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Artur da Nóbrega Carreiro

Dinâmica reprodutiva de *Pseudopaludicola pocoto* Magalhães, Loebmann, Kokubum, Haddad & Garda, 2014 na caatinga nordestina

Patos/PB
2022

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Tese submetida ao Programa de Pós-Graduação em Ciência e Saúde Animal, da Universidade Federal de Campina Grande, para obtenção do grau de Doutor em Ciência e Saúde Animal.

Orientador: Prof. Dr. Danilo José Ayres de Menezes

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ARTUR DA NÓBREGA CARREIRO

DINÂMICA REPRODUTIVA DE *Pseudopaludicola pocoto* MAGALHÃES, LOEBMANN, KOKUBUM, HADDAD & GARDA, 2014 NA CAATINGA NORDESTINA

Tese apresentada ao Programa de Pós-Graduação em Ciência e Saúde Animal como pré-requisito para obtenção do título de Doutor em Ciência e Saúde Animal.

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SUMÁRIO

RESUMO

O estudo da dinâmica reprodutiva de animais *in situ* pode fornecer dados acerca da fisiologia de espécies extremamente ligadas ao equilíbrio de seus respectivos biomas, como animais bioindicadores. A presente tese tem como objetivo mostrar a caracterização da biologia reprodutiva de machos e fêmeas da espécie *Pseudopaludicola pocoto* MAGALHÃES, LOEBMANN, KOKUBUM, HADDAD & GARDA, 2014, na Depressão Sententrional Sertaneja, correlacionando caraterísticas ambientais, comportamentais, morfológicas e fisiológicas. Para tanto, foram coletados 78 animais, sendo três machos e três fêmeas por mês, durante treze meses, em um fragmento de Caatinga localizada no município de Passagem, PB, Brasil. Os animais foram identificados através de sua morfologia externa e por seu DNA genômico, por meio de PCR. Os espécimes foram dissecados e as gônadas foram isoladas e processadas para estudos morfológicos. Dados ambientais durante o ano de coleta foram tomados com auxílio de aparelho *data logger* acoplado a um Globo Negro e a temperatura do solo e água dos corpos d'água, foi obtida através de uma câmera termográfica e termômetro infravermelho, bem como foi utilizado o banco de dados meteorológicos referentes ao ano de 2019 e 2020 do Programa de Monitoramento Climático em Tempo Real da Região Nordeste (Proclima). Os resultados obtidos foram compilados e divididos para a composição de três artigos científicos, com o primeiro referente aos dados ambientais e sua influência nos parâmetros corporais e morfológicos gerais das gônadas em machos e fêmeas. O segundo e o terceiro capítulos, consistem em dois estudos estereológicos para a identificação das modificações na espermatogênese e oogênese ocorridas ao longo do período de coleta, observando a correlação destas modificações com os fatores ambientais analisados em machos e fêmeas maduras da espécie *Pseudopaludicola pocoto* respectivamente.

PALAVRAS-CHAVE: Anfíbio; Reprodução; Semiárido; Sazonalidade.

ABSTRACT

The study of the reproductive dynamics of animals *in situ* can provide us with data on the physiology of species extremely linked to the balance of their respective biomes, such as bioindicator animals. This thesis aims to show the characterization of the reproductive biology of males and females of the species *Pseudopaludicola pocoto* MAGALHÃES, LOEBMANN, KOKUBUM, HADDAD & GARDA, 2014, in the Sertaneja Sententral Depression, correlating environmental, behavioral, morphological and physiological characteristics. For this, 78 animals were collected, three males and three females per month, during thirteen months, in a fragment of Caatinga located in the municipality of Passagem, PB, Brazil. The animals were identified through their external morphology and their genomic DNA, by means of PCR. The specimens were dissected and the gonads were isolated and processed for histomorphometric studies. The environmental data during the year of collection were obtained with the aid of a data recording device coupled to a black globe and the soil and water temperature of the water bodies was obtained by means of a thermographic camera and an infrared thermometer, as well as as the meteorological database for the year 2019 and 2020 of the Real Time Climate Monitoring Program for the Northeast Region (Proclima). The results obtained were compiled and divided for the composition of three scientific articles, the first referring to environmental data and its influence on the morphological and general body parameters of the gonads in males and females. The second and third chapters consist of two stereological studies to identify the changes in spermatogenesis and oogenesis that occurred during the collection period, observing the correlation of these changes with the environmental factors analyzed in mature males and females of the *Pseudopaludicola pocoto* species, respectively.

KEY-WORDS: Amphibian; Reproduction; Semiarid; Seasonality

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LISTA DE ABREVIATURAS E SIGLAS

IGS/ GI	Índice Gonadossomático/ Gonadossomatic Index
PCR	Polimerase Chain Reaction
S	Sul
O	Oeste
CO^2	Dióxido de Carbono
GLM	Generalized Linear Models
CRC	Comprimento Rostro-cloacal
CCA	Comprimento da Cabeça
LDC	Largura da Cabeça
DEO	Distância entre os Olhos
CDC	Comprimento da Coxa
LDCX	Largura da Coxa
CDT	Comprimento da Tíbia
LDT	Largura da Tíbia
CDP	Comprimento do Pé
CDB	Comprimento do Braço
LDB	Largura do Braço
CAB	Comprimento do Antebraço
LDAB	Largura do Antebraço
DON	Distância entre Olho e Narina
DDO	Diâmetro do Olho
TL	Testis Left/ Testículo Esquerdo
TR	Testis Right/ Testículo Direito
KL	Kidney Left/ Rim Esquerdo
KR	Kidney Right/ Rim Direito
FB	Fat Body/ Corpos Gordurosos
AP	Animal Polon/ Polo Animal
VP	Vegetative Polon/ Polo Vegetativo
GD	Gônada Direita
GE	Gônada Esquerda
TW/ PT	Testis Weight/ Peso Testicular
OW/ PO	Ovary Weight/ Peso Ovariano
SPG	Espermatogônias
SPC	Espermatócitos
EAr	Espermátides Arredondadas
EAl	Espermátides Alongadas
SPZ	Espermatozoides
CEUA	Comitê de Ética de Uso de Animais
UFCG	Universidade Federal de Campina Grande
IBGE	Instituto Brasileiro de Geografia e Estatística
ICMBio	Instituto Chico Mendes de Biodiversidade
I	Primeiro grau
II	Segundo grau
III	Terceiro grau
OCT	Oócito
VOCT	Volume do Oócito

LISTA DE SÍMBOLOS

®	Marca registrada
%	Porcentagem
km	Quilômetros
ºC	Graus Celsius
mm	Milímetros
g	Gramas
µm	Micrômetros
x	Multiplicação
/	Divisão
Hz	Unidade de medida de frequência hertz
±	Mais ou menos
mg	Miligrama
µm^2	Micrômetros quadrados
IV	Quarto grau
p1 –p14	Poças 1 a 14
R^3	Raio ao cubo
π	Número pi
H	Altura
∑	Somatório
+	Intersecções
Pt	Pontos

INTRODUÇÃO GERAL

Atualmente são conhecidos três grupos de anfíbios: as salamandras (Caudata), com 502 espécies, amplamente distribuídas, principalmente no hemisfério norte; as cobras-cegas (Gymnophiona), com 165 espécies, caracterizadas pela ausência de membros; e os anuros (Anura), com 4.873 espécies, conhecidas como sapos, rãs e pererecas, que constituem o principal grupo de anfíbios encontrados no Brasil (BASTOS et al., 2003). Os anfíbios apresentam uma variedade de adaptações fisiológicas, morfológicas e comportamentais que lhes permitem ocupar uma ampla gama de tipos de *habitat*. Diante disso, eles são considerados bons modelos para estudos ecológicos e evolutivos, fornecendo informações relevantes ao conhecimento do estado de conservação de regiões naturais, onde os quais funcionam como excelentes bioindicadores de níveis de alteração ambiental (DUELLMAN e TRUEB 1994; POUGH et al., 2008).

Dentre as diversas adaptações dos anfíbios, podemos destacar os seus ciclos reprodutivos, os quais estão sujeitos a controles hormonais que respondem a variáveis ambientais e produzem padrões específicos. Mudanças na temperatura, fotoperíodo e chuvas afetam a espermatogênese e a oogênese e estabelecem adaptações nos ciclos reprodutivos (JORGENSEN, 1992; HUANG et al., 1997).

Os ciclos reprodutivos dos anfíbios podem ser classificados em: descontínuo, potencialmente contínuo e contínuo (LOFTS, 1984). Os tipos descontínuos são comumente encontrados em espécies de zonas temperadas e geralmente têm um discreto período de reprodução com pronunciadas mudanças no tamanho das gônadas, na produção de gametas e nas estruturas sexuais acessórias. No tipo potencialmente contínuo ocorre uma interrupção parcial na atividade gametogênica durante algumas estações no ano, mas espermatogônias primárias nos machos permanecem sensíveis à estimulação gonadotrófica. Espécies que habitam regiões tropicais, normalmente apresentam o tipo contínuo de ciclo reprodutivo (SANTOS; OLIVEIRA, 2007), entretanto, o período reprodutivo pode ocorrer também na estação seca, quando há disponibilidade de ambientes apropriados como os corpos d’água permanentes (BASTOS et al.,2003). Nessas regiões, os maiores picos de produção de hormônios sexuais coincidem com a preparação fisiológica para o período reprodutivo (WILCZYNSK et al, 2005), onde as maiores taxas de produção de andrógenos determinam o desenvolvimento das características sexuais secundárias, a manutenção do comportamento sexual e o início da gametogênese dos anuros (MOORE, 2005).

Nos anuros os testículos são descritos como órgãos pares, arredondados, compactos, de cor geralmente amarelada, esbranquiçada ou branco-leitosa localizados na cavidade celomática próximo aos rins e ligados a parede dorsal do abdômen pelo mesórquio, no qual passa a *vasa efferentia*, conjunto de 10 a 12 ductos que ligam os túbulos seminíferos aos elementos néfricos que desembocam nos ductos mesonéfricos os quais podem conduzir a urina e os espermatozoides. Podem apresentar variações anatômicas de forma e peso de acordo com o período reprodutivo (DUELLMAN e TRUEB 1994; LOFTS, 1984), neste período as células germinativas primordiais começam a espermatogênese até se diferenciarem através de fases mitóticas e meióticas em espermatozoides aptos a fecundação.

Nos vertebrados existem dois padrões de espermatogênese, nos indivíduos amniotas, como os anfíbios a espermatogênese ocorre dentro lúmen dos túbulos seminíferos onde as espermatogônias se dividem de forma mitótica até a fase de espermatócitos primários, a partir deste, inicia-se a fase meiótica da divisão celular até a fase de espermátide, após isso, não ocorre mais divisões e sim apenas o processo de maturação celular. Nos indivíduos anaminiotas, os espermatozoides são produzidos em clones em “compartimentos” chamados de cistos, cada um apresentando células no mesmo estado de diferenciação celular, as espermatogônias são envolvidas por tecido conjuntivo chamadas de células foliculares ou células de *Sertoli* produzindo diversas cópias celulares dentro destes cistos, onde estas sofrem diversos processos de maturação celular (KARDONG, 2016).

No tocante as fêmeas na oogênese, as Oogônias se desenvolvem a partir das células germinativas primordiais que migram para as gônadas em desenvolvimento nos estágios iniciais da embriogênese. Após várias divisões mitóticas e meióticas, estas se diferenciam em folículos primários, estes sob influência de hormônios maturam-se até ficarem aptos para a fecundação (ALBERTS et al., 2009).

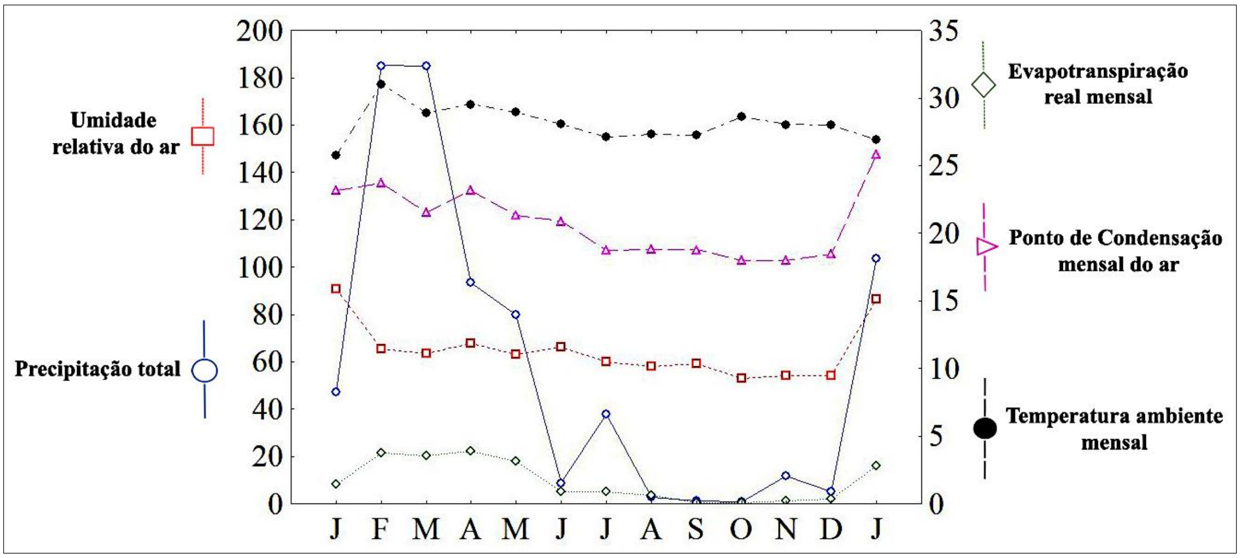
O gênero *Pseudopaludicola* compreende 18 espécies de pequenas rãs na América do Sul, ocorrendo do Norte da Argentina até a Venezuela e ao leste dos Andes (FROST, 2013), sendo frequentemente encontrados em formações abertas como: Chaco, Pampas, Cerrado e Caatinga (MAGALHÃES, 2014).

Pouco se sabe sobre a *Pseudopaludicola pocoto*, atualmente apenas dados morfológicos para identificação da espécie, tais como os de Magalhães et al. (2014), Pansonato et al. (2014), Andrade et al. (2015), De-Carvalho et al. (2015) e Silva *et al.* (2018) foram publicados, porém, dados sobre suas características reprodutivas e sua morfofisiologia em relação as mudanças climáticas na caatinga nordestina ainda são inexistentes.

A Caatinga é o único bioma exclusivamente brasileiro e compreende 70% da região com clima semiárido do Nordeste do Brasil, com uma porção no Estado de Minas Gerais e também alguns enclaves em outros biomas, como na Amazônia, Mata Atlântica e no Cerrado. A sua área estende-se por 734.478 km², representando as regiões do semiárido conhecidas convencionalmente como o "agreste" e o "sertão" com apenas 7,5% de seu território protegido e muito pouco estudada (MMA, 2013). O Zoneamento Agroecológico do Nordeste (ZANE) dividiu o bioma Caatinga em eco regiões: Complexo de Campo Maior, Complexo Ibiapaba, Araripe, Depressão Sertaneja Setentrional, Planalto da Borborema, Depressão Sertaneja Meridional, Dunas do São Francisco, Complexo da Chapada Diamantina, Raso da Catarina (VELLOSO et al., 2002).

Este estudo foi realizado em um fragmento de caatinga localizado na Fazenda Aba, (07º 05' 55.5" S 37º 01' 32.7" W) pertencente ao município de Passagem, Paraíba, com uma área aproximada de 350 hectares, dos quais 120 hectares são de reserva legal. Situada na depressão sertaneja sententrional na mesorregião do sertão paraibano e na microrregião do município de Patos, Paraíba, Brasil (IBGE, 2021). Segundo Köeppen a região que engloba a fazenda possui um tipo climático BSh, caracterizado como Semi-árido quente com escassez de chuvas e grande irregularidade em sua distribuição; baixa nebulosidade; forte insolação; índices elevados de evaporação e temperaturas médias elevadas (por volta de 27ºC). A umidade relativa do ar é normalmente baixa, e as poucas chuvas de 250 mm a 750 mm por ano concentram-se num espaço curto de tempo em enchentes torrenciais. Mesmo durante a época das chuvas (novembro a abril), sua distribuição é irregular, deixando de ocorrer durante alguns anos e provocando secas. A média de precipitação mensal ao longo do ano de 2019 foi de aproximadamente 25,2mm, com valores que variam bastante ao longo do ano (Gráfico 1) (PROCLIMA, 2019).

Gráfico 1 – Precipitação e Umidade relativa no município de Passagem, Paraíba, Brasil, janeiro de 2019 a janeiro de 2020.



A vegetação característica associada a este tipo de clima é a xerófila (Caatinga). A topografia dos terrenos possui relevo ondulado à fortemente ondulado com declividade mais elevada nas porções central-norte, nordeste e sul do município (BELTRÃO et al. 2005). A vegetação predominante da área é de caatinga arbustiva-arbórea densa, com alguns trechos de caatinga arbustiva aberta e formação de serrapilheira. Ocorre ainda área de mata ciliar nas margens de um rio temporário denominado Rio Caldeloso que, no período da estiagem ainda abriga corpos d’água menores com vegetação aquática peculiar além de briófitas, pteridófitas e algas (Figures 1 e 2). No interior da caatinga, a presença de cipós e plantas epífitas completa a fisionomia de vários trechos. Apesar da maior parte da caatinga, na área, estar situada sobre a planície, esta chega a atingir também as serras (MACIEL PEREIRA CORDEIRO et al., 2015). Dados preliminares de sua flora indicam a ocorrência de plantas raras, endêmicas e ameaçadas de extinção (FERNANDO et al. 2014).

Figura 1: Caracterização da região do Rio Caldeloso, Passagem, Paraíba, Brasil. (Fonte: Arquivo pessoal).

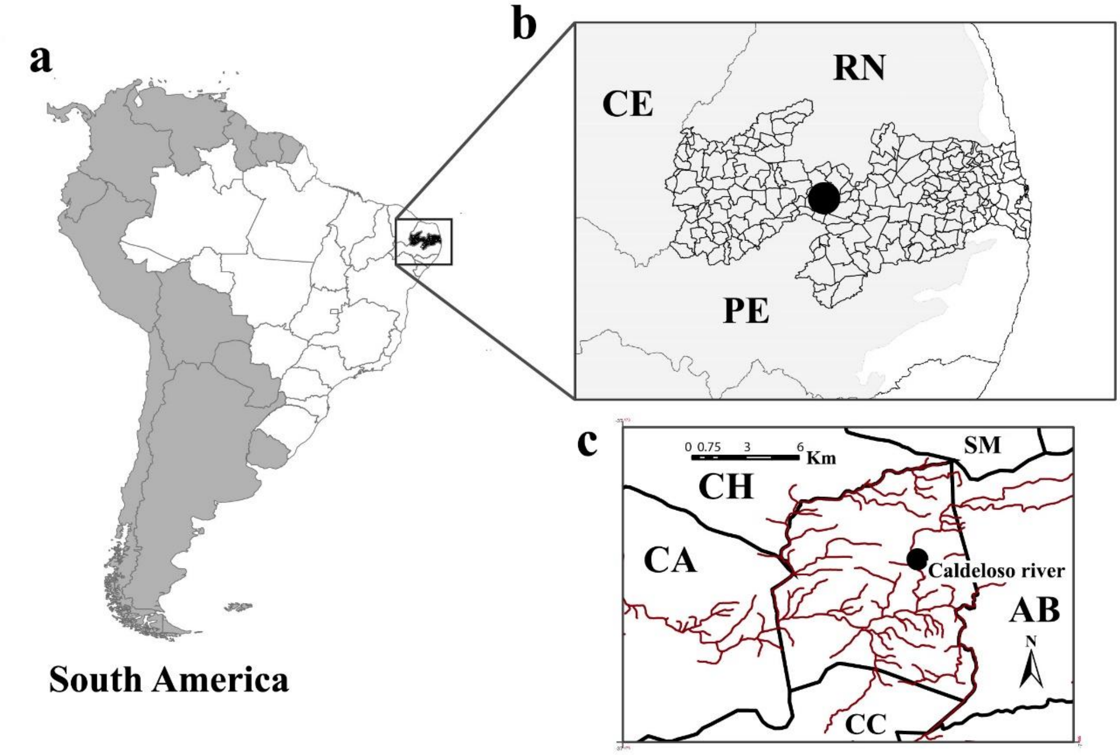


Figura 2: Área de coleta. (a) Mapa da América do Sul (cinza escuro) destacando a divisão geopolítica do Brasil (cinza) e do Estado da Paraíba (preto); (b) Município de Passagem (circulo preto) limitado pelos estados do Ceará (CE), Rio Grande do Norte (RN) e Pernambuco (PE), destacando os biomas Caatinga (cinza claro) e Mata Atlântica (branco). (c) Rio Caldeloso em destaque e seus respectivos afluentes circundado pelos municípios de Cacimba de areia (CA), Quixaba (CH), São Mamede (SM), Cacimbas (CC) e Areia de Baraúnas (AB).

Visto a escassez de informação, acerca da sua morfologia, a influência de fatores ambientais na biologia e reprodução de *Pseudopaludicola pocoto*, buscou-se trazer os primeiros resultados sob forma do Capitulo 1 dedicado a informações sobre a biometria corporal e morfologia dos órgãos genitais e a influência dos fatores ambientais sobre tais parâmetros. O segundo e o terceiro capítulo tem como objetivo descrever as modificações ocorridas no parênquima testicular e ovariano de machos e fêmeas adultas de *Pseudopaludicola pocoto* Magalhães, Loebmann, Kokubum, Haddad & Garda, 2014, ao longo do ano em um fragmento de caatinga nordestina.

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CAPÍTULO I:

The influence of abiotic factors on the reproductive biology of the *Pseudopaludicola pocoto* Magalhães, Loebmann, Kokubum, Haddad & Garda, 2014 in a fragment of northeastern caatinga

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The influence of abiotic factors on the reproductive biology of *Pseudopaludicola pocoto* Magalhães, Loebmann, Kokubum, Haddad & Garda, 2014 in a fragment of northeastern caatinga

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HIGHLIGHTS

- Males and females showed sexual dimorphism, which was not observed for the species until then;
- The abiotic factors analyzed showed a significant correlation for both males and females in the gonadal morphology;
- It was observed that individuals of the species *Pseudopaludicola pocoto* had a specific predilection for temperature and location in relation to water bodies;

ABSTRACT:

The aim of the present study was to characterize the reproductive morphology of adult individuals of the species Pseudopaludicola pocoto in a fragment of the caatinga biome in northeastern Brazil, highlighting the morphological adaptations and changes in their reproductive system resulting from the influence of abiotic factors such as environmental temperature, precipitationrainfall, relative humidity, air dew point, and evapotranspiration over 13 months. Seventy-eight adult animals of the species Pseudopaludicola pocoto Magalhães, Loebmann, Kokubum, Haddad & Garda, 2014, were collected monthly in groups with three males and three females, in a fragment of the caatinga biome in the municipality of Passagem, PB, Brazil throughout 2019. Through morphometric analysis, the urogenital tracts of males and females of Pseudopaludicola pocoto were described, as well as the changes caused by environmental factors in the reproductive biology of the species. In addition to the finding of dimorphism, hitherto not described for this species, with larger and heavier females, when compared to males. After selecting the less collinear variables, the analysis of Generalized Linear Models (GLM) showed that males and females displayed significant correlations between gonad weight and the Gonadosomatic Index with environmental predictor variables, such as rainfall precipitation and environmental temperature. Regarding the spatial arrangement of individuals, there was a selective distribution for areas with specific temperatures throughout the year, accentuated in periods with lower rainfall and lower availability of water bodies.

Keywords: Amphibian; Reproduction; semiarid; seasonality

1 INTRODUCTION

The interface between the terrestrial and the aquatic environments gives amphibians a peculiar reproductive behavior in relation to other vertebrates, the need for suitable places to perform amplexus, external fertilization, and oviposition, as well as their reproductive physiology being subject to extrinsic (environmental) and intrinsic (hormonal cycles) factors. Frogs from tropical regions tend to have continuous reproductive cycles; however, they breed in the wet season, which provides water bodies that allow for oviposition (Ryan, 1986). The Leptodactylidae family is divided into three subfamilies (Leuperinae, Leptocadactylinae, and Paratelmatobiinae), being generally represented by small and medium-sized, insectivorous, terrestrial, or semiaquatic animals, with nocturnal activity. They are found in places ranging from tropical forest regions to those with a semi-arid climate with diverse reproductive behaviors. The genus *Pseudopaludicola* is part of the subfamily Leuperinae, represented by 19 species of small frogs very similar to each other and with gaps in information about their reproductive biology as a whole. Regarding the species *Pseudopaludicola pocoto*, only morphological data were currently published and are available for identifying the species (De Carvalho et al., 2015; De Silva et al., 2018; Medeiros Magalhães et al., 2014; Pansonato et al., 2014; Silva et al., 2017). However, data on their reproductive characteristics and morphophysiology regarding climate change in the northeastern caatinga are still scarce. Thus, the present work aimed to characterize the morphology of the reproductive system of

Pseudopaludicola pocoto, correlating the data with environmental factors, to contribute to the understanding of its reproductive dynamics concerning its *habitat*.

2 MATERIAL AND METHODS

Study area

The study was conducted on the banks of the Caldeloso River in a fragment of the caatinga biome at Fazenda ABA, (7° 6'6.78"S and 37° 1'29.76"W) (Figures 1 and 2) in the municipality of Passagem, Paraíba, with approximately 350 hectares, and 120 of them integrate a conservation area. Located in the Depressão Sertaneja Sententrional in the mesoregion of the sertão of Paraíba and in the microregion of the municipality of Patos, Paraíba, Brazil (Velloso et al., 2001).

Figure 1: Fragment of caatinga located at Fazenda ABA, (7° 6'6.78"S and 37° 1'29.76"W). The formations of bodies of water along the river stand out.

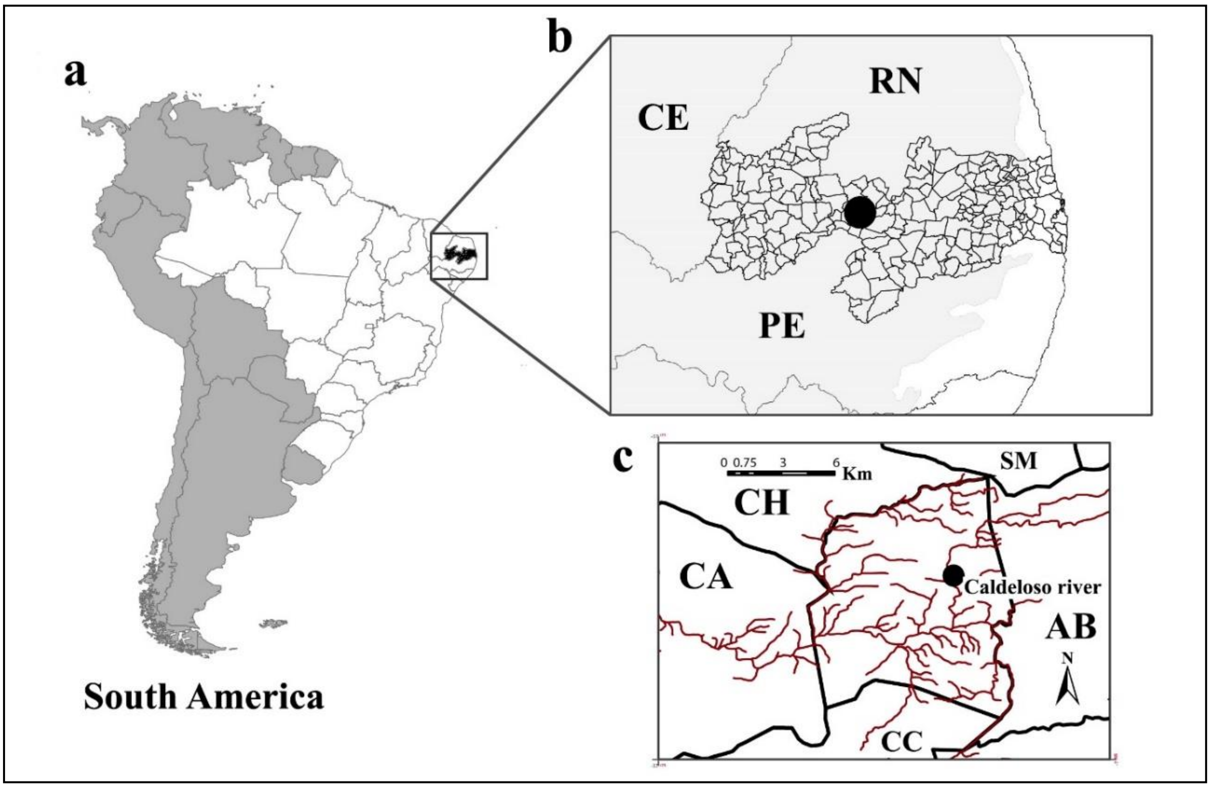

Figure 2: Collection area. (a) Map of South America (dark grey) highlighting the geopolitical division of Brazil (grey) and the State of Paraíba (black); (b) Municipality of Passagem (black circle) limited by the states of Ceará (CE), Rio Grande do Norte (RN) and Pernambuco (PE), with emphasis on the Caatinga (light gray) and Atlantic Forest (white) biomes. (c) Highlight of the Caldeloso River and its respective tributaries surrounded by the municipalities of Cacimba de Areia (CA), Quixaba (CH), São Mamede (SM), Cacimbas (CC) and Areia de Baraúnas (AB).

Environmental data collection and characterization of the habitat

We have collected environmental data such as relative air humidity, dew point, and environmental temperature with a data logger device, HOBO® brand, model U12 Temp/RH 2EX, coupled to an aluminum black globe positioned close to the analyzed water bodies. The temperature of the soil and water of the water bodies was obtained through a thermographic camera of the FLUKE® brand, model ti-400 9Hz Thermal Imager, and an infrared thermometer of the Benetech® brand, model GM-320.

The rainfall and evapotranspiration were obtained from the meteorological database for the year 2019 from the Real Time Climate Monitoring Program for the Northeast Region

(Proclima, 2012). The positioning of the water bodies of the study area was outlined by aerial photos from a drone of the DJI Phantom 4® brand, model CP.PT.234.01.

Collection and identification of specimens

Adult individuals of both sexes were collected monthly in groups with three males and three females over 13 months, through manual and active searches. Immediately after collection, the animals were euthanized with lidocaine cream in the abdominal region, followed by asphyxia in a CO^2 chamber, in agreement with the Directive of the European Parliament and of the Council of 22 September 2010, on protecting animals used for scientific purposes (2010/63/EU, 2010). The procedure was also approved by the Instituto Chico Mendes de Biodiversidade (Brazil, 2018) under protocol number 70895-1. The identification of the individuals was performed with the aid of a Leyca® brand stereomicroscope, model MZ 125, based on the description in the literature (Toledo, 2010), by their coloration and external morphometric characteristics, concomitantly with molecular analyzes using the Blood/Tissue DNA Mini kit from Mebep (Bioscience®).

External morphometry

The animals were weighed on an analytical scale with a precision of 0.00001g Model AUW-220D of the SHIMADZU® brand, placed in Petri dishes, and submitted to external morphometric measurement with a digital caliper (accuracy of 0.001mm) of the Digimess® brand, where the following measurements, already pre-established for anuran amphibians, were obtained: Snout–vent length (SVL), Head length (HL), Head width (HW), Interorbital distance (IOD), Thigh length (THL), Thigh width (THW), Tibia length (TL), Tibia width (TW), Foot length (FL), Arm length (AL), Arm width (AW), Forearm length (FLL), Forearm width (FW), Snout-vent length (EN), and Eye diameter (ED). [3]

Gonad morphology

A medial incision was made to open the coelomic cavity, where the organs of the urogenital tract were analyzed and morphologically described with the aid of a Leyca® brand MZ 125 stereomicroscope, with a graduated scale bar to perform the topographic description of the reproductive tract, as well as the morphological and morphometric aspects.

After macroscopic analysis and evaluation, the gonads were collected and submitted to a routine histological processing for light microscopy, according to the methodology described in the literature (Tolosa et al., 2003). Sections of 5µm were stained in Hematoxylin-eosin and the slides were analyzed under a microscope of the Leyca® brand, model E200.

In males, the classification of spermatogenic development stages was based on morphological aspects already described for frogs (Kaptan and Murathanoğlu, 2008), as well as the stereoscopic and microscopic classifications (Rodríguez-Gómez et al., 2010; Sretarugsa et al., 2001). The Gonadosomatic Index (GSI) of the specimens was obtained by Cavalieri's principle (Pasqualini et al., 1986), to obtain values such as volume, mass, and weight of both testes.

For females, an analytical scale was used with a precision of 0.00001g, Model AUW220D of the SHIMADZU® brand, and both values were submitted to the formula for obtaining the Gonadosomatic Index (GSI = Weight of the gonads / Weight of the individual) x 100, thus establishing their respective monthly GSI values. The identification of the stages of oocyte development was based on the aspects described for adult female frogs (Rodríguez-Gómez et al., 2010).

Data analysis

All sample data were submitted to normality and homoscedasticity tests, using the Kolmogorov-Smirnov and Shapiro-Wilk tests with $p \geq 0.05$. Pillai's trace was used in a multivariate analysis of variance (MANOVA) with a degree of freedom equal to 1 and $p \leq 0.05$ to identify sexual dimorphism among the specimens. To avoid multicollinearity between the predictor variables (environmental), the Spearman Correlation analysis was performed and then, the less collinear predictor variables were selected.

The relationship between morphometric and environmental variables was obtained by GLM (Generalized Linear Models) with Poisson distribution, for data with high overdispersion, and log linkage function, through the "MASS" package in the R software (Annex II) (Ripley et al., n.d.; Sugio et al., 2018). The presence of a temporal autocorrelation was tested from the residuals of the analysis with the Breusch-Godfrey test, using the “lmtest” package in R (Annex II) (Hothorn et al., 2012).

3 RESULTS

Specimen identification, external morphometry, and sexual dimorphism

The external morphology showed high similarity with the individuals already registered for the species *Pseudopaludicola pocoto* regarding their external morphology, with means and standard deviations within what was already observed, and coloration similar to that already described in the literature. The molecular analysis of the BLAST nucleotide showed that the individuals correspond to the species *Pseudopaludicola pocoto*. In the phylogenetic tree (Appendix II), all species of the genus *Pseudopaludicola* are reciprocally monophyletic and the posterior probability values are high for all species. Through the measurements, external morphometric means were obtained, to establish a margin of size and weight for adult males and females of the species. Regarding weight, males and females had averages of 265.48±40.75mg and 310.02±48.32mg, respectively.

Figure 3: An adult male specimen of the species *Pseudopaludicola pocoto* Magalhães, Loebmann, Kokubum, Haddad & Garda, 2014. (Scale bar = 10mm).

From the external morphometric measurements, the multivariate analysis of variance (MANOVA) showed sexual dimorphism for the species, with females being larger and heavier than males (Table 1).

Table 1 – Mean and standard deviation of morphometric measurements of males and females of *Pseudopaludicola pocoto* collected in a fragment of caatinga in the hinterland of Paraíba.

External Measure	Mean and Standard Deviation	
	Males	Females
*CRC	13,334±0,4349	14,092±0,4548
*CCA	5,302±0,2425	5,564±0,3956
LDC	4,755±0,1418	4,876±0,19796
DEO	1,362±0,1995	1,361±0,3327
*CDC	6,176±0,3113	6,442±0,3922
LDCX	2,767±0,2310	2,777±0,3134
CDT	6,802±0,3719	6,976±0,4272
LDT	2,031±0,2486	2,023±0,3362
CDP	6,712±0,5336	6,726±0,6445
CDB	2,27±0,3040	2,322±0,3295
*LDB	1,072±0,1463	1,121±0,1786
CAB	2,307±0,3302	2,343±0,3965
LDAB	0,939±0,4458	0,907±0,5353
DON	1,334±0,1142	1,3±0,10547
DDO	1,734±0,1666	1,731±0,1757

* Values of $p \leq 0.05$ for significant distinct measures between males and females (MANOVA).

Description of the urogenital tract

Males

The testes were located close to the roof of the coelomic cavity, cranioventrally to the kidneys and in the caudodorsal direction to the liver and intestines, and lateral to the abdominal aorta. The right testis was displaced more cranially in relation to the left one, with shapes similar to “blackberries”, composed of rounded units, called seminiferous locules, with a gradient color pattern, from brown on the periphery to whitish yellow in the center of each unit (Figure 4). Throughout the year, it was possible to observe color changes in the testicular parenchyma, where testes collected from individuals in June had a lighter color than those from previous months. Several spots of brownish pigmentation were observed in them, as well as in the serosa, with groups of melanocytes scattered throughout the entire coelomic cavity in all individuals.

Fat bodies were adhered to the gonads, with a more transparent appearance compared to the other structures that compose the tract (Figure 4).

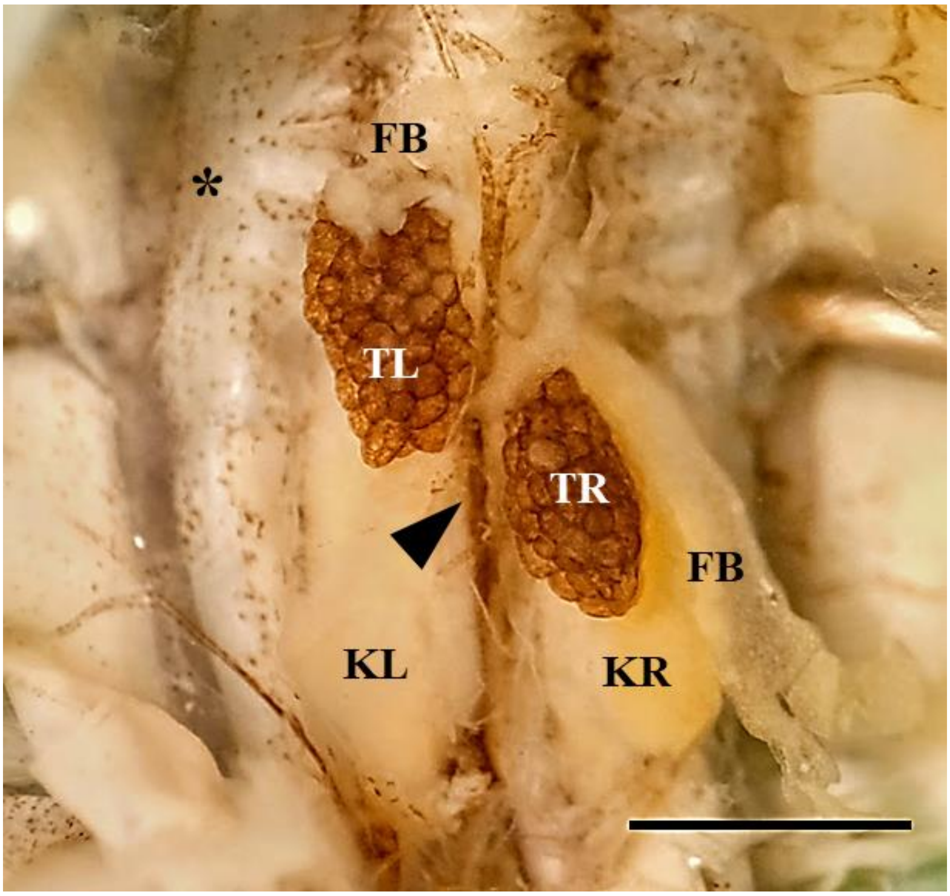

Figure 4: Male reproductive system of Pseudopaludicola pocoto. Open coelomic cavity with ventro-dorsal view and gastrointestinal tract removed. Fat bodies (FB); Right Testicle (TR); Left Testicle (TL); Aorta (Arrow); Right Kidney (KR); Left Kidney (KL); (*) Melanocytes. Leyca® magnifier model MZ 125. 16x magnification; Scale 0.5mm.

In the different serial sections of the gonads, the testicular parenchyma did not show any alteration in relation to the sectioned portion. It was possible to estimate six spermatogonial stages in a cystic conformation, where each cyst had a clear and well-grouped spermatogenic development lineage, in addition to an interstitial space formed by connective tissue with blood vessels, nerve endings, and *Sertoli* cells surrounded by the Tunica Albuginea (Figure 5)

It was possible to highlight, in all analyzed parenchymas, the conformation of the rounded to ellipsoidal shape of the seminiferous locules, surrounded by abundant layers of melanocytes and melanin (Figure 6), which explains the staining observed in the macroscopy.

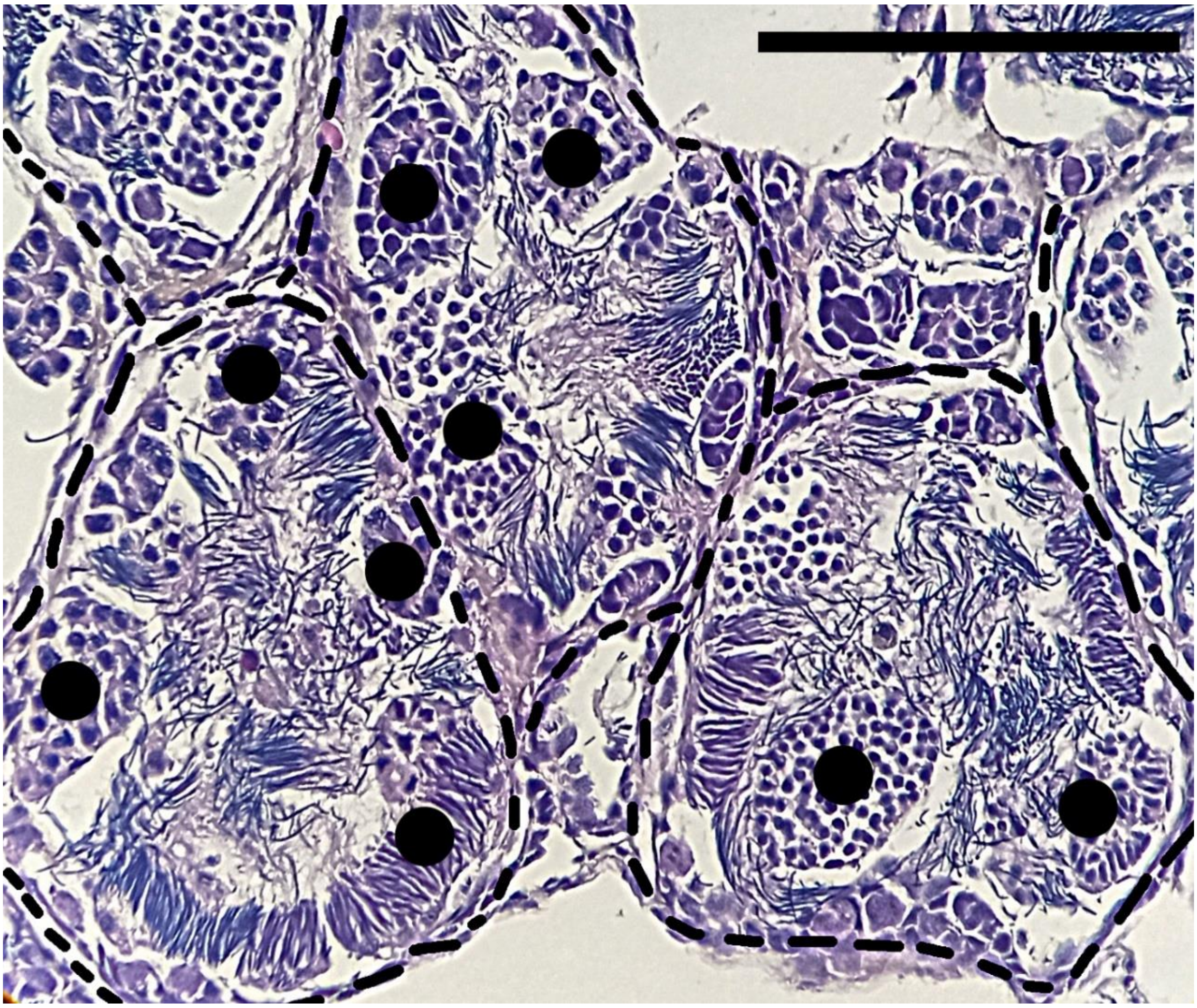

Figure 5: Conformation of the testicular parenchyma of *Pseudopaludicola pocoto*. Seminiferous locules and interstitial space; Germ cysts (circles) in the rainy season.(HE staining) (Scale bar = 50µm).

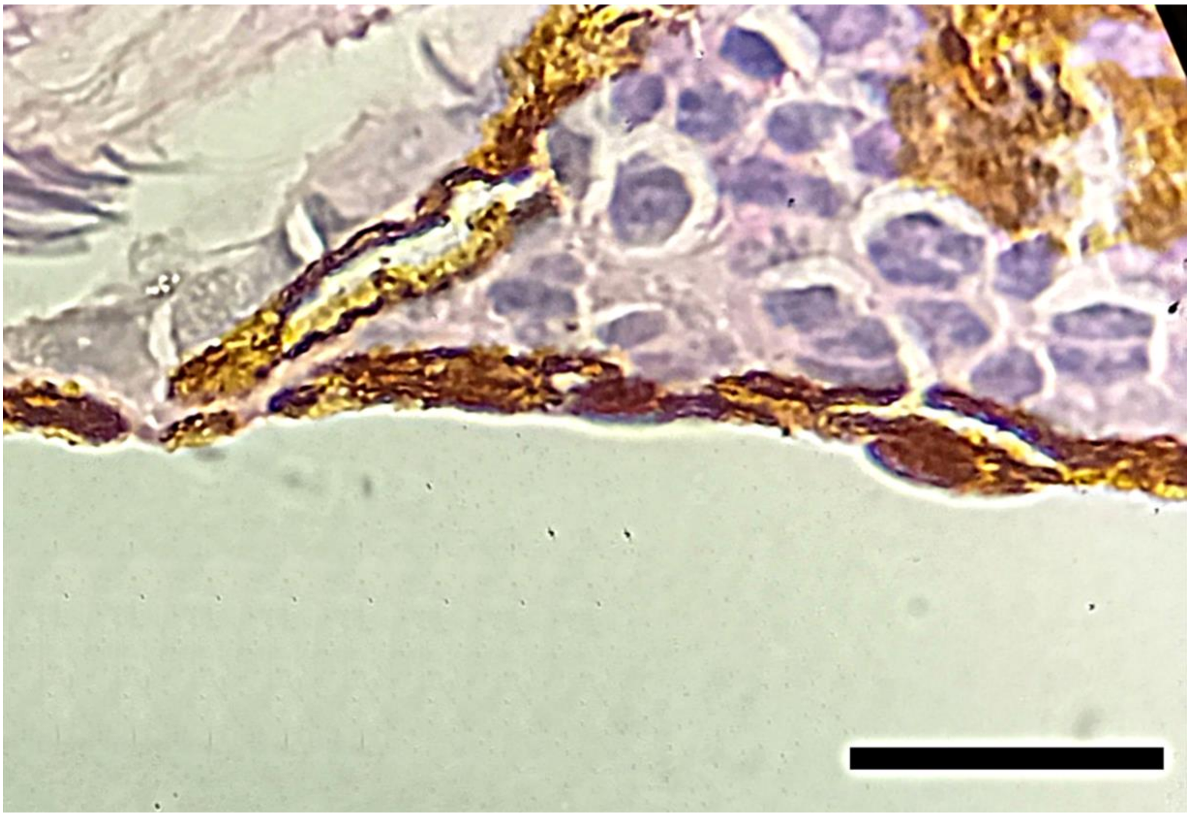

Figure 6: Interstitial portion of parenchyma cells. Melanocytes and Melanin surrounding the seminiferous locules in the rainy season. (Scale bar = 10µm).

The conformation and distribution of the locules were uniform to all other individuals, highlighting the size relationship between the locules and the entire testicular parenchyma, with seminiferous locules measuring an average of 505.158µm², with a standard deviation of ±16.831.

In relation to germline cells, type A spermatogonia can be seen arranged as rounded cells, with a well-defined nucleus when compared to those of type B, which have cytoplasm containing nucleoli of smaller size than those of type A (Figure 7 - A and B), both isolated or in small groups. In a smaller size, Spermatocytes had smaller cytoplasm and more condensed nuclei when compared to Spermatogonia (Figure 7 – C and D). Spermatids appeared as rounded and elongated, with practically imperceptible cytoplasm (Figure 7 – E and F), juxtaposed in bundles where, numerous times, their delimitation was impossible. Spermatozoa presented a well-defined flagellum and body, grouped in parallel, easily identifiable in relation to other cell lineages (Figure 7 – H), and arranged in cell bundles similarly to elongated spermatids. Each spermatogenic cyst is composed of a cell type of the germ line and surrounded by *Sertoli* cells (Figure 7 – G). It was common to observe that lineages in more advanced stages, such as elongated spermatids and spermatozoa, were positioned directed to *Sertoli* cells.

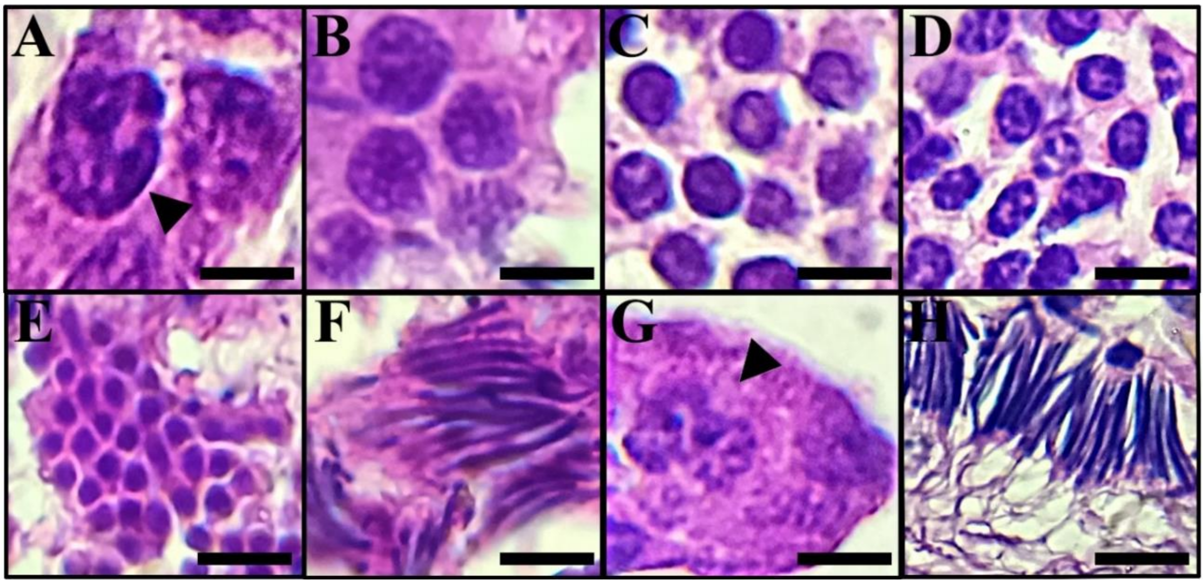

Figure 7: Different types of germ cells in *Pseudopaludicola pocoto* during the spermatogenic process: type A and type B spermatogonia (A/B); Primary spermatocytes in leptotene/zygotene (C); Primary diplotene spermatocytes (D); Rounded and elongated spermatids (E and F); Spermatozoa in the final stage of development (H); Sertoli cell (G) - arrow - in contact with elongated spermatids in its apical region (HE staining) (Scale = 5μm).

Females

During the analysis, it is observed stereoscopically that both ovaries occupy a significant portion of the coelomic cavity, located dorsally to the intestines, in a caudodorsal direction to the liver and ventral to the kidneys, encompassing the entire coelomic cavity, starting from the caudal portion of the liver. Both are quite symmetrical, their color ranges from brown to yellowish white, and they are arranged in small spherical units called Oocytes, resembling a “bunch of grapes”, in addition to the presence of fat bodies around them (Figure 8).

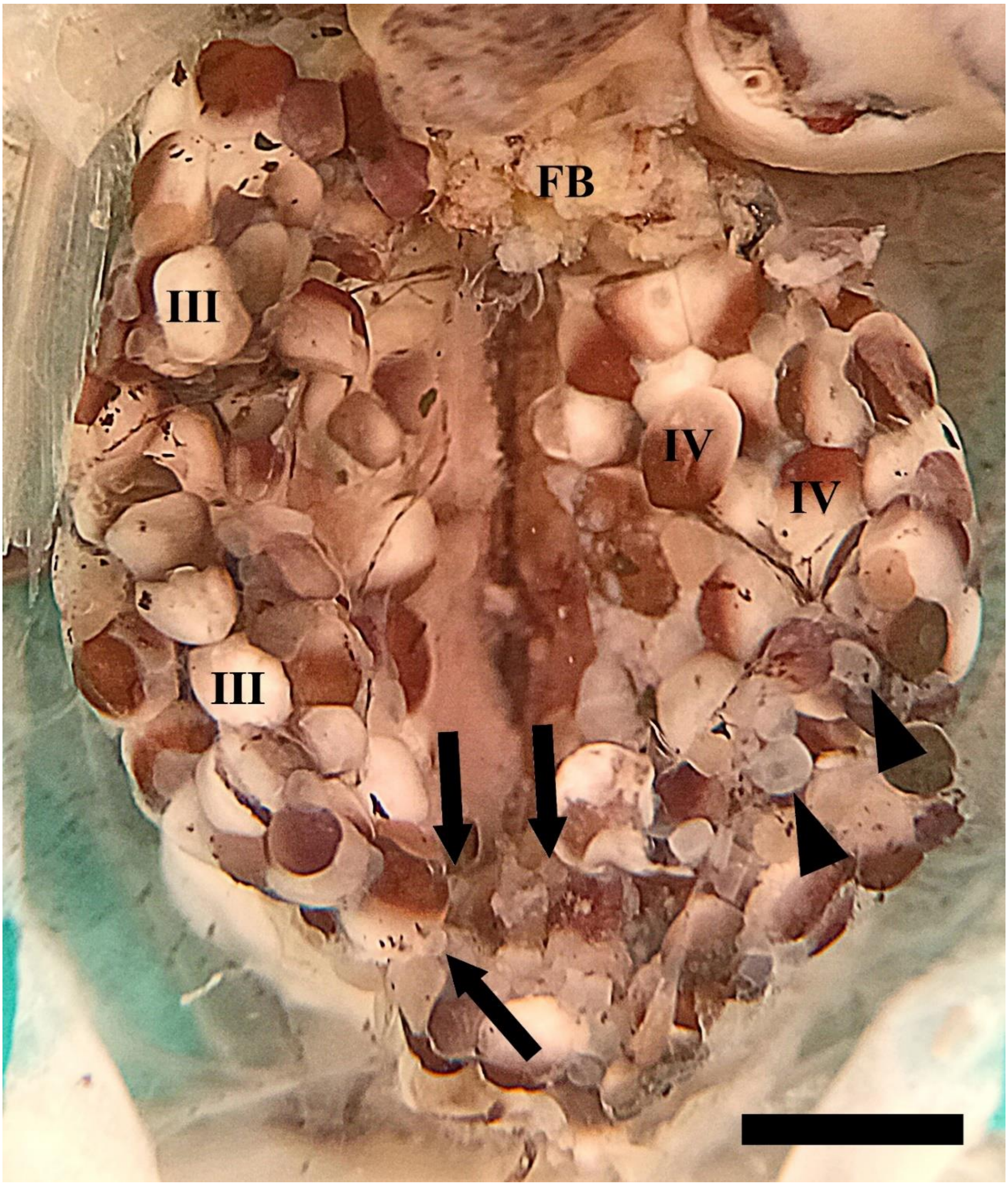

Figure 8: Stereoscopic photo of *Pseudopaludicola pocoto* ovaries. Open coelomic cavity with ventro-dorsal view and gastrointestinal tract removed; F.B – Fatty bodies. Oocytes classified into 4 stages of development: arrow-I, arrowhead-II, 3-III, 4-IV. 16x magnification; 2x 110AL lens. Scale 2 mm.

It was possible to observe all stages of oocyte development, both stereoscopically and microscopically, because of the thin layer of the Tunica albuginea. Grade I (previtellogenic) oocytes are smaller and yellow-translucent. Grade II (previtellogenic) oocytes are larger and more opaque than previous ones. Grade III (vitellogenic) oocytes are larger than the previous ones and are whitish-yellow and opaque. Grade IV (vitellogenic) oocytes have two poles, a vegetal one with a whitish-yellow color and the animal one with a brown color, being well delimited due to pigmentation under the oolemma of the animal pole (Figure 8).

As in the stereoscopy, it was possible to distinguish 4 stages of oocyte development. Grade I previtellogenic oocytes were reduced in size with a nucleus occupying a large part of it and the basophilic cytoplasm; Grade II were larger in size than the previous ones, with the formation of peripheral vacuoles called cortical alveoli and a smaller nucleus (Figure 9).

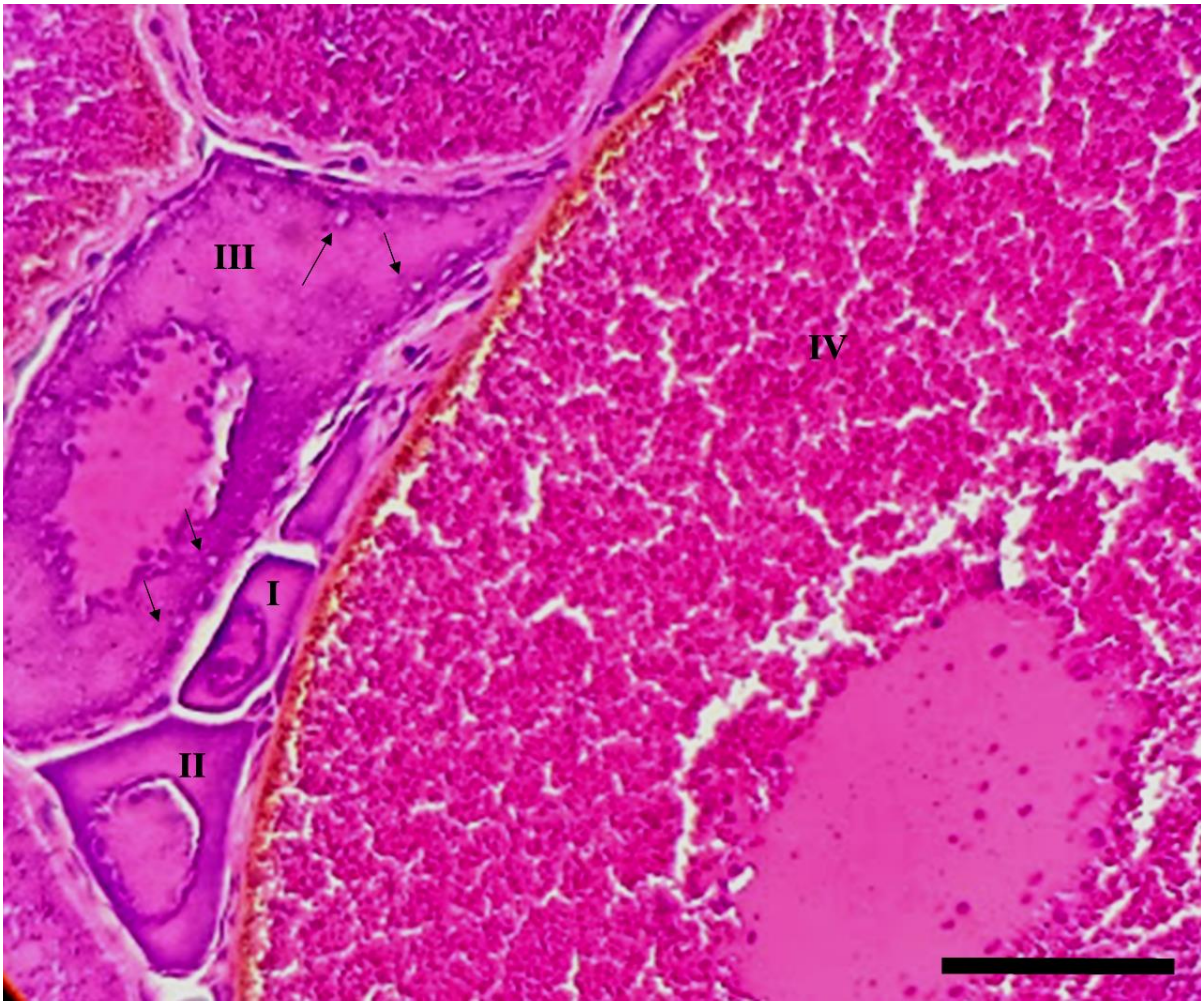

Figure 9: Micrograph of adult *Pseudopaludicola pocoto* ovarian parenchyma with different stages of oocyte development. Pre-vitellogenic oocytes deocytes I and II (I, II), vitellogenic oocytes of grades III and IV (III, IV). Layer of cortical alveoli in developing oocyte (Arrows). (Scale = 20µm).

In those from Grade III, the nuclear membrane is arranged in a more convoluted manner, with the presence of vitellogenic granules in the oocyte cytoplasm and of larger size compared to previtellogenic ones. Those in Grade IV are the largest in size compared to the other stages, with distinct vegetal and animal poles, full of vitellogenic granules and a more rounded nuclear membrane (Figures 10 and 11).

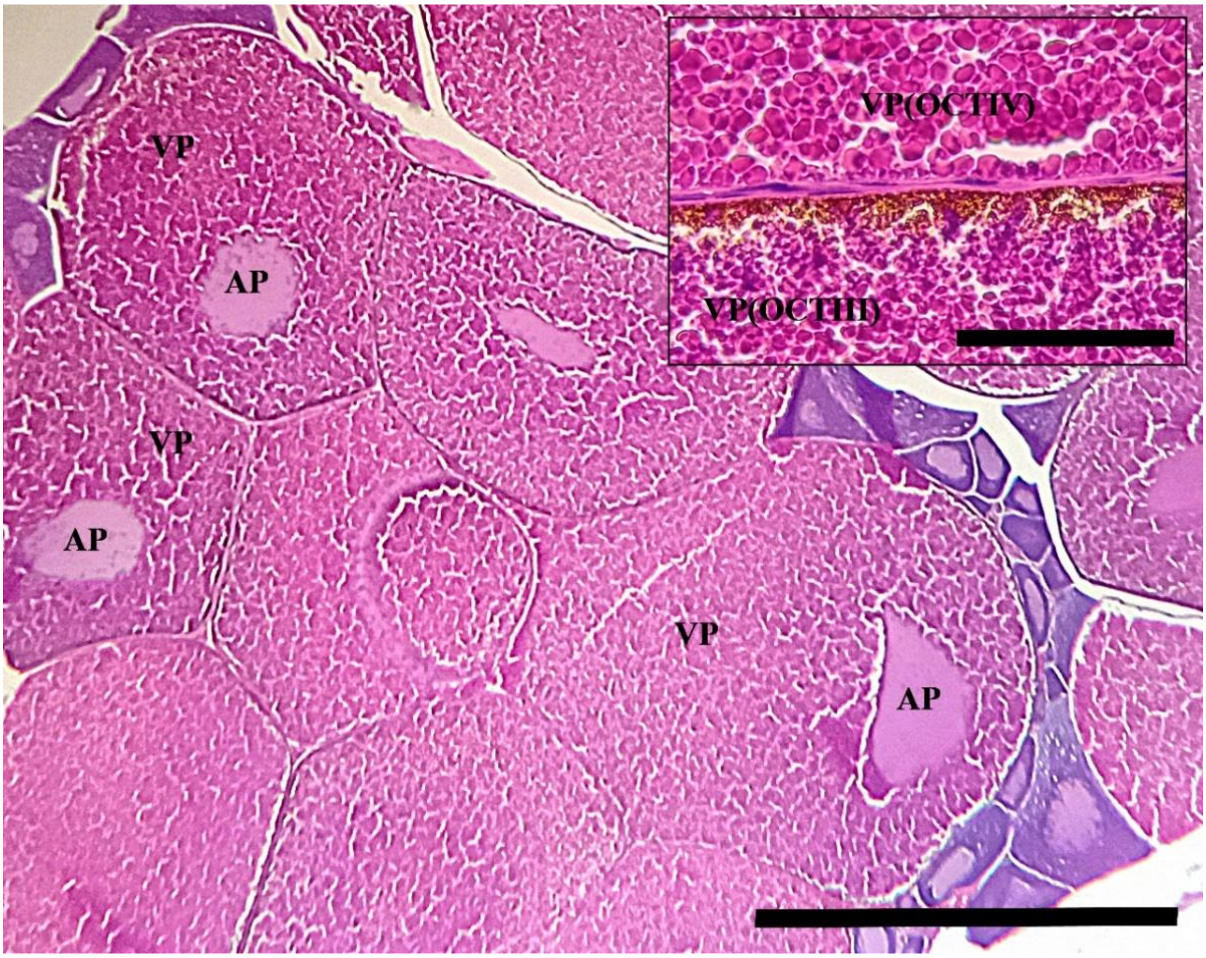

Figure 10: Vitellogenic oocytes of *Pseudopaludicola pocoto* in late-stage (AP) and vegetative (VP) animal development surrounded by a final layer of follicular cells (highlight). (Scale bar =10µm/150µm).

Gonad weight and Gonadosomatic Index (GSI)

In males, it is observed that the weights of the testis differed significantly between the antimeres, where the left testicle presented a larger size in relation to the right one ($p = 0.02$).

Regarding the Gonadosomatic Index (GSI) an average of 1.045% was observed in males. Regarding females, the difference between the sizes of the ovaries was quite clear in all individuals, where the left ovary was larger and heavier than the right one (p = 0.01), with an average Gonadosomatic Index (GSI) of 15.153% (Table 2).

Table 2 - Means and standard deviation codes of gonad weight and gonadosomatic index in *Pseudopaludicola pocoto* in a fragment of caatinga, Brazil, 2019

	Weight			GI
	GR (mg)	GL (mg)	Total (mg)	(%)
Machos	0,143±0,046^a	0,14±0,052^b	0,284±0,074	1,045±0,275
Fêmeas	19,193±7,981^a	28,789±11,971^b	47,981±19,95	15,153±5,055

(GR- Right Gonad; GL- Left Gonad; GI- Gonadosomatic Index). Different letters on the same line differ significantly ($p \leq 0.05$).

Environmental factors and morphology

Spearman's correlation showed the degree of collinearity between the environmental variables (predictors). After this, we selected the variables rainfall and environmental temperature, both of which had the lowest correlation coefficient, i.e., they were less collinear (Table 3) for analyzing Generalized Linear Models (GLM) with morphological variables.

Table 3 – Spearman correlation between environmental variables (independent) for collinearity test in a northeastern caatinga fragment, Brazil 2019/2020.

		Precip. total	Umid. Relativa	Evapotransp. real	Ponto de cond.	Temp. Ambiente
Precipitação	Coefic.	1,000	0,692	0,930	0,797	0,524*

(*) lowest correlation value between variables

The GLM analysis showed a significant correlation between the Gonadosomatic Index of males with rainfall and environmental temperature (Table 4), contrasting with what was observed in females, where only the weight of the ovaries showed a significant correlation with the same variables (Table 5).

Table 4 – Result of the Generalized Linear Model of Testicular Weight and Gonadosomatic Index of *Pseudopaludicola pocoto* with Total Precipitation and Ambient Temperature in a fragment of caatinga

Response variable	Selected term	Coeficient	Standard error	Z-value	P-value (>|z|)
TW	Intercept	1,83739	14	0,13	0,897
	Precipitation	0,00393	0,01025	0,383	0,702
	Ambient Temperature	-0,11831	0,51717	-0,229	0,819
GI	Intercept	26.873.783	4	6	p<0,01*
	Precipitation	0,010707	0,004285	2	0,0125*
	Ambient Temperature	-0,966474	0,167313	-6	p<0,01*

(TW) Peso testicular; (GI) Índice Gonadossomático; (*) valores significativos de p.

Table 5 – Result of the Generalized Linear Model of Ovarian Weight and Gonadosomatic Index of *Pseudopaludicola pocoto* with Total Rainfall and Ambient Temperature in a Northeastern Caatinga Fragment, Brazil 2019/2020.

Response variable	Selected term	Coeficient	Standard error	Z-value	P-value (>|z|)
OW	Intercept	5,5101	0,9892658	5,57	p<0,01*
	Precipitation	0,0036692	0,0007325	5,009	p<0,01*
	Ambient temperature	-0,0675725	0,0361411	-1,87	0,0515*
GI	Intercept	3,557	1,83331	1,94	0,0524*
	Precipitation	0,001895	0,001349	1,405	0,1601
	Ambient temperature	-0,036062	-0,066844	-0,539	0,5895

(TW) Testicular weight; (GI) Gonadosomatic Index; (*) significant values of p.

Although some water bodies remain throughout the year, only those with vegetation presented individuals of the species in amplexus or vocalizing in the rainy season. At the end of the year, only puddles p13, p9, p7, and p5 had water present, but only p5 had vegetation and individuals (Figure 11).

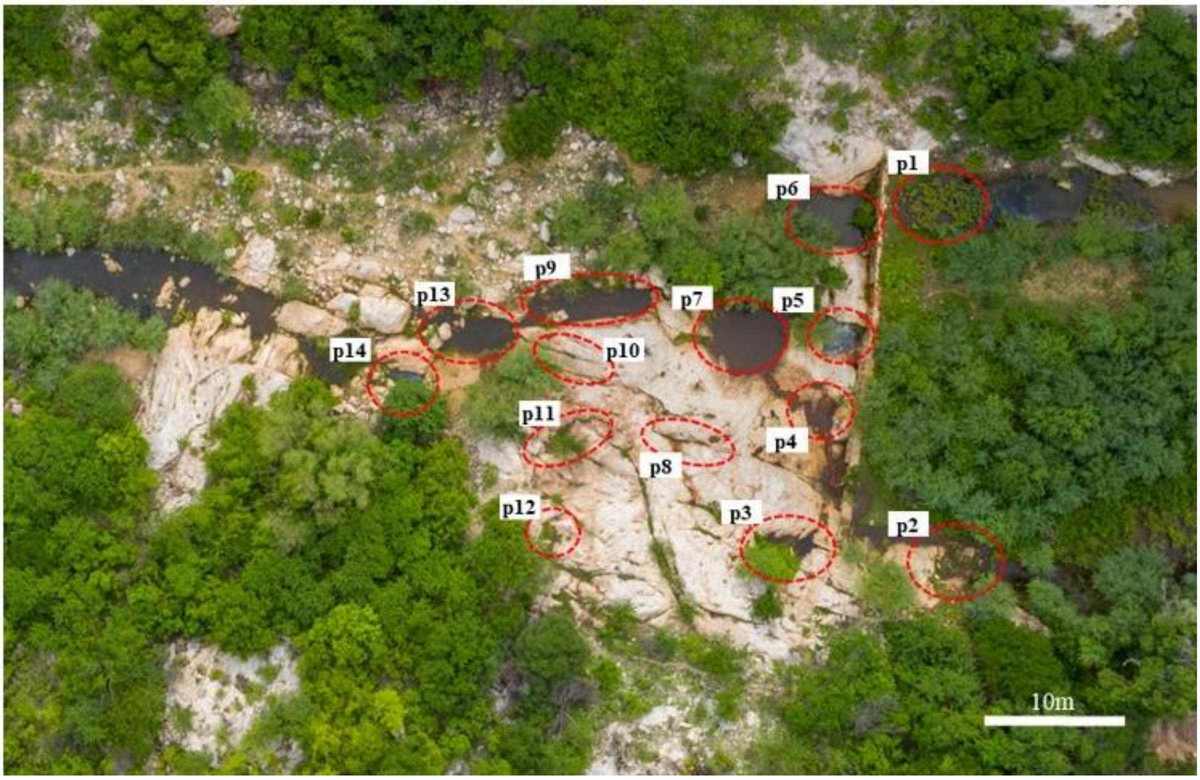

Figure 11: Panorama of water bodies (p1-p14) with vocalization of individuals of the species *Pseudopaludicola pocoto* in a fragment of caatinga in the hinterland of Paraíba. Aerial photo taken by a dronne equipment brand DJI Phantom 4® model CP.PT.PT.00000234.01.

After the thermographic photographs, the individuals were located in an average temperature range of 28.4°C in the soil of water bodies (Figure 12). Regarding the averages obtained for soil and water temperatures, an average difference of 4.2 °C was observed, where the water was warmer in almost all situations throughout the year.

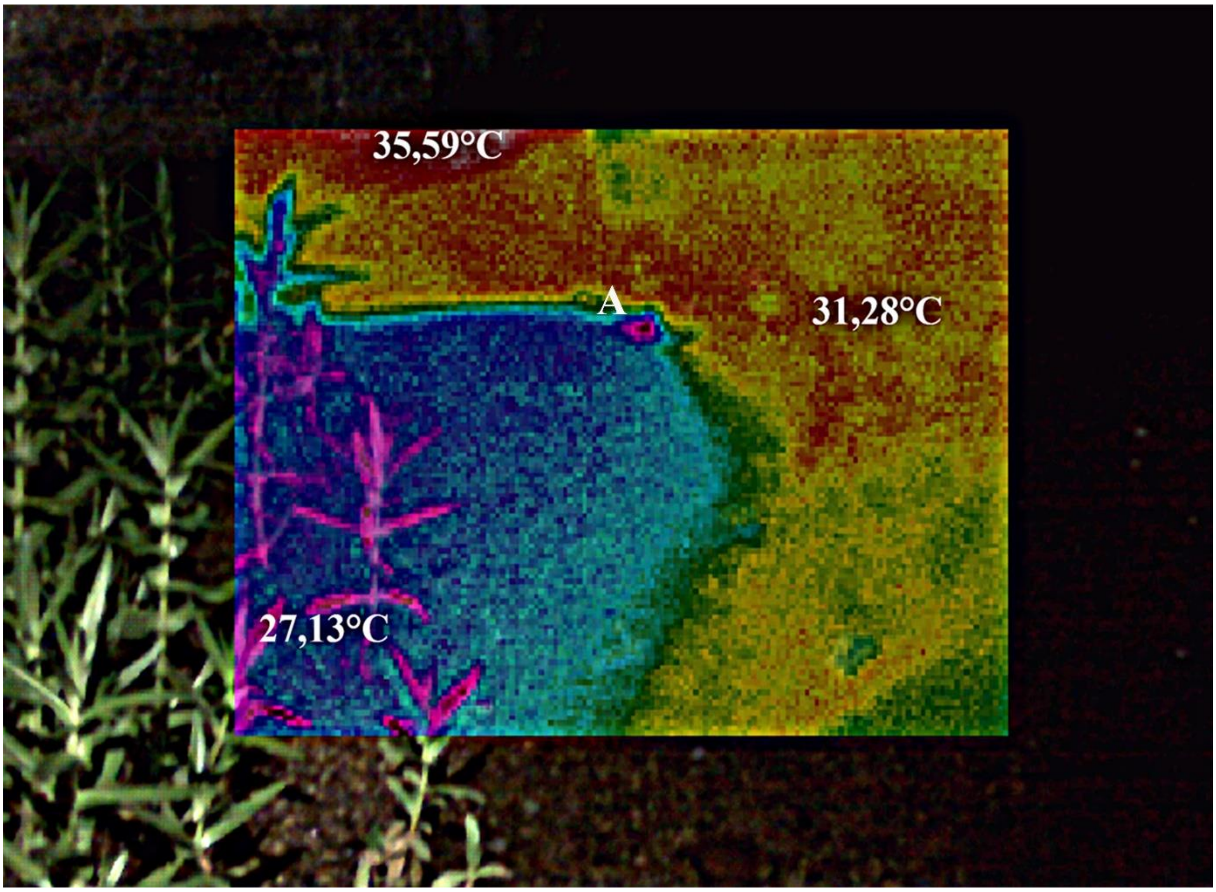

Figure 12: Thermographic image of water bodies with reproductive activity of individuals of the *Pseudopaludicola pocoto* species. (A) Male specimen of *Pseudopaludicola pocoto*. (Fluke Ti300+ 60Hz Thermal Imager).

4 DISCUSSION

The general external morphology observed in the collected individuals was very similar to that described for the species in the literature, differing in small morphometric aspects that can be attributed to the availability of water, food, competitiveness, and predation (Amaral, 2009; Grillo, 2011; Rodrigo dos Santos, 2015). Differences also observed in populations from urban or rural environments (De Andrade et al., 2016; Lantyer-Silva et al., 2016; Lehtinen et al., 1999; Medeiros Magalhães et al., 2014; Rezende et al., 2014; Rezende et al., 2021). However, molecular identification proved to be effective in elucidating any margins for errors in identification. Such analyzes enable this process especially when frogs are extremely similar in their external morphology, in addition to being fundamental for the definition of conservation strategies and sustainable use of these genetic resources (Fouquet et al., 2007).

Although the literature brings important information about the morphology of the species, no study found dimorphism between the genera like this one, where it was observed that females presented CRC, CCA, CDT, and LDB with higher values than males. Such contrast resulted from the morphometric data of individuals collected in other studies since they were in more isolated or focal periods (De Andrade et al., 2016; Lantyer-Silva et al., 2016; Medeiros Magalhães et al., 2014). Regarding *Pseudopaludicola pocoto*, this work presents data covering a longer time interval and a greater number of individuals collected, which can be considered an important factor to establish a morphometric mean true to what would be a body pattern of the species, particularly in amphibians, given their close correlation between the habitat and body score, which may vary in rainy and dry seasons, for example (Blaustein, 1994; De Oliveira and Alves, 1997; Frost, 2016; Huang et al., 1997; Jørgensen, 1981; Pierantoni et al., 2002; Reproduction and 1984, n.d.; Rosa and Lima, 2008)

In the internal mesoscopic description of the reproductive tract of *Pseudopaludicola pocoto*, the distribution, pattern of coloration, morphology, positioning, and the variable presence of fat bodies is repeated for several frog species for both males (Lehtinen et al., 1999; Leite et al., 2015; Pierantoni et al., 2002), and females (Ogielska et al., 2013; Rodríguez-Gómez et al., 2010; Säfholm et al., 2012) within the family Leptodactylidae, differing basically in the thickness of the tunica albuginea and in the number of melanocytes in the cortical region of the gonads, which have an unknown functional aspect and biological value.

Regarding the microscopic description of the testicular parenchyma of *Pseudopaludicola pocoto*, the cystic locular pattern appears to remain intact in several amphibian species, with well-defined cysts for each cell lineage throughout spermatogenesis (Kardong, 2016; Leite et al., 2015). Pierantoni et al., 2002; Villagra et al., 2014) with differences in the volumetric proportion between the locules and the testes. The same occurs with the ovarian parenchyma in female frogs, where the oocyte conformation seems to remain intact, not only in frogs but even in other groups such as teleost fish, for example, a characteristic that seems to be inherent to all anamniotes in both sexes (McMillan, 2007).

Some authors have already highlighted that environmental factors can significantly influence the reproductive cycle of several species of anuran amphibians, to the point that the same species has different reproductive cycles in different regions [57; 58; 59, 60; 49;61]. Among these factors, relative humidity, rainfall, and environmental temperature stand out (Chieffi et al., 1980; Donnelly and Guyer, 1994; Hödl and Hodl, 1990; Pombal et al., 1994; Ryan, 1986; Vitt and Caldwell, 2008). These facts reinforce the importance of using different techniques that allow a closer analysis of the real scenario of how a population interacts with external factors, and their action on its physiology, even more, when dealing with individuals such as amphibians (Begon et al., 1986; Ryan, 1986; Toledo et al., 2003; Wells, 2010). In reproduction, some variables may represent the changes resulting from the reproductive system interacting with environmental factors, for example. The Gonadosomatic Index (GSI) corresponds to a variable that represents, in numerical percentage, the reproductive potential of an individual or group of individuals, through the relationship between their weight and that of their gonads (Kenagy and Trombulak, 1986). This index has already been used in some works involving amphibians, presenting significant data when related to climate changes in a region, facilitating the identification of the period with the greatest gonadal activity (Díaz-Páez and Ortiz, 2001; Santos and Oliveira, 2007). In the present study, it was possible to observe significant correlations between environmental factors, such as rainfall and temperature, with morphometric variables, such as gonad weight for females and GSI for males, possibly due to responsiveness to other internal factors of hormonal cyclicity, which are more marked in females than males, as highlighted by some authors (Borah et al., 2019; Kaefer et al., 2007)

Some authors have already emphasized that atypical periods of rain, of isolated occurrence, do not interfere in the reproductive cycle of some frog species, clarifying that, for a significant relationship between both to occur, other abiotic factors need to be adequate to the needs of the species, e.g., temperature and humidity (Leão Pompeu et al., 2020). The same was observed in the present study, since June presented a single isolated rainy event in its duration. This fact raised the average rainfall, but apparently it did not influence the GSI for males and females.

The predilection for more humid sites observed in the present study; in which all individuals were close to water bodies, in an average temperature range of ±24.8°C, more specifically where there was vegetation and with lower temperature in relation to water bodies; is supported by considerations made by several authors regarding frogs (Díaz-Ricaurte et al., 2020; JØrgensen, 1997; Koyama et al., 2001; Toledo and Jared, 1993; Tracy, 1976), since their water absorption capacity in the external environment is already well reported and they can choose sites with higher moisture content, in addition to a chemosensory capacity to avoid hyperosmotic sources and, consequently, a negative fluid balance. This fact is reinforced by some authors (DíazRicaurte et al., 2020; Navas et al., 2004; Toledo and Jared, 1993) who claim that frogs in the caatinga biome have thinner skin and twice as much vascularity as those in the Atlantic Forest, for example, developing greater absorptive capacity due to the water scarcity in the region, besides selectivity for sites with milder temperatures, in order to fully exercise their metabolic activities in osmotic comfort with the environment (Díaz-Ricaurte et al., 2020; Tracy, 1976), to the point of generating a pattern of heterogeneous spatial distribution throughout the year regarding water bodies, as already observed in this and other studies (Díaz-Ricaurte et al., 2020; Tracy, 1976).

5 CONCLUSION

Many morphological aspects, despite being intact and similar in several species of amphibians, can change in a certain period due to climate change. The morphological description proved to be a useful tool in identifying differences between the genera, hitherto not observed, as in the nuances resulting from the interaction between individuals and habitat regarding the reproductive biology of the species. However, other influential factors of these processes must be analyzed since the intrinsic ones, such as hormonal curves, may alter them. The heterogeneous spatial distribution in water bodies and the predilection for mild temperatures observed throughout the year in reproductive sites reinforce the close link between the maintenance of a positive fluid balance and thermal comfort for the reproductive activity of the species, in addition to the conservation of these habitats for their preservation.

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CAPÍTULO II:

Testicular morphological variations of the *Pseudopaludicola pocoto* Magalhães, Loebmann, Kokubum, Haddad & Garda, 2014 in a fragment of the northeastern caatinga biome during the annual reproductive cycle

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Testicular morphological variations in a frog community in a fragment of the northeastern caatinga biome during the annual reproductive cycle

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ABSTRACT

The present study aimed to describe the changes in macroscopic and microscopic morphology, resulting from the interaction of environmental factors in a community of individuals of the species *Pseudopaludicola pocoto* Magalhães, Loebmann, Kokubum, Haddad & Garda, 2014. Thirty-nine mature male individuals were collected monthly in groups of three animals, in a fragment of the caatinga biome in the municipality of Passagem, PB, Brazil, from January 2019 to January 2020. Morphometric and stereological analyzes were performed on their gonads, where morphological changes were observed in volume and frequency of the germ cell lines and in the average amount of *Sertoli* cells in significant correlations with environmental factors, such as environmental temperature and rainfall, with more voluminous cells and a greater frequency in the rainiest months and with milder temperatures. The presence of all germ lines throughout the year characterized a continuous reproductive cycle adapted to areas with certain unpredictability in the environmental phenomena, which is typical of a semi-arid region.

Keywords: Amphibians, Reproduction, Spermatogenesis, Semiarid.

1 INTRODUCTION

Little is known about the species *Pseudopaludicola pocoto*, which was identified in 2014 by Magalhães and collaborators. Its genus comprises 18 species of small frogs in South America, from northern Argentina to Venezuela and east of the Andes (Frost, 2016), and it is frequently found in open formations such as Gran Chaco, Pampas, Cerrado, and Caatinga (Medeiros Magalhães et al., 2014). Data on this species are restricted only to morphology for its taxonomic identification (Medeiros Magalhães *et al.*, 2014; Pansonato *et al.*, 2014; De Carvalho *et al.*, 2015; de Andrade *et al.*, 2017), however, studies on reproductive aspects need attention and, in males specifically, testicular morphophysiology is an important indicator of reproductive activity, with spermatogenesis being directly linked to the morphology of the gonadal parenchyma (Chanda & Biswas, 1984; Kim *et al.*, 1998; Ferreira, Rosa & Mehanna, 2009; Rosati *et al.*, 2020).

The reproduction of many vertebrate species is influenced by environmental factors, such as the hydroperiod, and amphibians are more sensitive to these changes given their interface between the aquatic and terrestrial environment. Phenomena that can reduce the hydroperiod consequently interfere with the availability of reproductive sites, food, and the fluid balance of specimens, to the point of affecting metabolic cascades of cell development (Brannelly *et al.*, 2019; Nagel *et al.*, 2021).

There is a wide variety of works that study spermatogenesis in amphibians and its correlation with environmental factors (Paniagua, Fraile & Saez, 1990; Huang, Lin & Yu, 1997; Villagra *et al.*, 2014; Zeng *et al.*, 2014; Dendrobatidae *et al.*, 2015; Rheubert *et al.*, 2017; Poo *et al.*, 2018), however, there are a few works approaching neotropical frogs from the caatinga biome, which motivated this study aimed at analyzing the testicular morphological patterns of *Pseudopaludicola pocoto* during the annual reproductive cycle, subject to the rainy and dry seasons.

2 MATERIAL AND METHODS

Study area

The study was conducted in a fragment of the caatinga biome at Fazenda ABA, (07º 05' 55.5” S 37º 01' 32.7” W) belonging to the municipality of Passagem, Paraíba, with an approximate area of 350 hectares, and 120 of them are part of a conservation area. It is in the Depressão Sertaneja Setentrional (Northern Sertaneja Depression) in the mesoregion of the sertão of Paraíba and in the microregion of the municipality of Patos, Paraíba, Brazil (IBGE & Cláudio da Silva Júnior, 2000).

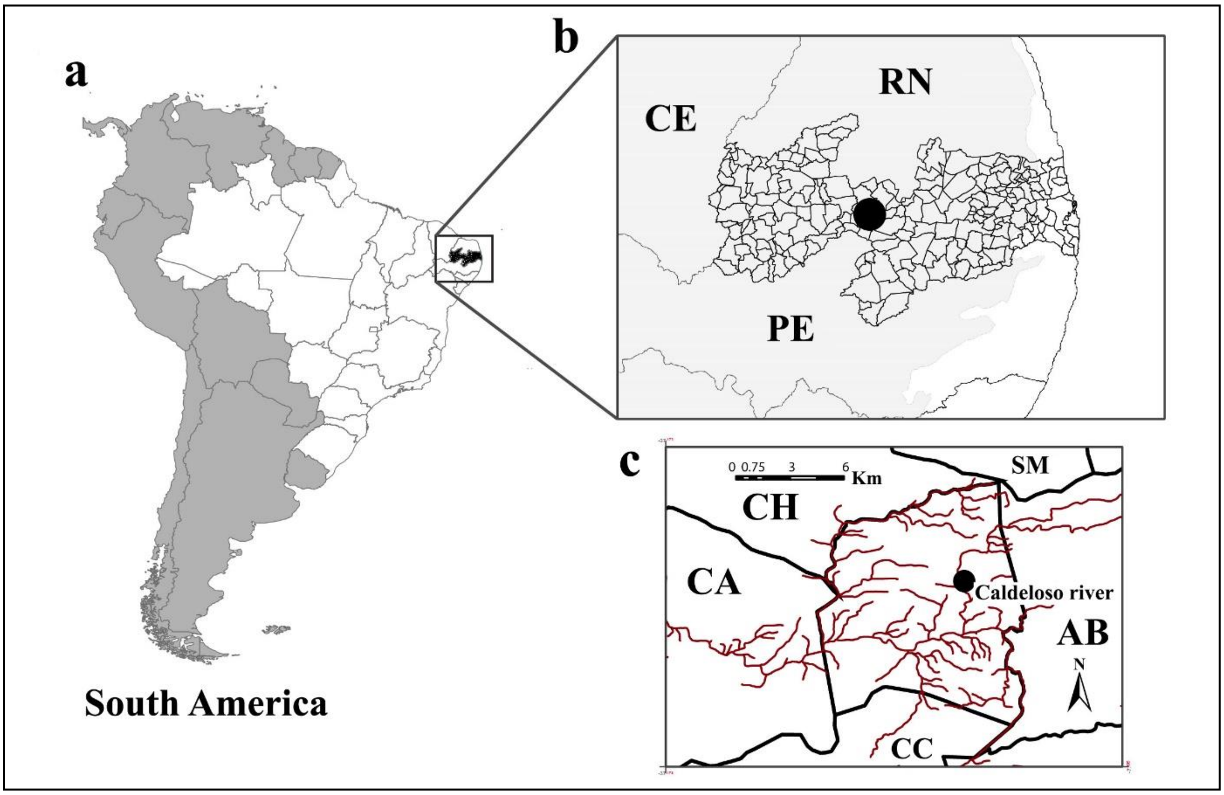


Figure 1: Collection area. (a) Map of South America (dark grey) highlighting the geopolitical division of Brazil (grey) and the State of Paraíba (black); (b) Municipality of Passagem (black circle) limited by the states of Ceará (CE), Rio Grande do Norte (RN) and Pernambuco (PE), highlighting the Caatinga (light gray) and Atlantic Forest (white) biomes. (c) Rio Caldeloso highlighted and its respective tributaries surrounded by the municipalities of Cacimba de Areia (CA), Quixaba (CH), São Mamede (SM), Cacimbas (CC) and Areia de Baraúnas (AB).

Specimen identification

The identification of specimens was performed through external morphological analysis in a Leyca® brand MZ 125 stereomicroscope, based on the description in the literature (Medeiros Magalhães et al., 2014), com base em sua coloração e características morfométricas com paquímetro digital (precisão de 0,001mm) da marca Digimess® e através de análises moleculares, com divergência genética inferior a 3% para a espécie.

Gonad capture and processing methodology

Thirty-nine specimens were collected through active manual search in water bodies. The monthly groups of 3 adult males of the species *Pseudopaludicola pocoto* were gathered from January 2019 to January 2020.

They were euthanized according to the guidelines described by the Directive of the European Parliament and the Council of the 22nd of September 2010 on protecting animals used for scientific purposes (Council, 2010) and authorized by the Chico Mendes Institute for Biodiversity (ICMBio) under protocol number 708951, with lidocaine cream applied under the skin, followed by a CO2 chamber (CFMV, 2012). After opening the coelomic cavity of the specimens, the testes were identified, analyzed, and morphologically described by stereoscopy. The gonads of the individuals were collected and fixed in a 10% buffered formaldehyde solution for 48 hours, washed in running water, and preserved in 70% ethyl alcohol for the routine histological processing under light microscopy, according to the methodology already established (Tolosa et al., 2003). We stained 5µm sections in a Hematoxylin-eosin solution and the slides were analyzed under a Leyca® microscope, model E200.

Testicular volume, locular and interlocular volumetric proportion, and melanin concentration

To establish the locular and interlocular volumetric proportions and interlocular melanin for each individual monthly, five serial isotropic sections were performed from different regions of the testes, and three of them were selected; and 20 random, systematic, and uniform fields were analyzed for each of them. Each field was analyzed in counting grids with 42 points (area per point 4000µm²) per image in the ImageJ software (area per point 4000µm²) (Mayhew & Olsen, 1991; SANDAU, 1999).

Classification and monthly frequency of germinal cysts

Germinal cysts were classified based on the stages of cell development in each cyst in the testicular parenchyma distributed in the seminiferous locules, being classified into cysts of Spermatogonia (SPG); Spermatocytes (SPC), Rounded Spermatids (EAr), Elongated Spermatids (EAl), and Spermatozoa (SPZ), through pre-established morphological criteria (Vilela et al., 2003; Leal et al., 2009b). Concomitantly, these cell types per cyst were counted for each monthly group of individuals analyzed using Cavalieri's Principle. (Mayhew & Olsen, 1991; SANDAU, 1999):

$$\mathbf{Vv} = \Sigma P\ \textbf{(C. Spermatogen)} / \Sigma P\ \textbf{(Pt)}$$

where ΣP (C. Spermatogenic) is the total number of points (of a quadratic test system with 42 points in a cross where the 4th quadrant with 100µm2 of area per cross was chosen) that touch the germinal cysts, and ΣP (Pt) is the total number of points (from the same test system) touching the entire structure of the sectional area. The value obtained was multiplied by 100 for its expression in percentage.

Germ cell and Sertoli cell volume

For each cell line, 30 cells of each lineage were measured randomly per field analyzed, the cell volume was obtained in μm^3 using the cell diameters, by the following formula (Mayhew & Olsen, 1991; SANDAU, 1999):

$$\textbf{Cell volume (spherical)} = 4/3\pi R^3$$

where R= Diameter/2. For cylindrical cells, the following formula was used:

$$\textbf{Cell volume (cylindrical)} = [(\pi R)^2]H,$$

where R=Diameter/2 and H correspond to height.

Environmental data collection and habitat characterization

Environmental data such as light intensity, relative air humidity, dew point, and environmental temperature were recorded with a HOBO® brand data logger device, model U12 Temp/RH 2EX, coupled to an aluminum Black Globe positioned equidistantly from all water bodies analyzed. The temperature of the soil and water of the water bodies was obtained through a thermographic camera of the FLUKE® brand, model ti-400 9Hz Thermal Imager, and an infrared thermometer of the Benetech® brand, model GM-320.

For the collection of data regarding rainfall and evapotranspiration, the meteorological database for the year 2019 of the Real Time Climate Monitoring Program of the Northeast Region (Proclima, 2012) was used. To obtain a panoramic view and schematize the positioning of the water bodies of the studied site, aerial photos were taken using a DJI Phantom 4® drone device, model CP.PT.234.01.

Statistical analysis

The values obtained in the present study were expressed as means and standard deviation, and submitted to normality and homoscedasticity tests.

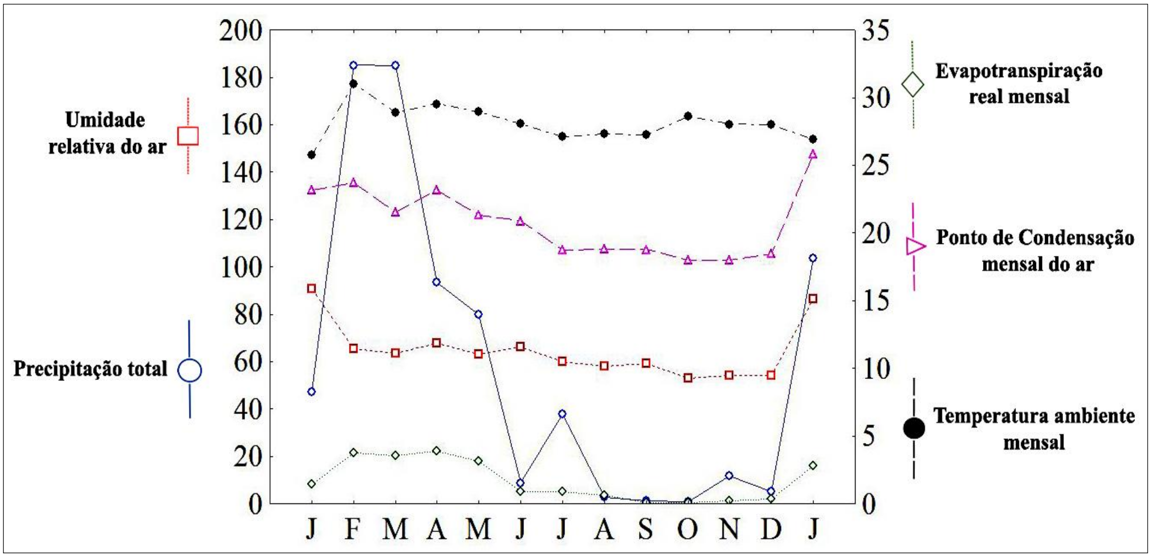
To avoid multicollinearity between the predictor variables, the Spearman Correlation analysis was performed and then the less collinear predictor variables were selected.

The relationship between morphometric and environmental variables was obtained from GLM (Generalized Linear Models) with Poisson distribution, for data with high overdispersion, and a log link function, through the “MASS” package in the R *software* (Tables 2 and 3) (Ripley *et al.*, n.d.; Sugio, Miyaguni & Tayasu, 2018). The presence of a temporal autocorrelation was tested from the residuals of the analysis with the Breusch-Godfrey test, using the “lmtest” package in R (Hothorn *et al.*, 2012) (See annex: tables 2a, 3a, 4a).

3 RESULTS

Throughout the collection period, it was possible to observe variations in the environmental factors of the analyzed fragment. Among them, monthly rainfall was the one that apparently presented the greatest contrast over the 13 months analyzed (Graph 1).

Graph 1 - Precipitation and relative humidity in the municipality of Passagem, Paraíba, Brazil, January 2019 to January 2020.



After the multicollinearity test between the environmental variables, the less collinear ones were selected, these being the monthly rainfall and the environmental temperature (See Annex: table 1a).

Although the locular structural arrangement of the testicular parenchyma was maintained, both testicular parenchyma presented larger and more rounded seminiferous locules in the wettest months and with lower temperatures, as well as the space between them (interlocular) was thicker, with more rounded peritubular cells. In contrast, in the drier months, both interlocular spaces and seminiferous locules significantly reduced in size, resulting in more ellipsoidal locules and flatter peritubular cells in the interlocular spaces. ($p<0,01$) (Table 2).

In all analyzed testicles, peripheral melanin layers were observed in the interlocular spaces. Interestingly, as the drier months approached, the interlocular melanin layers became significantly thicker and voluminous (Figure 2), in an inversely proportional relationship with rainfall and environmental temperature. ($p<0,01$) (Table 1).

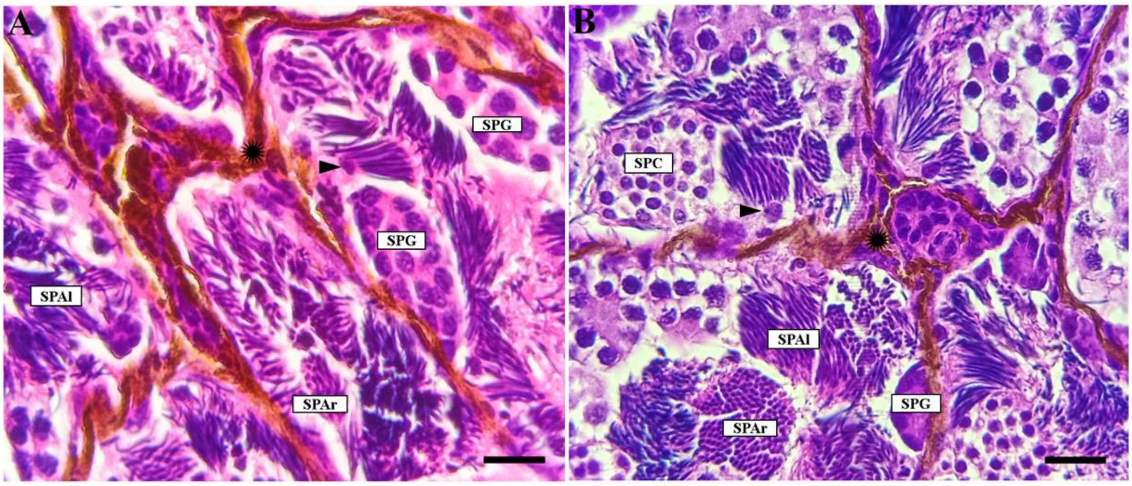


Figure 2 – Seminiferous locules of *Pseudopaludicola pocoto* surrounded by dense layers of melanin. Dry period of the year (A) and rainy period (B). Spermatogonia (SPG); Spermatocyte (SPC); Rounded spermatocyte (SPAr); Elongated spermatocyte (SPAl); (*) Interlocular melanin; (Arrow head) Sertoli cell. (Scale bar = 10µm).

Germ cysts of all developmental lineages were observed in all months of the analysis, however, some morphological changes were also observed, where the months with lower rainfall presented more condensed cysts with smaller cells, in relation to the rainiest ones (Table 1). It is worth noting that such changes were present in the cysts of more basal developmental lineages such as spermatogonia and spermatocytes, differing significantly in their respective volumes, which were higher in the wettest periods. Interestingly, the environmental temperature did not influence these cells significantly, as shown in Table 2.

Although all types of germinal cysts were observed throughout the year, their monthly frequency varied in relation to the months in terms of rainfall and temperature, with emphasis on cysts of lineages at the extremes of the development chain, the most basal ones (Spermatogonial cysts) and the most differentiated ones (sperm cysts). Spermatogonia cysts were present in smaller numbers in months with greater rainfall and mild temperatures, on the other hand, Sperm cysts were more numerous in this period (Table 1). Regarding the other ones, of the other developmental lineages, only rainfall had a directly proportional influence, that is, they became more frequent in the wettest months, except for Spermatocyte cysts, a more intermediate lineage of development (Table 2).

Regarding the cell volumes of the germinal cysts, it was possible to observe that only the cells of more basal developmental lineages showed significant variations over the observed months, where Spermatogonia and Spermatocytes were larger, with more apparent cytoplasm in the wettest months. Room temperature had no significant influence on their respective volumes. The other lineage showed no significant correlation with the environmental variables (Tables 1 and 2).

Table 1 - Means and standard deviation of testicular components in mature *Pseudopaludicola pocoto* in the rainy season and in the dry season in a fragment of northeastern caatinga, Brazil 2019/2020. (Analysis of Generalized Models – GLM).

	Rainy season	Dry season
V. Locular (%)	71,23±6,34	62,41±5,03
V. Interlocular (%)	28,77±6,34	37,59±5,03
Melanine Interloc.(%)	52,47±19,55	70,80±5,01
F.SPG(%)	13,39±10,09	41,79±6,75
F.SPC(%)	19,08±4,50	21,39±2,54
F.SPAr(%)	21,17±2,66	14,74±3,69
F.SPAl(%)	20,33±3,49	14,29±2,15
F.SPZ(%)	27,50±12,34	8,82±4,83
VSPG(µ)	1,56±0,47	1,26±0,19
VSPC(µ)	2,57±0,57	2,73±0,29
VSpAr(µ)	0,45±0,09	0,41±0,10
VSPAl(µ)	0,15±0,08	0,16±0,01
VSPZ(µ)	0,16±0,03	0,17±0,01

Frequency of Spermatogonia (FSPG); Frequency of Spermatocytes (FSPC); Frequency of rounded spermatids (FSPAr); Frequency of elongated spermatids (FSPAl); Frequency of Sperm (FSPZ); Spermatogonial volume (VSPG); Spermatocyte Volume (VSPC); Rounded Spermatid Volume (VSPAr) Elongated Spermatid Volume (VSPAl); Sperm Volume (VSPZ)Rainy Period (January to May 2019 and January 2020) and Dry Period (June to December 2019).

Table 2 – P values for correlation of structural morphological components of adult *Pseudopaludicola pocoto* testes with precipitation and ambient temperature in a fragment of northeastern caatinga, Brazil 2019/2020. (Analysis of Generalized Models – GLM).

	Precipitation	Ambient temperature
V. Locular	p<0,01*	s/a
V. Interlocular	p<0,01**	p<0,01*
Melnanine Interloc.	p<0,01**	p<0,01*
FSPG	p<0,01**	p<0,05**
FSPC	s/a	s/a
FSPAr	p<0,05*	s/a
FSPAl	p<0,05*	s/a
FSPZ	p<0,01*	p<0,01**
VSPG	p<0,01*	s/a
VSPC	p<0,01*	s/a
VSPAr	s/a	s/a
VSPAl	s/a	s/a
VSPZ	s/a	s/a

Frequency of Spermatogonia (FSPG); Frequency of Spermatocytes (FSPC); Frequency of rounded spermatids (FSPAr); Frequency of elongated spermatids (FSPAl); Frequency of Sperm (FSPZ); Spermatogonial volume (VSPG); Spermatocyte Volume (VSPC); Rounded Spermatid Volume (VSPAr) Elongated Spermatid Volume (VSPAl);

Sperm Volume (VSPZ); (*) values with positive correlation coefficient. (**) values with negative correlation coefficient. (y/n) without significant correlation.

Sertoli cells were arranged on the periphery of the germinal cysts during all months of collection, regardless of environmental changes. It was possible to observe and quantify them in cysts of Spermatogonia, Spermatocytes, and rounded Spermatids. Since the elongated Spermatids overlapped, arranged in bundles of clusters, it was not possible to observe them clearly.

The number of *Sertoli* cells per cyst of Spermatocytes and rounded Spermatids showed a significant correlation with rainfall, except for Spermatogonial cysts, in which the amount of *Sertoli* cells increased significantly and proportionally to the rainy months in the cysts of lineages subsequent to Spermatogonia. The temperature did not have a significant influence on this phenomenon (Table 3).

Table 3 - P-values for the correlation between the number of Sertoli cells by types of germinal cysts of *Pseudopaludicola pocoto* testes adults with Precipitation and ambient temperature in a fragment of northeastern caatinga, Brazil 2019/2020. (Analysis of Generalized Models – GLM).

	SRT/SPG	SRT/SPC	SRT/SAr
Precipitation	s/a	p<0,01*	p<0,01*
Ambient Temp.	s/a	s/a	s/a

Number of Sertoli cells per Spermatogonial cyst (SRT/SPG); number of Sertoli cells per Spermatocyte cyst (SRT/SPC); number of Sertoli cells per rounded spermatid cyst (SRT/SAr); (*) values with positive correlation coefficient. (**) values with negative correlation coefficient. (y/n) without significant correlation.

The number of Sertoli cells changed in relation to the number of cells within the cysts, where the cysts with the highest number of germ cells had a greater number of Sertoli cells, in relation to their respective conformations, in the serial cuts, it was not possible to observe changes significant morphology. Differently from the gonadosomatic index, the weight of the gonads showed a significant correlation with the number of

Sertoli cells, where the gonads with the highest amount of Sertoli cells had higher weight values (Table 4).

Table 4 – Spearman correlation between the number of Sertoli cells by the number of germ cells of the cysts, the gonadosomatic index and total weight of adult *Pseudopaludicola pocoto* gonads in a fragment of northeastern caatinga, Brazil 2019/2020 (Analysis of Generalized Models – GLM).

	STR/SPG	STR/SPC	STR/SPAr	SRT/IGS	SRT/PG
P value	p≤0,01*	p≤0,01*	p≤0,01*	s/a	p<0,05*

Number of Sertoli cells per Spermatogonial cyst (SRT/SPG); number of Sertoli cells per Spermatocyte cyst (SRT/SPC); number of Sertoli cells per rounded spermatid cyst (SRT/SAr); (*) values with positive correlation coefficient. (**) values with negative correlation coefficient. (y/n) without significant correlation.

4 DISCUSSION

Using Histology allied with Stereology it is possible to quantify data about spermatogenesis, and thus analyze possible changes in morphophysiological processes for different vertebrates in a more concrete way (Vilela *et al.*, 2003; Leal *et al.*, 2009a; Gomes *et al.*, 2012). Regarding *Pseudopaludicola pocoto*, it was possible to observe morphological changes in the qualitative aspect, where structures of the testicular parenchyma underwent modifications, allowing a proportional analysis of 342 components throughout the collection period.

The conformation of the locules, observed in the *Pseudopaludicola pocoto*, remained within the structural patterns observed in all anamniote vertebrates, with seminiferous locules ranging from ellipsoidal to a rounded shape, presenting cysts with cells in spermatogenic development stages, from more basal lineages, such as Spermatogonia, to those in the final stages of maturation, such as sperm (Santos & Oliveira, 2007; Rastogi *et al.*, 2011; Díaz-Ricaurte *et al.*, 2020).

The changes observed in terms of locular and interlocular volume in specimens of *Pseudopaludicola pocoto* are based on other studies with amphibians, where the increase in volume and change in the shape of these locules result from the increase in the number of cells in the germinal cysts, product of successive meiotic divisions in developing lineages (Lofts, 1964, 1978; Costa *et al.*, 1998; Villagra *et al.*, 2014; Chaves *et al.*, 2017), may be associated with periods of high rainfall, where there is a significant increase in locular volume in some amphibian species, given the high water availability in the *habitat* (VAN OORDTP & LOFTS, 1963; Lofts, 1964; Villagra *et al.*, 2014; Chaves *et al.*, 2017).

Regarding the changes in the volume of interlocular melanin observed in specimens of *Pseudopaludicola pocoto*, there are no available studies that have evaluated or justified them, despite their presence in the interlocular spaces in gonads of other amphibian species (Oliveira & Zieri, 2005; Goldberg, Valverde & Franco-Belussi, 2020). However, the inversely proportional correlation to periods with low rainfall rates and the associated hydrophobic capacity of melanin may suggest an adaptive mechanism for reducing the dehydration suffered by these individuals in periods of drought, when there is a shortage of water.

It was observed in all the analyzed gonads that, as the cells matured to more advanced stages of development, there was a reduction in their respective nuclei and cytoplasm, to assume a cylindrical shape close to that of mature sperm and ready for external fertilization, as observed for some species of frogs, not only free-living ones but also those in captivity, configuring a process inherent to all of them (Vilela *et al.*, 2003; Amaral, 2009; Leal *et al.*, 2009a; Chaves *et al.*, 2017). Although such cellular maturation processes follow a standard conformation of volume reduction until the most suitable stages for external fertilization, morphological studies allied to Stereology allow us to observe nuances between these maturation processes and associate them with several external factors (Wingfield, 1984; Díaz-Páez & Ortiz, 2001; Gribbins *et al.*, 2009; Assis, 2011; Leite *et al.*, 2015; Kesselring *et al.*, 2019).

In *Pseudopaludicola pocoto* it was possible to observe all types of spermatogenic lineage development, from the driest to the wettest period, which, according to the literature, configures a species that has a continuous reproductive cycle (Wassersug, Feder & Burggren, 1993; Huang *et al.*, 1997). However, despite all developmental lineages being present, it is possible to observe significant variations in the frequency of cysts of some of these lineages that may indicate periods with lower reproductive activity (FERREIRA *et al.*, 2008; Villagra *et al.*, 2014; Chaves *et al.*, 2017). These periods, which coincide with low rainfall and high temperatures, exhibit a certain influence of these environmental factors on the reproductive biology of the species, corroborating what has already been described for amphibians (Lofts, 1964; Faria De Oliveira *et al.*, 2007; Kaefer, Boelter & Cechin, 2007; Gribbins *et al.*, 2009). Such effects coincide with the changes observed in the morphology of the *Pseudopaludicola pocoto* germlines, where the cells of more basal lineages, such as Spermatogonia and Spermatocytes, presented smaller volumes in the hottest and less rainy months of the year and larger ones in those with the greatest rainfall and milder temperatures, in a directly proportional correlation.

Regarding the frequency of cysts for each cell developmental lineage observed for *Pseudopaludicola pocoto*, where the frequencies varied significantly in a directly proportional correlation, in which the rainiest months presented a higher frequency of cysts, except for spermatogonial ones that presented a lower frequency in the rainiest months. Interestingly, only the most extreme lineage frequencies in terms of cell development, such as Spermatogonia (basal) and Spermatozoa (differentiated), showed a significant correlation with temperature. According to some authors, very high temperatures can deleteriously influence the pituitary gonadrotopins and, consequently, the gonadal activities regulated by them, causing degranulation of pituitary gonadotropes and the development of several germ cell lines (Rastogi *et al.*, 1978; Minucci *et al.*, 1995).

The "low" influence exerted by the temperature in the frequency of germinative cysts of *Pseudopaludicola pocoto* may be justified because the rains in areas of the caatinga biome are more crucial for stimulating spermatogenesis in frogs from semi-arid environments when compared to temperature, which is also influenced by the rains. This was already verified in specimens of the Bufonidae and Leptodactilidae family in fragments of this biome (Madelaire & Gomes, 2016; Chaves *et al.*, 2017). In contrast, the influence of environmental temperature seems to be greater in species from temperate regions (Lofts, 1964; Borah, Renthlei & Trivedi, 2019; Di Fiore *et al.*, 2020)

The increase in the number of *Sertoli* cells proportional to the number of germ cells observed in *Pseudopaludicola pocoto* is justified by the fact that, as the number of germ cells increases in the cyst, the demand for nutrients and growth factors supplied by *Sertoli* cells increases proportionally; to meet this demand, they replicate proportionally to the number of germ cells in the cyst (Billard & Escaffre, 1969; Vilela *et al.*, 2003; Leal *et al.*, 2009b; MK Skinner, 2015). This phenomenon significantly interferes with testicular size and sperm production (Petersen & Söder, 2006; Uribe & Mejía-Roa, 2014; Chen *et al.*, 2020), as observed in *Pseudopaludicola pocoto,* where testicular weight presented a significant correlation with the number of *Sertoli* cells. The same was not observed for the gonadosomatic index, possibly because this factor is associated with other variables such as the weight of individuals.

Possibly, the correlation observed between Sertoli cells and monthly rainfall in the present study is related to the number of cells per cyst involved by them, which varied significantly in relation to changes in monthly rainfall, given its importance in mediating hormonal cascades in the development of cells within the cysts (Pudney, 1995; Díaz-Ricaurte *et al.*, 2020; Oielska, 2020).

It is worth noting that, although the collected males present mature sperm throughout the year, it does not suggest that the species is in fact in its reproductive period, since the maturation stage of females is fundamental for its occurrence (Díaz-Páez & Ortiz, 2001; Faria De Oliveira *et al.*, 2007).

5 CONCLUSION

The presence of sperm lines throughout all months for the species *Pseudopaludicola pocoto* characterizes its cycle as a continuous reproductive one. However, environmental factors had a significant influence on the spermatogenesis of the species, with modifications present in all components of the testicular parenchyma, better morphological indices suitable for reproduction in the period from January to May, and a higher frequency of cysts from mature lineages, *Sertoli* cells, and availability of environmental resources such as intense rainfall and milder temperatures.

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CAPÍTULO III:

Ovarian morphological variations of *Pseudopaludicola pocoto* Magalhães, Loebmann, Kokubum, Haddad & Garda, 2014 in a fragment of the caatinga bioma in the Northeast region of Brazil, in a one-year cycle

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Ovarian morphological variations of *Pseudopaludicola pocoto* Magalhães, Loebmann, Kokubum, Haddad & Garda, 2014 in a fragment of the caatinga bioma in the Northeast region of Brazil, in a one-year cycle.

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HIGHLIGHTS

- Oocytes were more voluminous in the months with more rainfall and milder temperatures;
- The frequency of oocytes was higher in the wettest months for those in more differentiated stages;
- Oogenesis occurred continuously throughout the thirteen months of collection.

1 ABSTRACT

The present study aimed to describe the changes in macroscopic and microscopic morphology, resulting from the interaction of environmental factors in a community of individuals of the species *Pseudopaludicola pocoto* Magalhães, Loebmann, Kokubum, Haddad & Garda, 2014. Thirty-nine mature females were collected in groups of three monthly, in a fragment of the caatinga biome in the municipality of Passagem, PB, Brazil throughout the year 2019. Histomorphometric and stereological analyzes were conducted in the gonads of the collected individuals, where significant morphological changes were observed in oocyte volume and its frequency at different stages of development, with more robust ones and a higher frequency of oocytes of more differentiated lineages in months with greater rainfall and mild temperatures, observing a cyclicity closely dependent on environmental factors such as rainfall and environmental temperature, despite showing all lineages of oocyte development throughout the months collected, characterizing a behavior adapted to the unpredictability of rainfall in semiarid regions.

Keywords: Amphibians, Reproduction, Oocytes, Semiarid.

2 INTRODUCTION

Oogenesis comprises a period preceding reproduction, therefore, crucial for the perpetuation of several species. It is characterized by meiotic divisions until the formation of a gamete mature enough for external fertilization, in the case of amphibians (Brachet, 1979). During this process of cell development and maturation, several morphological changes are observed macro and microscopically, these, in turn, can serve as markers for the identification and characterization of stages of oocyte development and maturation (Brachet, 1979; Jørgensen, 1981; Oliveira & Santos, 2004; Pancharatna & Saidapur, 1985).

Many factors can interfere in the development and consequently in the reproduction of amphibian species from temperate regions and tropical zones, such as rainfall, temperature, photoperiod, food availability, and breeding sites (Chaves et al., 2017; De Sá, Zina, & Haddad, 2014; Madelaire, Sokolova, & Gomes, 2017a). However, it is worth noting that several intrinsic factors, such as hormonal cyclicity and the presence of fat bodies, are crucial for gamete development (Petersen & Söder, 2006; Polzonetti-Magni et al., 1998).

Regarding Neotropical amphibian communities, studies regarding gametogenesis are scarce compared to those on species from temperate regions. When such a scenario is narrowed to the caatinga biome, this number significantly reduces. The present study aimed to describe oogenesis based on the macro and microscopic morphological changes in a community of amphibians of the *Pseudopaludicola pocoto* Magalhães, Loebmann, Kokubum, Haddad & Garda, 2014 species, resulting from their interaction with environmental factors in a fragment of the caatinga biome, based on morphological and stereological techniques to quantify such modifications with environmental changes over 13 months.

3 MATERIAL AND METHODS

The present study was previously evaluated and authorized by the Biodiversity Information System (SisBio) under protocol number 63207-1, and by the Animal Use Ethics Committee of the Federal University of Campina Grande (CEUA/UFCG) under protocol number 072-2018.

Study site and animals

Three female specimens of *Pseudopaludicola pocoto* were collected per month, through visual and active manual searches in a fragment of the caatinga biome at Fazenda “Aba” on the Caldeloso River (7° 6'6.78"S and 37° 1'29.76"W) (Figure 1), in the municipality of Passagem, in the state of Paraíba, Brazil (IBGE, 2016).

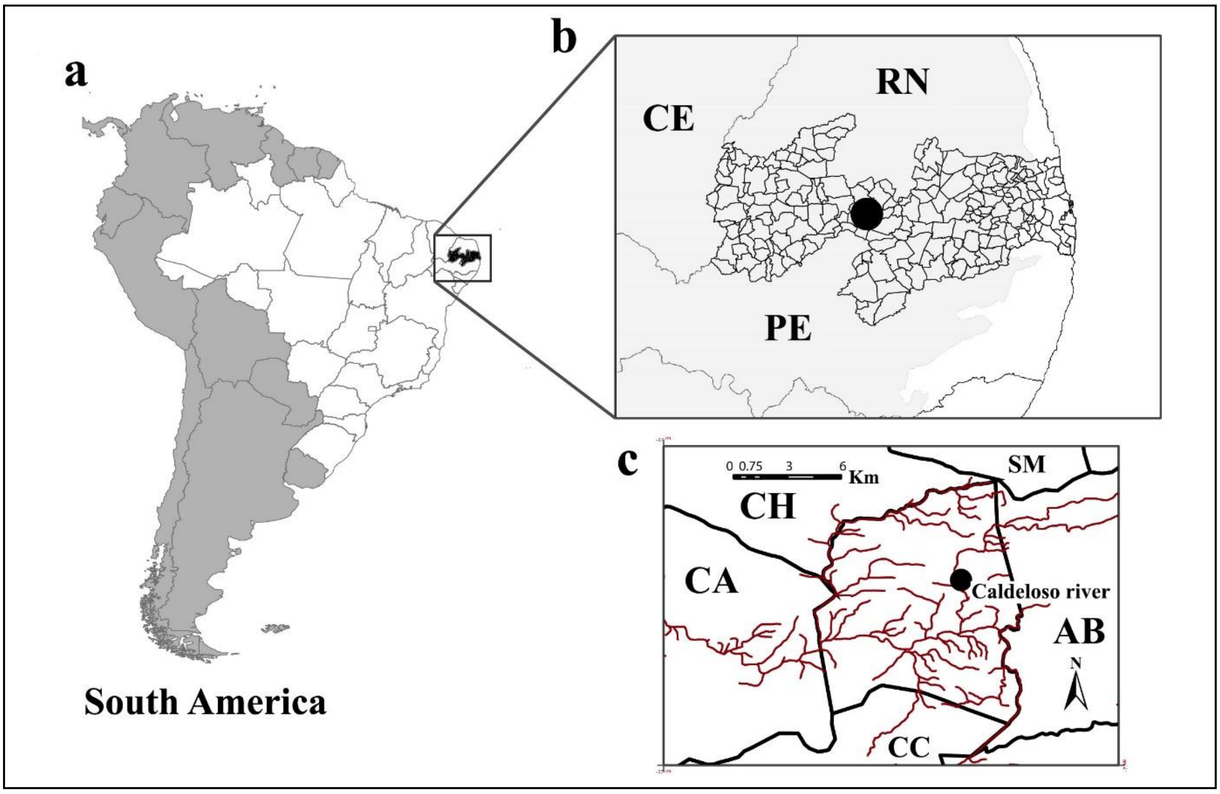

Figure 1: Collection area. (a) Map of South America (dark grey) highlighting the geopolitical division of Brazil (grey) and the State of Paraíba (black); (b) Municipality of Passagem (black circle) limited by the states of Ceará (CE), Rio Grande do Norte (RN)

and Pernambuco (PE), highlighting the Caatinga (light gray) and Atlantic Forest (white) biomes. (c) Rio Caldeloso highlighted and its respective tributaries surrounded by the municipalities of Cacimba de Areia (CA), Quixaba (CH), São Mamede (SM), Cacimbas (CC) and Areia de Baraúnas (AB).

Specimen identification

Through external morphological analysis with the aid of a Leyca® brand MZ 125 stereomicroscope, based on the description in the literature (Medeiros Magalhães, Loebmann, Kokubum, Haddad, & Garda, 2014), individuals of the species were identified by color. Their morphometric characteristics were obtained with a digital caliper (accuracy of 0.001mm) of the Digimess® brand and, through molecular analyses, genetic divergence of less than 3% was observed in relation to the specimens already identified in the literature.

Gonad capture and processing methodology

The specimens, after being collected in groups of three individuals, monthly, by active visual search, were euthanized according to the guidelines described by the Directive of the European Parliament and of the Council of 22 September 2010 on protecting animals used for scientific purposes (Council, 2010), authorized by the Instituto Chico Mendes de Biodiversidade (ICMBio) under protocol number 70895-1. After the analysis and stereoscopic morphological description, the gonads of the individuals were collected and fixed in a 10% buffered formaldehyde solution for 48 hours and preserved in 70% ethyl alcohol for routine histological processing for light microscopy, according to the methodology in the literature (Tolosa, Rodrigues, Behmer, & Freitas-Neto, 2003). Sections of 5µm were stained in a Hematoxylin-eosin solution and the slides were analyzed under a Leyca® microscope, model E200.

Interstitial and oocyte volumetric proportion

To establish the interstitial and oocyte volumetric proportion for each individual monthly, 20 random fields were photographed at 40x magnification on a Leica® brand microscope, model DM2500. Each field photographed was analyzed in the ImageJ software, with a reticle with 88 intersections (crosses), (area per point 10.000µm²) (Mayhew & Olsen, 1991; SANDAU, 1999).

Classification and monthly frequency of oocytes

Oocytes were classified from the developmental stages into four developmental stages according to the literature (Costa, Lima, Andrade, & Agostinho, 1998; Pancharatna & Saidapur, 1985; Rastogi et al., 1983; Rodríguez-Gómez, Sanz-Ochotorena, Segura-Valdés, Lara-Martínez, & Jiménez-García, 2010). Then, their types were counted for each group analyzed monthly, and the following formula was used, following Cavalieri's Principle (Mayhew & Olsen, 1991; SANDAU, 1999):

$$\mathbf{Vv} = \Sigma P(\mathbf{Oocyte}) \,/\, \Sigma P\ (\mathbf{Pt})$$

where $\Sigma P(\text{Oocyte})$ is the total number of points (from a quadratic test system) touching oocytes of the grade to be analyzed and ΣP (Pt) is the total number of points (from the same test system) touching the entire structure of the sectional area. The value obtained was multiplied by 100 for expressing it in percentage.

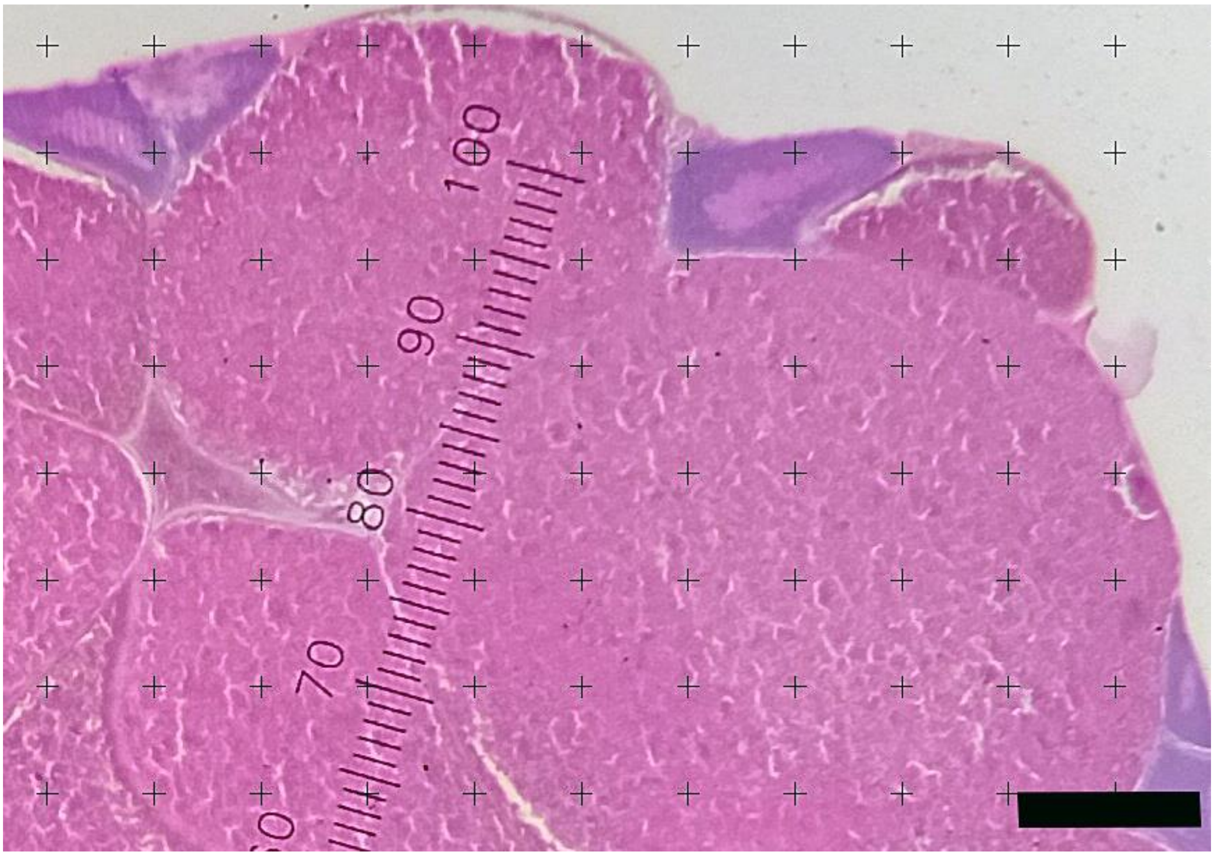

Figure 2 – Quadratic test system with 88 crosses on *Pseudopaludicola pocoto* Oocytes (10,000πm2 area per point). Scale bar 170µm.

Oocyte volume

For each cell line, 30 oocytes were measured randomly per field analyzed, the cell volume was obtained in μm^3 using the cell diameters, by the following formula, according to the literature (SANDAU, 1999):

$$\textbf{Cell volume (spherical)}= 4/3\pi R^3$$

where R= Diameter/2.

Environmental data collection and *habitat* characterization

Environmental data such as light intensity, relative air humidity, dew point, and environmental temperature were recorded with a HOBO® brand data logger device, model U12 Temp/RH 2EX, coupled to an aluminum black globe positioned equidistantly from all the water bodies analyzed. The temperature of the soil and water of the water bodies was obtained through a thermographic camera of the FLUKE® brand, model ti-400 9Hz Thermal Imager, and an infrared thermometer of the Benetech® brand, model GM-320.

For the collection of data regarding rainfall and evapotranspiration, the meteorological database for the year 2019 of the Real Time Climate Monitoring Program of the Northeast Region (Proclima, 2012) was used. In order to obtain a panoramic view and schematize the positioning of the water bodies of the study area, aerial photos were taken using a DJI Phantom 4® drone device, model CP.PT.234.01.

Statistical analysis

The values obtained in the present study were expressed as means and standard deviation and submitted to normality and homoscedasticity tests.

To avoid multicollinearity between the predictor variables, the Spearman Correlation analysis was performed and then the less collinear predictor variables were selected.

The relationship between morphometric variables and environmental variables was obtained from a GLM (Generalized Linear Models) with Poisson distribution, for data with high overdispersion, and a log linkage function, through the "MASS" package in the R software (Annex II) (RIPLEY et al.,; SUGIO; MIYAGUNI; TAYASU, 2018). The presence of the temporal autocorrelation was tested from the residuals of the analysis with the Breusch-Godfrey test, using the “lmtest” package in R (Hothorn, Zeileis, …, & 2015, 2012) (Appendix II).

4 RESULTS

All oocytes were apparently juxtaposed, shaped similarly to “bunches of grapes”, in a large chain that filled a great part of the coelomic cavity of the individuals collected in the wettest periods and, to a lesser extent, in the driest ones.

As the oocytes reached more advanced stages of maturation, those with translucent staining in more basal phases increased the opacity of their stains with the beginning of a characteristic polarization, forming an animal pole and another vegetal one in the more differentiated phases. Such morphological changes were very clear in all months of the year for *Pseudopaludicola pocoto*.

It was possible to stereoscopically observe four stages of oocyte development in all months of the collection period. Smaller translucent-looking ones measured 0.122mm^3, on average (Grade I); those with an opaquer color in relation to these, measured 0.271mm^3, on average (Grade II); opaque staining oocytes at the beginning of polarization measured 0.312mm^3, on average (Grade III); and oocytes with a clear polarization measured 0.651mm^3, on average (Grade IV) (Figure 3).

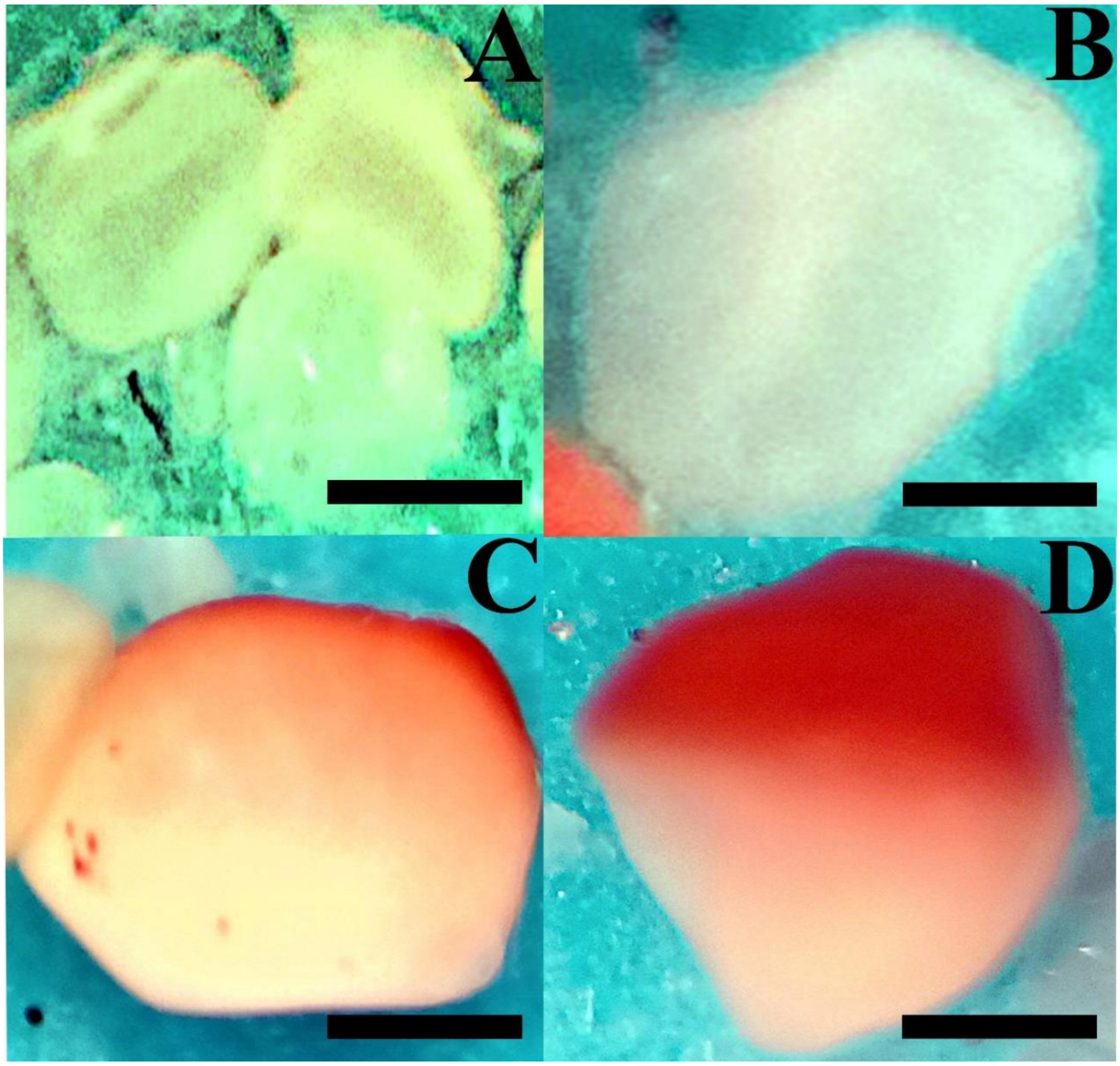

Figure 3 – Stereoscopy of *Pseudopaludicola pocoto* oocytes in different stages of development (Grade I – A; Grade II – B; Grade III – C; Grade IV – D). (0.5mm scale bar; 20X magnification).

Under microscopy, grade I oocytes presented a basophilic cytoplasm and an acidophilic nucleus grouped with grade II oocytes, the latter with nucleoli located on the periphery of the nucleus and of greater size. Grade III oocytes were highlighted by the yolk inclusions in their cortical portion. Grade IV oocytes were easily identified by being larger than those from the other grades observed, with a well-delimited nucleus and yolk inclusions arranged in the cortical portion in greater proportion to the nucleus (Figure 4).

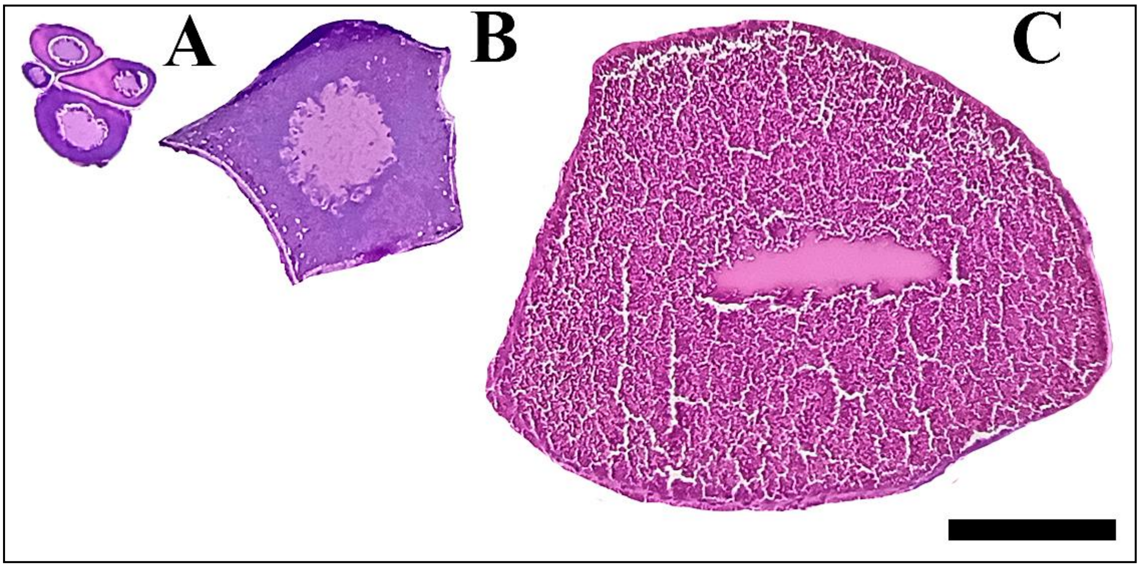

Figure 4 – Micrograph of *Pseudopaludicola pocoto* oocytes in different stages of development (Grades I and II – A; Grade III – B; Grade IV – C). (100µm scale bar; 40X magnification).

After the multicollinearity test between the environmental variables, the less collinear variables were selected, and the monthly rainfall and environmental temperature had the lowest coefficient of collinearity (Annex II).

Although all degrees of oocyte development were observed in *Pseudopaludicola pocoto* over the analyzed months, some variations occurred in this frequency at certain stages of development. In the wettest months and with milder temperatures, they presented higher frequencies of oocytes in more advanced stages of development (grades III and IV) in relation to those in more basal stages (grades I and II) (Table 1).

In summary, rainfall proved to be a common influencing factor for the frequency of all stages of oocyte development, however, temperature had a significant influence only in the most advanced stages of development (Table 2).

The months with more rainfall and milder temperatures had the highest oocyte volumes, with more rounded and larger shapes in all stages, except for grade I oocytes (Table 1). High temperatures associated with water scarcity promoted a reduction in oocyte volume, which was more susceptible to environmental changes in relation to their frequency (Table 2).

Table 1 – Means and standard deviation of Ovarian Components of *Pseudopaludicola pocoto* in a Northeastern Caatinga fragment, Brazil.

	Rain season	Dry season
OCTI %	2,75±3,21	15,17±5,82
OCTII%	5,75±3,91	14,22±5,51
OCTIII%	10,01±3,58	7,25±3,91
OCTIV%	71,29±17,45	50,31±12,15
VOCTI (µ)	6,31±15,02	6,91±17,01
VOCTII (µ)	39,07±15,74	31,65±72,01
VOCTIII (µ)	67,15±29,31	64,22±48,84
VOCTIV (µ)	88,34±22,70	81,37±53,63

(OCTI%) Percentage of stage I oocytes; (OCTII%) Percentage of stage II oocytes; (OCTIII%) Percentage of stage III oocytes; (OCTIV%) Percentage of stage IV oocytes; (VOCTI) Stage I oocyte volume; (VOCTII) Stage II oocyte volume; (VOCTIII) Stage III oocyte volume; (VOCTIV) Volume of stage IV oocytes. Rainy Season (January to May 2019 and January 2020) and Dry Season (June to December 2019).

Table 2 – P values for correlation of the frequency and volume of adult *Pseudopaludicola pocoto* oocytes with Precipitation and ambient temperature in a fragment of northeastern caatinga, Brazil 2019/2020 (Analysis of Generalized Models – GLM).

	Precipitation	Ambient temp.
%OCTI	p<0,001**	s/a
%OCTII	p<0,01**	s/a
%OCTIII	p<0,05*	p<0,01**
%OCTIV	p<0,01*	p<0,05**
VOCTI	s/a	s/a
VOCTII	p<0,01*	p<0,001**
VOCTIII	p<0,001*	p<0,001**
VOCTIV	p<0,001*	p<0,001**

(OCTI%) Percentage of stage I oocytes; (OCTII%) Percentage of stage II oocytes; (OCTIII%) Percentage of stage III oocytes; (OCTIV%) Percentage of stage IV oocytes; (VOCTI) Stage I oocyte volume; (VOCTII) Stage II oocyte volume; (VOCTIII) Stage III oocyte volume; (VOCTIV) Volume of stage IV oocytes. (*) values with positive correlation coefficient. (**) values with negative correlation coefficient. (y/n) without significant correlation.

Such morphological changes observed in the ovarian parenchyma showed a significant relationship with weight, in which only the volume of grade IV oocytes showed a directly proportional correlation, as well as for the gonadosomatic index (Table 3).

Table 3 – P-values for Spearman's correlation between oocyte volumes at various stages of development of *Pseudopaludicola pocoto* with weight and gonadosomatic index in a fragment of northeastern caatinga, Brazil 2019/2020.

	IGS	Peso ovariano
VOCTI	s/a	s/a
VOCTII	s/a	s/a
VOCTIII	s/a	s/a
VOCTIV	P<0,01*	p<0,05*

(VOCTI) Stage I oocyte volume; (VOCTII) Stage II oocyte volume; (VOCTIII) Stage III oocyte volume; (VOCTIV) Volume of stage IV oocytes. (*) values with positive correlation coefficient. (**) values with negative correlation coefficient. (y/n) without significant correlation.

5 DISCUSSION

The conformation in oocytes surrounded by follicular cells at various stages observed in the present study is an inherent characteristic of all anamniote individuals and, regardless of the developmental stage, the morphological conformation is similar in several groups of amphibians (Dumont, 1972; McMillan, 2007; Rodríguez-Gómez et al., 2010). Regarding the morphology of the stages of oocyte development, we found a variety in the classification and determination of these stages, divided between four to six stages of macro and micro development (Dumont, 1972; Newman, Aguero, & King, 2018; Oliveira & Santos, 2004; Rodríguez-Gómez et al., 2010), all based on the same oocyte maturation parameters, such as size, color, presence of nucleoli, nucleus shape and differentiation of vegetal and animal poles. These parameters were well defined for each of the four stages of oocyte development in *Pseudopaludicola pocoto*.

As observed for this species, as the oocytes reach more advanced stages of development, there is a greater differentiation between the nucleus and the cytoplasmic portion. Macroscopically, this development begins with translucent oocytes of extremely reduced size, and, as they develop, they begin to receive yolk or yolk deposits in their respective cytoplasms. This happens concomitantly with melanogenesis, resulting in the clear formation of two poles, the animal and the vegetal ones, divided by an equatorial line, accentuated in the last stage of development (Newman et al., 2018).

Microscopically, the morphological changes observed in *Pseudopaludicola pocoto* are perpetuated in stages already described for other frogs, where the oocyte nucleus begins to increase in volume and present an irregular contour with several dispersed nucleoli, as well as the number of yolks increases significantly, and a cortical melanin layer is present. In a second moment, there is a progressive reduction in the number of nucleoli dispersed in the nucleus, as well as its size stagnates and the cytoplasmic portion becomes more abundant as the oocyte reaches the IV stage of development, differentiating because of the animal and vegetal poles already seen at the acroscopy (Joshi & Udaykumar, 1998; Oliveira & Santos, 2004; Rodríguez-Gómez et al., 2010). These stages have also been observed in teleost fish (Nagahama, Yoshikuni, Yamashita, Tokumoto, & Katsu, 1995; Sokolov et al., 2019).

During the oocyte maturation process, several components are assimilated into the yolk through microvilli (Nagahama et al., 1995; Rodríguez-Gómez et al., 2010). However, this occurs according to the availability of these resources in the serum of individuals. Therefore, in situations where there is a shortage of these nutrients, the cell maturation process may undergo some morphological changes (Díaz-Ricaurte, Serrano, Guevara-Molina, Araujo, & Martins, 2020; Pérez-Rodríguez, Blas, Viñuela, Marchant, & Bortolotti, 2006). Such statements corroborate what was observed for *Pseudopaludicola pocoto*, where, despite showing basal and differentiated cells throughout the year, both the frequency and volume of oocytes underwent significant changes regarding rainfall and environmental temperature, since, in months with greater rainfall and mild temperatures, the oocytes showed higher volumes and frequencies compared to the driest ones with higher temperatures.

The abundance of mature oocytes observed in *Pseudopaludicola pocoto* in the wettest months is supported by the statements and results obtained in the literature (French, DeNardo, & Moore, 2007; Madelaire & Gomes, 2016), that a context of abundant resources is necessary for full reproductive activity in a community for both females and males. However, such changes are much more marked in females, given their large reproductive investment in relation to males, not only in amphibians but also in several other groups of vertebrates (Hayward & Gillooly, 2011).

When compared to tropical regions, the temperate ones exhibit more demarcated climatic seasons. Some authors defend the presence of reproductive seasonality only in amphibian species from these regions (Polzonetti-Magni et al., 1998; Pudney, 1995; Ryan, 1986). However, more recent works have demonstrated a seasonal cyclicity in the behavior and physiology of amphibians from tropical regions, with morphological changes observed during specific periods of the year (Anderson, Bovo, & Andrade, 2018; Madelaire & Gomes, 2016). These facts were observed in *Pseudopaludicola pocoto*, where morphologically we have a period with low gonadal activity, comprised of low rainfall and high temperatures.

Showing all stages of oocyte development throughout the year gives *Pseudopaludicola pocoto*, as in other tropical frog species, an adaptive mechanism for regions with unpredictable rainfall patterns so that they can be prepared to generate their respective offspring (Da Silva Vieira, Santana, & Arzabe, 2009).

Room temperature also had its share of influence on oocyte development and frequency, possibly associated with the photoperiod, as reported in some species of frogs (Delgado, Gutiérrez, & Alonso-Bedate, 1989; Godoy Pieri et al., 2014). The non-correlation observed between temperature and grade I oocytes suggests that, perhaps, these cells are under greater hormonal influence when compared to external factors such as environmental temperature and monthly rainfall, given the strong intrinsic influence of hormones on the hypothalamic-pituitary-gonadal axis (Madelaire, Sokolova, & Gomes, 2017b)

Much of the available work on the gametogenesis of neotropical frogs is limited to macroscopic morphometric measurements, such as Gonadosomatic Index and the Weight of the gonads (Bortolini, Gonçalves, & Zanella, 2018; Delia, Cisneros-Heredia, Whitney, & Murrieta-Galindo, 2010; Faria De Oliveira, Neves, Sérgio, & Pinto Da Matta, 2007; Gottsberger & Gruber, 2004; Santos & Oliveira, 2007). Both variables showed a strong correlation with the volume changes observed in oocytes at the IV degree of development in *Pseudopaludicola pocoto,* which corroborates with what was observed by some authors that, in fact, the amount of yolk and water increases substantially in the most differentiated phases of oocyte maturation (Madelaire & Gomes, 2016; Milone, Iela, Esposito, Rastogi, & Chieffi, 1978).

6 CONCLUSION

Despite rainfall being a crucial factor for the maintenance of oogenesis in *Pseudopaludicola pocoto*, these individuals exhibited all stages of oocyte development throughout the year, configuring an adaptive mechanism for the region given the unpredictability of rainfall. Their oogenesis is subject to morphological changes in periods where rainfall is scarce or absent, which can significantly influence their reproductive dynamics.

CONTRIBUTIONS OF THE AUTHORS

All authors have contributed significantly in all stages of production of the present study.

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CONCLUSÃO GERAL

As alterações morfológicas que ocorreram ao longo de todo o período de coleta foram cruciais para entender a dinâmica reprodutiva de Pseudopaludicola pocoto, tanto para machos quanto para fêmeas, volume e frequência de gametas, peso gonadal, índice gonadossomático, melanina interlocular e até mesmo a morfometria externa foram significativamente influenciadas por fatores externos. Fatores Ambientais.

A pluviosidade apresentou-se como variável líder nesse processo interativo juntamente com a temperatura, em que meses com maior pluviosidade e temperaturas amenas proporcionaram mudanças morfológicas significativas e provavelmente uma maior exibição da fisiologia e comportamento reprodutivo. Vale ressaltar que várias cascatas hormonais influenciam essas alterações morfológicas. Portanto, estudos sobre a ciclicidade hormonal desses indivíduos são necessários para explicar mais profundamente seus respectivos ciclos.

A forte influência observada entre a morfologia reprodutiva e fatores ambientais externos reforça ainda mais a importância de estudos como o presente para a conservação e preservação de espécies do bioma caatinga no Brasil, dado o cenário climático caótico atual.

ANEXOS

Tabela 1a – Correlação de *Spearman* entre variáveis ambientais (independentes) para teste de colinearidade

		Precip. Total*	Umid. Relativa	Evapotransp. real	Ponto de cond.	Temp. Ambiente*
Precipitação	Coefic.	1,000	0,692	0,930	0,797	0,524

(*) Variáveis menos colineares

Tabela 2a – Resultado do Modelo linear generalizado de Volumes interlocular, locular e melanina interlocular de *Pseudopaludicola pocoto* com Precipitação total e Temperatura Ambiente em um fragmento de caatinga, Brasil.

Variável resposta	Termos selecionados	Coeficiente	Erro padrão	Z-valor	Valor de p
Melanina Interlocular (%)	Intercepto	1,119	0,984	1,137	0,256
	Precipitação	-0,004	0,001	-5,308	p<0,01*
	Temp. Ambiente	0,114	0,036	3,198	p<0,01*
Volume Interlocular (%)	Intercepto	1	1,30444	0,807	0,41966
	Precipitação	-0,00255	0,000944	-2,701	0,00691*
	Temp. Ambiente	0,092493	0,047317	1,955	0,05061*
Volume Locular (%)	Intercepto	5,3365564	0,854684	6,244	p<0,01*
	Precipitação	0,0012087	0,000631	1,916	0,0554*
	Temp. Ambiente	-0,043145	0,031154	-1,385	0,1661

(*)valores significativos de p.

Tabela 3a - Resultado do teste de Breusch-Godfrey para autocorrelação temporal dos valores de Peso total das gônadas e IGS para fêmeas e machos com Precipitação total e temperatura ambiente.

Variável resposta		Breusch-Godfrey		
		Lmtest	df	p-value
GW	Machos	0,6852	1	0,4078
	Fêmeas	0,36281	1	0,547
GI	Machos	0,20325	1	0,6521
	Fêmeas	0,87173	1	0,3505

Tabela 4a - Resultado do teste de Breusch-Godfrey para autocorrelação temporal dos valores do percentual de Volume locular, interlocular e melanina interlocular para machos com Precipitação total e temperatura ambiente.

Variável resposta	Breusch-Godfrey		
	Lmtest	df	p-value
Melanina interlocular (%)	1,5995	1	0,206
Volume interlocular (%)	0,063889	1	0,8005
Volume locular (%)	0,063889	1	0,8005

Tabela 5a - Resultado do teste de Breusch-Godfrey para autocorrelação temporal de frequência de cistos germinativos para machos com Precipitação total e temperatura ambiente.

Variável resposta	Breusch-Godfrey		
	Lmtest	df	p-value
SPG (%)	0,28541	1	0,5932
SPC (%)	0,12982	1	0,7186
SPAr (%)	1,5222	1	0,2173
SPAl (%)	0,00066031	1	0,9795
SPZ (%)	0,53049	1	0,4664

Tabela 6a - Resultado do teste de Breusch-Godfrey para autocorrelação temporal de volume de células germinativas para machos com Precipitação total e temperatura ambiente.

Variável resposta	Breusch-Godfrey		
	Lmtest	df	p-value
VPG (%)	1,7231	1	0,1893
VPC (%)	0,24445	1	0,621
VPAr (%)	3,1965	1	0,07379
VPAl (%)	1,5572	1	0,2121
VPZ (%)	1,2799	1	0,2579

Tabela 7a - Resultado do teste de Breusch-Godfrey para autocorrelação temporal de frequência de oocitos para fêmeas com Precipitação total e temperatura ambiente.

Variável resposta	Breusch-Godfrey		
	Lmtest	df	p-value
OCTI (%)	2,5175	1	0,1126
OCTII (%)	1,618	1	0,2034
OCTIII (%)	0,85018	1	0,3565
OCTIV (%)	0,9172	1	0,3382

Tabela 8a - Resultado do teste de Breusch-Godfrey para autocorrelação temporal do volume de oócitos com Precipitação total e temperatura ambiente.

Variável resposta	Breusch-Godfrey		
	Lmtest	df	p-value
VOCTI	0,47114	1	0,4925
VOCTII	4,0488	1	0,0442
VOCTIII	0,017298	1	0,8954
VOCTIV	0,097886	1	0,7544

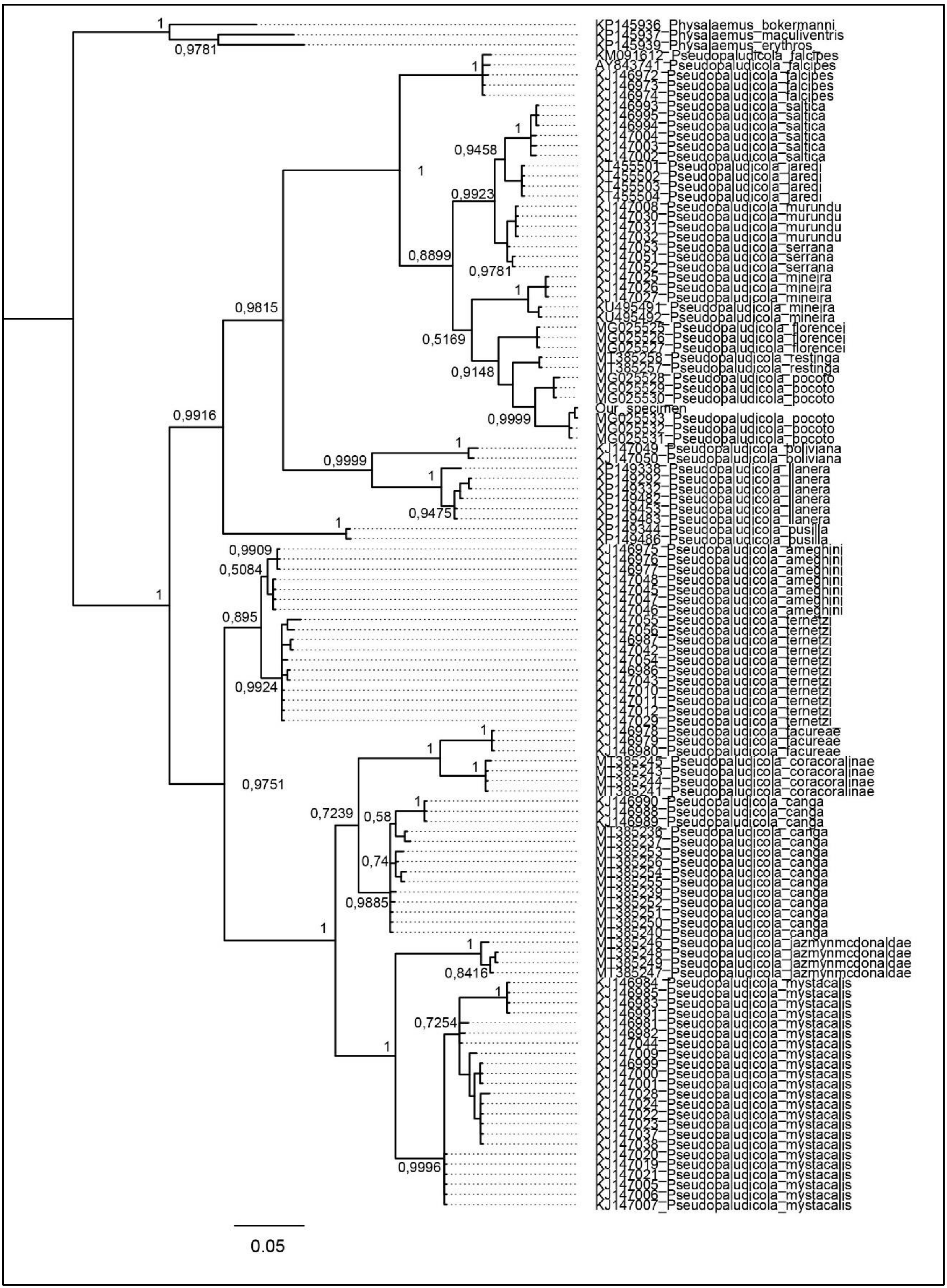

Figure 1a - Árvore filogenética Bayesiana de espécies de Pseudopaludicola baseada no gene 16s.