intercellular bridges as described in others species (Arsenault et al., 1980; Shigekawa and Clark, 1986).

Microscopically, the wall of the seminiferous tubules of the different *M. amazonicum* morphotypes contained the germinal epithelium as described in Palaemonidae (Papathanassiou and King, 1984; Poljaroen et al., 2010) and Penaedae (You-Hou et al., 2010). Spermatogonia of *M. amazonicum* displayed morphological characteristics similar to those observed in other species (Zhao et al., 1997; Poljaroen et al., 2010; Cobos et al., 2011; Feng et al., 2017). Moreover, the lower frequency of spermatogonia in the GC morphotype, in favor of more advanced germ cell stages, supports the notion that the most mature individuals are highly invested in reproductive activity. More advanced germ cell stages were characterized by the condensation of nuclear chromatin, formation of synaptonemal complexes, and disappearance of the nuclear membrane corroborating studies on *Enoplometopus occidentalis* (Haley, 1984), *M. rosenbergii* (Poljaroen et al., 2010), *Hipolito inermis* (Cobos et al., 2011), *Litopenaeus schmitti* (Fransozo et al., 2016), *Litopenaeus vannamei* (Alfaro-Montoya et at., 2016) and *Penaeus monodon* (Feng et al., 2017).

Notably, we established for the first time three spermatid subtypes, namely St_1, St_2, and St_3. While St_1 and St_2 displayed a similar size, St_1 were irregularly shaped and located in the germinal epithelium, whereas St_2 were sickle-shaped and located in the lumen of the seminiferous tubules. Importantly, the St_2 subtype was surrounded by PAS-positive material, suggesting that these glycoproteins support their final maturation. Spermatids matured as they advanced within the lumen of the seminiferous tubules ultimately becoming St_3, which were cup-shaped and fashioned an acrosomal complex. This contrasts with that previously reported in other species. For instance, in *M. rosenbergii* spermatids were limited only to the germinative epithelium (Chow et al., 1982; Lynn and Clark, 1983b, Poljaroen et al., 2010). To our knowledge, the observation of spermatids in the lumen of the seminiferous tubule has not been reported in any other species of the Palaemonidae family.

In this study we also detailed acrosomal vesicle formation in *M. amazonicum,* which occurred in the St_2 subtype. The acrosomal vesicle was shown to derive from the rearrangement of cytoplasmic organelles in other prawn species such as *Palaemon serratus* (Papathanassiou and King, 1984), *Sicyonia typica* and *Sicyonia dorsalis* (Camargo et al., 2015), *Litopenaeus vannamei* (You-Hou et al., 2010; Alfaro-Montoya et al., 2016), *Litopenaeus schmitti* (Fransozo et al., 2016), and *Panaeus monodon* (Feng et al., 2017). Attending to some reports, cytoskeletal rearrangement driven by actin filament organization guided the formation of the acrosomal vesicle (Sun et al., 2011; Hou and Yang, 2013; Wei and Yang, 2018). Moroever, we believe that in *M. amazonicum,* the cytoskeleton played a fundamental role in driving acrosomal complex formation. Once the spike displayed regular and compact cross-striations, the germ cell could be considered a mature spermatozoon.

The mature spermatozoon of *M. amazonicum* was cup-shaped, with very scant cytoplasm, condensed chromatin, and a long single spike. Similar sperm morphology was also reported in other

Palaemonidae family members including, *Palaemon elegans* (Pochon-Masson, 1969), *Palaemonetes paludosus* (Koehtler, 1979), *M. acanthurus* (Carvalho, 1980), *M. rosenbergii* (Chow et al., 1982), and *P. serratus* (Papathanassiou and King, 1984), as well as in Penaeidae family members including, *Penaeus aztecus* (Clark Jr. et al., 1973). We observed that in spermatozoa of *M. amazonicum* the spike derived from a condensed structure originating in the posterior and convex portion of the St_3. This acrosomal origin of the spike was also reported in other prawn species (Medina et al., 2006; Braga et al., 2013; Hou and Yang, 2013; Camargo et al., 2015; Fransozo et al., 2016). Moreover, in mature spermatozoa of *M. amazonicum* the spike was long and featured regular and compact cross-striations, similar to that reported in *M. rosenbergii* (Poljaroen et al., 2010). A spike organized in two different density layers of tubular-like structures was also observed in mature sperm from *Pandalopsis japonica* (Kim et al., 2003). In addition, it was reported that a spike with cross-striated appearance and radial fibrils could perforate the oocyte chorion during fertilization in *M. rosenbergii* (Lynn and Clark, 1983). Therefore, we propose that *M. amazonicum* sperm must be classified as natantian given the presence of an acrosome and long spike, structures that are likely involved in facilitating the fertilization process.

In the present study, somatic cells were observed in the germinal epithelium of *M. amazonicum*. Previous reports regarding gonadal organization in invertebrates also described other cell types beside germ cells. These cells were characterized by abundant mitochondria and endoplasmic reticulum in their cytoplasm, consistent with a supporting/nurturing cell role. Therefore, these cells have been termed intragonadal somatic cells (Franco et al., 2011), accessory, or Sertoli cells (Fransozo et al., 2016). Given their characteristics and arrangement, we believe that these cells are involved in the production of substrates responsible for the support, protection, nutrition, and maturation of spermatogenic cells. Thus, these supporting cells are the equivalent to Sertoli cells in vertebrates (Schulz et al., 2010; Hai et al., 2014).

Interestingly, we observed that both the lumen of the seminiferous tubules and vasa deferentia of *M. amazonicum* were filled with PAS-positive material. A secreting epithelium has been reported lining the vasa deferentia of *Panulirus penicillatus* (Matthews, 1951) and in other decapoda such as *Carcinus maenas* (Spalding, 1942), *Portunus sanguinolentus* (George, 1965) and *Sicyonia ingentis* (Subramoniam, 1995). Previous reports suggested that glycosaminoglycans within the vasa deferentia could play a role in maintaining sperm viability and hastening sperm maturation, in addition to providing antimicrobial activity (Subramoniam, 1991; Fransozo et al., 2016). In our study spermatid maturation occurred through transit within the lumen of the seminiferous tubules and vasa deferentia. Since both places were involved in secreting a glycoprotein-rich PAS-positive substance, we believe this provided an environment crucial in supporting differentiation and maturation of spermatozoa.

5. Conclusion

In this study we established differences in germ cell frequency and size within the seminiferous tubules, defining spermiogenesis stages in the different morphotypes of M. amazonicum. Notably, we characterized spermiogenesis with the novel description of three spermatid types. From these, the two more advanced stages are located and become spermatozoa within the lumen of the seminiferous tubules, rather than the germinal epithelium. Then, final sperm maturation occurs within the lumen of the vas deferens. Altogether, this information contributes to expanding our knowledge on taxonomy, phylogenetic relationships, and reproduction among Palaemonidae species.

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CHAPTER II

Is the hydrological cycle a limiting factor for the life-history traits of a freshwater prawn?

Gicelle M. F. Silva, Marcelo C. Andrade, Breno R. M. Silva, Ingrid S. Palheta, Yanne A. Mendes, Liziane B. Gonçalves, Rossineide M. Rocha and Maria A. P. Ferreira .

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Is the hydrological cycle a limiting factor for the life-history traits of a freshwater prawn?

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Abstract

1. In recent years, the species richness and diversity found in aquatic ecosystems has been declining as environments have become increasingly impacted. In freshwater systems, the hydrological cycle plays a key role in the evolutionary adaptation of species. In this context, crustaceans have an enhanced capacity to adapt and survival in distinct habitats.
2. The present study investigated the effects of the hydrological cycle and environmental conditions on the life-history traits of crustacean populations in distinct aquatic systems in eastern Brazilian Amazonia (river and estuary). A model species was used to explain the ecological factors that determine the susceptibility of crustacean populations.
3. The study found that (1) the hydrological cycle has a strong influence on the development of the Amazon freshwater prawn; (2) Crustacean populations from the Amazon estuary are longer and heavier than those from the river during the different periods of the hydrological cycle; (3) Crustacean populations are female-dominated, principally in the fluvial

environment; (4) there is significant variation in the weight-length ratios and condition factor; (5) negative allometric growth predominates in both populations.

4. The relative frequency of occurrence of the female maturation stages and male morphotypes in the two populations is related systematically to the abiotic parameters of the two environments.
5. In these two distinct aquatic systems, the abiotic parameters determined by the hydrological cycle have a profound influence on the development of the crustacean, despite its ecological plasticity. Overall, then, the study shows that the hydrological cycle plays a fundamental role in the determination of the life-history traits of *M. amazonicum* in distinct aquatic systems.

Keywords: Freshwater, Estuary, reproduction, abiotic factors

Introduction

In recent years, the species richness and diversity found in aquatic ecosystems has been declining as the impacts on these environments have grown (Pringle, 2001; Dudgeon *et al.*, 2006; Hughes, 2015). One prominent example of this type of impact is the impoundment of rivers, which leads to the formation of very deep artificial habitats that generally support an increase in primary productivity, a proliferation of floating macrophytes, a high availability of feeding resources, an abundance of aquatic fauna, and the transformation of lotic systems into lentic environments (Kubecka, 1993; Agostinho *et al.*, 1999, 2016; Von Sperling, 2012). These environments are characterized by thermal stratification, a reduction in dissolved oxygen concentrations in the deeper strata, the dispersal of species (Agostinho, Pelicice & Gomes, 2008; Wang *et al.*, 2013), and major alterations in the quality of the water (Manyari & de Carvalho, 2007; Cunha-santino, Bitar & Jr, 2013; Wohl, Lane & Wilcox, 2015). By contrast, estuaries represent natural environments that are

rich in sediments, nutrients, organic material, and have a marked gradient of salinity generated by the proximity of the sea (Boto & Wellignton, 1984; Mckenney Jr, 1996), which creates an enormous diversity of resources that supports the reproduction and growth of aquatic species (Telesh & Khlebovich, 2010).

In both types of environment, hydrological cycles may interfere in the life cycles of aquatic species, although there tend to be few data on the exact influence of this cycle as a factor limiting the potential for the survival or adaptation of the local species. In this case, it is important to remember that niche theory determines that environmental conditions and local variations associated with the intrinsic characteristics of a species will determine its adaptation to the environment. These conditions are related to the microhabitats, abiotic factors, resources, and predators, which may all be essential for the physiological and behavioral adaptation of the species (Grinnell, 1917). Based on this theory, the species composition of local assemblages should be determined by the environmental traits that will filter out the species capable of establishing local populations (Hutchinson, 1957). From this perspective, the physical and chemical characteristics of the environment may act as important factors determining the development of the strategies of reproduction, growth, and maintenance of the local species (Kubecka, 1993).

Freshwater prawn are among the aquatic organisms most capable of adapting their morphophysiological and behavioral characteristics for survival in a heterogeneous environment. Populations of crustaceans found in estuarine environments influenced by seawater, are larger and more fecund, whereas populations in rivers, which are not influenced by saline waters, are characterized by low fecundity and complete their whole life cycle in freshwater (Collart & Rabelo, 1996; Moraes-Riodades & Valenti, 2004). The Amazon River prawn, *Macrobrachium amazonicum* (Heller, 1862), is a valuable crustacean model that presents major variation in its body size and life cycle (Vergamini, Pileggi & Manelatto, 2011). The need to understand the ecological factors that

determine the susceptibility of crustacean populations in distinct environments has provided the inspiration for a number of studies (Anger, 2003; Rocha & Barbosa, 2017; Pantaleão *et al.*, 2018).

In this context, the biometric and reproductive features that consolidate the life history traits of a population are fundamental to the diagnosis of the growth patterns and life cycle of the species in that environment (Abohweyere & Williams, 2008; Deekae & Abowei, 2010). Data on the interference of the environment and the hydrological cycle on the life cycle of the animal and/or its life history traits provide an important tool for the understanding of the survival dynamics of the species in different aquatic systems. Based on these data, we tested the hypothesis that the hydrological cycle influences the characteristics of aquatic systems and the life cycle of crustacean populations. To test this hypothesis, the present study investigated how the hydrological cycle of two distinct aquatic systems (river and estuary) influence environmental conditions and the local populations of *M. amazonicum* in eastern Brazilian Amazonia.

Material and Methods

Study area

The data were collected every two months between June 2017 and May 2018 in two different aquatic systems in the eastern Amazon region of northern Brazil (Figure 1): **site I:** An estuary (01˚04’17.3” S, 48˚18’36.3” W) approximately 19 km long, with a mean depth of 6-7 m; **site II:** A river (03˚48’22.9” S, 49˚44’01.3” W), an area of approximately 2,917 km^2 and maximum depths of between 58 m and 74 m (Fearnside, 2015). Both sites have a hot, humid tropical climate.

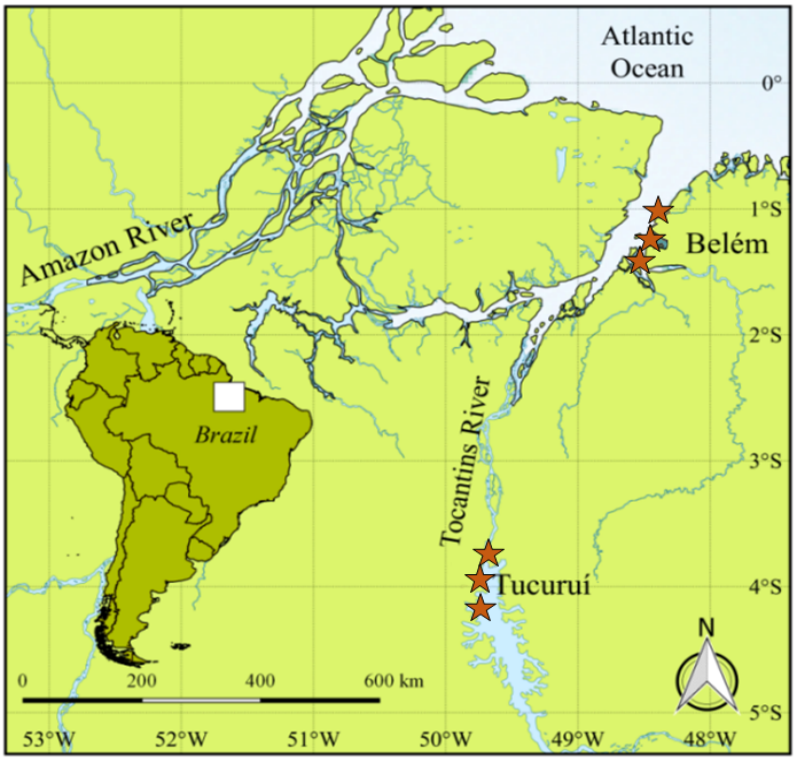

Figure 1. Location of the *Macrobrachium amazonicum* sampling points at the two study sites in the state of Pará, northern Brazil. Site I: The Furo das Marinhas Estuary in Belém. Site II: Tocantins River upstream from the Tucuruí hydroelectric dam.

Data collection

The abiotic characteristics (temperature, salinity, pH, turbidity, and dissolved oxygen concentration) of each site were measured *in situ* using a Horiba U-50 multiparameter quality checker during each field excursion. The precipitation data were obtained from the database of the Brazilian National Meteorological Institute (INMET, 2017). The hydrological cycle was composed of four distinct periods: rainy-dry (June-August), dry (September-November), dry-rainy (December-February), and rainy (March-May).

Capture of the specimens

Three sampling points were established at each site for the collection of *M. amazonicum* specimens. At each point, 10 wooden shrimp traps, known locally as "matapis" were used to collect the specimens, grated babaçu (*Orbignya speciosa*) fruit pulp was used to bait the traps at each sampling point (Simonian, 2006). The traps were set at a depth of 1-2 m for a standard period of 12 hours at both sites. Once collected, the specimens were transported to the laboratory, where they were identified, based on the taxonomic reference of Holthuis (1952) and sexed (Moraes-Riodades & Valenti, 2004). The total length (TL) of each specimen was measured (in centimeters), its total mass (TM) was determined (in grams), and the gonads were removed and fixed in Bouin solution, for 24 hours.

Light microscopy

Once fixed, the gonads were processed histologically for embedding in paraffin (Prophet *et al.*, 1995) for the extraction of a series of 5 μm sections, which were stained with hematoxylin and eosin before being analyzed and photographed under an Eclipse Ci-S light microscope attached to a Nikon S-Ri1 (Japan) digital camera. The ovarian stages of the females were classified according to the shape, coloration, and histology of the ovaries, based on the scheme of Chaves & Magalhães (1993) and Ferreira *et al.* (2012). In the case of the males, morphotypes were established based on the classifications of Moraes-Riodades & Valenti (2004) and Silva et al. (2009), which were modified for the present study.

Data analysis

An Analysis of Covariance (ANCOVA) was used to evaluate the variation in body mass recorded between sites and among periods of the hydrological cycle, relating the body mass

(dependent variable) and the sites and hydrological periods (independent variables), with the total length, dissolved oxygen, temperature, and precipitation as covariables.

Deviations in the sex ratio recorded at each site and during each period of the hydrological cycle were evaluated using Chi-square ($\alpha = 0.05$), based on Zar (1996). Differences in the mean body length and total mass between sexes, sites, and hydrological periods were tested using Student's *t*.

The body mass-length ratio was obtained using an equation adapted from the model proposed by Huxley (1924), that is, $TM = a*TL^b$, where TM is the total mass of the specimen, *a* is the coefficient of proportionality, TL is the total length of the specimen, and *b* is the allometric coefficient. In this analysis a value of *b* equal to 3 corresponds to isometric growth, where the body length of the animal increases in direct proportion to its mass. The mass-length ratios were compared between males and females in the same aquatic system using an ANCOVA, and between aquatic systems for the same sex. The condition factor (K) was calculated by $K = TM/TL^b$, where *b* is the coefficient of allometry determined *a priori* separately for each sex and site, and compared with a one-way Analysis of Variance (ANOVA), followed by Tukey's *post hoc* test (Zar, 1999).

The normality of the distribution of the data was evaluated using the Shapiro-Wilk test, and the homogeneity of variances was verified by Levene's test. The significance level for all analyses was established as $\alpha = 0.05$. All the analyses were run in the R program, version R 3.4.4 (Team R Core, 2018).

Results

The abiotic factors that varied most significantly during the hydrological cycle (Kruskal-Wallis, $p < 0.001$) at both sites I and II were dissolved oxygen, precipitation and temperature. However, no significant variation (Kruskal-Wallis, $p > 0.05$) was recorded in temperature at site I when comparing the dry-rainy/rainy period with the rainy-dry/dry period.

A similar lack of variation was recorded at site II for dissolved oxygen and precipitation, while temperature did not vary significantly between the dry-rainy/rainy periods (Figure 2). The factors associated with the hydrological period had a significant (ANCOVA, $p < 0.001$) influence on the body mass of the animals. As a whole, the variables were responsible for 96% of the log body mass of the *M. amazonicum* populations (Table 1).

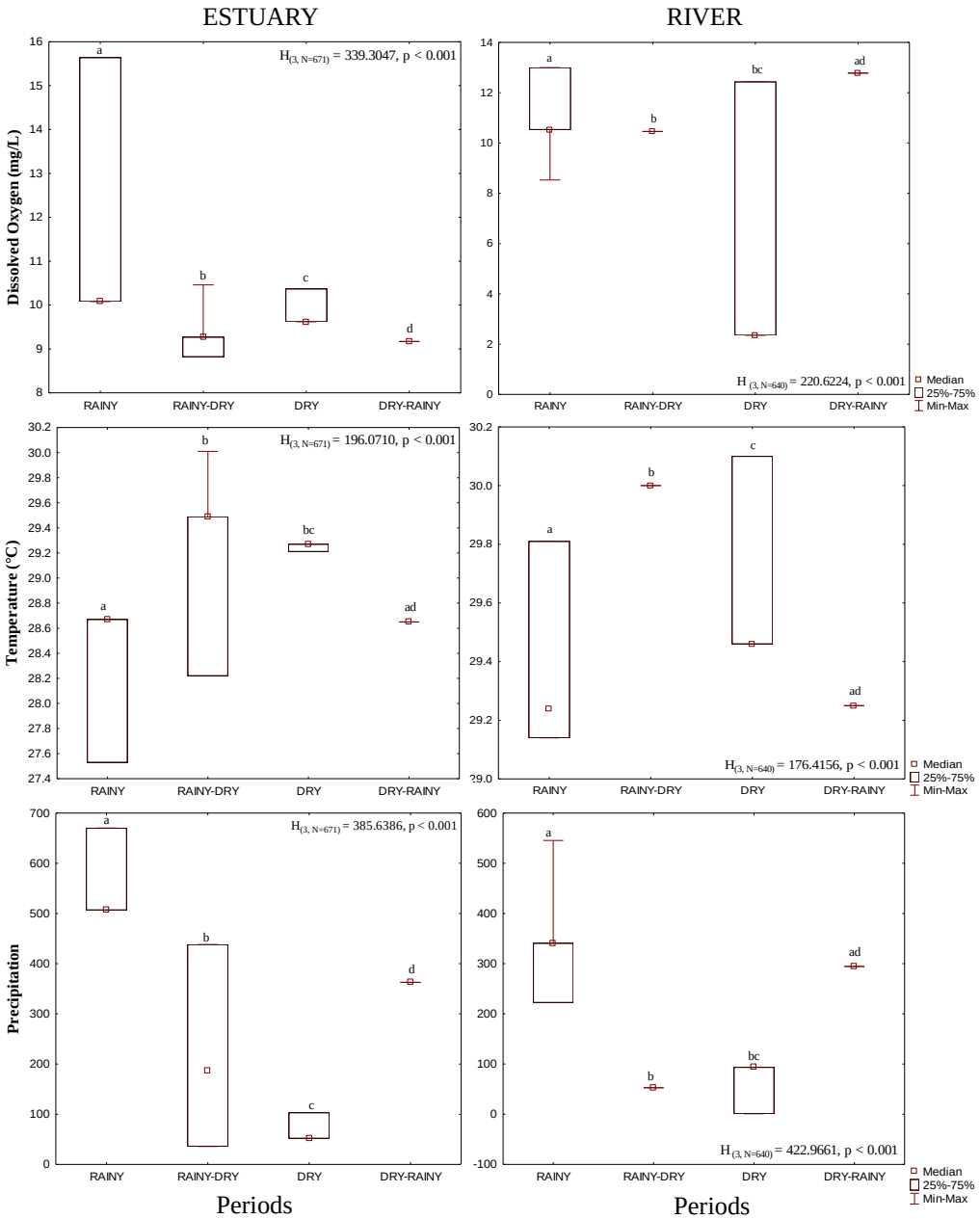

Figure 2. Mean±standard deviation, and the minimum and maximum values recorded for each environmental variable: dissolved oxygen, water temperature and precipitation at site I and site II. Denoted by different letters on the top of the bars. Significance of 5%.

Table 1. Covariance analysis (ANCOVA) of the relationship between total weight (g) and length (TL), site, period and abiotic factors for *Macrobrachium amazonicum*.

Relationship	Sum of Squares	df	Mean Square	F-ratio	*P*
Total lenght	192.482	1	192.482	7345.556	0.000*
Site	1.307	1	1.307	49.882	0.000*
Period	2.342	3	0.781	29.795	0.000*
Dissolved oxygen	0.123	1	0.123	4.686	0.031*
Temperature	0.568	1	0.568	21.673	0.000*
Precipitation	1952	1	1.952	74.511	0.000*
Site*Period	506	3	0.169	6.437	0.000*
Error	33.908	1294	0.026		

* = statistically significant values

A total of 1,311 *M. amazonicum* specimens were collected, 671 at site I (233 males and 438 females) and 640 at site II (63 males and 577 females). Most specimens at site I were collected during the rainy-dry and dry periods, whereas at site II, specimens were more abundant during the dry and rainy periods. A female-biased sex ratio was recorded at both sites during all periods, except the rainy-dry period at site I (Table 2).

Table 2. Number of females (F) and males (M) of *Macrobrachium amazonicum* at site I and site II, sex ratio, chi-square value (X^2). Statistically significance at 0.05 level.

SITE	SEASON	F	M	♂:♀	X^2	*p-value*
I	Rainy	84	35	1:2.4	20.2	**<0.001**
	Rainy-Dry	132	103	1:1.3	3.6	0.058
	Dry	145	61	1:2.4	34.3	**<0.001**
	Dry-Rainy	77	34	1:2.3	16.7	**<0.001**
II	Rainy	306	44	1:7.0	196.1	**<0.001**
	Rainy-Dry	30	5	1:6.0	17.9	**<0.001**
	Dry	148	9	1:16.4	123.1	**<0.001**
	Dry-Rainy	93	5	1:18.6	79	**<0.001**

At site I, the total length of the females ranged from 4.1 cm to 12.2 cm, and their total mass from 0.4 g to 12.4 g, whereas in the males, total length ranged from 5.0 cm to 13.5 cm, and total mass from 0.7 g to 18.5 g. At site II, the total length of the females was 3.0-7.0 cm and the total mass 0.3-2.7 g, while the males were 3.5-7.0 cm long, with a mass of 0.4-2.8 g (Table 3).

Table 3. Total length (TL) in centimeters, and weight (W) in grams for females (F) and males (M) *Macrobrachium amazonicum* at site I and II, among periods. SD = standard deviation.

SITE	PERIOD	Sex	TL range	TL mean±SD	W range	W mean±SD
I	Rainy-Dry	F	4.1–12.2	8.7±1.3	0.4–12.4	5.3±2.4
		M	5.0–13.5	8.8±1.9	0.7–18.5	5.3±4.1
	Dry	F	5.0–11.1	7.8±1.2	1.2–10.0	3.9±1.7
		M	5.5–11.7	7.4±1.1	1.3–11.3	3.2±1.8
	Dry-Rainy	F	5.5–10.6	7.7±1.1	1.1–8.0	3.8±1.7
		M	5.7–13.0	8.7±1.9	1.2–14.5	5.6±3.8
	Rainy	F	6.4–11.2	8.4±0.9	2.1–10.0	4.8±1.5
		M	6.2–13.5	9.0±1.6	1.8–18.5	5.8±3.6
II	Rainy-Dry	F	4.5–7.0	5.6±0.7	0.9–2.7	1.6±0.5
		M	6.0–6.8	6.3±0.3	1.3–2.5	1.7±0.5
	Dry	F	3.0–6.7	4.6±0.7	0.3–2.2	0.9±0.4
		M	3.5–7.0	5.0±1.1	0.4–2.2	0.9±0.6
	Dry-Rainy	F	3.5–6.5	4.7±0.6	0.4–1.9	0.8±0.3
		M	4.5–6.5	5.5±0.8	0.4–2.1	1.2±0.6
	Rainy	F	4.0–7.0	5.4±0.5	0.4–2.6	1.3±0.4
		M	4.5–7.0	5.7±0.7	0.4–2.8	1.5±0.6

Males were significantly longer (Student's *t*, $p < 0.05$) than females at site I in all hydrological periods except the ebb. At site II, however, a significant difference was recorded only during the rainy-dry period. Male body mass was significantly greater (Student's *t*, $p < 0.05$) than

that of the females at site I at dry and rainy-dry period, whereas no significant variation in body mass was recorded between the sexes at site II (Figure 3).

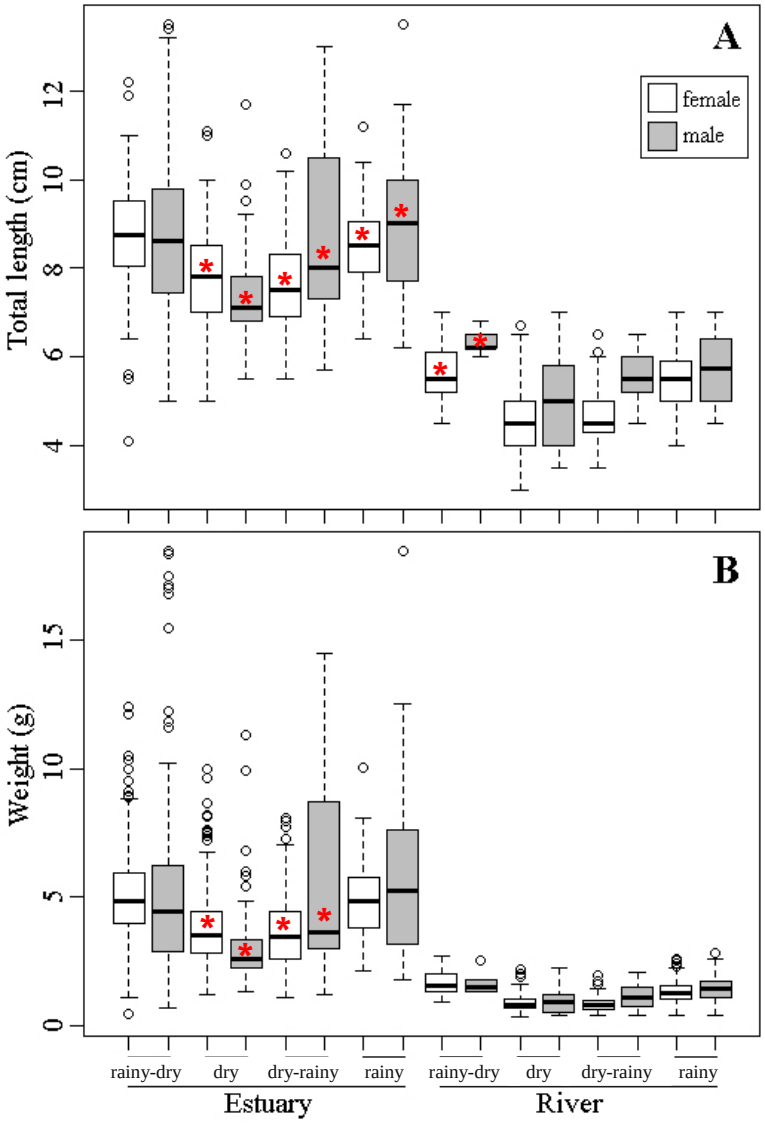

Figure 3. Characteristics of the male and female *Macrobrachium amazonicum* at sites I (estuary) and II (river) during hydrological periods, state of Pará, northern Brazil. (A) Total length, and (B) Total weigth. (*) Significant difference (5% significance level).

No significant variation (ANCOVA, $p = 0.48$) was found in the body mass/length ratio of the males at sites I and II, although the variation was significant (ANCOVA, $p < 0.001$) when the females were compared between sites, and all individuals (males and females) were combined for

analysis (Table 4). In all cases, the value of the *b* coefficient was less than 3, which indicates negative allometric growth (Table 4).

At site I, the condition factor (K) was 0.754 in the males and 1.228 in the females, whereas at site II, it was 0.681 in the males and 2.096 in the females (Table 4). The K values were significantly larger (ANOVA, $p < 001$) in the females at both sites in comparison with the males.

Table 4. Relationship Weight-length and condition factor (K) for females (F) and males (M) of the *Macrobrachium amazonicum* at site I (Estuary) and site II (River), N=1311.

SITE	SEX	*a*	95% CL (a)	*b*	95% CL (b)	r^2	*K*
I	F	0.0122	0.0102-0.0145	2.7818	2.6978-2.8658	0.907	1.228
	M	0.0075	0.0063-0.0089	2.9606	2.8773-3.0439	0.956	0.754
II	F	0.0207	0.0178-0.0241	2.4219	2.3293-2.5146	0.824	2.096
	M	0.0083	0.0051-0.0136	2.910	2.6249-3.1942	0.906	0.681

The relative frequency of occurrence of the different female maturation stages and the male morphotypes was related primarily to precipitation and turbidity in both environments. Mature female *M. amazonicum* were present in all the periods of the hydrological cycle at both sites. At site I, however, the occurrence of maturing and mature females varied according to the increase in precipitation and turbidity recorded during the dry-rainy and rainy periods. At site II, maturing and mature females were observed when turbidity was greatest during the rainy-dry period (Figure 4). In case of the males, at site I, the TC, CC, and GC morphotypes were observed during all the periods, although the dominant GC morphotype was less frequent during the dry period. The TC morphotype predominated during all periods at site II, while the GC morphotype was completely absent (Figure 4).

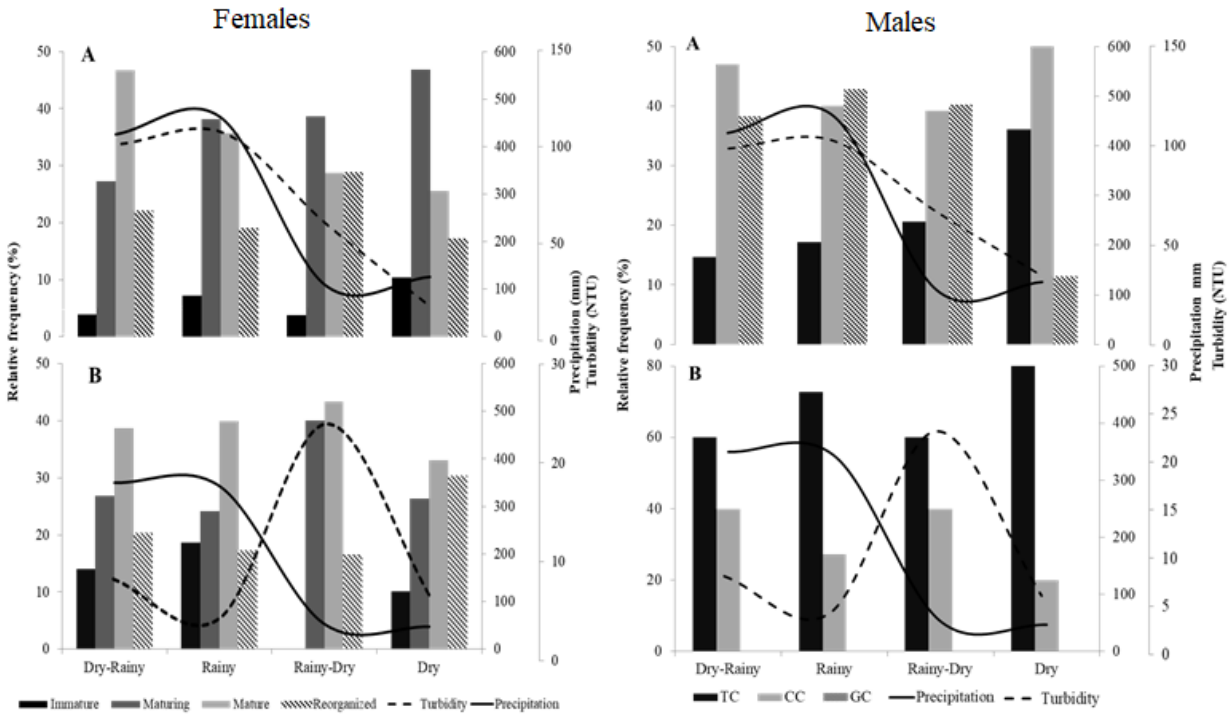

Figure 4. Relative frequency of occurrence (%) of the maturation stages females and morphotypes males *Macrobrachium amazonicum* at sites I (A) and II (B).

Discussion

In aquatic systems, abiotic factors have a fundamental influence on the life cycle of animal populations, in terms of their ecological, physiological, morphological, genetic, and reproductive characteristics (Augusto *et al.*, 2007; Maciel & Valenti, 2009; Vergamini *et al.*, 2011; Pantaleão, Hirose & Costa, 2012, 2014). In the two environments analyzed in the present study, however, one characterized by varying salinity (site I) and the other by freshwater (site II), these factors were clearly influenced by the hydrological cycle, which determines the prevailing conditions in the environment. The present study demonstrated that the growth and reproduction of the local populations of *M. amazonicum* in the estuary and the river varied considerably among the different hydrological periods. At both sites, dissolved oxygen concentrations, temperature, and precipitation

varied significantly. Precipitation is the primary determinant of nutrient concentrations, and the availability of ions and organic material (Maier, 1978).

At site I, higher precipitation rates coincided with higher frequencies of maturing and mature prawn, a pattern that is likely to interfere with the reproductive performance of these crustaceans (Costa & Negreiros-Fransozo, 1998; Costa, Negreiros-fransozo & Mar, 2013; Meireles, Valenti & Mantelatto, 2013). During the rainy season, the high concentrations of nutrients and particulate and dissolved organic matter contributed to an increase in turbidity (Tripathi & Pandey, 2014; Moura & Nunes, 2016). At site II, by contrast, precipitation and turbidity were inversely proportional, with the highest frequency of the reproductive stages of *M. amazonicum* occurring during periods of greater turbidity. This indicates that, in the river environment, which has deeper waters, the reduction in the volume of water that occurs during the rainy-dry period may result in a concentration of nutrient levels, which would, in turn, increase the availability of resources for the crustaceans. In this case, the availability of nutrients and other local factors may determine reproductive parameters (Pantaleão *et al.*, 2018).

Females predominated in the *M. amazonicum* populations at both sites throughout almost all of the study period. A female-biased sex ratio is common in crustaceans, given the importance of the females for the recruitment process, especially in populations that breed continuously (Mossolin & Bueno, 2002; Sampaio *et al.*, 2007; Silva, Frédou & Filho, 2007; Magalhães, Mossolin & Mantelatto, 2012; Castelo-Branco *et al.*, 2015; Mendes *et al.*, 2017). Even so, the males were larger than the females, which may reflect dominance behavior and territoriality in the population, which favor larger individuals (Barki, Karplus & Goren, 1992; Silva & de Fátima Arruda, 2015). The larger size of the crustacean males in comparison with the females probably confers greater reproductive success and provides advantages during intraspecific competition (Gherardi & Micheli, 1989; Andrade *et al.*, 2013; Alkalay *et al.*, 2014).

The *M. amazonicum* from site I were both longer and heavier than those at site II, although at site II, males and females only differed significantly in size during the rainy-dry period. Despite certain differences, the populations from sites I and II are part of the same monophyletic clade, and belong to a single species (Mishler & Theriot, 2000; Vergamini *et al.*, 2011). A wide range of factors may nevertheless contribute to variation in the characteristics of local populations, including the influence of coastal dynamics and the hydrogeographic characteristics of each locality, including the riparian vegetation and water quality (Telesh & Khlebovich, 2010; Barros-Alves *et al.*, 2012; Meireles *et al.*, 2013). In this context, deep water environments may be characterized by thermal stratification, a reduction in the availability of refuges where marginal vegetation has been lost, a decline in organic matter, low fertility, and an increase in predation (Agostinho *et al.*, 2008; Wang *et al.*, 2013). From this perspective, it seems likely that the animals at site II faced limitations of resources that demanded greater energetic investment in growth.

At both sites, however, the animals presented negative allometric growth in both sexes. Similar findings have been obtained in other studies of crustaceans (Freire, Marques & Silva, 2012), and contrast with the data on *M. rosenbergii*, which presented positive allometric growth in captivity (Sampaio & Valenti, 1996) and isometric growth in its natural environment (Kunda *et al.*, 2008). In peneid prawns, variation in the mass-length ratio are common between the sexes, and among habitats and seasons (Fontaine & Neal, 1971; Primavera, Parado-Estepa & Lebata, 1998; Kuris *et al.*, 2006), which indicates a systematic relationship between habitat and growth patterns in these crustaceans. The negative allometry observed in *M. amazonicum* may be associated with the cycle of gonadal maturation (Freire *et al.*, 2012). Even so, while the prawn from site I were larger and heavier, overall, only the females from site II had high K values, which may reflect greater investment in reproduction.

At site I, the mature females and all the male morphotypes were observed in all the hydrological periods, although peaks of maturation and reproduction were recorded during the dry-

rainy/rainy periods, when precipitation and turbidity increased. Previous studies have shown that the ovarian maturation of freshwater decapods is typically associated with the rainy season (Oh *et al.*, 2003). At site II, mature females were also common in all the hydrological periods, but were associated primarily with peaks of turbidity during the rainy-dry period, related to the greater concentrations of suspended organic matter in the water in this environment.

In freshwater decapod crustaceans, ovarian maturation is stimulated by environmental parameters in the natural habitat, and is closely associated with rainy periods (Oh *et al.*, 2003). In freshwater palaemonid prawns, by contrast, variation in the morphotypes of males occupying the same ecological niche has been described in *M. amazonicum* (Moraes-Riodades & Valenti, 2004; Silva *et al.*, 2009), *M. rosenbergii* (Ra'anan & Sagi, 1985; Kuris *et al.*, 2006), *M. dayanum* (Langer, 2004) and *M. grandimanus* (Wortham & Maurik, 2012), 2012). In the present study, while all the different morphotypes were recorded at site I (estuary), the GC morphotype was absent from site II (river). The individuals may not necessarily pass through all the different phases, however, and the transition from one morphotype to another may occur either through a single molt or a more gradual process (Moraes-Riodades & Valenti, 2004). It seems likely that the conditions found at site II are unfavorable to the investment in molting from one morphotype to another and, in this case, the males may tend to remain in the TC morphotype to guarantee reproductive success in the local environment.

Overall, then, the results of the present study have shown that hydrological cycle is characterized by distinct abiotic factors in the different environments. These factors interfer in the dynamics of the tolerance and survival of the crustacean under varying environmental conditions, which may determine shifts in the life-history traits of the species that are fundamental for the understanding of population-level processes.

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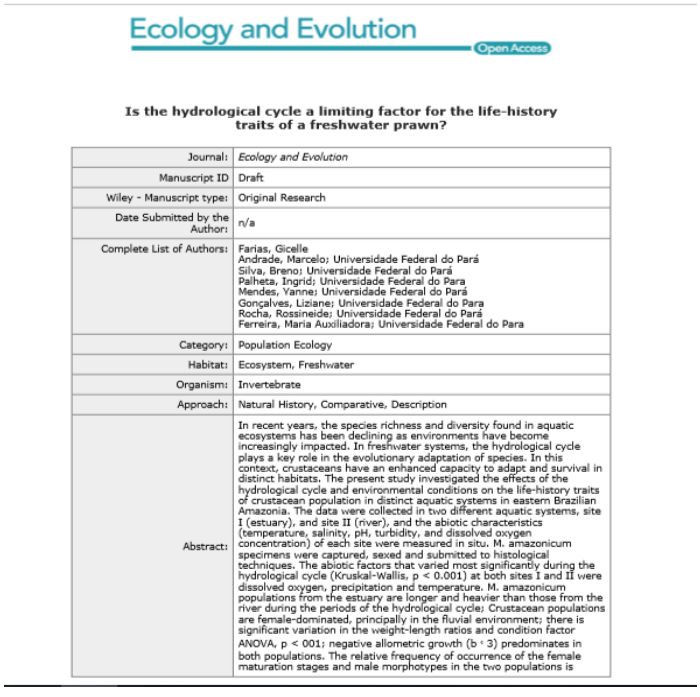
ANEXO I



Ecology and Evolution Open Access

Is the hydrological cycle a limiting factor for the life-history traits of a freshwater prawn?

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Category:	Population Ecology
Habitat:	Ecosystem, Freshwater
Organism:	Invertebrate
Approach:	Natural History, Comparative, Description
Abstract:	In recent years, the species richness and diversity found in aquatic ecosystems has been declining as environments have become increasingly impacted. In freshwater systems, the hydrological cycle plays a key role in the evolutionary adaptation of species. In this context, crustaceans have an enhanced capacity to adapt and survival in distinct habitats. The present study investigated the effects of the hydrological cycle and environmental conditions on the life-history traits of crustacean population in distinct aquatic systems in eastern Brazilian Amazonia. The data were collected in two different aquatic systems, site I (estuary), and site II (river), and the abiotic characteristics (temperature, salinity, pH, turbidity, and dissolved oxygen concentration) of each site were measured in situ. M. amazonicum specimens were captured, sexed and submitted to histological techniques. The abiotic factors that varied most significantly during the hydrological cycle (Kruskal-Wallis, $p < 0.001$) at both sites I and II were dissolved oxygen, precipitation and temperature. M. amazonicum populations from the estuary are longer and heavier than those from the river during the periods of the hydrological cycle; Crustacean populations are female-dominated, principally in the fluvial environment; there is significant variation in the weight-length ratios and condition factor ANOVA, $p < 001$; negative allometric growth ($b < 3$) predominates in both populations. The relative frequency of occurrence of the female maturation stages and male morphotypes in the two populations is

CHAPTER III

Energy allocation trade-off in *Macrobrachium amazonicum*, with no resting stage

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Energy allocation trade-off in *Macrobrachium amazonicum*, with no resting stage

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Abstract

Animals may employ a trade-off strategy to maintain themselves when environmental factors limit reproduction and growth. In this study, we investigated a trade-off strategy in populations of the prawn *Macrobrachium amazonicum* in estuarine and river environments. Abiotic factors and environmental characteristics were evaluated during a hydrological cycle. Precipitation, turbidity and dissolved oxygen showed differences between the estuary and river, assessed through PCA. Larger numbers of specimens were observed in the estuary. Based on biometric and histological features, females in the river population were smaller, and the maturing, mature and reorganized ovarian stages were frequent and occurred simultaneously with embryonic development, which suggests a high energy allocation to reproduction. In males, the GC morphotype was absent in the river, where the TC morphotype predominated and showed similar reproductive conditions to the GC morphotype in the estuary. As the populations of *M. amazonicum* did not have a resting stage, we suggest that trade-offs occur as a survival strategy. This is the first description of a trade-off model in studies of the reproductive ecology of decapod populations in different environments.

Introduction

Trade-off is a strategy of allocation or a negative relationship between two characteristics in an organism's life history, in which the amount of a resource allocated to one process increases and becomes unavailable for other processes [1]. In the evolutionary context, organisms have interrelated morphological, physiological, and behavioral characteristics, which, depending on biotic or abiotic conditions, may or may not be helpful [2,3]. Thus, the investment of energy in a given function involves a trade-off hierarchy, in which the total amount of energy initially available is dedicated to one function, and later, energy will be distributed to other functions or to the individual as a whole [4–6].

Reproduction and growth are considered antagonistic and competing processes because each activity requires a large amount of energy [7]. In crustaceans as in other animals, this energy is obtained from the environment, which has a strong influence on the ability to survive, grow, and reproduce [8–10]. For example, in ecological interactions, one important factor is the size of an organism, which is closely associated with reproductive maturation and offspring investment [1,11–13]; and food availability, which alters the reproductive behavior and various aspects of a species' life cycle [8,14,15]. With respect to reproductive effort, species can use trade-offs as a survival strategy [16–18].

The river prawn *Macrobrachium amazonicum* (Heller, 1862) is one of the most widespread species in neotropical water bodies. Its morphology, growth, and reproductive traits such as male morphotypes and ovarian maturation vary in different populations according to environmental factors [19–22]. This prawn shows ecological plasticity, but it is not clear when individuals change their energy allocation during the life cycle [20].

We hypothesized that the reproductive strategies of this prawn are environmentally dependent, and that in unfavorable conditions their fitness changes and the species would show

trade-offs in energy allocation. In this study, we investigated the presence of trade-offs between growth and reproduction in populations of *M. amazonicum* in aquatic systems with different environmental characteristics.

Materials and Methods

1- Study area

The specimens were collected bimonthly from June 2017 through May 2018, in two different aquatic environments: the Amazon estuary at Mosqueiro Island (01°04'17.3"S 048°18'36.3"W), and the Tocantins River upstream of the Tucuruí Hydroelectric Power Plant (UHE) (03°48'22.9"S 049°44'01.3"W), both in the eastern Amazon basin, northern Brazil. Abiotic characteristics (temperature, salinity, pH, turbidity, and dissolved oxygen concentration) at each site were measured *in situ*, using a Horiba U-50 multiparameter probe, during each field excursion. The precipitation data were obtained from the database of the Brazilian National Meteorological Institute (INMET, 2017). Water-level data were obtained from the National Water Agency (ANA) database.

2- *Capture of specimens*

Three sampling points were established at each site for collection of *M. amazonicum* specimens. At each point, 10 wooden shrimp traps, known locally as **"**matapis**"**, were set, baited with grated fruit pulp from the oil palm or babaçu (*Orbignya speciosa*) [23]. The traps were set at a depth of 1–2 m for 12 h at each site. Captured specimens were transported to the laboratory and identified by specific literature [24]. The prawns were sexed by observing the appendix on the second pair of pleopods in males, which is absent in females [25]. The total length (TL) of each specimen was measured (in centimeters), its total mass ™ was determined (in grams), and the gonads were removed and fixed in Bouin's solution for 24 h.

3. Light microscopy

Fixed gonads were processed histologically for embedding in paraffin [Prophet *et al.*, 1995] to cut a series of 5-μm sections. Sections were stained with hematoxylin and eosin before being analyzed and photographed under an Eclipse Ci-S light microscope fitted with a Nikon S-Ri1 digital camera (Nikon, Japan). The ovarian stages of females were classified according to the shape, coloration, and histology of the ovaries, based on the scheme of Ferreira *et al.* [2012]. Male morphotypes were established using a classification adapted from appropriate literature [25,26].

4. *Analysis of the relationship between embryonic phases and ovarian maturation*

Ovigerous females were collected and the eggs were isolated and immersed in a 5% sodium hypochlorite solution for 3 min to remove the gelatinous layer and chorion. Then, they were washed in PBS, fixed in Karnovsky's solution for 24 h, and stored in 70% ethanol for analysis and documentation with the aid of a Nikon Eclipse Ci-E light microscope fitted with a DS-Ir1 digital camera (Nikon, Japan). The embryonic phases were defined according to appropriate literature [27].

5. Data analysis

To analyze the environmental variation in each study area, a Principal Components Analysis (PCA) was performed [28]. Because the environmental data were collected in differents units of measurement, the variables were standardized.

A two-way variance analysis (ANOVA) was performed to detect differences in the IGS of ovigerous females and the morphotypes and condition factor in males from the estuary and river, followed by Tukey's *post-hoc* test [29]. To detect differences between the biometric parameters and reproductive profile of ovigerous females from the estuary and river, we performed the Mann-Whitney U test. The normality of the data distribution was evaluated using the Shapiro-Wilk test,

and the homogeneity of variances by the Levene test. The significance level for all analyses was set at $\alpha = 0.05$. All analyses were run in the R program, version R 3.4.4 [30] and Microsoft Office.

Results

A total of 1311 specimens were analyzed, including 433 females and 238 males from the estuary and 576 females and 64 males from the river (data summarized in Table 1).

Table 1 – Numbers of specimens of *Macrobrachium amazonicum* from the estuary and the river.

Study site	Male		Ovigerous		Non-ovigerous		Total	
	N	**%**	**N**	**%**	**N**	**%**	**N**	**%**
River	64	10	263	41.09	313	48.91	**640**	**100**
Estuary	238	35.47	184	27.42	249	37.11	**671**	**100**

Differences between the environmental variables in the estuary and river were analyzed by PCA. The first two axes explained 71.67% of this variation. Axis 1 explained 41.08% of the variation; turbidity, temperature, and precipitation contributed most to its formation. Axis 2 explained 30.59% of the remaining variation; pH contributed most to its formation. Turbidity, temperature, and precipitation were higher in the estuary, while pH was higher in the river (Fig 1).

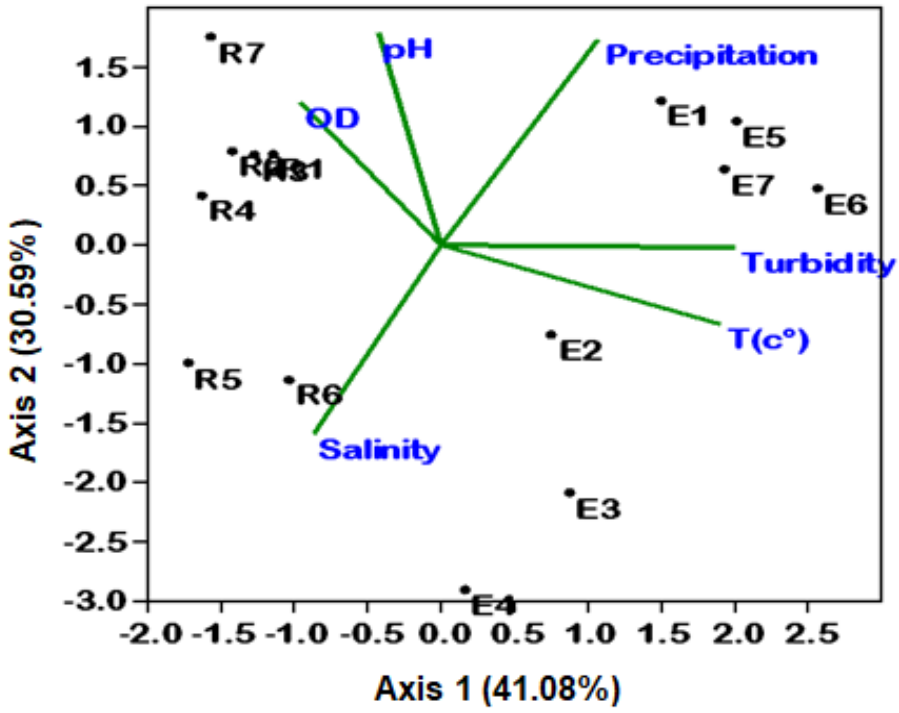

Fig 1. Environmental variation ordered according to the estuary and river environments by Principal Components Analysis (PCA).

The estuary and the river environments differed in water level, turbidity, and environmental heterogeneity. The estuary water level ranged from 6.2 to 6.5 m, with turbidity of 39.9 to 117.0, and extensive shoreline vegetation. The river had quite different characteristics, including low turbidity of 3.5 to 24.4 and water level from 64 to 75 m, with higher water transparency and sparse vegetation along the banks (Fig 2).

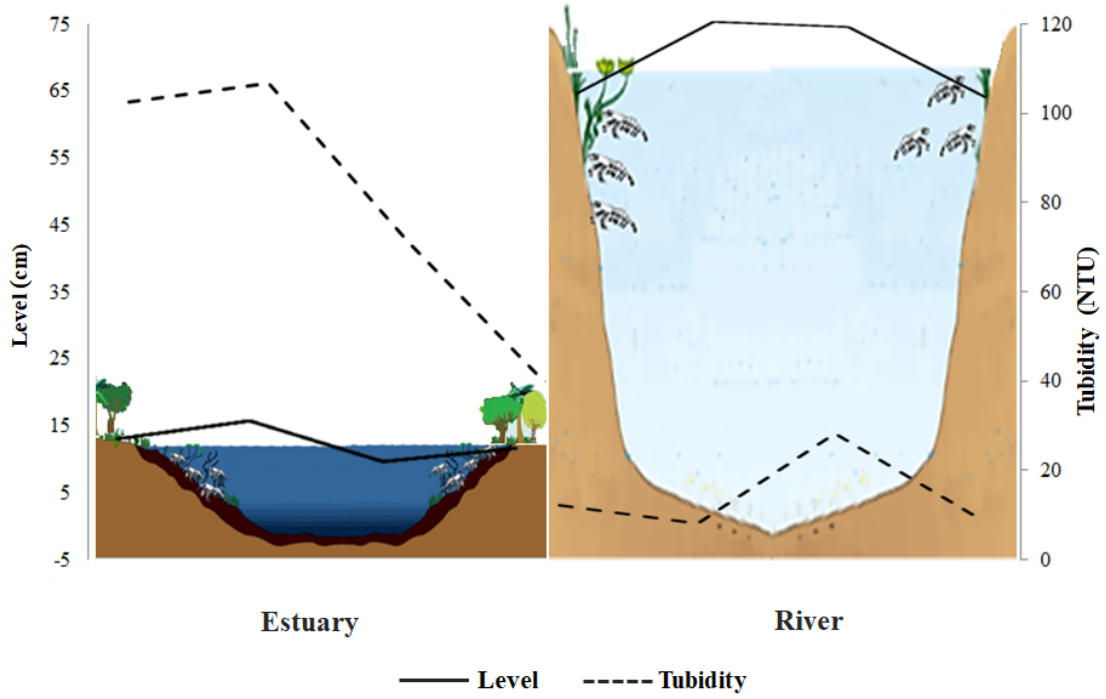

Fig 2. Schematic drawing showing the differences between the estuary and river ecosystems: depth, water transparency, turbidity, and shoreline vegetation.

The ovarian stages were similar in prawns from the two environments. Based on the coloration, the chromatophores present in the dorsal region, and the oocyte organization, three stages were determined: reorganized, maturing, and mature. Ovarian maturation occurred simultaneously with embryonic development in the incubator cavity. In the reorganized phase, the ovary appeared opaque and flaccid, with some chromatophores, empty follicles, atretic oocytes, and nests of oogonia forming the cell proliferation zone (Fig 3A and 3D). In the maturing phase, the ovary ranged from whitish to greenish, with evident chromatophores; a predominance of early vitellogenic oocytes, characterized by basophil cytoplasm and vesicles on the cell periphery; and advanced vitellogenic oocytes that contained acidophilic cytoplasm with yolk granules (Fig. 3B and 3E). In the mature phase, the ovaries were olive-green with many chromatophores and contained

vitellogenic oocytes with large amounts of yolk (Fig 3C and 3F). During the study period, the spawned phase was not observed in either environment. After they lay eggs, the females begin a new cycle of gonadal maturation.

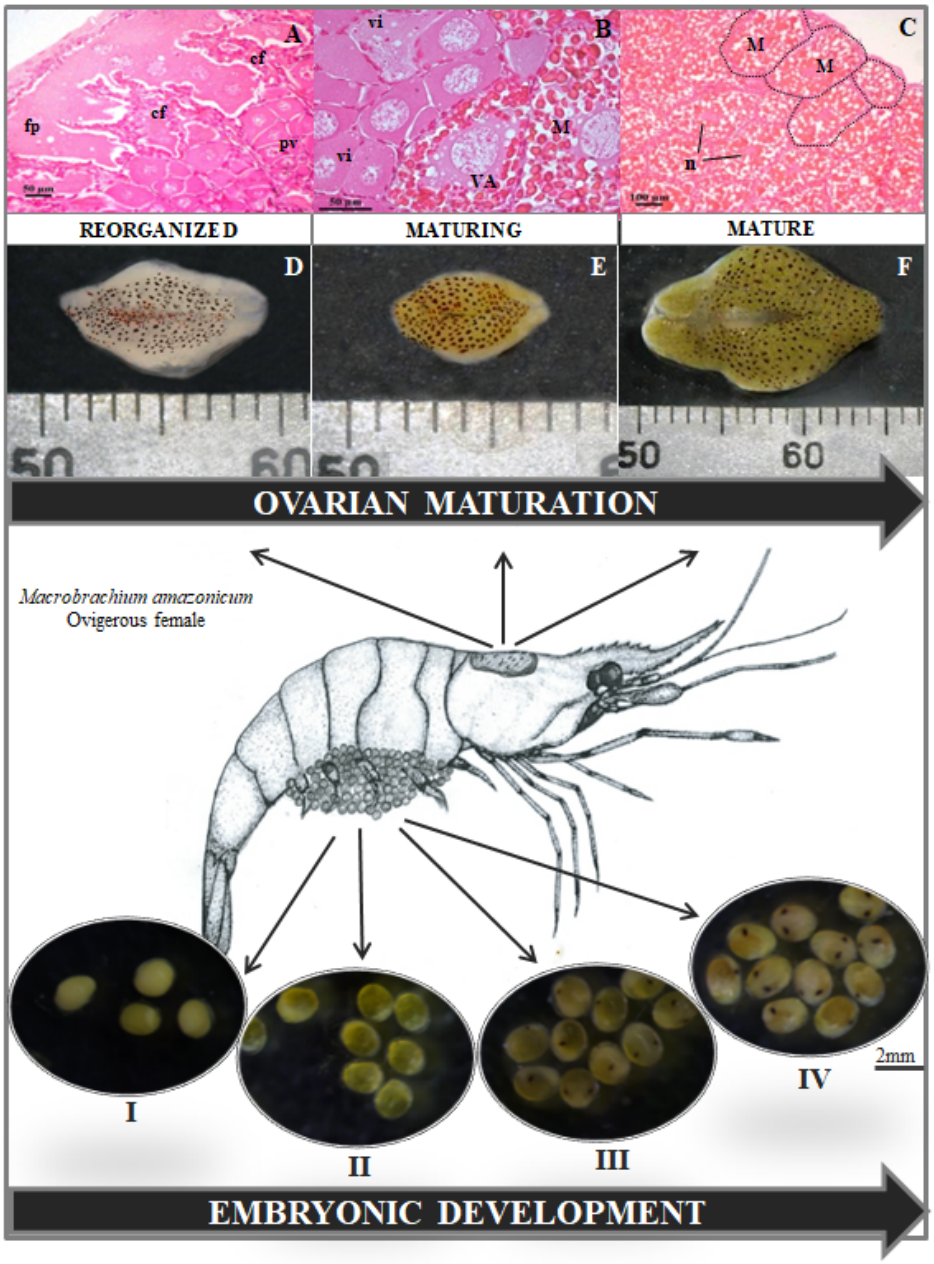

Fig 3. Photomicrographs of the relationship between ovarian and embryonic stages in ovigerous females of *Macrobrachium amazonicum*.

The relationship between the relative frequency of ovarian maturation stages and embryonic development in ovigerous females was demonstrated for both environments. Ovigerous females in the maturing and mature stages were found in higher frequency in the river, while females in the reorganized stage were found mainly in the estuary. Females were frequently found in the reorganized stage, with embryos in phase I; while females in the maturing and mature stages, with embryos in phases III and IV, respectively, were also observed (Figs 3 and 4).

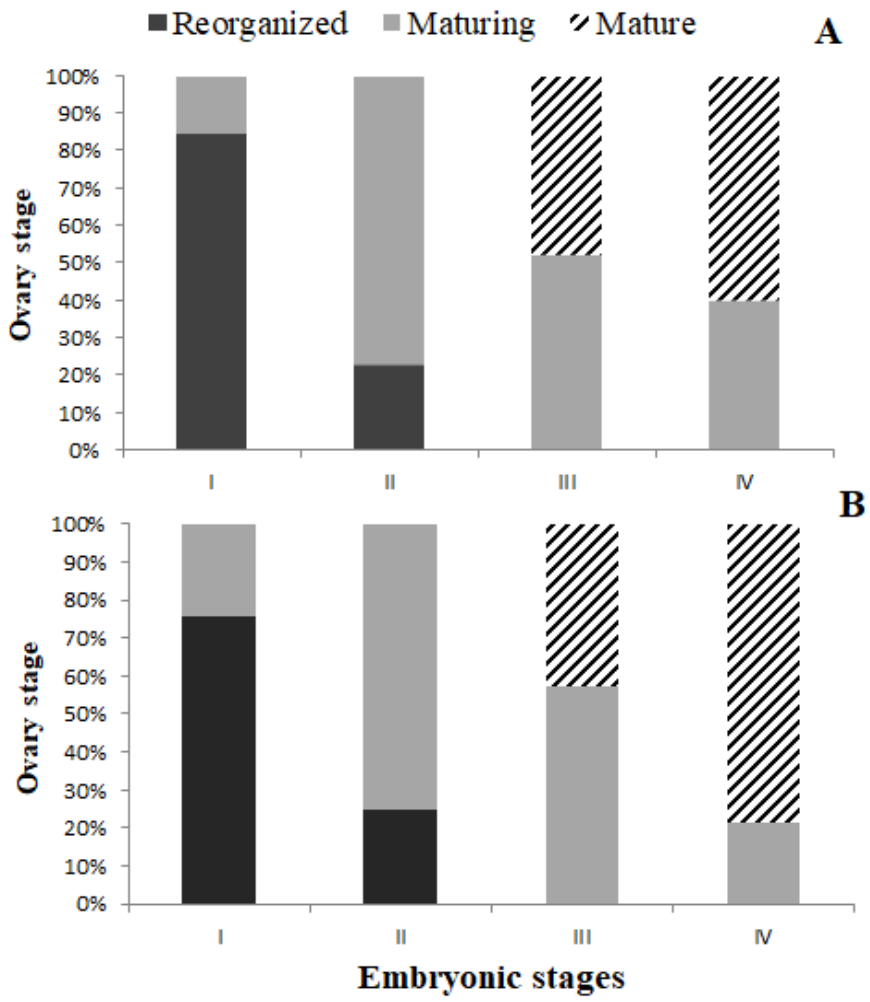

Fig 4. Relationship of percentage (%) of maturation stages of ovigerous females with embryonic stages of *Macrobrachium amazonicum* in the estuary (A) and river (B).

Based on biometric parameters (total length and weight and reproductive profile (ovarian weight and condition factor), ovigerous females from the estuary and river differed significantly (Mann-Whitney U test, $p < 0.001$), Table 2.

Table 2. Means (± standard deviation) of the biometric parameters and condition factors of ovigerous females of *Macrobrachium amazonicum* from the estuary and the river, 5% significance level.

Biometry	Estuary	River	p-valor
Total lenght (cm)	8.47 ± 3.9^{a}	5.23 ± 0.37^{b}	0.001
Total weight (g)	4.93 ± 1.8^{a}	1.31 ± 0.79^{b}	0.001
Ovarian weight (g)	0.073 ± 0.06^{a}	0.018 ± 0.01^{b}	0.001
Condition factor (K)	0.012 ± 0.001^{a}	0.023 ± 0.004^{b}	0.001

[a,b] Different superscripts within row denote significant differences between estuary and the river.

During egg development, the ovigerous females in the estuary and river showed significant differences in IGS (two-way ANOVA, $p < 0.001$). Females in the river showed higher IGS at the end of egg development than the IGS observed in females in the estuary (Fig 5).

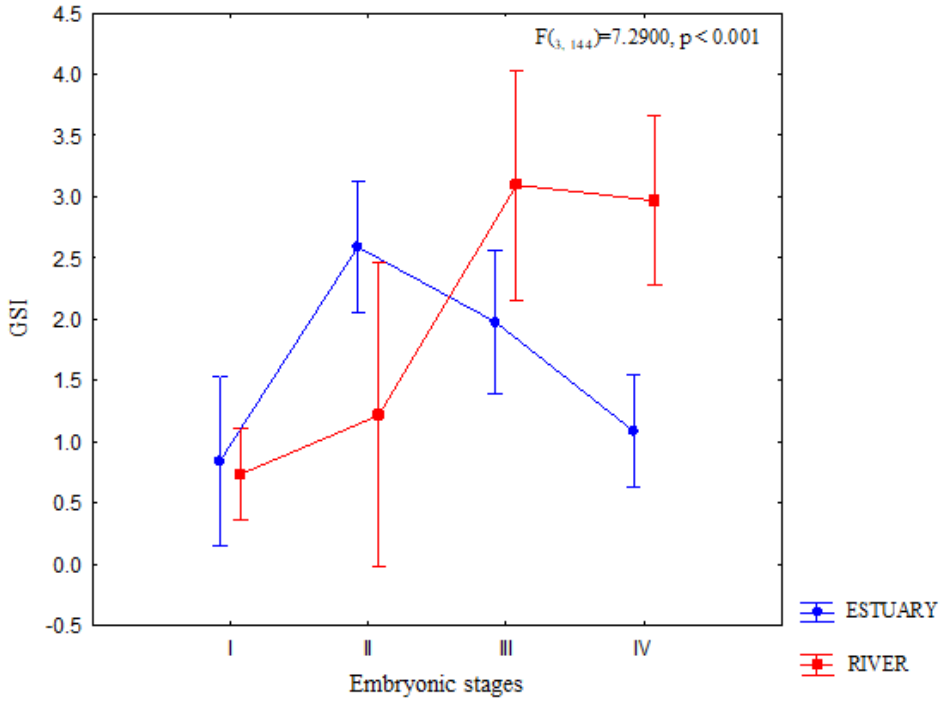

Fig 5. Gonadosomatic Index (IGS%) of ovigerous females of *Macrobrachium amazonicum* from the estuary and river environments, 5% significance level.

The relative frequency of all morphotypes of adult males was estimated for the estuary; only morphotypes TC and CC were found in the river (Fig 6). These morphotypes did not show significant differences between IGS% and condition factor K of the populations (two-way ANOVA, $p > 0.05$).

For both environments, morphotypes TC and GC showed testes formed of seminiferous tubules containing germinal epithelium with spermatogonia, spermatocytes, and sperm occupying the lumen of the tubule. In the CC morphotype, the germinal epithelium was smaller and the lumen contained cells in stages of division (Fig 6A – 6F).

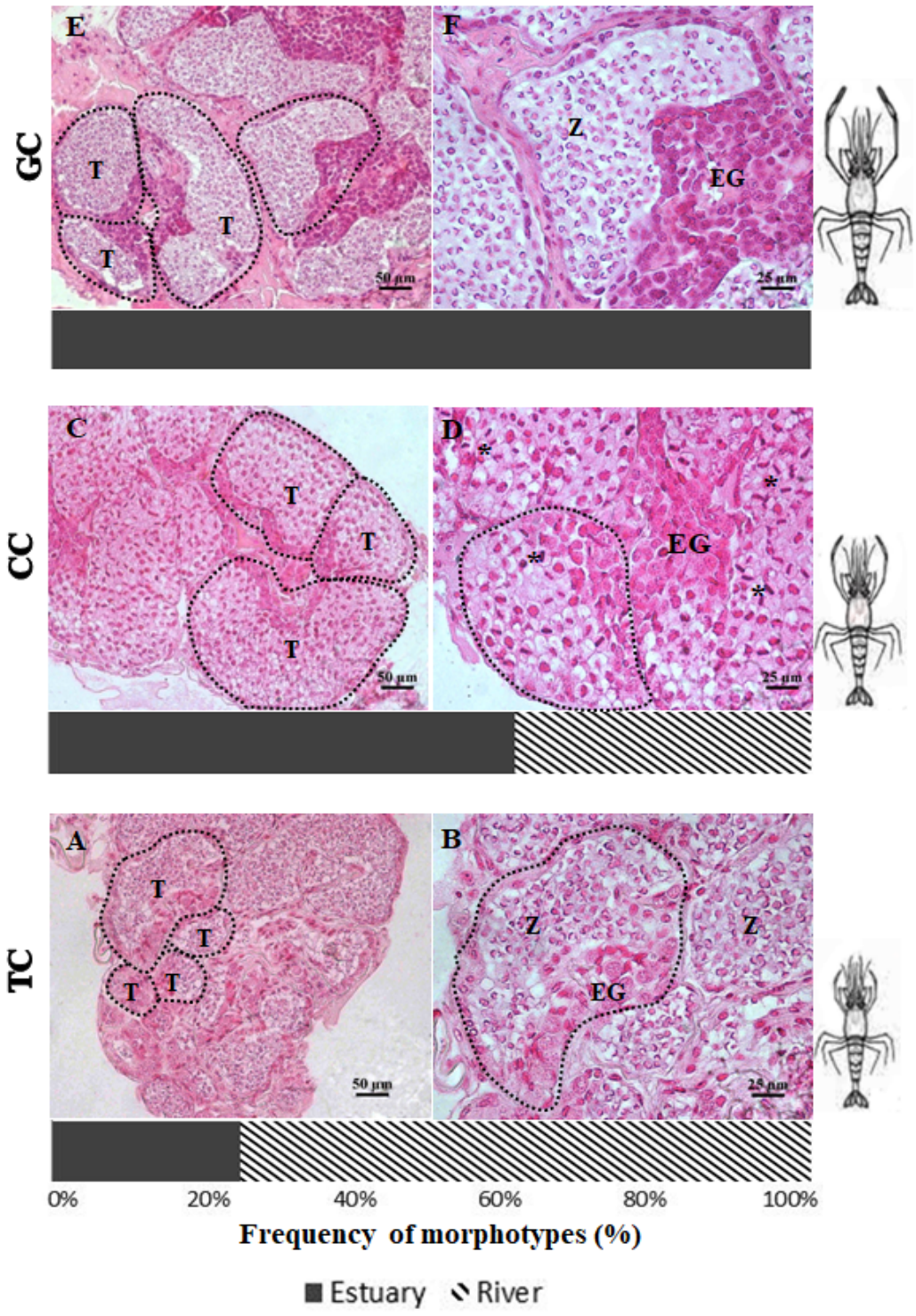

Fig 6. Photomicrograph and relative frequency of occurrence (%) of *Macrobrachium amazonicum* male morphotypes in the estuary and river environments. (A-B) TC morphotype, irregular seminiferous tubules containing germinal epithelium and sperm in lumen. (C-D) CC morphotype, dividing cells (*). (E-F) GC morphotype, predominance of sperm in the lumen. EG: Germinal epithelium; Z: Sperm; T: Seminiferous tubules.

Discussion

Crustacean populations show variations in reproduction and morphogenesis that are strongly influenced by environmental factors, resulting in adaptation to different environments and ecological plasticity [9,20,31,32]. An example observed here was the larger number of female specimens and smaller number of males of *M. amazonicum* in the river environment. This species tends to use reproductive strategies to maintain the population in the river, employing trade-offs [17]. Therefore, we believe that in the river environment, individuals exhibit their particular profile in response to the site, which does not interfere with their survival.

In our study we observed environmental heterogeneity, with differences between the estuary and river in several abiotic factors, particularly temperature, precipitation and turbidity. Environmental conditions profoundly impact the ecology of decapod populations [33–36]. We suggest that the environmental conditions are the main causes of the differences found in these *M. amazonicum* populations, and that they are related to reproductive and biometric aspects, but are independent of the hydrological period.

An important aspect of *M. amazonicum* females in both environments was the occurrence of the ovarian stage simultaneously with embryonic development, as well as the absence of the spawned stage. This situation differs from those described for various members of the genus *Macrobrachium* [37–39], but is similar to reports for other decapods [40,41]. In general, in crustaceans, females in the spawned stage correspond to an asynchronous phase, which is a period of energy recovery for the next reproductive cycle [42]. The relationship between the female's reproductive receptivity and the molting process is a common pattern, and females perform copulation within a short period after ecdysis [43–45]. It is possible that in river environments, where environmental conditions are different, these animals are allocating energy only for reproduction and do not have rest periods and/or invest in growth.

Ovigerous females in the two environments differed in some respects: females were larger in size in the estuary and smaller in the river, where they showed a higher IGS and K, at the end of egg development in the incubator cavity. Although smaller females incubate fewer eggs, this process occurs more often and over time their reproductive performance equals that of larger females [18,46–48]. It is possible that females in the river, even though they are smaller, reproduce continuously as a strategy to maintain the population in this environment.

Four male morphotypes were found in the estuary, but only TC and CC were found in the river. Our results contrast with those observed in the population in the hydroelectric reservoir, where all the morphotypes were found [49]. Morphotypes are an important factor in reproduction; the GC morphotype is the dominant male, responsible for the largest number of mated females [25]. However, we observed here that in the river environment the TC morphotype predominated, with no GC males. The similarity of the IGS and condition factor (K) between the TC males from the river and the GC males from the estuary suggests that these animals are in the same reproductive condition. Populations of this prawn in lentic environments have promiscuous mating systems, with little precopulatory interactions or inter-male agonistic behavior. As a result, males remain small and highly mobile, and do not need to grow or develop large chelipeds to fight other males and attract females [49–51]. These differences are closely related to the ecological characteristics of their environments.

A new reproductive profile was observed here in *M. amazonicum.* This observation helps to understand the morphological and reproductive plasticity in these populations in two environments, and that females and males develop strategies that allow them to perpetuate the species regardless of the conditions to which they are exposed.

Conclusion

This is the first time that a trade-off model has been described for studies on the reproductive ecology of decapod populations in different environments. *Macrobrachium amazonicum* may serve

as a model for studies on behavior and differences between decapod populations that perform a trade-off between growth and reproduction. These changes can be attributed to site characteristics: in the river, the females reproduce more continuously, and TC morphotype males show reproductive conditions similar to the dominant CG morphotypes males in the estuary.

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5- CONCLUSÃO GERAL

Em nossos estudos elucidamos aspectos morfológicos de *Macrobrachium amazonicum* através da análise do aparelho reprodutor dos morfotipos de machos e estabelecemos diferenças na frequência e tamanho das células germinativas nos túbulos seminíferos, definindo os estágios da espermiogênese. Notadamente, caracterizamos a espermiogênese com a nova caracterização de três tipos de espermátides e propomos uma nova descrição da espermiogênese nesse gênero.

Quanto aos aspectos ecológicos, nossos resultados mostraram que o ciclo hidrológico é um fator limitante para os traços da história de vida dessa espécie, logo os fatores abióticos distintos de cada ambiente interferem na dinâmica da tolerância e sobrevivência do animal, o que pode determinar variações entre as populações.

As variações entre as populações de *M. amazonicum* exibem variações quanto às características morfológicas e reprodutivas dependem do ambiente onde estão inseridas. Desta forma quando em condições desfavoráveis realiza trade-off para a sua sobrevivência no ambiente, momento em que a espécie apresenta mudanças na alocação de energia no seu ciclo de vida. No rio as fêmeas se reproduzem continuamente sem pausa para repouso, e os machos do morfotipo TC apresentam condições reprodutivas similares aos morfotipos dominante CG do estuário.

Nossos estudos demonstraram que *M. amazonicum* representa um modelo para estudos sobre a compreensão dos processos em nível populacional, do comportamento, da taxonomia e da reprodução em diferentes ecossistemas.

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Research paper

Morphometry, frequency and ultrastructure of male germ cells in morphotypes of the freshwater prawn *Macrobrachium amazonicum* (Decapoda: Palaemonidae)

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BRIEF COMMUNICATION

Reproductive characteristics of pike-characids *Boulengerella cuvieri* (Ctenoluciidae) in the middle Xingu River, Eastern Amazon

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