



Programa de Pós-Graduação
Ecologia Aquática
e Pesca



**UNIVERSIDADE FEDERAL DO PARÁ
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
NÚCLEO DE ECOLOGIA AQUÁTICA E PESCA DA AMAZÔNIA
PROGRAMA DE PÓS-GRADUAÇÃO EM ECOLOGIA AQUÁTICA E PESCA**

IVANA KERLY DA SILVA VIANA



**DESCRIÇÃO DA ESTRUTURA TESTICULAR E DO COMPLEXO FOLICULAR NOS
OÓCITOS DE PEIXES SILURIFORMES DA REGIÃO AMAZÔNICA**



**BELÉM, PARÁ
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Tese de Doutorado apresentado ao Programa de Pós-Graduação em Ecologia Aquática e Pesca da Universidade Federal do Pará, como requisito para obtenção do título de Doutora em Ecologia Aquática e Pesca.

Orientadora: Dr^a. Rossineide Martins Rocha

Co- Orientadora: Dr^a. Maria Auxiliadora Pantoja Ferreira

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ÓOCITO EM PEIXES SILURIFORMES DA REGIÃO AMAZÔNICA**

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eu mesma não acreditava.

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*“Não importa o quão poderoso você se torne
nunca tente fazer tudo sozinho, caso contrário
irá falhar”
Uchiha Itachi, Naruto*

RESUMO

Conhecer a morfologia das células da linhagem germinativa em peixes teleósteos é o primeiro passo para estabelecer diversas relações entre as espécies e as estratégias reprodutivas. Nesse contexto foram utilizadas espécies de peixes de importância socioeconômico para a região amazônica, tanto ornamentais como para o consumo humano. Então, o objetivo do presente estudo foi descrever pelas análises morfológicas a estrutura testicular de *Hypancistrus zebra*, *Peckoltia oligospila* e *Baryancistrus xanthellus* e verificar as similaridades e diferenças entre a família. Analisar as diferenças morfológicas no complexo folicular dos oócitos de *Peckoltia oligospila*, *Baryancistrus xanthellus*, *Panaqolus tankei*, *Auchenipterichthys longimanus*, *Ageneiosus ucayalensis* e *Hypophthalmus marginatus* e comparar com as estratégias de reprodução de cada espécie. Para tanto, os peixes foram coletados em quatro rios amazônicos. Após a coleta, as gônadas foram submetidas as técnicas de microscopia de luz, microscopia eletrônica de transmissão e varredura. Na sequência foram realizadas análises morfométricas das células da linhagem espermatogênica e das camadas que recobrem os oócitos maduros que formam o complexo folicular. Pelas análises morfológicas dos testículos de *H. zebra*, *P. oligospila* e *B. xanthellus* apresentaram diferenças morfológicas nas células da linhagem espermatogênica, apesar de fazerem parte de uma mesma família. Também há diferenças nas estruturas que formam o complexo folicular dos óocitos, como a espessura da zona radiata, células foliculares e camada da teca entre *P. oligospila*, *B. xanthellus*, *P. Tankey*, *A. longimanus*, *A. ucayalensis* e *H. marginatus*, sugerindo que as diferenças nas estruturas do complexo folicular podem estar relacionadas com as diferentes estratégias reprodutivas das espécies. O resultado deste estudo pode subsidiar o desenvolvimento de pacotes tecnológicos que auxiliem na fertilização in vitro e por conseguinte à manutenção dessas espécies em cativeiro.

Palavras-chave: Auchenipteridae, Espermatogênese, Histologia, Loricaridae, Morfologia, Oócitos, Peixe, Pimelodidae.

ABSTRACT

Knowledge of the morphology of germline cells in teleost fish is the first step to establish different relationships between species and reproductive strategies. In this context, fish species of socioeconomic importance for the Amazon region, ornamental and for human consumption were used. Therefore, the aim of the present study was to describe by morphological analysis the testicular structure of *Hypancistrus zebra*, *Peckoltia oligospila* e *Baryancistrus xanthellus* and to verify the similarities and differences between the family. To verify the morphological differences in the follicular complex of the oocytes of *Peckoltia oligospila*, *Baryancistrus xanthellus*, *Panaqolus tankei*, *Auchenipterichthys longimanus*, *Ageneiosus ucayalensis* and *Hypophthalmus marginatus* and compare with the reproduction strategies of each species. For that, the fish were collected in three Amazonian rivers. After collection, the gonads were submitted to light microscopy, transmission electron microscopy and scanning techniques. Subsequently, morphometric analyzes of the spermatogenic lineage cells and the layers that cover the mature oocytes that form the follicular complex were performed. The morphological analysis of the testes of *H. zebra*, *P. oligospila* and *B. xanthellus* showed that they have differences in the cells of the spermatogenic lineage, despite being part of the same family. There are also differences in the structures that form the follicular complex of oocytes, such as the thickness of the zona radiata, follicular cells and theca layer between *P. oligospila*, *B. xanthellus*, *P. Tankey*, *A. longimanus*, *A. ucayalensis* and *H. marginatus*, suggesting that the differences in the structures of the follicular complex may be related to the different reproductive strategies of the species. The result of this study can support the development of technological packages that assist in in vitro fertilization and therefore the maintenance of these species in captivity.

Key words: Auchenipteridae, Spermatogenesis, Histology, Loricaridae, Morphology, Oocytes, Fish, Pimelodidae.

LISTA DE FIGURAS

Introdução Geral

Figura 1. Esquema da espermatogênese em peixes.....	14
Figura 2. Esquema oogênese em peixes	16
Figura 3. Exemplos de peixes siluriformes.....	20

Capítulo 1. SUBFAMILIA HYPOSTOMINAE SIMILARIDADES E DIFERENÇAS NA ESTRUTURA TESTICULAR DE PEIXES AMAZÔNICOS

Figura 1. species morphology and testicular structure.....	33
Figura 2. Analysis and morphometry of the germ cell and seminiferous tubule.....	34
Figura 3. Photomicrography of spermatogenic cells during the germinative phases.....	36
Figura 4. Photomicrography of sperm in the tubule lumen.....	38
Figura 5. Relation of loricarid species based on differences and similarities in organization of germinative cells.....	39

Capítulo 2. O COMPLEXO FOLICULAR PODE PREVER TÁTICAS REPRODUTIVAS EM PEIXES

Figura 1. Morphology of the follicular complex in type III and IV oocytes in six species of the order Siluriformes.....	55
Figura 2. Scanning electron microscopy of mature oocytes in species.....	56
Figura 3. Graphic representation of the mean thickness of the follicular complex.....	57
Figura 4. Schematic drawing of the main differences in the follicular complex between the groups.....	60

LISTA DE ABREVIATURAS E SIGLAS

Capítulo 1. SUBFAMILIA HYPOSTOMINAE SIMILARIDADES E DIFERENÇAS NA ESTRUTURA TESTICULAR DE PEIXES AMAZÔNICOS

BV: blood vessels

LE: Leydig cells

L: lumen

Mi: mitochondria

MY: myoid cells

N: nuages

n: nucleus

S: Sertoli cells

Sc: spermatocytes

pa: spermatocyte in pachytene

le: spermatocyte in leptotene

di: spermatocyte in diplotene

SgA: spermatogonia A

SgB: spermatogonia B

St: spermatids

Sz: spermatozoa

h: head

Ip: midpiece

f: principal piece

v: vesicles

Capítulo 2. O COMPLEXO FOLICULAR PODE PREVER TÁTICAS REPRODUTIVAS EM PEIXES

IV: type IV oocyte

Fc: follicular complex

Tc: theca layer

F: follicular cell

Zr: zona radiata

P: pores

Y: compacted yolk globules

CONSIDERAÇÕES GERAIS

A tese está no formato de um capítulo geral integrador e dois capítulos que correspondem a um artigo publicado e um manuscrito. Esta organização obedece ao Regimento Geral do Programa de Pós-Graduação em Ecologia Aquática e Pesca, Resolução nº 4.782/2016 CONSEPE/UFPa. O capítulo geral apresenta tópicos sobre o estado do conhecimento geral sobre biologia reprodutiva de espécies amazônicas, morfologia testicular e ovariana e ainda informações sobre as espécies de estudo. Formatado de acordo com as regras atuais vigentes da Biblioteca Central da UFPa. O artigo publicado segue as normas da revista BMC Zoology e o manuscrito está de acordo com as normas da revista Scientific Reports.

Sumário

1- INTRODUÇÃO	14
1.1 Aspectos gerais da biologia reprodutiva em peixes Amazônicos	14
1.2 Morfologia Testicular e Desenvolvimento das células germinativas masculinas.....	15
1.3 Morfologia Ovariana e Complexo Folicular	17
1.4 A Ordem Siluriforme	19
2- ESTRUTURA DA TESE.....	23
3- OBJETIVOS	25
3.1 - Geral.....	25
3.2 – Específicos	25
4 MATERIAL E METODOS	25
4.1- Coleta do Material Biológico	25
4.2 - Técnicas Histológicas.....	26
4.3- Microscopia óptica (MO).....	25
4.4- Análise morfométrica	25
4.5 - Microscopia eletrônica de transmissão (MET) e de varredura (MEV)	26
4.6- Análise estatística.....	26
5- REFERÊNCIAS.....	27
6- CAPITULO 1	31
7- CAPITULO 2	42
8- CONSIDERAÇÕES FINAIS.....	59

1- INTRODUÇÃO

1.1 Aspectos gerais da biologia reprodutiva em peixes Amazônicos

Nos peixes, a reprodução é um momento único cujas espécies utilizam todos os esforços necessários para garantir gametas viáveis e a sobrevivência de sua prole (SOUTO et al., 2017). Esse fenômeno em espécies neotropicais depende de alguns fatores ambientais como o fotoperíodo, precipitação e temperatura (DZYUBA et al., 2019) e de fatores fisiológicos como hormônios inibidores e liberadores de gonadotropinas e esteroides sexuais que são controlados pelo eixo hipotálamo-hipófise-gônada (MASSAAKI HONJI; GUIMARÃES MOREIRA, 2017). Para essa condição, o sistema nervoso através de receptores sensoriais capta os estímulos ambientais e os traduz, iniciando assim uma cascata de eventos em que ocorre a síntese e liberação do Hormônio Liberador de Gonadotropinas (GnRH), que estimula a adeno-hipófise a sintetizar e liberar os hormônios Folículo Estimulante (FSH) e luteinizante (LH) que atuam diretamente nas gônadas para a produção dos hormônios esteroides gonadais, que propiciam o início do ciclo reprodutivo, o desenvolvimento gonadal, a maturação e a liberação dos gametas femininos e masculinos (LUBZENS et al., 2010).

A bacia amazônica possui uma complexa e extensa rede fluvial, sendo considerada o maior sistema hídrico do mundo (FERREIRA et al., 2021b). Esses fatores associados a condições abióticas dos rios e a variação do nível de água possibilita um aumento na riqueza e na diversidade íctica presente neste ambiente (JÉZÉQUEL et al., 2020; LEAL; FREITAS; SOUZA-SIQUEIRA, 2018). Nesse cenário, os peixes teleósteos representam o maior grupo com cerca de 27.000 espécies registradas, compreendendo a maior porcentagem de vertebrados vivendo em diferentes habitats. Porém, o maior número de espécies é encontrado em ambiente dulcícola, o qual possui diversas adaptações as mais variadas condições ambientais (NELSON, JOSEPH S.; GRANDE; WILSON, 2016). Essas adaptações se refletem no comportamento, no ciclo de vida e no modo de reprodução das espécies.

Diversas estratégias estão envolvidas para a reprodução dos peixes como, a fecundidade, a migração reprodutiva, o tipo de fecundação (interna e externa), o cuidado parental, as áreas de desova, a proporção sexual, a idade da primeira maturação sexual (FREITAS et al., 2011; MENDES et al., 2021; VAZZOLER, 1996). No entanto, essas estratégias estão relacionadas as mudanças intrínsecas nos diferentes órgãos do animal, principalmente na gônada, sendo determinante nos padrões da diferenciação sexual, na gametogênese, estrutura dos gametas femininos e masculinos e no tipo de fertilização (ALMEIDA, 2013).

Desse modo, estudos sobre a morfologia das células de linhagem germinativa, especificamente a maturação e a diferenciação das células durante a espermatogênese e oogênese são determinantes para

conhecer a biológica básica das espécies, inferir nos diversos padrões de reprodução e estabelecer relações de proximidade entre espécies de peixes.

1.2 Morfologia Testicular e Desenvolvimento das células germinativas masculinas

Em peixes, os testículos se localizam na cavidade abdominal ventralmente aos rins e dorsalmente ao canal alimentar, geralmente são órgãos pares que podem se unir na extremidade caudal para formar o ducto comum, que desemboca na papila urogenital. Possuem variação de coloração discreta, oscilando entre o transparente na fase não reprodutiva até esbranquiçado na fase reprodutiva (RATTON; BAZZOLI; SANTOS, 2003). Histologicamente os órgãos são envolvidos por um tecido conjuntivo, chamado de túnica albugínea, que emite projeções para o interior do órgão, delimitando os túbulos seminíferos que sustentam o tecido epitelial germinativo (GRIER, 1981). Os testículos possuem dois compartimentos, o germinativo que é apoiado por uma membrana basal e o intersticial (GRIER, 1993).

O compartimento intersticial é formado por tecido conjuntivo, onde se encontram fibroblastos, células mióides, macrófagos, linfócitos, vasos sanguíneos, fibras colágenas, nervos e células de Leydig. Essas células são responsáveis por produzir testosterona e geralmente são encontradas próximas aos vasos sanguíneos. O compartimento germinativo é formado por um epitélio constituído por células germinativas (espermatogônias, espermatócitos, espermatídes e espermatozoides) e por células somáticas de Sertoli. Estas últimas possuem função de fagocitose, de suporte estrutural e nutricional para as células germinativas e por sintetizar o fluido que dá origem ao lúmen do túbulo espermático (GRIER, 1981; SCHULZ et al., 2010) (Figura 1). Existem dois tipos testiculares, o tipo tubular anastomosado e o tipo lobular. Se os compartimentos germinativos estão conectados ao longo de toda extensão do órgão formando ramos, onde é possível observar a junção de dois ou mais tubulos são descritos como tubular anastomosado, são comuns e encontrados em espécies mais basais. Porém, se os compartimentos estão bem delimitados e os túbulos seminíferos terminam na periferia do órgão são chamados de lobular e estão presentes nas espécies mais derivadas (GRIER, 1993; PARENTI; GRIER, 2004).

O compartimento germinativo pode ser classificado em contínuo, onde se observa a presença de células germinativas e de Sertoli ao longo de todo o epitélio, e descontínuo quando apresenta espaços sem células aparentes, ou seja, não apresenta a continuidade das células ao longo do compartimento (DE SIQUEIRA-SILVA et al., 2013; GRIER; TAYLOR, 1998). De acordo com GRIER (1981), há dois tipos de classificação dos testículos em relação a distribuição das espermatogônias: a)

espermatogonial irrestrito, que possui espermatogônias em toda extensão do túbulo seminífero e b) espermatogonial restrito, no qual as espermatogônias estão restritas na porção distal do túbulo.

No interior dos túbulos seminíferos ocorre o desenvolvimento das células de linhagem germinativa, que tem início quando uma espermatogônia isolada do tipo A (também chamada de célula tronco) é envolvida por projeções do citoplasma da célula de Sertoli e forma uma estrutura denominada de cisto, onde passa por sucessivas mitoses e se tornam espermatogônias do tipo B. Estas células, posteriormente, passarão por meiose e irão se diferenciar em espermatócito primário (primeira divisão da meiose) e espermatócito secundário (segunda divisão da meiose). Por fim, após as divisões meióticas os espermatócitos se diferenciam em espermatídes que se tornarão espermatozoides, processo chamado de espermiogênese (GRIER, 1981, 1993; SCHULZ et al., 2010). É importante ressaltar que dentro dos cistos (localizados dentro do túbulo seminífero) as células germinativas se dividem de forma sincrônica, devido a presença de pontes citoplasmáticas existentes entre elas (SCHULZ et al., 2010).

A espermatogênese para teleósteos pode ser apresentada de duas formas: cística quando ela ocorre completamente dentro dos cistos, que se rompem para liberar espermatozoides na luz dos túbulos seminíferos (processo ocorre dentro dos cistos germinativos) ou semi cística quando os cistos se rompem quando as células germinativas estão em estágio de espermatócito ou espermatíde e a maturação se completa na luz dos túbulos seminíferos (desenvolvimento ocorre parcialmente fora do cisto) (MAGALHÃES et al., 2011). Enquanto a espermiogênese é classificada de três formas: espermiogênese do tipo I, onde ocorre uma série de eventos que culmina com a formação de uma fossa nuclear próxima ao segmento inicial do flagelo e a rotação de 90° do núcleo em relação ao eixo flagelar. Na espermiogênese do tipo II, há a formação da fossa nuclear, porém o núcleo não rotaciona, e por esse motivo, o eixo flagelar permanece paralelo ao núcleo (MATTEI, 1970), e por último a espermiogênese do tipo III, onde não há formação de fossa nuclear e nem rotação do núcleo (QUAGIO-GRASSIOTTO; OLIVEIRA, 2008).

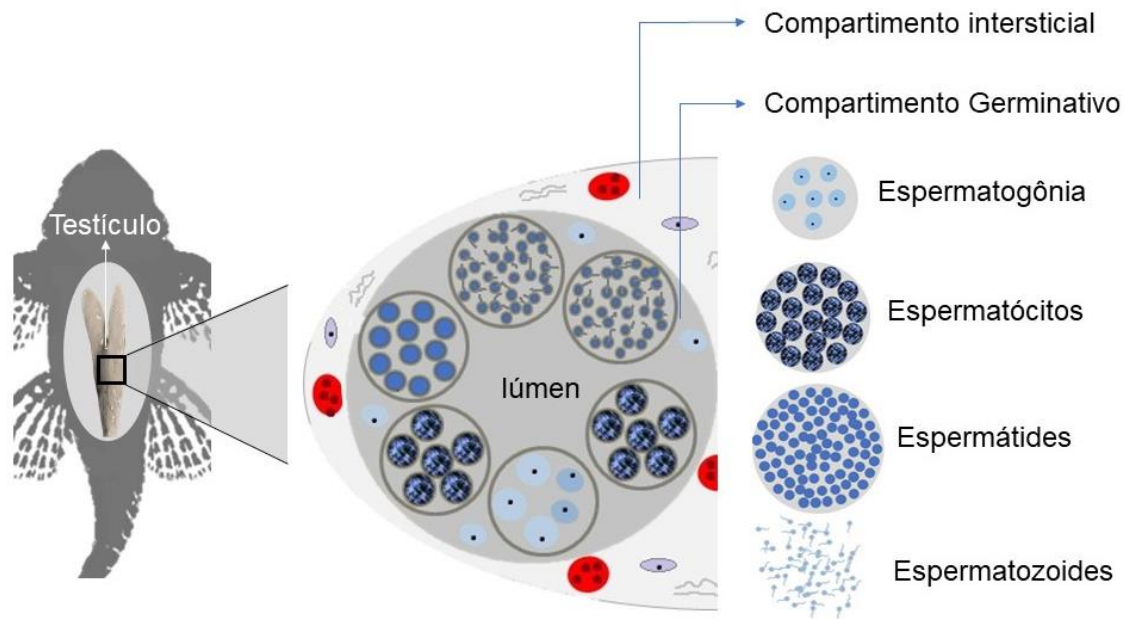


FIG. 1. Esquema da espermatogênese em peixes Fonte: autoria própria

1.3 Morfologia Ovariana e Complexo Folicular

Os ovários nos peixes teleósteos estão localizados na cavidade abdominal, predominantemente são órgãos pares com exceção de algumas espécies como por exemplo a espécie amazônica *Arapaima gigas* que possui apenas o desenvolvimento de um ovário (CAMPOS-BACA, 2001). Esses se unem em direção ao poro urogenital (Figura 2). É possível observar uma gama de coloração, que depende da espécie e da fase de maturação que as fêmeas se encontram (BAYONA PEREZ; MAZZONI; QUAGIO-GRASSIOTTO, 2020a; DOS SANTOS et al., 2019; FAGUNDES et al., 2021; RODRIGUEZ-PULIDO et al., 2020). Em alguns peixes apesar do ovário ser um órgão par, apenas um lado do ovário é funcional e o outro é vestigial. Essa característica é bem evidente na classe dos elasmobrânquios (PRATT, 1988). Em se tratando de teleósteos, a maioria das espécies possuem dois ovários.

Ainda que macroscopicamente os ovários sejam diferentes, microscopicamente na maioria dos teleósteos é possível observar um padrão de organização tecidual bem conservado. As gônadas são revestidas por uma cápsula de tecido conjuntivo espesso chamada de túnica albugínea, a qual emite septos para o interior do órgão delimitando compartimentos chamados de lamelas ovígeras, estrutura que serve de suporte para as células germinativas durante seu desenvolvimento (ARANTES et al., 2017; BAYONA PEREZ; MAZZONI; QUAGIO-GRASSIOTTO, 2020a; MELO et al., 2011). Essas

lamelas são constituídas por um epitélio germinativo que é formado por células somáticas e germinativas (oogônias e oócitos) as células germinativas se encontram em diferentes fases de desenvolvimento gonadal (GRIER, 2000).

O desenvolvimento dos oócitos, chamado de oogênese, é um processo que tem início com as oogônias que passam por sucessivas mitoses e posterior meiose dos oócitos até chegar a formação de oócitos maduros. Nessa diferenciação inicial as células foliculares envolvem a oogônia, posteriormente, esses elementos (oogônia + célula folicular) são circundadas por uma camada de células tecais de natureza conjuntiva. A partir dessa organização são originados os oócitos que de acordo com as características distintas são classificados nos tipos I, II, III e IV segundo a classificação de NÚÑEZ; DUPONCHELLE, 2009. Vale ressaltar que o período de desenvolvimento dos oócitos é dividido em crescimento primário, onde as oogônias originam os primeiros oócitos e crescimento secundário que ao final culmina com a ovulação do oócito maduro (JALABERT, 2005; MAZZONI et al., 2020; MEIJIDE et al., 2016).

Durante a oogênese é possível observar o aparecimento de algumas camadas ao redor do oócito (de fora para dentro: camadas da teca, membrana basal, camada de células foliculares e zona radiata). A camada da teca é subdividida em duas (teca externa e interna) constituídas de fibras colágenas e células com formato achatado (VIANA et al., 2018), que possuem características de células esteroidogênicas, ou seja que produzem esteroides essenciais para a maturação oocitária (GRIER, 2012). A camada de células foliculares apresenta diferentes morfologias que depende da espécie e da fase de crescimento do oócito. Por exemplo, no crescimento primário é comum essas células apresentarem formato mais achatado, ao longo do desenvolvimento elas podem adquirir o formato colunar ou cubóide (DOS SANTOS et al., 2019). A zona radiata é a camada mais interna, essa estrutura possui canais também chamados de poros que permitem a comunicação entre as células foliculares e o oócito, sua função está relacionada a proteção mecânica e também a adesividade do ovo ao substrato (RIZZO et al., 2002). A junção de todas essas camadas mais o oócito maduro é denominado de complexo folicular (GRIER, 2012) (Figura 2).

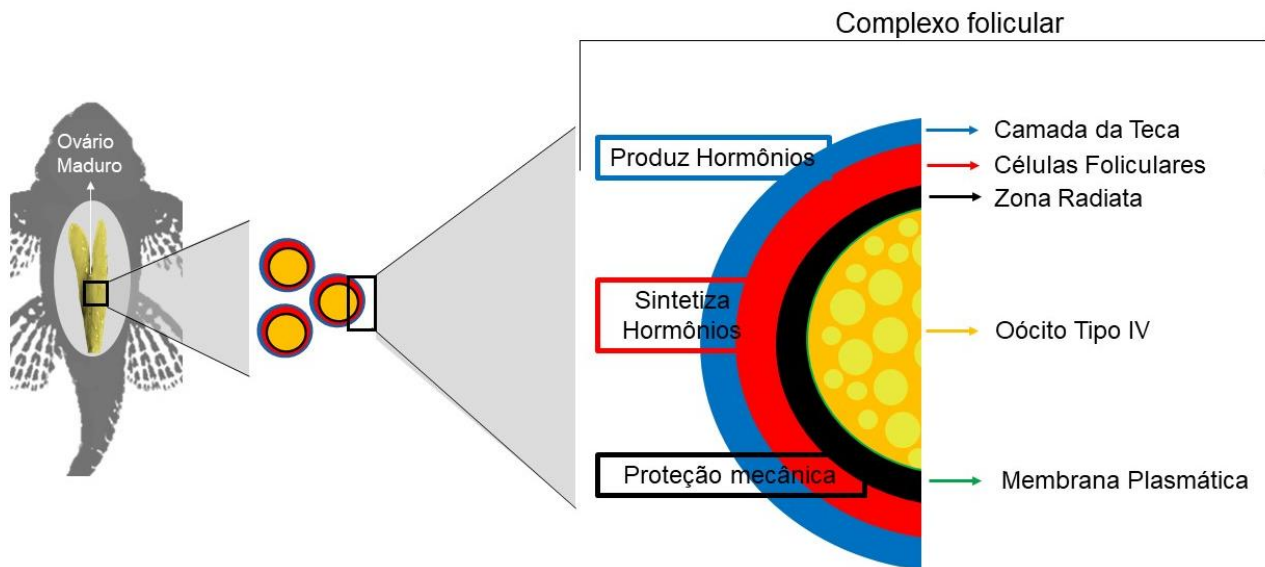


FIG. 2. Esquema oogênese em peixes. Fonte: autoria própria

As camadas que compõem o complexo folicular desempenham um papel fundamental no desenvolvimento e manutenção do oócito no ovário. Aspectos importantes como morfologia (BAYONA PEREZ; MAZZONI; QUAGIO-GRASSIOTTO, 2020b; DOS SANTOS et al., 2019; FAGUNDES et al., 2021; FELICIO et al., 2023) e atresia celular (CORRIERO et al., 2021; MAZZONI et al., 2020) vem sendo amplamente abordados ao longo dos anos. Outro fato interessante é que a morfologia das camadas que compõem o complexo folicular podem indicar alguns aspectos sobre a tática reprodutiva que as espécies realizam, como a presença de cuidado parental, ovos adesivos ou não, ovos livres ou agrupados em ninhos etc. (MELO et al., 2011; RIZZO et al., 2002; SUZUKI; AGOSTINHO; WINEMILLER, 2000). Dessa forma torna-se essencial a correlação entre a morfologia do complexo folicular com as diferentes estratégias reprodutivas realizadas pelas diferentes espécies de peixe, inclusive de espécies pertencentes a ordem Siluriforme.

1.4 A ordem Siluriforme

Os peixes siluriformes, também chamados de peixes gatos, bagres, mandis, peixes lisos e cascudos, representam a mais diversa ordem dentro da Superordem Ostariophysi. A ordem possui 39 famílias onde estão inseridas cerca de 3.894 espécies válidas de peixe (FERRARIS, 2007; FRICKE, RON; ESCHMEYER; VAN DER LAAN, 2021). As espécies dessa ordem possuem o corpo cilíndrico, achatado ventralmente com ausência de escamas, sendo revestido por pele espessa ou placas ósseas,

possuem nadadeira adiposa e barbilhões que estão localizados no focinho com funções sensoriais (BRUTON, 1996; NELSON, JOSEPH S.; GRANDE; WILSON, 2016).

A ordem é amplamente distribuída na América do sul, as espécies estão dispersas desde a Argentina, Colômbia e Panamá (FERRARIS, 2007), seus integrantes, na grande maioria, são dulcícolas, porém existem relatos de espécies marinhas (NELSON, JOSEPH S.; GRANDE; WILSON, 2016). Diversas famílias que estão inclusas nessa ordem apresentam espécies com grande importância econômica e ecológica.

Nesse estudo destacamos as famílias Auchenipteridae, Pimelodidae e Loricaridae que possuem particularidades quanto a ecologia reprodutiva, modo de vida e ambientes em que estão inseridas. Com exceção de Auchenipteridae, as demais são provenientes de rios antropizados pela instalação de barragens hidroelétricas. O funcionamento de Hidrelétricas pode representar um fator de risco para a sobrevivência das espécies, considerando a influência que elas exercem no ciclo hidrológico do rio, na disponibilidade de alimentos e conseqüentemente sobre ciclo de vida das espécies (WINEMILLER et al., 2016).

A família Auchenipteridae apresenta uma ampla variação de tamanho corporal, com valores de comprimento padrão que variam de 30 a 600 mm (FREITAS et al., 2021). Devido ao tamanho que podem alcançar diversas espécies dessa família são bastante apreciadas pelo comércio local, como por exemplo *Ageneiosus ucayalensis* Castelnau, 1855 (Figura 3A). Conhecido pela população ribeirinha como Mandubé ou Fidalgo, é um recurso pesqueiro utilizado para alimentação das comunidades Amazônicas (SÁ-OLIVEIRA et al., 2014). A espécie *A. ucayalensis* é carnívora tendo sua alimentação baseada principalmente em crustáceos e outros peixes (SÁ-OLIVEIRA et al., 2014), alcança a maturidade sexual entre 11,8 cm e 12,8 cm, onde as fêmeas são predominantemente maiores que os machos, e a maior atividade reprodutiva é encontrada em períodos de alta pluviosidade (OLIVEIRA et al., 2017).

Auchenipterichthys longimanus Günther, 1864 (Figura 3B) vulgarmente conhecido como bagre da meia-noite. Possui importância ecológica, sendo dispersora de sementes, inclusive de espécies vegetais em risco de extinção como é o caso da espécie *Virola surinamensis* (DA SILVA FREITAS; DA CONSOLAÇÃO ALMEIDA; DE ASSIS MONTAG, 2018). *A. longimanus* possui um longo período de desova, com atividade reprodutiva no período de cheia (FREITAS et al., 2011).

Outra família que é destaque pela sua importância comercial para pesca de consumo é a família Pimelodidae, que comporta grandes espécies de bagres. Algumas de suas espécies são conhecidas por apresentarem migração reprodutiva (VILLAMIL-RODRÍGUEZ; CORTÉS-ÁVILA; RODRÍGUEZ-PULIDO, 2018). Neste trabalho destacamos a espécie *Hypophthalmus marginatus*, vulgarmente

conhecida como mapará (Figura 3C), essa espécie tem importante papel como fonte de renda e de nutrição de diversas comunidades ribeirinhas da região amazônica (JURAS, ANASTÁCIO AFONSO; CINTRA, ISRAEL HIDENBURGO ANICETO; LUDOVINO, 2004). Apresenta hábito alimentar do tipo planctófago (CUTRIM; BATISTA, 2005). A espécie possui uma ampla distribuição geográfica que vai da bacia Amazônica até o Suriname (CUTRIM; BATISTA, 2005). As fêmeas são maiores que os machos, e apresenta um curto período de vitelogênese durante o ciclo reprodutivo (HAINFELLNER et al., 2019), além de realizar migração reprodutiva (CARVALHO, J. L., & MÉRONA, 1986).

A família Loricaridae é valorizada mundialmente pela indústria ornamental desde o ano de 1980, quando foram encontradas e descritas as primeiras espécies nos rios Xingu e Tocantins. Muitas espécies pertencentes à família vêm sendo exploradas na bacia amazônica devido seu valor no mercado de peixes ornamentais (CAMARGO, MAURICIO.; GHILARDI, 2009) e entre essas diversas espécies que são comercializadas na região amazônica destacam-se *Baryancistrus xanthellus*, *Hypancistrus zebra*, *Peckoltia oligospila* e *Panaqolus tankei*.

Baryancistrus xanthellus, vulgarmente conhecido como "amarelinho" ou "golden plug nugget" (Figura 3D) é uma espécie de porte médio a grande, apresenta coloração preta com manchas amarelas pelo corpo e uma faixa amarela mais acentuada na parte distal das nadadeiras dorsal e caudal quando jovem. É uma espécie reofílica e sua distribuição está fortemente ligada as corredeiras do rio Xingu (PY-DANIEL; ZUANON; DE OLIVEIRA, 2011). *Baryancistrus xanthellus* na fase juvenil é comercializado como peixe ornamental. No ano de 2016 chegou a representar 47% das espécies comercializadas na região (IBAMA 2016). Na fase adulta pode alcançar até 29,6 cm e 510 g, sendo importante item na dieta das comunidades ribeirinhas (CARVALHO JÚNIOR et al., 2011; GIARRIZZO et al., 2015).

Outro loricarideo de importância no mercado ornamental é a espécie *Hypancistrus zebra* ISBRÜCKER E NIJSSEN 1991, conhecido popularmente por acari zebra, possui pequeno tamanho, sua coloração lembra a zebra, por apresentar faixas brancas e pretas (Figura 3E). Alimenta-se de algas, de pequenos invertebrados aquáticos e de detritos encontrados sobre blocos rochosos (ISBRUCKER, I. J. H., NIJSSEN, 1991; ROSA; LIMA, 2008). *Hypancistrus zebra* é endêmico da região do médio rio Xingu, e sua extinção é provável tendo em vista que a espécie tem um histórico de forte comercialização na região norte. A pesca desordenada e a baixa fecundidade (menos de 20 ovos por desova) ocasionaram uma diminuição no tamanho das populações (ROSA E LIMA, 2008). Seja pela alta demanda, pelo mercado ornamental que gera uma sobre-exploração ou pelo risco de extinção causado por fatores biológicos e interferências humanas, é necessário ter medidas eficazes para a conservação das espécies.

Peckoltia oligospila Günther, 1864 vulgarmente conhecido como acari bola (Figura 3F), possui coloração exuberante com círculos de tonalidade que varia do castanho ao marrom em toda a extensão corporal, habita ambientes de água doce com pH entre 6,5 e 7,2 e temperatura de 23° a 27°C. São onívoros e se alimentam de raspas de madeira, microalgas e pequenos invertebrados aquáticos, devido a sua coloração exuberante. *Peckoltia oligospila* é altamente explorado pela pesca ornamental e sua comercialização chegou a representar 13% das exportações das espécies ornamentais na região (TORRES, 2007).

Panaqolus tankei Cramer & Sousa, 2016 (Figura 3G) possui uma ampla distribuição geográfica, espécimes já foram encontrados no Brasil, Equador, Guiana Francesa, Colômbia, Peru e Venezuela (FERREIRA et al., 2021a). Essa espécie apresenta barras escuras e mais claras ao longo de todo o corpo, podendo apresentar variações de coloração (CRAMER; DE SOUSA, 2016). Em relação à reprodução *P. tankei* tem seu período de desova relacionado a maiores níveis de precipitação, desova parcelada e fecundidade baixa (37 ovos por desova), porém com ovos grandes o que indica cuidado parental (MENDES et al., 2018).

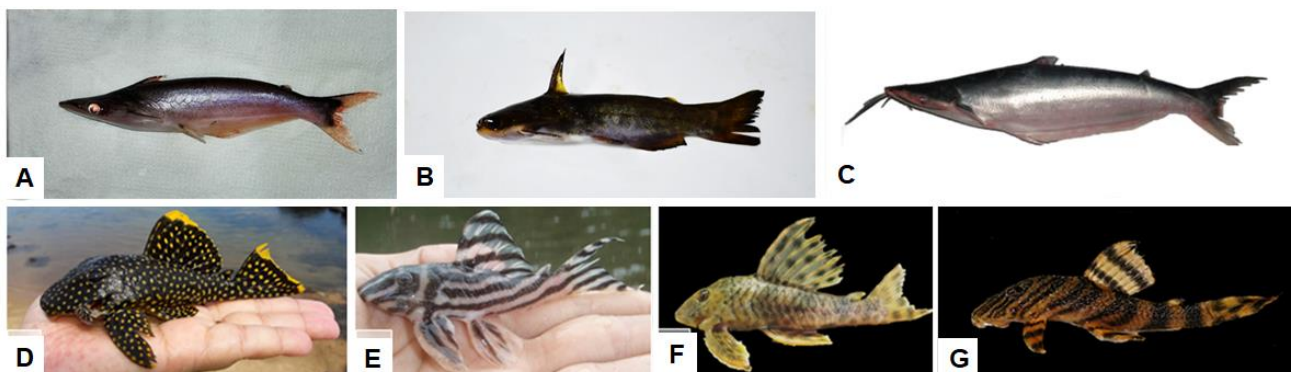


FIG. 3. Exemplos de peixes siluriformes A) *Ageneiosus ucayalensis*, B) *Auchenipterichthys longimanus*, C) *Hypophthalmus marginatus* D) *Baryancistrus xanthellus*, E) *Hypancistrus zebra* F) *Peckoltia oligospila* e G) *Panaqolus tankei*. Fonte: autoria própria

Dessa forma, seja pela alta demanda da pesca local, pela importância ecológica, pelo mercado ornamental que gera uma sobre exploração, pelo risco de extinção causado por interferências humanas, é necessário ter medidas eficazes para a conservação das espécies. Ainda mais quando se trata de espécies de alto valor econômico e ecológico como é o caso das espécies de siluriformes reportadas nesse estudo.

Nesse aspecto a caracterização morfológica dos testículos vem como uma ferramenta fundamental para preencher lacunas na literatura e embasar estudos que visam a criação em cativeiro e preservação de material genético das espécies. Pois conhecer as características biológicas do desenvolvimento de células germinativas é o primeiro passo a seguir quando se pretende fazer o manejo e reprodução induzida. Da mesma maneira estudos sobre as diferentes morfologias do complexo folicular de peixes siluriformes da região Amazônica, são inovadores e altamente necessários. Assim este estudo visa a caracterização das células de linhagem germinativa de *B. xanthellus*, *H. zebra* e *P. oligospila* e a análise do complexo folicular em *B. xanthellus*, *P. tankey*, *P. oligospila*, *A. ucayalensis*, *A. longimanus* e *H. marginatus*.

2– ETRUTURA DA TESE

A tese foi elaborada no formato de artigos, organizada em capítulos, conforme abaixo proposto:

2.1- Capítulo I: Subfamilia hypostominae similaridades e diferenças na estrutura testicular de peixes amazônicos.

Hipótese: As espécies *B. xanthellus*, *H. zebra* e *P. oligospila* por pertencerem a uma mesma subfamília não possuem diferenças em sua estrutura testicular.

2.2- Capítulo II: O complexo folicular pode prever táticas reprodutivas em peixes.

Hipótese: O complexo folicular das espécies *A. ucayalensis*, *A. longimanus*, *H. marginatus*, *B. xanthellus*, *P. oligospila* e *P. tankei* possuem diferenças morfológicas que indicam estratégias reprodutivas.

3- OBJETIVOS

3.1 - Geral

Descrever, analisar, comparar a estrutura testicular e a espermatogênese de três espécies pertencentes à subfamília Hypostominae, *B. xanthellus*, *P. oligospila* e *H. zebra*, para estabelecer a relações de proximidade entre as espécies. Descrever e analisar o complexo folicular de *A. ucayalensis*, *A. longimanus*, *H. marginatus*, *B. xanthellus*, *P. oligospila* e *P. tankei* e relacionar as diferenças morfológicas com as possíveis táticas reprodutivas dessas espécies

3.2 – Específicos

Capítulo 1:

- ✓ Caracterizar a morfologia dos testículos de *B. xanthellus*, *H. zebra* e *P. oligospila* em relação ao padrão de distribuição das espermatogônias;
- ✓ Caracterizar a espermatogênese de *B. xanthellus*, *H. zebra* e *P. oligospila*;
- ✓ Analisar morfometricamente os túbulos e células da linhagem germinativa para *B. xanthellus*, *H. zebra* e *P. oligospila*;
- ✓ Comparar o desenvolvimento estrutural dos testículos entre *B. xanthellus*, *H. zebra* e *P. oligospila*;

Capítulo 2:

- ✓ Caracterizar a morfologia das camadas que compõem o complexo folicular em *A. ucayalensis*, *A. longimanus*, *H. marginatus*, *B. xanthellus*, *P. oligospila* e *P. tankei*;
- ✓ Analisar morfometricamente as camadas que compõem o complexo folicular em *A. ucayalensis*, *A. longimanus*, *H. marginatus*, *B. xanthellus*, *P. oligospila* e *P. tankei*;
- ✓ Comparar as diferenças e semelhanças no complexo folicular de *A. ucayalensis*, *A. longimanus*, *H. marginatus*, *B. xanthellus*, *P. oligospila* e *P. tankei*.

4 MATERIAL E MÉTODOS

4.1- Coleta do Material Biológico

As coletas de *B. xanthellus*, e *H. zebra* foram realizadas no rio Xingu, *P. oligospila* foi coletado no rio Guamá, *A. ucayalensis* e *A. longimanus* no rio Anapu, *H. marginatus* no rio Tocantins e *P. tankei* no Lago Bolonha. Todos os locais ficam no Estado do Pará. Foram utilizados 60 espécimes para as espécies *Peckoltia oligospila* e *Baryancistrus xanthellus*, e 30 animais para as demais espécies. Os animais foram coletados por pescadores locais utilizando a técnica de mergulho com auxílio de um cilindro de ar para os Loricarídeos e por meio de redes de espera para Auchenipteridae e Pimelodidae. Após as coletas os animais foram acondicionados em caixas de isopor com água do próprio rio e levados ao laboratório, onde foi realizada a biometria. Todo o procedimento realizado foi aprovado pelo Conselho Nacional de Controle de Experimentação Animal da Universidade Federal do Pará (CONCEA/CEUA nº 5557211118 (ID 001065)).

4.2 - Técnicas Histológicas

Para cada animal foi realizada uma incisão ventro-longitudinal para retirada das gônadas. Posteriormente, as gônadas foram pesadas e fragmentos foram fixados para as técnicas de microscopia óptica, de microscopia eletrônica de transmissão e de varredura. As fases de maturação gonadal foram classificadas de acordo com adaptação de MENDES et al., 2018, onde foi possível identificar testículos imaturos, em maturação, maturo e espermiado e para fêmeas ovários maduros.

4.3- Microscopia óptica (MO)

Fragmentos da porção média das gônadas foram fixados em solução de Bouin por 24 horas e posteriormente desidratadas em concentrações crescentes de etanol (de 70% a 100%), diafanizadas em xilol, infiltradas e incluídas em parafina. Cortes de 5 µm de espessura foram obtidos através do micrótomo (Leica 2245) corados com Hematoxilina e Eosina.

4.4 - Análise morfométrica

Para a análise morfométrica somente gônadas maduras foram utilizadas para ambos os sexos. Para os machos foi realizada a medida do diâmetro dos túbulos seminíferos (n=58) e das células de linhagem germinativa (n=200) (espermatogônias a e b, espermatócitos, espermatídes e espermatozoides). Totalizando 58 túbulos e 1000 células de linhagem espermatogênica.

Para as fêmeas foi realizada a medida da espessura das camadas que compõem o complexo folicular (zona radiata, camada folicular e teca) de oócito do tipo III (n=50) e IV (n=50) em 4 espécimes por espécie, totalizando 1200 oócitos.

4.5 - Microscopia eletrônica de transmissão (MET) e de varredura (MEV)

Fragmentos de gônadas foram fixados em solução de Karnovsky (paraformaldeído 4%, glutaraldeído 2% em tampão cacodilato de sódio 0,1M PH 7,4) durante 24 horas. Após, foram lavados com tampão cacodilato de sódio (0,1M PH 7,4) e pós fixados em tetróxido de ósmio 1% em tampão cacodilato de sódio (0,1M PH 7,4) por 2 horas, desidratados em série crescente de acetona, embebidos e incluídos em Epon 812. Cortes ultra finos de 0,1 μ m de espessura foram feitos em ultra micrótomo e contrastados com citrato de chumbo e acetato de uranila e posteriormente analisados em microscópio eletrônico de transmissão.

Para MEV, após a fixação as amostras foram lavadas em tampão cacodilato de sódio 0,1M pH 7,4 e pós-fixadas em tetróxido de ósmio 1% acrescido de 0,8 de ferricianeto de potássio em um tampão cacodilato de sódio 0,1M pH 7,4 por 2 horas em temperatura ambiente. Em seguida as amostras foram desidratadas em concentrações crescentes de etanol (30% a 100%) e submetidas a secagem em ponto crítico em CO₂. Posteriormente, colocadas em stubs e submetidas a metalização com cobertura de ouro de 10 nm de espessura para análise em microscópio eletrônico de varredura.

4.6- Análise estatística

Para ambas as linhagens de células germinativas (espermatogênicas e oogênicas) as médias foram avaliadas quanto à normalidade pelo teste de Shapiro-Wilk e analisadas pelo teste de Kruskal-Wallis (H) e Mann-Whitney (U) (apenas oócitos) ($P < 0,05$) (Zar, 1999).

Apenas para os oócitos a espessura total e a espessura da camada em relação ao grupo de espécies foram analisadas usando análise de coordenadas principais (PCoA), análise de Permutação (Permanova) foi usada para testar se a variação observada no gráfico era significativa. Todas as análises foram realizadas usando o R Development Core Team Program (2016).

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6- CAPITULO 1

SUBFAMILIA HYPOSTOMINAE SIMILARIDADES E DIFERENÇAS NA ESTRUTURA TESTICULAR DE PEIXES AMAZÔNICOS

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RESEARCH ARTICLE

Open Access



Subfamily hypostominae: similarities and differences in testicular structure of amazonian fish

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Abstract

Background: Hypostominae is a subfamily of the family Loricariidae that has a great diversity of species. Accordingly, testicular studies in fish can contribute to the phylogeny and taxonomy of species and to the comparison of reproductive aspects between species. Thus, this work aimed to characterize the testicular morphology and spermatogenesis of the *Hypostominae* species *Baryancistrus xanthellus*, *Peckoltia oligospila* and *Hypancistrus zebra*.

Results: *B. xanthellus*, *P. oligospila* and *H. zebra* had an anastomosed tubular type of testis. The germinal epithelium was continuous with unrestricted spermatogonia, and the proliferative, meiotic and spermiogenic phases were defined in all species. In the spermiogenic phase, spermatids were classified as initial, intermediate and final. Only in *B. xanthellus* in the final phase was there nuclear rotation. The sperm for the three species had a head with an oval shape and a single flagellum composed of the short midpiece, principal piece and end piece. *B. xanthellus* and *P. oligospila* showed a cylindrical flagellum and *H. zebra* showed projections that produced a flattened appearance.

Conclusions: On the basis testicular structure and ultrastructural characteristics of the germ cells, there was a greater relationship between *B. xanthellus* and *P. oligospila*, while *H. zebra* had particular characteristics. These aspects show that despite belonging to the same subfamily, the species have distinct biological characteristics.

Keywords: Loricariidae, Amazonian fish, Testis, Spermatogenesis, Histology

Background

For several decades, knowledge of the testicular structure and spermatogenesis of fish has been important to understand the reproductive biology of the species and the mechanisms involved in the maturation of sperm [1–3]. More recently, these data have established different reproductive strategies, evolutionary adaptations to aquatic environments [4–7] and a broad understanding of phylogenetic and systematic relationships [8, 9]. However, there are few studies that address the morphological

differences between species of the same family [9–11], or between subfamilies [12, 13]. These characteristics can help to understand the relationships between species and support the permanence of a species in a family or its removal.

In general, fish testes can be classified according to morphology as filiform, fringed or lobular [4] and as types regarding the organization of the germinal epithelium, i.e., anastomosed tubular when seminiferous tubule anastomosis occurs, and lobular because the epithelium is in a closed compartment and because the seminiferous tubules end at the periphery of the testis [14]. However, during the development of the germinal epithelium, spermatogenesis is subdivided into three phases: proliferative,

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where there are successive mitotic divisions of type A and B spermatogonia; meiotic, where primary and secondary spermatocytes pass through meiotic divisions giving rise to spermatids; and spermiogenesis where spermatids give rise to spermatozoa [15].

The family Loricariidae is endemic to South America and widespread in the Amazon region, and it is made up of many species that are exploited due to their high value in the aquarium market [16]. This family is subdivided into the subfamilies Delturinae, Hypoptopomatinae, Hypostominae, Lithogeninae, Loricariinae and Neoplecostominae, where Hypostominae stands out because it contains a greater number of species, ecologically and morphologically diverse, which hinders a more comprehensive phylogeny revision [17]. The main studies that address the morphological differences in this subfamily have focused on the external anatomy of the digestive tract and osteology [18–20], and there is no information as to whether the testicular characteristics represent a relationship between species. Among the species of this subfamily are: *Baryancistrus xantheilus* Rapp Py-Daniel et al. 2011, *Peckoltia oligospila* Günther 1864 and *Hypancistrus zebra* Isbrücker & Nijssen 1991. All have a high value in the ornamental fish market, but *H. zebra* is the species that stands out for being on the Red List of species threatened with extinction, which confers greater vulnerability. This is even more so because of the anthropic influence in the Amazon region, such as the establishment of hydroelectric dams, which cause damage to natural habitats and interfere with the life cycle, resulting in a great loss of species diversity [21].

Although they are species of the same subfamily, there is no information as to whether there are differences or similarities in testicular structure and organization of the germ cells. These basic biology parameters are of fundamental importance because they help to understand the life history of the species [22]. On the basis of this thinking, the present study aimed to compare the testicular structure and spermatogenesis of three species belonging to the subfamily Hypostominae, namely *B. xantheilus*, *P. oligospila* and *H. zebra*, to establish the proximity relations.

Results

Testis morphology

Macroscopically, the testes of the species *B. xantheilus*, *P. oligospila* and *H. zebra* (Fig. 1a–c), showed a color that varied from transparent to whitish. The testes were paired, lobular organs of equal size, and they were fused caudally, forming a single sperm duct leading to the urogenital papilla (Fig. 1d). The organ was covered by the tunica albuginea, which produces septa in the organ, forming two compartments. The interstitial

compartment had blood vessels, myoid cells, interstitial or Leydig cells and connective tissue. The germinal compartment consisted of two cell types, Sertoli or somatic cells and germinal cells (spermatogonia, spermatocytes, spermatids and spermatozoa) (Fig. 1 e,f).

In the mature stage, *B. xantheilus*, *P. oligospila* and *H. zebra* showed testicular organization of the tubular anastomosis type, where the tubules are previously separated and then anastomosed (Fig. 2a–c).

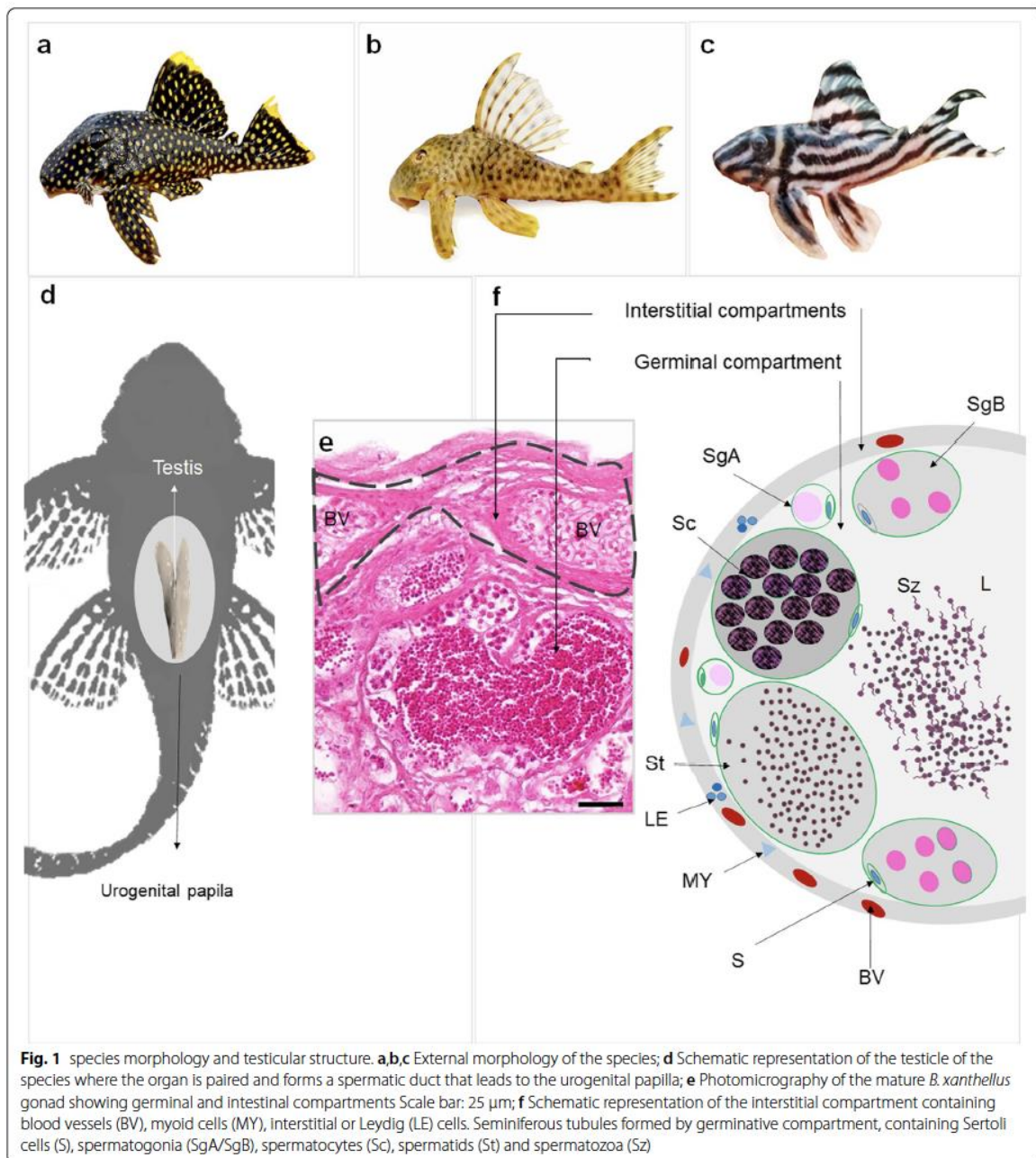
According to the size of the seminiferous tubules, *B. xantheilus* had the largest diameter ($174.37 \pm 70 \mu\text{m}$), followed by *H. zebra* ($102.97 \pm 33.68 \mu\text{m}$) and *P. oligospila* ($101.54 \pm 46.59 \mu\text{m}$), and significant differences were observed only between *H. zebra* and *P. oligospila* ($H=209.23$; $df=2$; $p>0.05$) (Fig. 2d). The proliferative, meiotic and spermiogenic phases were defined according to the organization of the germinal epithelium.

Proliferative phase

When analyzing the germinal epithelium of *B. xantheilus*, *P. oligospila* and *H. zebra*, a continuous epithelium was observed with the presence of Sertoli cells and spermatogenic cells: spermatogonia, spermatocytes, spermatids and spermatozoa (Fig. 2e–j). Sertoli cells showed the characteristics of spermatogenic cells, forming spermatid cysts within the seminiferous tubule (Fig. 2e); they had an elongated body and irregular electron-dense nucleus (Fig. 3e).

Spermatogonia were observed as two types, A and B, in all species, and were not restricted to a single position in the seminiferous tubule, characterizing an unrestricted spermatogonial epithelium (Fig. 3 a–g). Type A spermatogonia (SgA) were observed isolated in the germinal epithelium (Fig. 3 a–d). Ultrastructurally, they had a clear cytoplasm, with the presence of mitochondria and electron-dense structures known as nuages close to the nuclear envelope, and they had a nucleus with a condensed chromatin and a single nucleolus (Fig. 3c, d). In relation to the nuclear envelope, this structure was more evident in *P. oligospila* (Fig. 3c). The cell diameter of SgA in *B. xantheilus*, *P. oligospila* and *H. zebra* was 6.59 ± 0.97 , 7.59 ± 0.86 and $8.21 \pm 0.92 \mu\text{m}$, respectively, and significant differences were observed between the three species. ($H=511.71$; $df=2$; $p<0.05$) (Fig. 2d).

Type B spermatogonia (SgB) appeared smaller and formed cell nests, and the cytoplasm had few mitochondria. The nucleus contained condensed chromatin with heterochromatin adhered to the nuclear envelope, and a central or peripheral nucleolus (Fig. 3 e–g), and these spermatogonia showed a cell diameter for *B. xantheilus*, *P. oligospila* and *H. zebra* of 4.99 ± 0.54 , 5.75 ± 0.67 and $6.05 \pm 0.56 \mu\text{m}$, respectively, with significant differences being observed between the three species. ($H=297.13$;

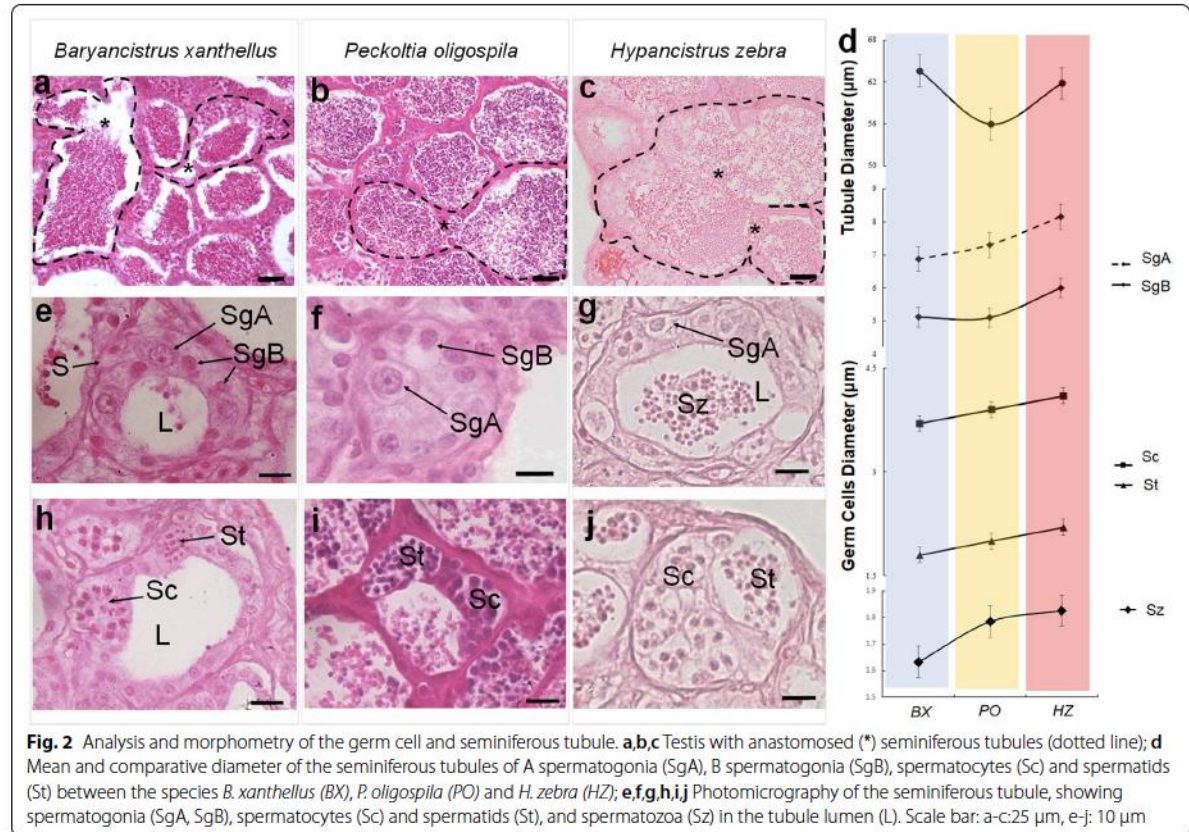


$df=2$; $p<0.05$). Overall, for spermatogonia A and B, *H. zebra* displayed the largest spermatogonia (Fig. 2d).

Meiotic phase

The primary spermatocytes formed nests near the wall of the seminiferous tubules, which are seen in

different phases of prophase I (Fig. 3 h-j): leptotene is characterized by an ovoid nucleus with little condensed chromatin, showing some points of compaction and cytoplasm with little delimitation (Fig. 3i); in pachytene, the delimitation of the nucleus and nucleolus is not visible, and the condensed chromatin



has a filamentous appearance (Fig. 3h); and in diplotene, chromosomes appear compacted with spots evident in the nucleus (Fig. 3j). Secondary spermatocytes were not seen. The diameter of the spermatocytes in *B. xanthellus*, *P. oligospila* and *H. zebra* was 3.92 ± 0.45 , 4.35 ± 0.43 and 4.43 ± 0.47 μ m, respectively, with no differences being observed between *H. zebra* and *P. oligospila* ($H = 283.15$; $df = 2$; $p > 0.05$) (Fig. 2d).

Spermiogenic phase

In the three species, spermatids showed specific cellular characteristics that revealed a subdivision into phases, i.e., initial, intermediate and final (Fig. 3 k-m). The initial spermatids formed cysts, in which the cells are connected by cytoplasmic bridges, and have a spherical nucleus, with condensed chromatin and nuclear fossa, and cytoplasm with vesicles and mitochondria (Fig. 3l). The intermediate ones had a nucleus with condensed chromatin with electron-lucid regions and nuclear fossa, and there was little cytoplasm (Fig. 3m). In the final spermatids,

(See figure on next page.)

Fig. 3 Photomicrography of spermatogenic cells during the germinative phases. **a,b,c,d,e,f,g** Proliferative phase: **a** schematic design demonstrating the similarities of spermatogenesis between the species and differences in the spermatozoa; **b,c,d** spermatogonia (SgA) with mitochondria (Mi) and nuages (N); **e,f,g** B spermatogonia (SgB) forming cell nest and Sertoli cell (S) with presence of nucleus (n); **h,i,j** meiotic phase: **h** spermatocyte in pachytene (pa); **i** spermatocyte in leptotene (le); **j** spermatocyte in diplotene (di); **k,l,m,n,o,p** spermiogenic phase: **k** final spermatids (St), in detail, the presence of the proximal (p) and distal (d) centrioles and mitochondria (Mi); **l** initial spermatids (St), nest cell, nucleus (n), vesicles (v) and mitochondria (Mi); **m** intermediate spermatids with spherical nucleus (n); note intermediate spermatid showing deep nuclear fossa (arrowhead); **n,o,p** spermatozoa with head (h), midpiece (lp) and flagellum (f), secretion (*); note, longitudinal view of Sz, showing a nuclear fossa; spermatozoa with oval nucleus (n), detail of head (h). Scale bar: light microscopy-b,f: 10 μ m; TEM- c-e, g-j: 5 μ m, k-m: 2 μ m, insert: 500 nm; SEM—n,p: 2 μ m, insert: 1 μ m

xantheus, *P. oligospila* and *H. zebra* was 1.81 ± 0.29 , 2.09 ± 0.20 and 2.21 ± 0.22 μm , respectively, where significant differences were observed between the three species. ($H = 302,15$; $df = 2$; $p < 0.05$) (Fig. 2d).

The spermatozoa were characterized as having compact chromatin, absence of acrosome, loss of residual bodies and formation of a single flagellum (Fig. 3 n-p). In *B. xantheus*, the head of the spermatozoa was oval, with a single flagellum consisting of a short midpiece with the presence of mitochondria, long principal piece and short

end piece (Fig. 3n). In *P. oligospila*, the nucleus had an oval shape and showed the presence of electron-lucid zones, and the midpiece was elongated and possessed mitochondria, while the principal piece was cylindrical (Fig. 3o). In *H. zebra*, the nucleus was half-sphere shaped and showed electron-lucid regions, and the midpiece appeared bulky and possessed mitochondria (Fig. 3p), while the principal piece displayed lateral projections which gave it a flattened shape (Fig. 4 b-e). In *P. oligospila* and *B. xantheus* lateral projections were not observed in

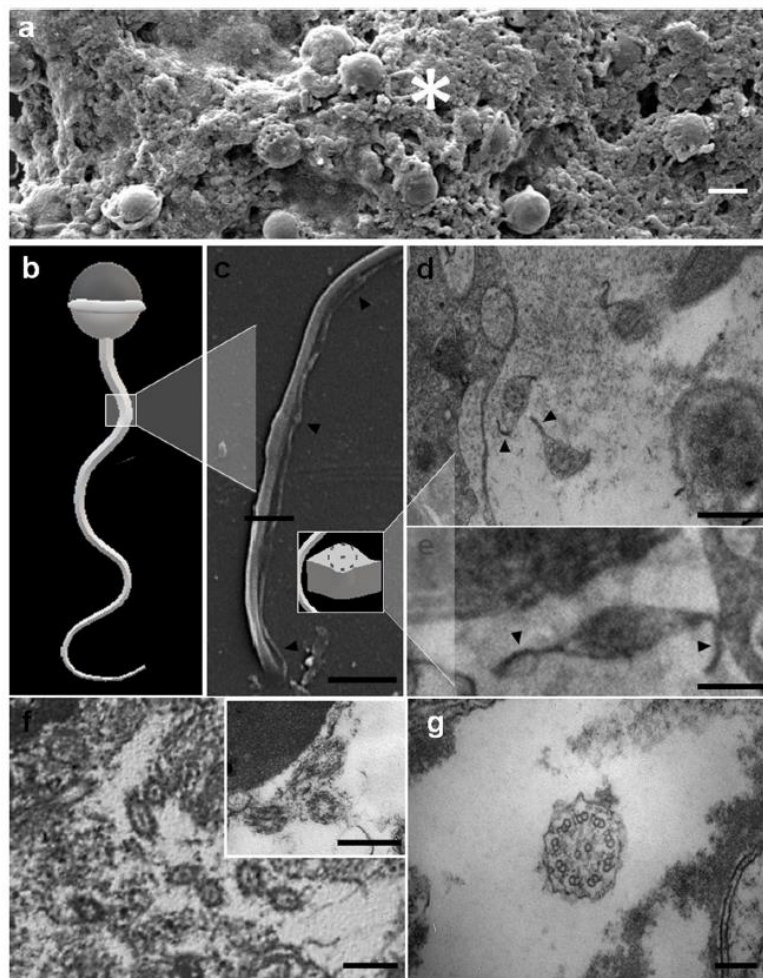


Fig. 4 Photomicrography of sperm in the tubule lumen. a-e *H. zebra*: a Sperm immersed in secretion (*); b schematic design of sperm; c detail of the flagellum showing lateral projections (arrowhead) by SEM; d and e detail of the cross-section of the flagellum showing the lateral projections (arrowhead). f *B. xantheus* g *P. oligospila*. Detail of the cross-section of the flagellum in both species. Scale bar: SEM- a: 2 μm , c: 3 μm ; TEM- d,e: 1 μm

principal piece of the flagellum (Fig. 4f, g) In all species, the principal piece of the flagellum showed a 9 + 2 axoneal pattern and the end piece 9 + 0 pairs of microtubules (Fig. 3a). TEM and SEM demonstrated that the sperm of *B. xanthellus* (Fig. 3n: insert) and *H. zebra* (Fig. 3p: insert and 4a) were immersed in a secretion. The diameter of the spermatozoa head in *B. xanthellus*, *P. oligospila* and *H. zebra* was 1.63 ± 0.13 , 1.78 ± 0.13 and 1.82 ± 0.12 μm , respectively, and significant differences were observed between the three species. ($H=168.28$; $df=2$; $p<0.05$). Overall, *H. zebra* had the largest spermatozoa head (Fig. 2d).

The testicular structure and the organization of the cells established the similarities and differences between the species studied in the subfamily (Fig. 5).

Discussion

In this study, we compared testicular structure and spermatogenesis in three species of the family Loricariidae, subfamily Hypostominae. Despite the morphological diversity of testicular structures among the neotropical teleosts [23], *B. xanthellus*, *P. oligospila* and *H. zebra* have paired, lobular or saculiform testes, with color varying according to the gonadal stage. These characteristics are similar to those observed in the family Loricariidae [24–26].

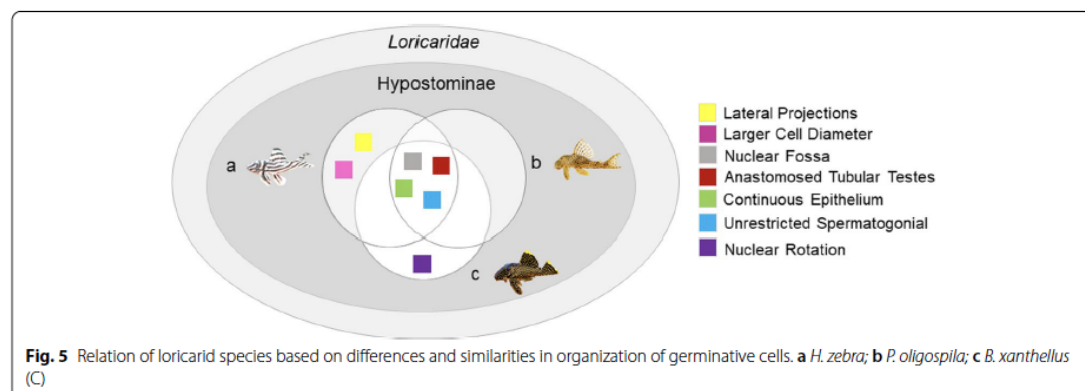
The testicular structure for all species is of the anastomosed tubular type. This observed testicular structure is considered to be more basal within the phylogenetic classification of Teleostei [14]. Anastomosed tubular has also been seen in *Pterygoplichthys disjunctivus* [25], *Hypostomus francisci* [27], and *Harttia torrenticola* [28], species that belong to the order Siluriforme, family Loricariidae.

Here, we emphasize that the development of the germinal cells of the three species occurs within cysts present throughout the seminiferous tubule. This characteristic

considered as continuous epithelium is in accordance with the literature [29] and has been observed in other species of the family Loricariidae, belonging to different subfamilies: Loricariinae (*Loricaria lentiginosa* [30] and *Harttia torrenticola* [28]), Hypoptopomatinae (*Pseudotocinclus tietensis* [31]), and Hypostominae (*Hypostomus francisci* [27]). It is worth mentioning that this characteristic of the epithelium varies according to the degree of development of the testis, where a species can have both types of epithelium as seen in *Rachycentron canadum* (L.) [32], *Padogobius martensi* [33] and *Serrasalmus spilopleura* [8]. We believe that this characteristic is related to the capacity to renew the gametogenic lineage; considering the species studied, because they have a continuous epithelium, it is possible to observe in the whole reproductive cycle the presence of germinal cells occupying the entire wall of the seminiferous tubule.

In the present study, types A and B spermatogonia differed in size between species, where the largest diameter was found in *H. zebra*. Although the spermatogenesis process is similar among fish, characteristics such as cell diameter differ between species, and in the case of the subfamily Hypostominae, this difference is even more evident when compared with species such as *Pterygoplichthys disjunctivus*, which have A spermatogonia with a diameter of 14.87 ± 0.22 μm [25] and *Hypostomus francisci* with a diameter of 8.2 ± 1.1 μm [27]. Possibly this characteristic may be associated with the reproductive biology of each species.

We found that *B. xanthellus*, *P. oligospila* and *H. zebra* had a distribution of spermatogonia along the seminiferous tubule, being characterized as unrestricted spermatogonial. According to the literature [15], this type is common in anastomosed testes, whereas restricted spermatogonia are often seen in lobular testes. Only primary spermatocytes with chromatin at different levels of



condensation have been observed. Similar results have been reported for other teleosts, showing that at this stage of division, secondary spermatocytes are difficult to visualize due to rapid cell division [5, 34].

In spermiogenesis, we showed peculiarities in spermatid development. The nuclear fossa was evident in *B. xantheus*, *P. oligospila* and *H. zebra*. The presence of the nuclear fossa was observed in the Loricariidae in the subfamilies Hypoptopomatinae (*Hypoptopoma guentheri* and *Schizolecis guntheri*), Neoplecostominae (*Kronichthys heylandi* and *Neoplecostomus paranensis*) and Loricariinae (*Farowella oxyrryncha* and *Loricaria lata*) [13]. This suggests that most of the members of the family Loricariidae have this biological characteristic.

In general, the spermatids from *B. xantheus*, *P. oligospila* and *H. zebra* also showed similarities and differences related to the head and flagellum. In the observations reported in literature [35], characteristics such as round or oval head, short midpiece with few mitochondria and single or double flagellum are observed in the spermatozoa of the aquasperm type, considered primitive, adapted to external fertilization. In most fish with external fertilization, spermatozoa without an acrosome penetrates the oocyte, which is facilitated by the presence of the micropyle [36–38].

The flagellum had a standard 9+2 microtubular arrangement, but in *H. zebra*, lateral projections were observed, which gave the flagellum a flatter shape. These projections have also been observed in other species of loricariids [13]. The morphology of the flagellum as well as its molecular structure accounts for spermatic motility [39], and differences in spermatid morphology are useful tools for systematic analysis of different habitats and reproductive modes in fish [34]. We believe that these lateral projections help in the efficiency of motility in relation to energy expenditure, in view of the different characteristic habitats of the species. As an example, spermatozoa of *H. zebra* have intermediate vigor and a long sustained motility that provides less energy expenditure [40]. This condition can help in reproduction since this species is endemic to the Xingu River and lives in stream rapids.

A relevant fact was the presence of a secretion in the lumen of the seminiferous tubules of *H. zebra* and *B. xantheus*, which made it difficult to visualize the plasma membrane of these sperm. This result differs from *Pterygoplichthys disjunctivus* [25], subfamily Hypostominae. However, for other Loricariidae subfamilies such as Hypoptopomatinae and Otothyriinae [13], the same difficulty in visualization the sperm membrane has been reported due to the presence of this secretion. According to the literature [41], these substances for fish can assist

in the fertilization process and in increasing seminal volume.

Conclusions

On the basis of gonadal morphology, testicular structure, spermatozoa location and germ cell ultrastructural characteristics, we found that there is a greater relationship between *B. xantheus* and *P. oligospila*. *H. zebra* has characteristics that differ from the others, although they are in the superorder Ostariophysia, in the same subfamily (Hypostominae) and are endemic species in the Amazon region. These results contribute to the understanding of spermatogenesis of neotropical species, Therefore, these findings are important for understanding the phylogeny of this very diverse group for enabling biotechnological studies such as cryopreservation of semen and induced reproduction of species in captivity, to guarantee a better strategy to survive in their environment.

Methods

Sample collection

The samples were collected between January 2019 and July 2019 in the middle Xingu River (3°12'52" S, 51°11'23" W). We collected 30 specimens of each species, namely *B. xantheus*, *P. oligospila*, *H. zebra*, with the help of local fishermen, who sampled the fish by scuba diving. Captured fish were anesthetized with benzocaine hydrochloride (0.1 g.L⁻¹) and euthanized with sodium pentobarbital solution (60–100 mg/kg). Subsequently, gonads were removed by ventral incision.

Light microscopy

Fragments of gonads in the maturing and mature stage of *B. xantheus*, *P. oligospila* and *H. zebra* were fixed in Bouin's solution for 24 h. The samples were then dehydrated in increasing concentrations of ethanol, cleared in xylene and infiltrated and embedded in paraffin [42]. Sections 5 µm thick were cut and stained with hematoxylin and eosin (HE), and stained sections were examined under a Carl Zeiss light microscope (AxioStar Plus 1,169,151).

Morphometry and statistical analysis

For morphometry only, testes in the mature stage of *B. xantheus*, *P. oligospila* and *H. zebra* were evaluated, because this stage has the largest seminiferous tubules. A total of five specimens/species were analyzed, and for each specimen, the mean diameter of the 58 seminiferous tubules were measured. In each specimen, the mean diameter ($n=200$) for each germ cell type (spermatogonia A and B, spermatocytes, spermatids and spermatozoa) were analyzed. Only cells that contained a nucleus were measured. Serial sections were cut, and the slides

were evaluated under a photomicroscope with the software NIS-elements BR (4.00.07-bit), and measurements were made at 40X magnification. Each cell was overlaid with two dashed lines crossing at right angles in the middle of the cell, and the length of the segment of the line over that diameter of the cell was measured. The mean length of the two measurements was taken as the approximate diameter of the cell. Means were assessed for normality using the Shapiro–Wilk test and analyzed using the Kruskal–Wallis test ($P < 0.05$) (Zar, 1999). All analyses were performed using the R Development Core Team Program (2016).

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM)

Fragments of testis were fixed in Karnovsky's solution (4% paraformaldehyde, 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4) for 24 h. After fixation, the fragments were washed in 0.1 M sodium cacodylate buffer, pH 7.4 and post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.4 for 2 h. For TEM analysis, the fragments were dehydrated in an ascending acetone series and embedded in Epon 812. Ultra-thin sections were cut in a microtome and contrasted with uranyl acetate and lead citrate, followed by examination in a JEOL (JEM -100CX II) electron microscope. For SEM analysis, testicular sections and 500- μ l aliquots of raw semen were fixed in Karnovsky's solution and then, a drop of the fixed semen sample was placed on a poly-L-lysine-coated coverslip. The samples were dehydrated in a graded ethanol series (30 to 100%) and critical-point dried using CO₂ and mounted on stubs, coated with gold and examined using a LEO 1430 SEM.

Abbreviations

BV: Blood vessels; LE: Leydig cells; L: Lumen; Mi: Mitochondria; MY: Myoid cells; N: Nuages; n: Nucleus; S: Sertoli cells; Sc: Spermatoocytes; pa: Spermatoocyte in pachytene; le: Spermatoocyte in leptotene; di: Spermatoocyte in diplotene; SgA: Spermatoogonia A; SgB: Spermatoogonia B; St: Spermatoids; Sz: Spermatozoa; h: Head; Ip: Midpiece; f: Principal piece; v: Vesicles.

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Authors' contributions

I.K.S.V., M.A.P.F. and R.M.R. conceived and designed the study. I.K.S.V., Y.A.M and R.S.O. carried out the field and laboratory activities. I.K.S.V. planned the study, analyzed and interpreted the data and wrote the manuscript. J.C.D.P., G.M.F.S. and J.L.G.N. reviewed data analysis and contributed to the revision of the

manuscript. M.A.P.F. and R.M.R. contributed reagents/materials/analysis tools. All authors read and approved the final paper.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

All applicable institutional guidelines for the care and use of animals were followed per National Council for Control of Animal Experimentation (CONCEA) and were performed in accordance with approved guidelines, CEUA No. 5557211118 (ID 001065).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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THE FOLLICULAR COMPLEX CAN PREDICT REPRODUCTIVE TACTICS IN FISH

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Abstract

In this study, we evaluated the morphology and morphometry of the layers that make up the follicular complex surrounding mature oocytes in the six fish species *Auchenipterichthys longimanus*, *Ageneiosus ucayalensis*, *Hypophthalmus marginatus*, *Baryancistrus xanthellus*, *Panaqolus tankei* and *Peckoltia oligospila*, belonging to the order Siluriformes, which inhabit the Amazon basin. The aim of this study was to analyze the follicular complex of the six species and relate the morphological differences to the possible reproductive tactics of these species. On the basis of the morphology and thickness of the layers of the follicular complex, the species were divided into two groups: 1- *A. longimanus*, *A. ucayalensis* and *H. marginatus* and 2 – *B. xanthellus*, *P. tankei* and *Peckoltia oligospila*. Group 1 showed a columnar and thicker layer of follicular cells in *A. longimanus* and *A. ucayalensis* and cuboidal in *H. marginatus*. Meanwhile, Group 2 displayed a layer of cuboidal-shaped follicular cells, the zona radiata layer and thicker theca layer. These differences may be related to the environment, as group 1 migrates without parental care and eggs are generally smaller and abundant. While group 2, represented by loricerids, inhabit lotic environments, have reproductive tactics of parental care and eggs that are generally large and in small numbers. Therefore, we can infer that the follicular complex in mature oocytes can predict the reproductive tactics of the species.

Keywords: Fish, histology, oocyte layer, SEM, Siluriforme and reproduction.

Introduction

Teleost fish have different reproductive tactics, for example, migration¹⁻³, different types of spawning⁴⁻⁶, parental care that occurs in different ways such as the storage of fertilized eggs in the mouth⁷⁻⁹, the construction of nests and the maintenance of continuous aeration of the eggs¹⁰⁻¹².

In females, mature oocytes indicate some reproductive tactics such as the reproductive period^{13,14} and type of spawning^{15,16}, and the animal's fecundity can be estimated^{5,17,18}.

The follicular complex comprises the mature oocyte, zona radiata (Zr), follicular (F) and theca (Tc) layers present in the fish ovary¹⁹. In recent years, the follicular complex has been studied with a focus on morphological description^{15,20,21}, in the chemical composition of the layers^{22,23} and in the process of oocyte atresia during gonadal maturation^{24,25}.

The structures of the follicular complex stand out for their intrinsic interaction with oocytes and may be related to different habitats and reproductive behaviors. For example, the zona radiata is a complex extracellular matrix, and its thickness is related to the protection of eggs against abrasion²⁶. In this sense, it is important to analyze the involvement of the follicular complex in different reproductive tactics or behavior used by different fish species.

The order Siluriforme has a wide geographic distribution with representatives on all continents, but the highest percentage of freshwater siluriformes is concentrated in South America²⁷. This order is the second highest in diversity of freshwater species that have different body morphology, feeding habits and reproductive strategies^{28,29}

We highlight the species *Auchenipterichthys longimanus* Guenther, 1864; *Ageneiosus ucayalensis* Castelnau, 1855 and *Hypophthalmus marginatus* Valenciennes, 1840, which are of socioeconomic and ecological importance in the Amazon region *Auchenipterichthys longimanus* is a seed disperser along rivers³⁰. *Ageneiosus ucayalensis* is of ecological importance, where it has a carnivorous habit and exerts direct and indirect effects on the aquatic biota³¹. *Hypophthalmus marginatus*, which is important for artisanal, subsistence fishing, shows reproductive migration³².

Meanwhile, *Baryancistrus xanthellus* Rapp Py-Daniel et al. 2011, *Panaqolus tankei* Cramer & Sousa 2016 and *Peckoltia oligospila* Günther 1864 are species belonging to the family Loricaridae; they are sedentary, show low fecundity, and have large eggs, which indicates parental care³³⁻³⁵. The representatives of this family are highly valued in the world aquarium trade, where the species are widely sold because of their exuberant color pattern.³⁶

Given the above, the aim of this study was to analyze the morphology and morphometry of the follicular complex in mature oocytes in six fish species from Siluriformes order and associate the morphological differences with the possible reproductive tactics of these species.

Results

Morphology of follicular complex

The follicular complex in the six species (Fig 1 A-F) is formed by three enveloping layers, the outermost theca, followed by the follicular cell layer and the zona radiata. These layers showed morphological differences between oocyte type III and IV (Fig 1 A^I-F^{II}). In type IV oocytes, the different characteristics of the layers were more evident between species (Fig 1 G).

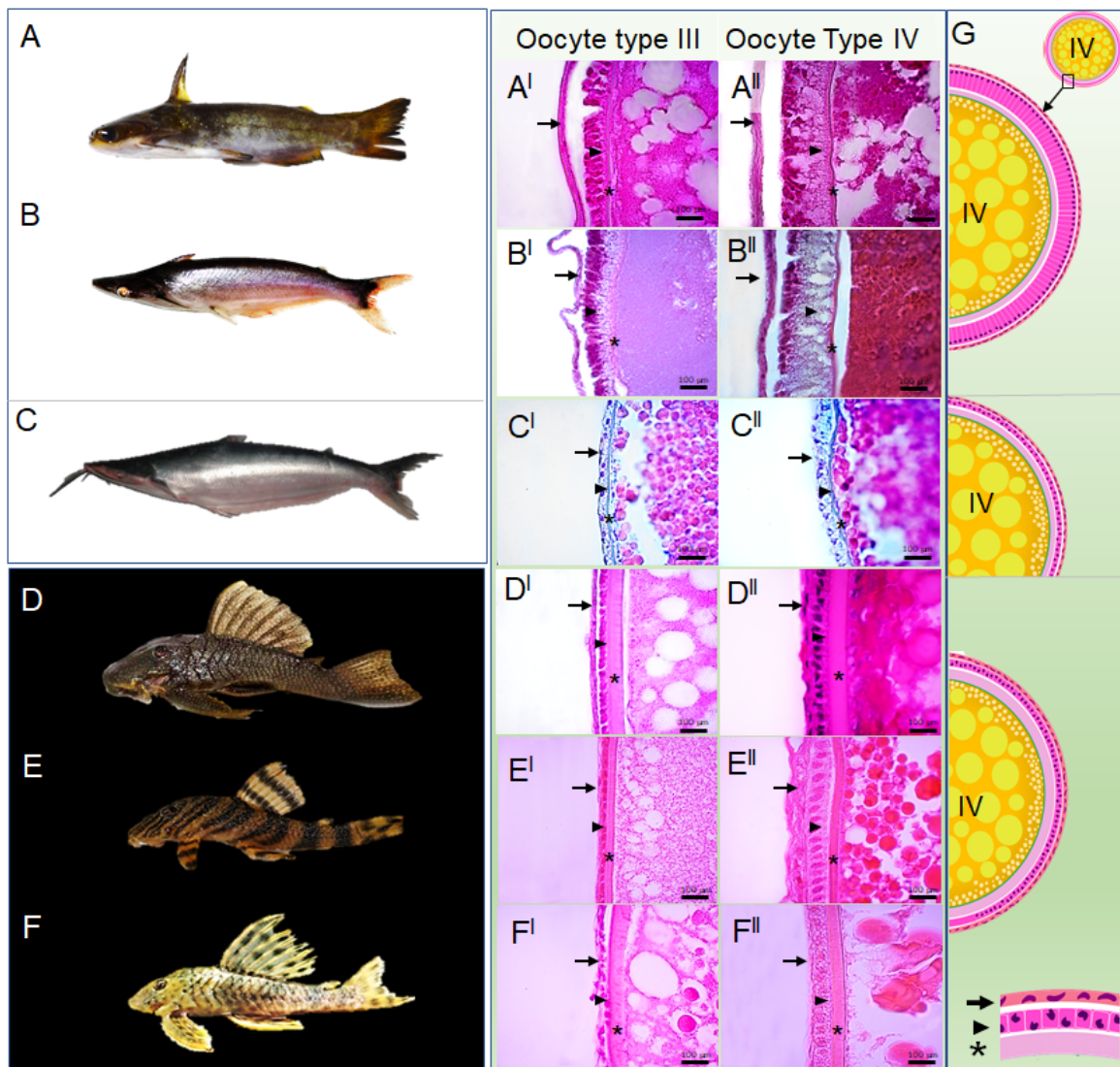


Figure 1- Morphology of the follicular complex in type III and IV oocytes in six species of the order Siluriformes. (A-A^{II}) *Auchenipterichthys longimanus* (B-B^{II}) *Ageneiosus ucayalensis* (C-C^{II}) *Hypophthalmus marginatus* (D-D^{II}) *Baryancistrus xanthellus* (E-E^{II}) *Panaqolus tankei* (F-F^{II}) and *Peckoltia oligospila* (G). Schematic drawing of the follicular complex of type IV oocytes of the species. Abbreviations: IV: type IV oocyte, arrow: theca layer, arrowhead: follicular cell, asterisk: zona radiata.

In the species *A. longimanus*, *A. ucayalensis* and *H. marginatus*, the theca layer of type III and IV oocytes (Fig 2 A) was thin, formed by squamous cells (Fig 1 A^I -C^{II}). In type IV oocytes, there is an increase in theca thickness of (Fig 1 A^{II} -C^{II}). SEM showed the basement membrane and connective fibers (Fig 2 B, C). *Hypophthalmus marginatus* displayed a thinner theca layer when compared to the other species. It was possible to observe a thick layer formed by yolk globules compacted near the cytoplasmic membrane of the oocyte (Fig 2 F). The follicular cell layer in type III and IV oocytes in *A. longimanus* and *A. ucayalensis* showed a columnar shape with the nucleus located in the basal pole of the cell (Fig 1 A^I -B^{II} and 2D). Meanwhile, in *H. marginatus*, they showed a cuboidal shape with a globose nucleus (Fig 1 C^I, C^{II}), where the SEM showed a thin layer with poorly defined cell boundaries (Fig 2G). The zona radiata in type III oocytes was thinner and more homogeneous. However, in type IV oocytes, it was thicker, displaying channels or pores connecting the oocyte and follicular cell (Fig 1 A^{II}-C^{II}). In *A. longimanus*, cytoplasmic projections were very evident inside these channels (Fig 2 E). However, in *H. marginatus*, the zona radiata was continuous and homogeneous (Fig 2G).

In the species *B. xanthellus*, *P. tankei* and *P. oligospila*, the theca layer of type III and IV oocytes (Fig 2H) was thin, formed by squamous cells (Fig 1 D^I-F^{II}). In *P. oligospila*, the theca layer displayed an extensive fibrillar network (Fig 2I). The follicular cell layers in types III and IV oocytes showed a cuboidal shape and distinct cell boundaries, especially in type IV oocytes (Fig 1 D^{II}-F^{II} and 2J). The zona radiata in type III oocytes showed connecting channels or pores (Fig 1 D^I-F^I); these structures increased in type IV oocytes (Fig 1 D^{II}-F^{II}). SEM demonstrated pores with an amorphous material inside in *B. xanthelus* oocytes (Fig 2 J-L).

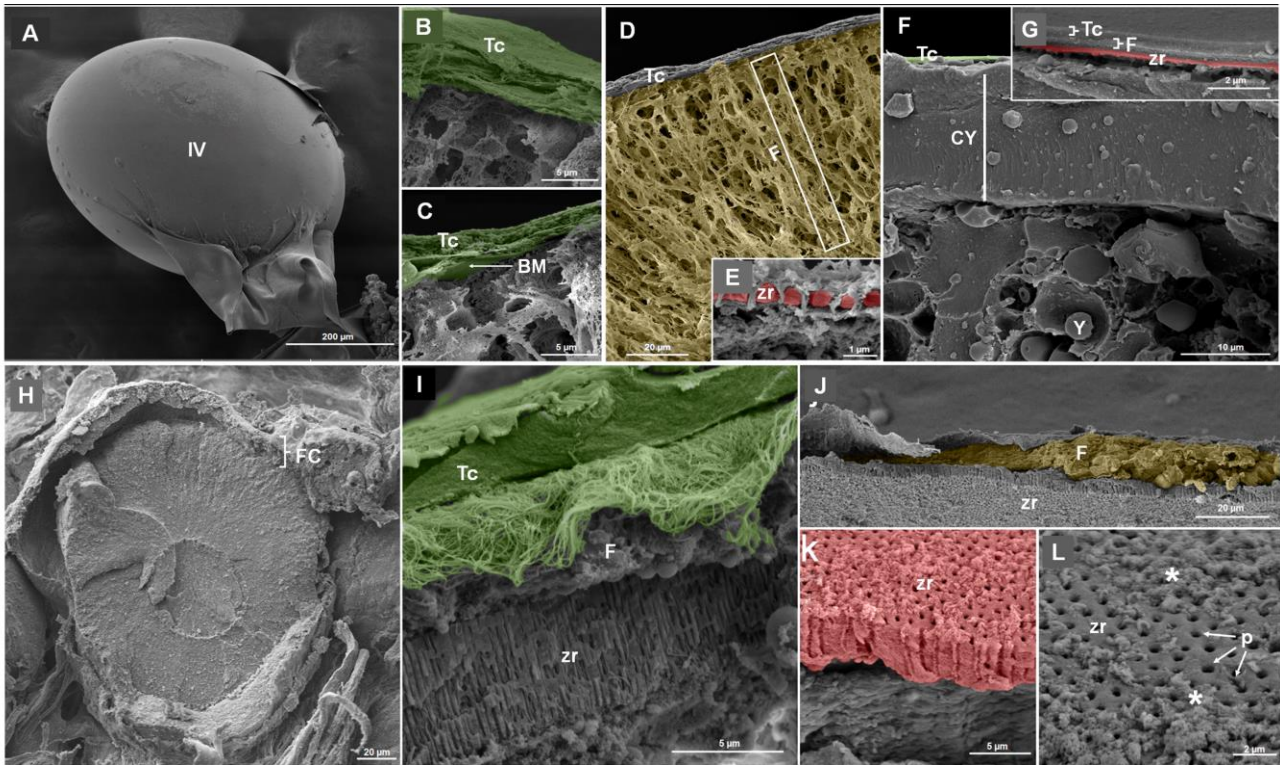


Figure 2- Scanning electron microscopy of mature oocytes in species. *H. marginatus*: (A) mature oocyte. (B) *A. ucayalensis* theca layer consisting of connective fibers. *A. longimanus*: (C) theca layer represented by connective fibers and basement membrane. (D) columnar follicular cell layer, (E) zona radiata with cytoplasmic projections inside. *H. marginatus*: (F) cross-section of oocyte showing yolk globules (G) follicular complex. (H) mature oocyte Abbreviations. (I) cross-section of *P. oligospila* oocyte showing theca layer consisting of connective fibers, follicular cell and zona radiata. (J) follicular complex in *B. xanthellus*. (K-L) zona radiata of *B. xanthellus* showing pores and amorphous material - IV: type IV oocyte, Fc: follicular complex, Tc: theca layer, F: follicular cell, Zr: zona radiata, P: pores, Y: compacted yolk globules, asterisk: amorphous material. Illustrative colors.

Morphometry of follicular complex

The species analyzed showed a different mean thickness for the layers that make up the follicular envelope in the mature oocyte. In the total layer's thickness there were significant differences among *A. longimanus*, *A. ucayalensis* and *H. marginatus* ($H = 185.06$; $D. F=5$; $p < 0.05$). Regarding the theca layer, there were significant differences only in *H. marginatus* compared to the other species ($H = 54.63$; $D. F=5$; $p < 0.05$). In the follicular cell layer, a significant difference was observed among *H. marginatus* and all species in the study and between *A. ucayalensis* and *P. tankei* ($H = 133.15$; $D. F=5$; $p < 0.05$). In the zona radiata layer, difference was not observed among *A. longimanus*, *A. ucayalensis* and *H. marginatus*, where these species had the lowest means with significant differences

in relation to the other species and also *P. tankei*, which differed from all species ($H = 189.40$; $D. F=5$; $p < 0.05$) (Table 1)

Table 1. Means and standard deviations of the layers that make up the follicular complex among the siluriform species.

Species	Total Thickness (μm)	Theca Thickness (μm)	Follicular Thickness (μm)	Zona radiata Thickness (μm)
<i>Auchenipterichthys longimanus</i>	43.23 ^a \pm 13.02	4.49 ^a \pm 1.18	34.03 ^a \pm 13.03	2.54 ^a \pm 0.94
<i>Ageneiosus ucayalensis</i>	29.78 ^b \pm 5.72	3.84 ^a \pm 1.3	22.97 ^{aA} \pm 5.00	1.67 ^a \pm 0.63
<i>Hypophthalmus marginatus</i>	10 ^c \pm 2.22	2.3 ^b \pm 1.18	5.38 ^b \pm 1.54	2.13 ^a \pm 0.72
<i>Baryancistrus xanthellus</i>	26.26 ^a \pm 6.11	4.69 ^a \pm 1.61	12.64 ^a \pm 4.94	8.65 ^b \pm 1.805
<i>Panaqolus tankei</i>	25.93 ^a \pm 8.69	4.08 ^a \pm 1.42	15.25 ^{aB} \pm 9.27	4.31 ^c \pm 1.05
<i>Peckoltia oligospila</i>	27.26 ^a \pm 6.74	5.89 ^a \pm 1.58	8.58 ^a \pm 5.64	5.89 ^b \pm 1.58

Different uppercase and lowercase superscripts in the same column indicate significant differences

PCoA showed that there were significant differences (PERMANOVA: Pseudo $F= 281.51$, $p= 0.001$) among species in relation to the thickness of the different layers (zona radiata, follicular and theca layers), in which this variation was 91.94 % explained in the first two axes (Fig 3A). The first axis corresponded to 62.36% of the data variation, where the variables that most negatively contributed to the formation of this axis were the total thickness of the layers and thickness of follicular cell layer. The second axis corresponded to 29.58% of the variation, where the variable that most contributed to this variation was the zona radiata. Through PcoA, we observed a separation into two groups, group 1 comprising *A. longimanus*, *A. ucayalensis* and *H. marginatus* and group 2 formed by the species *B. xantellus*, *P. tankei* and *P. oligospila* (Fig 3A).

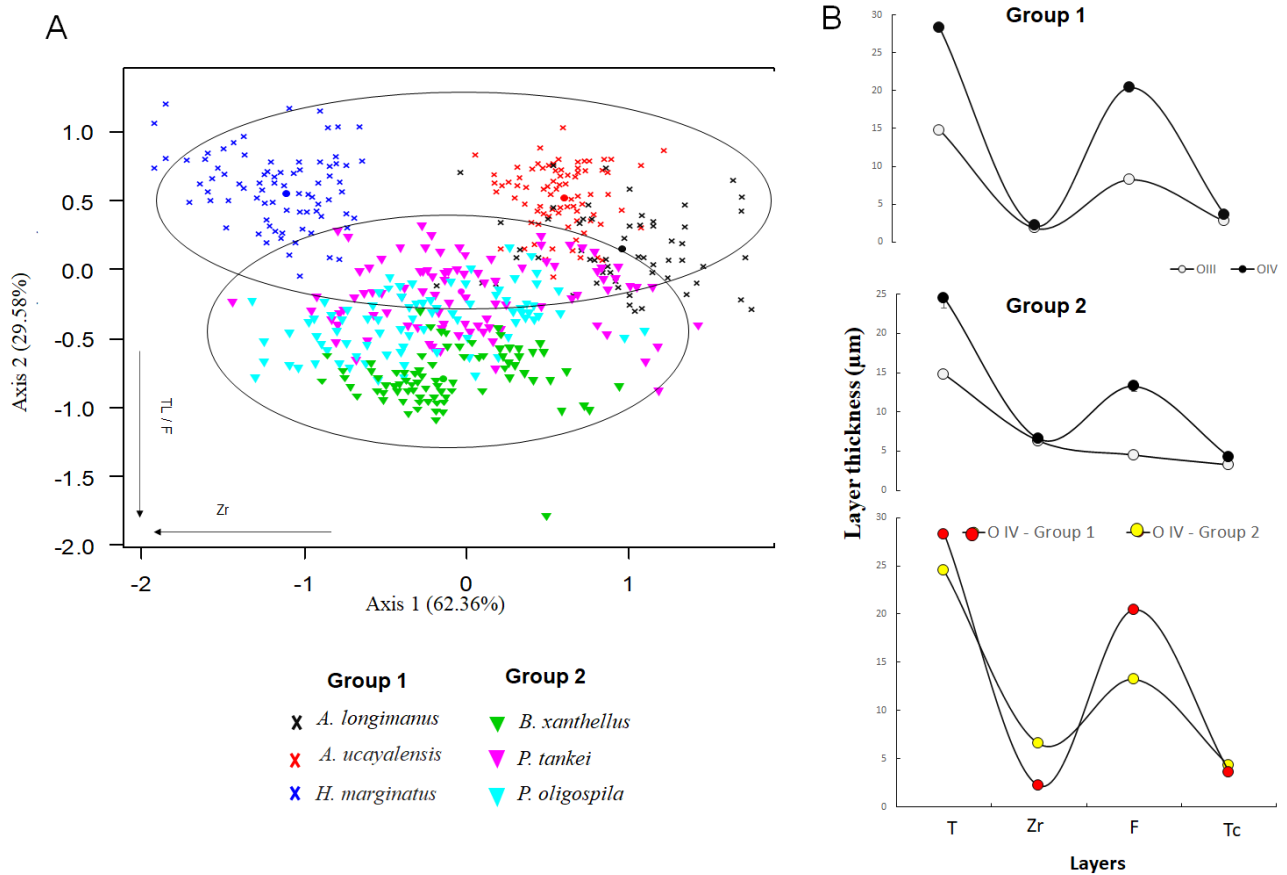


Figure 3- Graphic representation of the mean thickness of the follicular complex. (A) Principal coordinate analysis (PCoA) showing the morphological variables of the siluriform species. (B) Mean thickness of type III and IV oocytes in groups and among groups. Abbreviations: T: total layer, Tc: theca layer, F: follicular cell, Zr: zona radiata

In group 1, comparing the total thickness of the follicular complex between type III and IV oocytes, significant differences were evidenced ($U = 11.97$; $p < 0.05$); layer thickness was smaller for type III oocytes ($14.75 \mu\text{m}$) and greater for type IV oocytes ($28.55 \mu\text{m}$). In type III oocytes, the layers of zona radiata, follicular cells and theca had a mean thickness of 1.84 , 8.24 and $2.73 \mu\text{m}$, respectively. Meanwhile, in type IV oocytes, there was an increase in thickness in the zona radiata with $2.18 \mu\text{m}$, follicular cells with $20.44 \mu\text{m}$ and theca layers, with $3.60 \mu\text{m}$. In both oocytes, the greatest thickness was observed in the follicular cell layer and the smallest in the zona radiata layer. There were significant differences between layers of oocyte III and IV, zona radiata ($U = 4.43$; $p < 0.05$), follicular cells ($U = 11.72$; $p < 0.05$) and theca ($U = 6.72$; $p < 0.05$) (Fig 3B).

In group 2, the total thickness of the follicular complex also showed significant differences ($U = 18.01$; $p < 0.05$) between oocyte III ($14.75 \mu\text{m}$) and IV ($24.50 \mu\text{m}$). Type III oocytes showed thicker layers when compared to group 1, with a mean thickness of 6.24 , 4.46 and $3.21 \mu\text{m}$ for the zona radiata,

follicular cells and theca, respectively, while type IV oocytes showed 6.54, 13.26 and 4.26 μm for the zona radiata, follicular cells and theca, respectively. The highest value was found in the zona radiata layer in type III oocyte and in the type IV follicular cell layer. There was a significant difference in the zona radiata layer between the oocyte types ($U = 2.12$; $p < 0.05$); follicular cells ($U = 19.00$; $p < 0.05$) and theca ($U = 7.96$; $p < 0.05$) (Fig 3B).

We analyzed the type IV oocytes (oocyte ready for spawning) between the two groups and found that the total thickness of the follicular complex for group 1 was 28.25 μm , compared to 24.50 μm for group 2, but there was no significant difference ($U = 1.59$; $p > 0.05$). The theca and zona radiata layers were larger in group 2 (Theca: 4.26 μm ; Zona radiata: 6.54 μm) compared to group 1 (Theca: 3.60 μm ; Zona radiata: 2.18 μm). However, the follicular cell layer was thicker in group 1 (20.44 μm) than in group 2 (13.26 μm). There were significant differences for all values observed in the layers, namely zona radiata, follicular cells and theca ($U = -20.45$; $U = 5.78$; $U = -3.80$; $p < 0.05$, respectively) (Fig 3B).

Discussion

This study characterized the morphology of the follicular complex in six Siluriformes species. Through PcoA, it was possible to observe the presence of two distinct groups, group 1 formed by the species *A. longimanus*, *A. ucayalensis* and *H. marginatus*, and group 2 formed by the species *B. xantellus*, *P. tankei* and *P. oligospila*.

The species showed a significant increase in thickness in all layers that make up the follicular complex in type IV oocytes compared to type III oocytes, where there was variable thickness depending on the species. These data confirm the pattern that occurs in Siluriformes in relation to oogenesis, where the main events occur in the secondary growth phase of the oocyte, such as thickening of the layers and the total increase in cell volume^{4,21,37}.

The theca layer of the type IV oocyte of *H. marginatus* showed significant differences in the pattern of thickness and morphology between the species in the present study, unlike what is observed among teleosts, where there are no major morphological variations in this layer; its appearance is observed in the early stages of oogenesis, being one of the first layers of the follicular complex to form^{38–41}. Additionally, an extensive fibrillar network was observed in the theca layer in *A. ucayalensis* and *A. longimanus* (group 1) and *P. oligospila* (group 2). We believe that this fibrillar network assists in the remodeling of the theca layer during oocyte development. Since the cells that make up the theca layer are steroidogenic, they aid in the development of the oocyte and are surrounded by type IV collagen^{21,42,43}.

In group 1, the thickness of the follicular cell layer in type IV oocytes increased the total thickness of the follicular complex, but it was not significantly different between groups. However, there were significant differences among species. It is known that follicular cells are responsible for the conversion of hormones, mainly 17β -estradiol which helps in the maintenance of oocytes throughout ovarian development^{24,44}. In *A. ucayalensis* and *A. longimanus*, the columnar shape of the follicular cells is similar to that observed in *Trachelyopterus galeatus*⁴ and *Schizodon knerii*²⁰. Similar values for layer thickness have been observed in species of other families such as *Lophiosilurus alexandri*⁴⁵ and *Bryconops affinis*⁴⁶. During the reproductive cycle of these species, more than one spawning peak has been observed⁴⁷⁻⁴⁹. Possibly, in the present study, follicular cells were metabolically active, favoring an increase in cell volume and a rapid maturation of oocytes.

In group 2, the zona radiata of oocyte IV was thicker, with similar results being observed in representatives of the family Loricariidae as in *Ancistrus dolichopterus*, *Ancistrus dolichopterus*⁵⁰, and *Loricariichthys sp.*, *Loricariichthys platymetopon* and *Loricaria sp.*²⁶. These species display parental care, where the males carry the eggs on their abdomen until the larvae hatch, also noted in *P. tankey* which exhibits parental care¹⁷. *Baryancistrus xanthellus*, an endemic species that inhabits environments characterized by stream rapids, showed amorphous material inside the channels of the zona radiata and greater thickness of this structure. Similar characteristics were demonstrated in *H. zebra*, an endemic loricarid that inhabits stream rapids rivers and shows parental care²¹. The morphology and thickness of the zona radiata can reflect adaptations to different environments where the eggs are deposited⁵¹; this structure acts in the protection of eggs and their adhesion to different substrates²⁶. Accordingly, we can infer a relationship between the zona radiata and the environment, where the latter determines the characteristics and greater thickness of the zona radiata, besides these species displaying parental care.

Meanwhile, a thin zona radiata was observed in *H. marginatus*, which shows reproductive migration without any specialization. Although there are no reports of *A. longimanus* and *A. ucayalensis* showing reproductive migration, *A. longimanus* has a greater ability to move during feeding periods⁵². Migratory species have eggs with specific characteristics such as free and weakly adhesive eggs and even thinner zona radiata⁵³. Thus, we can suggest that the smaller thickness of the zona radiata in species of group 1 can indicate spawning with free eggs and possible migration.

Therefore, the follicular complex in mature oocytes can predict the reproductive tactics of the species under study, being evidenced by the morphological and morphometric differences between the groups (Fig 4). Highlighted in group 1 (*A. longimanus*, *A. ucayalensis* and *H. marginatus*) were the thinner zona radiata, lack of parental care and display of reproductive migration. Meanwhile, group 2

(*B. xanthellus*, *P. tankei* and *P. oligospila*) showed a zona radiata with greater thickness and specialization, parental care and sedentarism.

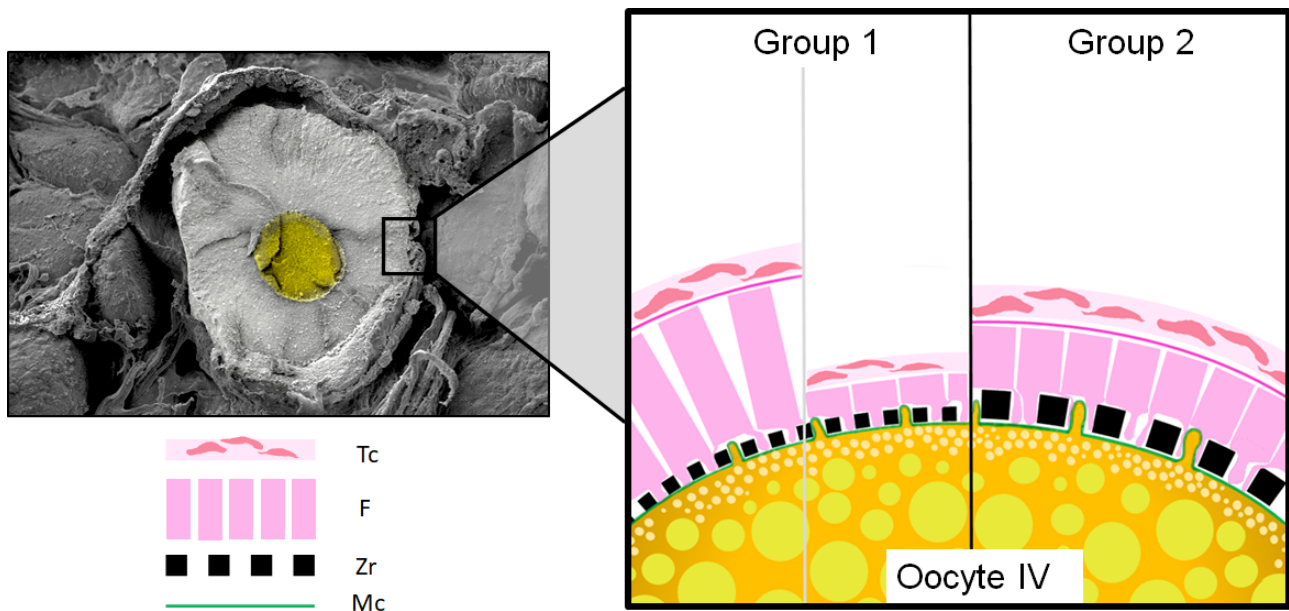


Figure 4- Schematic drawing of the main differences in the follicular complex between the groups. Abbreviations: Tc: theca layer, Zr: zona radiata, F: follicular cell and Mc: cytoplasmic membrane

Methods

Sample collection

The species were collected in the Amazon rivers (Xingu, Anapu, Tocantins, Guamá and Lago Bolonha) in 2012 and 2018 using different fishing gear (mesh net, longline, diving). Thirty specimens of mature females were collected per species, namely *Auchenipterichthys longimanus*, *Ageneiosus ucayalensis*, *Hypophthalmus marginatus*, *Baryancistrus xanthellus*, *Panaqolus tankei* and *Peckoltia oligospila*. Captured fish were anesthetized with benzocaine hydrochloride (0.1 g.L^{-1}) and euthanized with sodium pentobarbital solution (60–100 mg/kg). Subsequently, gonads were removed by ventral incision. All animal experiments were approved by the National Council for Control of Animal Experimentation (CONCEA), were performed in accordance with approved guidelines, CEUA No. 5557211118 and follows the recommendations in the ARRIVE guidelines.

Light microscopy

Mature gonad fragments for all species were fixed in Bouin's solution for 24 h. The samples were then dehydrated in increasing concentrations of ethanol, cleared in xylene and infiltrated and embedded in paraffin (Prophet et al. 1995). Sections 5 µm thick were cut and stained with hematoxylin and eosin (HE) and examined under a Carl Zeiss light microscope (AxioStar Plus 1169151).

Morphometry and statistical analysis

Four mature females were used, and measurements were taken of the total thickness of the follicular complex, theca, follicular cells and the zona radiata of 100 type III and IV oocytes per species of each group, totaling 1,200 oocytes analyzed. Serial sections were cut and the slides were evaluated under a photomicroscope with the NIS-elements BR software (4.00.07-bit), and measurements were made at 100X magnification. Measurements were performed at four different points. Means were assessed for normality using the Shapiro-Wilk test and analyzed using the Mann-Whitney (U) and Kruskal-Wallis (H) test ($P < 0.05$) (Zar, 1999). All analyses were performed using the Core Team Program (2016).

Total thickness and layer thickness in relation to the species group were analyzed using principal coordinate analysis (PCoA)⁵⁴, Permutation analysis (Permanova) was used to test whether the variation observed in the graph was significant⁵⁵. All analyses were performed using the R Development Core Team Program (2016).

Scanning electron microscopy (SEM).

Fragments of ovaries were fixed in Karnovsky's solution (4% paraformaldehyde, 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4) for 24 h. After fixation, the fragments were washed in 0.1 M sodium cacodylate buffer, pH 7.4 and post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.4 for 2 h. For SEM analysis, the specimens were dehydrated in a graded ethanol series (30 to 100%) and critical-point dried using CO₂. Fragments of ovaries were mounted on stubs, coated with gold and examined using a LEO 1430 and TESCAN Mira3 scanning electron microscope.

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Author contributions

I.K.S.V., M.A.P.F. and R.M.R. conceived and designed the study. I.K.S.V. planned the study, analyzed and interpreted the data and wrote the manuscript. I.K.S.V., Y.A.M, L.A.B.G and B.R.M.S reviewed data analysis and contributed to the revision of the manuscript. Y.A.M, B.R.M.S and L.A.B.G carried out the field and laboratory activities. M.A.P.F. and R.M.R. contributed reagents/materials/analysis tools. All authors read and approved the final paper.

Additional Information

The authors declare no competing interests.

CONSIDERAÇÕES FINAIS

Estudos sobre a biologia básica das espécies como a estrutura testicular, espermatogênese e a análise do complexo folicular nos permitem entender muitos aspectos da biologia reprodutiva dos peixes. Através dos resultados do capítulo 1, observamos que apesar de espécies pertencerem a uma mesma família e por conseguinte subfamília existe muitas diferenças ao longo do desenvolvimento testicular, o que conferiu uma maior relação entre *B. xantheus* e *P. oligospila*, enquanto *H. zebra* possui características que diferem das demais. Além disso, no capítulo 2 foram evidenciados dois grupos: 1- formado por *A. longimanus*, *A. ucayalensis* e *H. marginatus* apresenta zona radiata mais fina, não apresenta o cuidado parental e possui migração reprodutiva e o grupo 2- formado por *B. xanthellus*, *P. tankei* e *P. oligospila*, apresentou zona radiata com maior espessura e canais numerosos possui cuidado parental e sedentarismo. Esses resultados contribuem para entender a espermatogênese e a morfologia do complexo folicular das espécies de peixes tropicais, além de serem dados importantes para a filogenia dos loricarídeos e traçar relações entre a morfologia oocitária e a estratégia reprodutiva nos peixes.