UNIVERSIDADE ESTADUAL PAULISTA

"Júlio de Mesquita Filho"

INSTITUTO DE BIOCIÊNCIAS DE BOTUCATU

Rafael Oliveira Bordin

Morfofisiologia testicular e sazonalidade durante o ciclo reprodutivo anual de *Leptodactylus podicipinus* (Anura: Leptodactylidae) do Centro-sul paulista

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Tese apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, para obtenção do título de doutor no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração *Ciências Biológicas I.*

BOTUCATU – SP
Ano 2025

B729m

Bordin, Rafael Oliveira Alves

Morfofisiologia testicular e sazonalidade durante o ciclo reprodutivo anual de Leptodactylus podicipinus (Anura: Leptodactylidae) do Centro-sul paulista / Rafael Oliveira Alves Bordin. -- Botucatu, 2025

82 p.

Tese (doutorado) - Universidade Estadual Paulista (UNESP), Instituto de Biociências, Botucatu

Orientadora: Raquel Fantin Domeniconi

Coorientador: Classius Oliveira

1. Herpetologia. 2. Imunohistoquimica. 3. Espermatogênese em animais. 4. Reprodução animal. 5. Aparelho geniturinário. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Dados fornecidos pelo autor(a).

Impacto potencial dessa pesquisa

A presente pesquisa tem o potencial de gerar um impacto significativo ao contribuir com um registro detalhado das características estruturais, fisiológicas e metabólicas da espécie estudada, integrando aspectos ecológicos e funcionais. Essa abordagem fornece informações essenciais para a compreensão da diversidade funcional e do papel que ela desempenha na manutenção da biodiversidade, além de apoiar a formulação de estratégias mais eficazes de conservação. Paralelamente, o estudo pode ampliar a compreensão científica sobre as complexidades da reprodução em anuros. Focando na interação entre características histológicas, fisiológicas e reprodutivas da espécie neotropical *Leptodactylus podicipinus*. Acreditamos que as análises fornecem insights valiosos sobre a estrutura e a função do sistema genital masculino em anuros.

Potential impact of this research

This research has the potential to generate a significant impact by contributing a detailed record of the structural, physiological, and metabolic characteristics of the studied species, integrating ecological and functional aspects. This approach provides essential information for understanding functional diversity and its role in maintaining biodiversity while supporting the development of more effective conservation strategies. Additionally, the study may enhance scientific understanding of the complexities of anuran reproduction by focusing on the interplay between histological, physiological, and reproductive traits of the neotropical species *Leptodactylus podicipinus*. We believe the analyses will offer valuable insights into the structure and function of the male reproductive tract in anurans.

ATA DA DEFESA PÚBLICA DA TESE DE DOUTORADO DE RAFAEL OLIVEIRA BORDIN, DISCENTE DO PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA GERAL E APLICADA, DO INSTITUTO DE BIOCIÊNCIAS - CÂMPUS DE BOTUCATU.

Aos 01 dias do mês de agosto do ano de 2025, às 14h30min, por meio de Videoconferência, realizou-se a defesa de TESE DE DOUTORADO de RAFAEL OLIVEIRA BORDIN, intitulada **Morfofisiologia testicular e sazonalidade durante o ciclo reprodutivo anual de *Leptodactylus podicipinus* (Anura: Leptodactylidae) do Centro-sul paulista**, sob orientação da Profa. Dra. Raquel Fantin Domeniconi. A Comissão Examinadora foi constituída pelos seguintes membros: Profa. Dra. RAQUEL FANTIN DOMENICONI (Orientador(a) - Participação Virtual) do(a) Departamento de Biologia Estrutural e Funcional / Instituto de Biociências de Botucatu Unesp, Pós-doutoranda AMANDA JAMILLE CARVALHO BRUM (Participação Virtual) do(a) Departamento de Biodiversidade / Instituto de Biociências, Câmpus Rio Claro, Prof. Dr. RODRIGO ZIERI (Participação Virtual) do(a) Departamento de Ciências Biológicas / Instituto Federal de Educação, Ciência e Tecnologia de São Paulo, Profa. Dra. TAIS HARUMI DE CASTRO SASAHARA (Participação Virtual) do(a) Departamento de Biologia Estrutural e Funcional / Instituto de Biociências de Botucatu - UNESP, Profa. Dra. MAELI DAL PAI (Participação Virtual) do(a) Departamento de Biologia Estrutural e Funcional / Instituto de Biociências de Botucatu - Unesp. Após a exposição pelo doutorando e arguição pelos membros da Comissão Examinadora que participaram do ato, de forma presencial e/ou virtual, o discente recebeu o conceito final:_ _ _APROVADO_ _ _ . Nada mais havendo, foi lavrada a presente ata, que após lida e aprovada, foi assinada pelo(a) Presidente(a) da Comissão Examinadora.

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Resumo

Nas últimas décadas, a diversidade dos anuros tem sofrido mudanças com a descrição de novas espécies, o que torna necessário o conhecimento da diversidade funcional desses animais através da investigação dos traços reprodutivos das espécies. Esses traços são fundamentais para o esforço de preservação da diversidade. Devido a capacidade adaptativa do grupo, é importante avaliar a dinâmica reprodutiva das espécies, considerando sua distribuição geográfica, bem como as condições climáticas do seu habitat, especialmente em um cenário de mudanças ambientais globais. A espécie *Leptodactylus podicipinus* é amplamente distribuída, e apresenta modo de vida com certa terrestrialidade. Tais características representam uma dinâmica reprodutiva interessante para avaliar a morfofisiologia genital, com foco no ciclo reprodutivo da espécie. Diversos aspectos da reprodução de *L. podicipinus* e sua relação com a umidade e sazonalidade foram avaliados através de métodos variados como histomorfométria, imunohistologia e microscopia eletrônica. A anatomia do aparelho reprodutor consiste em ductos e dúctulos testiculares responsáveis pelo transporte espermático, e ainda foi encontrada variação sazonal na proliferação celular testicular. Também foram identificadas as aquaporinas 1, 2 e 9 no tecido testicular da espécie, com diferenças sazonais na sua localização. Além disso, caracterizou-se a dinâmica germinativa e o comportamento reprodutivo anual da espécie e propôs-se um padrão reprodutivo interespecífico, com possível influência do comportamento poliândrico. Esses resultados ampliam o entendimento sobre a reprodução dos anuros e reforçam dados morfofisiológicos de *L. podicipinus*, destacando sua dinâmica reprodutiva e contexto ambiental, características essenciais para os esforços de mitigação de danos e proteção da biodiversidade frente às mudanças ambientais atuais.

Palavras-chaves: Herpetologia, Urogenital, Poliandria, Células germinativas.

Abstract

In recent decades the diversity of anurans has changed with the discovery of new species, making it necessary to know the functional diversity of these animals. Investigating the reproductive traits of species is fundamental to the effort to preserve diversity. Due to the adaptive capacity of the group, it is crucial to assess the reproductive dynamics of the species, taking into account their geographical distribution and the climatic conditions of their habitat, particularly in the context of global environmental change. The species *Leptodactylus podicipinus* is widely distributed and has a somewhat terrestrial lifestyle. These characteristics present an interesting reproductive dynamic for evaluating genital morphophysiology, focusing on the species' reproductive cycle. Various aspects of *L. podicipinus* reproduction and its relationship with humidity and seasonality were evaluated using various methods such as histomorphometry, immunohistology, and electron microscopy. The anatomy of the reproductive system consists of testicular ducts and ductules responsible for sperm transport, and seasonal variation in testicular cell proliferation has also been found. Aquaporins 1, 2, and 9 were also identified in the species' testicular tissue, with seasonal differences in their location. In addition, the germ dynamics and annual reproductive behavior of the species were characterized, and an interspecific reproductive pattern was proposed, with the possible influence of polyandrous behavior. These results broaden our understanding of anuran reproduction and reinforce morphophysiological data on *L. podicipinus*, highlighting its reproductive dynamics and environmental context, which is essential for efforts to mitigate damage and protect biodiversity in the face of current environmental changes.

Keywords: Herpetology, Urogenital, Polyandry, Germinative cells.

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Introdução

Os anuros são os tetrápodes que apresentam a maior diversidade reprodutiva, a qual está relacionada aos diferentes locais possíveis para reprodução desses animais, que são tanto terrestres como aquáticos (Nunes-de-Almeida et al 2021). Sendo a reprodução vital para a perpetuação das espécies (Vitt & Caldwell 2014), o grupo conta com uma complexidade de modos reprodutivos bem caracterizados, que descrevem os vários locais e processos de sua reprodução (Haddad & Prado 2005; Nunes-de-Almeida et al 2021). Essa ampla diversidade de modos reprodutivos reflete a capacidade adaptativa dos anuros, que se desenvolvem em diferentes condições ambientais, ajustando-se às variações presentes ao longo do ciclo reprodutivo (Prado et al 2000; Haddad & Prado 2005; da Silva et al 2012; Nunes-de-Almeida et al 2021).

Nas últimas décadas, a compreensão da diversidade de anfíbios tem mudado drasticamente, com a descoberta de novas espécies e mudanças nas relações filogenéticas do grupo. No entanto, à medida que avança a exploração taxonômica e ecológica dos anuros, maior se torna a necessidade de estudos funcionais que elucidem características relacionadas à sua fisiologia e ao ciclo de vida (Catenazzi 2015), uma vez que traços funcionais são fundamentais para a avaliação de biodiversidade e para o delineamento de estratégias de conservação (Mouillot et al 2013; Zhao et al 2018; Liu et al 2021).

De modo geral, a reprodução dos anuros concentra-se na estação mais úmida; entretanto, como já discutido, essas espécies conseguem lidar com condições ambientais adversas adotando diferentes estratégias (Heyer 1969; Santos et al 2011; Toledo et al 2012). Há combinações dos traços de reprodução, que leva a modos reprodutivos singulares, com variadas características de amplexo (Zamudio et al 2016), oviposição, desenvolvimento de girinos (Nunes-de-Almeida et al 2021), além de, comportamentos com relação ao cuidado parental, que podem estar presentes ou ausentes em muitas espécies (Haddad & Prado 2005; Toledo et al 2012). A capacidade adaptativa que anuros demonstram, a partir dos modos reprodutivos complexos, é fundamental para o desenvolvimento do grupo, principalmente em relação às rápidas alterações climáticas e espaciais da atualidade (Liu et al 2021; Nunes-de-Almeida et al 2021).

Essas alterações são especialmente preocupantes diante do declínio observado há décadas nas populações de anuros (Stebbins & Cohen 1995; Catenazzi 2015). Como todas as espécies, os anuros desempenham um papel fundamental no equilíbrio ecológico

(Stebbins & Cohen 1995). Suas funções incluem o controle populacional de invertebrados e a transferência de energia para predadores de níveis tróficos superiores (Stebbins & Cohen 1995; Bernarde 2012). Nesse último aspecto, os anuros se destacam por sua baixa taxa de gasto energético e alta eficiência na predação de invertebrados, o que os torna uma fonte alimentar de alto valor nutricional para diversos vertebrados maiores, como serpentes, peixes e mamíferos (Stebbins & Cohen 1995; Vitt & Caldwell 2014; Jorgewich-Cohen et al 2020). Essas características reforçam a importância de se aprofundar o conhecimento sobre esse grupo, especialmente diante dos riscos que enfrentam em um cenário de mudanças globais cada vez mais intensas.

Anatomia e fisiologia reprodutiva

O sistema genital no grupo dos anuros é um tópico de interesse para vários estudos, especialmente pela já descrita variabilidade reprodutiva (Blüm 1986; Lombardi 1998; Oieslka 2009; Oliveira & Franco-Belussi 2012; Rheubert et al 2017). Esses animais possuem um aparelho urogenital bem conhecido e variável. Embora muitas espécies apresentem estruturas anatomicamente semelhantes, existem padrões diferentes dentro do grupo, especialmente em relação à íntima conexão entre os testículos e os rins nos anuros (Oieslka 2009).

Os testículos de anuros são estruturas ovoides, pares, esbranquiçadas e encontradas na cavidade abdominal, posicionadas ventralmente em relação ao rim e conectadas a este através do mesórquio (Oielska 2009; Oliveira & Franco-Belussi 2012). Externamente, o testículo é revestido por uma camada fibrosa, denominada túnica albugínea, devido à sua transparência, é possível observar estruturas com aparência de grânulos por toda a superfície das gônadas. Estes grânulos são os lóculos seminíferos, nos quais as células germinativas estão organizadas e se desenvolvem em cistos sustentados por Sertoli (Oliveira & Franco-Belussi 2012; Bordin et al 2022).

Em relação às estruturas compartilhadas entre os testículos e os rins, existe no grupo uma variação entre espécies. De forma geral, o parênquima seminífero é drenado por dúctulos internos, e a conexão urogenital se dá por vasos eferentes, originados a partir da fusão desses dúctulos, que atravessam o mesórquio em direção aos rins. Esses vasos se conectam lateralmente aos dúctulos coletores renais, que transportam os espermatozoides através do espaço de Bowman até o ducto wolfiano (Hiragond & Saidapur, 2017; Rheubert et al., 2017). No entanto, essa estrutura pode variar entre

espécies, inclusive apresentando padrões nos quais os rins não participam do transporte de gametas (Figura 1) (Blum 1986; Oielska, 2009).

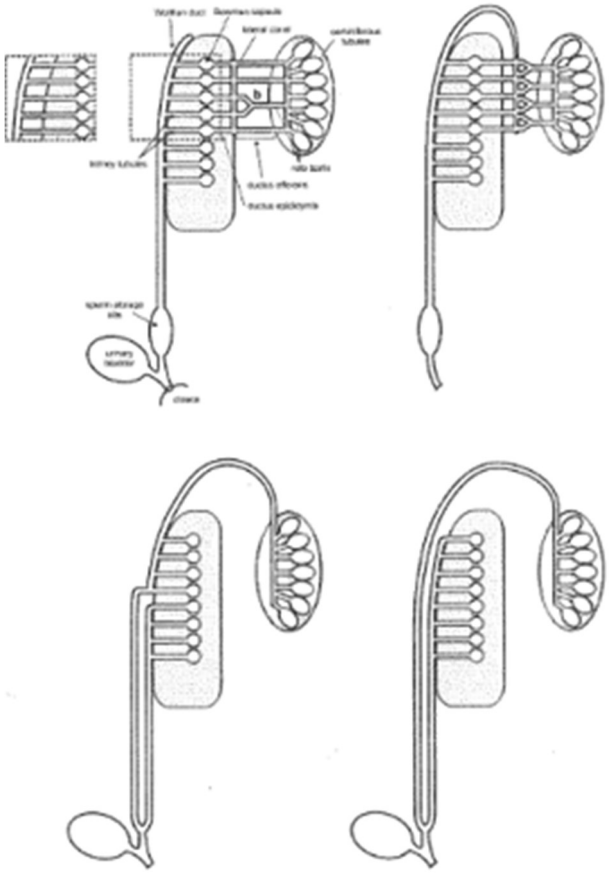


Figura 1. Esquema ilustrando a diversidade estrutural da relação testículo e rins em anuros. Observa-se diferenças anatômicas que podem ser encontradas em diferentes espécies. Adaptado de Oieslka M, 2009.

De forma geral, são conhecidos quatro padrões de organização estrutural do aparelho urogenital masculino em Anura (Blum, 1986; Oielska, 2009; Hiragond & Saidapur, 2000). Três desses padrões foram descritos com base em espécies paleárticas, nas quais se observa um processo de separação funcional entre os sistemas urinário e genital (Blum, 1986). Nesse modelo de desenvolvimento, a porção urogenital, inicialmente integrada por um sistema comum de canais que conecta testículos e rins, passa por modificações que, em algumas espécies, resultam na perda dessa conexão. Como consequência, os testículos e rins passam a ser drenados por ductos distintos, caracterizando sistemas independentes para as funções reprodutivas e excretoras (Blum 1986; Oielska 2009).

Por outro lado, estudos com espécies da região Indo-Malaia revelam um padrão distinto, que não coincide com os três anteriormente descritos (Hiragond & Saidapur

2000). Nessas espécies, observa-se uma interação íntima entre testículos e rins, em que os espermatozoides são transportados por vasos eferentes até os glomérulos renais, de onde seguem pelo ducto wolffiano. Esse tipo de conexão também é encontrado no aparelho urogenital da espécie neártica *A. catesbeiana* (Rheubert et al 2017).

A organização em cistos germinativos é uma característica comum de alguns vertebrados, sobretudo peixes e anfíbios (Figura 2) (Santos & Oliveira 2008; Schulz & Nóbrega 2011; Leite et al 2015). Nessa estrutura, as células germinativas dentro dos lóculos seminíferos estão arranjadas em cistos que são envoltórios formados pelas células de Sertoli, que apenas se desfazem quando as células atingem a fase de espermátides alongadas (Santos & Oliveira 2008). As células germinativas em cistos podem ser observadas em diferentes estágios de maturação: **espermatogônias**, que se dividem por mitose e originam **espermatócitos primários**; estes, por meio da meiose I, dão origem aos **espermatócitos secundários**, os quais sofrem a meiose II e formam as **espermátides**No lúmen locular geralmente se encontram espermatozoides livres já maduros após o processo de espermiogênese das espermátides, prontos para serem transportados para a cloaca (Oliveira et al 2003; Oieslka 2009).

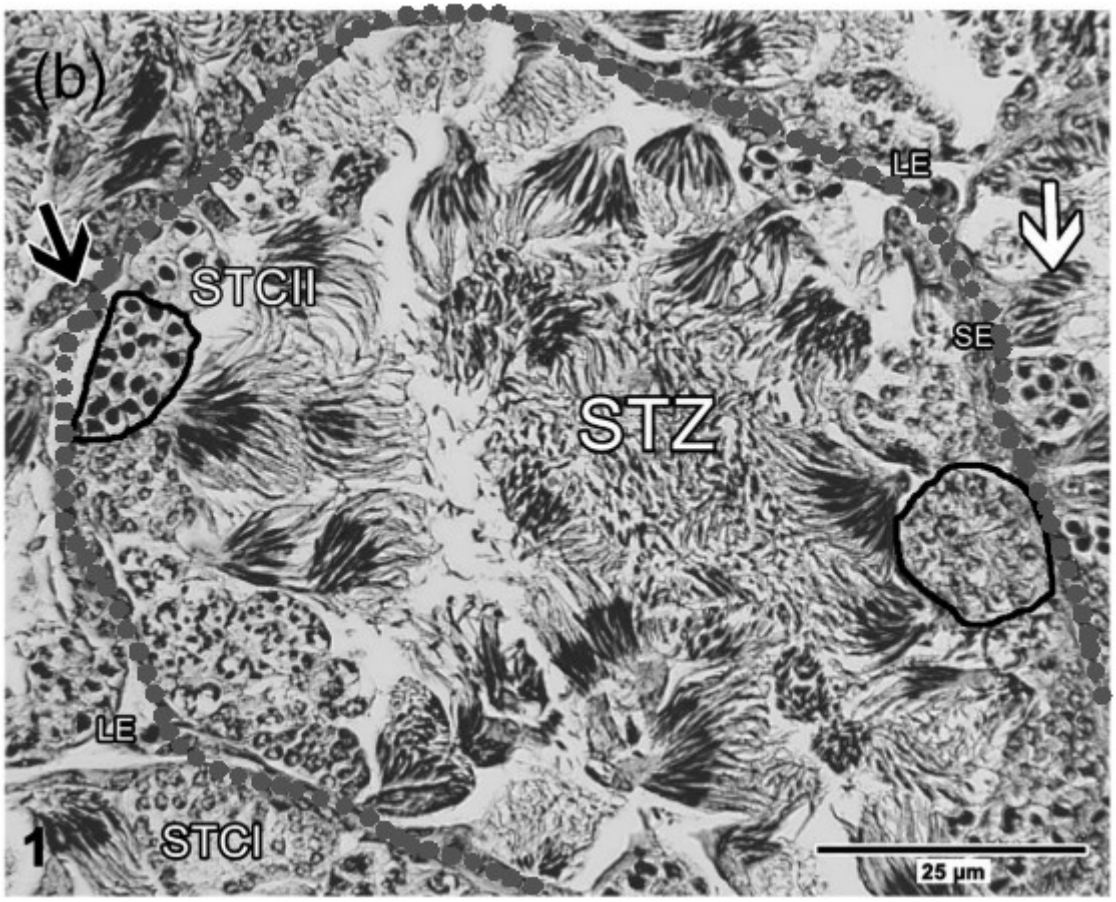


Figura 2. Histologia do testículo de anuro demonstrando a organização cística. Linha pontilhada: Delimitação do lóculo; Linha contínua: cistos germinativos; STZ: espermatozoides; STCI: espermatócitos primários; STCII: espermatócitos secundários; SE: células de Sertoli; LE: células de Leydig; Setas brancas: espermátides; Setas pretas: espermatogônias. Adaptado de Bordin et al 2022.

Grande parte dos trabalhos a respeito da reprodução de anuros focam em características ecológicas, celulares ou evolutivas específicas (Santos et al 2011; Pucci et al 2020; Bordin et al 2022). Entretanto, apesar dos diversos estudos sobre a reprodução de anuros, ainda há carência de informações sobre sua anatomia e a biologia celular reprodutiva em um contexto mais amplo, uma vez que são escassos os trabalhos que descrevem em detalhes a estrutura e relações do aparelho urogenital desses animais (Hiragond & Saidapur, 2000; Rheubert et al., 2017). A ausência de informações detalhadas sobre a anatomia básica dificulta a compreensão das relações entre a estrutura do aparelho urogenital e o funcionamento do ciclo reprodutivo dos anuros, sobretudo considerando a grande diversidade desse grupo de vertebrados.

Comportamento reprodutivo em anuros

O comportamento reprodutivo consiste em uma gama de estratégias e características intrínsecas das espécies animais, que são em grande parte influenciadas e moduladas por fatores externos. Em anuros, esse comportamento reprodutivo é especialmente interessante devido a sua diversidade (Prado et al 2002; Gomez-Mestre et al 2012; Nunes-de-Almeida et al 2021). Tendo em vista a complexidade de estratégias e características, os modos reprodutivos dos anuros têm sido amplamente discutidos e caracterizados para a maioria das espécies (Nunes-de-Almeida et al 2021).

Os modos reprodutivos podem ser mais bem definidos como uma combinação de características do que um caráter específico, sendo assim, uma combinação de características que incluem tipo de reprodução, local e maneira de oviposição, nutrição e desenvolvimento larval e ainda cuidado parental (Haddad & Prado 2005; Nunes-de-Almeida et al 2021). A definição e informações sobre modos reprodutivos são fundamentais para a compreensão de padrões evolutivos (Gomez-Mestre et al., 2012). Entretanto, o comportamento reprodutivo dos anuros engloba também traços além daqueles definidos para os modos reprodutivos, como atividade de vocalização, comportamentos de corte, competição reprodutiva, esforço e ciclos reprodutivos diversos (Lofts 1964; Smith et al 2003; Zamudio et al 2016).

O gerenciamento de energia destinado às atividades reprodutivas é crucial para os anuros, estando o esforço aplicado na reprodução intimamente relacionado às características corporais e fisiológicas, como o desenvolvimento gonadal e espermático (Prado et al., 2002; Bordin et al., 2022), bem como às atividades, como vocalização, que afetam diretamente o sucesso reprodutivo dos indivíduos (Lima et al., 2022).

Além disso, comportamentos como poliandria e poliginia também acrescentam a complexidade da reprodução desses animais. A poliandria ocorre em várias espécies de anuros (Zamudio et al 2016; Bordin, Alves et al 2024) e consiste na competição entre machos para fertilizarem a desova de uma fêmea. A competição modula várias características tais como o tamanho testicular e espermático (Haddad & Prado 2003; Byrne et al 2002; Bordin et al 2022). Já a poliginia em geral está relacionada à competição por locais de reprodução, onde machos monopolizam locais e fêmeas para reprodução exclusiva (Ficetola et al., 2010; Sá et al., 2020). Dessa forma, essas características juntamente com os modos reprodutivos são fundamentais para a compreensão do comportamento reprodutivo em anuros.

Sazonalidade e fatores climáticos

A sazonalidade é um fator de grande relevância no ciclo de vida dos anuros, pois as interações entre os fatores fisiológicos e ambientais exercem grande influência na reprodução desses animais (Sasso-Cerri et al 2004; Santos et al 2011). Características ambientais, como temperatura e umidade, podem influenciar a dinâmica reprodutiva de comunidades de anuros (Pereira et al 2020), a diversidade de estratégias reprodutivas (Todd et al 2011; Cayuela et al 2012; da Silva et al 2012; Pereira et al 2020) e até mesmo modular o desenvolvimento germinativo e a estrutura testicular das espécies (Santos et al 2011; Villagra et al 2014; Chaves, de Moura et al 2017).

A temperatura é um parâmetro ambiental que pode influenciar o ciclo anual de reprodução dos anuros, variando amplamente entre as espécies em função das adaptações às condições climáticas de seus habitats (Rastogi 1976). Essa variação espécie-específica torna-se evidente na dinâmica reprodutiva. Enquanto algumas espécies são influenciadas pela temperatura (Paniagua et al 1990; Todd et al 2011; Borah et al 2019), outras parecem não sofrer influência desse parâmetro climático ou apresentam apenas efeitos parciais sobre os traços reprodutivos (Rastogi 1976; Chaves, de Moura et al 2017).

As variáveis hídricas do ambiente são determinantes para o comportamento e diversidade reprodutiva de anuros, podendo explicar parte da riqueza de seus traços e modos reprodutivos (Haddad & Prado 2005; da Silva et al 2012; Nunes-de-Almeida et al 2021). A reprodução de anuros é modulada por fatores hídricos, como níveis de precipitação e disponibilidade de água, dos quais muitas espécies dependem para o sucesso na colonização, oviposição e sobrevivência da prole (Santos et al 2011; Cayuela et al 2012). Espécies que se reproduzem ao longo do ano, mesmo em épocas menos úmidas, também apresentam relação de dependência com disponibilidade de corpos d'água (Prado et al 2000; Santos et al 2011).

Na história evolutiva da reprodução dos anuros, a água é um componente vital, pois os ovos desses animais são frágeis e, por serem permeáveis, dependem da umidade para evitar dessecação (Vitt & Caldwell 2014). Durante a história evolutiva dos anuros, foram originados os mais diferentes modos reprodutivos que se conhecem atualmente, de modos aquáticos para terrestres e até reversões de modos terrestres para aquáticos (Haddad & Prado 2005; Altig & McDiamind 2007; Pereira et al. 2015). Com a transição para o ambiente terrestre, torna-se importante a evolução de características para tornar a manutenção do equilíbrio hídrico mais eficiente, como mudanças no tamanho e camadas dos ovos, adição de espuma para evitar dessecação e reprodução preferencial em locais mais úmidos (Pucci et al 2020). Assim, o equilíbrio osmótico é um aspecto vital para o sucesso do modo reprodutivo (Gomez-mestre et al 2012; Lion et al 2018).

Considerando tais relações, é clara a modulação do ciclo reprodutivo dos anuros pela interação entre a fisiologia e os fatores externos (Sasso-Cerri et al 2004; Chaves, de Moura et al 2017) e a dinâmica hídrica local e sazonal têm papel fundamental no desenvolvimento desses animais (Jakob et al 2003; Cayuela et al 2012). Dessa forma, é necessária a compreensão fisiológica relacionada à regulação osmótica e equilíbrio hídrico em anuros, considerando o contexto ambiental (Lemenager et al., 2022).

Proteínas de regulação osmótica

Como já mencionado, a regulação osmótica é essencial para a sobrevivência dos anuros. Nesse contexto, as aquaporinas (AQPs) desempenham papel fundamental no processo de manutenção do controle hídrico, contribuindo para o equilíbrio de fluidos internos deste grupo (Krane & Goldstein 2007; Miller 2018; Lemenager et al 2022). Essas

proteínas atuam facilitando o processo de transporte de água e apresentam permeabilidade variada (Yang & Verkman 1997; Pastor-Soler et al 2001).

As AQPs constituem família de proteínas pequenas "membrane-spanning", cujos monômeros têm de 26 a 34 kDa. Essas proteínas são expressas na membrana plasmática de tipos celulares envolvidos no transporte de fluidos (Figura 3). As proteínas dessa família organizam-se nas membranas como homotetrâmeros. Cada monômero consiste em seis domínios "membrane-spanning" α-hélice, com as extremidades carboxi e aminoterminal orientadas para o citoplasma, contendo um poro de água distinto, dessa forma, são canais de água que facilitam e regulam o transporte de água através das membranas celulares (Verkman & Mitra, 2000). Estão presentes nas membranas de organelas intracelulares, regulando assim o volume da célula e da organela (Nozaki et al 2008). As AQPs estão envolvidas em uma grande variedade de funções fisiológicas, como a homeostase de água e solutos, função secretória, facilitando o transporte transepitelial de fluidos, migração celular e neuroexcitação. Além de participarem de muitos processos patológicos como glaucoma, epilepsia, obesidade e câncer (Verkman 2009).

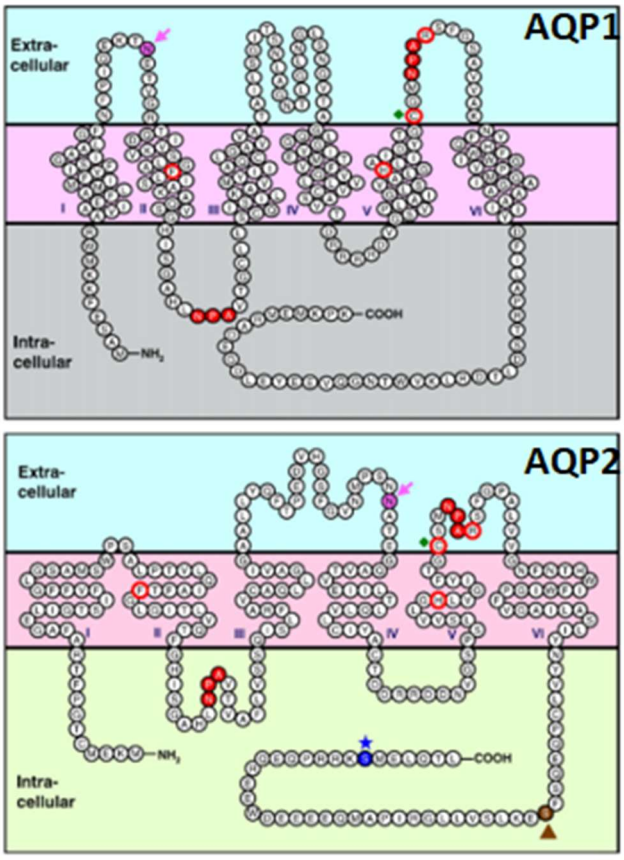


Figura 3. Esquema da estrutura peptídica de duas aquaporinas. Observa-se sua expressão na membrana celular, possuindo estruturas dentro e fora da célula. Adaptado de Suzuki et al 2007.

No sistema genital masculino, a água é necessária para a manutenção do ambiente luminal onde os espermatozoides estão localizados, pois serve como veículo para transportar esses espermatozoides do testículo para o ducto deferente (Hermo & Smith, 2011). Diferentes tipos de aquaporinas são expressos em células e regiões específicas ao longo dos ductos extratesticulares de mamíferos. Segundo Hermo & Smith (2011), as AQPs estão localizadas em domínios de membrana específicos, como microvilos, membranas apical, lateral e basal, bem como, endossomos, e a expressão destas AQPs pode ser controlada por hormônios e/ou fatores luminais.

As AQPs também podem ser reguladas e influenciadas por fatores internos e externos, como variações de pH, fosforilação e ligação de proteínas auxiliares (Brown et al 1995, 1998; Deen & van Os 1998; Echevarria & Ilundain 1998; Engel et al 2000; Verkman & Mitra 2000). Há também, evidência para uma relação direta entre a variação da expressão e a disponibilidade de água no meio (Suzuki et al 2007; Castellar et al 2015; Lemenager et al 2022).

O sucesso dos processos biológicos em anuros está ligado intimamente à dinâmica hídrica, regulação osmótica e, além disso, ao controle fisiológico do fluxo hídrico através dos órgãos (Krane & Goldstein 2007; Lemenager et al 2022). Evidências sugerem que a dinâmica de concentração de fluidos entre o meio interno e externo exercem papel fundamental em processos biológicos gerais e específicos, como na reprodução, atuando no sucesso da fertilização externa, especialmente para ativação da motilidade dos espermatozoides de anuros (Byrne et al 2015; Miller 2018). Dessa forma, a água é importante na dinâmica do sistema genital masculino e sua regulação no testículo por meio das aquaporinas, é processo essencial para a concentração do esperma (Huang et al 2006; Belleannée et al 2012) e consequentemente para a fertilidade masculina (Domeniconi et al 2008; Klein et al 2013).

O gênero *Leptodactylus* e a espécie *Leptodactylus podicipinus*

O gênero *Leptodactylus* (Cope, 1862) possui comportamento reprodutivo característico, geralmente envolvendo atividade em beiras de lagoas e poças, com oviposição em ninhos de espuma gerada por secreção glandular das fêmeas durante o amplexo (Prado et al 2002; Prado & Haddad 2003; Pereira et al 2015; Pucci et al 2020). O grupo se destaca por apresentar diferentes modos reprodutivos desde a deposição de ovos em ninhos de espuma sobre a água, até a construção de bacias ou tocas subterrâneas

onde as desovas são depositadas (Heyer 1969; Prado et al 2002; Pucci et al 2020). Além disso, parte das espécies possui morfologia especializada para o hábito escavador, que auxilia na construção das tocas subterrâneas, além de especializações como os tubérculos nos tarsos e um rostro mais rígido adaptado para cavar (Ponssa et al 2011; Salles et al 2017).

A espécie *Leptodactylus podicipinus* (Fitzinger, 1826) possui ampla distribuição geográfica, desde formações abertas do Paraguai, Argentina, Bolívia, Uruguai, até a porção central do Brasil (Frost 2024). A espécie possui dieta generalista e variada, de acordo com a disponibilidade de presas no ambiente (Heyer 1994; Prado et al 2000; Rodrigues et al 2004). Os indivíduos de *L. podicipinus* vivem em áreas abertas, comumente encontrados em ambientes antropizados (Eterovick & Sazima 2020). As fêmeas cuidam tanto da desova quanto dos girinos, os quais são encontrados em ambientes aquáticos rasos (Figura 4) (Prado et al 2002; Carrillo et al 2022). Os sítios de vocalização são, geralmente, bacias superficiais naturais ou construídas pelos machos, localizadas na beira de corpos d'agua, cobertos por vegetação da margem ou folhagem da serapilheira (Figura 4) (Prado et al 2000).

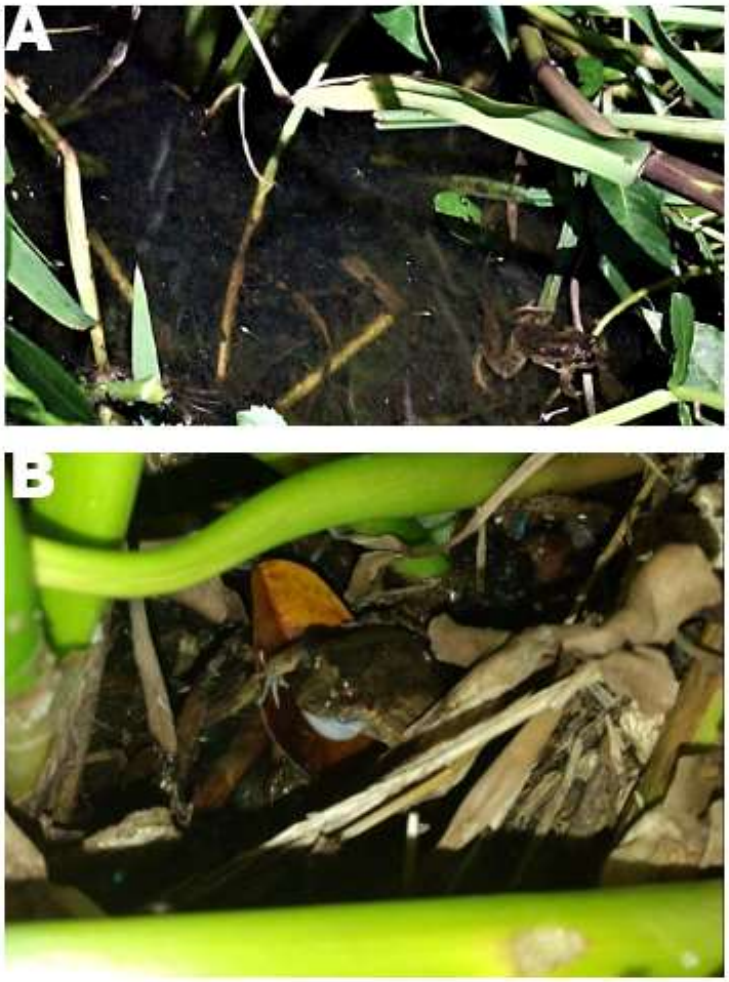


Figura 4. Fotos de indivíduos da espécie *Leptodactylus podicipinus*. A – Fêmea próxima a um grupo de girinos, demonstrando o cuidado parental presente na espécie. B – Macho vocalizando em uma depressão em vegetação marginal de um corpo d'agua. Acervo pessoal.

A anatomia dos testículos de *L. podicipinus* é semelhante ao descrito para anuros em geral, estruturas pares, esbranquiçadas e ovoides, localizadas ventralmente aos rins (Oielska 2009; Oliveira & Franco-Belussi 2012). Externamente o testículo é revestido pela túnica albugínea, através da qual é possível observar as estruturas dos lóculos seminíferos semelhantes a grânulos pela superfície testicular (Oliveira & Franco-Belussi 2012). As células germinativas se organizam em cistos que populam o interior dos lóculos (Bordin et al 2022). Tal organização é semelhante à vista em outras espécies de anuros (Leite et al 2015).

O comportamento reprodutivo de *L. podicipinus* pode ser descrito como oportunista, como observado na região do Pantanal (Prado et al 2000). Desta forma, a espécie possui a capacidade de se reproduzir o ano todo caso haja condições climáticas favoráveis e disponibilidade de locais propícios para oviposição (Prado et al 2000; Ferreira et al 2009). Essa descrição é usada para várias espécies de região tropical que se

reproduzem regularmente após intensas chuvas ao longo do ano (Duellman & Trueb 1994). No entanto, *L. podicipinus* possui registros que variam de uma reprodução demarcada, dentro do período de chuvas, (Rossa-Feres & Jim 1994) até atividade reprodutiva comportamental e testicular fora desse período, se estendendo ao longo do ano (Prado et al 2000; Bordin et al 2022). No último caso os registros foram feitos na região Centro-Oeste do país, porém ainda não há dados acerca do desenvolvimento de células germinativa ao longo de todo seu o ciclo reprodutivo. Tal variação na descrição da atividade reprodutiva é também observada em diferentes espécies, como *Dendropsophus minutus* (Santos et al 2011; Ferreira & Mehanna 2012), *L. macrosternum* (Villagra et al 2014; Chaves, de Moura et al 2017), *Physalaemus cuvieri* e *Pseudopaludicola falcipes* (Moresco 2013; Brum et al 2022).

A oviposição de *L. podicipinus* em ambiente aquático, os traços do modo reprodutivo (ninho de espuma e bacias naturais ou construídas), sugerem que a espécie possui um caráter intermediário em relação ao modo de vida aquático e terrestre em *Leptodactylus* (Prado et al 2002; Ponssa et al 2011). Essa característica transicional de *L. podicipinus* é interessante para compreender a relação entre sazonalidade e dinâmica hídrica no sistema genital masculino. A influência sazonal é bem evidente no ciclo reprodutivo dos anfíbios (Sasso-Cerri et al 2004), e esses são considerados bons modelos para o estudo da dinâmica de regulação hídrica mediada por aquaporinas (Krane & Goldstein 2007). Considerando o contexto apresentado, é evidente a necessidade de compreender melhor a situação reprodutiva dessas populações, além da avaliação de dados de animais provenientes de ambientes com diferentes tipos de uso (Borges et al., 2019; Rezende et al., 2021). Existem informações sobre a reprodução de *Leptodactylus podicipinus* em ambientes da região Centro-Oeste do Brasil, tanto urbanos quanto preservados (Ferreira et al., 2009; Bordin et al., 2022). No entanto, observa-se a ausência de monitoramento mais abrangente e de uma investigação mais detalhada sobre a condição reprodutiva dessas populações, especialmente em outras áreas de distribuição da espécie, onde o regime climático difere daquele das regiões já estudadas.

Dessa forma, o presente estudo teve como objetivo investigar a dinâmica entre a morfofisiologia testicular e sua relação com a sazonalidade ambiental ao longo do ciclo reprodutivo de *Leptodactylus podicipinus*, com ênfase na expressão de proteínas reguladoras dos testículos e em parâmetros germinativos de indivíduos da região centro-sul do estado de São Paulo. Foram testadas as seguintes hipóteses: (I) a proliferação

testicular e os aspectos anatômicos do trato urogenital variam sazonalmente; (II) fatores sexuais e climáticos atuam de forma sinérgica na modulação do padrão germinativo observado na espécie; e (III) a expressão de aquaporinas no testículo difere entre indivíduos de diferentes períodos do ano.

Capítulo I

Seasonal analysis of the male reproductive tract and germ cell proliferation in *Leptodactylus podicipinus* (Anura: Leptodactylidae)

Published in Journal of Morphology

Accepted in 08/2025

DOI: https://doi.org/10.1002/jmor.70072

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RESEARCH ARTICLE OPEN ACCESS

Seasonal Analysis of the Male Reproductive Tract and Germ Cell Proliferation in *Leptodactylus podicipinus* (Anura)

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Received: 16 April 2025 | **Revised:** 30 July 2025 | **Accepted:** 2 August 2025

Funding: This study was funded in part by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brasil (CAPES), grant number Finance Code 001. Classius De Oliveira has been continuously supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), grant #309722/2023-3.

Keywords: PCNA | reproductive anatomy | spermatogenesis | testis

ABSTRACT

In the reproductive dynamics of anurans, the male gonads have a fundamental relationship with the kidneys. Although reproductive aspects have been widely studied in this group, there are still considerable gaps in understanding the morphology and physiology of the reproductive system of neotropical anurans. Most research has emphasized aspects such as spermatogenesis and reproductive ecology, without information on the structure of the male reproductive tract and the dynamics of spermatogenesis in different species. To better understand the reproductive diversity of anurans, it is essential to comprehend reproductive morphology in a broad sense and simultaneously at different organizational levels. In this context, the present study aimed to characterize the components of the male reproductive tract and assess testicular cell proliferation in *Leptodactylus podicipinus* throughout its reproductive cycle, using histological, immunohistochemical, and computerized microscopy techniques. The male reproductive tract of this species comprises intratesticular ducts that converge into a longitudinal collecting duct, which gives rise to extratesticular efferent vessels entering the kidneys through lateral ducts. These ducts take the sperm through the glomeruli to the collecting ducts, leading to the Wolffian duct. Differences were observed in the intratesticular ducts of individuals in the reproductive and nonreproductive periods. Additionally, the proliferation of the initial germ cells (spermatogonia and spermatocyte I) exhibited positive PCNA staining, with distinct differences between the two periods analyzed.

1 | Introduction

The reproductive tract of vertebrates has been widely studied due to the anatomical and functional variability between different zoological groups (Lombardi 1998). From an evolutionary perspective, the origin and development of the reproductive tract in vertebrates occur in close association with the urinary tract, rendering them practically inseparable in many groups. This relationship can be explained by the fact that both originate from adjacent mesodermal structures during embryonic development (Oielska 2009). From these mesodermal elements originates the most ancestral nephric portion, i.e., the pronephros, which develops into a structure with glomeruli, i.e., the dorsal kidneys or opistonephros, present in anamniotes (cyclostomes, aquatic gnathostomes ['fish'] and amphibians (Lombardi 1998). The male reproductive tract is characterized by the production of sperm in the testes and the transport of these cells through a network of canals located inside and outside the gonads. These channels run

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through the testes and kidneys and end in the cloaca (Oielska 2009; Rheubert et al. 2017). The interaction between the kidneys, testes, and genital processes is a common feature in many anuran species. For example, the cranial part of the kidneys, known as the reproductive kidney, plays a crucial role in sperm transport. (Oielska 2009; Hiragond and Saidapur 2000; Rheubert et al. 2017).

However, this association can vary between species, including patterns in which the kidneys do not participate in gamete transport (Blüm 1986; Oielska 2009). The connection between the urinary and genital portions, especially in males, occurs during sexual differentiation (Lombardi 1998). In amphibians specifically, this connection also occurs through the connection of the primordial seminiferous parenchyma with the ducts called *vasa efferentia* that run from the testes through the mesorchium or mesothelium towards the kidneys (Oielska 2009; Rheubert et al. 2017; Serrano-Perez and Ramírez-Pinilla 2021).

Among the three orders of amphibians, Gymnophiona, Caudata, and Anura, the morphological patterns of the testes and the sperm duct show both particularities and similarities. In apodes (Gymnophiona), the lobed testes are connected to the kidneys and the Wolffian duct by several transverse efferent vessels, which originate from a central canal in the testes (Gomes et al. 2012; Serrano-Pérez and Ramírez-Pinilla 2021). In urodeles (Caudata), the testes are organized internally into seminiferous tubules that connect to a central canal, similar to the apodes, which branch into several efferent vessels, that run from the testes towards the kidneys. These vessels transport the sperm to the renal corpuscles or directly to the proximal renal ducts, following through the renal canals to the Wolffian duct (Siegel et al. 2014).

Although there are several studies on the male reproductive tract of amphibians, especially anurans, it is important to note that the morphology and histology of these structures vary widely between different species (Hiragond and Saidapur 2000; Rheubert et al. 2017). Despite a large amount of research on anuran reproduction, many focus on ecological, evolutionary, or cellular aspects (Santos et al. 2011; Pucci Alcaide et al. 2020; Bordin et al. 2024b), leaving gaps in knowledge about the detailed anatomical description of the reproductive tract of various species (Rheubert et al. 2017).

The morphology of reproduction in anurans is a complex and promising field with great potential for discovery. The male reproductive process involves a number of factors, including the development of the gonads, spermatogenesis, the maintenance of spermatozoa, and their transportation to ensure reproductive success (Méndez-Tepepa et al. 2023). Several studies have investigated the dynamics of spermatogenesis in different anuran species (Santos et al. 2011; Chaves et al. 2017; Pucci Alcaide et al. 2020; Bordin et al. 2022). In this context, cell proliferation in the gonads is a fundamental aspect of understanding testicular dynamics in anurans, especially concerning the type of cell involved in spermatogenesis (Chieffi et al. 2000).

The use of proliferating cell nuclear antigen (PCNA) has been effective in analyzing cell proliferation in anuran testes, and this tool has been related to the species' seasonal reproductive cycles (Chieffi et al. 2000; Chianese et al. 2015; Olea et al. 2021). Understanding cell biology is one of the essential aspects for comprehending the integral functioning of reproduction. Furthermore, knowledge of cellular processes is fundamental to understanding the biological patterns observed in the reproductive cycles of species.

Considering the importance of anatomical and cellular information in the study of the reproductive system and the scarcity of data relating these aspects to reproduction in the anuran group, this study aims to describe the structure of the male reproductive tract of *Leptodactylus podicipinus* (Leptodactylidae). It will be compared with available data on other amphibian species, and cell proliferation in the testes of this species will be analyzed using PCNA immunolocalization throughout its reproductive cycle. We hypothesize that the anatomical and cellular aspects of the individuals of this species vary seasonally.

2 | Methods

2.1 | Study Species

L. podicipinus (Cope 1862) is widely distributed in the Neotropical region, being found in Brazil, Bolivia, and Paraguay, northeastern Argentina (Sá et al. 2014). In terms of reproductive behavior, the species is commonly found in open areas on the edges of ponds or pools with undergrowth, where it breeds in natural or constructed shallow basins (de Almeida Prado et al. 2000; Bordin et al. 2024a).

2.2 | Sampling

Collections were carried out over 2 years (2021–2023). Individuals were located and captured in the field using active search methods and listening at vocalization sites. Field trips were made from dusk until late at night (6 pm to 11 pm). Sexual maturity was confirmed through secondary sexual characteristics such as the presence of vocal sacs and spines on the hands. The individuals were separated into two groups according to the different periods of the species' reproductive cycle (Bordin et al. 2024a), a reproductive period from October to February (REP—11 individuals) and a nonreproductive period from March to September (NREP—9 individuals).

The sites where the individuals were obtained were Porto Said (22°40′28.0“S 48°20′00.8” W) and Rio Bonito (22°40′49.1“S 48°19′37.0” W), both located in the municipality of Botucatu—São Paulo, Brazil. The locations have an average annual temperature of 21°C and annual rainfall of 1500 mm (Franco et al. 2023). The specimens were euthanized through the topical application of 5% lidocaine to both the dorsal and ventral surfaces.

The animals used in this experiment were kept under the Ethical Principles in Animal Experimentation, adopted by the Brazilian College of Animal Experimentation (CEUA No. 4478221021), and all collections were carried out under a license to collect and keep amphibians in the laboratory (No. 79953-3 SISBIO/IBAMA).

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2.3 | Scanning Electron Microscopy

To obtain scanning microscopy images, an independent collection of 3 adult males from the reproductive period was made. The testes were collected and fixed in buffered glutaraldehyde (2.5%) at room temperature for 3 h. They were then dried to the critical point using acetone at a temperature of 4°C. After the drying process, the samples were sputter-coated with gold and taken to the scanning electron microscope (Quanta Series 200—FEI Company), using an accelerating voltage of 12.5 KeV. The technique was performed at the Electron Microscopy Center of the Universidade Estadual Paulista, Botucatu campus.

2.4 | Histology

Histological analyses were carried out to evaluate the architecture of the reproductive tract of *L. podicipinus*. Twenty sexually mature adult males were used (REP—11; NREP—9). The testes and kidneys were extracted and fixed in buffered formalin (10%) and embedded in Paraplast (Sigma–Aldrich) following standard protocols (Canene-Adams 2013). Non-serial 3 μm-thick sections of the testes and kidneys were obtained using a rotary microtome (longitudinal sections). The material was stained with Hematoxylin-Eosin (HE) and analyzed using light microscopy (Axiophot 2 microscope with AxioCam HR, Zeiss, Germany) at the Anatomy Laboratory of the Instituto de Biociências de Botucatu—Unesp.

The sections from individuals from both reproductive periods were compared qualitatively and classified into two categories based on the morphology of the intratesticular ductules: (1) dilated with sperm, and (2) closed or empty. This classification was performed to identify morphological changes in the testes throughout the reproductive cycle of the species.

2.5 | Immunohistochemistry

Histological sections of the testes were deparaffinized in xylene and hydrated in ethanol. Antigen retrieval was carried out by incubating the sections in citrate buffer (pH: 6.0) at 100°C for 15 min in a microwave oven (power 750 W). Endogenous peroxidases were blocked in methanol (3% H2O2) for 15 min. To reduce nonspecific protein-protein bonds, the sections were incubated in a blocking solution with bovine serum albumin (3%) in PBS-T buffer for 1 h at room temperature. Subsequently, the Anti-PCNA antigen was localized using the primary antibody diluted at a concentration of 1:100 (MAB424—MiliporeUSA) in 1% BSA and incubated overnight at 4°C. After washing with PBS-T buffer, the sections were incubated with the HRP secondary antibody (1:100 anti-mouse Abcam) for 1 h at room temperature and then revealed with diaminobenzidine (DAB) and counterstained with Mayer's hematoxylin. Sections with the primary antibody replaced by 0.01 mol l^{-1} PBS were used as negative controls.

The sections were dehydrated and coverslipped for imaging and analysis using an Axiophot 2 photomicroscope equipped with an AxioCam HR digital camera (Zeiss, Jena, Germany), from the Department of Structural and Functional Biology, Anatomy Sector, Instituto de Biociências de Botucatu, UNESP-Universidade Estadual Paulista, Brazil.

2.6 | Proliferating Cell Nuclear Antigen Quantification

The images obtained from the slides using the immunohistochemistry technique were analyzed using Image ProPlus software (v6.0, Media Cybernetics). The cells marked by the PCNA antibody were quantified using Weibel's graticulated method (Weibel 1963). For this, the values were obtained by counting points, and a total of 50 images were analyzed, approximately 3 to 6 images per individual analyzed.

2.7 | Comparison With Other Species

To establish a framework for comparative anatomy, data were compiled from existing literature on amphibian species with detailed descriptions of their reproductive systems. The anatomical features compared included internal testicular morphology, the presence of a central intratesticular duct, and the structure of the extratesticular ducts. The species included in the comparison were *Aquarana catesbeiana* (Ranidae, Anura), *Caecilia thompsoni* (Caeciliidae, Gymnophiona), and *Ambystoma maculatum* (Ambystomatidae, Caudata).

2.8 | Statistical Analysis

The Mann-Whitney non-parametric test was used to compare the means of the groups. Before applying the test, the data were analyzed for normality using the Shapiro–Wilk test. The statistical analyses were carried out using the R software (version 4.3.2).

3 | Results

3.1 | Male Reproductive Tract

The reproductive tract of males of the species *L. podicipinus* consists of two whitish ovoid testes located in the abdominal cavity (Figure 1). The kidneys are located dorsally to the testes, and both the kidneys and the ducts and blood vessels observed are supported by a visible fibrous tunic, i.e., the mesorchium, which also surrounds the testes (Figure 1). The testes within the mesorchium, in turn, are covered by another fibrous tunic, the tunica albuginea, which, due to its transparency, makes it possible to identify the peripheral testicular architecture by visualizing the seminiferous loculi (parenchyma) being delimited by the arrangement of the stroma.

Internally, the germinative epithelium develops within the sac-like parenchyma, that is, the seminiferous loculi. Within the same locule, there are different clusters of germ cells at varying stages of maturation, these clusters are germ cysts and are supported by Sertoli cells from the periphery of the loculi. Between the loculi of the parenchyma are somatic support cells that form the stroma, including fibroblasts that delimit the loculi and Leydig cells.

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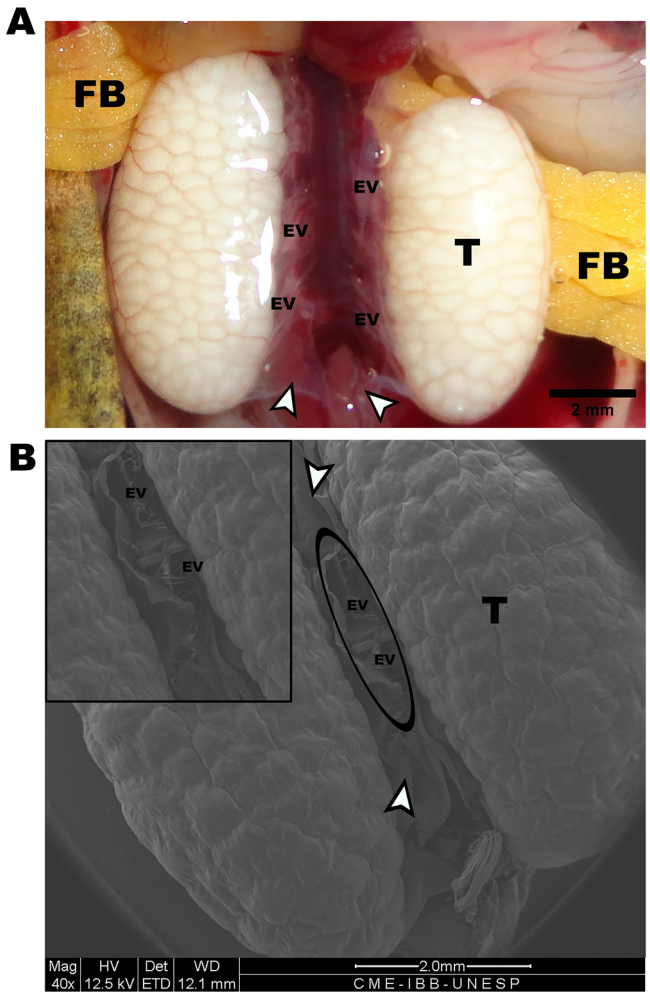


FIGURE 1 | *Leptodactylus podicipinus*, anatomy of the reproductive tract. (A) Macro-photograph of efferent vessels leaving the testis in the direction of the dorsal kidneys; the testis is surrounded by the mesorchium (arrowheads); cranial fat bodies can be observed in the cranial part of the testes. (B) Scanning electron micrograph of the reproductive tract; testicular surface; the efferent vessels exposed after cutting the mesorchium. Arrowheads – mesorchium; EV – efferent vessels; T – testicular surface; FB – fat bodies.

After spermatogenesis, mature free sperm are found in the locular lumen. Intratesticular ductules (ID) can be seen draining the seminiferous loculi (Figure 2A,B). These ductules converge into a larger common channel, called the longitudinal collecting duct (LCD—Figure 2C,D; Figure 4). The sperm are then transported to the extratesticular ducts, i.e., efferent vessels (EV —Figure 2E,F; Figure 4), which cross the mesorchium and can be seen between the testes (Figure 1A).

The efferent vessels (EV) then connect to the lateral ductules of the kidneys (LD—Figure 3A,B) and finally enter the reproductive part of the kidneys (Figure 3C–F; Figure 4), where the sperm are transported through the network of lateral ductules to the renal collecting ducts (CD), which take the sperm to the Wolffian duct (WD—Figure 3E,F; Figure 4).

By comparing the male reproductive tract of *L. podicipinus* with data from the literature on other amphibian species, including representatives of the orders Gymnophiona and Caudata, a shared morphological feature was identified between *L. podicipinus* and the apode and urodele species (Figure 5). Specifically, the longitudinal collecting duct (LCD—*L. podicipinus*), longitudinal testicular duct (LTD—*Ambystoma maculatum*) and spermatic collecting duct (CD—*C. thompsoni*) is a similar morphological aspect shared by the three species. *A. catesbeiana* and *L. podicipinus* present a similar organization of seminiferous loculi (SL) and intratesticular ducts (ID), in contrast with the seminiferous lobules (LB) in *Ambystoma maculatum*; however, *A. catesbeiana* does not present a longitudinal duct. A comparable structure found in all three species is the branching extratesticular ducts towards the kidneys (EV and SD in *C. thompsoni*, Figure 5).

Concerning the periods during the reproductive cycle (REP and NREP) of the species, it was possible to observe differences in the intratesticular ductules of the individuals studied. Most of the individuals from the nonreproductive period (Table 1) had closed ducts and ductules without many spermatozoa (Figure 6). In contrast, individuals from the reproductive period had the ductules (Table 1) dilated and filled with sperm varying in quantity (Figure 6). In general, the pattern of the animals from the reproductive period was of dilated intratesticular ductules and longitudinal canal with sperm, while the animals from the nonreproductive period had a pattern of ductules and longitudinal canal closed, empty, or with few sperm.

3.2 | Proliferating Cell Nuclear Antigen Analysis

In the analysis of PCNA immunoreactivity in the testes of *L. podicipinus* individuals, the germ cells of earlier stages were positively marked, such as spermatogonia and spermatocytes (Figure 7), while later cells such as spermatids and spermatozoa showed no prominent immunoreactivity. Positive labeling was observed in both the nucleus of the cells and the cytoplasm.

Immunohistochemistry was carried out on the testes of animals from different periods of the species' reproductive cycle. When the amount of PCNA was compared between individuals from the two periods studied, there was a significant difference (Mann–Whitney $U = 17$, $P < 0.05$ two-tailed) between the periods. Individuals of *L. podicipinus* from the nonreproductive period (NREP) had a higher average number of positive PCNA markings in the testis than animals from the reproductive period (Figure 8).

4 | Discussion

The external anatomy of the male reproductive tract in *L. podicipinus* is similar to patterns already described for the species (Bhaduri 1953). The testes are paired, whitish, and located ventrally to the kidneys. They are lined by the tunica albuginea and supported by another fibrous tunic, the mesorchium. Between the testes, EV can be seen leaving the testes in the direction of the kidneys.

4.1 | Patterns and Variations

The internal morphology of the reproductive tract in *L. podicipinus* exhibits structural patterns similar to those observed in

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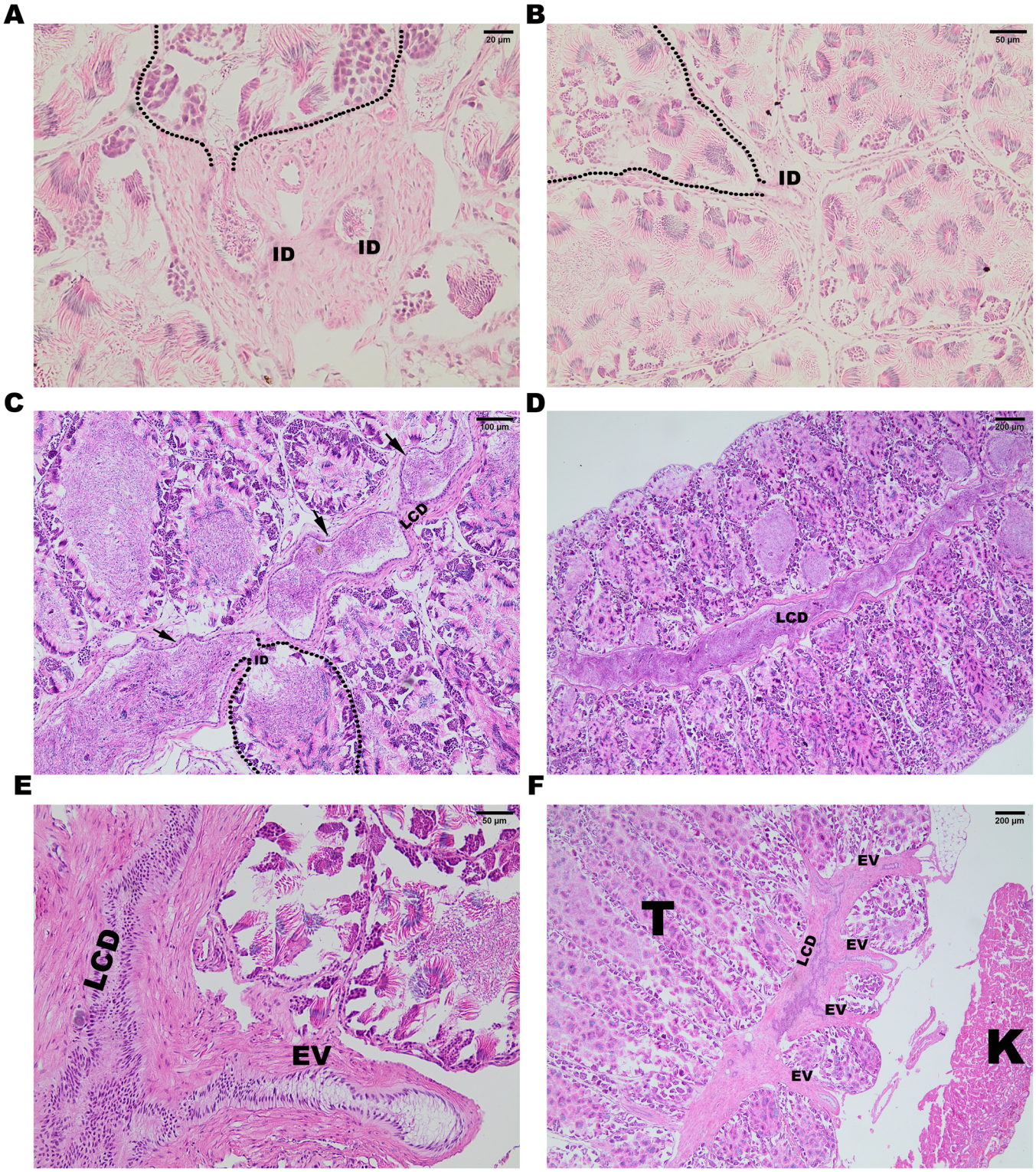


FIGURE 2 | *Leptodactylus podicipinus*, photomicrograph of the histological details of the reproductive tract. (A and B) Intratesticular ductules drain the sperm from the seminiferous locules. (C and D) The draining intratesticular ductules fuse, creating larger ductules that later fuse in the longitudinal collecting duct. (E and F) Efferent vessels, which branch from the longitudinal collecting duct and cross the mesorchium towards the kidney. Dotted lines – Loculi delimitation; ID – intratesticular ducts; Arrows – Large IDs before fusion; LCD – longitudinal collecting duct; EV – efferent vessels; K - renal corpuscle and T - testis. A bar: 20 µm/B and E bar: 50 µm/D bar: 200 µm/F bar: 200 µm/C and F bar: 50 µm. Dotted line—delimitation of the locules. Hematoxylin and Eosin (HE) staining.

other species within the group (Hiragond and Saidapur 2000). In general, anurans show several different patterns in the organization of the male reproductive tract and the network of sperm transport channels. These patterns can range from channels with a joint urogenital function to single channels for genital and urinary functions (Blüm 1986). Among different species, structures, such as the seminiferous loculi and EV, are mostly similar, but there are also points of divergence, for

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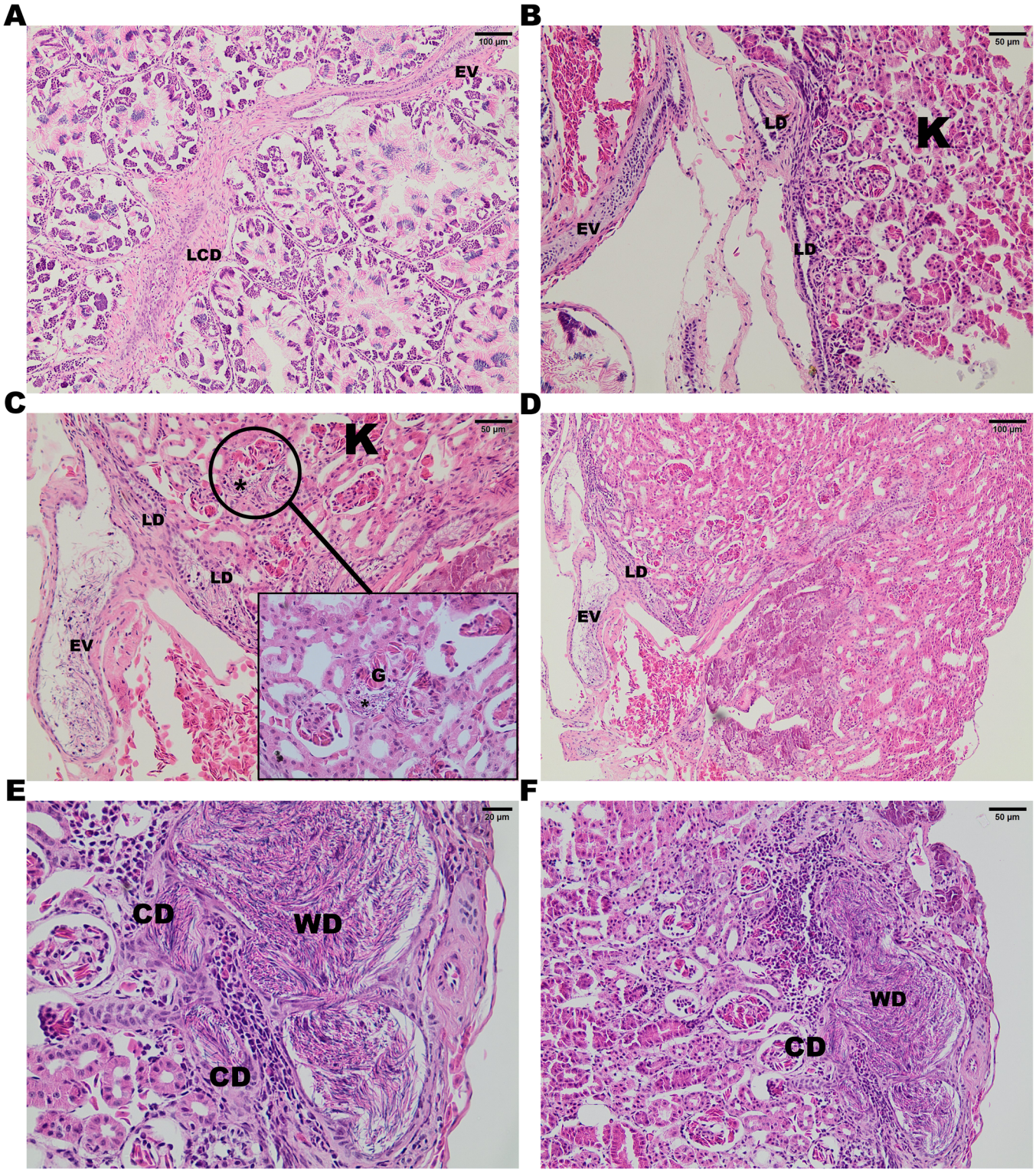


FIGURE 3 | *Leptodactylus podicipinus*, photomicrograph of the histological details of the reproductive tract in *L. podicipinus*. (A and B) The longitudinal collecting duct branches in efferent vessels towards the kidney, where it connects to the lateral ductules of the kidney. (C and D) The lateral ductules carry the sperm through the glomerulus. (E and F) The renal collecting duct transports the sperm to the Wolffian Duct. LCD – longitudinal collecting duct; EV – efferent vessel; LD – lateral ductule; K – Kidney; G – glomerulus; ***** - sperm; CD – renal collecting duct and WD – Wolffian duct. A and D bar: 100 µm/B, C and F bar: 50 µm/E bar: 20 µm. Hematoxylin and Eosin (HE) staining.

example, the absence of ductules as the ID or LD, and even the presence of LCD (Hiragond and Saidapur 2000). These variations highlight the morphological variability present in the reproductive tract of anurans (Rheubert et al. 2017).

In the works of Blüm (1986), Oielska (2009), and Hiragond and Saidapur (2000), four patterns of structural organization of the male reproductive system in Anura were observed. Three of these patterns defined by Blüm (1986) are based on the Palearctic

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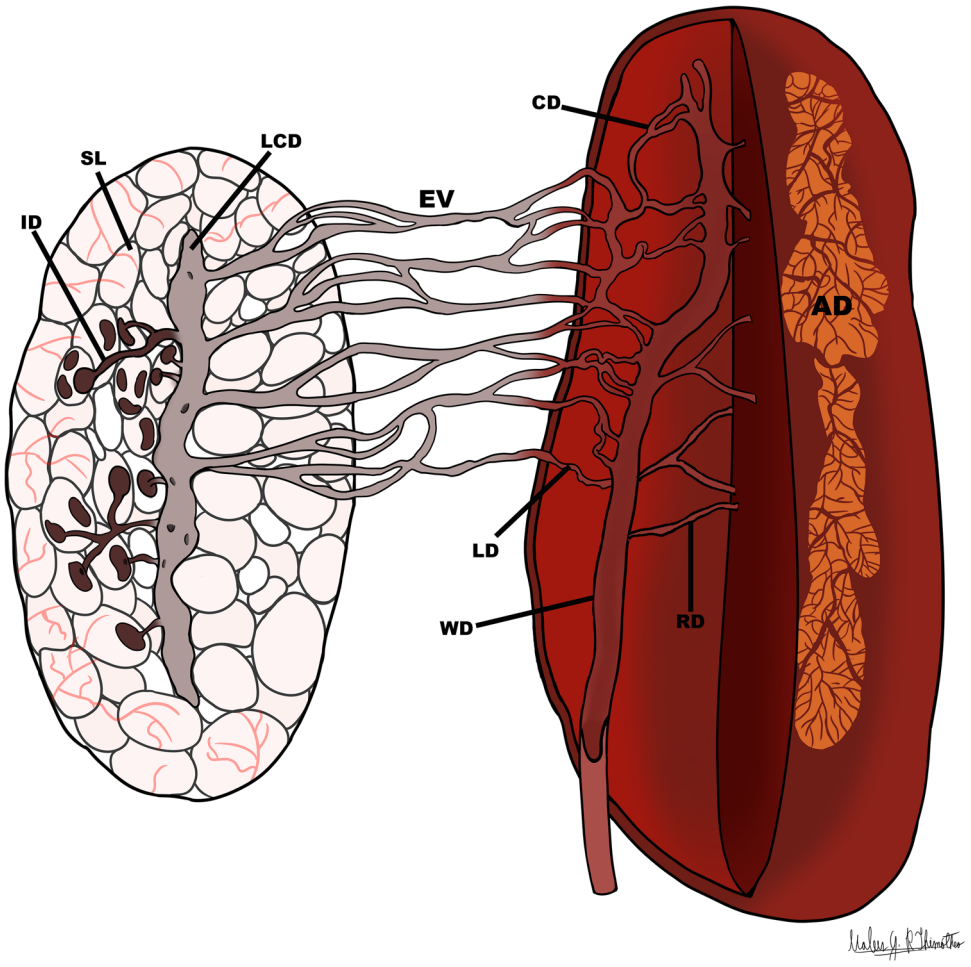


FIGURE 4 | *Leptodactylus podicipinus*, anatomical drawing of the reproductive tract. The illustration depicts the sperm transport pathway, beginning in the seminiferous locule. Sperm is first drained by the intratesticular ductules, which convey it to the longitudinal collecting duct. From there, sperm pass into the efferent ducts that traverse the mesorchium and enter the kidney, ultimately reaching the lateral ductules of the kidney. These ducts channel the sperm through the glomeruli and into the collecting ducts, which lead to the Wolffian duct. SL – Seminiferous locule; ID – intratesticular ductules; LCD – longitudinal collecting duct; EV – efferent vessels; LD – lateral ductules; CD – collecting ducts; RD – renal ducts; WD – Wolffian duct; AD – Adrenal gland.

species of the genera *Bombina*, *Discoglossus*, and *Alytes*, where a developmental pattern of separation of the urinary and genital functions is indicated. In *Bombina*, the urogenital portion is connected through the canal system of both the genital and urinary portions; in *Discoglossus*, there is a loss of connection between testes and kidneys, and only the WD is shared; while in *Alytes*, there are different ducts that drain the organs (testes and kidneys) separately. Hiragond and Saidapur (2000) investigated six species from the Indo-Malayan region, and the patterns found did not coincide with any of the three reported by Blüm (1986), since the eastern species have an intimate interaction between testes and kidneys, with the sperm being transported through the EV to the renal glomeruli, where they are taken to the WD. This connection was also found by Rheubert et al. (2017) in the male reproductive tract of the Nearctic species *A. catesbeiana*. The pattern observed in the Indo-Malayan and Nearctic species appears to be closer to that found in the Neotropical species *L. podicipinus*.

4.2 | Male Reproductive Tract in *L. Podicipinus*

In *L. podicipinus*, the seminiferous locules are drained by the ID, which emerge within the testicular stroma. These IDs have been observed in several species, such as *Euphlyctis cyanophlycts* (Dicroglossidae), *Fejervarya limnocharis* (Dicroglossidae) and *A. catesbeiana* (Ranidae) (Hiragond and Saidapur 2000; Rheubert et al. 2017). However, in other species, such as *Polypedates maculatus* (Rhacophoridae) and *Duttaphrynus scaber* (Bufonidae), IDs are absent, and the seminiferous locules instead empty directly into the EV network (Hiragond and Saidapur 2000).

In addition to the IDs, *L. podicipinus* possesses the LCD, a canal that extends longitudinally throughout the testis. This structure is similar to the one described for the species *P. maculatus* (Hiragond and Saidapur 2000) and is not present in the other species *E. cyanophlyctis*, *F. cyanophlyctis*, *F. limnocharis*, *A. catesbeiana* (Hiragond and Saidapur 2000; Rheubert et al. 2017). However, in *L. podicipinus*, the seminiferous locules do not radiate from the LCD as they do in *P. maculatus*. Instead, the IDs drain the locules and transport sperm until they converge in the LCD. The pattern observed in *P. maculatus* appears to resemble more closely the organization of the reproductive tract described for caecilian species (Wake 1968). In the species studied, the EV exits the testis and crosses the mesorchium toward the kidneys, in a similar way to that observed in other species (Hiragond and Saidapur 2000; Rheubert et al. 2017).

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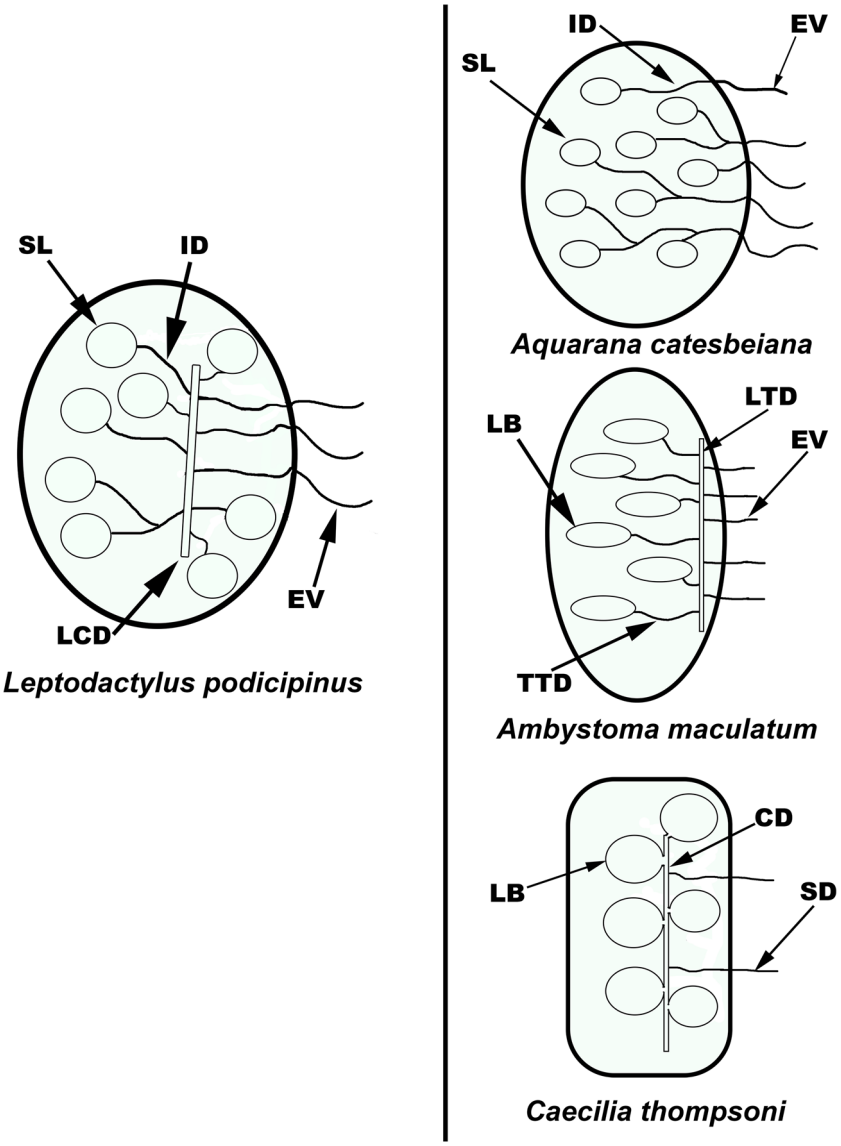


FIGURE 5 | Comparative diagram of the reproductive tract of amphibian species. Schematic representing the male reproductive tract of *L. podicipinus*, *A. catesbeiana*, *Ambystoma maculatum*, and *C. thompsoni*. SL - seminiferous loculus; ID - intratesticular ductules; LCD - longitudinal collecting duct; EV - efferent vessels; LB - seminiferous lobule; TTD - transverse testicular duct; LTD - longitudinal testicular duct; CD - spermatic collecting duct; SD - spermatic duct. **Schemes based on and adapted from** Wake 1968; Siegel et al. 2013; Rheubert et al. 2017; Serrano-Perez and Ramírez-Pinilla 2021.

TABLE 1 | Proportion of testicular ducts observed in *L. podicipinus* in the different periods studied.

	Testicular ductules	
Period (n=20 individuals)	**Dilated with sperm**	**Closed or empty**
REP ($n = 11$)	63.6%	36.4%
NREP ($n = 9$)	22.3%	77.7%

Abbreviations: NREP, nonreproductive; REP, reproductive.

Another characteristic observed *in L. podicipinus* and other species is the presence of sperm in the glomerulus. These traits appear to be well-conserved, as all species with available histological descriptions in the literature exhibit these features. In the analysis of urogenital interaction in *L. podicipinus*, the EV network connects to the LDs, which are part of the reproductive kidney. Unlike what is observed in Ranidae and Dicroglossidae (Rheubert et al. 2017; Hiragond and Saidapur 2000), only a single lateral renal canal is present, where sperm are deposited before entering the renal corpuscles. However, the structure observed in *L. podicipinus* is similar to that found in other species, such as *P. maculatus* and *D. bengalensis* (Bufonidae) (Hiragond and Saidapur 2000). Histological analysis of the spermatic transport ducts in *L. podicipinus*, in addition to the description for *P. maculatus*, reveals that both species possess an LCD, a structure not reported in other anuran species described to date (Rheubert et al. 2017; Hiragond and Saidapur 2000).

4.3 | Comparison Between Amphibian Species

In caecilians (Gymnophiona) and urodeles (Caudata), such as *C. thompsoni* and *A. maculatum*, the seminiferous lobules drain into a central LCD, which then branches into EV or sperm ducts that connect the testes to the kidneys (Figure 5). In contrast,

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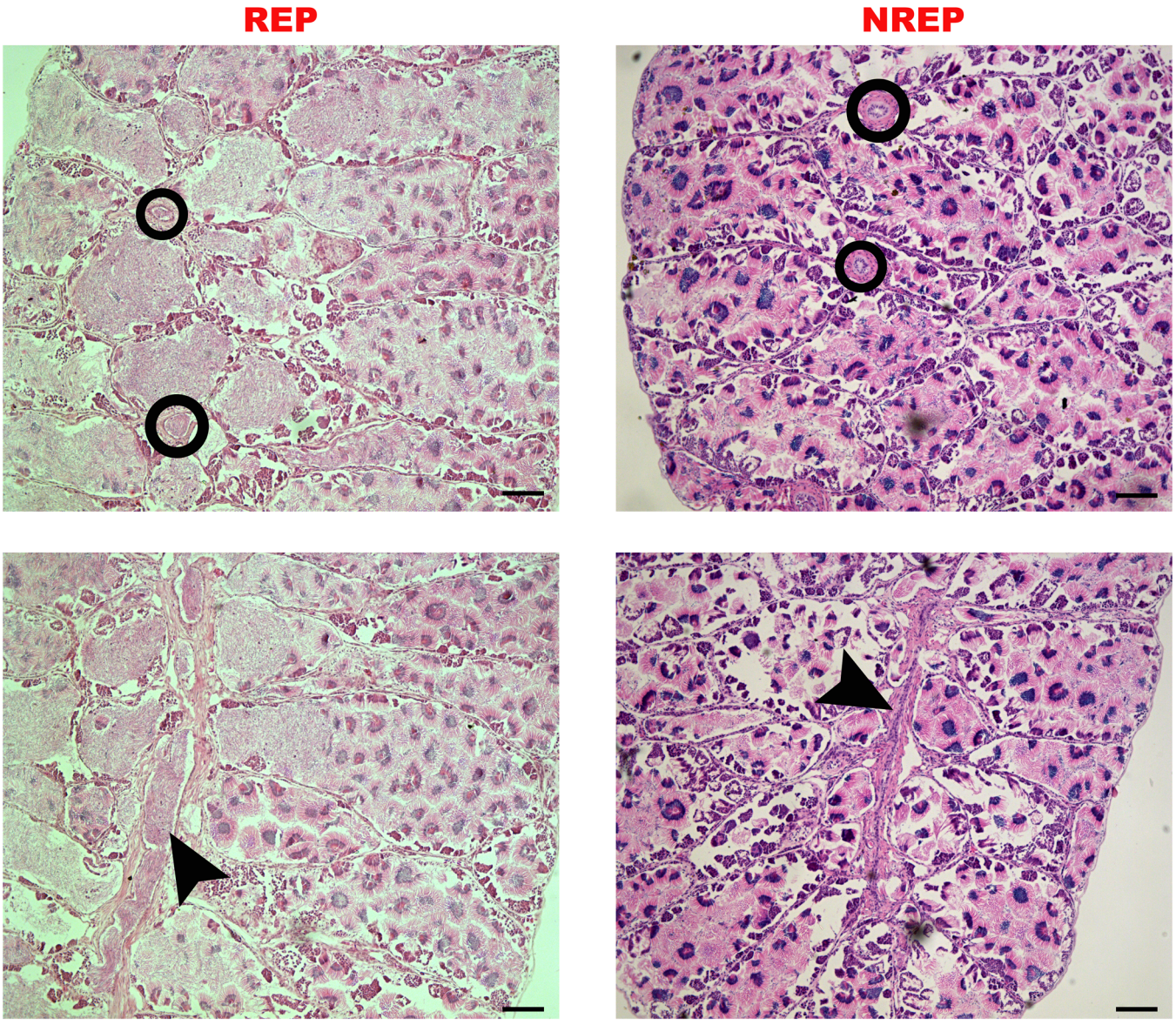


FIGURE 6 | *Leptodactylus podicipinus*, comparison of the histological characteristics of the intratesticular ducts in different reproductive periods. Bar: 100 μm. Circles - Intratesticular ducts; Arrowheads - Longitudinal collecting ducts. REP - reproductive, NREP - nonreproductive.

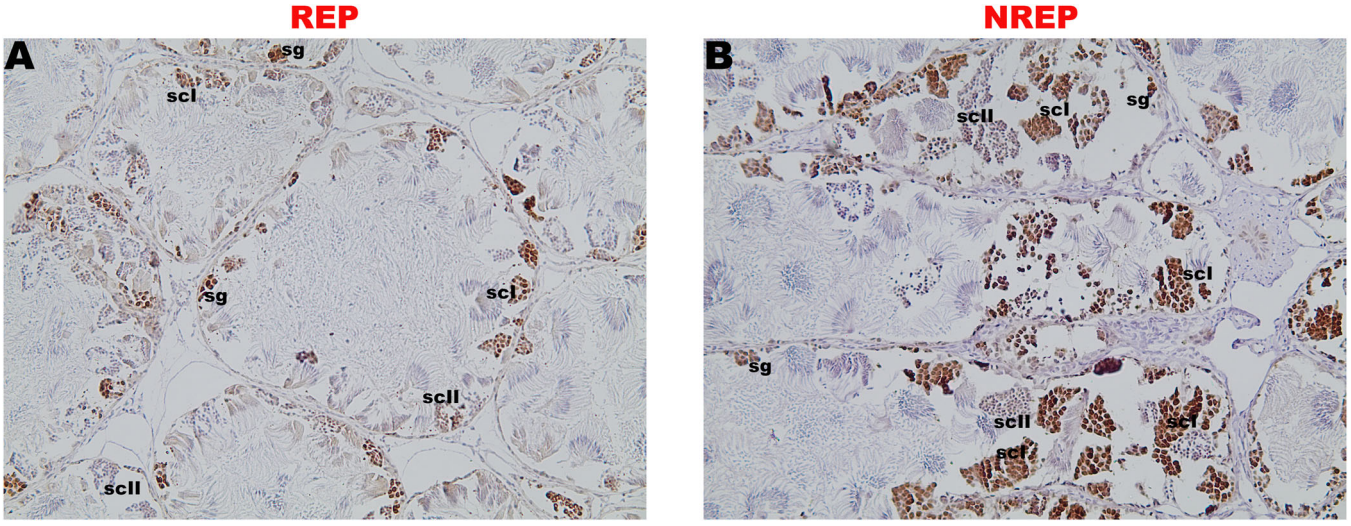


FIGURE 7 | *Leptodactyluspodicipinus*, PCNA immunolocalization in the testes. Animals from different periods were used REP - reproductive and NREP - nonreproductive. sg - spermatogonia; scI - primary spermatocyte; scII - secondary spermatocyte.

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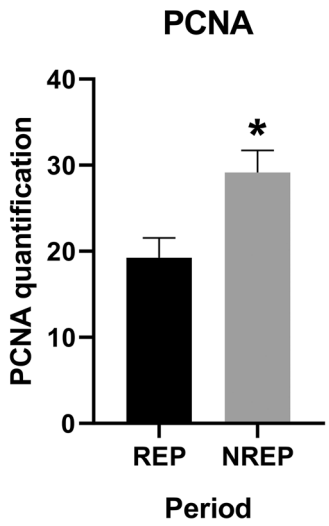


FIGURE 8 | *Leptodactylus podicipinus*, graph of PCNA quantification of individuals from the periods tested. Mann-Whitney test; significance $p < 0.05$; values expressed as the mean and standard error of the mean. REP – reproductive period, NREP – nonreproductive period.

anurans exhibit a different configuration, in which the IDs drain directly into the EV, with no LCD present. Interestingly, the branching of the central canal within the EV, a feature characteristic of both Caudata and Gymnophiona (Siegel et al. 2014; Serrano-Pérez and Ramírez-Pinilla 2021), is also observed in *L. podicipinus* and *P. maculatus*, but not in other anurans such as *A. catesbeiana*. Considering these findings, *L. podicipinus* shows morphological traits shared with apodes and urodele species (Figure 5). This contrasts with findings in other anuran species, where neither an LCD nor a central canal is present before the spermatic duct exits the testis. However, additional information on different species is needed to clarify how much variation exists among amphibians.

Polypedates maculatus and *L. podicipinus* species share some similarities in their reproductive behaviors. Both are foam-nest breeders, and both the Rhacophoridae and Leptodactylidae families exhibit species with large testes and engagement in sperm competition (Kanamadi and Jirankali 1992; Girish and Saidapur 1999; Prado and Haddad 2003). While foam-nest breeding may not be directly linked to multimale spawning (Prado and Haddad 2003), the similarities in reproductive conditions and behaviors may be associated with comparable morphological and physiological traits related to testicular sperm transport. It is known that reproductive characteristics can influence the morphology and physiology of anuran species (Furness et al. 2010; Byrne et al. 2015; Chen et al. 2023).

4.4 | Seasonality and PCNA

Seasonal differences among individuals from the different periods analyzed were observed in the dilation of the IDs and in the amount of sperm present within them. In *S. acuminatus* (Hylidae), the reproductive structures of the kidneys showed anatomical changes during periods of higher reproductive activity (Olea et al. 2021). This finding may suggest a seasonal influence on the reproductive cycle of the species, similar to that observed in the IDs of the species studied. However, in *L. podicipinus*, the canals associated with the kidneys showed no anatomical changes between the periods analyzed, except for the variation in the amount of sperm in the lumen. The scarcity of information on the anatomy of the male reproductive tract in neotropical species during different periods of the reproductive cycle makes it difficult to obtain clear patterns for comparison.

Immunohistochemical analysis of proliferative cell activity was assessed by detecting positive cells for Proliferating Cell Nuclear Antigen (PCNA); PCNA-positive cells indicate active cell proliferation. These cells were detected in individuals of the studied species throughout the year, regardless of the season, suggesting continuous proliferative activity. This result is similar to that observed in *Pelophylax esculentus* (Ranidae), *A. catesbeiana*, and *S. acuminatus* (Chieffi et al. 2000; Caneguim et al. 2013; Olea et al. 2021). In *P. esculentus*, spermatogonia and spermatocytes I were positive for PCNA labeling; in *L. podicipinus*, all early germ cells showed positive labeling. The prolonged labeling of PCNA, as well as its presence in the cytoplasm beyond the nucleus, may be related to its role in DNA repair reactions and interactions with other cytoplasmic proteins not yet identified, possibly kinases (Chieffi et al. 2000).

In *S. acuminatus*, PCNA-positive labeling remained constant throughout the reproductive cycle. In contrast, *L. podicipinus* exhibited significant variation in PCNA labeling intensity, suggesting fluctuations in germinative cell activity. This occurred despite the species maintaining continuous testicular activity, as evidenced by the persistent presence of spermatozoa in the lumen across the reproductive cycle (Bordin et al. 2024b). Such fluctuations in germinative activity were also observed in *P. esculentus*, where cell proliferation increased during the summer and fall, periods of higher reproductive activity, unlike in *L. podicipinus* (Chieffi et al. 2000; Chianese et al. 2015).

In *A. catesbeiana*, there was an increase in PCNA labeling in spermatogonia in spring-summer, associated with an increase in testosterone levels, which inhibit cell proliferation (Caneguim et al. 2013). This variation may be attributed to interspecific differences, methodological approaches used for immunolabeling quantification, and the origin of the individuals, which in *A. catesbeiana* were obtained from captivity (Caneguim et al. 2013), while those of *L. podicipinus* were collected from the wild. Although it is interesting to note, that while both species have continuous testicular activity, *A. catesbeiana* shows an increase in testicular size during the reproductive period (Sasso-Cerri et al. 2004), which may influence the proliferation of spermatogonia due to seasonal testosterone levels. In contrast, *L. podicipinus* shows no variation in testicular size throughout the reproductive cycle, possibly due to its opportunistic reproductive behavior (Bordin et al. 2024b).

4.5 | State of Reproductive Morphology in Anura

As discussed, genital morphology and urogenital connections exhibit considerable variation among anuran species. The scarcity of detailed information and the limited focus on these structures have hindered full exploration of their comparative potential and the identification of underlying patterns that need to be

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elucidated. Currently, the information available on the urogenital structure of anurans is limited, covering around 64 species from 21 families (Bhaduri 1953; Oielska 2009; Rheubert et al. 2017). However, the available information lacks histological details.

5 | Conclusion

This study provides novel insights into the reproductive morphology of *L. podicipinus*, with a focus on cell proliferation and histological details of urogenital interactions, thereby expanding current knowledge about the morphology of the species. The findings highlight the complexity and variability within the group, emphasizing both similarities and differences in comparison to data available for other amphibian species. The reproductive characteristics observed in *L. podicipinus* offer valuable contributions to the understanding of anuran reproductive biology, an ecologically important group increasingly threatened by climate change and anthropogenic activities.

Author Contributions

Rafael O. A. Bordin: conceptualization, methodology, formal analysis, writing – original draft, writing – review and editing, validation. **Classius de Oliveira:** conceptualization, methodology, validation, writing – original draft, writing – review and editing, project administration, supervision. **Raquel F. Domeniconi:** project administration, supervision, writing – original draft, writing – review and editing, conceptualization.

Acknowledgments

The authors thank the technicians, Gelson Rodrigues and Rinaldo Ortiz, for histology processing. This study was funded in part by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brasil (CAPES), grant number Finance Code 001. Classius De Oliveira has been continuously supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), grant #309722/2023-3. The Article Processing Charge for the publication of this research was funded by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) (ROR identifier: 00x0ma614).

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data is contained within the study.

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Capítulo II

Germinative dynamic, seasonality and polyandry: A dive in neotropical point-belly frog reproduction

This is the accepted version of the following article: Bordin, R. O. A., de Oliveira, C., & Domeniconi, R. F. (2024). Germinative dynamic, seasonality, and polyandry: A dive in neotropical point-belly frog reproduction. The Anatomical Record. https://doi.org/10.1002/ar.25627, which has been published in final form at https://anatomypubs.onlinelibrary.wiley.com/doi/10.1002/ar.25627. This article may be used for non-commercial purposes in accordance with the Wiley Self-Archiving Policy (http://www.wileyauthors.com/self-archiving).

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Title Page

Germinative dynamic, seasonality and polyandry: A dive in neotropical point-belly frog reproduction

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Conceptualization, methodology, validation, formal analysis, R.O.A.B, and C.d.O; writing – original draft preparation, writing – review and editing, R.O.A.B, C.d.O., and R.F.D; supervision, project administration – C.d.O and R.F.D.

Funding

This study was funded in part by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brasil (CAPES), grant number Finance Code 001.

Conflict of Interest

The authors (R.O.A.B, C.d.O., R.F.D.) declare no conflict of interest.

Data Availability

The data is contained within the study

Manuscript

Abstract

Complexity and reproductive plasticity are very well-known characteristics of anurans. The group is being continuously explored taxonomically and phylogenetically, which leads to a great potential diversity not yet uncovered. This is particularly evident due to the lack of investigation regarding species-specific traits, even the ones well distributed in the lineages as polyandry. Although the climate and environment affect reproductive variability, each species has unique dynamics, and a deeper understanding of their reproductive cycles is essential for meaningful comparisons and recognition of patterns within the extensive variability observed in anurans. Given this context, the present study examined the reproductive and environmental parameters of the polyandrous species *Leptodactylus podicipinus* throughout its reproductive cycle over two consecutive years. The objective was to characterize the species' reproductive dynamics and compare them with phylogenetically related species and other polyandrous species, aiming to explore patterns and the influence of polyandry on anuran reproduction. The main reproductive activity of *L. podicipinus* was during the months with greater rainfall, from October to February. The seminiferous epithelium presented a higher mean of final germinative cells (spermatozoa) in the reproductive period (October-February) and early germinative cells (spermatocytes I and spermatids) in the non-reproductive period (March-September). Among the climatic parameters tested, rainfall was the only one significantly related to reproductive outcomes. Two distinct germinative patterns were identified between polyandric and non-polyandric species compared. Polyandry is certainly a trait that plays a significant role in shaping gametogenesis and the evolution of animals with this reproductive attribute.

Keywords: testis; reproduction; anura; polyandry; *Leptodactylus podicipinus*

Introduction

Anurans comprise the vertebrates with the greatest reproductive diversity, often related to the different reproductive sites, which are both terrestrial and aquatic (Nunes-de-Almeida et al 2021). Since reproduction is vital for the perpetuation of species, the group has a complexity of well-characterized reproductive modes (Haddad & Prado 2005; Nunes-de-Almeida et al 2021). This broad reproductive richness reflects the adaptive capability of anurans to environmental variations throughout their reproductive cycle (Prado et al 2000; Haddad & Prado 2005; da Silva et al 2012; Nunes-de-Almeida et al 2021). In recent decades, the understanding of amphibian diversity has advanced with the discovery of new species and adjustments to the group's phylogenetic relationships (Catenazzi 2015). However, these advances increase the need for functional knowledge to clarify characteristics of the physiology and life cycle of these species (Catenazzi 2015), since functional traits are important in assessing biodiversity and conservation strategies (Mouillot et al 2013; Zhao et al 2018; Liu et al 2021).

Seasonality is a crucial factor in the life cycle of anurans and, adaptation is fundamental for the development of the group, especially given the current rapid climatic and spatial changes (Liu et al 2021; Nunes-de-Almeida et al 2021). Thus, interactions between physiological and environmental factors modulate the reproduction of these animals (Sasso-Cerri et al 2004; Santos et al 2011). Climatic variables, such as rainfall and temperature, can affect the dynamic of the annual reproductive cycle of anurans and even modulate the germinal development and testicular structure of the species (Santos et al 2011; Villagra et al 2014; Chaves et al 2017; Pereira et al 2020). Climatic influence can be highly variable among species, as well as determining the reproductive behavior and diversity of anurans, since these variables are responsible for part of the richness of reproductive traits and modes (Haddad & Prado 2005; da Silva et al 2012; Nunes-de-Almeida et al 2021; Brum et al 2022). However, it is known that combinations of reproductive traits can lead to unique reproductive modes, with varied characteristics of complex reproductive behaviors such as amplexus (Mating of anuran pair), oviposition, tadpole development, parental care (Zamudio et al 2016; Nunes-de-Almeida et al 2021) and also behaviors such as competition and polyandry that can be present or absent in many species

(Haddad & Prado 2005; Toledo et al 2012; Zamudio et al 2016). These behaviors are closely related to ensuring reproductive success, especially synchronous polyandry (Roberts & Byrne 2011; Buzatto et al. 2017).

Polyandry is a relevant influencing factor in anuran reproduction (Roberts & Byrne 2011; Zamudio et al 2016) and this behavior is a fundamental factor in testicular and sperm development (Byrne et al 2003; Prado & Haddad 2003; Bordin et al 2022), especially in Leptodactylids this influence has already been reported and discussed (Prado & Haddad 2003; Pucci et al 2020; Bordin et al 2022). The sperm competition to which males of species that exhibit polyandry behavior are subjected is associated with a greater expense in testicular dynamics, both in sperm morphology and in the size of the gonad (Byrne et al 2002,2003; Zeng et al 2014; Zamudio et al 2016), but there is not much information about the germinative pattern of these species and the implications that this behavior may have to cellular dynamics within the testicular cycle of anurans.

Considering the complexity of anuran reproduction and the need for functional knowledge, especially because of scarce knowledge of behaviors such as polyandry, this study analyzed the reproductive cycle of the polyandric species *Leptodactylus podicipinus* over two years to clarify the reproductive dynamics of the species. We hypothesize that sexual factors such as polyandry synergistically with climatic influences help to understand the germinative distribution pattern present in the reproductive cycle of *L. podicipus*.

Materials and Methods

Species and Study site

Leptodactylus podicipinus is a widely distributed leptodactylid, commonly found in open areas with short vegetation in both preserved and urbanized environments (Prado et al 2000; Bordin et al 2022). This species is a foam-nest breeder with documented polyandrous behavior, as described in the literature (Prado & Haddad, 2003). Reproduction generally occurs along the margins of water bodies in natural or constructed depressions (Prado et al., 2000). *L.*

podicipinus broad distribution and extended breeding season of *L. podicipinus* make it an excellent model for studying the anuran reproductive cycle, as well as the impact of climate and polyandry on reproductive dynamics. Adult males of the species (number of individuals = 31) were collected from open areas favorable to the species' breeding (Prado et al., 2000; Cohen et al., 2020). The collection sites were Rio Bonito (22°40'28.0”S; 48°20'00.8”W) and Porto Said (22°40'49.1”S; 48°19'37.0”W), both located in the Neotropical region of Botucatu municipality, São Paulo, Brazil. The climate of the region is tropical, with a dry winter (Aw) according to the Köppen classification, with an average temperature of 21°C and annual precipitation of 1500 mm (Franco et al., 2023).

Sampling

The field samplings took place between 2021 and 2023. Field trips were also made every two weeks from dusk until late night (6 pm to 11 pm) to observe the dynamics of the species occurrence and record the abundance of vocalizing individuals throughout the species' reproductive cycle, the field observations were approximately 200h. The collection period was averaged in a single year for better graphical representation. Individuals were located and captured in the field by active search and listening at the vocalization site. The animals used in this experiment were kept under the Ethical Principles in Animal Experimentation, adopted by the Brazilian College of Animal Experimentation (CEUA No. 4478221021), and all collections were carried out under a license to collect and keep amphibians in the laboratory (No. 79953-3 SISBIO/IBAMA).

Biometry and Climate data

After sampling the animals were brought to the laboratory and euthanized with a topical application of 5% benzocaine. Subsequently, the SVL (mm) and body mass (mg) were determined. The gonads were extracted, weighed (mg), and measured (mm). Temperature and rainfall data were obtained from the meteorological station at Fazenda Lageado, Faculty of Agronomic Sciences, São Paulo State University, Botucatu, São Paulo, Brazil.

Histology and Testicular morphometry

The extracted testis was fixed for 24 hours by immersion in 10% Tamponade Formalin (90% PBS, 10% formaldehyde) at room temperature. Following fixation, the material underwent inclusion in Paraplast (Sigma-Aldrich®), and three non-consecutive longitudinal sections (3 µm thick, 50 µm apart) were made per animal (number of individuals = 31). Histological slides of the testes were prepared using the Hematoxylin-Eosin staining protocol. Qualitative histological analyses were carried out to assess the internal architecture of the testis and the composition of seminiferous loculi populated by the germinative cells throughout the reproductive cycle of *L. podicipinus*. The histological slides were photographed using a light microscope (Axiophot 2) equipped with a digital camera AxioCam HR (Zeiss, Jena, Germany) from the Department of Structural and Functional Biology, Anatomy sector, Biosciences Institute, UNESP—State University of São Paulo, Brazil. The analyses of the testis loculi included the measurement of the locular area, morphological identification of the main cell types of different generations of germ cells (spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, and spermatozoa), using parameters already well established by Oliveira et al. (2003; 2012), as well as important cells of the somatic component that contribute to gametogenesis, the Sertoli cells next to the seminiferous epithelium and the Leydig cells in the interlocular tissue. The image analyses were performed using the Image-Pro Plus v.6.0 software.

For the morphometric analyses, the dynamics of the germ cells were determined according to the locular volume occupied by the cells, in which the volume of 10 loculi of each individual was measured and an average of the volume of these loculi was obtained (n=100). The average area occupied by the germ cell cysts was obtained using Weibel's graticulated diagram method (Weibel, 1963), through which points from each germinative cyst were identified. In this way, the area occupied by each cyst was obtained, based on 252 points, representing the locular area of the species.

Comparison between species

To assess the reproductive patterns and explore the influence of polyandry on the reproductive dynamics of anuran species, available literature data on reproductive, biometric, and climatic parameters of polyandrous and non-

polyandrous species were compiled. This facilitated a comparative analysis with data from *L. podicipinus* in the present study.

Statistical Analysis

Statistical analyses were conducted to assess the relationships between climatic and reproductive parameters. The choice of correlation test (Spearman or Pearson) was based on the data distribution determined by the Shapiro-Wilk normality test results. Pearson's correlation was used for normally distributed data, while Spearman's correlation was applied for non-normally distributed data. These tests investigated the associations between reproductive, biometric, and climatic parameters. Similarly, to compare the means of reproductive and biometric data between the two study periods (Reproductive and Non-reproductive), either the Student's t-test or the Mann-Whitney U test was performed, depending on the results of the normality test for each parameter. The Student's t-test was used for normally distributed data, while the Mann-Whitney U test was applied for non-normally distributed data. The Rayleigh test was conducted to analyze whether the vocalization data exhibited a seasonal distribution. Statistical significance was determined at $p<0.05$. All analyses were performed using R software (version 4.3.2).

Results

Natural history and reproductive behavior of *L. podicipinus*

The population studied showed reproductive activity beginning in September (spring) and lasting until March (Fig. 1). The vocalization and occurrence of males were observed in several months throughout the year, only in June and July were no occurrences or vocalizations recorded (Fig. 1). The presence of females and pools of tadpoles (Fig. 1) were mostly recorded during the months of greatest vocalization activity (October to February); there was only one field record of a female outside this reproductive period.

The vocalization activity of *L. podicipinus* exhibited a seasonal pattern throughout the year (Rayleigh p-value < 0.001; Fig. 2). Based on vocalization activity, as well as the presence of females and tadpoles, the reproductive period of the studied *L. podicipinus* population was found to be concentrated from

October to February, with a period of reduced (non-reproductive) activity from March to September.

In this study, individuals with a preference for basins in the roots of aquatic plants further from the shore were observed during the reproductive months, while during the non-reproductive period, the animals were observed predominantly in open areas with little aquatic vegetation, and the largest gatherings of vocalizing males were in places further from the shore.

Climatic variables influence

In the population studied, rainfall was the climatic variable that was most influential on the reproduction of *L. podicipinus* (Table 1). There was no significant relationship between the biometric parameters (body weight, CRC) and the climatic variables (rainfall and temperature).

Comparison between species

The pattern of the germinative parameters evaluated in *L. podicipinus* is similar to those of other species within the Leptodactylus genus with reported polyandry, such as *L. macrosternum* and *L. bufonius* (Table 2). Despite these similarities in the polyandrous species, there are differences in reproductive parameters when compared to *L. fuscus* and *L. labyrinthicus*, two other species in the genus that do not present polyandric behavior (Table 2).

L. podicipinus also shows a reproductive pattern in common with another polyandrous species, *Rana dalmatina*, which, although distributed in a different environment and climate, has similar testicular dynamics to *L. podicipinus* during the non-reproductive period (Table 2).

Analysis of the available spermatogenesis data across the compared species reveals two distinct patterns (Table 2). In polyandric species, the germinative pattern features a high population density of spermatozoa during the reproductive period, followed by a high density of initial germ cells in the non-reproductive period. In contrast, non-polyandric species exhibit an inverse pattern

during the reproductive period, characterized by a low population density of spermatozoa and a high density of initial germ cells.

Biometric parameters differed between two leptodactylid species, *L. podicipinus* and *L. macrosternum*. A significant relationship between testicular size, and body weight was not found in *L. macrosternum* as in *L. podicipinus* of the present study (Table 3), however, there is no information on the relationship between gonadal status and germ cells for *L. macrosternum*. The relationship between testes, germ cells, and body size was reported in another species with polyandry from southwestern Australia, *Crinia georgiana* (Table 3).

Annual reproductive pattern of *L. podicipinus*

The individuals showed a histological and anatomical pattern in line with that already described for the species, which consists of ovoidal, whitish testicles organized in globular or ovoidal compartments called seminiferous loculi, where the germ cells are contained in spermatogenic cysts supported by Sertoli cells (Fig. 3). There was no difference between the means of the biometric parameters (CRC, body weight, locular area, testicular weight and length) studied for the two periods.

Throughout the year, all the individuals showed all the germ cell types in cystic organization (Fig. 3), but there were variations in the abundance of cell types throughout the reproductive cycle (Fig. 4).

Analyzing the germinal portion, animals from the reproductive period had a higher average ($p=0.03$) of final germ cells (spermatozoa) than individuals from the non-reproductive period. However, animals from the non-reproductive period had a higher average number of spermatocytes I ($p=0.005$) and spermatids ($p=0.04$). In the testicular portion of non-germinative cells associated with gametogenesis, individuals from the non-reproductive period had a higher average number of Sertoli cells ($p=0.04$) and Leydig cells ($p=0.01$) when compared to animals from the reproductive period.

Thus, animals in the non-reproductive period have a higher average number of non-germ cells (Leydig and Sertoli) than animals in the reproductive period. However, the latter have a higher average number of final germ cells (Spermatozoa).

Correlations between reproductive parameters

In terms of cell structure, significant relationships were only found in the reproductive group (Fig. 5). Testicular mass (r^2=0.4; p=0.01) is positively related to the number of spermatozoa. While the number of spermatogonia is negatively related to the testicular mass (r^2= -0.5; p=0.001) and locular area (r^2=0.4; p=0.03). Concerning non-germ cells, the biometric parameters testicular mass (r^2=0.4; p=0.04) and locular area (r^2=0.5; p=0.01) were positively related to the number of Sertoli cells.

Discussion

The reproductive dynamics of *L. podicipinus* are distributed throughout the year (Prado et al 2000; Bordin et al 2022), however, we found a seasonal trend in the species, where individuals do not show any vocalization activity during June and July and low occurrence in May and August, which comprises 25% of the annual period without reproductive events. This variation can be explained by the climatic characteristics of the region since the reproductive description of the species is based on populations in central-western Brazil (Prado et al 2000; Ferreira et al 2009; Bordin et al 2022) and the area studied in southeastern Brazil has a different climate regime. This pattern has already been reported in the literature, as in *Dendropsophus minutus* and *Physalaemus cuvieri*, which have different reproductive dynamics throughout their distribution (Santos et al 2007; Ferreria & Mehanna 2012; Brum et al 2022).

The relationship between the reproductive biology of anurans and the climatic regime has been well described in the literature (Byrne 2002; Smith et al 2003; Santos et al 2011; Chaves et al 2017a). In *L. podicipinus*, only rainfall was significantly related to the parameters tested. Similarly, for *L. macrosternum*, rainfall was the main parameter related to the spermatogenesis of individuals in the Brazilian semi-arid region (Chaves et al 2017a). In both cases, the average temperature was constant throughout the cycle of the two species (*L. podicipinus* and *L. macrosternum*), and in general, the reproductive dynamics of anurans are more influenced by the variable climatic factor than by factors that remain constant, as explained by the relationship reported for the leptodactylids *P. cuvieri*, *P. riograndensis* and *Pseudopaludicola falcipes*, where rainfall, being

constant throughout the species' annual cycle, showed no significant relationship with any reproductive parameter (Brum et al 2022).

Concerning the cellular dynamics of the testicle in the studied specimens, the histological organization was consistent with the pattern previously documented for the species (Bordin et al., 2022), where the testicle showed all the germinative and non-germinative cell types throughout the cycle. The variation observed between the cell averages across the studied periods followed an expected pattern. During the reproductive period, individuals exhibited a higher average of spermatozoa, whereas, in the non-reproductive period, a higher average of initial germ cells (spermatids and spermatocytes I) and somatic cells (Sertoli and Leydig cells) was observed.

Comparing this pattern of testicular cell population dynamics to that described for other neotropical species, and especially species that have polyandry behavior, such as *L. macrsoternum* and *L. bufonius*, we observed a pattern of high spermatozoa density in the months of reproductive activity (Chaves et al 2017a; Pucci et al 2020), and a non-reproductive period with a lower density of spermatozoa in the loculi (Villagra et al 2014).

Given the importance of polyandrous behavior in analyzing the testicular dynamics of the species (Prado & Haddad, 2003; Zamudio et al., 2016; Pucci et al., 2020; Bordin et al., 2022), and its potential influence on germline dynamics, we observed similarities between the polyandrous frog species *Rana dalmatina* and *L. podicipinus*. Specifically, the non-reproductive period of both species was marked by the presence of numerous initial germ cysts and Leydig cells. Even though they are distributed in very different climates, with *R. dalmatina* being from a temperate climate and *L. podicipinus* a neotropical species, the two species share a germinative pattern in the non-reproductive period.

The germinative patterns observed in both leptodactylids and *R. dalmatina* reinforce the importance of polyandry as an influential factor in the annual reproductive dynamics of these animals, in addition to the climatic regime in which these anurans are found.

Another factor that corroborates this idea is when we compare the germinative patterns found with those described for two other species of

leptodactylids that do not show polyandrous behavior, *L. fuscus,* and *L. labyrinthicus*. The germinative profile of *L. fuscus* in southern Brazil indicates a low reproductive effort, with less than half of the individuals captured during the reproductive period being males with spermatozoa in the loculi, even though there was an increase in the gonads indicating spermatogenic activity (Maragno et al 2009). In northern Argentina, another population showed a germinative pattern with few spermatozoa in the testes and many initial cells in the reproductive period (Pucci et al 2020).

In *L. labyrinthicus*, a germinative pattern was described for a population in southeastern Brazil, during the reproductive period, with spermatozoa present in the loculi, even in the lumen, but in small quantities, and a large part of the loculi contained more initial germ cells than spermatozoa (Prado et al 2004). Considering this information and the results of the germinative pattern found in the *L. podicipinus* individuals studied, it can be hypothesized that polyandry behavior contributes to the germinative organization of the reproductive cycle, as seen in other reproductive aspects in anurans, such as testicular size and sperm morphology (Prado & Haddad 2003; Byrne et al 2003; Bordin et al 2022).

Regarding the biometric parameters of *L. podicipinus*, there was no difference between the individuals from both periods in terms of weight and length, both body and testicular. However, the animals' body weight was significantly related to gonadal size, indicating a parallel investment between body and gonadal development, a relationship not found in individuals from the central-western Brazilian population during the non-breeding period (Bordin et al 2022). This difference can be explained by the fact that the population in this study was analyzed throughout the species' cycle in a different occurrence gradient, clarifying and uncovering relationships not previously found.

Testicular parameters were also positively related to spermatozoa, and the same parameters plus the locular area are negatively related to spermatogonia, so in this study, larger testes tend to have more spermatozoa and fewer spermatogonia. In two other anuran species, testicular parameters were also tested: in *L. macrosternum*, no significant relationship was found between the body condition of the individuals and the testicular index (Chaves et al 2017b),

while in *C. georgiana*, both body weight and spermatozoa were found significantly related to testicular weight (Hettyey & Roberts 2007). A possible explanation for the difference in relationships between the two species of leptodactylids could be the environment in which they occur, with the population of *L. macrosternum* coming from a semiarid region and, that of *L. podicipinus* occurring in a tropical climate, another alternative that can be inferred is about the type of polyandry described for the three species.

In *L. macrosternum* a behavior is described where the amplexus pair is surrounded by other males who try to push their ejaculate toward the amplexus couple without making direct contact (Prado & Haddad 2003), while in *L. podicipinus* and *C. georgiana* multiple amplexus of a female with two or more males occur (Roberts et al 1999; Prado & Haddad 2003). In *C. georgiana* it has been reported that smaller individuals are more prone to multiple amplexus, thus indicating a relationship between size and multiple amplexus (Roberts & Byrne 2004; Hettyey & Roberts 2007). In addition, the size of the testicle also increases fertilization success depending on the position of the amplexus (Buzatto et al 2017), thus the body weight-testicular relationship existing in *L. podicipinus* and *C. georgiana* and, not found in *L. macrosternum* corroborates the idea of the influence of polyandry behavior on the reproductive dynamics of the species. However, the lack of information regarding the relationship between gonadal and germ cells condition in *L. macrosternum* makes it difficult to obtain a clearer pattern of comparison between these species.

Reproduction is a crucial factor in the survival and success of a species, involving a myriad of strategies and processes adapted over millions of years. The influence of climatic and environmental factors on anuran reproduction has been widely studied (Sasso-Cerri et al 2004; Chaves et al 2017a; Santos et al 2011), and these factors can vary and produce different effects on the reproductive parameters of other species (Brum et al 2022). However, reproductive characteristics include various behavioral and physiological processes and environmental ones, such as polyandry, a trait so widely distributed in the group (Prado & Haddad 2003; Zamudio et al 2016).

Conclusion

We characterized the reproduction of a population of *L. podicipinus*, with a multifactorial view of what can modulate the reproductive dynamics of individuals, including polyandry as a relevant factor for the testicular pattern. There is still much to be unraveled about the relationship between histological, physiological, and behavioral characteristics of anuran reproduction. We believe that the information here contributes to the advancement of functional knowledge in the group, including a little-explored trait such as polyandry, to which we attribute a value as an attribute that reciprocally modulates gametogenesis and the evolution of animals with these properties.

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Figure Legends

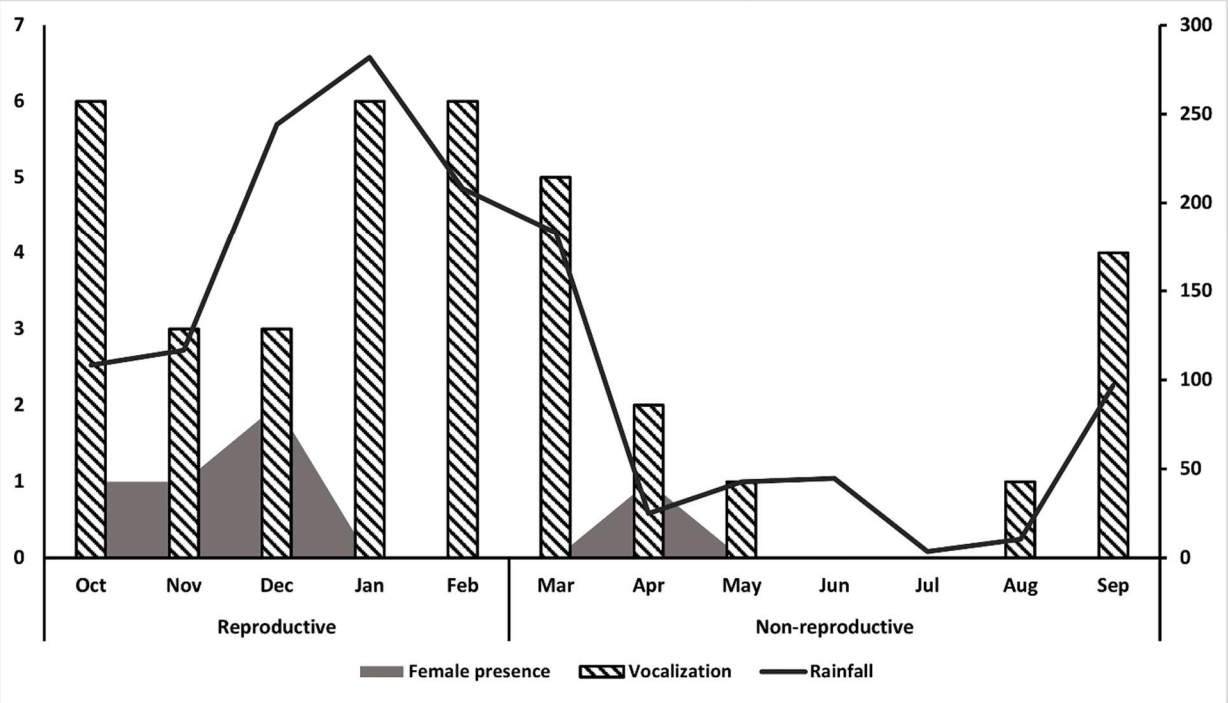


Fig 1. Graph showing rainfall and vocalization activity of individuals of the *species L. podicipinus* throughout their reproductive cycle, left axis – number of observations; right axis – precipitation (mm).

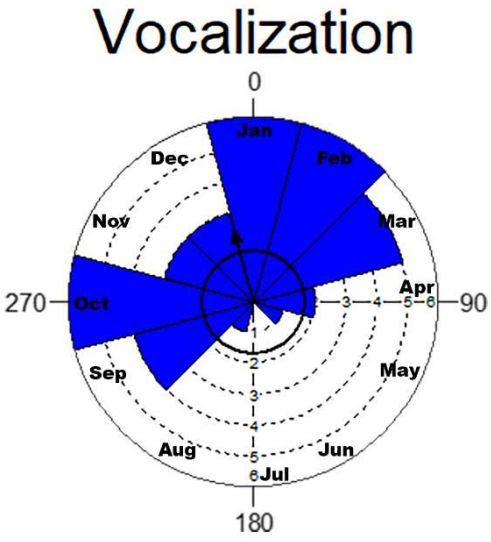


Fig 2. Rose diagram presenting monthly vocalization activity of *L. podicipinus*.

Parameter	Rainfall
	p
Vocalization	0,003*
Spermatogonia	0,40

Spermatocyte I	0,12
Spermatocyte II	0,03*
Spermatid	0,08
Spermatozoa	0,006*
Sertoli	0,008*
Leydig	0,05

Table 1. Relationships between rainfall and reproductive parameters in *L. pocipinus*. *indicates significance.

Species	Climatic environment	Polyandry	Germinative pattern	Authors
Leptodactylus podicipinus	Tropical	**Present**	Reproductive period - High density of spermatozoa Non-reproductive period – High density of initial germ cells and **Leydig cells**	**Prado & Haddad 2003** **Bordin et al 2022** **Present study**
L. macrosternum	Semiarid	**Present**	Reproductive period - High density of spermatozoa Non-reproductive period - High density of initial germ cells	**Chaves et al 2017[a]** **Villagra et al 2014**
L. bufonius	Tropical	**Present**	Reproductive period - high density of spermatozoa Non-reproductive period - High density of initial germ cells	**Pucci et al 2020**
Rana dalmatina	Temperate	**Present**	Reproductive period - (insufficient data) Non-reproductive period - High density of initial germ cells and **Leydig cells**	**Guarino & Bellini 1998**
Crinia georgiana	Mediterranean	**Present**	Insufficient data	**Hettyey & Roberts 2007**
L. fuscus	Subtropical	Absent	Reproductive period - Low density of spermatozoa and high density of initial germ cells Non-reproductive period (insufficient data)	**Maragno & Cechin 2009** **Pucci et al 2020**
L. labyrinthicus	Tropical	Absent	Reproductive period - Low density of spermatozoa and high density of initial germ cells Non-reproductive period (insufficient data)	**Prado et al 2004**

Table 2. Comparative table of reproductive parameters between species. Data were obtained from the available literature.

Species	Climatic environment	Amplexus	Biometrics	Authors
L. podicipinus	Tropical	Multiple	Germ cells related to body and testicular size	**Prado & Haddad 2003** **Bordin et al 2022**

				Present study
L. macrosternum	Semiarid	Single	Germ cells (insufficient data) No relationship between body and testicular size	**Chaves et al 2017b** **Prado & Haddad 2003**
C. georgiana	Mediterranean	Multiple	Germ cells related to body and testicular size	**Roberts et al 1999** **Hettyey & Roberts 2007**

Table 3. Comparative table of biometric parameters between polyandrous species. Data were obtained from the available literature.

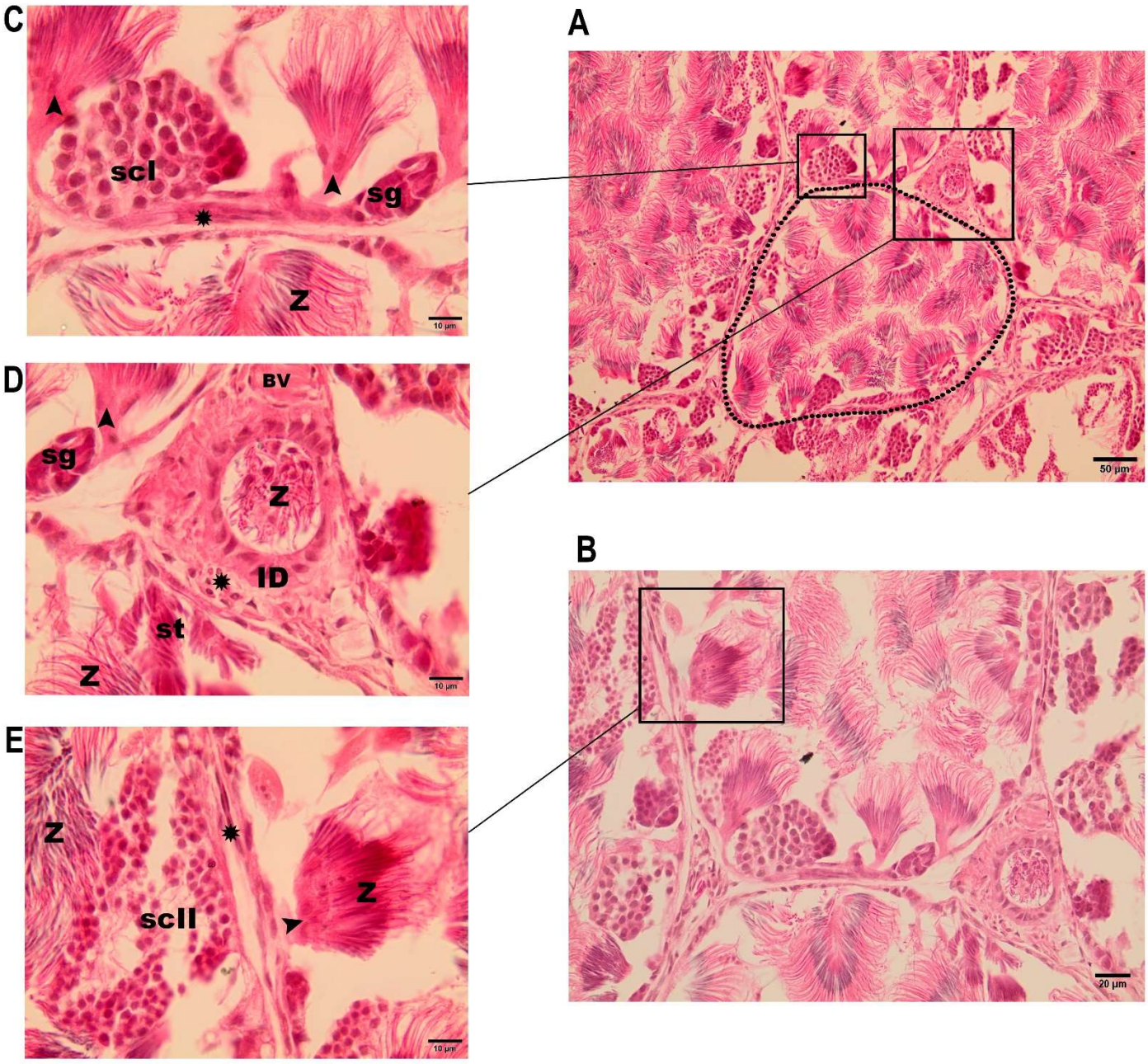


Fig 3. Testicular epithelium of *L. podicipinus*. A and B – dotted line: seminiferous locule (20x bar=50µm, 40x bar=20µm); C,D and E - arrowhead: Sertoli cell, star: Leydig cell, sg: spermatogonia, scI: spermatocyte I, scII: spermatocyte II, st: spermatid, z: spermatozoon, ID: intratesticular duct (100x bar=10µm).

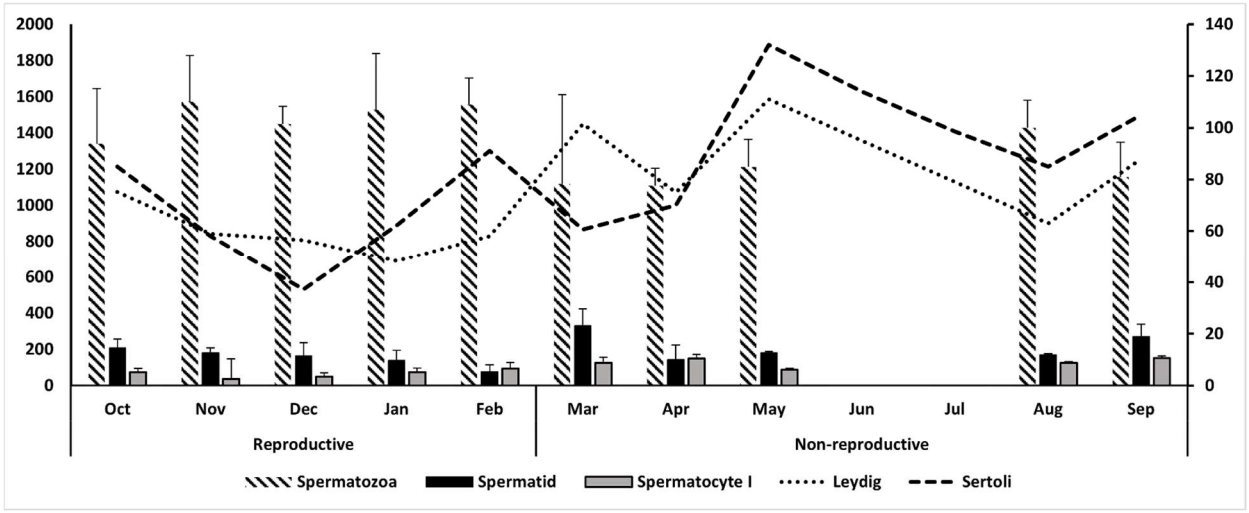


Fig 4. Graph showing the differences between the number of germ and somatic cells in the two studied periods. Column values are expressed as mean and standard deviation. Line values expressed in mean.

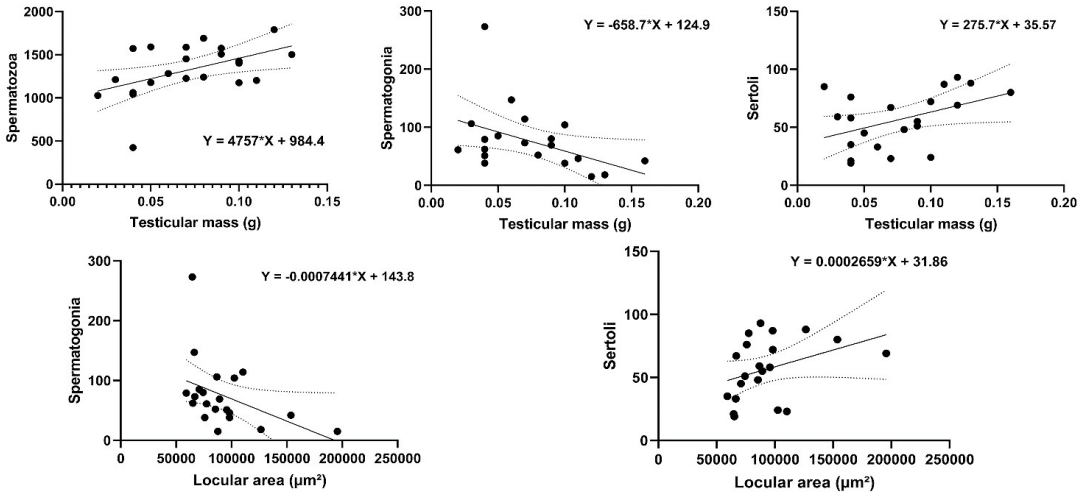


Fig 5. Graphs of the relationships between biometric and cellular parameters in *L. podicipinus*.

Capítulo III

Immunolocalization of Aquaporin 1, 2, and 9 in Anuran Testis of the Neotropical Pointedbelly Frog *Leptodactylus podicipinus*

Published in Current Issues in Molecular Biology

Accepted in 08/2024

DOI: https://doi.org/10.3390/cimb46090594

current issues in molecular biology

MDPI

Article

Immunolocalization of Aquaporin 1, 2, and 9 in Anuran Testis of the Neotropical Pointedbelly Frog *Leptodactylus podicipinus*

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Abstract: Many anuran survival strategies involve hydric regulation, and reproduction is not different. The aquaporin (AQP) family plays an important role in water transport and regulation in many tissues, including the male gonad. The testes undergo various stages of change during the reproductive cycle, and water balance is an important factor for ensuring reproductive success. Considering the relevance of water control in testicular development in anurans and the lack of research regarding the tissue localization of AQP in the male gonad, the present study investigated the expression of three AQPs (1, 2, and 9) in the testis of the neotropical anuran species *Leptodactylus podicipinus* during two different periods of the reproductive cycle (reproductive and non-reproductive). AQP1 and 2 immunoreactions were found in early germ cells, spermatozoa, Leydig cells, and Sertoli cells, which were more frequently expressed within the reproductive period. AQP1 was also found in the testicular blood vessels. AQP9 was identified predominantly in the epithelium of the intratesticular ducts of reproductive-period individuals. This study presents, for the first time, the localization of AQP1, AQP2, and AQP9 in the testes of an anuran species and the differences in their location during two distinct periods of the reproductive cycle.

Keywords: anura; aquaporin; immunohistochemistry; spermatogenesis

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Citation: Bordin, R.O.A.; Oliveira, C.d.; Domeniconi, R.F. Immunolocalization of Aquaporin 1, 2, and 9 in Anuran Testis of the Neotropical Pointedbelly Frog *Leptodactylus podicipinus*. *Curr. Issues Mol. Biol.* **2024**, *46*, 9958–9969. https://doi.org/10.3390/cimb46090594

Academic Editor: Huafeng Wang

Received: 8 July 2024
Revised: 28 August 2024
Accepted: 30 August 2024
Published: 10 September 2024

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1. Introduction

Water plays a crucial role in the reproductive process of Anura, as these amphibians are highly vulnerable to desiccation due to their skin permeability and reliance on humid environments. In addition, frog eggs are delicate and permeable, making water an essential component for their survival [1]. The natural history of anurans delineates the evolution of diverse reproductive modes, ranging from aquatic to terrestrial, and includes instances of reversion from terrestrial to aquatic habitats [2–4]. In the transition of organisms from aquatic to terrestrial environments, the establishment of effective hydric regulation mechanisms is crucial for maintaining optimal water balance. These mechanisms encompass adaptations, including the modulation of egg layer quantity and size, production of foam to prevent dehydration, and preference for reproductive activities in humid locales [5]. Therefore, proper osmotic management is essential for successful reproductive modes [6,7].

The mechanisms responsible for water dependence and regulation are crucial for survival and reproductive success in offspring and female eggs [8–10]. However, little information is available regarding the cellular physiology involved in the process of spermatogenesis, which contributes morphologically by producing viable spermatozoa. Understanding the intricate processes of spermatogenesis at the cellular level is essential for gaining comprehensive insight into reproductive biology. This involves not only the production of viable spermatozoa but also the regulation of their development, differentiation, and eventual function during fertilization.

The interaction between physiology and external factors modulates the reproductive cycles of frog species, as reported previously [11,12]. Factors such as temperature, humidity, and seasonal changes significantly influence reproductive patterns and success rates. Moreover, seasonal and local water availability dynamics are fundamental to the development of anuran species [13,14]. These dynamics can affect breeding sites, larval development, and adult survival, highlighting the importance of water as a critical resource. To fully understand the complexity of anuran reproduction, it is necessary to understand the physiology of how anurans regulate and balance osmotic levels in their environment [15]. This includes studying the osmoregulatory mechanisms that allow homeostasis despite fluctuations in external conditions.

Anuran survival is heavily dependent on the process of osmotic regulation, with aquaporins (AQPs) assuming a pivotal role in facilitating osmoregulation. AQPs participate in the management and control of water flux both inside and outside cells, thereby preserving the internal fluid balance of this taxonomic group [15–17]. These proteins function via active transport and show different permeabilities to water and other metabolic molecules [18,19].

AQPs comprise a family of small "membrane-spanning" proteins with monomers of 26 to 34 kDa. These proteins are expressed in the plasma membranes of cells involved in fluid transport. Proteins belonging to the aquaporin family are homotetramers in the membrane. Each monomer consists of six domains of "membrane-spanning" α-helix with carboxy and aminoterminal extremities oriented to the cytoplasm containing a distinct water pore acting as a water channel that facilitates and regulates the passage through the cell membrane [20]. Aquaporins are present in the membranes of intracellular organelles, regulating the volume of both the organelles and the overall cell [21]. AQPs are involved in many physiological processes such as water and solute homeostasis, secretion, facilitation of transepithelial transport, cellular migration, and neuroexcitation, and they also participate in a variety of pathological processes such as glaucoma, epilepsy, obesity, and cancer [22,23].

In the male genital system, water is necessary for maintaining the luminal environment in which spermatozoa are located. Water functions as a vehicle to transport spermatozoa from the testis to the vas deferens, ensuring that they remain viable and capable of fertilization [24]. The presence and proper functioning of water channels, such as AQPs, are crucial for this process. AQPs facilitate water movement across cell membranes, thereby maintaining the appropriate hydration levels within the reproductive ducts. Different types of aquaporin are expressed in specific regions or cells throughout the extratesticular ducts of mammals. Hermo and Smith [24] reported that AQPs are located in specific membrane domains, including microvilli, apical, basal, and lateral regions of the cell, as well as in endosomes. This strategic localization allows AQPs to efficiently regulate water transport and maintain the osmotic balance required for sperm maturation and mobility. The expression of these AQPs can be controlled by hormonal or luminal factors, indicating a complex regulatory mechanism that ensures optimal reproductive function.

The regulation of AQPs is a multifaceted process that can be influenced by both external and internal factors. AQPs may also be regulated and influenced by external and internal factors such as variations in pH, phosphorylation, and auxiliary proteins [20,25–30]. There is evidence of a direct relationship between aquaporin differential expression and environmental water availability [15,31,32]. These regulatory mechanisms are essential for adapting to changing physiological conditions and maintaining reproductive health.

The success of the biological processes of anurans is intimately bound to the hydric dynamics, osmotic regulation, and physiological control of water flux through organs [15,16]. Evidence indicates that the dynamics of fluid concentrations between the internal and external mediums plays a fundamental role in biological processes such as reproduction and in the success of external fertilization, especially regarding the activation of sperm motility in frogs [17,27]. Considering this information, water is important in the dynamics of the male genital system, and water regulation in the testis by aquaporin is an essential process for sperm concentration [33,34] and consequential to male fertility [35,36].

In light of the pivotal role aquaporin play in water regulation in the male reproductive system [19] and the lack of research on the expression of aquaporin across different animal groups and environmental context, particularly in anuran reproduction [15], this study seeks to investigate the expression patterns of three distinct aquaporin proteins (AQP1, 2, and 9) in the testis of the neotropical anuran species *Leptodactylus podicipinus* throughout its reproductive cycle. Therefore, this study aimed to elucidate the localization of these proteins within testicular tissue and determine whether differences exist in the labeling of aquaporins throughout the species' reproductive cycle.

2. Materials and Methods

2.1. Sampling and Location

Animals were obtained from specific areas for the reproduction of species [37,38]. Rio Bonito (22°40′28.0″ S 48°20′00.8″ W) and Porto Said (22°40′49.1″ S 48°19′37.0″ W) are both located in the city of Botucatu state of São Paulo, Brazil.

The sampling was carried out through monthly expeditions over two years (2021–2023), totalizing 8 mature males separated into two groups (reproductive, n = 4; non-reproductive, n = 4) representing different periods of the species' reproductive cycle. The periods were determined according to species vocalization activities and seasonal rainfall at the study site. The presence of secondary sexual characters such as an apparent vocal sac and nuptial thorns in the thoracic limb confirmed sexual maturity. The location and capture of specimens were performed via active searches at vocalization sites during the crepuscular period. The specimens used in the experiment were treated according to the Ethical Principles of Animal Experimentation adopted by the Colégio Brasileiro de Experimentação Animal (CEUA N° 4478221021). All expeditions and sampling were conducted with a sampling license (n° 79953-3 SISBIO/IBAMA).

2.2. Immunohistochemistry

After euthanasia with topical application of 5% benzocaine, the gonads were fixed for 24 h by immersion in Tamponade Formalin 10% (90% PBS, 10% Formol) at room temperature. After fixation, the animals were submitted to inclusion in Paraplast (Sigma-Aldrich®, St. Louis, MO, USA), and 3 non-consecutive longitudinal sections were made per animal (3 µm thick, 50 µm distance between the sections).

Histological slides of the testes were prepared, first being deparaffinized in xylol followed by dehydration in ethanol. Antigenic retrieval was performed by subjecting the slides to immersion in tamponade citrate solution (pH 6.0) and subsequent exposure to a temperature of 100 °C for 15 min in a microwave (750 W). To ensure optimal results, peroxidase blockade was performed in methanol containing 3% H_2O_2 for 15 min. To reduce the nonspecific link, slides were incubated in a blocking solution comprising bovine serum albumin (BSA) at a concentration of 3% in PBS-T for 1 h at room temperature. Following preparatory steps, the detection of AQP1, AQP2, and AQP9 was meticulously carried out using selected antibody dilutions at a concentration of 1:100 (Anti-AQP1 AB2219 Sigma-Aldrich; Anti-AQP2 AB3066 Sigma-Aldrich; Anti-Aqp9 AQP91-A Alpha Diagnostics, San Antonio, TX, USA) diluted in PBS, with overnight incubation at 4 °C. Subsequent to thorough washing with PBS, the slides were incubated with a secondary HRP antibody (Anti-rabbit IgG—1:100 AB6721 Abcam, Waltham, MA, USA) for 1 h at room temperature, followed by revelation using diaminobenzidine (DAB) and coloration with Mayer Hematoxilyn. Negative controls were generated by substituting the primary antibody for 0.01 M of PBS. Positive controls were employed to validate specific labeling using slides containing mouse and rat tissues (Figure A1, Appendix A).

Commercially available antibodies were used because aquaporin from anuran tissue presents a high homology to mammalian aquaporins (AQP1 60% similarity [16,39]; AQP2 70% similarity [16,40]).

After immunohistochemistry, the slides were coded for blinded analysis. The evaluation of the AQP1, 2, and 9 reaction results outcomes entailed meticulous comparison of

reaction intensities across the two designated period groups. This analysis was conducted by Bordin and was independently corroborated by Dr. Domeniconi to ensure rigor and reliability. Subsequently, the coded slides were photographed under a light microscope (Axiophot 2) equipped with a digital camera AxioCam HR (Zeiss, Jena, Germany) from the Department of Structural and Functional Biology, Anatomy sector, Biosciences Institute, UNESP—State University of São Paulo, Brazil.

2.3. Western Blotting

Frozen testicles were homogenized (n = 9; 5 reproductive and 4 non-reproductive) using a sonicating homogenizer (Q125 Sonicator Qsonica Inc., Newtown, CT, USA), with RIPA lysis buffer (Bio-Rad, Hercules, CA, USA) enhanced with a protease inhibitor mix (Sigma, St. Louis, MO, USA) for 5 min, with a cycle of 1 min per pulse at 40% amplitude while maintaining the temperature at 4 °C. Following this process, the samples were centrifuged to separate the cellular debris, and the protein fraction was obtained from the resulting supernatant. Protein concentrations in the samples were then determined using the Bradford method. Subsequently, the samples (60 µg per sample), with the addition of Laemmli buffer (1:1 dilution), were heated in a dry bath at 96 °C for 15 min to denature the proteins. These samples were then loaded onto 10% SDS-polyacrylamide gels for electrophoresis under reducing conditions. Post-electrophoresis, the separated protein bands were transferred onto a nitrocellulose membrane (Sigma). The membrane was blocked with 3% non-fat dry milk in TBST (comprising 10 mM TRIS-HCl pH 7.5, 150 mM NaCl, 0.1% Tween-20) for 1 h to prevent non-specific binding. The blot was then incubated overnight at 4 °C with primary antibodies against AQP1 (AB2219, Sigma-Aldrich), AQP2 (AB3066, Sigma-Aldrich), or AQP9 (Aqp91-A, Alpha Diagnostic), each diluted at a ratio of 1:1000 in 3% bovine serum albumin. Following this incubation, the blot membrane was washed with TBST seven times for 5 min to remove unbound antibodies. The samples were then incubated for 1 h at room temperature with peroxidase-conjugated goat anti-rabbit-IgG secondary antibody (AB6721, Abcam). After a subsequent series of seven 5 min washes in TBST, the proteins were visualized using an ECL prime detection reagent (Sigma). Beta-actin was used as a positive control for quality control and to ensure equal loading (Figure A1; Appendix A).

2.4. Statistical Analysis

The blot images were analyzed and quantified using ImageJ v1.54. The mean of each lane was compared between periods using Student's *t*-test or Mann–Whitney test, based on data distribution, with a significance level of $p < 0.05$. All statistical analyses were performed using GraphPad Prism 8 software.

3. Results

The cellular localization of the three studied aquaporins was determined by immunohistochemistry. AQP1 localization was detected throughout the entire testicular tissue. The reaction was observed in all germline cells, even in the tails of bundled or free spermatozoa in the locular lumen. AQP1 was also detected in the Leydig and Sertoli cells (Figure 1A,B). Immunohistochemical images from both groups were compared qualitatively. AQP1 was labeled in spermatozoa in most images from the reproductive group (Figure 1A), whereas labeling was mostly absent in the non-reproductive group (Figures 1B and A3). AQP1 was also observed in testicular blood vessels, with no apparent differences in labeling between the groups (Figure 1A,B).

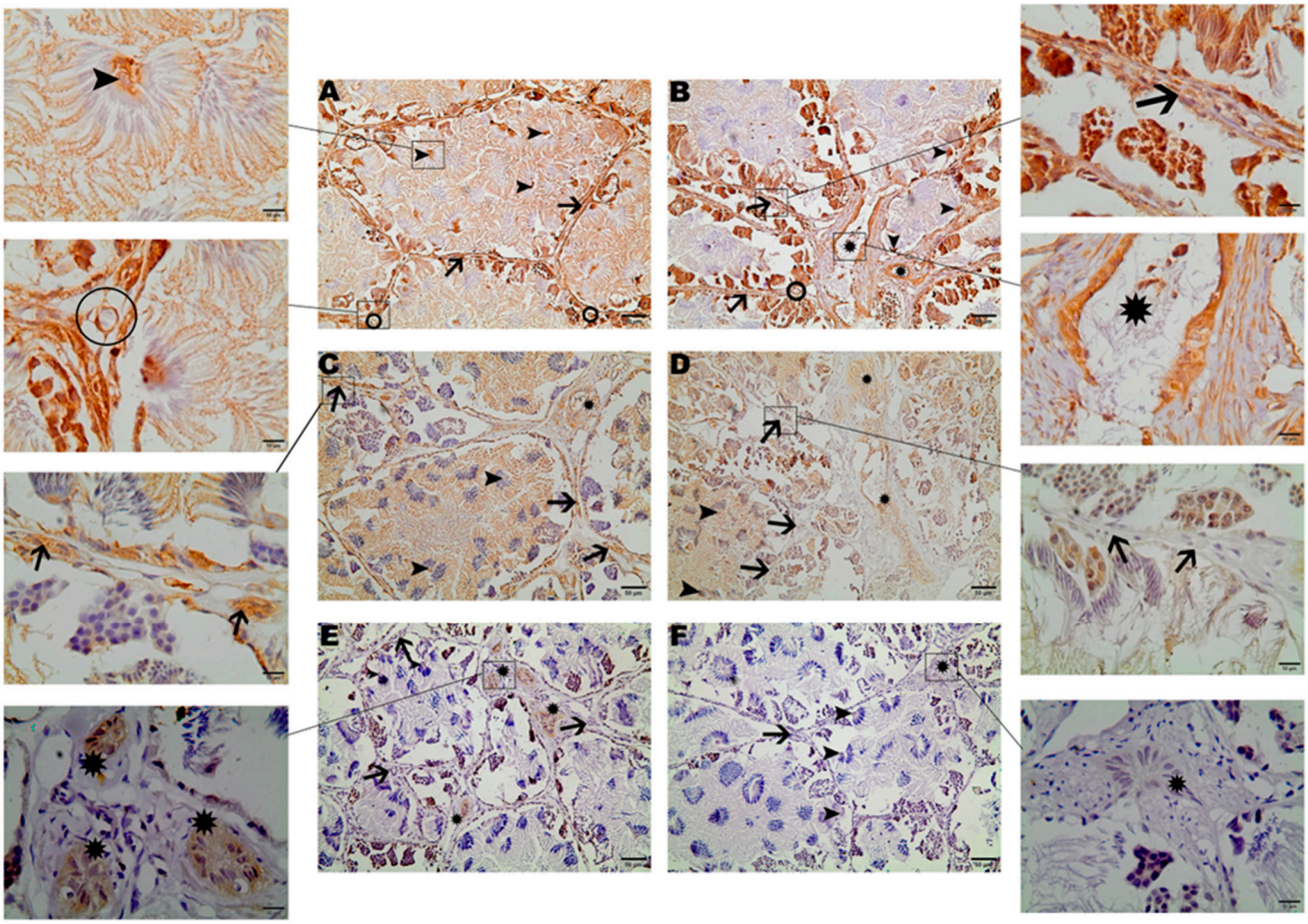


Figure 1. Immunohistochemical staining of AQP1, AQP2, and AQP9 in the testicular tissue of *Leptodactylus podicipinus*. (**A**,**C**,**E**): reproductive period; (**B**,**D**,**F**): non-reproductive period. AQP1 staining can be seen in germinative cells, Leydig, Sertoli, blood vessels (**A**), and in the intratesticular ducts (**B**). AQP2 labels germinative cells, Leydig cells (**C**), and intratesticular ducts (**D**). AQP9 immunoreaction can be seen only in intratesticular ducts of the reproductive period (**E**). Arrowheads = Sertoli cells; arrows = Leydig cells; circle = blood vessels; stars = intratesticular ducts. Magnification: 20× bar = 50 μm; 100× bar = 10 μm.

Similar to AQP1 labeling, AQP2 exhibited immunoreactivity in the tail of spermatozoa, as well as in Sertoli cells. Leydig cell immunoreactivity for AQP2 was observed only in animals from the reproductive group (Figure 1C), whereas in the non-reproductive group, Leydig cells labeling was not detected in most of the images (Figures 1D and A3). Additionally, AQP2 was detected in the epithelial cells of the intratesticular ducts of both groups (Figure 1C,D).

Immunoreactivity of AQP9 was predominantly observed in the epithelial cells of intratesticular ducts in animals from the reproductive group, with no distinct labeling evident in other regions of testicular tissue (Figures 1E,F and A3).

Western blotting analysis for AQP1, AQP2, and AQP9 of the testicle extracts showed bands at 25–29 kDa in the majority of the samples analyzed (Figure 2). This finding corroborates the presence of these aquaporin within the testicular tissue of *L. podicipinus*, as further evidenced by the results obtained from the immunohistochemistry analysis. Notably, despite a thorough examination, there were no statistically significant differences in the mean levels of the aquaporins between the two periods studied.

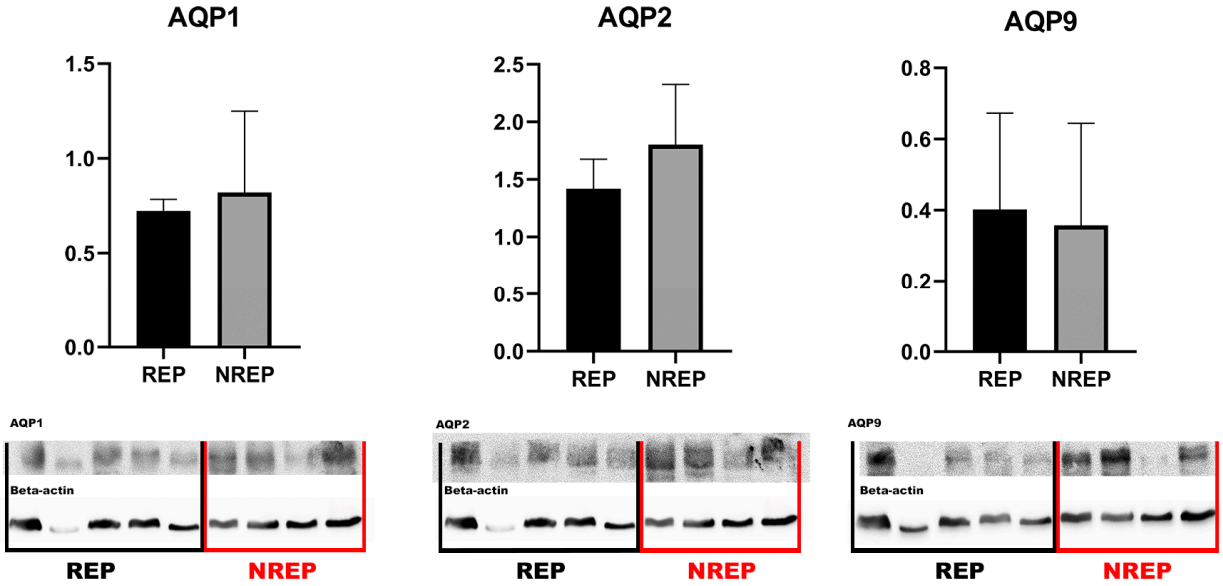


Figure 2. Western blot analysis of AQP1, AQP2, and AQP9 in *Leptodactylus podicipinus* testicle. Values are expressed as mean and standard deviation. REP (reproductive period), NREP (non-reproductive period).

4. Discussion

In this study, we not only elucidated the immunolocalization of AQP1, AQP2, and AQP9 in anuran testicular tissue but also incorporated Western blotting analysis, thus providing a multifaceted approach to characterizing aquaporin expression in this context. It is noteworthy that, while the aquaporin family has been investigated across various zoological groups, including fish, amphibians, birds, and mammals [31,41–44], our study makes a distinct contribution by integrating immunolocalization and Western blotting techniques to examine aquaporin in amphibian testes.

In the anuran species *Dryophytes chrysoscelis*, AQP1 was detected in the testis, expressed in testicular tissue and Leydig cells but not in germ cells and ejaculated spermatozoa, indicating that AQP1 is related to support cells and testicular development but not closely associated with mature spermatozoa [17]. In *L. podicipinus*, the findings contrast those for *D. chrysoscelis* because AQP1 is closely linked to both germinative and testicular tissue, indicating its presence in both support and spermatogenic cells. The difference seen could be a result of the interspecific variability, as this is common in anuran reproduction with a wide range of modes and strategies. Also, the two species could be differently influenced by factors such as climate, as temperature parameters are responsible for changes in aquaporin dynamics in *D. chrysoscelis*, a temperate species, encountered in North America (Canada and U.S), whereas *L. podicipinus*, a tropical species, predominantly inhabits South America (Brazil, Argentina, Bolivia, Paraguay, and Uruguay), and environmental factors such as temperature, rainfall, and photoperiod are commonly related to changes in frog reproduction [11,12].

In the fish species *Sparus aurata*, AQP1 appears to be specifically associated with early germ cells like spermatids and spermatozoa. The association suggests a potential role in addressing the metabolic requirements during the advanced stage of the spermatogenic process [44]. This study also identified AQP1 as being associated with germinative cells and frequently observed in the tails of spermatozoa. These observations support the hypothesis that AQP1 plays a pivotal role in the metabolic processes of spermatogenesis, particularly in spermiogenesis. Additionally, the strong labeling of AQP1 in spermatozoa from the reproductive group indicates its potential involvement in sperm maturation and motility.

Aquaporins exhibit varied characteristics denoting conservation and variation across the zoological groups; in goose testes, AQP1 is expressed in testicular blood vessels but not in Leydig cells [43]. In the tested anuran species, AQP1 was present in both the testicular

blood vessels and Leydig cells, underscoring the conservation of AQP1's role in blood regulation. However, there is variation in its expression in anuran Leydig cells, such which was not detected in birds.

Regarding AQP2, this aquaporin has been described in the turkey reproductive ducts and is considered to be involved in the maturation of avian spermatozoa [45]. Similar to birds, *L. podicipinus* AQP2 is present in spermatozoa; however, AQP1 seems to play a more significant role in the maturation process. This is evidenced by the strong labeling observed in both early and late germ cells, as well as in supporting cells such as Sertoli, along with the absence of labeling in sperm from the non-reproductive periods. These findings suggest that AQP1 in anurans may serve as a marker for the final stage of sperm maturation.

AQP2 was exclusively found in Leydig cells in animals from the reproductive group. This result may be attributed to the seasonal variation of vasotocin, a peptide that regulates AQP2 expression in anurans [39,41,46]. In addition to its involvement in various reproductive activities, such as vocalization, amplexus, and male agonistic behavior, vasotocin is predominantly expressed during the reproductive period of anurans, varying with steroid sex hormone levels that are regulated by somatic cells such as Leydig cells [46,47]. The observation of AQP2 labeling in Leydig cells only during the reproductive period suggest that vasotocin, which has been previously shown to regulate AQP2 in anurans [39,41,46], may play a role in this regulation within the reproductive tract. Although vasotocin was not directly tested in this study, these findings point to a potential connection between AQP2 and seasonal behaviors throughout the reproductive cycle of *L. podicipinus*.

AQP9 was detected in the interstitial tissues of *S. aurata* testis, specifically in Leydig cells [44]. However, the function of AQP9 regarding the Leydig cells remains unknown. In contrast, in *L. podicipinus* testis, AQP9 was found only on the intratesticular ducts, with no presence observed in interstitial tissue or germ cells. According to the results, AQP9 may be more closely associated with the transport of spermatozoa rather than participating in their maturation process. Specifically, the majority of AQP9 labeling in *L. podicipinus* testis was found in animals from the reproductive period, indicating that the function and localization of AQP9 may be closely related to the transportation and regulation of mature spermatozoa, as seen in the reproductive tract of mammals, where it is found in the epididymis of rodents and carnivores [19,42].

The localization of proteins is crucial for their function, especially in dynamically regulated processes such as reproduction. Our immunohistochemical observations indicate that aquaporin localization changes during the reproductive cycle. This suggests that aquaporins play specific and contextual roles in reproductive physiology, with high expression in critical locations during reproductive periods. The differential localization of these proteins can offer valuable insights into the molecular mechanisms regulating reproduction in anuran species, thereby aiding hypothesis formulation and guiding future research in this area.

The Western blot results for the studied aquaporin showed bands at 29–30 kDa, which align closely with existing results in the literature. In *D. chrysoscelis*, AQP1-type aquaporin was reported at 26 kDa [17], slightly lower than the results observed in *L. podicipinus*. For *Hyla japonica*, an AQP2-type aquaporin was described in urinary bladder tissue with bands at 30–31 kDa, similar to findings in *Rana catesbeiana*, *R. nigromaculata*, and *Bufo japonicus* [39,48]. The observed variations in the molecular weights of aquaporins may arise from differences in amino acid sequences, post-translational modifications, or tissue-specific isoforms. Additionally, limited data are available to describe the characteristics of AQP9 in anurans. Although the molecular weights of aquaporins provide valuable insights into their structural features, comprehensive studies encompassing functional assays, protein interactions, and expression patterns are necessary to unravel the complexities of AQP biology across different species and tissues.

Regarding Western blotting results, no statistically significant differences were observed between the reproductive and non-reproductive seasons, indicating that the differences observed were related to the location of the AQPs and not their quantity, as confirmed

by the IHC analysis. Western blotting is effective for measuring global protein levels in tissue samples, but it does not detect variations in protein localization that are more apparent with immunohistochemistry. Furthermore, aquaporin expression can be regulated post-translationally, affecting their localization and functionality without altering the total levels detectable by Western blotting.

The protein levels obtained from the blot analysis showed no different values of AQP1 and AQP2 in animals from the non-reproductive period, which aligns with the immunohistochemical labeling results of spermatozoa and Leydig cells, as there are no statistical differences. The immunohistochemical differences were specific to specific cells and not to entire tissues. Because the labeling was predominantly found in other cells, the quantification of these aquaporins between the stages of reproduction could be related to the water conservation function rather than their reproductive and hormonal function, as these two functions are not mutually exclusive.

The aquaporin family in anurans has been studied for a long time, starting with the hypothesis related to the cellular water channels [16]. The present molecular results contribute to the understanding of aquaporin diversity within the anuran group by providing the molecular weight of AQPs from *L. podicipinus* and supplementing the previously reported molecular weights of the aquaporins HC-1 and AQPh2 from *D. chrysoscelis* and *H. japonica* [40,48]. This diversity is also related to the range of environmental behavior within the group, as reflected in physiological mechanisms such as water balance via aquaporins influenced by environmental or hormonal factors [16]. This is evident in the thermally specific expression of *D. chrysoscelis* HC-1 and HC-3 in osmoregulation organs [16,40]. Conversely, in *L. podicipinus* testicles, there was no significant difference in the quantity of AQP expression between periods, despite tissue expression variation. Although the organs originate from different systems, the lack of variation in *L. podicipinus* may reflect the stable and tropical environment that the individuals inhabit.

Furthermore, extended research and testing are necessary to determine whether the difference is related only to the localization of the aquaporins or if there is indeed a differential expression of these proteins throughout the reproductive cycle of the species.

5. Conclusions

This study is the first to describe the expression of AQP1, AQP2, and AQP9 in the testis of an anuran species. These AQPs were expressed in spermatozoa, Leydig cells, and intratesticular ducts mainly during the reproductive season. These observations underscore the impact of reproductive status on aquaporin expression. In conclusion, while this study advances our understanding of reproductive dynamics in anurans, it also underscores the need for further research to fully elucidate the cellular and physiological roles of aquaporin in this context.

Author Contributions: Conceptualization, R.O.A.B. and R.F.D.; formal analysis, R.O.A.B. and R.F.D.; investigation, R.O.A.B. and R.F.D.; methodology, R.O.A.B.; project administration, R.F.D.; supervision, C.d.O. and R.F.D.; visualization, R.O.A.B., C.d.O. and R.F.D.; writing—original draft, R.O.A.B., C.d.O. and R.F.D.; writing—review and editing, R.O.A.B., C.d.O. and R.F.D. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded in part by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brasil (CAPES, grant number Finance Code 001) and Pró-reitoria de Pós-graduação da Universidade Estadual Paulista (PROPG-UNESP).

Institutional Review Board Statement: The animal study protocol was conducted following the Ethical Principles of Animal Experimentation adopted by the Colégio Brasileiro de Experimentação Animal (CEUA N° 4478221021). All expeditions and sampling were conducted with a sampling license (n° 79953-3 SISBIO/IBAMA).

Informed Consent Statement: Not applicable.

Data Availability Statement: The Data is contained within the article.

Acknowledgments: The authors thank Gelson Rodrigues, a technician, for histology processing.

Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

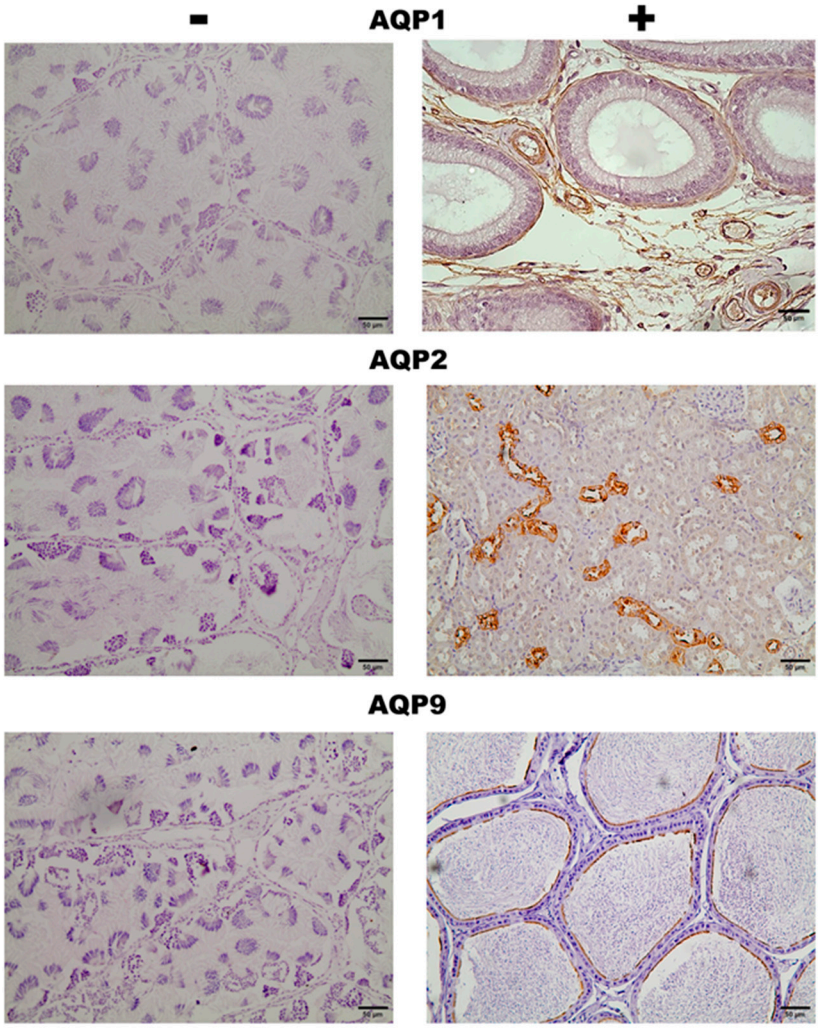


Figure A1. Immunohistochemistry staining of positive and negative controls. Negative controls: AQP1, AQP2, and AQP9 were performed using testis tissue from *Leptodactylus podicipinus*. Positive controls: AQP1: Rat epididymis presenting specific labeling on blood vessels; AQP2: Rat kidney with labeling on the collecting ducts; AQP9: Mouse epididymis with labeling on the tubule stereocilia. Magnification 20× bar = 50 µm.

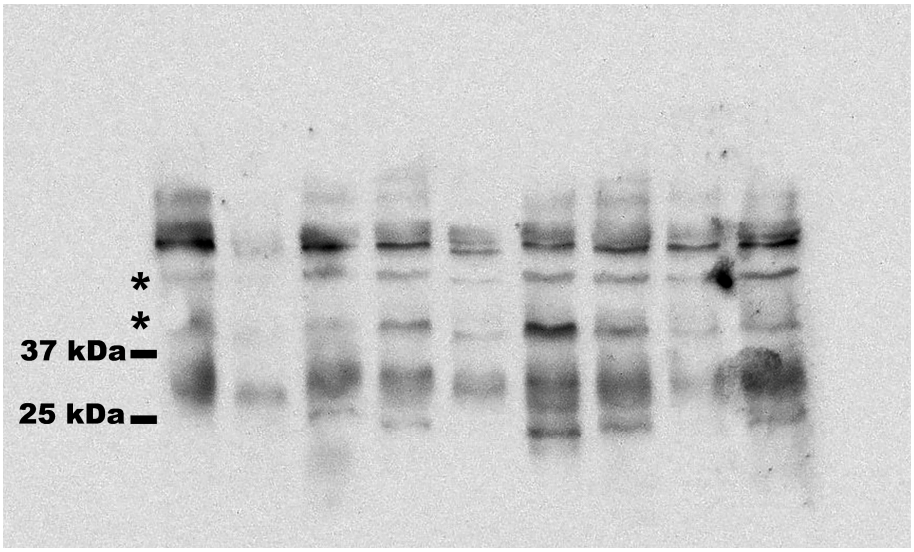


Figure A2. Full-range Western blot membrane of AQP1 from testis tissue of *L. podicipinus*. "*" unspecific bands.

Aquaporin	Parameters	Percentage per period	
		REP (n=4)	NREP (n=4)
AQP1	Spermatozoa	75.0	25.0
AQP2	Leydig cells	100	-
AQP9	Intratesticular ducts	50.0	-

Figure A3. Percentage of testicular parameters with distinct aquaporin label per group of *L. podicipinus*. The percentage indicates the presence of the parameter in the individuals of each group (n = 4 per group); "-" indicates parameter absence.

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Considerações finais e Conclusões

Este trabalho abordou diferentes aspectos da reprodução de *Leptodactylus podicipinus*, incluindo a descrição do testículo e sua relação com o rim, a imunolocalização das AQPs no tecido testicular e a proposição de um padrão germinativo interespecífico, com ênfase no papel da poliandria.

A anatomia do aparelho urogenital da espécie *L. podicipinus* revelou organização similar à observada em outras espécies, mas com particularidades importantes, como o ducto coletor longitudinal e os ductos laterais nos rins. Além disso, diversas diferenças foram observadas na estrutura e fisiologia urogenital ao longo do ciclo reprodutivo, evidenciando uma dinâmica diferencial nas gônadas dessa espécie, influenciada por fatores sazonais.

A caracterização reprodutiva da população estudada também revelou um padrão de atividade e comportamento sazonal, distinto do descrito para a espécie em outras regiões de sua ocorrência. Esses achados reforçam a hipótese de que as características reprodutivas de *L. podicipinus* são moduladas por condições ambientais locais. Além disso, nosso estudo amplia essa perspectiva ao mostrar que essa influência se estende aos processos celulares, como demonstrado pela expressão diferencial das AQPs entre os diferentes períodos analisados.

Adicionalmente, observamos que o comportamento de poliandria aparenta desempenhar um papel crucial no desenvolvimento reprodutivo dessa espécie, reforçando a influência desse traço nos aspectos reprodutivos. Esses resultados aprofundam o entendimento sobre a reprodução de anuros, destacando a complexidade ainda pouco explorada dessa área. Para avançar no conhecimento da diversidade funcional desses animais, é fundamental adotar uma investigação abrangente, que combine técnicas tanto tradicionais quanto inovadoras, ampliando as perspectivas sobre os processos biológicos que regem suas estratégias reprodutivas.

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