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**CARACTERIZAÇÃO MORFOLÓGICA E IMUNO-HISTOQUÍMICA DE
RECEPTORES HORMÔNIO-ESTEROIDAIS NO SISTEMA REPRODUTIVO DE
MACHOS DE *Potamotrygon wallacei* (Chondrichthyes - Potamotrygoninae)**

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Sinopse:

Esta tese investigou as células esteroidogênicas de testículos maduros de *Potamotrygon wallacei* e seu potencial na produção de testosterona. Além disso, traz informações sobre a morfologia e a imunolocalização de receptores de progesterona dos ductos anexos do sistema reprodutivo, mostrando a potencial função deste hormônio na maturação espermática.

Palavras-chave: Célula de Leydig, célula de Sertoli, espermatozoide, testosterona, progesterona

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*Let us leave the past, live the present, create our future
and always move forward in the direction of our dreams!*

Vasanth B. Pai

RESUMO

A arraia cururu *Potamotrygon wallacei* é endêmica da bacia do médio Rio Negro e a menor espécie entre os potamotrygoníneos. Machos apresentam atividade reprodutiva durante o período de cheia e vazante do rio. Estudos prévios mostraram a influência dos hormônios esteroides no desenvolvimento gonadal e dos ductos anexos de machos adultos. A testosterona (T) se apresenta como o principal esteroide no controle do desenvolvimento testicular e a produção de espermatozoides. Por outro lado, a progesterona (P_4) parece agir durante a espermatogênese e nos processos da maturação e armazenamento espermático. No entanto, essas associações morfológicas e hormonais foram realizadas correlacionando os níveis dos esteroides no plasma com as mudanças morfológicas observadas, sem atribuir uma função clara as células esteroidogênicas e o real alvo dos esteroides. Nesse contexto, a presente tese investigou a ultraestrutura do sistema reprodutivo de machos maduros explorando ao longo de dois capítulos a presença e capacidade das células esteroidogênicas na produção de T nos testículos. Além disso, analisou a relação da P_4 com os ductos anexos para maturação espermática e formação de agregados espermáticos em machos de *P. wallacei*. Foram obtidas amostras do sistema reprodutivo de machos adultos em diferentes estágios reprodutivos e processadas para análises de sua ultraestrutura e análises de imuno-histoquímica. No primeiro capítulo foi analisada a ultraestrutura das células esteroidogênicas e a imunolocalização da enzima 17β -hidroxiesteroide desidrogenase tipo 3 (17β -HSD3) para observar a capacidade destas células na produção de T. No segundo capítulo foi descrita a morfologia dos diferentes ductos anexos do sistema reprodutivo, analisando os receptores de progesterona (PR) e relacionando-os com os processos de maturação espermática e a formação de agregados espermáticos. Por fim, os resultados obtidos nessa tese ampliaram o conhecimento sobre os processos endocrinológicos envolvidos na reprodução das arraias neotropicais de água doce (subfamília Potamotrygoninae). Estes dados, poderão ajudar a elucidar o mecanismo reprodutivo nestas espécies, ampliando as informações sobre a endocrinologia reprodutiva dos elasmobrânquios.

Palavras-chave: arraias de água doce; esteroidogênese; células de Leydig; célula de Sertoli; agregados espermáticos; morfologia; testosterona; progesterona.

ABSTRACT

The cururu Stingray *Potamotrygon wallacei* is endemic to the Middle Rio Negro basin and the smallest species among the potamotrygonins. Males present reproductive activity during the high and descending waters of the river. Previous studies have shown the influence of steroid hormones on gonadal and attached ducts development in adult males. Testosterone (T) is the primary steroid controlling testicular development and sperm production. On the other hand, progesterone (P₄) seems to act during spermatogenesis and in the processes of maturation and sperm storage. However, these morphological and hormonal associations were performed by correlating the levels of steroids in plasma with the observed morphological changes without attributing a clear function to the steroidogenic cells and the real target of the steroids. In this context, this thesis investigated the ultrastructure of the reproductive system of mature males, exploring over two chapters the presence and capacity of steroidogenic cells in the production of T in the testis. In addition, analyzed the relation of P₄ to attached ducts for sperm maturation and sperm aggregates formation in *P. wallacei* males. Samples of the reproductive system of adult males in two different reproductive stages were obtained and processed for analysis of their ultrastructure and immunohistochemical analyses. In the first chapter, the ultrastructure of steroidogenic cells and the immunolocalization of the enzyme 17-beta-hydroxysteroid dehydrogenase type 3 (17 β -HSD3) were analyzed, observing the ability of these cells to T production. The second chapter describes the morphology of the different accessory ducts of the reproductive system, analyzing the progesterone receptors (PR) and relating them to sperm maturation processes and the formation of sperm aggregates. Finally, the results obtained in this thesis expanded the knowledge about the endocrinological processes involved in the reproduction of Neotropical freshwater stingrays (subfamily Potamotrygoninae). These data may help to elucidate the reproductive mechanism of this species, expanding information on the reproductive endocrinology of elasmobranchs.

Keywords: freshwater stingrays; Leydig cells; morphology; progesterone; steroidogenesis; Sertoli cell; sperm aggregates; testosterone.

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Introdução Geral

Os elasmobrânquios apresentam uma ampla variedade de estratégias reprodutivas, podendo ser encontradas espécies vivíparas e ovíparas, com sistemas reprodutivos que variam amplamente (Wourms, 1977; Pratt, 1988). As variações nos sistemas e modos reprodutivos têm relação com o tipo de estratégia que as espécies utilizam para obter o seu sucesso reprodutivo, apresentando ciclos reprodutivos igualmente diversos, como contínuos, anuais com um ou dois períodos de atividade reprodutiva, além de ciclos anuais ou bianuais definidos (Wourms, 1977).

Com a diversidade dos ciclos e sistemas reprodutivos, se preconiza que os elasmobrânquios desenvolveram um sistema essencial para a regulação da esteroidogênese no controle da reprodução (Koob e Callard, 2005). Alguns estudos neste grupo mostraram a função do sistema endócrino na regulação do eixo hipotálamo-hipófise-gônadas (HHG), demonstrando a existência de produção de gonadotrofinas (GTH) e seu papel na produção de hormônios, como o $\beta 1$ análogo do hormônio folículo estimulante (FSH) associado às fases iniciais do crescimento das gônadas, e o $\beta 2$ análogo do hormônio luteinizante (LH), que atua na regulação dos eventos maturacionais (Awruch, 2013).

Em testículos de elasmobrânquios, as células de Sertoli são apresentadas como a fonte principal de esteroides (Pudney e Callard, 1985; Callard, 1992; Koob e Callard, 1999). Estas células estão localizadas dentro dos espermatocistos (unidades funcionais do testículo) associadas à linhagem germinativa (Wourms, 1977), controlando a proliferação de células germinativas, produção de hormônios, alongamento da espermatíde e a espermição (Tyminski et al., 2015). As células de Sertoli incrementam as atividades biossintéticas nas zonas mitóticas para as meióticas, regulando a produção de andrógenos (Callard et al., 1985) e consequentemente a progressão espermatogênica (Engel e Callard, 2005; Tyminski et al., 2015).

As células de Leydig são responsáveis pela biossíntese e secreção de andrógenos, principalmente testosterona (Zirkin e Papadopoulos, 2018; Zhou et al., 2019). Reguladas pelo LH, estas células metabolizam o colesterol em testosterona regulando os níveis de expressão de enzimas envolvidas na esteroidogênese (Zirkin e Papadopoulos, 2018). No entanto, a participação ou presença das células de Leydig em elasmobrânquios tem sido questionada (Pudney e Callard, 1984; Koob e Callard, 1999; Awruch, 2016).

Autores como Pudney e Callard, (1985) contestam a presença dessas células nos testículos de tubarão galhudo (*Squalus acanthias*), no entanto, sugerem a presença de células

análogas (Leydig-like) no interstício dos espermatocistos sem um evidente potencial para a produção de esteroides. Alguns estudos desmitificam que os elasmobrânquios não apresentam estas células e pelo contrário, vêm demonstrando sua participação ativa na produção de andrógenos, incluindo a testosterona para a regulação da espermatogênese (Prisco et al., 2002; Prisco et al., 2008; Kassab et al., 2009; Tyminski et al., 2015).

Em células Leydig-like, células análogas as de Leydig do tubarão martelo *Sphyrna tiburo*, receptores de andrógenos foram encontrados e associados a estas (Tyminski et al., 2015). Kassab et al. (2009) e Gewaily et al. (2021), demonstraram a progressão da glicosilação no decorrer da espermatogênese em tubarão bambu *Chiloscyllium punctatum*, usando diversos receptores de lectinas (HRP), comprovando a associação de diferentes lectinas às células de Leydig, o que pode indicar atividade androgênica. Para representantes de batóides, como a raia torpedo *Torpedo marmorata*, Prisco et al. (2002) identificaram a presença de células de Leydig no espaço intersticial entre os espermatocistos. Prisco et al. (2008) descreveram o potencial esteroidogênico destas células, localizando imunologicamente enzimas chave para a produção de esteroides como a 3-beta-hidroxiesteroide desidrogenase (3 β -HSD) e a 17-beta-hidroxiesteroide desidrogenase (17 β -HSD).

A enzima 3 β -HSD catalisa a conversão da dehidroepiandrosterona em androstenediona (4-diona), o precursor imediato da testosterona (Labrie et al., 1997). Por outro lado, a 17 β -HSD controla a última etapa na formação de todos os andrógenos e todos os estrogênios (Andersson et al., 1995; Labrie et al., 1997). Esta enzima apresenta pelo menos quatro isoenzimas nos testículos com expressão específica da célula, do substrato e dos mecanismos de regulação e atividade catalítica redutora ou oxidativa (Labrie et al., 1997; Mindnich et al., 2005; Zhang et al., 2021). O tipo 1 catalisa a interconversão do 17 β estradiol. O tipo 2 degrada o 17 β estradiol em estrona e a testosterona em androstenediona. Características semelhantes apresenta o tipo 4, degradando o estradiol em estrona e androst-5-eno-3 β ,17 β -diol em dehidroepiandrosterona (DHAE). Finalmente, a 17-beta- hidroxiesteroide desidrogenase tipo 3 (17 β -HSD3) controla a conversão de androstenediona em testosterona (Labrie et al., 1997; Saloniemi et al., 2012; Zhang et al., 2021), sendo este tipo principalmente encontrada nas células de Leydig (Ge et al., 2006).

A compreensão da produção de esteroides nas gônadas de raias e tubarões tem se fundamentado em associações entre o desenvolvimento gonadal e do sistema reprodutivo (histologia) e o perfil plasmático de hormônios esteroides (Manire e Rasmussen, 1997; Maruska et al., 1996; Koob e Callard, 1999; Tricas et al., 2000; Sulikowski et al., 2004). Por exemplo, nos testículos as células de Sertoli, as células de Leydig e, possivelmente, as células

germinativas, contribuem para a síntese de esteroides, onde a testosterona e o estradiol possuem um papel importante no desenvolvimento e maturação dos espermatocistos, enquanto a progesterona atua como precursor dos hormônios androgênicos (Engel e Callard, 2005).

Além de controlar a espermatogênese, os esteroides produzidos nos testículos regulam os ductos extra testiculares (epidídimos, glândulas de Leydig, ductos deferentes e vesículas seminais) (Tricas et al., 2000; Marcon et al., 2021). Esses ductos além de transportarem e armazenarem o esperma, tem a função de nutrir e capacitar os espermatozoides, processo chamado de maturação espermática (Dzyuba et al., 2018; Dzyuba et al., 2019), que permite que os espermatozoides adquiram capacidade fecundante durante seu trânsito até a vesícula seminal (Dzyuba et al., 2018).

Entretanto, a nutrição e capacitação espermática dos elasmobrânquios envolve não só a secreção dos ductos extra testiculares (Dzyuba et al., 2018), também depende da formação de agregados espermáticos (Pratt e Tanaka, 1994). Os agregados espermáticos podem ser do tipo espermatozeugmata, que consistem na agrupação lateral de espermatozoides associados a uma matriz composta por secreção dos ductos e citoplastos de células de Sertoli constituídos por fragmentos citoplasmáticos que apresentam organelas associadas a produção de esteroides (Pudney e Callard, 1986; Jones e Lin, 1993; Jones e Hamlett, 2006); ou tipo espermatóforo nos quais os espermatozoides são agrupados aleatoriamente e incorporados por uma matriz (Pratt e Tanaka, 1994).

As características dos agregados espermáticos se somam à capacidade do esperma dos elasmobrânquios de sintetizar hormônios esteroides. Os espermatozoides além de possuírem receptores de andrógenos (Tyminski et al., 2015), se assegura que o esperma pode sintetizar esteroides por meio de enzimas para converter o colesterol em 11-desoxicorticosterona via pregnenolona e progesterona (Simpson et al., 1964; Jones et al., 1984).

Contudo, os diferentes estudos envolvendo endocrinologia reprodutiva ainda são limitados e generalizados a apenas algumas espécies de elasmobrânquios. Estas informações se apresentam como base para o entendimento do sistema endócrino na regulação fisiológica da reprodução. No entanto, em potamotrigoníneos ainda há controversias sobre a presença e funcionalidade das células esteroidogênicas, principalmente as células de Leydig, questionando o controle hormonal neste grupo de elasmobrânquios. Até o presente momento, apenas alguns estudos envolvendo endocrinologia reprodutiva de machos desta subfamília foram conduzidos em uma única espécie, *Potamotrygon wallacei* (Morales-Gamba, 2018; Marcon et al., 2021; Morales-Gamba et al., 2021).

Potamotrygon wallacei Carvalho Rosa e Araújo 2016 é uma arraia endêmica da bacia do médio Rio Negro, vivípara matotrófica com histotrofia lipídica, que atinge maturação sexual por volta dos dois anos de vida, com larguras de disco em torno de 160 mm (Araújo, 1998; Carvalho et al., 2016). Os machos possuem um par de testículos, que histologicamente apresentam lóbulos diametricamente dispostos, compostos por espermatocistos que se desenvolvem de forma radial (Araújo, 1998; Morales-Gamba, 2018; Marcon et al., 2021). Os testículos estão ligados por meio dos epidídimos aos ductos anexos: glândulas de Leydig e vesículas seminais, onde ocorre a nutrição e armazenamento espermático (Araújo, 1998; Zaiden et al., 2010; Morales-Gamba, 2018; Marcon et al., 2021).

Esta espécie apresenta alta fidelidade às áreas de floresta inundada (igapós), sendo este um habitat essencial para a reprodução e alimentação da mesma (Shibuya et al., 2009; Carvalho et al., 2016). Esta grande afinidade ao igapó, parece incidir no perfil hormonal de *P. wallacei*, uma vez que a sincronização que esta espécie apresenta com o nível do Rio Negro se correlaciona com a produção dos hormônios esteroides, principalmente, progesterona e testosterona (Morales-Gamba, 2018; Marcon et al., 2021; Morales-Gamba et al., 2021).

Nos machos de *P. wallacei*, a testosterona está correlacionada com o desenvolvimento gonadal e do trato reprodutivo. Sem correlações claras, a produção de estradiol, que segue o comportamento da testosterona, pode participar no desenvolvimento de diversas estruturas do sistema reprodutivo. Altas concentrações de progesterona também foram encontradas, sugerindo a importância deste hormônio na produção de testosterona e nos processos espermiogênicos e da maturação espermática (Morales-Gamba, 2018; Morales-Gamba et al., 2019; Marcon et al., 2021).

Em potamotrigoníneos, descrições da morfologia testicular são escassas. Em *P. wallacei* Zaiden et al. (2010) usando histologia com inclusão em parafina não descreveram a presença destas células na composição da estrutura testicular. Em *Potamotrygon magdalenae*, usando a mesma técnica analítica, Pedreros-Sierra e Ramirez-Pinilla (2015) citam a presença de células de Leydig no interstício dos espermatocistos, sem, no entanto, atribuírem uma função clara as mesmas. Entretanto, o uso desse tipo de técnica, podem levar a interpretações equivocadas sobre a identificação e a localização destas estruturas (Pudney e Callard, 1985; Engel e Callard, 2005). Sem evidências claras da presença de células de Leydig nos potamotrigoníneos, surge a questão se de fato estas células existem em *P. wallacei* e qual seria o seu envolvimento na produção de hormônios esteroides.

O conhecimento sobre o controle endócrino dos hormônios esteroides em elasmobrânquios ainda é incipiente e, em muitos casos especulativos, uma vez que as

diferenças nos sistemas e modos reprodutivos são evidentes. Tendo em conta o estudo prévio sobre o perfil dos hormônios sexuais em *P. wallacei* por Morales-Gamba (2018) e Marcon et al. (2021), surge a necessidade de realizar análises de ultraestrutura testicular, para a identificação das células esteroidogênicas. Além disso, é necessário entender o potencial da progesterona nos ductos do sistema reprodutivo dos machos desta espécie, uma vez que este esteroide apresenta uma possível relação com a maturação espermática.

Nesse contexto, a presente tese investigou as células esteroidogênicas testiculares e caracterizou, através de dados morfológicos e de imuno-histoquímica, o sistema reprodutivo de machos maduros de *P. wallacei*. Os resultados alcançados encontram-se organizados em dois capítulos:

- **Capítulo 1** apresenta a descrição morfológica da ultraestrutura das células esteroidogênicas de *P. wallacei* e mostra o seu potencial na produção de testosterona.
- **Capítulo 2** proporciona informações sobre os receptores de progesterona nos ductos extra testiculares de *P. wallacei* e traz associações deste hormônio com a maturação espermática e a formação dos agregados espermáticos.

Capítulo 1

Morales-Gamba, R.D., Araújo, M.L.G., Rêgo, M.G., Barcellos, F.M., Dias, L.C., Landines-Parra M.A., Jaydione, L.M. **Ultrastructure of steroidogenic cells and immunolocalization of 17-beta-hydroxysteroid dehydrogenase type 3, in mature testes of *Potamotrygon wallacei* (Chondrichthyes - Potamotrygoninae).**

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Ultrastructure of steroidogenic cells and immunolocalization of 17-beta-hydroxysteroid dehydrogenase type 3 (17 β -HSD3) in mature testes of *Potamotrygon wallacei* (Chondrichthyes - Potamotrygoninae)

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Highlights

- Leydig cells are present in *P. wallacei* testicles and can produce testosterone.
- Sertoli cells present 17 β -HSD3 enzymes contributing for testosterone production.
- Changes in 17 β -HSD3 expression occur in Sertoli cells during spermatogenesis.

Abstract

In elasmobranchs, spermatogenesis is regulated by sex steroids secreted by Sertoli and Leydig cells present in the testis. However, in South American freshwater stingrays, knowledge regarding the presence or contribution of these cells in this process is still limited. This study aimed to describe the ultrastructure of the steroidogenic cells in mature testes of *Potamotrygon wallacei* and examine the localization of 17-beta-hydroxysteroid dehydrogenase type 3 (17 β -HSD3) as a marker of the site of testosterone (T) production. Using transmission electron microscopy and polyclonal antibodies, we examined the steroidogenic cells of *P. wallacei* testes in three different reproductive stages. The ultrastructural study showed the presence of Sertoli cells and Leydig cells, which boast a typical organelle associated with steroid production. The Sertoli cells change their morphology according to the spermatogenic stage, thus increasing the organelles needed for steroid production from mitotic to spermiogenic zones. Sertoli cells show immunoreactivity to 17 β -HSD3 and have the ability to produce T in the spermatogonia, spermatid and spermatozoan zones. In the spermatogonia cyst of all the analyzed males, the reactivity of 17 β -HSD3 was observed, which suggests that spermatogonia, in association with Sertoli cells, can produce testosterone in males with the absence of spermiogenic zones, as seen in regressing males. The Leydig cells showed reactivity to 17 β -HSD3 in all the analyzed males. These findings demonstrated the active participation of Leydig, Sertoli and spermatogonia cells in T production in *P. wallacei* testes.

Keywords: freshwater stingrays, Leydig cell, Sertoli cell, spermatocyte, spermatogenesis, spermiogenesis.

1.0. Introduction

Spermatogenesis is regulated by sex steroids secreted from steroidogenic cells in the vertebrate testes. Enzymes, such as 17-beta-hydroxysteroid dehydrogenase (17 β -HSD), control the intracellular concentration of sex steroids (Labrie et al., 1997). In the testes, this enzyme has four series of isoenzymes with individual cell-specific expression, substrate specificity, and regulatory mechanisms (Labrie et al., 1997). Type 1 catalyzes the interconversion of 17 β estradiol, and type 2 degrades 17 β estradiol into estrone and testosterone (T) into androstenedione. Type 4 has similar characteristics to type 2, and degrades the estradiol into estrone and androst-5-ene-3 β , and 17 β diol into dehydroepiandrosterone. Finally, 17-beta-hydroxysteroid dehydrogenase type 3 (17 β -HSD3) controls the conversion of androstenedione into T (Labrie et al., 1997; Saloniemä et al., 2012; Zhang et al., 2021), and plays a key role in the production of spermatozoa.

In vertebrates, T is an essential steroid that regulates spermatogenesis and the production of mature sperm (Smith and Walker, 2014). This androgen is produced by the Leydig cells present in the interstitial space between the seminiferous tubules of the testes (Ge et al., 2006). In contrast, in elasmobranchs, the Sertoli cells are the main site for the production of steroids, including T (Callard et al., 1985; Callard et al., 1989). However, controversial studies suggest the absence of functional Leydig cells in elasmobranchs (Callard et al., 1985; Callard et al., 1989).

Despite the advances in our knowledge regarding steroidogenic cells in the testes of elasmobranchs, what we do know is still limited to only a few marine species (Callard et al., 1985; Callard et al., 1989; Prisco et al., 2008; Tyminski et al., 2015). More detailed studies have demonstrated the presence and active participation of Leydig cells or analogous (Leydig-like cells) cells in the control of steroidogenesis (Prisco et al., 2002; Prisco et al., 2008; Tyminski et al., 2015).

In the marbled torpedo ray (*Torpedo marmorata*), activity of 3-beta-hydroxysteroid dehydrogenase (3 β -HSD) and 17 β -HSD has been observed in the Sertoli, Leydig cells, and spermatogonia cells, but with distinct actions during spermatogenesis: the Leydig cells participated in the meiosis phases, while the role of Sertoli cells were more evident in the mitosis and spermiogenesis phases (Prisco et al., 2008). In the bonnethead shark (*Sphyrna tiburo*), androgen receptors are expressed in multiple cell types of the testes, including Leydig-like cells, Sertoli cells, and peritubular myoid cells (PMCs), thus demonstrating the crucial roles of androgens in regulating spermatogenesis (Tyminski et al., 2015).

In potamotrygonins, the only freshwater-restricted group of stingrays in South America (Carvalho et al., 2016b), the presence and functionality of Leydig cells are unclear. Although there are currently more than 38 described species of potamotrygonins (Fontenelle et al., 2021), studies involving morphological aspects of the testes are scarce. Pedreros-Sierra and Ramirez-Pinilla (2015) mention the presence of Leydig cells in the interstitium of spermatocysts of *Potamotrygon magdalenae*; however, they did not assign them a clear function. On the other hand, the presence of Leydig cells is not mentioned in the description of the testicular structure of *Potamotrygon wallacei* (Zaiden et al., 2010; Marcon et al., 2021). These studies involved conventional histology, and this technique can lead to misinterpretations about the identification and location of some cellular structures (Pudney and Callard, 1985; Engel and Callard, 2005).

The cururu stingray (*Potamotrygon wallacei* Carvalho, Rosa and Araújo 2016), is an endemic species of the Rio Negro basin (Carvalho et al., 2016b). Males have paired oval compound type testes (Pratt, 1988) associated with an epigonal organ (Marcon et al., 2021). The testicular surface has a series of lobes that are developed diametrically and are composed of spermatocyst that develop radially from the germinal zone to the periphery (efferent duct area) (Zaiden et al., 2010; Marcon et al., 2021). Males of this species have limited periods of reproductive activity during high and falling water periods of the flood pulse of the middle Rio Negro basin (Charvet-Almeida et al., 2005; Marcon et al., 2021). During spermatogenesis, the male stingrays show increases in testosterone plasma levels during the progress of spermatogenesis (Marcon et al., 2021).

Therefore, considering the lack of knowledge regarding the control of steroidogenesis in male potamotrygonins and the lack of a clear evidence of the presence and functionality of Leydig cells, this study aimed to describe the ultrastructure of the steroidogenic cells in the testes of *P. wallacei*, and corroborate the existence and active functionality of the Leydig cells by examining the immunolocalization of 17 β -HSD3 as a marker of the T production site during spermatogenesis in this Amazonian stingray species.

2. Material and methods

2.1. Samples

In order to maximize the use of sampled biological material of this endemic stingray, a single set of testes previously collected and analyzed by Marcon et al. (2021), was used. This

study correlated the plasma steroid hormone levels with the morphological aspects of the reproductive tract of *P. wallacei* males.

Eight males with mean disk width (mean $\pm$ SEM) of 196.5 ± 0.69 mm, with calcified clasper lengths of 58.7 ± 0.12 mm were captured at night by professional fishermen using a typical hand net (rapiché) and headlight, during the flooding period January to May 2017 at Igarapé Zamula ($0^{\circ}50'28''\text{S}$, $62^{\circ}46'5''\text{W}$), a tributary of the middle Rio Negro basin (Barcelos, Amazonas, Brazil). Subsequently, were anesthetized via immersion in a eugenol solution (75 mg L⁻¹; Lima et al., 2021), and then euthanized by spinal section for the removal of the reproductive system. Specimens were classified at three maturational stages: regression (n = 2), capable to reproduce (n = 2), and active (n = 4).

This investigation had prior authorization (SISBIO No. 18285–1) from the Chico Mendes Institute for Biodiversity Conservation (ICMBio) - Ministry of the Environment, and was conducted in accordance with the guidelines of the Ethics Committee for the Use of Animals in Research of the Federal University of Amazonas (Protocol CEUA/UFAM #002/2017).

2.2. Transmission electron microscopy

Fragments of the testes were fixed in Karnowsky solution for 24 h. Subsequently, samples were treated with 1% osmium tetroxide for 2 h in the dark, contrasted with an aqueous solution of 5% uranyl acetate for a further 2 h, dehydrated and then finally embedded in resin (Durkupan, ACM Fluka, Switzerland). Thin sections of the testes were cut using a ultramicrotome (Leica UC7, Leica Microsystems-Wetzlar, Germany), stained with uranyl acetate, and their morphological features documented via a transmission electron microscope (JEM-1400, JEOL, Tokyo, Japan) at 80 kV.

2.3. Histological and Immunohistochemistry analysis

Testes previously fixed in Bouin solution were dehydrated in a graded series of alcohol (70–100%), cleaned in xylene and processed for routine paraffin histology. Sections of 5 μm were prepared using a microtome (Leica RM 2125RT, Leica Microsystems-Wetzlar, Germany) and subsequently, stained with hematoxylin-eosin (HE).

For the immunohistochemical analysis, sections of testes were adhered to microscope slides coated with 0.01% poly-L-lysine (Sigma Chemical Co., St. Louis, USA). The deparaffinization, rehydration, and unmasking epitopes were carried out by boiling the slides in a pressure cooker containing Trilogy™ (Cell Marque Corporation, Rocklin, USA) for 15

min. For the inhibition of the peroxidase activity, sections were treated with 3% hydrogen peroxide for 30 min in the dark. After a TBS-T buffer rinse, the slides were treated with a protein blocker (3% non-fat powdered milk in TBS) for 2 hours, and then incubated with the primary antibody (17 β -HSD3 Polyclonal Antibody; Thermo Scientific Pierce, Rockford, USA, product number PA5-30063) diluted to 1:100 and left for 24 h at room temperature. Negative control sections were treated with TBS instead of the primary antibody (Figure S1). Positive controls were performed on sections of mice testes, whose Leydig cells test positive for a histochemical reaction for 17 β -HSD3 (Figure S2) (Sipilä et al., 2020). After a 15-min rinse in TBS-T, antibody-antigen complexes were revealed with EnVision+/HRP, Dual Link Rabbit/Mouse kit (Dako) for 1.5 hours and the 3,3'-diaminobenzidine (DAB) was used as chromogen (Pierce Biotechnology, Rockford, USA) following the manufacturer's instructions. Counterstaining was performed with Mayer's hematoxylin. For analysis and photographic documentation, an optical microscope (Leica DM500, Leica Microsystems, Wetzlar, Germany) with an integrated camera (Leica ICC50W, Leica Microsystems, Wetzlar, Germany) was used.

3.0. Results

3.1. Gonadal morphology

Potamotrygon wallacei males present paired oval testes associated with an epigonal organ, which are situated ventrally to the vertebral column and suspended by the mesorchium. Histologically, the testicular surface shows a compound testis with a series of lobules that develop diametrically. In each lobule, spermatocysts with somatic Sertoli cells and germ cells, which develop radially from the germinal zone to the efferent duct area, were found (Figure 1). Spermatocysts were surrounded by peritubular myoid cells (PMCs) (Figure 2). The interstitium among the spermatocysts was comprised by loose connective tissue, Leydig cells, and blood vessels (Figure 2-3).

The spermatogenesis is developed in zones containing spermatocysts composed of the same type of germ cells. Five zones were analyzed, namely the germinal, spermatogonia, spermatocyte, spermatid, and spermatozoa zones (Figure 1A, B). The germinal zone has cysts with germ cells accompanied by developing pre-Sertoli cells (Figure 1C; 2A, B). In mitotic zones, the spermatogonia zone was formed by cysts containing spermatogonia undergoing division, and Sertoli cells positioned at the center or the periphery; the spermatocysts may contain a lumen that eventually disappears as the mitotic division continues (Figure 1D). The spermatocyte zone was composed of cysts containing spermatocytes at the same stage of

meiotic division: spermatocyte I and spermatocyte II. The spermatocyte I complete the first meiotic division and become smaller spermatocyte II, always accompanied by Sertoli cells (Figure 1E).

The spermiogenesis zones were classified into spermatid and spermatozoan zones. The spermatid zone consisted of spermatocysts containing spermatids in different phases. These cells change from being round to being spiral in shape, and their nuclei are associated with Sertoli cells (Figure 1F). Finally, the completion of spermiogenesis occurs in the spermatozoid zone that presents groups of spermatozoa associated with large peripheral Sertoli cells (Figure 1G).

3.2. Peritubular Myoid cells (PMCs)

The PMCs presented an elongated elliptical nucleus with perinuclear chromatin clumps (Figure 2A, C, D). The cytoplasm contains a rough endoplasmic reticulum (RER), mitochondria with tubular cristae, glycogen particles, and various filaments commonly arranged in bundles in a parallel arrangement (Figure 2D). These filaments surround and are attached to the basal lamina of the spermatocyst (Figure 2D). Immunoreactivity with 17 β -HSD3 was not evident in these cells.

3.3. Sertoli cells

The ultrastructural characteristic of the Sertoli cells of adult *P. wallacei* males changed drastically from mitotic to spermiogenic zones (Figure 2). In the germinal zone, pre-Sertoli cells develop a small nucleus with an irregular form and cytoplasm with some organelles such as mitochondria and RER (Figure 2A). In cysts of spermatogonia and spermatocyte zones, the nucleus of Sertoli cells has an indented shape (irregular form), with a prominent nucleolus and perinuclear chromatin clumps (Figure 2A-C). The cytoplasm displays a reduced number of organelles engaged in steroid hormone production, such as the smooth endoplasmic reticulum (SER) and mitochondria with tubular cristae (Figure 2B).

In the spermatogonia zone, a positive reaction to 17 β -HSD3 was evident at the Sertoli cell and spermatogonia levels (Figure 4A). No positive reaction to 17 β -HSD3 was evident at the level of Sertoli cells in a cyst containing spermatocytes (Figure 4B). Spermatogonia was the only germinal cell that presented immunoreactivity; it presented steroidogenic organelles such as SER, and mitochondria with tubular cristae and lipid droplets (Fig 5).

In the spermatid and spermatozoa zones, the Sertoli cell nucleus was regular and oval-shaped (Figure 2G, H). Their cytoplasm contains abundant SER, mitochondria with tubular

cristae, Golgi complexes, and lipid droplets (Figure 2H). In these zones, the Sertoli cells showed immunoreactivity to 17 β -HSD3 (Figure 4C, D).

3.4. Leydig cells

The Leydig cells were observed in all spermatogenic zones of interstitial space (Figure 2D; Figure 3). Leydig cells appear round to triangular-shaped, with a size of ~6 μ m (Figure 3). Their nucleus is round and presents big patches of heterochromatin (Figure 3), and the cytoplasm shows SER, lipid droplets, and mitochondria with tubular cristae (Figure 3B). The SER was composed of tubular elements with vesicles distributed throughout the cytoplasm (Figure 3B). The immunohistochemical analysis showed Leydig cells having immunoreactivity to 17 β -HSD3 in the spermatogonia, spermatocyte and spermiogenic zones (Figure 4).

4.0. Discussion

This study investigated the ultrastructure of the steroidogenic cells of the testes of *P. wallacei* and demonstrated that, as in other vertebrates (Grier 1981; Zhou et al., 2019), the Leydig cells are present in the testes of this freshwater stingray species. Our findings are of particular significance since the presence of Leydig cells has been questioned in the past (Pudney and Callard, 1984), although they have been found among the spermatocyst of marine elasmobranchs (Prisco et al., 2002; Tyminski et al., 2015; Sephid et al., 2021; Coetzee et al., 2021). Now, these cells are confirmed as being present in potamotrygonins.

Leydig cells are responsible for the biosynthesis and secretion of androgens, principally T (Zirkin and Papadopoulos, 2018; Zhou et al., 2019). Regulated by luteinizing hormone, Leydig cells metabolize cholesterol to T and regulate the expression levels of enzymes involved in steroidogenesis (Zirkin and Papadopoulos, 2018). In *P. wallacei*, the ultrastructural analysis revealed that Leydig cells have the typical characteristics of steroidogenic cells, and present mainly SER, mitochondria with tubular cristae, and lipid droplets (Prisco et al., 2002; Haider, 2004; Kang et al., 2013). In addition, the immunohistochemical analysis demonstrated the presence of the 17 β -HSD3 enzyme, and thus a T production capacity.

The findings of this study coincide with those for *T. marmorata* testes, in which Leydig cells present similar components (Prisco et al., 2002); however, with different enzyme expressions. In *T. marmorata*, 17 β -HSD was observed only in the Leydig cells from meiotic zones (Prisco et al., 2008). On the other hand, in *P. wallacei*, the expression of 17 β -HSD3 was observed in meiotic, mitotic and spermiogenic zones. These differences may result from the

enzyme specificity used by Prisco et al. (2008), which did not describe the specific enzyme type, since the 17 β -HSD group has different isozymes with different functional roles, such as type 3, that catalyzes the formation of T from androstenedione (Labrie et al., 1997). Future studies are required for a better understanding of the activity of Leydig cells in T production during the different phases of spermatogenesis.

The role of peritubular myoid cells (PMCs) has been described in the testes of other male vertebrates, and it is said to give structural support to the spermatogenic tubules (Unsicker and Burnstock, 1975; Maekawa et al., 1996) and participate in the communication with the Leydig and Sertoli cells via paracrine mechanisms (Unsicker and Burnstock, 1975; Welsh et al., 2009; Tyminski et al., 2015; Zhou et al., 2019). In *P. wallacei* testes, the role of PMCs seems to be no different from those described in other male vertebrates. The protein filaments of the PMCs surrounding the basal lamina of the spermatocyst, despite not having the characteristics of a steroidogenic cell, were attached to the basal lamina of the spermatocyst adjacent to the Sertoli cell regions, thus showing possible direct paracrine signaling between these cells (Maekawa et al., 1996; Smith and Walker, 2014; Tyminski et al., 2015). These observations support the findings of Tyminski et al. (2015), who found androgen receptors in PMCs capable of mediating the contractile movement of these cells and influence Leydig and Sertoli cells via paracrine signaling in *S. tiburo* testes.

The morphology of the Sertoli cells of *P. wallacei* males corroborate the typical organization of steroidogenic cells in elasmobranchs (Dubois and Callard, 1989; Prisco et al., 2002; McClusky, 2018). These cells modify their structural organization through the different spermatogenic zones in a similar way to those described in most elasmobranch species, such as *T. marmorata* (Prisco et al., 2002), *S. acanthias* (Dubois and Callard, 1989; McClusky, 2015), *Prionace glauca* and *Isurus oxyrinchus* (McClusky, 2018), and result from changes in metabolic and steroidogenic enzyme activity (Cuevas and Callard, 1992; Prisco et al., 2002).

In the spermatogonia zones of *P. wallacei* testes, the expression of 17 β -HSD3 in Sertoli cells appears to be maintained for the connection of spermatogonia that have similar characteristics of steroidogenic cells and also present this enzyme. Our findings corroborate similar observations found in *T. marmorata* spermatogonia, which presents 3 β -HSD and 17 β -HSD enzymes that are required for producing androgen hormones (Prisco et al., 2008). The presence of 17 β -HSD3 in spermatogonia may result from the need to produce T in *P. wallacei* regressing males that have a greater proportion of mitotic zones, such as spermatogonia zones, and without spermiogenic zones (Marcon et al., 2021). These findings suggest that germ cells

can produce steroids, thus making the control of steroid production in elasmobranchs more complex than in other vertebrates.

In the spermiogenic zones, (spermatid and spermatozoa) of *P. wallacei*, the morphological adaptations of the Sertoli cell nucleus and the high content of cytoplasm organelles associated with steroid production are linked to the immunoreactivity of 17 β -HSD3 for T production. These features are similar to those observed in marine sharks and ray species, such as *S. acanthias* (Callard et al., 1984; Callard, 1992), *Necturus maculosus* (Callard, 1992) and *T. marmorata* (Prisco et al., 2002). Even though in the majority of vertebrate species the Sertoli cells are known for diffusing the T binding to the androgen receptors to support the germ cells (Smith and Walker, 2014), in elasmobranchs Sertoli cells from spermiogenic zones are capable of regulating androgen production (Callard et al., 1985) for spermatid elongation and spermiation (Tyminski et al., 2015).

Our results demonstrate the potential of Sertoli and Leydig cells of *P. wallacei* for maximal T biosynthesis in meiotic and spermiogenic zones as observed in other elasmobranchs (Callard et al., 1985; Prisco et al., 2008). The highest serum T concentration measured in males of this species that contained large proportions of spermiogenic zones (Marcon et al., 2021) is associated with the morphological features of both steroidogenic cells in these zones. These findings may help to partially elucidate the hormonal control of reproduction in elasmobranchs, particularly in potamotrygonins.

5.0. Conclusions

The ultrastructural study of the testes of the cururu stingray *P. wallacei* shows that the Leydig and the Sertoli cells, as well as the spermatogonia, have typical steroidogenic cell characteristics. These cells presented enzymatic expression of 17 β -HSD3, which demonstrates that they are engaged in the production of T.

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Declaration of Competing Interest

The authors declare no conflict of interest.

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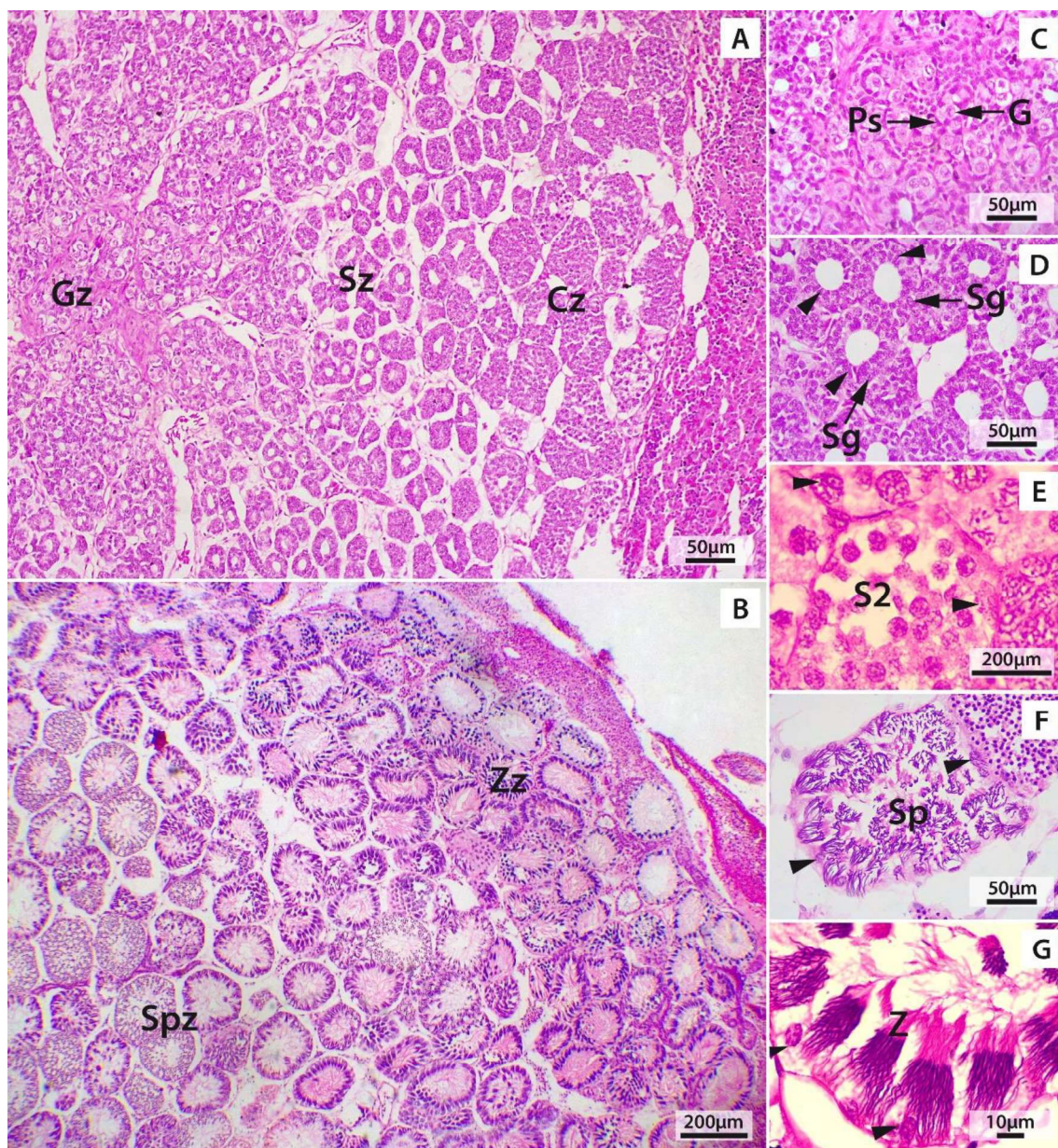


Figure 1. Photomicrographs of testicular lobules of *Potamotrygon wallacei* showing the spermatogenic zones (A-B), and the details of the cyst of each zone (C to G). Germinal zone (Gz); spermatogonia zone (Sz); spermatocyte Zone (Cz); spermatid zone (Spz); spermatozoa zone (Zz); germinative cells (G); spermatogonia (Sg); Cyst with meiotic cells, spermatocyte I (S1) and spermatocyte II (S2); spermatids (Sp); spermatozoa (Z); Sertoli cell precursor (Ps); black arrowhead - Sertoli cell; round spermatid (*).

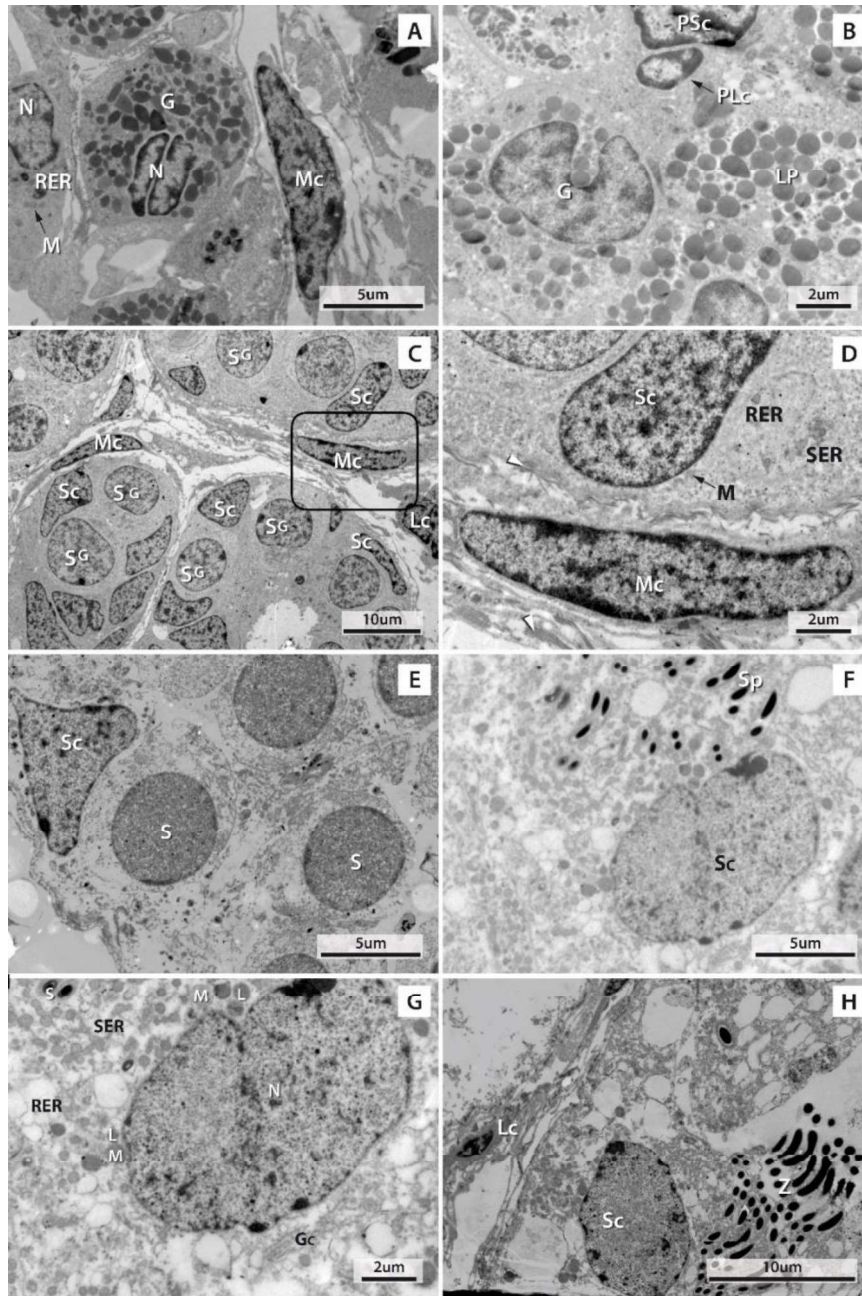


Figure 2. Transmission electron micrographs showing how the Sertoli cells changes during the spermatogenesis of *Potamotrygon wallacei*. Sertoli cells develop from the germinal zone with a nucleus that has perinuclear chromatin clumps (A-B). Sertoli cells of a spermatogonia cyst showing a fusiform shape (C); The myoid cells (Mc) attached with filaments (white arrow head) that surround and attach to the basal lamina of the spermatocyst near to Sertoli cell area (D); Sertoli cell in the cyst of spermatocytes (S) (E); the Sertoli cell of a spermatid cyst change to a round nucleus and present an increase in cytoplasmic organelles (F-G); Sertoli cell attached to spermatozoa group (F); Leydig cell (Lc); mitochondria with tubular cristae (M); spermatogonia (Sg); smooth endoplasmic reticulum (SER); rough endoplasmic reticulum (RER).

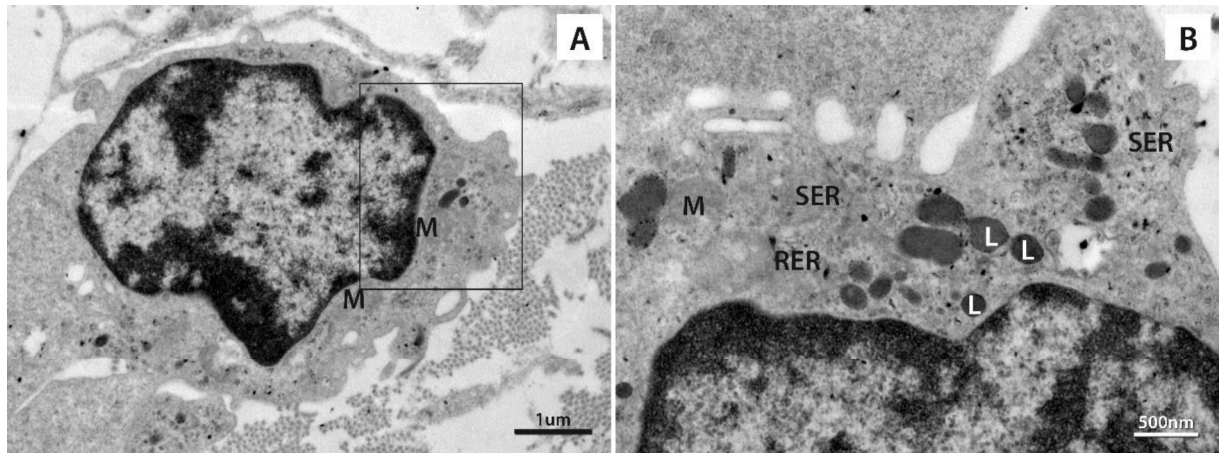


Figure 3. Transmission electron micrographs of *Potamotrygon wallacei* Leydig cell (A). Magnification of cytoplasm showing organelles associated with steroid production (B). mitochondria with tubular cristae (M); smooth endoplasmic reticulum (SER); rough endoplasmic reticulum (RER).

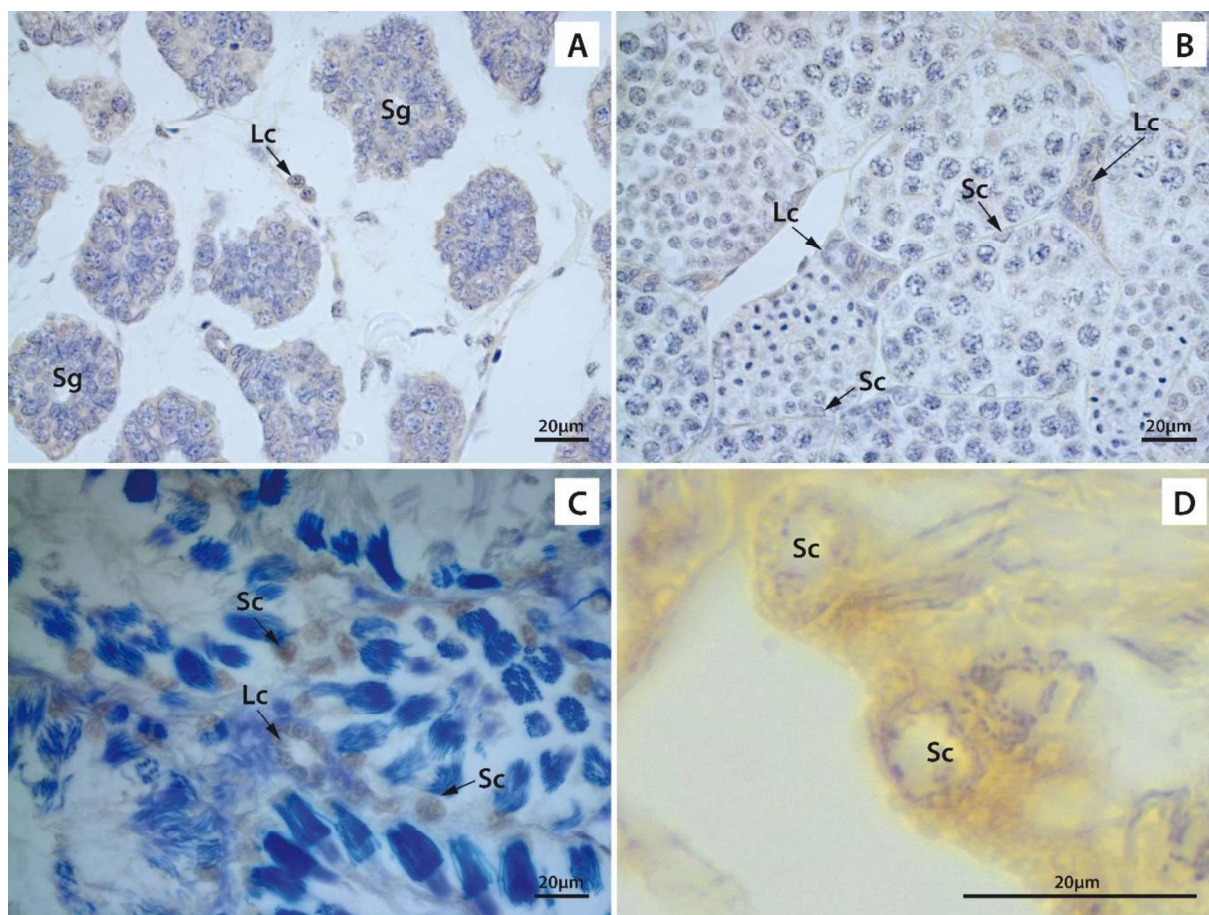


Figure 4. Immunolocalization of 17-beta-hydroxysteroid dehydrogenase type 3 (17 β -HSD3) in *Potamotrygon wallacei* testes. Cysts containing spermatogonia (Sg), Sertoli cells and Leydig cells positive for 17 β -HSD3 (A). Leydig cells of spermatocyte zone with 17 β -HSD3 reactivity (B). Sertoli and Leydig cells of spermatid and spermatozoa zones are immunolabelled with 17 β -HSD3 (C). Cyst containing spermatozoa (Z) Sertoli cells immunolabelled with 17 β -HSD3 (D). Leydig cell (Lc); Sertoli cell (Sc); spermatocyte (S); spermatid (Sp); spermatozoa (Z).

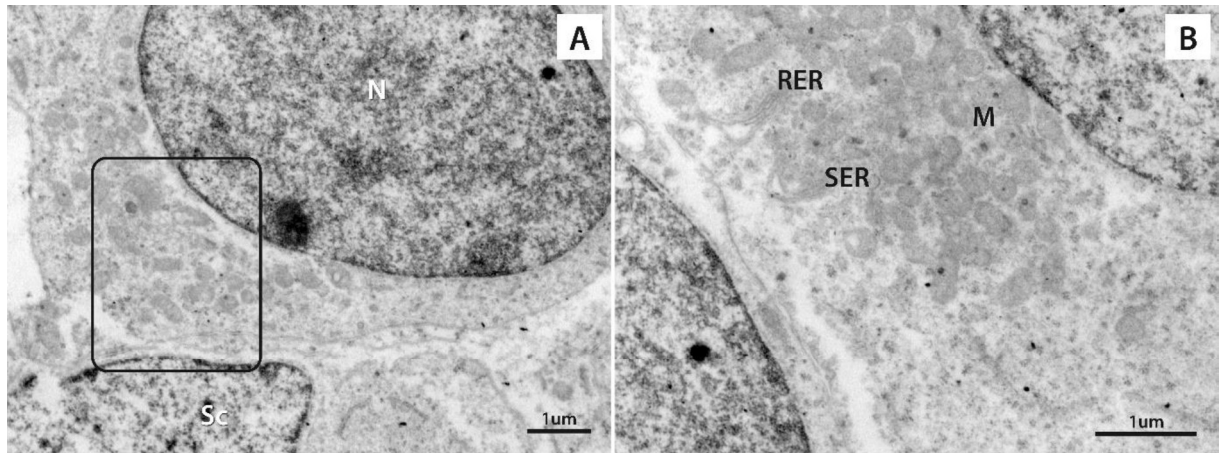


Figure 5. Electron microscopy of *Potamotrygon wallacei* spermatogonia (A). Magnification of cytoplasm showing mitochondria with tubular cristae (M), smooth endoplasmic reticulum (SER) and rough endoplasmic reticulum (RER) (B).

Supplementary material

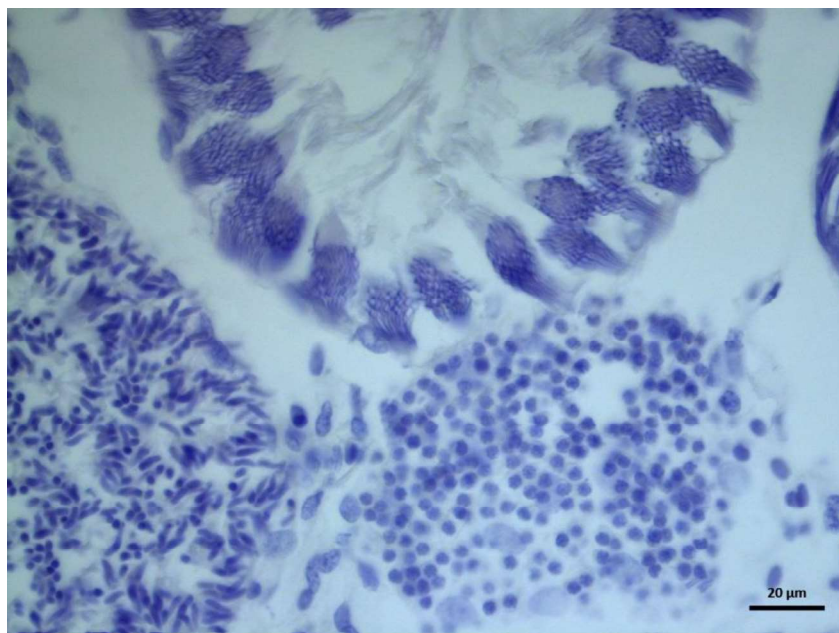


Figure S1. Negative control. Section of *Potamotrygon wallacei* testis treated with TBS instead of primary antibody.

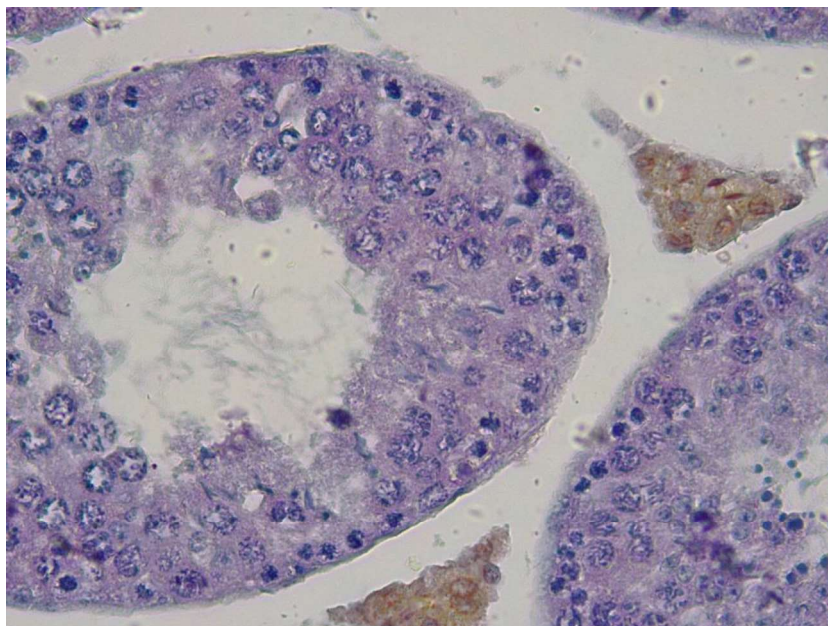


Figure S2. Positive control showing a section of mice testis whose Leydig cells are positive for 17 β -HSD3.

Capítulo 2

Morales-Gamba, R.D., Araújo, M.L.G., Rêgo, M.G., Barcellos, F.M., Dias, L.C., Jaydione, L.M. **Progesterone in extra testicular ducts of the Amazonian stingray *Potamotrygon wallacei*: a potential role in sperm maturation and aggregate formation.**

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Progesterone in extra testicular ducts of the Amazonian stingray *Potamotrygon wallacei*: a potential role in sperm maturation and aggregate formation

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Abstract

In cururu stingray (*Potamotrygon wallacei* Carvalho, Rosa and Araújo 2016) males, plasma progesterone (P₄) levels appear to be associated with spermiation events. However, the specific contribution of P₄ in sperm maturation via extra testicular ducts in this stingray species is unknown. With the aim of filling this knowledge gap, this study examined the morphology and the presence of progesterone receptors (PR) in the ducts, and analyze the relationship of progesterone (P₄) with sperm maturation and formation of aggregates. Morphological analysis showed that a columnar pseudostratified epithelium with stereocilia lined all the attached ducts. In active males, the secretory cells of the epididymis and the Leydig glands presented PR; however, these receptors were not found in the distal region of the epididymis (essential for nurturing and capacitation events) of regressing males. In the seminal vesicles of active males, the spermatozoa are parallelly aligned and embedded in a matrix to form the spermatozeugmata. The matrixes are formed by proteins secreted by the ducts and Sertoli cell cytoplasm. These structures presented PR, which indicates that P₄ engages sperm metabolism during storage. Our findings demonstrate the potential role of P₄ in regulating the development and function of the attached ducts in different reproductive phases. Furthermore, P₄ seems to be an essential component for regulating sperm progress, protein secretion, aggregate formation, and maintenance of sperm during storage in this freshwater stingray.

Keywords: epididymis, Leydig gland, seminal vesicles, spermatozeugmata, spermatozoa.

1.0. Introduction

In males of different elasmobranch species, the ducts of the reproductive system play an important role in the maturation and storage of spermatozoa (Hamlett, 1999; Jones and Hamlett, 2002; Dzyuba et al., 2018). After release from the testes, the sperm travels through the different ducts (efferent ducts, epididymes, deferent ducts, seminal vesicles) of the reproductive tract and are stored until its releasing during the copulation event.

The epididymis secretes a wide range of glycoproteins necessary for sperm maturation and capacitation (Jones et al., 1984; Hamlett et al., 2002; Jones and Hamlett, 2002; Dzyuba et al., 2018; Dzyuba et al., 2019). Furthermore, it can absorb most of the extracellular fluid leaving the testes (Jones et al., 1984). The epididymis, as well as the deferent duct, receives a viscous fluid that is secreted by the adjacent Leydig gland, which contributes to the final composition

of seminal fluid (Hamlett, 1999). Lastly, the sperm travels through the deferent duct and reach the seminal vesicle where they are stored as aggregates (Pratt and Tanaka, 1994; Hamlett, 1999).

In the seminal vesicles, individual spermatozoa adhere laterally and become aggregate in seminal fluid to form the spermatozeugmata, or randomly clumped sperm isare embedded in a matrix to form spermatophores (Pratt and Tanaka, 1994). Spermatozeugmata is classified into three types. Type I features clumps of 60-200 sperm that are unbound in a supporting matrix, type II consists of single-layered spheres formed of sperm clumps with the sperm heads bound in a common supporting matrix, while type III is represented by large structures formed by the accumulation of several single-layered aggregates (Pratt and Tanaka, 1994).

The morphological changes and the physiological events observed in the male reproductive tract of elasmobranchs are also a consequence of the influence of sex steroids (Manire and Rasmussen, 1997; Tricas et al., 2000; Marcon et al., 2021). Increases in progesterone (P₄) and testosterone (T) levels during spermatogenesis and spermiation have been described by several studies, with special attention on the role of P₄, which is a substrate for the biosynthesis of T and other steroid hormones in the testes and in sperm metabolism (Simpson and Wright 1964; Manire and Rasmussen, 1997; Tricas et al., 2000; Sulikowski et al., 2005; Marcon et al., 2021).

In potamotrygonins, such as the cururu stingray (*Potamotrygon wallacei*), an endemic species of the middle Negro River, the registered morphological and physiological changes of the extra testicular ducts seem to be a response to the incremental P₄ and T plasma levels (Marcon et al., 2021). The levels of P₄ in this species were over ten times higher compared to the other analyzed steroids (Marcon et al., 2021), and seemed to regulate the sperm maturation and the production of high-quality spermatozoa (Morales-Gamba et al., 2019).

Although the basic morphology and some functional properties of spermatozoa are already known for a few potamotrygonin species (Dzyuba et al., 2018; Morales-Gamba et al., 2019; Dzyuba et al., 2019; Dzyuba et al., 2020;), the hormonal control of sperm maturation and capacitation deserve further investigations. Thus, the present study aimed to examine the morphological aspects observed in the extra testicular ducts of the reproductive tract and the relationship of P₄ to sperm maturation and the formation of sperm aggregates in *P. wallacei* males.

2.0. Material and methods

2.1. Study site and sampling

Field collections were conducted from January to May 2017 in the Igarape Zamula (0°50'28" S, 62°46'5" W), a tributary of the middle Negro River basin (Barcelos, Amazonas, Brazil). Ten males at two reproductive stages, regressing males ($n = 5$) and active males ($n = 5$), were analyzed. The specimens were anesthetized via immersion in a eugenol solution (75 mg L⁻¹; Lima et al., 2021), and subsequently euthanized by spinal section for the removal of the reproductive system. These samples were also analyzed in the parallel investigation of Marcon et al. (2021), which was designed to correlate the morphology of the reproductive tract with plasma steroid hormone profiles in adults of this species during different phases of the reproductive cycle.

This investigation had prior authorization (SISBIO No. 18285–1) from the Chico Mendes Institute for Biodiversity Conservation (ICMBio) - Ministry of the Environment, Brazil, and was conducted in accordance with the guidelines of the Ethics Committee for the Use of Animals in Research of the Federal University of Amazonas (Protocol CEUA/UFAM # 002/2017).

2.2. Scanning electron microscopy

Samples of the attached reproductive ducts (epididymes, deferent ducts, Leydig gland and seminal vesicles) from each individual were fixed in Bouin's solution for 12 h and then transferred to 70% alcohol. After this period, samples were dehydrated in an alcohol series (70–100%), diaphanized with xylol, and embedded in paraffin wax at 60 °C. Sections of 10 µm were placed on a coverslip and deparaffined with xylol (3x for 5 min each). Samples were attached with double-sided adhesive tape to an aluminum stub, coated with gold using a sputter coater (SCD050 BAL-TEC-Witten, Germany) and then examined using a scanning electron microscope (Tescan VEGA3, Tescan-Brno, Czech Republic).

2.3. Transmission electron microscopy of the sperm

Sperm samples of active males were collected *in loco* from the seminal vesicle region using a disposable syringe and were then fixed in Karnowsky solution for 24 h. Subsequently, they were centrifuged at 5,000 rpm for 5 min and separated from the solid phase. The material was post-fixed in 1% osmium tetroxide for 2 h in the dark, contrasted with an aqueous solution of 5% uranyl acetate for 2 h, then dehydrated and embedded in resin (Durkupan, ACM Fluka,

Switzerland). Thin sections were cut on an ultramicrotome (Leica UC7, Leica Microsystems-Wetzlar, Germany), then stained with uranyl acetate, and photodocumented using a transmission electron microscope (JEM-1400, JEOL, Tokyo, Japan) operating at 80 kV.

2.4. Immunohistochemistry

Samples of the attached reproductive ducts previously mounted in paraffin were used. Sections of 5 μ m were mounted on poly-L-lysine-coated slides (Sigma Chemical Co, St. Louis, USA) and dried overnight at room temperature. The steps of deparaffinization, rehydration, and unmasking of epitopes were performed by boiling slides in a pressure cooker containing Trilogy™ (Cell Marque Corporation, Rocklin, USA) for 15 min. For the inhibition of peroxidase activity, sections were treated with 3% hydrogen peroxide for 30 min in the dark. After a TBS-T buffer rinse, slides were treated with a protein blocker (3% non-fat powdered milk in TBS) for 2 h, and subsequently incubated with the primary antibody (Progesterone Receptor Polyclonal Antibody; Thermo Scientific Pierce, Rockford, USA, product number PA5-82163) at a 4:100 dilution for 24 h at room temperature. Negative control sections were treated with TBS instead of primary antibody (Figure S1). Positive controls were performed on sections of mice uterus, which shows PR (Figure S2) (Kurita et al., 2001). After a 15 min rinse in TBS-T, antibody-antigen complexes were revealed with EnVision+/HRP, Dual Link Rabbit/Mouse kit (Dako) for 1.5 h and 3,3'-diaminobenzidine (DAB) was used as chromogen (Pierce Biotechnology, Rockford, USA) following the manufacturer's instructions. Counterstaining was performed with Mayer's hematoxylin. For analysis and photographic documentation, an optical microscope (Leica DM500, Leica Microsystems-Wetzlar, Germany) integrated with a digital camera (Leica ICC50W, Leica DM500, Leica Microsystems-Wetzlar, Germany) was used.

3.0. Results

The reproductive system of *P. wallacei* consists of two lobulated testicles independently linked to the epididymis, Leydig gland, deferent duct, and seminal vesicles (Figure 1). The morphological and PR immunolocalization data of each duct section are described in the following sections.

3.1. Epididymis

The epididymis comprises of a system of tubes dispersed in loose connective tissue, blood vessels, and a layer of fibrous connective tissue that covers the organ. Each tubule is lined with a pseudostratified columnar epithelium, the lamina propria of connective tissue, and a layer of smooth muscle. Two well-defined epididymal regions were observed, namely the proximal and distal regions (Figure 2A, 3A-D).

The proximal epididymis consists of tubules composed of a columnar pseudostratified epithelium with stereocilia, the lamina propria of connective tissue, and a layer of smooth muscle (Figure 2A; Figure 3A, B). The secretory cells have a basal nucleus and an apical cytoplasm with the presence of vesicles (Figure 2A; 3B). In active males, proteins are secreted into the lumen that presents masses of spermatozoa (Figure 2A). In regressing males, this secretory activity is not observed, and the lumen of the tubules was empty or had the remains of spermatozooids and macrophages (Figure 3A). Immunocytochemical localization of the PR was confirmed by an evident brown staining that marked the secretory cells of both active and regressing males (Figure 2B).

The distal epididymis exhibits similar morphological characteristics to the proximal epididymis; however, the tubular epithelium presents folds (Figure 2A; Fig 3C, D), and the epithelial cells appear to be hypertrophied by an increase of cytoplasm in active males when compared to regressing males (Figure 3C, D). PR were observed only in the cytoplasm from tubular secretory cells of active males (Figure 3D).

3.2. Leydig gland

This gland is located on the distal epididymis and the deferent duct (Figure 1). It features a tubular system of pseudostratified epithelium with a basal nucleus (Figure 3E, F). In the lumen of the tubules, round bodies were observed as a result of the apocrine secretion (Figure 2B). In this gland, the immunohistochemical localization of PR was evident in the cytoplasm of secretory cells in males with both reproductive stages, but with apparently less reactivity in regressing males (Figure 3E, F).

3.3. Deferent duct

In the renal middle region, the epididymis becomes straight and widens, and gives rise to the deferent duct, which is embedded in the Leydig gland (Figure 1). The deferent duct shows a pseudostratified epithelium with stereocilia, a lamina propria of connective tissue, and a multilayer of smooth muscle (Figure 2C, D). The epithelium is projected in the direction of the

lumen. Secretions of the Leydig gland are also deposited through this duct (Figure 2D). Immunocytochemical staining of the PR was not evident in this region (Fig 3E, F).

3.4. Seminal vesicle

In the posterior renal region, the vas deferens undergo an enlargement that gives rise to the seminal vesicle. The lining of the seminal vesicle is constituted by a pseudostratified epithelium with stereocilia that is located over a lamina propria of loose connective tissue. The epithelium exhibits long projections in the direction of the lumen (Figure 2E; 3G, H). Active males presented a lumen full of spermatozoa when compared to regressing males (Figure 3G, H). Scanning electron microscopy of a cross-section showed large protein chains secreted to the lumen (Figure 2E). This vesicle was not marked for PR, except for the lumen content in active males, which revealed these receptors in the sperm matrixes (Figure 3H).

3.5. Transmission electron microscopy of sperm

The spermatozoa of *P. wallacei* were helical in shape and composed by a head, the midpiece, and a flagellum (Figure 4). The head had a short acrosome with a cone shape and was $\sim 1.8 \mu\text{m}$ in length (Figure 4A). Posterior to the acrosome, the nucleus has dense material (Figure 4B, C), and the transverse sections show chromatin and parachromatin areas surrounded by the plasma membrane (Figure 4B). The midpiece has a cylindrical shape connected to the nucleus by the axial rod in the center (Figure 4D, E). Internally, the midpiece has packages of mitochondria along its whole length, and is surrounded by a fibrous mitochondrial sheath and more externally by the plasma membrane (Figure 4D). In the posterior part of the midpiece, the cytoplasmic sheath covers this region and the anterior part of the flagellum (Figure 4E, G). Internally, the distal centriole covers the axoneme (Figure 4G). The flagellum consists of a microtubular axoneme (9+2) that is accompanied by two longitudinal columns on the two sides, and is surrounded by the plasma membrane (Figure 4F, H).

In the lumen of the seminal vesicles, the sperm were aligned in parallel to each other and embedded in the matrix to form single-layered aggregates (Figure 5A, B). The matrix comprises proteins secreted for the ducts, such as the Leydig gland round bodies that present electron-dense materials (Figure 5C), and other components, such as Sertoli cell cytoplasm, which are remnants of Sertoli cells. The Sertoli cell cytoplasm consists of cytoplasmic fragments without a nucleus and present some organelles such as mitochondria and smooth endoplasmic reticulum (Figure 5D).

4.0. Discussion

The current study is the first to document the ultrastructure of the spermatozoa and of each segment of the genital ducts of *P. wallacei* males. It also provides evidence that P₄ could play an important role in the maturation of sperm and the formation of spermatozeugmata.

As in other male elasmobranchs, the reproductive tract of *P. wallacei* is composed of testicles, epididymis, deferent ducts, Leydig glands, and seminal vesicles (Jones and Hamlett, 2002; McClusky, 2015; Pedreros-Sierra and Ramírez-Pinilla, 2015; Marcon et al., 2021). While the sperm travel through these ducts, a sequence of events like maturation, capacitation and storage occurs (McClusky, 2015; Dzyuba et al., 2018), all of them being regulated by testicular steroids that acts as signaling agents to provide the differentiation and functioning of these structures (Manire and Rasmussen, 1997; Snelson et al., 1997; Tricas et al., 2000; Marcon et al., 2021).

Increases in steroid hormones were observed in the hammerhead shark (*Sphyrna tiburo*) (Manire and Rasmussen, 1997), the Atlantic stingray (*Hypanus sabinus*) (Snelson et al. 1997; Tricas et al., 2000), and also in the freshwater cururu stingray (*P. wallacei*) (Marcon et al., 2021). In all species, the active role of P₄ and T in the sperm transit in these ducts has been suggested. In active *P. wallacei* males, P₄ seems to act in the spermiation events (Marcon et al., 2021) and contributes to the high percentage of a high-quality sperm produced by these animals (Morales-Gamba et al., 2019).

The epididymis is the first duct to receive sperm, and plays an important role at the beginning of maturation and in capacitation events (Dzyuba et al., 2018). In the proximal segment of the epididymis, the sperm plasma is reabsorbed, and the sperm become more concentrated (Jones and Jones, 1982). Our findings suggest that the presence of PR in the distal epididymis of *P. wallacei*, in addition to the resorption of sperm plasma to concentrate the spermatozoa, could be due to the participation of P₄ in the resorption of unreleased spermatozoa in the regressing males.

In the distal epididymis, the folds in the epithelium increase the area available for the nutrition and physical support of the spermatozoa (Pratt and Tanaka, 1994). In this duct, the expression of PR observed in the secretory cells in active *P. wallacei* males could be associated to the hypertrophy of the epithelium cells due to increased activity of cytoplasmic organelles involved in the protein secretion and glycosylation process (Goss, 1966; Jones and Hamlett, 2002). The glycosylation is regulated by the action of P₄, which promotes the N-linked and O-linked glycosylation of epididymal proteins that support and sustain the sperm (Boettcher and

Kay, 1973; Medvedová and Farkas, 2004), since glycan expression is related to the nurturing and maturation of the spermatozoa in other elasmobranch species (Jones and Hamlett, 2002). Thus, in *P. wallacei*, as well as in other freshwater stingray species, such as *Potamotrygon motoro*, the distal epididymis is essential for nurturing and capacitation events (Dzyuba et al., 2018), and seems to be regulated by P₄ levels. This steroid acts as a substrate for 5 α -reductases for dihydroprogesterone substrate production in further stages of sperm metabolism (Levy et al., 1995; Vigil et al., 2011).

The tubular system of the Leydig gland has an apocrine secretion, as observed in other elasmobranchs (Jones and Hamlett, 2002). In mammals, the apocrine secretions of the prostate (an analog of the Leydig gland in elasmobranchs) are regulated by steroids, including P₄ (Wales and Ebling, 1971; Gesase and Satoh, 2003; Oettel and Mukhopadhyay, 2004). The same seems to occur in *P. wallacei* males, which have PR in the secretory cells of the Leydig gland, showing the P₄ to be a substrate for the apocrine secretion that occurs in this gland.

The functional characteristics of seminal vesicles of *P. wallacei* are similar to those observed in other elasmobranchs (Chatchavalvanich et al., 2005; Jones and Hamlett, 2006; McClusky, 2015). The long epithelial projections in the direction of the lumen, in addition to secreted large protein chains, increase the area, provide physical support to the sperm, and may aid in aggregate formation by providing a cilia-lined “tumbling mill” (McClusky, 2015) into which sperm is aligned and embedded in the matrix to form the spermatozeugmata.

The morphology of the spermatozoa of *P. wallacei* is composed of a helical head, a mid-piece, cytoplasmic sleeve, and a flagellum that resembles those of other elasmobranch species (Stanley, 1983; Jamieson, 2005; Chatchavalvanich et al., 2005; Morales-Gamba et al., 2019; Penfold and Wyffels, 2019; Muñoz-Baquero et al., 2021). Their elongated helical nucleus presents a conical acrosome fitted over the nuclear tip, which is formed during nuclear elongation (Chatchavalvanich et al., 2005). These findings differ from what was observed in the spermatozoon of the small-spotted catshark (*Scyliorhinus canicula*), which have a spiral acrosome with several ridges on the plasma membrane forming an acrosome-type crown (Muñoz-Baquero et al., 2021). These differences may result from the morphological adaptations to the acrosome that enable the spermatozoon to penetrate the zona pellucida of the egg (Austin and Bishop, 1958; Bedford, 2006).

The cytoplasmic sleeve of the spermatozoon in *P. wallacei* is a residual spermatid cytoplasm (Pudney and Callard, 1986) that encases the junction of the midpiece and the flagellum. In addition, the rigid spiral head and the soft flagellum linked to a cylindric midpiece allow the dual helical propulsion style known in the ray sperm (Wang et al., 2021). This

propulsion style appears to be important for the motile adaptations of the spermatozoon in viscous solutions of the reproductive system in females (Wang et al., 2021), such as the lipid histotrophy that is characteristic of Myliobatiformes (Musick and Ellis, 2005; Kina et al., 2021).

In *P. wallacei* males, the sperm stored as spermatozeugmata formed sperm clumps with the sperm heads bound in a common supporting matrix, which is consistent with the description of Pratt and Tanaka (1994) that indicates this spermatozeugmata to be of type II. However, in *P. wallacei*, the sperm have their heads bound in a matrix and are also joined to each other at the midpiece. Similar characteristics were observed in the Spotted ratfish (*Hydrolagus colliei*), in which the spermatozoa are closely adhered along the midpiece in the region of the cytoplasmic sleeve to the supportive matrix (Stanley, 1983).

The spermatozeugmata matrix composition of *P. wallacei* is similar to what is found in species like *Squalus acanthias* (Pudney and Callard, 1986), *Heterodontus portusjacksoni* (Jones and Lin, 1993), *Chiloscyllium plagiosum* (Penfold and Wyffels, 2019) and *Raja eglanteria* (Jones and Hamlett, 2006), which consist of the cytoplasm of Sertoli cells and protein secretions from other extra testicular ducts. The proteins that compose this structure participate in cell-cell interaction, which causes the spermatozoon to recognize other cells (Björkgren and Sipilä, 2019), and thus permits the agglutination of sperm (Boettcher and Kay, 1973) and, consequently, the spermatozeugmata formation. On the other hand, the cytoplasm of Sertoli cells presents organelles coupled to steroid production and appears to continue their activity in the lumen ducts, as observed in other elasmobranchs (Pudney and Callard, 1986; Jones and Lin, 1993; Jones and Hamlett, 2006).

As occurs with the proteins, the steroids can also regulate the alignment and agglutination of the sperm (Boettcher and Kay, 1973). The PR observed in the matrixes of *P. wallacei* can be a result of the presence of cytoplasm of the Sertoli cells that, in addition to the high levels of P₄ found in the plasma of these males (Marcon et al., 2021), could link the action of P₄ to the alignment of sperm and the formation of spermatozeugmata. The action of P₄ in the *P. wallacei* spermatozeugmata seems to engage the sperm metabolism during the storage until the copulation period takes place. These findings may be associated with the ability of the elasmobranch sperm to synthesize steroid hormones through enzymes for converting cholesterol into 11-deoxycorticosterone via pregnenolone and P₄ (Simpson et al., 1964; Jones et al., 1984).

The sperm aggregates appear necessary for maintaining spermatozoa until the copulation period in species with seasonal reproduction (Pratt and Tanaka, 1994). Species with reproductive cycles and spatial distribution invest in sperm storage to take advantage of rare or

opportunistic encounters (Pratt and Tanaka, 1994; Chatchavalvanich et al., 2005). *Potamotrygon wallacei* presents restricted periods of reproductive activity that coincide with the high-water period of the flood pulse (Marcon et al., 2021), in which the species increase their longitudinal distribution and sex segregation throughout the flooded forest (Araújo, M.L.G., personal communication). These characteristics suggest that, in this species, the sperm aggregates are necessary for sperm maintenance during storage until the copulation period.

5.0. Conclusions

This study established differences in the immunoreactivity of the PR of attached ducts between active and regressing *P. wallacei* males. In the distal region of the epididymis, the immunolocalization of the PR in active males suggested the role of P₄ in sperm nutrition and capacitation events. In addition, this steroid seemed to participate in the formation of spermatzeugmata aggregates and sperm metabolism during storage.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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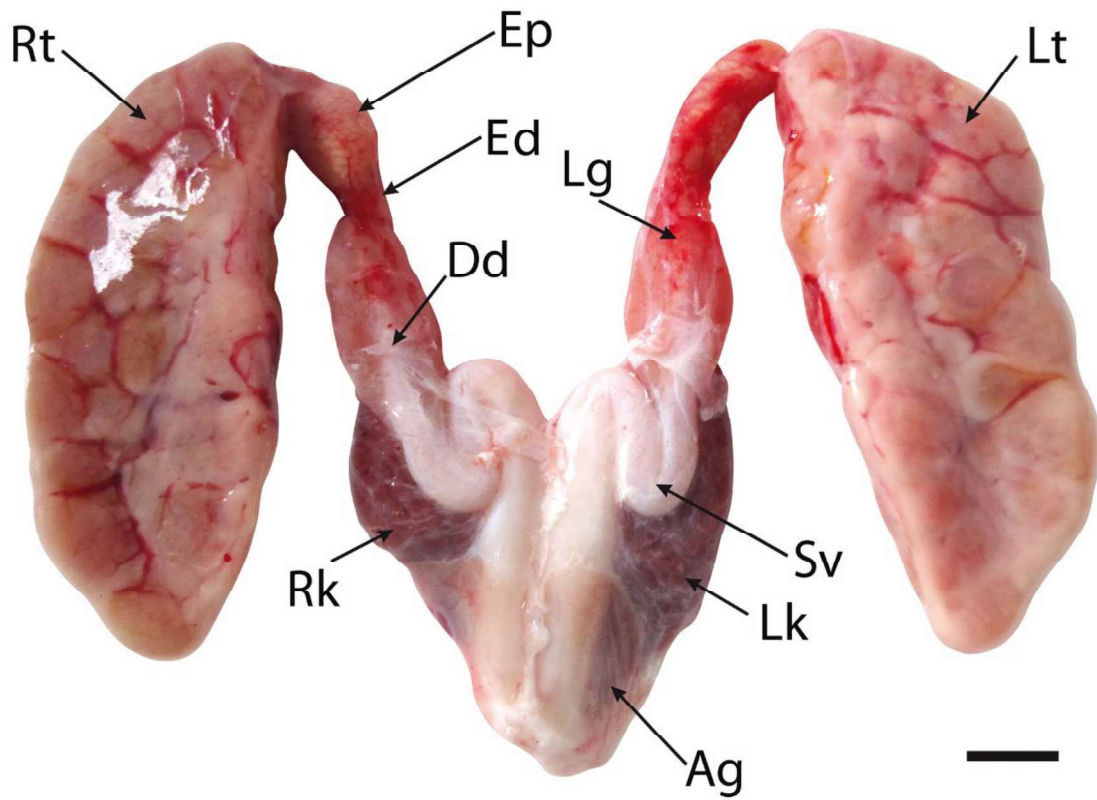


Figure 1. Components of the reproductive system of *Potamotrygon wallacei* males. Alkaline gland (Ag); deferent duct (Dd); proximal epididymis (Ep); distal epididymis (Ed); left testicle (Lt); right testicle (Rt); Leydig gland (Lg); left kidney (Lk), seminal vesicle (Sv); right kidney (Rk).

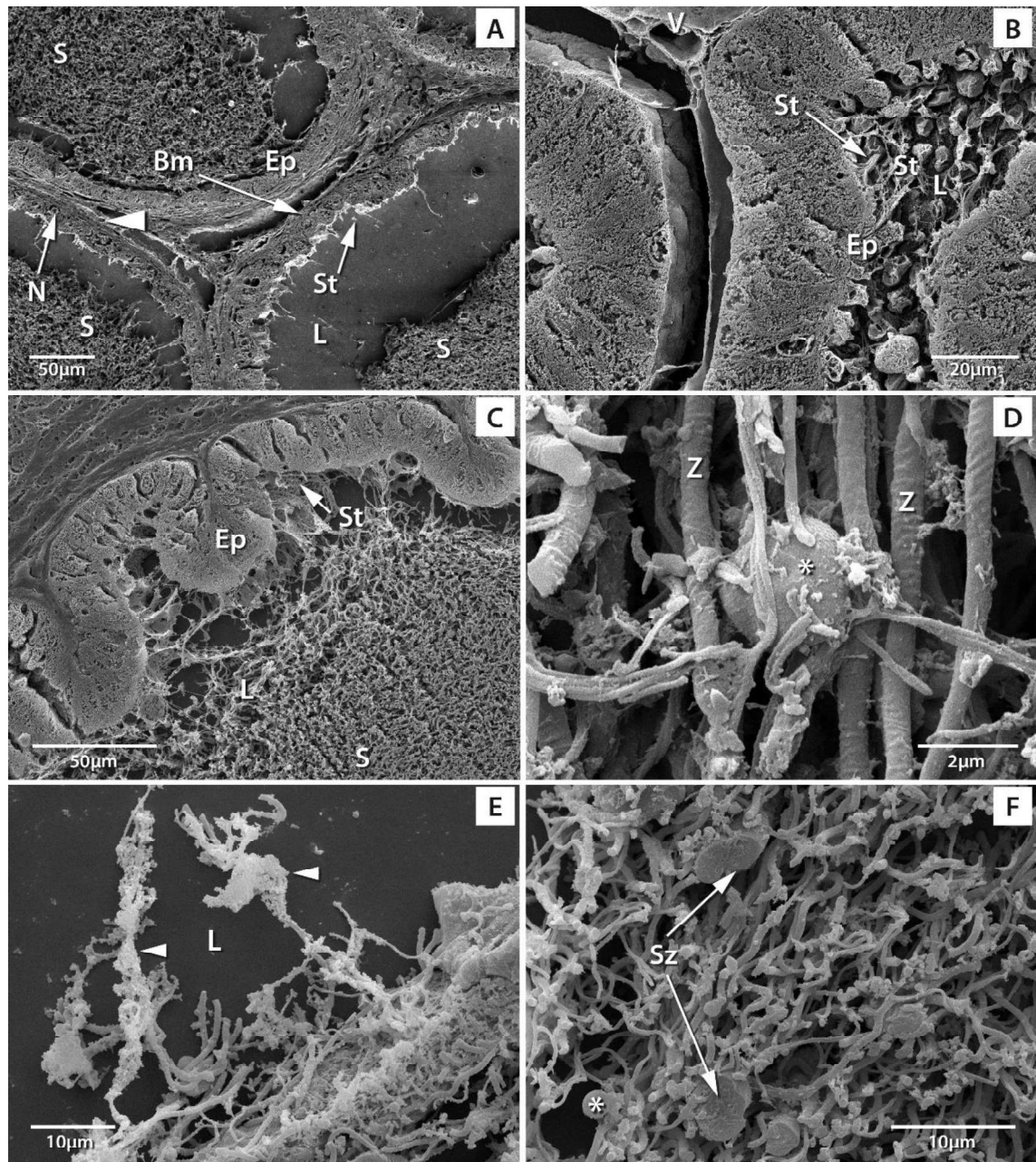


Figure 2. Electron micrographs of the attached genital ducts of *Potamotrygon wallacei* males. A) tubules of the proximal epididymis; B) Leydig gland showing apocrine secretion; C) deferent duct; D) magnification of the lumen (L) of distal epididymis showing the Leydig gland secretion (round bodies*) on the sperm (S); E) seminal vesicle epithelia secreting long protein chains (white arrowhead); F) seminal vesicle lumen showing spermatozeugmata (Sz); sperm basal membrane (Bm); epithelia (Ep); spermatozoa (Z); stereocilia (St).

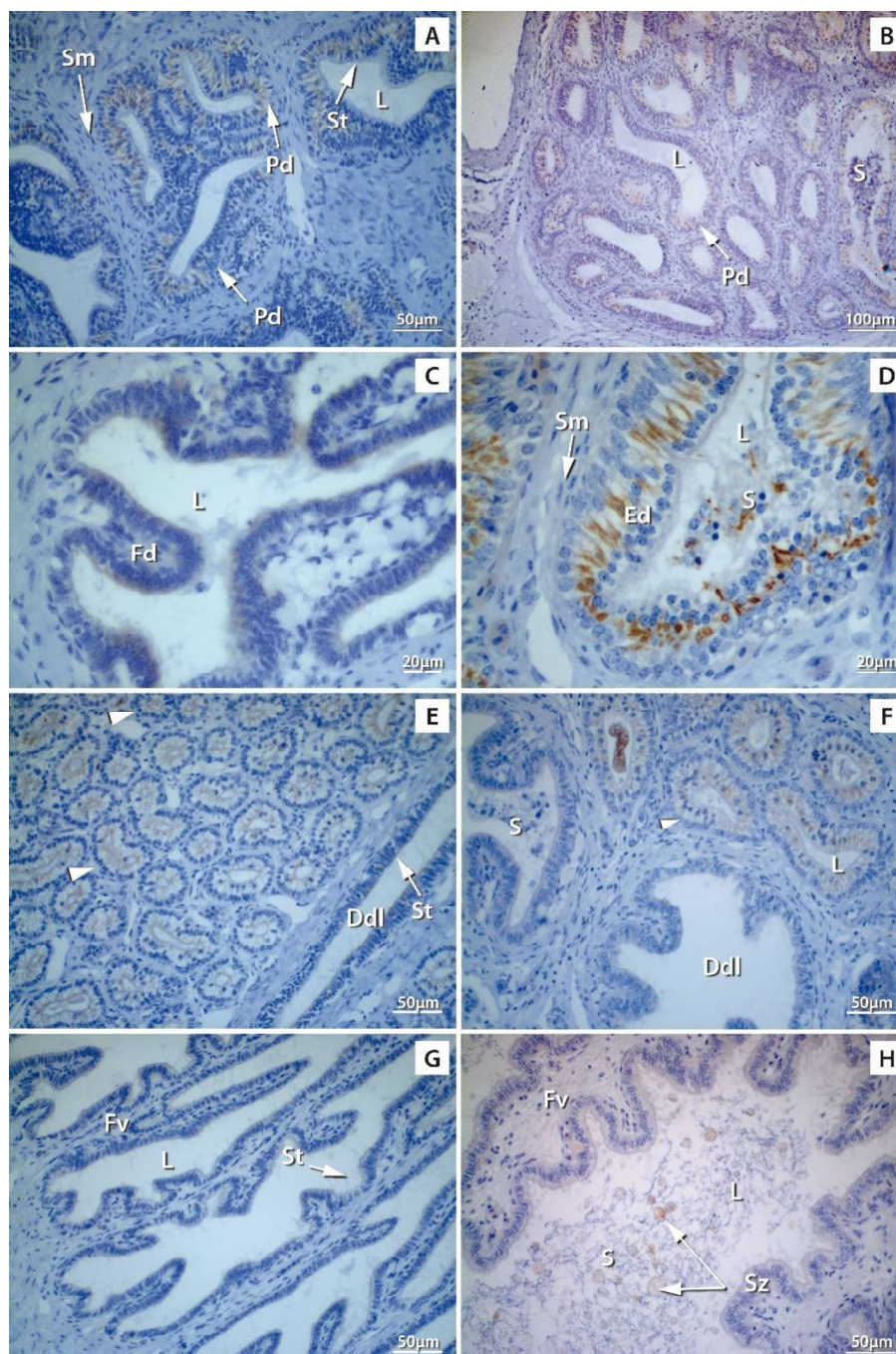


Figure 3. Localization of progesterone receptor protein in the attached ducts of active and regressing males of *Potamotrygon wallacei* via immunocytochemistry and using the progesterone receptor polyclonal antibody. Left and right panels refer to regressing and active males respectively. Positive immunostaining was indicated by an evident brown color. A-B) proximal epididymis; C-D) distal epididymis; E-F) Leydig gland tubules (white arrowhead) cover the deferent duct; G-H) seminal vesicles. Duct of deferent lumen (Ddl); epithelium folds of distal epididymis (Fd); epithelium folds of seminal vesicle (Fv); lumen (L); proximal epididymis duct (Pd); smooth muscle (Sm); sperm (S); spermatozeugmata (Sz); stereocilia (St);

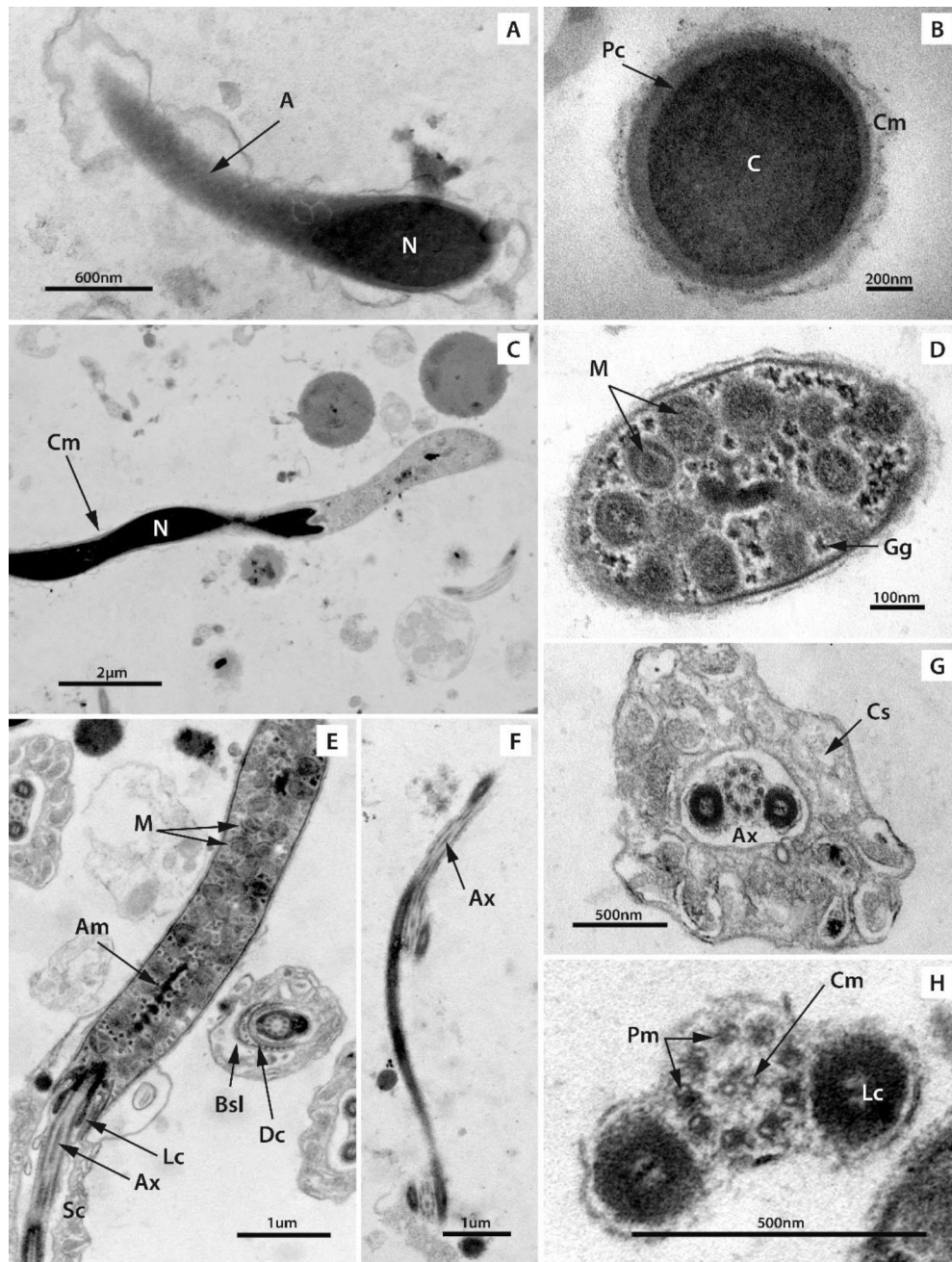


Figure 4. Transmission electron microscopy of *Potamotrygon wallacei* spermatozoon. A) acrosome; B) transversal section of nuclei and midpiece; C) longitudinal sections of nuclei and midpiece; D) transversal section of midpiece; E) longitudinal section of midpiece with cytoplasmic sleeve; F) longitudinal section of flagellum; G) transversal section of cytoplasmic sleeve; H) transversal section of flagellum. Axoneme (Ax); axial midpiece rod (Am); central microtubule (Cm); chromatin (C); cytoplasmic membrane (Cm); cytoplasmic sheath (Cs); distal centriole (Dc); glycogen granules (Gg); longitudinal column (Lc); mitochondria (M); nuclei (N); parachromatin (Pc); peripheral microtubule (Pm); persistent base of cytoplasmic sleeve (Bsl).

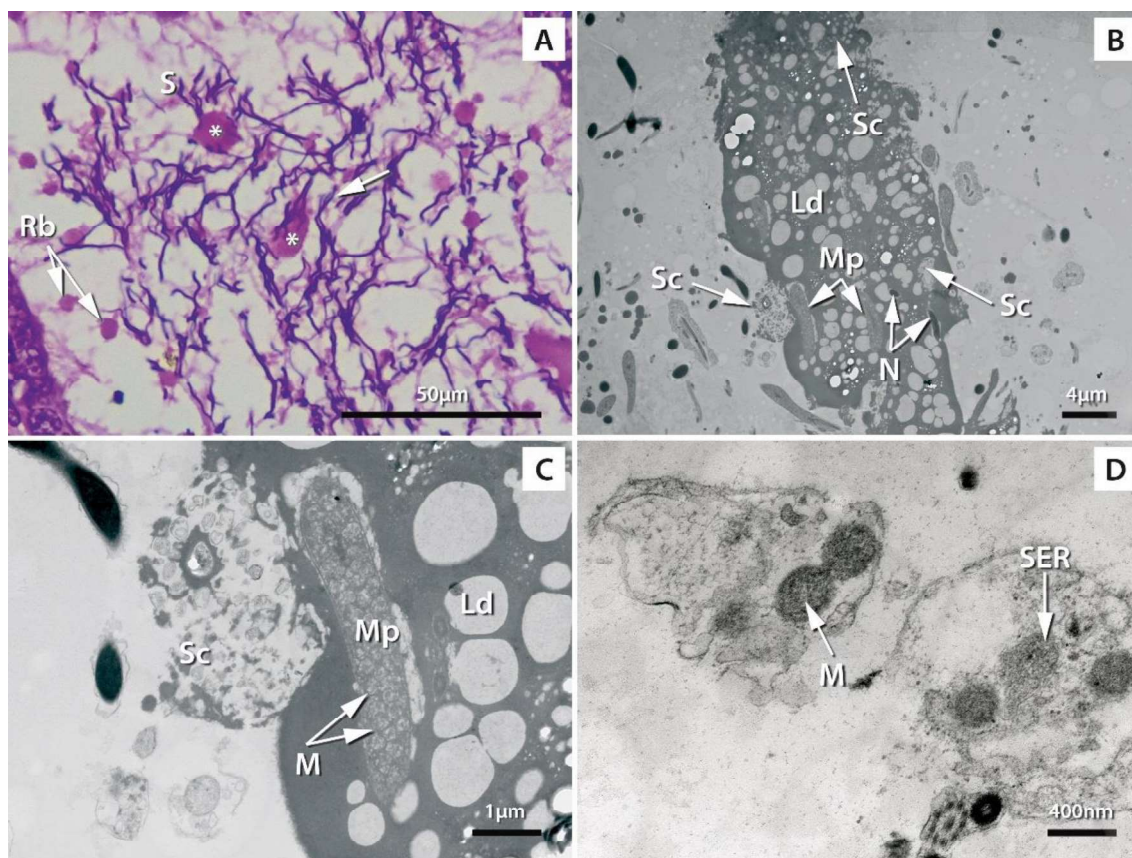


Figure 5. Micrographs of *Potamotrygon wallacei* sperm. A) light microscopy of seminal vesicles showing spermatozoa aligned to the matrix to form the spermatozeugmata (*); B-C) transmission electron microscopy of spermatozeugmata components; D) the electron microscopic examination of the cytoplasm of the Sertoli cells (Sc) showing organelles associated with steroid production as smooth endoplasmic reticulum (SER) and mitochondria (M). Lipid droplets (Ld); midpiece (Mp); round bodies (Rb); spermatozoa (Z). Hematoxylin-Eosin staining.

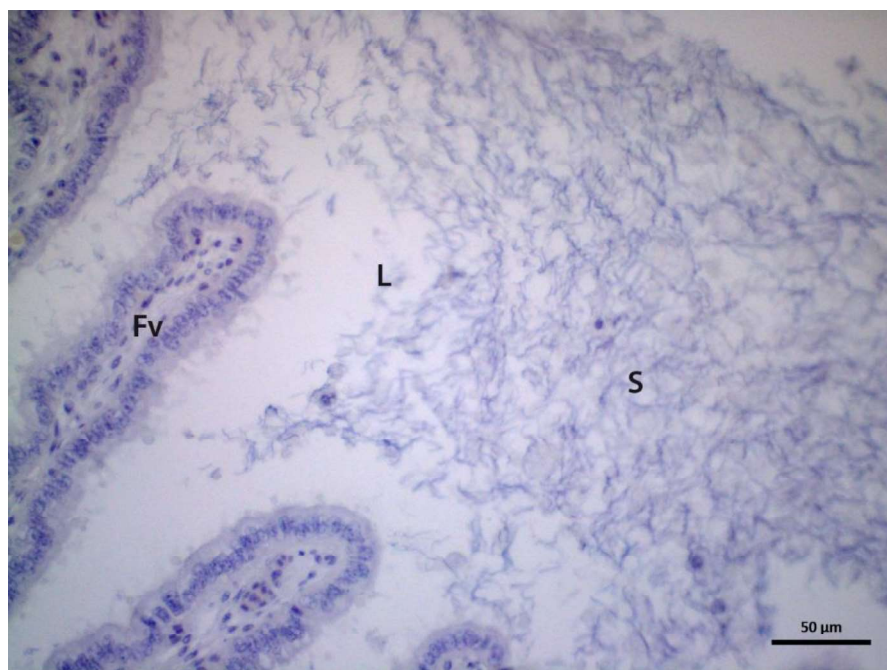
Supplementary material

Figure S1. Negative control. Seminal vesicle of active male of *Potamotrygon wallacei* containing sperm. No labelling was evident after omission of the progesterone receptor antibody. Lumen (L); seminal vesicle folds (Fv); sperm (S).

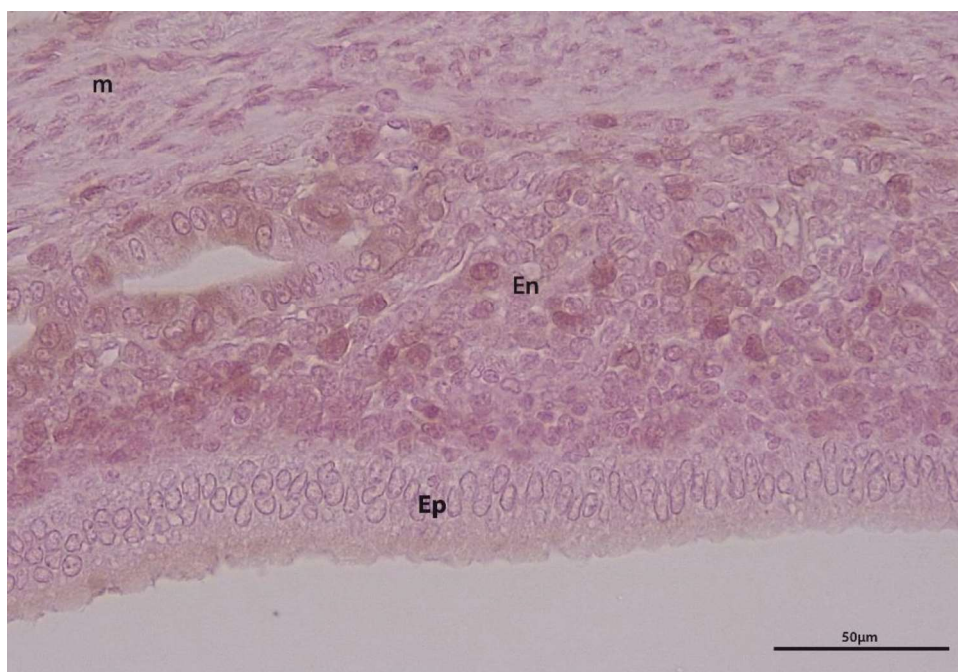


Figure S2. Positive control. Section of mouse uterus showing progesterone receptors in the epithelia (Ep) and endometrium (En). Myometrium (m).

CONSIDERAÇÕES FINAIS

Os resultados obtidos nesta tese contribuíram para ampliar o conhecimento sobre a morfologia e funcionalidade das células esteroidogênicas dos testículos em potamotrigoníneos. Adicionalmente, este estudo fornece informações inéditas sobre a participação da progesterona sobre a morfologia dos ductos anexos do sistema reprodutivo e dos espermatozoides.

No **Capítulo 1**, foi realizado o primeiro estudo da ultraestrutura testicular da arraia cururu, *Potamotrygon wallacei*, demonstrando a presença de células de Leydig, que em conjunto com as células de Sertoli, participam ativamente na produção de testosterona, principal hormônio para o desenvolvimento da espermatogênese.

Com relação ao **Capítulo 2**, novos dados morfológicos dos ductos anexos de machos adultos de *P. wallacei*, assim como da ultraestrutura do esperma foram fornecidos. Além disso, foi descrita a formação, composição e o tipo dos agregados espermáticos. A imunolocalização de receptores de progesterona nos ductos mostra a participação ativa da progesterona nos processos de secreção para a maturação espermática, assim como a função deste hormônio na atividade esteroidogênica da espermatogênese.

As concentrações circulantes dos hormônios esteroides que influenciam o trato reprodutivo somado a estas novas informações, poderão no futuro contribuir para a avaliação não letal do estágio reprodutivo desta espécie. Além disso, os achados sobre a morfologia dos espermatozoides assim como dos agregados espermáticos de *P. wallacei*, poderão servir como base para o entendimento e estudo dos processos de armazenamento espermático em fêmeas destas espécies.

Por fim, esses dados poderão contribuir para um melhor entendimento dos processos endocrinológicos envolvidos na reprodução nas arraias de água doce da América do Sul. Adicionalmente, poderão ajudar a elucidar o mecanismo reprodutivo, ampliando as informações sobre a endocrinologia reprodutiva dos elasmobrânquios.

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