TALITA DE OLIVEIRA FARIAS

AVALIAÇÕES CELULARES, MOLECULARES E ENDÓCRINAS NA REPRODUÇÃO DE *Myotis levis* MACHOS (CHIROPTERA: VESPERTILIONIDAE)

Instituto de Ciências Biológicas
Universidade Federal de Minas Gerais
Abril/2021

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Tese apresentada ao Programa de Pós-Graduação em Biologia Celular do Departamento de Morfologia, do Instituto de Ciências Biológicas, da Universidade Federal de Minas Gerais, como requisito parcial para a obtenção do título de Doutor em Ciências.

Área de concentração: Biologia Celular

Orientador: Dr. Guilherme Mattos Jardim Costa
Coorientadora: Dr.ª Sônia Aparecida Talamoni

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ATA DA DEFESA DE TESE DE DOUTORADO DE

TALITA DE OLIVEIRA FARIAS

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Às **quatorze horas** do dia **29 de abril de 2021**, reuniu-se, no Instituto de Ciências Biológicas da UFMG, a Comissão Examinadora da Tese, indicada pelo Colegiado do Programa, para julgar, em exame final, o trabalho final intitulado: **"AVALIAÇÕES CELULARES, MOLECULARES E ENDÓCRINAS NA REPRODUÇÃO DE MYOTIS LEVIS MACHOS (CHIROPTERA: VESPERTILIONIDAE)"**, requisito final para obtenção do grau de Doutora em Biologia Celular. Abrindo a sessão, o Presidente da Comissão, **Dr. Guilherme Mattos Jardim Costa**, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra à candidata, para apresentação de seu trabalho. Seguiu-se a arguição pelos examinadores, com a respectiva defesa da candidata. Logo após, a Comissão se reuniu, sem a presença da candidata e do público, para julgamento e expedição de resultado final. Foram atribuídas as seguintes indicações:

Prof./Pesq.	Instituição	Indicação
Dr. Guilherme Mattos Jardim Costa	UFMG	APROVADA
Dra. Sônia Aparecida Talamoni	PUC-MG	APROVADA
Dra. Mariana Machado Neves	UFV	APROVADA
Dra. Leticia Zoccolaro Oliveira	UFMG	APROVADA
Dra. Danielle Barbosa Morais	UFRN	APROVADA
Dra. Ludmilla Moura de Souza Aguiar	UnB	APROVADA

Pelas indicações, a candidata foi considerada: APROVADA

O resultado final foi comunicado publicamente à candidata pelo Presidente da Comissão. Nada mais havendo a tratar, o Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora. **Belo Horizonte, 29 de abril de 2021.**

Dr. Guilherme Mattos Jardim Costa (Orientador) _____

Dra. Sônia Aparecida Talamoni (Coorientadora) _____

Drª. Mariana Machado Neves _____

Drª. Leticia Zoccolaro Oliveira _____

Dra. Danielle Barbosa Morais _____

Dra. Ludmilla Moura de Souza Aguiar _____

Fernanda RCL Almeida

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Esta tese foi desenvolvida no Laboratório de Biologia Celular do Departamento de Morfologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, sob a orientação do Prof. Dr. Guilherme Mattos Jardim Costa e coorientação da Prof.ª Dr.ª Sônia Aparecida Talamoni, e com o auxílio das seguintes instituições:

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“Bats are truly amazing creatures, and we should be proud to have them as our neighbors.”

(Don E. Wilson)

LISTA DE ABREVIATURAS

3-Beta-HSD: Enzima esteroidogênica 3-Beta-Hidroxiesteróide Desidrogenase

AGM: Massa das glândulas sexuais acessórias (*Accessory sex gland mass*)

BAT: Massa do tecido adiposo marrom (*Brown adipose tissue mass*)

BrdU: Marcador temporal de proliferação celular 5-bromo-2′-deoxiuridina

Caspase-3: Marcador de apoptose celular; Membros da família de proteases cisteína-aspartato

D: Espermatócito primário em diplóteno (*Diplotene spermatocyte*)

DIFF: Espermatogônia diferenciada (*Differentiated spermatogonia*)

E: Espermátide em alongamento/alongada (*Elongating/elongated spermatid*)

EM: Massa dos epidídimos (*Epididymis mass*)

GATA-4: Fator de Transcrição e marcador de células de Sertoli; Quarto membro da família de proteínas de ligação GATA

GSI: Índice Gonadossomático (*Gonadosomatic index*)

HA: Alterações histopatológicas (*Histopathological alterations*)

IC: Compartimento intertubular (*Intertubular compartment*)

II: Espermatócito secundário (*Secondary spermatocyte*)

Ki-67: Marcador de proliferação celular MKI67

M: Figura de divisão meiótica (*Figure of meiotic division*)

P: Espermatócito primário em paquíteno (*Pachytene spermatocyte*)

Pl: Espermatócito primário em pré-leptóteno (*Pre-leptotene spermatocyte*)

R: Espermátide arredondada (*Round spermatid*)

Rb: Corpos residuais (*Residual bodies*)

RPPN: Reserva Particular do Patrimônio Natural

S: Célula espermatogonial (*Spermatogonial cell*)

SC: Célula de Sertoli (*Sertoli cell*)

SE: Epitélio seminífero (*Seminiferous epithelium*)

SEC: Ciclo do epitélio seminífero (*Seminiferous epithelium cycle*)

ST: Túbulo Seminífero (*Seminiferous tubule*)

TM: Massa dos testículos (*Testis mass*)

UND: Espermatogônia indiferenciada (*Undifferentiated spermatogonia*)

Z: Espermatócito primário em zigóteno (*Zygotene spermatocyte*)

RESUMO

O vespertilionídeo *Myotis levis* é um morcego insetívoro neotropical que apresenta padrão reprodutivo sazonal, caracterizado por uma flutuação cíclica dos órgãos reprodutivos ao longo do ano. Devido a sua importância ecológica e singularidade de estratégias reprodutivas, o objetivo desse estudo foi investigar os aspectos celulares, moleculares e endócrinos envolvidos na reprodução de *M. levis*. Machos adultos foram capturados na colônia de *M. levis*, localizada na RPPN Santuário do Caraça, Minas Gerais, Brasil. As gônadas, epidídimos, glândulas sexuais acessórias, tecido adiposo marrom e sangue foram coletados para a realização de análises biométricas, histológicas, morfométricas, de imunomarcação e hormonais. Além disso, fragmentos testiculares e tecidos adiposos marrons foram utilizados para a realização de xenoenxerto em camundongos imunodeficientes. Como resultados, após pico de precipitação, ocorre um aumento significativo da massa testicular e o desenvolvimento da espermatogênese de *M. levis* nos estágios de Maturação e Maduro. No estágio Maduro, uma rápida e elevada produção espermática ocorre associada ao aumento do diâmetro nuclear e volume das células de Leydig, máxima expressão da enzima esteroidogênica 3-Beta-HSD e elevados níveis séricos de testosterona de *M. levis*. Durante esses períodos, o tecido adiposo marrom é consumido, favorecendo a síntese de testosterona (comprovada por meio do xenoenxerto do tecido adiposo marrom). No estágio de Regressão, as massas dos epidídimos e glândulas sexuais acessórias possuem os maiores valores. Os espermatozoides ficam armazenados na cauda do epidídimo por oito meses. A concentração, vitalidade e motilidade espermática apresentam maiores taxas no estágio de Regressão, mas a maior incidência de gametas morfologicamente normais ocorre no período do acasalamento (estágio de Repouso). Após realizar o xenoenxerto de fragmentos testiculares, percebemos que a espermatogênese progride até a fase espermiogênica em todos os estágios reprodutivos em um modelo receptor com níveis hormonais estáveis. Esses dados reforçam a influência da sazonalidade e variação hormonal na reprodução de *M. levis*. As novas informações sobre o ciclo reprodutivo de *M. levis* e o uso de xenoenxerto de tecidos são vitais para compreender a reprodução de vespertilionídeos neotropicais. Estudos sobre a reprodução de morcegos são cruciais nos aspectos ecológico e econômico. Além de serem bioindicadores, os morcegos podem controlar populações de insetos que causam prejuízos em culturas agrícolas e que promovem a disseminação de doenças na população.

ABSTRACT

The vespertilionid *Myotis levis* is a neotropical insectivorous bat, which presents a seasonal reproductive pattern, characterized by a cyclic fluctuation of reproductive organs along the year. Considering its ecological importance and unique reproductive strategies, this study aimed to investigate the cellular, molecular, and endocrine aspects involved in *M. levis* reproduction. The adult male bats were captured from *M. levis* colony located in RPPN Santuário do Caraça, Minas Gerais, Brazil. The gonads, epididymides, accessory sex glands, brown adipose tissue, and blood were collected to perform biometric, histological, morphometric, immunostaining and hormonal analyses. Moreover, testis fragments and brown adipose tissue were used for xenografting in immunodeficient mice. As results, after a rainfall peak, a significant increase of testis mass and spermatogenesis development of *M. levis* occurs in the Maturing and Mature stages. In the Mature stage, a fast and elevated sperm production occurs associated with an increase in nuclear diameter and volume of Leydig cells, maximum expression of the steroidogenic 3-Beta-HSD enzyme, and elevated testosterone serum levels of *M. levis*. During these periods, the brown adipose tissue is consumed, supporting testosterone synthesis (proven through brown adipose tissue xenograft). In the Regressed stage, epididymis and accessory sex gland masses reach higher values. The spermatozoa remain stored in epididymis cauda for eight months. The sperm concentration, vitality, and motility presented high rates in the Regressed stage; however, the higher incidence of normal morphological gametes occurred in the mating period (Rest stage). After performing the xenograft of testis fragments, we realize that spermatogenesis progressed until the spermiogenic phase in all reproductive stages in a recipient model with stable hormonal levels. These data reinforced the influence of seasonality and hormonal variation in *M. levis* reproduction. The new information concerning the reproductive cycle of *M. levis* and the use of tissue xenografts are vital for understanding the reproduction of neotropical vespertilionids. The studies about bat reproduction are crucial for the ecological and economic aspects. Besides being bioindicators, bats can control insect populations that cause damage to crop and promote disease dissemination in the population.

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1. INTRODUÇÃO

1.1 Morcegos

Os morcegos destacam-se entre os mamíferos, por apresentar grande diversidade, >1400 espécies no mundo, e elevada variedade de hábitos alimentares e hábitats (Nowak, 1994; Wilson & Mittermeier, 2019). Além disso, as características de sua história de vida e biologia os tornam potencialmente importantes como bioindicadores, pois além de serem altamente reativos a estressores ambientais, são provedores de importantes serviços ecossistêmicos, como polinização e dispersão de sementes (Fleming *et al*., 2009; Jones *et al*., 2009; Kunz *et al*., 2011; Russo & Jones, 2015; Zukal *et al*., 2015). Além de sua importância ecológica, os morcegos são potenciais reservatórios de mais de 200 vírus pertencentes a 27 famílias, sendo que poucos vírus são responsáveis por doenças em humanos e há grande variação das respostas imunes de morcegos de diferentes espécies a infeções virais (Moratelli & Calisher, 2015). Portanto, é de extrema relevância estudos para compreender a fisiologia dos morcegos.

Na ordem Chiroptera, a família Vespertilionidae é a mais diversa em número de espécies, possuindo espécies distribuídas em regiões tropicais e temperadas do planeta, sendo que o gênero *Myotis* possui a mais ampla distribuição geográfica dentre os morcegos (Nowak, 1994; Simmons, 2005). *Myotis levis* (I. Geoffroy Saint-Hilaire, 1824) é um vespertilionídeo insetívoro neotropical que forrageia em áreas abertas próximas à água e captura insetos em pleno voo. Entre as características morfológicas, destacam-se o pequeno tamanho (peso de ~6g), pelagem curta de coloração que varia de tons acinzentados a castanhos, rostro alongado e ausência de ornamentos faciais (LaVal, 1973; Nowak, 1994; Eisenberg & Redford, 1999). Em relação à sua distribuição geográfica, ocorre no Uruguai, sul e sudeste do Brasil, sul da Bolívia, Paraguai e norte da Argentina (Wilson, 2007; Stevens *et al*., 2010; Araújo *et al*., 2013).

Morcegos insetívoros são provedores de importantes serviços ecossistêmicos como controle de pragas agrícolas e vetores de doenças (Jones *et al*., 2009; Kunz *et al*., 2011; Maine & Boiles, 2015; Russo & Jones, 2015; Russo *et al*., 2018; Rodríguez-San Pedro *et al*., 2020). Dessa forma, contribuem para a manutenção do equilíbrio dos ecossistemas, consumindo uma grande quantidade de insetos por noite dependendo da espécie, estação climática e fase do ciclo reprodutivo (Anthony & Kunz, 1977; Kurta *et al*.,1989; Kunz *et al*., 1995; Kunz *et al*., 2011).

A reprodução de quirópteros pode ser regulada por diversos fatores ambientais como temperatura, precipitação, fotoperíodo e disponibilidade de recursos alimentares (Heideman, 2000).

Vespertilionídeos que vivem em regiões temperadas apresentam geralmente padrão reprodutivo monoéstrico sazonal (Gustafson, 1979; Entwistle *et al*., 1998; Krutzsch, 2009). Durante o período de escassa disponibilidade de insetos e baixas temperaturas, os morcegos de regiões temperadas hibernam ou entram em torpor por longos períodos e utilizam mecanismos fisiológicos para garantir o sucesso reprodutivo (Barbour & Davis, 1969; Weir & Rowlands, 1973). Dentre esses mecanismos, podemos citar a regressão testicular e estocagem de espermatozoides na cauda do epidídimo em machos (Weir & Rowlands, 1973; Gustafson, 1979; Racey, 1979; Crichton, 2000; Krutzsch, 2009; Pfeiffer & Mayer, 2012).

Em regiões tropicais, os vespertilionídeos podem apresentar padrão reprodutivo monoéstrico sazonal (Medway, 1972; McWilliam, 1988; Myers, 1977), assim como padrão reprodutivo poliéstrico (Dwyer, 1970; Myers, 1977) em virtude da reduzida variação sazonal que não restringe a disponibilidade de insetos. O padrão reprodutivo do vespertilionídeo neotropical *M. levis* é monoéstrico sazonal (Araújo *et al*., 2013). A maturação das gônadas de machos e fêmeas é assíncrona (Araújo *et al*., 2013) e ocorrem eventos de regressão testicular e estocagem de espermatozoides na cauda do epidídimo (Araújo *et al*., 2013; Farias *et al*., 2015), estratégias reprodutivas semelhantes à de espécies de regiões temperadas (Racey & Entwistle, 2000) porém sem a ocorrência de hibernação ou torpor. Os machos acasalam com fêmeas receptivas na metade da estação seca (abril a setembro), sendo este comportamento reprodutivo confirmado pela presença de espermatozoides alojados nas criptas uterinas, e fêmeas lactantes são encontradas no início da estação chuvosa (outubro a novembro) (Araújo *et al*., 2013).

1.2 Espermatogênese

A espermatogênese é um processo cíclico, altamente complexo e organizado que ocorre nos túbulos seminíferos de animais sexualmente maduros (França & Russell, 1998; França *et al*., 1998; Godinho, 1999; Almeida, 2002). Baseado em características morfológicas e funcionais, o processo espermatogênico pode ser dividido em três fases: (a) fase espermatogonial (proliferativa), caracterizada por várias e sucessivas divisões mitóticas dos diferentes tipos de espermatogônias; (b) fase espermatocitária (meiótica),

na qual ocorre a duplicação do DNA, a recombinação gênica e duas divisões meióticas (reducional e equacional) que resultam na formação de células haploides denominadas espermátides; e (c) fase espermiogênica (diferenciação), onde as espermátides arredondadas passam por drásticas alterações morfológicas e funcionais tais como a formação do acrossoma, do flagelo e a condensação nuclear, resultando numa célula altamente especializada, o espermatozoide, o qual estará apto para o processo de capacitação (Russell *et al*., 1990; Sharpe, 1994; Hess & França, 2007; Lara *et al*., 2018).

O processo espermatogênico tem sido investigado em diferentes espécies de morcegos neotropicais (Beguelini *et al*., 2009, 2013a; Morais *et al*., 2012, 2013b, 2014; Notini *et al*., 2015; Farias *et al*., 2018; Silva *et al*., 2019, 2020), assim como em vespertilionídeos (Beguelini *et al*., 2009, 2013b, 2013c; Araújo *et al*., 2013; Farias *et al*., 2020). Em *Myotis levis* a espermatogênese é marcada pela sazonalidade e caracterizada por quatro estágios (Araújo *et al*., 2013). Os estágios de atividade reprodutiva denominados Repouso, Maturação, Maduro e Regressão apresentam grande variação na composição do epitélio seminífero (Farias *et al*., 2015), semelhante ao padrão encontrado em vespertilionídeo de regiões temperadas (Miller, 1939; Pearson *et al*., 1952; Racey & Tam, 1974; Krutzsch, 1975; Gustafson, 1979; Krutzsch, 2009). Além disso, a duração da espermatogênese de diferentes espécies de mamíferos (França *et al*., 2005; Lara *et al*., 2018) e morcegos neotropicais tem sido caracterizada (Morais *et al*., 2013a; Morais *et al*., 2017; Silva *et al*., 2020).

Diferentes parâmetros espermáticos são analisados para avaliar a qualidade dos espermatozoides e prever defeitos na espermatogênese e maturação espermática (WHO, 2010). Dentre eles podemos citar a vitalidade, em que os espermatozoides são identificados como mortos ou vivos de acordo com a integridade da membrana celular; a motilidade, que consiste na habilidade de deslocamento do espermatozoide para que seja capaz de chegar ao ovócito; a morfologia, em que a avaliação das anormalidades nas estruturas dos espermatozoides (cabeça, peça intermediária e cauda) é necessária para predizer seu desempenho; e a concentração, que pode refletir a produção espermática testicular (WHO, 2010; Vieira, 2019). Em espécies de morcegos das famílias Pteropodidae (Jong *et al*., 2005; Melville *et al*., 2015; Abiaezute *et al*., 2020), Hipposideridae (Marina *et al*., 2003), Phyllostomidae (Álvarez-Guerrero *et al*., 2014; Brito *et al*., 2020) e Vespertilionidae (Fawcett & Ito, 1965; Sharifi & Javanbakht, 2016) esses parâmetros têm sido caracterizados possibilitando uma melhor compreensão da biologia reprodutiva dos quirópteros. Entretanto, em relação à família Vespertilionidae

destaca-se a escassez de dados referentes à duração do processo espermatogênico e de parâmetros seminais em espécies neotropicais.

1.3 Função endócrina

As células de Leydig estão presentes no compartimento intersticial testicular e são responsáveis pela síntese de testosterona, essencial para o desenvolvimento da espermatogênese (Hess & França, 2007; Lara *et al*., 2018; Zirkin & Papadopoulos, 2018). O eixo hipotalâmico-hipofisário-gonadal influencia na regulação da produção de testosterona testicular e consequentemente na função das células de Leydig (Zirkin & Papadopoulos, 2018). O hormônio liberador de gonadotrofina (GnRH) é secretado no hipotálamo e estimula a liberação do hormônio luteinizante (LH) pela hipófise, em que o LH atua diretamente nas células de Leydig estimulando a sua função esteroidogênica (Zirkin & Papadopoulos, 2018). Dessa forma a flutuação de gonadotrofinas, observada em vespertilionídeos sazonais (Anthony & Gustafson, 1984; Bernard *et al*. 1991), interfere diretamente na síntese de testosterona (Zirkin & Papadopoulos, 2018).

Em diferentes espécies de mamíferos ocorre grande variação no volume e proporção das células de Leydig (Lara *et al*., 2018). Alterações morfológicas e funcionais dessas células, em relação ao diâmetro nuclear (Racey, 1974; Bernard *et al*. 1991; Hosken *et al*. 1998), níveis de testosterona sérica e intratesticular (Racey & Tam, 1974; Gustafson & Shemesh, 1976; Gustafson & Damassa,1985; Hosken *et al*. 1998), e expressão da enzima esteroidogênica 3-Beta-HSD (Racey & Tam, 1974; Kurohmaru *et al*. 2002) foram descritas em vespertilionídeos sazonais ao longo do ciclo reprodutivo.

Além dos órgãos reprodutivos, outros tecidos especializados podem apresentar um papel importante na reprodução de morcegos. O tecido adiposo marrom está presente em muitas espécies de mamíferos e é abundante naqueles que hibernam (Rasmussen, 1923; Joel, 1965; Smith & Horwitz, 1969; Trayhurn *et al*., 1991). Além disso, pode ser encontrado em morcegos que não hibernam (Wimsatt, 1955).

Sabe-se que o tecido adiposo marrom contribui na produção de calor durante o despertar da hibernação (Smalley & Dryer, 1963; Hayward & Ball, 1966; Lyman, 1970), pode ser importante na manutenção da temperatura corporal durante a atividade de voo em baixas temperaturas (O'Farrell & Schreiweis, 1978) e acredita-se que possua atividade androgênica tendo um papel importante no ciclo reprodutivo de machos e na função testicular (Krutzsch & Wells 1960). Além disso, sugere-se que os hormônios

sexuais podem regular a função do tecido adiposo marrom, em que grandes mudanças nos níveis e atividade dos hormônios sexuais estão associadas com alterações na presença e atividade metabólica desse tecido adiposo (Quarta *et al*., 2012). Soma-se a isso a presença de diferentes receptores de esteroides sexuais (receptor de andrógeno) em adipócitos marrom *in vitro* (Rodriguez-Cuenca *et al*., 2007). Dessa forma, é sugerido que os andrógenos podem regular a sua capacidade termogênica e homeostase energética (Quarta *et al*., 2012).

1.4 Justificativa

A compreensão da reprodução de morcegos insetívoros é essencial considerando sua importância ecológico-econômica, sendo que o controle biológico de pragas agrícolas é considerado como uma das maiores contribuições dos morcegos para os ecossistemas, com destaque para a valoração desse serviço ecossistêmico ao redor do mundo (Cleveland *et al*., 2006; Boiles *et al*., 2011; Maine & Boiles, 2015; Puig-Montserrat *et al*., 2015; Taylor *et al*., 2017; Rodríguez-San Pedro *et al*., 2020).

Considerando a biologia reprodutiva dos vespertilionídeos neotropicais, investigamos o vespertilionídeo neotropical *Myotis levis* que se destaca pela complexidade biológica e diversidade de estratégias reprodutivas. O estudo morfológico e molecular das gônadas desse morcego ao longo do ano se fez necessária para determinar a influência de fatores ambientais na fisiologia das células testiculares dessa espécie. Ainda, a investigação da produção espermática diária bem como a estocagem de espermatozoides e parâmetros seminais fez-se importante para elucidar as estratégias adaptativas desenvolvidas para garantir o sucesso reprodutivo da espécie, considerando que existe uma assincronia na atividade das gônadas de machos e fêmeas.

Numa vertente diferente, desenvolvemos experimentos envolvendo o enxerto de tecidos para verificar a importância da estabilidade das gonadotrofinas para o desenvolvimento da espermatogênese de *Myotis levis*, bem como para elucidar a real importância fisiológica do tecido adiposo marrom na reprodução de machos dessa espécie.

2. OBJETIVOS

2.1 Objetivo geral

Investigar os aspectos celulares, moleculares e endócrinos na reprodução de machos adultos do morcego insetívoro *Myotis levis*.

2.2 Objetivos específicos

- Investigar os aspectos celulares e morfofisiológicos que regulam a espermatogênese nos diferentes estágios reprodutivos (repouso, maturação, maduro e regressão) associados a fatores ambientais;
- Investigar os aspectos moleculares da espermatogênese nos estágios reprodutivos, em relação à dinâmica de morte e proliferação celular, e a atividade esteroidogênica das células de Leydig;
- Descrever a duração da espermatogênese e a produção espermática;
- Caracterizar a morfologia dos espermatozoides e os parâmetros espermáticos, e registrar o período de estocagem de espermatozoides no epidídimo ao longo do ano;
- Avaliar o desenvolvimento da espermatogênese, nos diferentes estágios reprodutivos, na ausência de fatores ambientais por meio do xenoenxerto de fragmentos testiculares;
- Realizar o xenoenxerto de tecido adiposo marrom para verificar a sua função androgênica.

3. RESULTADOS

3.1 Artigo 1

Male reproductive morphofunctional evaluation of a Neotropical sperm-storing vespertilionid bat (*Myotis levis*) in an environmental context (Farias *et al*., 2020)

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REGULAR ARTICLE

Male reproductive morphofunctional evaluation of a Neotropical sperm-storing vespertilionid bat (*Myotis levis)* in an environmental context

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Abstract

Myotis levis (yellowish myotis) is a small Neotropical insectivorous vespertilionid bat that provides valuable ecosystem services, such as control of disease vectors and agricultural pests. Aiming to describe the fluctuations of the reproductive organs throughout the year, the gonads and epididymis from 124 adult bats were histologically evaluated. These animals were captured in Santuário do Caraça, Minas Gerais, Brazil. After the initial screening, six bats per reproductive stage (in a representative month) had specific organs harvested for further investigation. The gonads, epididymis, accessory sex gland and brown adipose tissue were collected for biometric analyses. Furthermore, yellowish myotis testis was evaluated through histomorphometric and molecular assays, whereas blood samples were collected for hormonal analyses. The data were compared among the reproductive stages and correlated with rainfall distribution. As a result, we demonstrated that yellowish myotis presented a seasonal reproduction showing testis regression and rest, resembling the pattern exhibited by temperate-zone vespertilionid bats. During the Mature stage, after the peak of rainfall distribution, yellowish myotis testicles were fully developed for gamete production and maximum testosterone synthesis. These findings indicate a significant influence of this environmental factor on yellowish myotis reproduction. Following that, the accessory sex gland, brown adipose tissue and epididymis weights increased in the Regressed stage. The epididymis sperm storage occurred for at least 8 months and was observed in the Regressed, Rest and beginning of the Maturing stage. This reproductive fluctuation is interesting because the reactivation of the gonads coincided with the least amount of sperm in the epididymis.

Keywords Chiroptera · Seasonal reproduction · Rainfall · Spermatogenesis · Sperm storage · Testosterone

Introduction

Chiroptera is the second order, among mammals, regarding the number of species, and these bats have a high variety of feeding habits and habitats (Nowak 1994). In this order, the Vespertilionidae family is the most diverse in terms of the number of species, distributed in tropical and temperate regions (Nowak 1994; Simmons 2005). The genus *Myotis* has the widest geographical distribution among bats (Nowak 1994). The present study subject, yellowish myotis, *Myotis levis* (I. Geoffroy Saint-Hilaire, 1824), is a small insectivorous bat (weight of ~6 g) that forages in open areas near water by capturing insects in flight (LaVal 1973; Nowak 1994; Eisenberg and Redford 1999). In general, the characteristics of bats' life and biology make them important bioindicators, since they are highly reactive to environmental stressors (Jones et al. 2009; Zukal et al. 2015). Moreover, bats provide important ecosystem services, such as pollination, seed dispersal, control of disease vectors and agricultural pests (Fleming et al. 2009; Jones et al. 2009; Kunz et al. 2011; Russo and Jones 2015; Zukal et al. 2015). The valuation of the latter ecosystem service provided by bats is estimated to be around US$ 1 billion per year, considering their activities around the world (Maine and Boyles 2015).

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Vespertilionid bats, living in temperate and tropical regions, usually have a seasonal monoestrous reproductive pattern (Medway 1972; Myers 1977; Krutzsch 1979; McWilliam 1988; Entwistle et al. 1998; Krutzsch 2009). During the period of low availability of insects and low temperatures, bats from temperate regions hibernate or go into torpor for long periods (Barbour and Davis 1969; Weir and Rowlands 1973). Differently, bats from tropical regions do not present hibernation and may exhibit monoestrous reproductive pattern as described above, as well as a polyestrous reproductive pattern (Dwyer 1970; Myers 1977) when the reduced seasonal variation does not restrict the availability of insects.

There are similar reproductive strategies on species living in temperate and tropical regions (Racey and Entwistle 2000). Among them, testicular regression and sperm storage in the cauda of the epididymis in males can be cited (Weir and Rowlands 1973; Gustafson 1979; Racey 1979; Crichton 2000; Krutzsch 2009; Pfeiffer and Mayer 2012). Regarding male reproduction, the spermatogenic process of yellowish myotis is marked by seasonality, composed of four stages, characterized by Araújo et al. (2013). The reproductive stages are listed as Rest, Maturing, Mature and Regressed, and significant differences among stages were observed in the cell composition of seminiferous epithelium (Farias et al. 2015). It is believed that the brown adipose tissue acts as a key element in gonad changes since its structure presents androgenic activities, which play a role in the male reproductive cycle and testis function (Krutzsch and Wells 1960). The fertility capacity of *M. levis* is challenging since sperm is only produced during the Mature stage, while the females are receptive in a different period, i.e. during the middle of the dry season (Araújo et al. 2013; Farias et al. 2015).

Based on the unique features of yellowish myotis male reproduction, we aimed to investigate the cellular, molecular and morphophysiological aspects regulating spermatogenesis of *M. levis* during the different reproductive stages, linked to rainfall pattern. By doing so, we believe that the knowledge herein described would help the biological control of agricultural pests and the development of different biotechnologies associated with the reproduction of this species, such as sperm cryopreservation, germ cell transplantation and testis tissue xenograft.

Material and methods

Study area

The colony of yellowish myotis is located in Santuário do Caraça, a preserved area in Serra do Caraça, southeastern Brazil (20°04′30″ S, 43°24′28″ W). The reserve belongs to the Iron Quadrangle geomorphological domain, with an area of 10.187 ha with elevations of 750 to 2.072 m (Moreira and Pereira 2004; Abreu and Palú 2008). It has a great diversity of fauna and flora because it is located in a transition region of the biomes Atlantic Forest and Cerrado, with different ecosystems such as seasonal semideciduous forest, gallery forest, riparian forest, rocky fields and altitudinal fields (Giulietti et al. 1997; Moreira and Pereira 2004).

The climate of the region is seasonal with a rainy summer (rainy season—October to March) and dry winter (dry season—April to September) (Sá et al. 2012). The month of August is considered the coldest (9–18 °C), and the months of February and March are considered the warmest (23–25 °C) (Araújo et al. 2013). The rainfall distribution occurs mainly during the rainy season, corresponding to 81.5% of the annual average of 1.373 mm, and the remaining percentage of precipitation occurs in the dry season (Sá et al. 2012). Rainfall data were obtained from the Santuário do Caraça weather station from January 2017 to December 2018.

Animal processing and data analysis

In the initial step of this study, 124 males were collected from January 2017 to December 2018 (Supplementary Table 1) for histological examinations of the gonads and epididymis. These analyses allowed the establishment of the reproductive stages of *M. levis* according to the most characteristic features of the testis and epididymis. The relative frequency of the reproductive stages was also obtained for the identification of the most prevalent reproductive stage per month. Considering the aforementioned, six bats per reproductive stage (Rest, Maturing, Mature, Regressed) had the gonads, epididymis, accessory sex gland, brown adipose tissue and blood samples collected for additional investigation. Bats were captured from October to April, which is the shortest time window (7 months) to obtain samples from all four reproductive stages. The bats were then subdivided into four groups, i.e. (i) Rest stage (captured in October), (ii) Maturing stage (captured in December), (iii) Mature stage (captured in March) and (iv) Regressed stage (captured in April), and evaluated for biometric, testicular, morphometric, hormonal and immunostaining parameters.

Bats were captured through two mist nets installed in the attic of Santuário do Caraça church, from 18:00 to 00:00 h. The captured bats were identified (Gardner 2008; Miranda et al. 2011) and measured to obtain their forearm length and body mass and to determine their sex. Only adult males were used in the study, while captured females and subadult males were immediately released back to the colony. The differentiation between adults and subadults was determined through the visualization of the finger epiphyseal cartilages in the metacarpus of the subadults, whereas those of the adults were ossified (Anthony 1988) and they have complete testicular descent (Duarte and Talamoni 2010).

The bats were euthanized with an overdose injection of sodium thiopental intraperitoneally (0.9 mg/g body weight). The gonads, epididymis, accessory sex gland complex and brown adipose tissue from the interscapular region were collected, weighed and fixed in Bouin solution (for histomorphometric analysis). The testes were also fixed in Methacarn solution (for immunohistochemical analysis). The samples were then routinely processed and included in Paraplast®. Before histological procedures, the mean of the following parameters was calculated: testis mass (TM), gonadosomatic index (GSI = mass of testes × 100/body mass), epididymis mass (EM), accessory sex gland mass (AGM) and brown adipose tissue mass (BAT).

All specimens are deposited in the reference collection of the Pontifical Catholic University of Minas Gerais. Captures were performed under licence (#28120–4) granted by the Brazilian Chico Mendes Institute for Biodiversity Conservation, and access to animal genetic legacy was granted by licence n° A8CA63C of the Genetic Legacy Management Council by the Brazilian Ministry of Environment (SISGen). The procedures used in this study were previously approved by the Ethics Committee on Animal Use from the Federal University of Minas Gerais (CEUA document 386/2017).

Spermatogenesis evaluation

For the evaluation of *M. levis* spermatogenesis, monthly descriptions of the seminiferous epithelium composition and testicular morphometric analyses were performed. The analyses were not performed in June because the colony left their roost. The seminiferous epithelium composition was characterized according to the presence of different germ cell layers associated with Sertoli cells. The mean diameter of the seminiferous tubules and the mean height of the seminiferous epithelium were obtained from random measurement of 15 round cross sections of seminiferous tubules per animal.

The volume densities (%) of the testicular components were estimated using 475 points (intersection grid) in each field, at 200 × magnification, through ImageJ software (Rasband 2014). For each animal, 15 fields were used, summing up 7125 points. From the tubular compartment, tunica propria, seminiferous epithelium and lumen were evaluated, while Leydig cells, connective tissue cells and blood vessels were analysed in the intertubular compartment. The volume of Leydig cells was obtained using the nuclear volume and the proportion between the nucleus and cytoplasm. The nuclear volume of Leydig cells was calculated using the sphere formula ($4/3\pi R^3$, in which R = nuclear diameter/2), where 30 nuclei were measured per bat. The proportion between nucleus and cytoplasm was estimated counting 1000 points over Leydig cells for each bat. The number of Leydig cells was estimated from Leydig cell size and the total volume occupied by these cells in the testis parenchyma.

Hormonal analyses

After bat anaesthesia induction, blood samples were collected by cardiac puncture. Plasma was separated through centrifugation (2.000 rpm, for 10 min, at 4 °C) and stored at −20 °C for subsequent hormone evaluation. The samples were processed in the automated Cobas e 411 (Roche Diagnostics Inc., Indianapolis, IN, USA) platform for direct assessment of testosterone. Plasma testosterone was measured by electrochemiluminescence assay using commercial kits (Roche Diagnostics Inc., Indianapolis, IN, USA) with 2.5-ng/dL sensitivity. Testosterone coefficients of variation (CV) intra- and inter-assay were, respectively, 1.1% and 1.5%. The procedures were performed by Licenced Laboratory specialized in Animal Health (Belo Horizonte, Brazil).

Immunostaining analyses

Immunostaining analyses were performed using the immunoperoxidase and immunofluorescence methods. For immunohistochemical analysis, deparaffinized sections were dehydrated, and the endogenous peroxidase activity was blocked by incubating the sections in a 3% hydrogen peroxide solution (Sigma, St. Louis, MO, USA). Subsequently, the antigens were exposed to heating in buffered sodium citrate (pH 6.0) at 96 °C for 10 min. The sections were treated with Protein Block (ab93697, Abcam Inc., USA) for Ki-67, GATA-4 and Cleaved Caspase-3 for 10 min. For 3-Beta-HSD, the protein was blocked using 10% normal rabbit serum (Sigma, St. Louis, MO, USA, #R9133) in PBS for 30 min.

The slides were incubated overnight (4 °C) with specific primary antibodies against the cell proliferation marker Ki-67 (1:100, BD Biosciences Pharmingen, USA, mouse monoclonal antibody, 550,609), the Sertoli cell marker GATA-4 (1:100, Santa Cruz Biotechnology, USA, mouse monoclonal antibody, sc-25,310), the cell apoptosis marker Cleaved Caspase-3 (1:100, Imuny, BR, rabbit polyclonal antibody, IM-0035) and the steroidogenic enzyme 3-Beta-HSD (1:100, Santa Cruz Biotechnology, USA, goat polyclonal antibody, sc-30,820). Once the antibodies were raised against human proteins, the protein homology between human and *Myotis* species was tested through in silico analysis (Basic Local Alignment Search Tool). For Ki-67, GATA-4, Cleaved Caspase-3 and 3-Beta-HSD, the homologies were 90.3%, 94.07%, 87.7% and 76.7%, respectively.

The reactions were developed using the Biotinylated Goat Anti-Polyvalent Plus (ab93697; Abcam Inc., USA) for Ki-67, GATA-4 and Cleaved Caspase-3 IgG secondary antibodies. For 3-Beta-HSD, we used rabbit pAb to goat IgG secondary antibody (ab6740; Abcam Inc., USA). Diaminobenzidine (DAB) was used as chromogen, and the negative controls had the primary antibodies omitted.

Immunofluorescence for Ki-67 and Cleaved Caspase-3 was performed to corroborate the dynamics of cell proliferation and apoptosis. For that reason, reactions were visualized using 633 anti-mouse, and Alexa-488 anti-rabbit conjugated secondary antibodies (1:100 dilution; Thermo Fisher Scientific) visualized using a Nikon fluorescence microscope (EclipseTi).

To evaluate the expression of the steroidogenic enzyme 3-Beta-HSD, protein labelling by immunostaining was quantified. For this analysis, ten random images (50 cells) were captured from the testicular parenchyma of bats using an Olympus BX60 microscope with a coupled camera. The images were treated to convert into greyscale in Photoshop CS6 v13.0, and the pixel intensity was measured from the labelled cells. The pixel intensity was normalized by the ratio between the pixel intensity obtained from the labelled cells and obtained in the background of the image (lumen of seminiferous tubules or blood vessels).

Statistical analysis

All quantitative data were tested for normality and homoscedasticity of variances by the D'Agostino and Pearson tests. Rainfall distribution, biometric data, testis morphometric parameters, testicular parenchyma volume density and the morphometric parameters of Leydig cells presented normal distribution. These parameters were submitted to the analysis of variance (one-way ANOVA), and the means of the reproductive stages were compared with the Newman-Keuls test. For nonparametric data, i.e. epididymis mass, tunica propria, seminiferous epithelium and connective tissue volume density, 3-Beta-HSD pixel intensity and serum testosterone levels, the Kruskal-Wallis test was applied for the analysis of variance, followed by the Dunn's test to compare the means of the reproductive stages. Furthermore, Spearman correlation was performed between the biometric and reproductive parameters of yellowish myotis and rainfall distribution in Santuário do Caraça. The data obtained were expressed as the mean ± standard error of the mean (SEM). Statistical analyses were performed through the program GraphPad Prism 6 (GraphPad Software, Inc). The level of significance considered was $P < 0.05$.

Results

Yellowish myotis shows four reproductive stages according to the morphology of their reproductive organs

The testis parenchyma presents a substantial variation in its cellular composition (Fig. 1a–k) while the epididymis shows the highest sperm storage when the spermatogenic process is interrupted (Fig. 2a–k).

In the Rest stage (May to October), the seminiferous tubules (Fig. 1a–e) have absented or reduced lumen, and the seminiferous epithelium presents numerous Sertoli cells without evident nucleoli (white arrowhead) and undifferentiated spermatogonia (black arrowhead). At the beginning of this reproductive stage, the epididymis (Fig. 2a–e) presents spermatozoa (*) from a previous Regressed stage, which is stored until mating occurs. Considering all analysed bats, within the period of study, 35.5% of them were in the Rest stage.

The Maturing stage (November to February) is characterized by cellular proliferation. The seminiferous tubules (Fig. 1f–i) have reduced lumen, and the seminiferous epithelium presents Sertoli cells (white arrowhead), undifferentiated (black arrowhead) and differentiated (grey arrowhead) spermatogonial cells, primary spermatocytes in pre-leptotene (blue arrowhead), leptotene, zygotene (green arrowhead) and few pachytenes (yellow arrowhead). It should be mentioned that a vast number of primary spermatocytes are trapped in zygotene (green arrowhead). Few individuals present spermatozoa (*) in the epididymis cauda (Fig. 2f–i), probably belonging to the previous Regressed stage. Considering all analysed bats within the period of study, 34.7% of them were in the Maturing stage.

The Mature stage (March) occurs only in the rainy season and is characterized by a well-defined seminiferous tubules lumen (Fig. 1j), presence of Sertoli cells (white arrowhead) and undifferentiated spermatogonia (black arrowhead). Although the seminiferous epithelium is developed, there are missing germ cell layers in its basal compartment, from undifferentiated spermatogonia to primary spermatocytes in zygotene. In the adluminal compartment, few primary spermatocytes in zygotene (green arrowhead), primary spermatocytes in pachytene (yellow arrowhead), secondary spermatocytes, and round (orange arrowhead) and elongated (red arrowhead) spermatids are observed. In the early Mature stage, the spermatogenic process is not fully developed in all individuals, during which few tubules were releasing spermatids in the seminiferous tubule lumen with few spermatozoa (*) found in the epididymis cauda (Fig. 2j). However, in the late Mature stage, spermiation is a frequent event in the seminiferous tubules with a large amount of spermatozoa found in the epididymis cauda. Considering all analysed bats within the period of study, 14.5% of them were in the Mature stage.

The Regressed stage (April) occurs only in the dry season and is characterized by wide seminiferous tubules lumen (Fig. 1k) and reduced number of germ cells and comprised mainly of undifferentiated spermatogonia (black arrowhead) and elongated spermatid (red arrowhead). There is an evident gap between spermatogonial cells and spermatids, as a consequence of seminiferous epithelium discontinuity and loss of germ cell generations, indicating blockage of the differentiation process. Furthermore, large vacuoles (purple arrowhead), cell sloughing and residual bodies (pink arrowhead) are most frequently observed. The epididymis cauda (Fig. 2k) is a replenished of

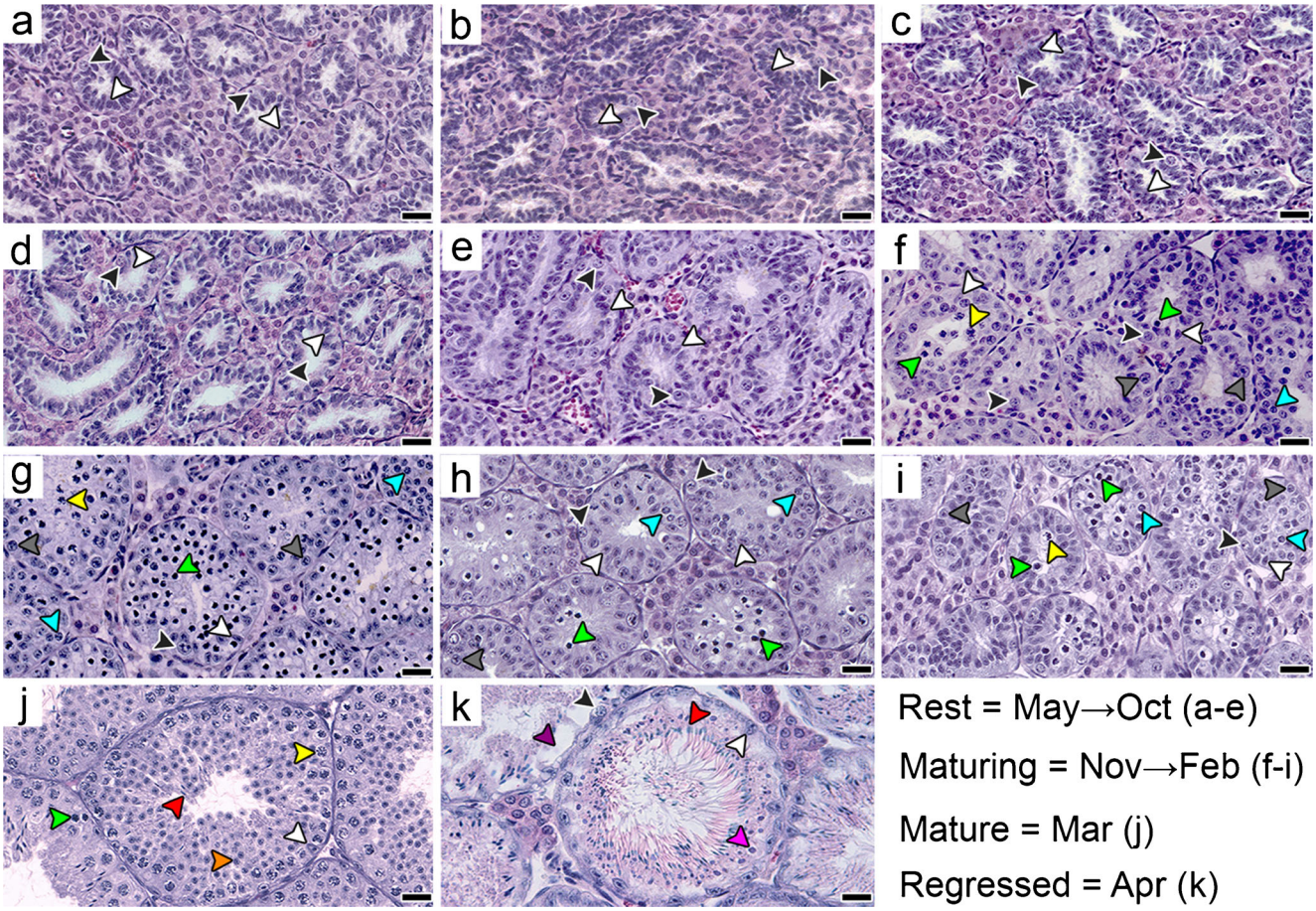

Fig. 1 Testis morphofunctional characteristics along the yellowish myotis reproductive cycle. The images represent the variation of the cellular composition of testis parenchyma (**a** to **k**) throughout the reproductive stages, i.e. Rest (May to October, **a** to **e**), Maturing (November to February, **f** to **i**), Mature (March, **j**) and Regressed (April, **k**) from 2017 to 2018. The month of June is not represented because the colony left their roost. Arrowheads: white (Sertoli cell), black (undifferentiated spermatogonia), grey (differentiated spermatogonia), blue (pre-leptotene spermatocyte), green (zygotene spermatocyte), yellow (pachytene spermatocyte), orange (round spermatid), red (elongated spermatid), pink (residual bodies), purple (vacuoles). Bar: 20 μm

spermatozoa (*). Considering all analysed bats within the period of study, 15.3% of them were in the Regressed stage.

Furthermore, there is an individual variation of the reproductive stages in the yellowish myotis colony throughout the year (Fig. 3a and b). The prevalence of the Rest stage is concentrated in the months from May to October, following by the Maturing stage in the months from November to February. The Mature stage shows a higher prevalence in March while the Regressed stage in April. After this initial in-depth description, the following data were obtained using bats collected in a representative month of each reproductive stage ($n = 6$ per group; Supplementary Table 1).

Rainfall distribution is concentrated from October to March

Higher levels of rainfall in Santuário do Caraça are reached from October to March (rainy season), summing up to a mean of 209.06 mm per month (Santuário do Caraça weather station). On the other hand, during the dry season (April to September), rainfall reaches a mean of 37 mm per month. The distribution of mean rainfall shows that gonadal maturation at the Maturing stage coincides with higher levels of rainfall (Rest = 132.00 ± 20.60 mm; Maturing = 1130 ± 219.7 mm; Mature = 192.1 ± 110.00 mm; and Regressed = 47.60 ± 36.80 mm) (ANOVA, $F = 16.53$, $P = 0.0102$) (Fig. 4).

Testis mass reaches its maximum value at the Mature stage, while the epididymis mass does so at the Regressed stage

The body mass of *M. levis* did not show a significant difference throughout the year or among the reproductive stages (Supplementary Table 2, Fig. 5a). Contrarily, testis mass (0.0600 ± 0.0060 g) and GSI (1.0800 ± 0.1262%) reached the highest values at the Mature stage during the rainy season, while the epididymis mass (0.0124 ±

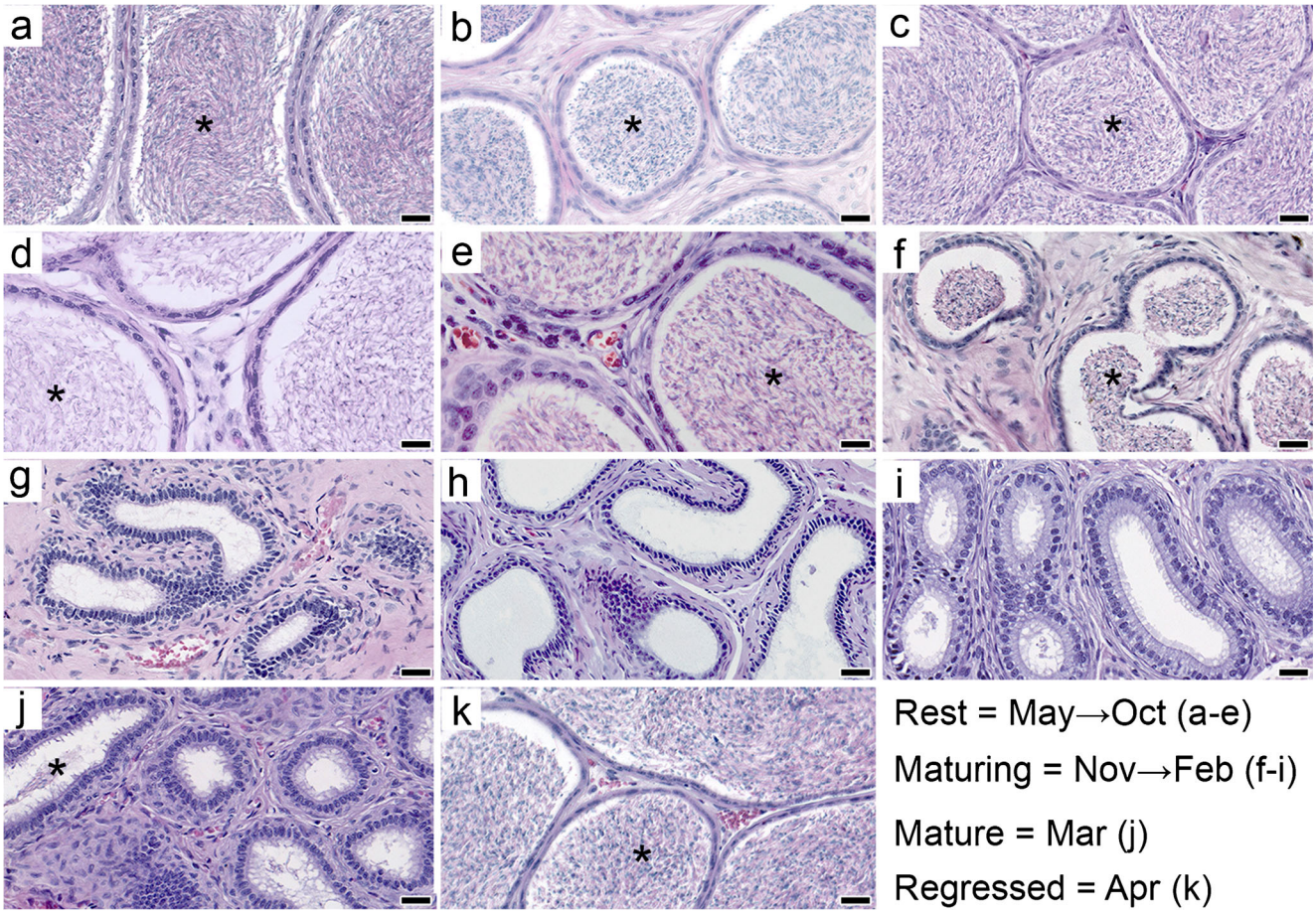

Fig. 2 Epididymis morphofunctional characteristics along the yellowish myotis reproductive cycle. The images represent the occurrence of sperm (*) in the epididymis lumen (**a** to **k**) throughout the reproductive stages, i.e. Rest (May to October, **a** to **e**), Maturing (November to February, **f** to **i**), Mature (March, **j**) and Regressed (April, **k**) from 2017 to 2018. The month of June is not represented because the colony left their roost. Bar: 20 μm

0.0017 g) presented the highest absolute values in the Regressed stage during the dry season (Supplementary Table 2, Fig. 5b–d).

The storage of sperm in the epididymis cauda starts in the Mature stage (March) due to the progression of spermatogenesis and increases during the Regressed stage (April). Surprisingly, three individuals still had spermatozoa in their epididymis cauda in the Maturing stage (November), characterizing 8 months of sperm storage.

The brown adipose tissue mass and accessory sex gland mass present the highest values in the dry season

Similar to the epididymis mass, the accessory sex gland mass (0.0619 ± 0.0056 g) also showed higher values in the Regressed stage (Fig. 5e). Besides, the brown adipose tissue mass reached higher absolute values in the Regressed and Rest stages, followed by a decrease in the following reproductive stages, coinciding with the development of spermatogenesis (Fig. 5f). Both parameters displayed different patterns when compared with the testis mass and GSI (Fig. 5b and c).

The diameter of seminiferous tubules and height of seminiferous epithelium reached the highest values in the Mature stage

The testis morphometric parameters exhibited huge variations between the reproductive stages, reaching the highest values in the Mature stage during the rainy season, similarly to the testis mass and GSI (Fig. 5b and c). Regarding the tubular compartment, the diameter of the seminiferous tubules (Fig. 6a) increased from the Rest to the Mature stage, followed by a significant reduction in the Regressed stage (Rest = 46.54 ± 3.30 μm; Maturing = 59.74 ± 3.47 μm; Mature = 136.00 ± 9.65 μm; Regressed = 77.82 ± 6.55 μm) (ANOVA, $F = 39.13$, $P < 0.0001$). Similarly, the height of the seminiferous epithelium (Fig. 6b) also increased until the Mature stage and decreased in the Regressed stage (Rest = 14.82 ± 1.37 μm; Maturing = 20.65 ± 1.89 μm; Mature = 45.69 ± 3.06 μm; Regressed = 21.06 ± 2.93 μm) (ANOVA, $F = 32.23$, $P < 0.0001$).

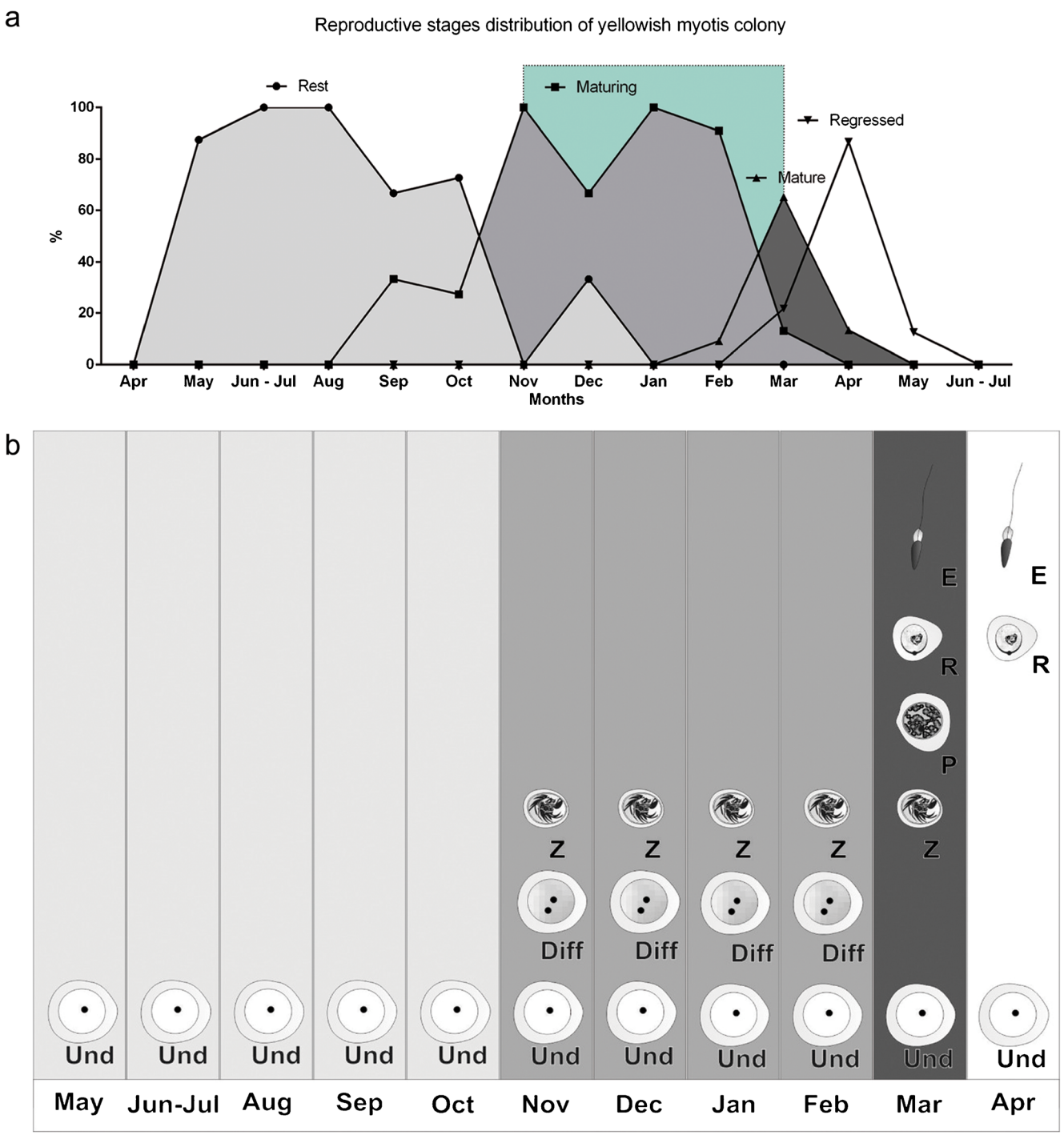

Fig. 3 Variation of the reproductive stages and seminiferous epithelium cellular composition of yellowish myotis colony throughout the year. Note that the testes remain in the Rest stage for 6 months of the year (**a**), and seminiferous epithelium shows only Sertoli and undifferentiated spermatogonial cells (Und) (**b**). The Maturing stage coincides with the beginning of the rainy season (**a**), presenting differentiated spermatogonial cells (Diff) and zygotene spermatocytes (Z) in the seminiferous epithelium (**b**). The Mature stage occurs in a short period (**a**), and the seminiferous epithelium is composed of advanced germ cells as pachytene spermatocytes (P), round (R) and elongated spermatids (E). The Regressed stage coincides with the beginning of the dry season (**a**) and shows undifferentiated spermatogonial cells (Und), and round (R) and elongated spermatids (E) in the seminiferous epithelium. The coloured area represents the rainy season

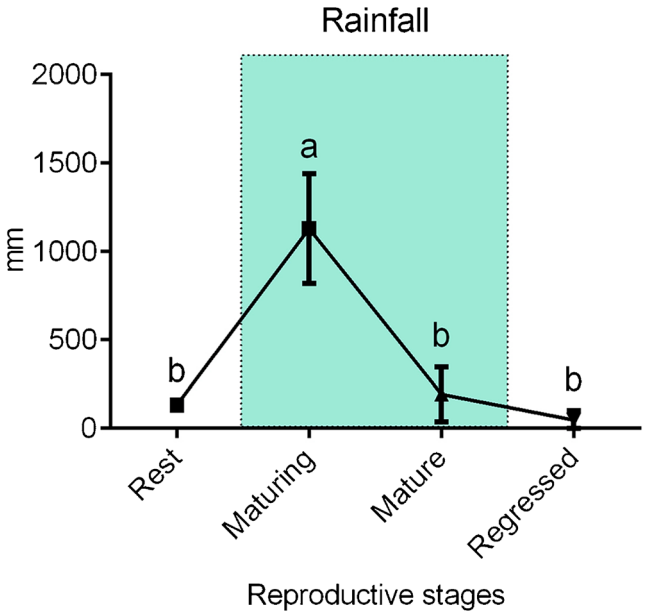

Fig. 4 Rainfall distribution (mm ± SEM) in Santuário do Caraça from January 2017 to December 2018. Note that the peak of rainfall coincides with the Maturing stage. Different letters show statistically significant differences, $P < 0.05$. The coloured area represents the rainy season

The volumetric proportion shows large variation between the Rest and Mature stages

The tubular compartment presented a 30% variation between the Rest and Mature stages (Table 1). The seminiferous epithelium and lumen were the parameters responsible for that high variation (Table 1). Contrarily, tunica propria, Sertoli and spermatogonial cell proportion were reduced during the Mature stage (Table 1). A remarkable difference among the reproductive stages was the presence of seminiferous epithelium vacuoles observed in the Regressed stage (Fig. 1, Table 1).

Regarding the intertubular compartment, Leydig cells presented the highest proportion in the Rest and Maturing stages (Table 1), coinciding with the lowest tubular diameter in these stages (Fig. 6a). The blood vessels (%) were decreased only in the Mature stage (Table 1).

The Leydig cell parameters presented structural and molecular alterations for the maximum production of testosterone in the Mature stage

The morphometric parameters of Leydig cells, such as nuclear diameter (5.36 ± 0.09 μm), cellular volume (414.00 ± 43.24 μm^3), cytoplasmic volume (333.20 ± 39.68 μm^3) and nuclear volume (80.77 ± 3.94 μm^3), reach their maximum value in the Mature stage during the rainy season (Supplementary Table 3, Fig. 6c–f).

The steroidogenic enzyme 3-Beta-HSD is expressed in the cytoplasm of Leydig cells in all reproductive stages (Fig. 7). The pixel intensity evaluation revealed significant difference among the reproductive stages, reaching the highest expression of 3-Beta-HSD in the Mature stage (Rest = 2.46 ± 0.14; Maturing = 2.68 ± 0.18; Mature = 4.47 ± 0.23; Regressed = 2.94 ± 0.18) (Kruskal-Wallis test, $H = 13.54$, $P = 0.0036$) (Fig. 6g).

Serum testosterone levels varied significantly among the reproductive stages, reaching the highest value in the Mature stage (Rest = 345.1 ± 95.6 ng/dL; Maturing = 1829.0 ± 755.5 ng/dL; Mature = 3146.0 ± 663.6 ng/dL; Regressed = 1301.0 ± 371.5 ng/dL) (Kruskal-Wallis test, $H = 13.15$, $P = 0.0043$) (Fig. 6h).

Cell apoptosis is a remarkable event in the Regressed stage

Cell apoptosis was evaluated through Caspase-3 staining and differed among the reproductive stages (Fig. 8). In the Rest stage (Fig. 8a and b), no apoptosis was detected in the tubular compartment. In the Maturing stage (Fig. 8c and d), apoptosis occurred in few Sertoli cells, few primary spermatocytes in pachytene and many in zygotene. In the early Mature stage, apoptosis occurred in pachytene, zygotene spermatocytes and round spermatids (Fig. 8e and f). In the late Mature stage (Fig. 8g and h), round and elongated spermatids were observed in apoptosis. In the early Regressed stage, apoptosis occurred most frequently in the round and elongated spermatids and beyond that in spermatozoa present in the lumen of seminiferous tubules (Fig. 8i and j). In the late Regressed stage, apoptosis occurred in the remaining spermatids in the seminiferous epithelium (Fig. 8k and l).

Cell proliferation was prominent during the Maturing stage

Cell proliferation was evaluated through Ki-67 staining and differed among the reproductive stages (Fig. 9). In the Rest stage, proliferation occurred in Sertoli cells (Fig. 9a and b). Confirmation of proliferative Sertoli cells was achieved through the GATA-4 immunostaining in seriated histological sections. In the Maturing stage, germ cell proliferation occurred in spermatogonial cells, pre-leptotene, zygotene and few pachytene spermatocytes (Fig. 9c and d). In the Mature stage, proliferation occurred in few zygotene, pachytene, diplotene and secondary spermatocytes (Fig. 9e and f). In the Regressed stage, no cell proliferation was observed (Fig. 9g and h).

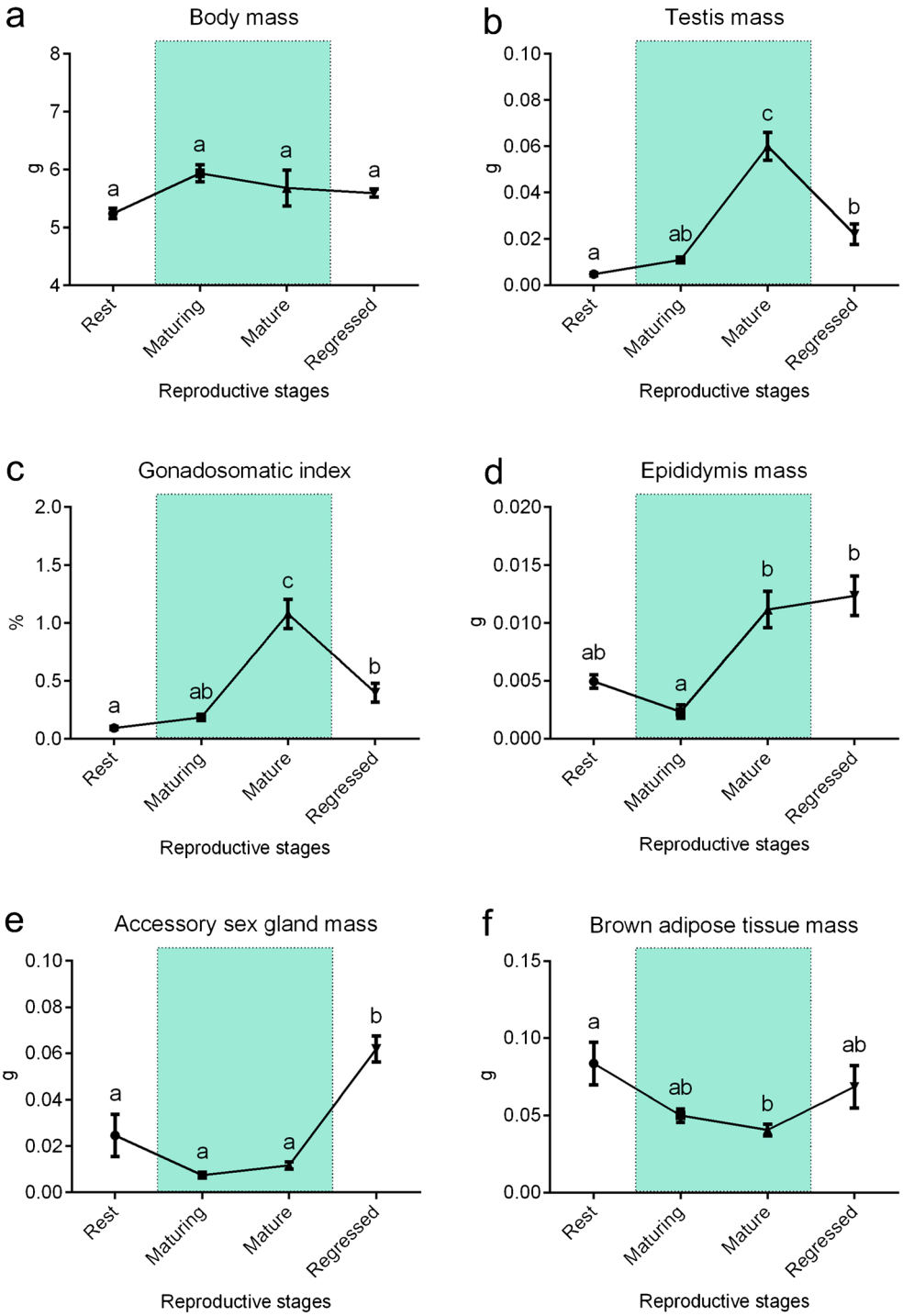

Fig. 5 Mean (± SEM) values of biometric data of yellowish myotis in the reproductive stages. The body mass does not differ among the reproductive stages (**a**). As expected, the testis mass (**b**) and GSI (**c**) present higher weights in the Mature stage, while the epididymis mass (**d**) in the Regressed stage. The higher values of accessory sex gland mass (**e**) and brown adipose tissue mass (**f**) are concentrated in the dry season. Different letters show statistically significant differences, $P < 0.05$. The coloured area represents the rainy season

There was a strong correlation between several testicular parameters and rainfall

There was a strong positive correlation between rainfall and testis mass, GSI, tubular compartment volume density, diameter of the seminiferous tubules, height of seminiferous epithelium, lumen, cellular volume of Leydig cells, serum levels of testosterone and 3-Beta-HSD pixel intensity (Table 2, part a). On the other hand, a strong negative correlation was observed between rainfall and intertubular compartment volume density, Leydig cell volume density, Spermatogonial cells volume density and Sertoli cells volume density (Table 2, part b).

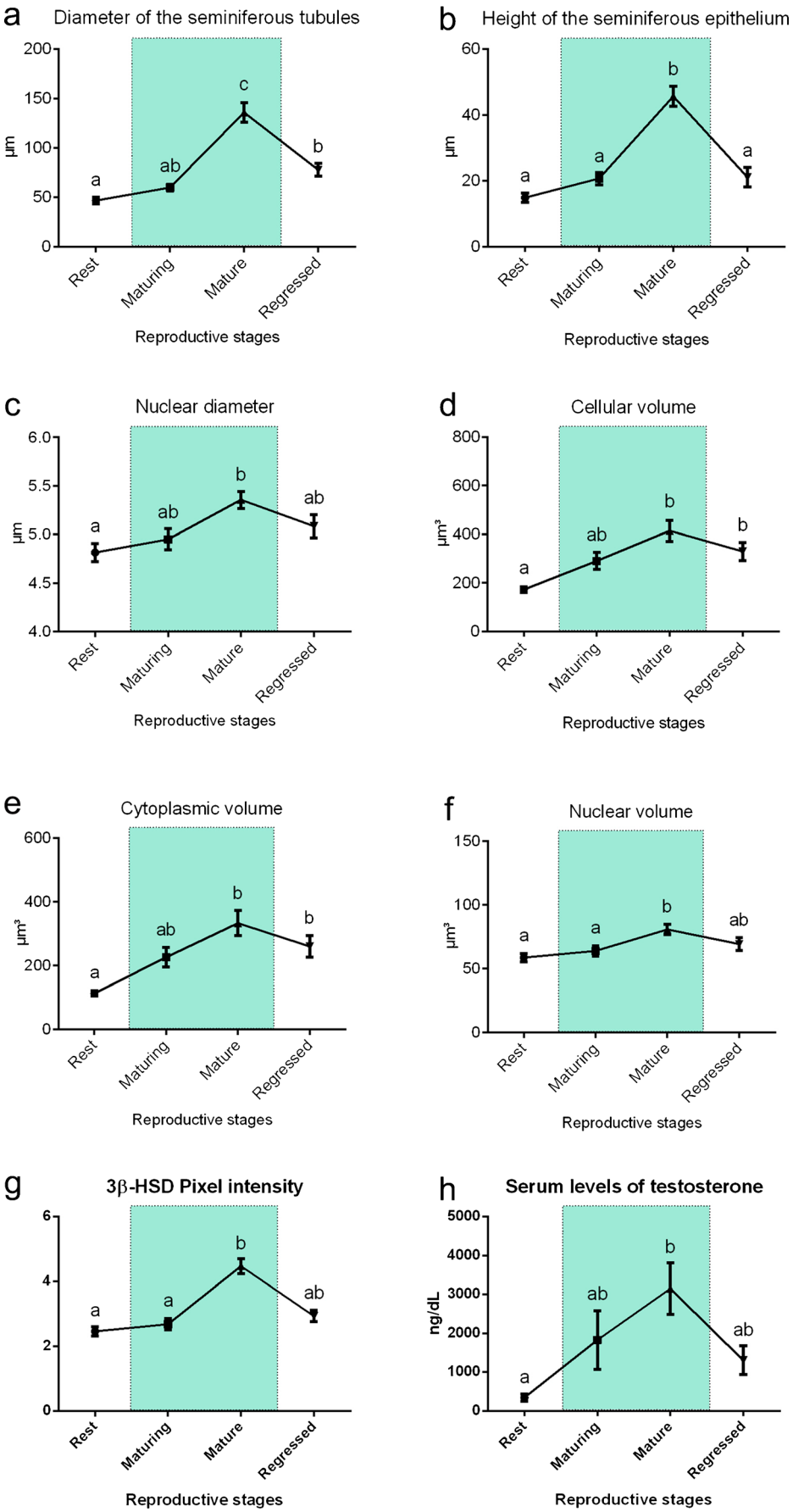

Fig. 6 Mean (± SEM) values of testicular morphometric data of yellowish myotis in the reproductive stages. Note that the highest values of seminiferous tubule morphometric data, such as the diameter of the seminiferous tubules (**a)** and height of the seminiferous epithelium (**b**), are concentrated in the Mature stage. The values of Leydig cell parameters, such as nuclear diameter (**c**), cellular volume (**d**), cytoplasmic volume (**e**), nuclear volume (**f**), 3-Beta-HSD pixel intensity (**g**) and serum testosterone levels (**h**), are concentrated in the Mature stage. Statistically significant differences are represented by different letters, $P < 0.05$. The coloured area represents the rainy season

Table 1 Mean (% ± SEM) values of the volumetric proportion of testicular parenchyma of yellowish myotis in the reproductive stages

Parameters	Rest ($n = 6$)	Maturing ($n = 6$)	Mature ($n = 6$)	Regressed ($n = 6$)	ANOVA *(F)* *Kruskal-Wallis *(H)*	*P* value
Tubular compartment	64.7 ± 4.8^{a}	74.9 ± 4.6^{a}	95.6 ± 0.5^{b}	89.2 ± 2.6^{b}	15.10	< 0.0001
Tunica propria	9.1 ± 0.8^{a}	9.5 ± 0.5^{a}	3.2 ± 0.1^{b}	8.1 ± 0.9^{ab}	14.01*	0.0029
Seminiferous epithelium	53.9 ± 4.0^{a}	62.6 ± 4.7^{ab}	75.1 ± 2.0^{b}	64.2 ± 3.9^{ab}	11.21*	0.0107
Sertoli cell	31.4 ± 2.6^{a}	17.2 ± 1.6^{b}	4.3 ± 0.6^{c}	17.0 ± 3.3^{b}	24.25	< 0.0001
Spermatogonial cells	2.5 ± 0.5^{a}	2.5 ± 0.5^{a}	0.1 ± 0.0^{b}	1.1 ± 0.2^{ab}	10.83	0.0002
Vacuole	n. d.	n. d.	n. d.	3.2 ± 1.5	-	-
Lumen	1.7 ± 1.0^{a}	2.8 ± 0.7^{a}	17.3 ± 2.1^{b}	16.9 ± 1.0^{b}	45.72	< 0.0001
Intertubular compartment	35.3 ± 4.8^{a}	25.1 ± 4.6^{a}	4.4 ± 0.5^{b}	10.8 ± 2.6^{b}	15.10	< 0.0001
Leydig cell	30.7 ± 4.7^{a}	23.0 ± 4.6^{a}	1.3 ± 0.3^{b}	8.2 ± 1.8^{b}	15.66	< 0.0001
Blood vessels	2.2 ± 0.4^{a}	1.4 ± 0.2^{ab}	0.2 ± 0.1^{b}	2.1 ± 0.9^{ab}	3.55	0.0330
Connective tissue	2.4 ± 0.4^{a}	0.7 ± 0.1^{ab}	2.9 ± 0.3^{a}	0.5 ± 0.1^{b}	18.83*	0.0003

Different line superscript letters show statistically significant differences, $P < 0.05$. N.d. = non-detected

Regarding testis mass, there was a strong positive correlation with the tubular compartment volume density, the diameter of the seminiferous tubules and the height of the seminiferous epithelium (Table 2, part c). Regarding the testosterone serum levels, a positive correlation was observed with epididymis mass, accessory sex gland mass, the cellular volume of Leydig cells and 3-Beta-HSD pixel intensity (Table 2, part d). Considering only the Rest and the Mature stages, in which the testosterone serum levels differed significantly, a negative correlation ($r = -0.7$) was also observed between hormone level and the brown adipose tissue.

Discussion

The present study reinforced that yellowish myotis presents a seasonal pattern of reproduction since sperm production occurs within a restricted time (1 month) of the year. The spermiogenic phase is initiated in the Mature stage (March) and concluded in the Regressed stage (April). Moreover, the gonads persist in the Rest and Maturing stage for 6 and 4 months, respectively. Considering that analyses in Rest and Maturing stages were carried out in only 1 month, future

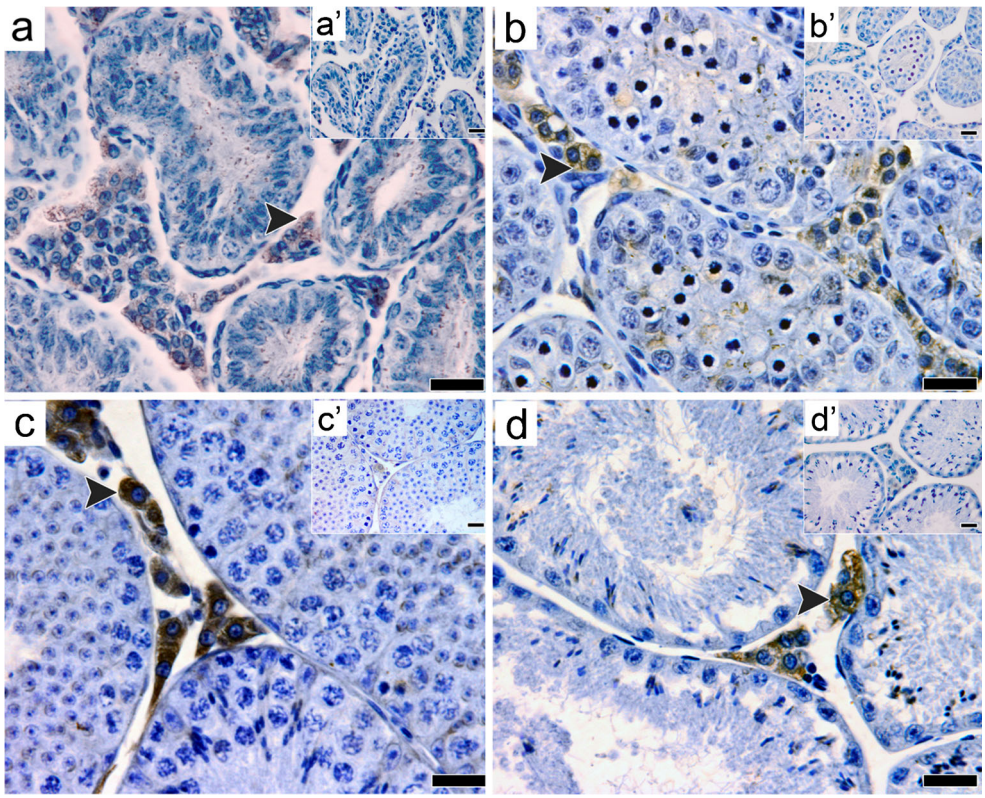

Fig. 7 3-Beta-HSD staining in the reproductive stages of yellowish myotis. 3-Beta-HSD enzyme was observed at different levels in Leydig cells cytoplasm (black arrowheads) in the Rest (**a**), Maturing (**b**), Mature (**c**) and Regressed (**d**) stages. Inserts (a' to d') show the negative controls. Bars: 20 μm

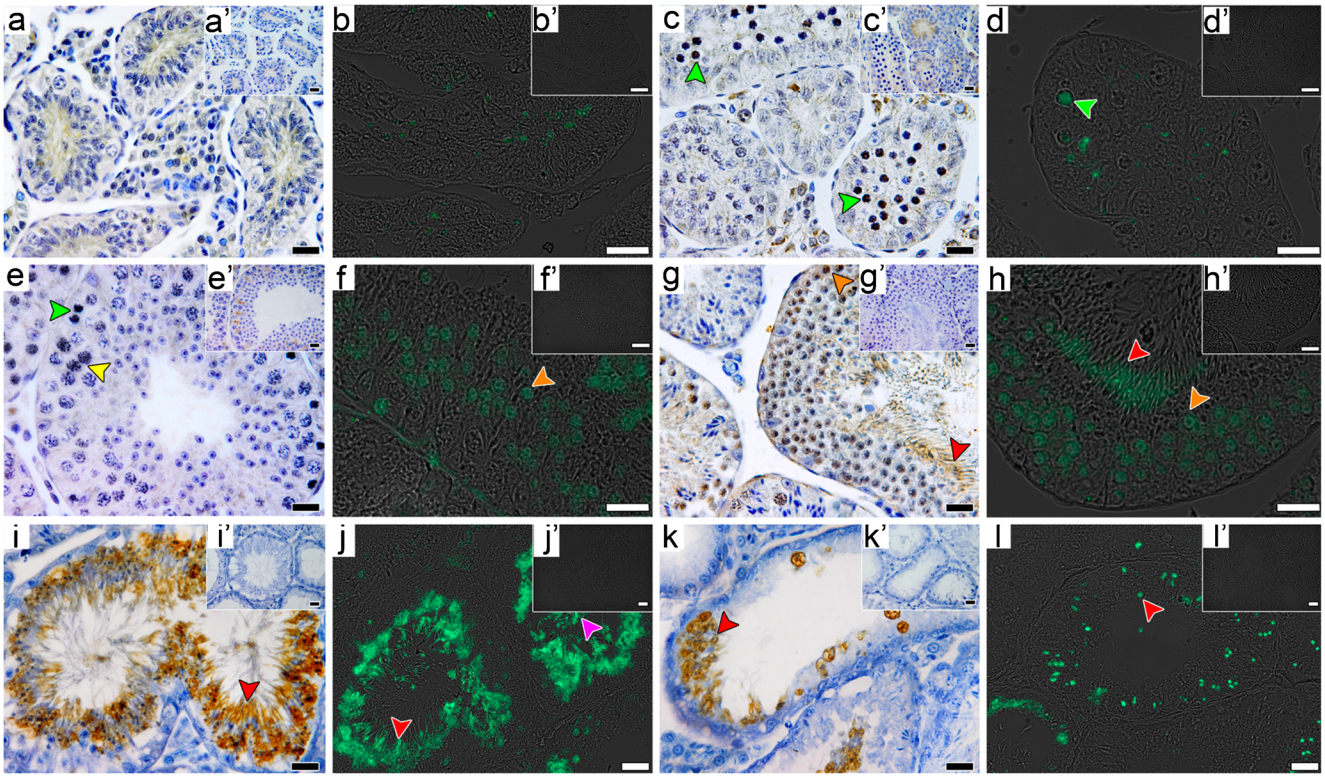

Fig. 8 Caspase-3 staining in the reproductive stages of yellowish myotis. There is an absence of cell apoptosis in the Rest stage (**a** and **b**). In the Maturing stage, green arrowheads show apoptotic zygotene spermatocytes (**c** and **d**). In the early Mature stage, apoptosis was seen in pachytene (**e**, yellow arrowhead) and zygotene spermatocytes (**e**, green arrowhead) and round spermatids (**f**, orange arrowhead). In the late Mature stage, apoptotic round (**g** and **h**, orange arrowhead) and elongated spermatids (**g** and **h**, red arrowhead) were observed. In the early Regressed stage, apoptotic round and elongated spermatids (**i** and **j**, red arrowhead) and spermatozoa (**j**, pink arrowhead) were found. In the late Regressed stage, apoptosis was seen in remaining spermatids (**k** and **l**, purple arrowheads). Inserts (a' to l') show the negative controls. Bars: 20 μm

investigations should be performed in other months (within these stages) to evaluate possible cellular, molecular and hormonal variations. However, it is worth mentioning that samples obtained for the in-depth evaluation were collected from October to April. This period corresponds to the shortest time (7 months) needed to investigate all reproductive stages of yellowish myotis. In general, we demonstrated that gonadal reactivation of yellowish myotis occurred concurrently with the maximum testosterone production after a rainfall peak distribution. Furthermore, testosterone levels were positively correlated with Leydig cell 3-Beta-HSD expression and seemed to be associated with the brown adipose tissue mass. These events were followed by the hypertrophy of the accessory sex gland and at least 8 months of sperm storage in epididymis cauda, coinciding with the beginning of the next reproductive cycle (Fig. 10).

Reproduction involves costs of energy and individual body condition (Clutton-Brock et al. 1989), which is dependent on the availability of food resources and presents a strong relationship with sexual development (Speakman and Racey 1986; Crichton and Krutzsch 2000). Herein, as observed for other vespertilionid bats in the tropics (McWilliam 1988; Racey and Entwistle 2000), it was demonstrated that the seasonal reproductive pattern of male *M. levis* was strongly related with rainfall, instead of with temperature (Araújo et al. 2013). The availability of insects follows the rainfall pattern, reaching a peak in the rainy season (Janzen and Schoener 1968; Racey 1982; McWilliam 1988; Rautenbach et al. 1988; Racey and Entwistle 2000). Differently, in the case of seasonal vespertilionid bats from temperate zones, temperature cycles and food availability are the main environmental factors regulating their reproduction (Wilson 1979).

Several studies have shown the influence of rainfall on the reproduction of Neotropical insectivorous bats (Wilson 1979; O'Shea and Vaughan 1980; McWilliam 1988; Happold and Happold 1989, 1990; Cumming and Bernard 1997); however, as the yellowish myotis present high testicular plasticity (cell remodelling) during their reproductive stages, the effect of this environmental factor is even more emphasized. Therefore, yellowish myotis present gonadal reactivation and development of several testicular parameters coinciding with the peak of rainfall. Rainfall and possibly the increase in food availability promote a rise in testis mass, number of germ cells and androgen production. As observed in several studies (Racey

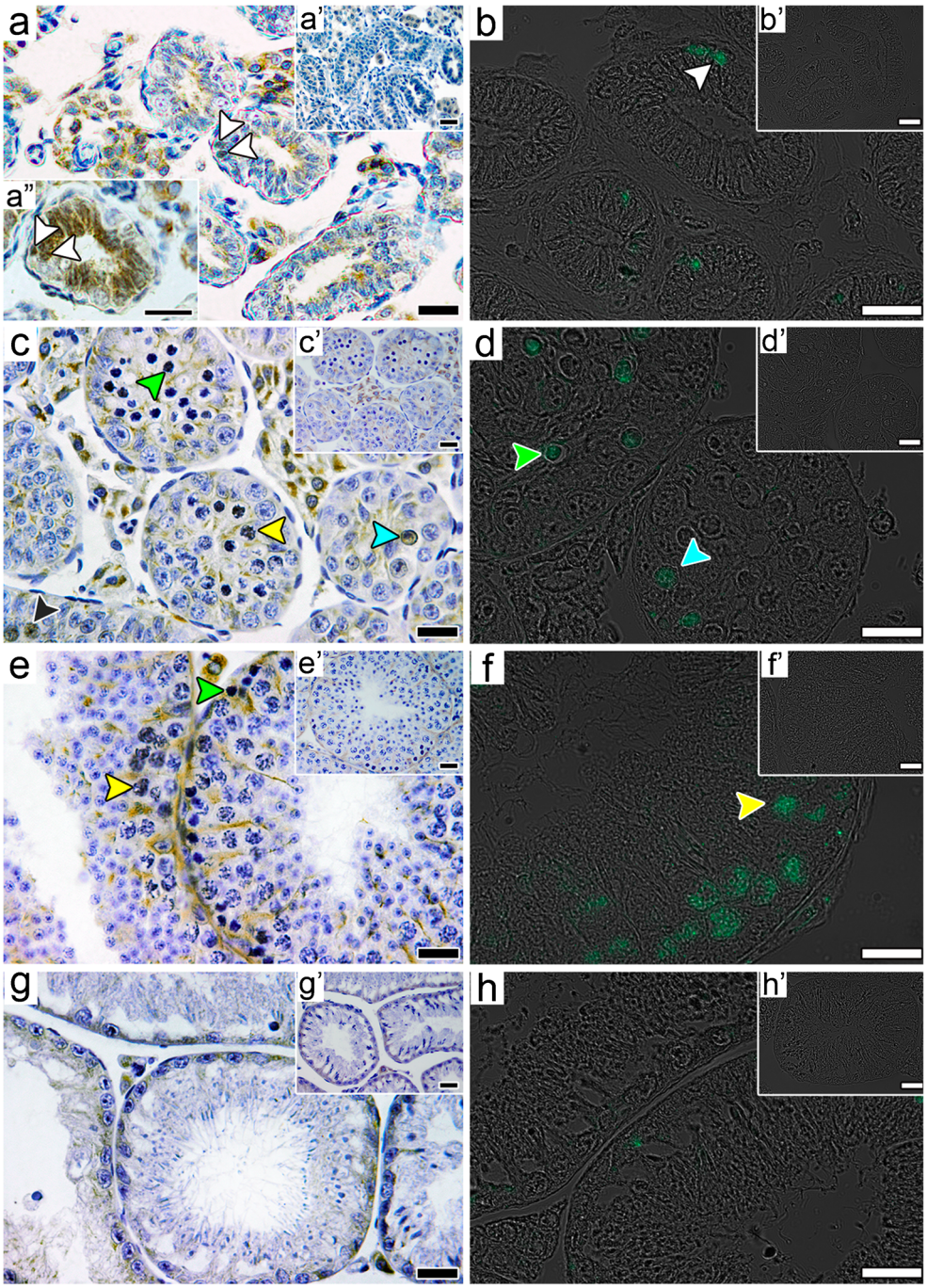

Fig. 9 Ki-67 staining in the reproductive stages of yellowish myotis. In the Rest stage, cellular proliferation was observed in Sertoli cells (**a** and **b**, white arrowheads). In the Maturing stage, arrowheads show proliferation in pre-leptotene (**c** and **d**, blue arrowheads), zygotene (**c** and **d**, green arrowheads) and pachytene spermatocytes (**c**, yellow arrowheads). In the Mature stage, cellular proliferation was observed in zygotene (**e**, green arrowhead) and pachytene spermatocytes (**e** and **f**, yellow arrowheads). There was an absence of cell proliferation in the Regressed stage (**g** and **h**). Top inserts (a' to h') show the negative controls. The bottom insert (a") shows the GATA-4 immunolabelling. Bars: 20 μm

1974; Gustafson and Shemesh 1976; Crichton 2000; Krutzsch 2000), androgen support promotes the maintenance of epididymal sperm and hypertrophy of accessory sex glands.

While testis mass and GSI peak occur in the Mature stage, when the development of the spermatogenic process occurs, the epididymis mass peak occurs in the Regressed stage due to sperm storage, similar to vespertilionids in tropical and temperate regions (Miller 1939; Gustafson 1979; Racey 1979; Krutzsch and Crichton 1986; Bernard et al. 1997; Hosken et al. 1998; Sharifi et al. 2004, 2008; Krutzsch 2009; Pfeiffer and Mayer 2012). Sperm storage in Neotropical vespertilionids may occur from 1 to 3 months (Medway 1972; Myers 1977; Racey 1979; Bernard et al. 1997), whereas, in hibernating vespertilionids, it occurs for a longer period, i.e. from 5 (Racey 1972) to 10 months (Racey 1979). Although the yellowish myotis is not a hibernating bat, it was demonstrated, for the first time in this study, that its sperm storage also occurs for a long period, i.e. 8 months.

Accessory sex glands are androgen-dependent (Racey 1974; Krutzsch 2000; Puga et al. 2016), and the data of the present study suggest that positive stimuli begin in the Mature stage in which levels of serum testosterone are elevated. The

Table 2 Spearman correlation among biometric and reproductive parameters of yellowish myotis and rainfall distribution in Santuário do Caraça

		Parameters	Correlation
A	Rainfall	Testis mass	$r = 0.92$
		Gonadosomatic index	$r = 0.92$
		Tubular compartment volume density	$r = 0.87$
		Diameter of the seminiferous tubules	$r = 0.92$
		Height of seminiferous epithelium	$r = 0.78$
		Lumen	$r = 0.84$
		Cellular volume of Leydig cells	$r = 0.76$
		Serum levels of testosterone	$r = 0.79$
		3-Beta-HSD pixel intensity	$r = 0.82$
B	Rainfall	Intertubular compartment volume density	$r = -0.87$
		Leydig cell volume density	$r = -0.90$
		Spermatogonial cells volume density	$r = -0.83$
		Sertoli cells volume density	$r = -0.84$
C	Testis mass	Tubular compartment volume density	$r = 0.79$
		Diameter of the seminiferous tubules	$r = 0.83$
		Height of seminiferous epithelium	$r = 0.79$
D	Serum levels of testosterone	Epididymis mass	$r = 0.72$
		Accessory sex gland mass	$r = 0.82$
		Cellular volume of Leydig cells	$r = 0.70$
		3-Beta-HSD pixel intensity	$r = 0.73$

hypertrophy (alteration in mass) of the epididymis and accessory sex gland is observed in the Regressed stage, depicting a temporal asynchrony with spermatogenesis in the Mature stage. This pattern is also observed in many vespertilionid species (Gustafson and Shemesh 1976; Gustafson 1979; Racey 1979; Hosken et al. 1998; Encarnação et al. 2004; Krutzsch 2009). Although further studies are necessary to confirm the involvement of the brown adipose tissue in the reproductive cycle of yellowish myotis, the fluctuation of its mass along the reproductive stages suggests a possible androgenic role (Krutzsch and Wells 1960). Observing the negative correlation observed between testosterone serum levels and the brown adipose tissue mass in the Rest and Mature stages, one can say that the brown adipose tissue may be linked to testosterone synthesis since this process depends on recruitment and conversion of cholesterol molecules (Zirkin and Papadopoulos 2018).

The testicular parameters of tubular compartment volume density, tubular diameter and height of seminiferous epithelium present a positive correlation with rainfall. Fluctuations of germ cell composition in non-hibernating yellowish myotis are described in detail along the reproductive stages in the present study and present a similar pattern of seminiferous epithelium cytoarchitecture in vespertilionids from temperate regions (Miller 1939; Pearson et al. 1952; Racey and Tam 1974; Krutzsch 1975; Gustafson 1979; Krutzsch 2009). Besides, germ cell arrest in zygotene spermatocyte for a long time (in the Maturing stage) regulates the kinetics and development of other germ cells. Probably, a cellular checkpoint occurs at this stage and may be involved in the preparation for maximum sperm production. The presence of primary spermatocytes as the most advanced germ cells in the seminiferous epithelium, after a testis rest, occurs in the vespertilionids *Myotis lucifugus*, *M. grisescens*, *M. velifer, Pipistrellus pipistrellus* and *P. hesperus*, but for a shorter period (Miller 1939; Racey and Tam 1974; Krutzsch 1975; Gustafson 1979; Krutzsch 2009).

There is a positive correlation among testis mass, tubular diameter and height of seminiferous epithelium, and the fluctuation of these parameters is similar to many vespertilionids during the reproductive cycle (Medway 1972; Racey 1974; Racey and Tam 1974; Gustafson 1979; Bernard et al. 1991, 1997). The development of the seminiferous epithelium occurs due to the increase of germ cell differentiation during Maturing and Mature stages. Possible factors, such as retinoic acid and testosterone, may be involved in spermatogonial cell differentiation in the Maturing stage and spermatocyte differentiation in the Mature stage (Ismail and Morales 1992; van Pelt et al. 1995; Ogawa et al. 1998; Dobrinski et al. 2001; Hess and França 2007; Tanaka et al. 2016; Lara et al. 2018). Herein, the increase in serum testosterone levels coincides with the differentiation of spermatogonial cells. The differentiation of spermatogonial cells may occur for a short period since missing germ cell layers are observed throughout the spermatogenic process. Interestingly, even in the Mature stage, when the spermatogenic process is fully developed, not all germ cells are present.

The intertubular compartment presented a negative correlation with rainfall due to enlargement of the seminiferous tubules. Although this compartment was reduced, for the first

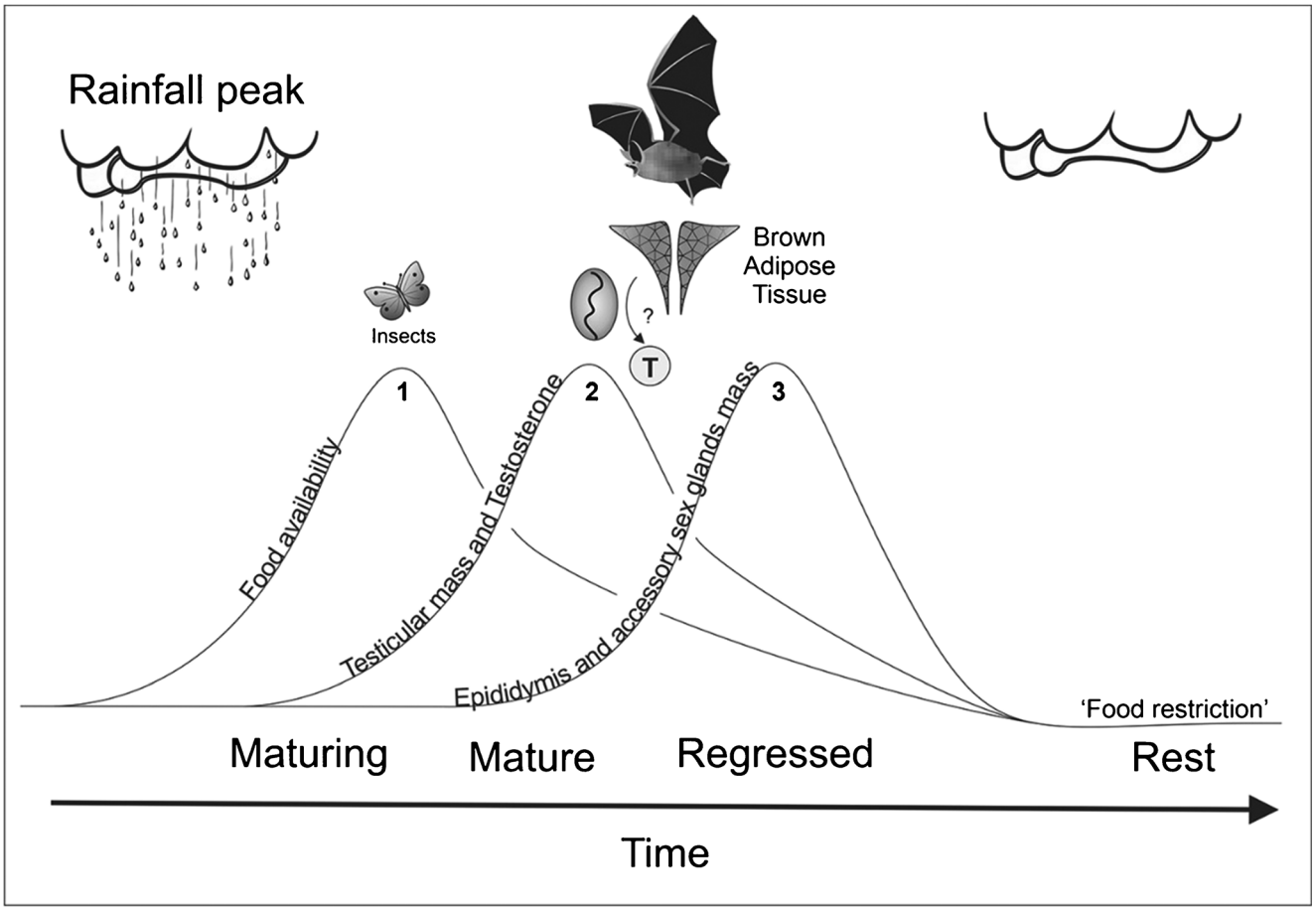

Fig. 10 Reproductive function of yellowish myotis linked to rainfall distribution. Based on literature, it is expected an increase in food availability (e.g. insects, line 1) after periods of rain. Herein, after a rainfall peak, we observed a sequence of events, being the increment of testis mass and testosterone levels (line2) followed by the epididymis and accessory sex glands hypertrophy (line 3)

time, it was demonstrated that Leydig cell nuclear diameter and cellular volume of Leydig cells, expression of 3-Beta-HSD and serum levels of testosterone were increased during the spermatogenic activity in the Mature stage of yellowish myotis. In other seasonal vespertilionid bats, spermatogenic activity coincided with morphological and functional changes in Leydig cells. An increase in nuclear diameter (Racey 1974; Bernard et al. 1991; Hosken et al. 1998), testicular testosterone concentrations (Racey and Tam 1974; Hosken et al. 1998) and serum levels of testosterone (Gustafson and Shemesh 1976; Gustafson and Damassa 1985) has been reported in these species. Expression of 3-Beta-HSD occurred in Leydig cells cytoplasm of seasonal pipistrelle and rhinolophid bats throughout the year (Racey and Tam 1974; Kurohmaru et al. 2002); however differences in intensity were observed in the latter species only. In the Rhinolophid bat, strong intensity occurred just before the beginning of spermatogenesis, while the weak intensity occurred prior to the interruption of spermatogenesis and the hibernation period (Kurohmaru et al. 2002). Differently, the strongest intensity of 3-Beta-HSD expression in yellowish myotis was achieved in the Mature stage and the weakest intensity in the Rest stage.

For the first time, the balance between cell apoptosis and proliferation dynamics was investigated in Neotropical vespertilionid yellowish myotis using molecular markers. Germ cell apoptosis was evident in the Regressed stage, coinciding with the reduction of serum testosterone levels. It is known that testosterone prevents apoptosis in germ cells, mainly in those located in the spermiogenic phase of spermatogenesis (Gendt et al. 2005; Hess and França 2007). There is an apoptosis regulation in the Regressed stage to prepare testis for the next reproductive stage. In the other reproductive stages, apoptosis was observed in the Maturing and Mature stages, indicating a possible density control of germ cells supported by Sertoli cells (Griswold 1998; Lara et al. 2018). Herein, *M. levis* post-meiotic germ cell apoptosis was observed when bats reach late Mature and Regressed stage. These data were different from what was observed for the Neotropical *Myotis nigricans*, in which the incidence of apoptosis was higher during the peak periods of spermatogenesis (Beguelini et al. 2013). In yellowish myotis, cell proliferation was rarely observed in Sertoli cell in the Rest stage. In this period, Sertoli cells present immature characteristics, such

as less evident nucleolus, known as pre-pubertal-like morphology (Gustafson 1979, 1987). Similarly, proliferation, as well as morphological changes in Sertoli cells, occurs in other adult seasonal animals (Johnson et al. 1991; Tarulli et al. 2006). In the Maturing and Mature stages, cell proliferation occurs mainly in primary spermatocytes. In the Regressed stage, this event is absent due to the presence of few germ cells in the seminiferous epithelium. Testicular factors may be regulating the balance of germ and somatic cells proliferation along the reproductive stages of yellowish myotis (Griswold 1998; Hess and França 2007; Lara et al. 2018).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (Ethics Committee on Animal Use from the Federal University of Minas Gerais - CEUA document 386/2017).

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Cell and Tissue Research

Male reproductive morphofunctional evaluation of a neotropical sperm-storing vespertilionid *Myotis levis* in an environmental context

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Supplementary tables

Supplementary Table 1. Yellowish myotis sampling from January 2017 to December 2018.

Reproductive stages	Months	Number of animals used for histological analyses (n=124)	Number of animals used for specific assays (n=24)
Rest	May	8	-
	June-July	8	-
	August	15	-
	September	3	-
	October	11	6
Maturing	November	8	-
	December	12	6
	January	10	-
	February	11	-
Mature	March	23	6
Regressed	April	15	6

Supplementary Table 2. Mean (± SEM) values (in g except for GSI) of body mass (BM), testis mass (TM), gonadosomatic index (GSI), epididymis mass (EM), accessory gland mass (AGM) and brown adipose tissue mass (BAT) of yellowish myotis in the reproductive stages.

Reproductive stage	BM	TM	GSI	EM	AGM	BAT
Rest (n=6)	5.24 ± 0.09	0.0049 ± 0.0006^{a}	0.0931 ± 0.0131^{a}	0.0050 ± 0.0006^{ab}	0.0246 ± 0.0092^{a}	0.0835 ± 0.0138^{a}
Maturing (n=6)	5.93 ± 0.15	0.0110 ± 0.0011^{ab}	0.1834 ± 0.0142^{ab}	0.0023 ± 0.0006^{a}	0.0075 ± 0.0005^{a}	0.0499 ± 0.0043^{ab}
Mature (n=6)	5.68 ± 0.31	0.0600 ± 0.0060^{c}	1.0800 ± 0.1262^{c}	0.0112 ± 0.0016^{b}	0.0117 ± 0.0015^{a}	0.0405 ± 0.0038^{b}
Regressed (n=6)	5.59 ± 0.07	0.0221 ± 0.0043^{b}	0.3990 ± 0.0810^{b}	0.0124 ± 0.0017^{b}	0.0619 ± 0.0056^{b}	0.0684 ± 0.0135^{ab}
ANOVA *(F)*	2.538	43.79	34.83	16.52*	20.72	3.604
P value	0.0856	< 0.0001	< 0.0001	0.0009	< 0.0001	0.0367

Different column superscript letters show statistically significant differences, $p < 0.05$. *Kruskal-Wallis test.

Supplementary Table 3. Mean (µm ± SEM) values of nuclear diameter of Leydig cell (NDL); and mean (µm³ ± SEM) values of volume of Leydig cell (VL), cytoplasmic volume of Leydig cell (CVL) and nuclear volume of Leydig cell (NVL) of yellowish myotis in the reproductive stages.

Reproductive stage	NDL	VL	CVL	NVL
Rest (n=6)	4.81 ± 0.09^{a}	172.01 ± 10.57^{a}	113.40 ± 8.76^{a}	58.74 ± 3.34^{a}
Maturing (n=6)	4.95 ± 0.11^{ab}	290.40 ± 35.17^{ab}	226.50 ± 31.27^{ab}	63.91 ± 4.07^{a}
Mature (n=6)	5.36 ± 0.09^{b}	414.00 ± 43.24^{b}	333.20 ± 39.68^{b}	80.77 ± 3.94^{b}
Regressed (n=6)	5.08 ± 0.12^{ab}	329.6 ± 37.01^{b}	260.20 ± 33.93^{b}	69.36 ± 4.95^{ab}
ANOVA *(F)*	5.085	8.810	8.867	5.265
P value	0.0089	0.0006	0.0006	0.0077

Different column superscript letters show statistically significant differences, $p < 0.05$.

3.2 Artigo 2

Sperm production and seminal analyses in a Neotropical sperm-storing vespertilionid bat yellowish myotis (*Myotis levis*)

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Sperm production and seminal analyses in a Neotropical sperm-storing vespertilionid bat yellowish myotis (Myotis levis)

--Manuscript Draft--

Manuscript Number:	
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Abstract:	Yellowish myotis is a neotropical vespertilionid bat that presents a seasonal reproduction. The sperm is produced in the Mature stage, stored in the Regressed stage and released in the Rest stage (mating period). Aiming to understand, for the first time, the relationship between testis and epididymis physiology in yellowish myotis reproduction, the spermatogenesis length, sperm production, and seminal parameters were herein evaluated. Fifty-one adult male bats were captured in Santuário do Caraça, Minas Gerais, Brazil. The gonads were collected in the Maturing and Mature stages for histomorphometric and immunohistochemical analyses, whereas the epididymis was evaluated in all reproductive stages for seminal studies. Our results demonstrated that the yellowish myotis spermatogenic process is fast, lasting 31.70 ± 0.15 days. Despite the low Sertoli cell efficiency (6.60 ± 1.23), the high numbers of Sertoli cells per testis enable an elevated sperm production in the Mature stage. The sperm concentration, vitality, and motility presented the highest values in the Regressed stage; however, in this period, an increased incidence of sperm morphological defects was detected. In the following period (Rest stage), a drastic reduction of defective sperm was observed, suggesting that the epididymis may act as a sperm quality control organ before the mating period. Furthermore, the epididymis ability to maintain a long-term sperm-storage was observed in 26.7% of the bats in the Maturing stage. In summary, yellowish myotis presented a fast and high sperm production during the Mature stage, undergoing epididymal sperm selection and storing before the mating period.

To the Editor-in-Chief
Theriogenology
Belo Horizonte, Brazil, 13th of April, 2021

Dear Editor,

I am sharing with you the original manuscript entitled "**Sperm production and seminal analyses in a Neotropical sperm-storing vespertilionid bat yellowish myotis (*Myotis levis*)**" by Farias et al., which is being submitted for publication in the "Theriogenology".

This original MS gives a detailed description of yellowish myotis male spermatogenesis. In this MS, the spermatogenesis duration, description of the seminiferous epithelium cycle stages, and seminal characteristics were investigated along the reproductive stages. Our data demonstrate that yellowish myotis reproductive success depends on the synchronous process between testis and epididymis activity throughout the year. We hope our study will meet the standards required. We confirm that this original study was not submitted to any other journal.

Sincerely yours,

Dr. Guilherme Mattos Jardim Costa

Sperm production and seminal analyses in a Neotropical sperm-storing vespertilionid bat yellowish myotis (*Myotis levis*)

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Abstract

Yellowish myotis is a neotropical vespertilionid bat that presents a seasonal reproduction. The sperm is produced in the Mature stage, stored in the Regressed stage and released in the Rest stage (mating period). Aiming to understand, for the first time, the relationship between testis and epididymis physiology in yellowish myotis reproduction, the spermatogenesis length, sperm production, and seminal parameters were herein evaluated. Fifty-one adult male bats were captured in Santuário do Caraça, Minas Gerais, Brazil. The gonads were collected in the Maturing and Mature stages for histomorphometric and immunohistochemical analyses, whereas the epididymis was evaluated in all reproductive stages for seminal studies. Our results demonstrated that the yellowish myotis spermatogenic process is fast, lasting 31.70 ± 0.15 days. Despite the low Sertoli cell efficiency (6.60 ± 1.23), the high numbers of Sertoli cells per testis enable an elevated sperm production in the Mature stage. The sperm concentration, vitality, and motility presented the highest values in the Regressed stage; however, in this period, an increased incidence of sperm morphological defects was detected. In the following period (Rest stage), a drastic reduction of defective sperm was observed, suggesting that the epididymis may act as a sperm quality control organ before the mating period. Furthermore, the epididymis ability to maintain a long-term sperm-storage was observed in 26.7% of the bats in the Maturing stage. In summary, yellowish myotis presented a fast and high sperm production during the Mature stage, undergoing epididymal sperm selection and storing before the mating period.

Key-words: Chiroptera**,** seasonal reproduction, spermatogenesis duration, sperm production, seminal parameters.

1. Introduction

Yellowish myotis (Myotis levis I. Geoffroy Saint-Hilaire, 1824) is a Neotropical insectivorous vespertilionid bat that presents a seasonal reproductive pattern, testis regression and epididymal sperm storage [1-3], which are reproductive strategies similar to those found in vespertilionids from temperate regions [4-8]. Spermatogenesis is a highly complex germ cell differentiation event that occurs inside the seminiferous tubules and is divided into three phases named spermatogonial (proliferative), spermatocytary (meiotic) and spermiogenic (differentiation) [9-12]. To date, several researchers are investigating spermatogenesis in Neotropical Chiropteran species [13-21] as well as in the neotropical Vespertilionids *Eptesicus furinalis*, *Histiotus velatus*, *Lasiurus blossevillii*, *Myotis albescens*, *Myotis nigricans* and *Myotis levis* [1,3,13,22,23]. The spermatogenic process of yellowish myotis is different from that of several species of Neotropical bats, which present continuous spermatogenesis throughout the year [14,16-19,24]. The seasonal spermatogenic process of yellowish myotis comprises four reproductive stages classified as Rest, Maturing, Mature, and Regressed [2,3].

The testes of yellowish myotis remain in the Rest stage for six months in a year and the reactivation of spermatogenesis occurs in the Maturing stage, which takes four months and coincides with the peak of rainfall distribution [3]. In this stage, cellular proliferation and the emergence of primary spermatocytes in the seminiferous epithelium occur [3]. Germ cell proliferation and differentation leads to the development of spermatogenesis and sperm production in the Mature stage, when the bats present the highest testicular weight and testosterone serum levels [3]. The produced sperm is stored for at least eight months a year inside the epididymal cauda until the surge of the next spermatogenic activity [3]. Therefore, the spermatocitary and spermiogenic phases occur in specific periods of the year and the produced sperm is stored in the epididymides to guarantee female fertilization [1,3].

Germ cells are organized in specific cellular associations giving rise to the stages of the seminiferous epithelium cycle and the duration of their differentiation is considered species-specific, being controlled by the germ cell genotype [11,12,25-27]. In several mammalian species, spermatogenesis takes about 40 to 78 days to be completed [12,28]. Specifically, in neotropical bats, the spermatogenic cycle is usually faster (15 to 37 days), resulting in high sperm production [21,24,29]. Although unique characteristics of

seasonal reproduction in vespertilionid bats have been recorded [6,30], there is a lack of information about the spermatogenesis duration in this family.

Concerning gametes, Chiroptera spermatozoa are similar to that of mammals [31], but there are some morphological differences amongst the families [32] and, in the Vespertilionidae family, the spermatozoa present variations among the genera, especially regarding the relative size and format of the head and acrosome, arrangement and symmetry of the tail components [32]. In the typical bat spermatozoa pattern, the head is dorsoventrally flattened with arrow-like morphology and the nucleus base presents a concavity where the flagellum is attached [33]. The other features of spermatozoa, such as concentration, vitality, motility and abnormalities have been described for phyllostomids, pteropodids and temperate-zone vespertilionid bats [34-39]. Sperm storage in the order Chiroptera occurs in seasonal species of different bat families [4-6,40,41], including seasonal vespertilionid bats from temperate and tropical regions in the world [5,7,8,42-48]. This reproductive strategy in male bats occurs due to temporal differences between the testis and epididymis activity [4,30,49] and, therefore, involves the retention of spermatozoa within the reproductive tract, mainly in the epididymis cauda, for more extended periods [3,5,6,32,44,50].

The knowledge of insectivorous bats reproduction can help conservational programs aiming for the conservation of these animals in nature, promoting the environmental balance, control of disease vectors and agricultural pests [51-56]. Although essential for the species survival, there is little data on spermatogenesis and semen of neotropical vespertilionid bats. In this context, this study aimed to investigate and describe the duration of spermatogenesis, sperm production, the occurrence of long-term sperm storage, sperm morphology and seminal parameters to unveil new reproductive data in yellowish myotis.

2. Material and Methods

2.1. Study area and capture of bats

We studied a colony of yellowish myotis located in Santuário do Caraça, a preserved area in Serra do Caraça, in the southeastern part of Brazil (20°04’30” S, 43°24’28” W). This area is located in the Iron Quadrangle geomorphological domain, with 10,187 ha and elevations of 750 to 2,072 meters [57,58]. The reserve has a great diversity of fauna and flora due to its location in a transition region of the biomes Atlantic Forest and

Cerrado [57,59].

Bats were captured from January 2019 to November 2020 using mist-nets installed in the attic of Santuário do Caraça church, from 18:00h to 00:00h. Twenty bats in the Maturing and Mature stages were used for gonad histomorphometric and immunohistochemical evaluations. Additionally, 31 bats in all reproductive stages were used for seminal investigations (Table 1). Forearm length, body mass, age class and sex of each individual were recorded, and only adult males were used in the study, being differentiated from subadults by the presence of ossified finger epiphyseal cartilages in the metacarpus [60] and complete testicular descent in adults [61].

All specimens were deposited in the reference collection of the Pontifical Catholic University of Minas Gerais. Captures were performed under license (#28120-4) granted by the Brazilian Chico Mendes Institute for Biodiversity Conservation and access to animal genetic legacy was granted by license nº A8CA63C of the Genetic Legacy Management Council by the Brazilian Ministry of Environment (SISGen). The procedures used in this study were approved by the Ethics Committee on Animal Use of the Federal University of Minas Gerais (CEUA document 386/2017).

2.2 Maintenance of bats, BrdU injections and tissue preparation

For the analysis of spermatogenesis duration, 14 males of *M. levis* were collected in January and February, during the Maturing stage of reproduction (Table 1). The bats were placed in steel cages covered with black mesh, protected from the light, at room temperature and humidity. Yellowish myotis were fed with mealworms (Tenebrio coleopteran larvae, *Tenebrio molitor*) and water was provided *ad libitum*. The spermatogenic duration was estimated through intraperitoneal injections of 5-bromo-2′-deoxyuridine (BrdU; 150 mg/kg), a temporal marker for germ cells that were synthesizing DNA at the time of injection. Because of that, the spermatogenesis duration in yellowish myotis can only be researched in the Maturing stage due to the presence of primary spermatocytes in this phase [3]. The captured bats were euthanased with an overdose injection of anesthesics at five time intervals (one hour, three days, five days, seven days and ten days) following BrdU injections to evaluate spermatogenesis duration. All animals received the BrdU injection one-hour before euthanasia in order to demonstrate that the bats were in the same reproductive phase (Maturing stage). The testes and epididymis were collected, weighed and fixed in

Methacarn solution and the samples were then routinely processed and included in paraplast® (Sigma).

2.3 Immunostaining for BrdU and duration of spermatogenesis

Immunostaining analysis for BrdU was performed using the immunoperoxidase method. Deparaffinized sections were dehydrated and endogenous peroxidase activity was blocked by incubating sections in a 3% hydrogen peroxide solution (Sigma, St. Louis, MO, USA). Subsequently, antigens were exposed by heating in buffered sodium citrate (pH 6.0) at 96 °C for 10 min and the sections were treated with Protein Block (ab93697, Abcam Inc., USA) for 10 min. The slides were incubated overnight (4 ºC) with specific primary antibody against BrdU (1:100, Santa Cruz Biotechnology, USA, mouse monoclonal, sc-32323). Afterward, Biotinylated Goat Anti-Polyvalent Plus (ab93697; Abcam Inc., USA) was used against BrdU IgG secondary antibody. The diaminobenzidine (DAB) was used as a chromogen and the primary antibodies were omitted from the negative controls. Thus, the sections were analyzed by light microscopy to detect the most advanced germ cell labeled at different time intervals (mentioned above) after BrdU injection. The spermatogenic cycle duration was estimated based on the most advanced germ cell type labeled at different times following BrdU injections. The total duration of spermatogenesis took into account that approximately 4.5 cycles would be necessary from type A spermatogonia to spermiation [11,26].

2.4 Stages and duration of the seminiferous epithelium cycle

The stages of the seminiferous epithelium cycle were described using six males of *M. levis*, collected in March during the Mature reproductive stage (Table 1). Bats were euthanized through intraperitoneal injection of ketamine (240 mg/kg body weight) and xylazine (30 mg/kg body weight). The testes and epididymides were collected, weighed and fixed in Bouin solution for histomorphometric analysis and the samples were then routinely processed and included in paraplast® (Sigma).

The stages of the seminiferous epithelium cycle were characterized based on the development of the acrosomic system and morphology of the spermatids nuclei [9]. The relative stage frequencies were determined by evaluating 90 round seminiferous tubule cross-sections per bat, at ×400 magnification [62-64]. The acrosome angle measurement

on the spermatid nuclear surface was obtained from 150 germ cells per bat and per stage at ×400 magnification [15]. Regarding the acrosome development in elongating/elongated spermatids, the ratio between the length of nuclei longer and shorter axis was measured.

2.5 Daily sperm production

The histomorphometric analyses were performed in the same six males of *M. levis* collected in March, during the Mature reproductive stage (Table 1). The quantification of different testicular cells was performed in ten randomly chosen round seminiferous tubule cross-sections in the stage immediately after spermiation, at ×200 magnification. The following cell types were counted: spermatogonial cells, primary spermatocytes in pachytene, round spermatids and Sertoli cells. These counts were corrected for nuclear or nucleolar diameter and section thickness (5 μm) according to Abercrombie [65] and modified by Amann and Almquist [66]. For this purpose, ten nuclei or nucleoli diameter were measured per animal for each cell type analyzed. Cell ratios were obtained from the corrected counts obtained.

The total length of seminiferous tubules per testis, expressed in meters, was calculated based on their mean volume density (95.6 ± 0.5 %) and mean tubular diameter (136.00 ± 9.65 μm), which were already described by our group [3]. The full length was obtained by dividing the tubular volume by the tubular cross-section area (πR^2, R = tubular diameter/2) [67,68]. The length of seminiferous tubules per gram of testis corresponds to the ratio between the total length of seminiferous tubules and testis liquid mass.

The total number of Sertoli cells per testis and per gram of testis was determined from the corrected counts of Sertoli cell nucleoli and the full length of seminiferous tubules [63,69]. The Sertoli cell efficiency (SCE) was estimated from the ratio between the corrected counts of round spermatids and Sertoli cells. Finally, the daily sperm production (DSP) per testis and per gram of testis were determined using the following formula: DSP = total number of Sertoli cells per testis x SCE x Stage VII relative frequency (%)/Stage VII duration (days) [70].

2.6 Sperm analyses

For the seminal analyses, 31 bats of all reproductive stages were collected. The bats

were sampled in March (Mature stage), April (Regressed stage), August (Rest stage) and November (Maturing stage) (Table 1), and were euthanized through intraperitoneal injection of ketamine (240 mg/kg body weight) and xylazine (30 mg/kg body weight). The epididymides were collected, weighed and manually dissected on a Petri dish containing commercial Dulbecco's modified eagle's medium (#12500-062; DMEM/F12 - Gibco, Grand Island, NY) to obtain sperm suspensions for evaluation. The samples were collected and homogenized for analysis under a light microscope with a controlled temperature of 35.5°C, at 400x magnification [71].

The sperm count was performed in a Neubauer chamber (4x4; Lo-Laboroptik, United Kingdom), evaluating at least 200 spermatozoa. The sperm concentration was calculated using the following formula: C = (N/n) x (1/25) x dilution. (N) represents the number of spermatozoa counted and (n) the number of rows examined, while 1/25 refers to the Newbauer chamber correction. The dilution factor refers to the quantity of DMEM used in the sperm solution [71,72], which was 1:10 in the Mature and Rest reproductive stages, and 1:20 in the Regressed stage. No dilution was performed in the Maturing stage.

Sperm vitality and motility were analyzed as described by Vieira [71]. For the vitality analysis, the sample was mixed with Eosin solution and examined under a light microscope. The stained spermatozoa were counted as dead, while the non-stained spermatozoa were counted as alive. In the motility analysis, the sample was evaluated under a light microscope in which the spermatozoa motility was categorized as progressive, non-progressive and immotile. The sperm motility percentage was considered as the sum of progressive and non-progressive motilities. In both analyses, at least 200 spermatozoa were evaluated per animal. Sperm morphology was investigated through sperm smears stained with Hematoxylin-Eosin (HE) by assessing 200 spermatozoa regarding the head, midpiece and tail morphology. The sperm defects were identified, counted and classified [71].

2.7 Statistical analysis

All quantitative data were tested for normality and homoscedasticity of variances by the D'Agostino & Pearson tests. The parametric data (body mass, seminal and sperm morphology parameters) were submitted to a one-way ANOVA test and the means of the reproductive stages were compared using Tukey's test. For the non-parametric data

(testis mass, epididymis mass, tail defects, and sperm without midpiece and tail), the Kruskal-Wallis test was applied, followed by the Dunn's test to compare the reproductive stages means. The data obtained were expressed as the mean ± standard error of the mean (SEM), and the statistical analyses were performed through the GraphPad Prism 6 program (GraphPad Software, Inc). The level of significance considered was $P < 0.05$.

3. Results

3.1 Yellowish myotis presents a fast spermatogenic process

The most advanced germ cell types labeled one hour after BrdU injections were primary spermatocytes in pre-leptotene (Fig. 1a, c, e, g, i). After three days, labeled primary spermatocytes in zygotene were identified (Fig. 1b and i), while five days post-injection, stained primary spermatocytes in pachytene were seen (Fig. 1d and i). Following, seven days after the BrdU injection, labeled pre-leptotene and pachytene spermatocytes were observed (Fig. 1f and i) and, finally, at the ten-days interval, marked zygotene and pachytene spermatocytes were found (Fig. 1h and i). Based on the most advanced germ cell type labeled in these time intervals, each spermatogenic cycle lasted 7.05 ± 0.08 days and the entire spermatogenic process lasted 31.70 ± 0.15 days (Fig. 1i).

3.2 Ten stages of the seminiferous epithelium cycle were described in yellowish myotis

The complete spermiogenic phase of yellowish myotis spermatogenesis occurs only in the Mature stage (Farias et al., 2020). In the present study, we determined that the seminiferous epithelium cycle of yellowish myotis presents ten stages according to the acrosomic system (Fig. 2).

Stage I presented Sertoli and undifferentiated spermatogonial cells in contact with the basal lamina. Zygotene and pachytene spermatocytes, and two generations of spermatids (round and elongated spermatids) were observed in the seminiferous epithelium. The acrosomal system was not observed in spermatids by conventional light microscopy (Fig. 2a and k).

Stage II was characterized by the presence of Sertoli and undifferentiated spermatogonial cells on the basal lamina. Primary spermatocytes in pachytene, round

and elongated spermatids were observed in the seminiferous epithelium. Two small acrosomal vesicles were found on the nuclear surface of round spermatids, and, at the end of this stage, these small acrosomal vesicles fuse, resulting in a single round acrosomal vesicle in contact with the nucleus. The acrosome vesicle subtended an angle on the nuclear surface of 29 ± 0.76º (Fig. 2b and l).

Stage III presented Sertoli cells, undifferentiated spermatogonial cells, primary spermatocytes in pachytene, round and elongated spermatids. A large acrosomal vesicle was present and started flattening over the round spermatids nucleus surface, in an angle of 39 ± 0.78º (Fig. 2c and m).

Stage IV presented Sertoli cells, undifferentiated spermatogonial cells, primary spermatocytes in pachytene, round and elongated spermatids. The large acrosomal vesicle flattened over the nucleus of round spermatids and subtended an angle on the nuclear surface of 56 ± 1.26º (Fig. 2d and n).

Stage V was characterized by Sertoli cell presence, undifferentiated spermatogonial cells, primary spermatocytes in pachytene, round and elongated spermatids. A continued acrosome expansion was observed in round spermatids. The acrosome subtended an angle on the nuclear surface of 71 ± 1.93º. The elongated spermatids were situated close to the luminal border (Fig. 2e and o).

The occurrence of the spermiation event characterized stage VI. The seminiferous epithelium presented several residual bodies and elongated spermatids in the border, ready for spermiation. Sertoli cells, undifferentiated spermatogonial cells, primary spermatocytes in pachytene and several layers of round spermatids were observed in this stage. The acrosome subtended an angle on the round spermatid nuclear surface of 94 ± 2.55º (Fig. 2f and p).

Stage VII presented Sertoli cells, undifferentiated spermatogonial cells, few primary spermatocytes in zygotene, many primary spermatocytes in pachytene and several layers of round spermatids in the upper part of the seminiferous epithelium. The acrosome subtended an angle on the round spermatid nuclear surface of 106 ± 1.95º (Fig. 2g and q).

Stage VIII presented Sertoli cells, undifferentiated spermatogonial cells, primary spermatocytes in zygotene and pachytene and one generation of spermatids. The round nuclei of the spermatids began to elongate and the acrosome expanded over the dorsal surface, resulting in ovoid nuclei with a longer and shorter axis ratio of 1.8 ± 0.04 (Fig. 2h and r).

Stage IX presented Sertoli cells, undifferentiated spermatogonial cells, primary spermatocytes (in zygotene, pachytene, and diplotene) and elongated spermatids. The elongated spermatids were organized in bundles in the seminiferous epithelium, and their nuclei longer and shorter axis ratio was 3.6 ± 0.12 (Fig. 2i and s).

The presence of cellular meiotic divisions characterized stage X. There were also Sertoli cells, undifferentiated spermatogonial cells, primary spermatocytes (in pachytene and diplotene), secondary spermatocytes, and elongated spermatids. The latter were organized in bundles and the ratio between the longer and shorter axis of their nuclei was 4.2 ± 0.14 (Fig. 2j and t).

Concerning the frequencies of the stages, Stage VI presented the highest frequency (21.78%), while Stage X showed the lowest frequency (2.99%) (Fig. 2u). The frequency of pre-meiotic stages (VII to IX), meiotic stage (X) and post-meiotic stages (I to VI) of the seminiferous epithelium cycle were 39.96 %, 2.99 % and 57.05 %, respectively.

3.3 The elevated number of Sertoli cell results in high sperm production in yellowish myotis

The biometric data of yellowish myotis in the Mature stage and in all reproductive stages were presented in Table 2 and Suplementary Table 1, respectively. Testis mass showed the highest value in the Mature stage (Supplementary Table 1). The cell numbers of yellowish myotis were calculated in the Mature stage and are described in Table 2. The number of round spermatids was more than nine times higher than that of pachytene spermatocytes, and the Sertoli cell efficiency revealed that 6.60 ± 1.23 round spermatids were supported per Sertoli cell. The length of the seminiferous tubules per testis and per testis gram were 2.44 ± 0.28 m and 71.24 ± 10.54 m/g, respectively. The Sertoli cell number per testis and per testis gram were 8.11 ± 0.30 million and 251.45 ± 46.53 million, respectively, while the daily sperm production 7.71 ± 1.62 million per testis and 196.68 ± 21.45 million per gram of testis (Table 2).

3.4 The highest values of sperm concentration, vitality and motility were observed in the Regressed stage

The sperm production of yellowish myotis begins in the Mature reproductive stage, followed by its storage in the epididymis cauda during the Regressed, Rest and Maturing stages. The gamete storage was observed in 26.7% of the individuals in the

Maturing stage in a very low sperm concentration (37.68 ± 16.86 x10^6/ml). The seminal parameters are presented in Figure 3 and Table 3 and it can be observed that the sperm concentration increased in the Mature stage and reached the highest value in the Regressed stage ($p>0.05$; Fig. 3a, Table 3). Furthermore, as the seminal parameters were evaluated in only 13.3% of the Maturing stage samples, their data were not included in the statistical analyses. However, it should be mentioned that this pattern of low sperm concentration and long-term sperm storage at this stage repeated for three consecutive years (data from our research group).

The vitality of spermatozoa presented greater values in the Mature and Regressed stages ($p<0.05$), whereas the presence of dead sperm reached the highest percentage in the Rest stage ($p<0.05$; Figure 3b, Table 03). Sperm motility and progressive (linear) motility showed the highest values in the Regressed stage ($p<0.05$; Figure 3c, Table 03), while non-progressive motility showed no significant differences among the reproductive stages ($p>0.05$; Table 03). The immobile sperm percentage was higher in the Rest stage ($p<0.05$; Table 03). It should be mentioned that dead and immobile sperm were also highly observed in the Maturing stage.

3.5 The higher frequency of normal spermatozoa occurred in the Rest stage

During the sperm morphology analyses, we identified that the spermatozoa head of yellowish myotis presents a spatulate form (Fig. 3i). The head defects were classified as longer, pyriform, macrocephalous, double-headed, headless, and amorphous (Fig. 3j to o, respectively). Midpiece defects were identified as midpiece only, thicker, thinner and smaller (Fig. 3p to s, respectively). Tail defects were double-tailed, folded and coiled (Fig. 3t to v, respectively), and there were also spermatozoa without midpiece and tail (Fig. 3x).

The number of normal spermatozoa varied between the Regressed and Rest stages, showing the highest values in the Rest stage ($p<0.05$; Fig. 3d, Table 3). Head defect percentages were higher in the Mature stage ($p<0.05$; Fig. 3e, Table 3), while midpiece defects showed no variation among the reproductive stages ($p>0.05$; Fig. 3f, Table 3). Tail defects were the most predominant (56 to 66%), but there was no significant difference among the reproductive stages ($p>0.05$; Fig. 3g, Table 3). The number of spermatozoa without midpiece and tail was low (1.4 to 3%) and no variation was observed among the reproductive stages ($p>0.05$; Fig. 3h, Table 3).

4. Discussion

Yellowish myotis is a neotropical vespertilionid that presents a seasonal reproductive pattern with unique features, such as testicular regression and long-term sperm storage [3]. Regarding its spermatogenic activity, the spermiogenic process begins in the Mature stage and is completed in the Regressed stage. Ten stages of the seminiferous epithelium cycle were herein described and the high number of Sertoli cells associated with the short duration of spermatogenesis guaranteed a high daily sperm production in this species. In the Regressed stage, the period devoted to spermatids release, sperm concentration, vitality, and motility reached a peak in the epididymis cauda. It is essential to mention that a high concentration of defective sperm was identified in this period. Interestingly, coinciding with the mating period [1], the number of defective sperm decreased significantly in the Rest stage. After the mating period, in the Maturing stage, few live sperm were still present in the epididymis cauda (Fig. 4), reaffirming the epidydimal ability of sperm storing in a long-term pattern.

The spermatogenic cycle length of yellowish myotis, described for the first time in this article, lasts 31.7 days from spermatogonia to complete differentiated spermatozoa. Yellowish myotis spermatogenesis takes a more extended period to fully complete when compared with other described chiropteran species of the Phyllostomidae family, such as *Sturnira lilium* (15.52 days) and *Artibeus lituratus* (29.61 days) [21,29], but it is faster than *Desmodus rotundus* (37.02 days) [24]. However, the spermatogenesis length of yellowish myotis is shorter when compared to several mammals [12,28]. This short spermatogenic cycle of yellowish myotis enables fast sperm production before complete testicular regression [3]. Thus, it ensures male gamete production and reproductive success regardless of the gonad asynchrony between males and females [1]. The storing of sperm in the epididymis cauda is crucial to maintain sperm viability, a critical feature to fertilize the female egg during the receptive period, which occurs a few months later, in the dry season [1,3].

The seminiferous epithelium cycle of yellowish myotis presents ten stages based on the acrosomic system. Most studies on the seminiferous epithelium cycle of bat species, such as *Artibeus lituratus*, *A. planirostris*, *Carollia perspicillata*, *Platyrrhinus lineatus*, *S. lilium*, *D. rotundus*, and *Diphylla ecaudata* [13,18,20,21,24,29] were conducted following the tubular morphology method [73]. Moreover, an association between the

latter approach and acrosomal development was performed to study the *Molossus molossus* spermatogenic process [15]. Interestingly, except for the vespertilionid *Myotis nigricans* [13], the cellular composition of pre-meiotic, meiotic and post-meiotic stages found in these bats was similar to what was observed in yellowish myotis. In the Mature stage, yellowish myotis presented a gap between undifferentiated spermatogonial cells and zygotene spermatocytes in the seminiferous epithelium. The lower number of pachytene spermatocytes per round spermatids suggests that the spermatogonial differentiation is blocked during this period. Differently, other bat species present a continuous spermatogonial differentiation, allowing the identification of all germ cell types from undifferentiated spermatogonia to elongated spermatids in the seminiferous epithelium [13,15,18,20,21,24,29]. The difference in the cellular composition of the seminiferous epithelium and spermatogenesis length among bat species can reflect several reproductive patterns, mating systems, and environmental conditions [30,74,75].

Concerning the frequencies of the seminiferous epithelium stages, yellowish myotis presented a higher frequency of the post-meiotic stages (57.05 %) compared to the pre-meiotic stages (39.96 %), similar to the phyllostomid bats *A. lituratus*, *A. planirostris*, *C. perspicillata*, *P. lineatus* and *S. lilium* [13,18,29]. The stage of spermiation was the most frequent in the seminiferous epithelium cycle of yellowish myotis, while this occurred right after spermiation in other bat species [16,21,24,29]. In contrast, the meiotic stage of yellowish myotis showed the lowest frequency, which is similar to the pattern found in other bat species and this feature would be explained by the short life span of the secondary spermatocytes in bats [13,16,18,20,21,24,29]. However, it should be mentioned that the distribution of the frequencies in pre-meiotic and post-meiotic stages presents significant variation among bats, even among those belonging to the same family [13,18,20,21,24,29].

As classically known, the Sertoli cells play important roles in the spermatogenic process, such as physical support of germ cells, nutrition and delivery of growth factors [11,12]. The Sertoli cell efficiency of yellowish myotis is higher than that observed in other bat species, such as *M. molossus*, *S. lilium*, *Anoura geoffroyi*, and *A. lituratus* [16,29,76], but lower when compared to *D. rotundus* and *D. ecaudata* [20,24]. The large Sertoli cell numbers per testis gram found in yellowish myotis enables a better investment in spermatogenesis and sperm production during the Mature stage. The daily sperm production per testis gram of yellowish myotis, in the Mature stage, is 2.2 times higher than the *D. rotundus* bat [24], but a little smaller than the *S. lilium* bat [17]. The

high spermatogenic efficiency of yellowish myotis in the Mature stage is also explained by the high proportion of the tubular compartment (95%) [3] and the short spermatogenic cycle length demonstrated in the present work.

Therefore, yellowish myotis's reproductive success depends on the spermatozoa's ability to remain alive during the storage period in the epididymis until mating occurs several months later. The life span of mammalian spermatozoa lasts about a few weeks and sperm storage for a long time is atypical [6,77]. In the order Chiroptera, the sperm storage in males from temperate regions occurs for more extended periods, while it occurs for shorter periods in tropical species [5,42,44,45,47]. The sperm concentration in yellowish myotis epididymis presented the highest value in the Regressed stage due to the completion of spermiation in this stage. It should be mentioned that a small concentration of live spermatozoa was still observed in the epididymis cauda in the Maturing stage, indicating eight months of sperm storage [3]. The sperm concentration of yellowish myotis in all reproductive stages is higher than that of the microbats *Hipposideros larvatus*, *A. jamaicensis* and *S. lilium* [36,78], and the low concentration in the phyllostomid bats *A. jamaicensis* and *S. lilium* can be related to their continuous reproductive pattern throughout the year [17,79-81]. Some flying fox species presented higher sperm concentrations only when compared with the yellowish myotis Maturing stage [34,82].

The sperm vitality of yellowish myotis presented the highest values in the reproductive stages Mature and Regressed (65% and 74%, respectively). Considering the Regressed stage, the vitality value was smaller than *S. lilium* (84%), similar to *A. jamaicensis* (73%) and higher than the seasonal pteropodid *Eidolon helvum* (63%) [36,38]. Interestingly, the seasonal vespertilionid *Pipistrellus kuhlii* presented a remarkable higher sperm vitality (99%) when spermatogenesis was interrupted [37]. Concerning sperm motility, the highest percentages were observed in the Mature and Regressed stages. In contrast, the seasonal megabat *Eidolon helvum* presents low sperm motility when testicular regression occurs [38]. Herein, yellowish myotis did not show the highest sperm motility in the Rest stage (during the mating period) [1], which is different from several species of flying foxes in the breeding season [34,82]. Progressive motility showed an average of 63% and 76% in the Mature and Regressed stages, respectively. These values were higher than *A. jamaicensis* (36%) and *S. lilium* (60%) [36], and lower than the sperm storing vespertilionid *P. kuhlii* that presented 98% of progressively motile spermatozoa [37]. Furthermore, the non-progressive sperm

motility of yellowish myotis in these reproductive stages (7%) was lower than *A. jamaicensis* (36%) and *S. lilium* (27%) [36]. Although the vitality and motility of yellowish myotis sperm decreased based on storage time, the highest percentage of sperm with normal morphology occurred in the Rest stage, when mating takes place [1]. Therefore, these parameters indicate the excellent quality of yellowish myotis sperm during the mating period.

The sperm morphology and defects of yellowish myotis were described for the first time in this study. The yellowish myotis spermatozoa head presented a spatulate form similar to that observed in the vespertilionids *M. lucifugus* and *Eptesicus fuscus* [83]. The percentage of normal sperm in all reproductive stages of yellowish myotis was lower than that of microbats, such as *A. lituratus, P. lineatus, S. lilium* (35-60%) [39], *A. jamaicensis*, and *S. lilium* (62% and 70%, respectively) [36]. It was also lower than what was observed for the flying foxes *Pteropus scapulatus*, *P. poliocephalus*, *P. alecto*, *P. hypomelanus*, *P. rodricensis* and *P. vampyrus* (56%, 59%, 79%, 53%, 58%, and 71%, respectively) [34,82]. The highest rate of yellowish myotis sperm defects occurred in the Regressed stage (80%). A similar pattern was found in the seasonal *Eidolon helvum* during testicular regression, although at a lower percentage (29%) [38]. High rates of sperm defects (reaching a peak of 59%) were also reported in the insectivorous bat *Hipposideros larvatus* [78].

Several head defects were described in yellowish myotis spermatozoa in the present study. Similarly, *A. jamaicensis* and *S. lilium* bats presented spermatozoa without a head [36], while the insectivorous bat *H. larvatus* showed macrocephalic, double, and amorphous head [78]. Other head abnormalities were reported, such as filiform head in *A. lituratus, P. lineatus,* and *S. lilium* [39], a pyriform head in megabat species [34], and a microcephalic head in *H. larvatus* and *P. vampyrus* [78,82]. Similar to yellowish myotis, isolated heads (no midpiece and tail) were also found in *A. jamaicensis*, *S. lilium*, *E. helvum*, *P. scapulatus*, *P. poliocephalus*, and *P. alecto* [34,36,38]. The midpiece defects of yellowish myotis sperm occurred at a low frequency, being lower than the values described in flying fox species [34]. Considering the tail defects, yellowish myotis sperm presented a high incidence along the reproductive stages. Among the tail defects described, folded and coiled tail were also reported for the microchiropterans *A. lituratus*, *P. lineatus*, *S. lilium*, *H. larvatus*, and for the megachiropterans *E. helvum* and several *Pteropus* species [34,38,39,78,82]. Similar to yellowish myotis, the coiled tail was also present in *A. jamaicensis* and *S. lilium* sperm

[36], and the double tail was reported for *H. larvatus* and *P. vampyrus* [78,82]. Although cytoplasmic droplets in the sperm midpiece and tail were not found in yellowish myotis, they were observed in microchiropteran and megachiropteran species [34,36,38,39,78,82]. It is believed that the percentage of sperm defects differs among bat species and may be influenced by several factors, such as health, food habits, age, environment, and exposure to contaminants like pesticides and herbicides [36,78,84].

The yellowish myotis daily sperm production per gram of testis was in accordance with the values of the sperm concentration in the epididymis in the Mature stage, considering the time of bat collection. In seasonal animals, headless sperm, spermatozoa degradation and spermiophagy were described in the epididymis during testis regression [85-87]. According to the obtained data, we suggest that yellowish myotis epididymis eliminates defective and dead spermatozoa [88] prior to the breeding period (Rest stage). The higher percentage of normal spermatozoa observed in the Rest stage suggests a possible sperm quality control in the epididymis acting as a filter in the Regressed stage [87,88], being crucial to ensure the reproductive success of the species. Interestingly, spermatids and spermatozoa elimination also occurred in testis, through apoptosis, in the same reproductive stage [3]. Regarding sperm storage, there were a few live spermatozoa in the epididymis cauda after the mating period, in the Maturing stage, indicating that the epididymis is able to maintain the spermatozoa over time.

5. Conclusions

Yellowish myotis presented fast and elevated sperm production in the Mature stage, followed by epididymal sperm storage in the Regressed, Rest and Maturing stages. Furthermore, a possible epididymis sperm selection occurs before mating in the Rest stage.

6. Competing interests

The authors ensure that there are no conflicts of interest.

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10. Figure caption

Figure 1. Duration of yellowish myotis spermatogenesis. The length of spermatogenesis was evaluated in the Maturing stage considering the most advanced labeled germ cells at 1 hour (a, c, e, g), 3 days (b), 5 days (d), 7 days (f) and 10 days (h) after BrdU injections. Black arrowheads indicate pre-leptotene spermatocytes (Pl); yellow arrowheads show zygotene spermatocytes (Z); red arrowheads represent pachytene spermatocytes (P). SEC (7.05 days) = seminiferous epithelium cycle. Bar: 20 µm.

Figure 2. Stages of the seminiferous epithelium cycle of yellowish myotis. The seminiferous epithelium cycle presents ten stages (a to j), based on the development of the acrosomic system (k to t), with the occurrence of spermiation in stage VI (f). The acrosome angle values of spermatids are depicted below spermatid nuclei images (k to t). Stages VI-VIII presented the highest frequencies (u). Abbreviations: SC - Sertoli cell; S - spermatogonial cell; Z - zygotene spermatocyte; P - pachytene spermatocyte; D - diplotene spermatocyte; M - a figure of meiotic division; II - secondary spermatocyte; R - round spermatid; E - elongating/elongated spermatid; Rb - residual bodies. Bars from a-j: 12 µm. Bars from k-t: 4 µm.

Figure 3. Seminal parameters and sperm morphology of yellowish myotis. Sperm concentration (a), vitality (b), motility (c) and morphology (d) were compared among the reproductive stages. The standard yellowish myotis sperm head is spatulate (i). Head defects were higher in the Mature stage (e) and were classified as longer (j), pyriform (k), macrocephalous (l), double-headed (m), headless (n) and amorphous (o to o”). Midpiece defects (f) were classified as midpiece only (p), thicker (q), thinner (r) and smaller (s). Tail defects (g) were classified as double-tailed (t and t’), folded (u and u’) and coiled (v and v’). Spermatozoa without midpiece and tail were identified (x and x’) but in low numbers (h). Sperm images present a 1000x magnification.

Figure 4. Yellowish myotis sperm production and seminal parameters along the reproductive stages. a) The sperm production occurred in the Mature stage and the sperm release ends in the Regressed stage. b) The sperm concentration in epididymis reached the peak in the Regressed Stage. After the mating period, the concentration of

sperm in epididymis cauda significantly diminishes during the Maturing stage. c) The sperm vitality and motility decreased from the Regressed to the Rest stage. d) The percentage of normal spermatozoa significantly increased in the Rest stage, coinciding with the mating period.

11. Tables

Table 1. Yellowish myotis sampling from January 2019 to November 2020.

Reproductive stages	Months	Number of animals	Analyses
Maturing	November - December	15	Seminal analyses
	January - February	14	Duration of the seminiferous epithelium cycle
Mature	March	6	Characterization and frequencies of the seminiferous epithelium cycle stages
			Daily sperm production
		5	Seminal analyses
Regressed	April	6	Seminal analyses
Rest	May - June	-	-
	July - August (Mating Period)	5	Seminal analyses
	September - October	-	-

Table 2. Yellowish myotis biometric and testicular data in the Mature stage.

	Parameters (n =6)	Mean (± SEM)
Biometric data	Body mass (g)	5.53 ± 0.14
	Testis mass (g)	0.0546 ± 0.0066
	Epididymis mass (g)z	0.0097 ± 0.0015
Cell numbers per seminiferous tubule cross-sections	Sertoli cell	17.26 ± 1.09
	Undifferentiated spermatogonial cell	0.60 ± 0.06
	Primary spermatocyte in Pachytene	11.51 ± 3.51
	Round spermatid	109.57 ± 17.94
Tubular parameters and sperm production	Length of seminiferous tubules per testis (m)	2.44 ± 0.28
	Length of seminiferous tubules per g of testis (m/g)	71.24 ± 10.54
	Sertoli cell number per testis (10^6)	8.11 ± 0.30
	Sertoli cell number per g of testis (10^6)	251.45 ± 46.53
	Sertoli cell efficiency	6.60 ± 1.23
	Daily sperm production per testis (10^6)	7.71 ± 1.62
	Daily sperm production per g of testis (10^6)	196.68 ± 21.45

977 Table 3. Seminal parameters of yellowish myotis in the reproductive stages.

Seminal Parameters	Mature stage (n = 5)	Regressed stage (n = 6)	Rest stage (n = 5)	Maturing stage (n= 2)	ANOVA *(F)* *Kruskal-Wallis *(H)*	*P* value
Sperm Concentration (x10^6/ml)	2231.15 ± 1263.01^a	8824.94 ± 3807.53^a	6958.47 ± 4054.72^a	37.68 ± 16.86	1.105	0.3626
Sperm vitality (%)	64.55 ± 4.45^{ab}	74.49 ± 1.57^a	51.09 ± 11.34^b	52.82 ± 7.18	3.898	0.0525
Dead sperm	35.45 ± 4.45^{ab}	25.51 ± 1.57^a	48.91 ± 11.34^b	47.18 ± 7.18	3.898	0.0525
Sperm motility (%)	70.05 ± 0.85^{ab}	82.50 ± 1.85^a	54.42 ± 13.29^b	50.83 ± 12.37	4.672	0.0340
Progressive	62.97 ± 1.31^{ab}	75.52 ± 2.42^a	51.78 ± 12.36^b	41.07 ± 4.53	3.736	0.0578
Non-progressive	7.08 ± 0.49^a	6.98 ± 1.90^a	2.64 ± 0.98^a	9.76 ± 7.84	2.465	0.1304
Immobile	29.95 ± 0.85^{ab}	17.50 ± 1.86^a	45.58 ± 13.29^b	49.17 ± 12.37	4.672	0.0340
Normal sperm (%)	22.10 ± 2.42^{ab}	19.85 ± 1.36^a	32.80 ± 4.94^b	29.20 ± 0.03	5.607	0.0210
Sperm defects (%)	77.90 ± 2.42^{ab}	80.15 ± 1.36^a	67.20 ± 4.94^b	70.80 ± 0.03	5.607	0.0210
Head defects	11.09 ± 1.75^a	5.65 ± 0.96^b	3.67 ± 1.16^b	5.78 ± 1.16	8.147	0.0067
Midpiece defects	5.07 ± 0.81^a	5.56 ± 1.18^a	3.44 ± 0.65^a	5.93 ± 1.76	1.124	0.3596
Tail defects	59.65 ± 2.64^a	65.93 ± 3.06^a	58.72 ± 4.66^a	56.47 ± 1.86	2.881*	0.2577
Without midpiece and tail	2.09 ± 0.70^a	3.01 ± 0.74^a	1.37 ± 0.66^a	2.62 ± 1.23	1.133*	0.5964

978 Different line superscript letters show statistically significant differences, $p < 0.05$. The seminal parameters of the Maturing stage were not
979 included in statistical analyses.

Theriogenology

Belo Horizonte, Brazil, 13th of April, 2021

Highlights

- Yellowish myotis presents a fast spermatogenesis length.
- Yellowish myotis has a high sperm production at a restricted time.
- Seminal parameters of yellowish myotis were described in all reproductive stages.
- The epididymis of yellowish myotis maintains long-term sperm storage.
- Epididymis eliminates defective sperm prior to the mating period.

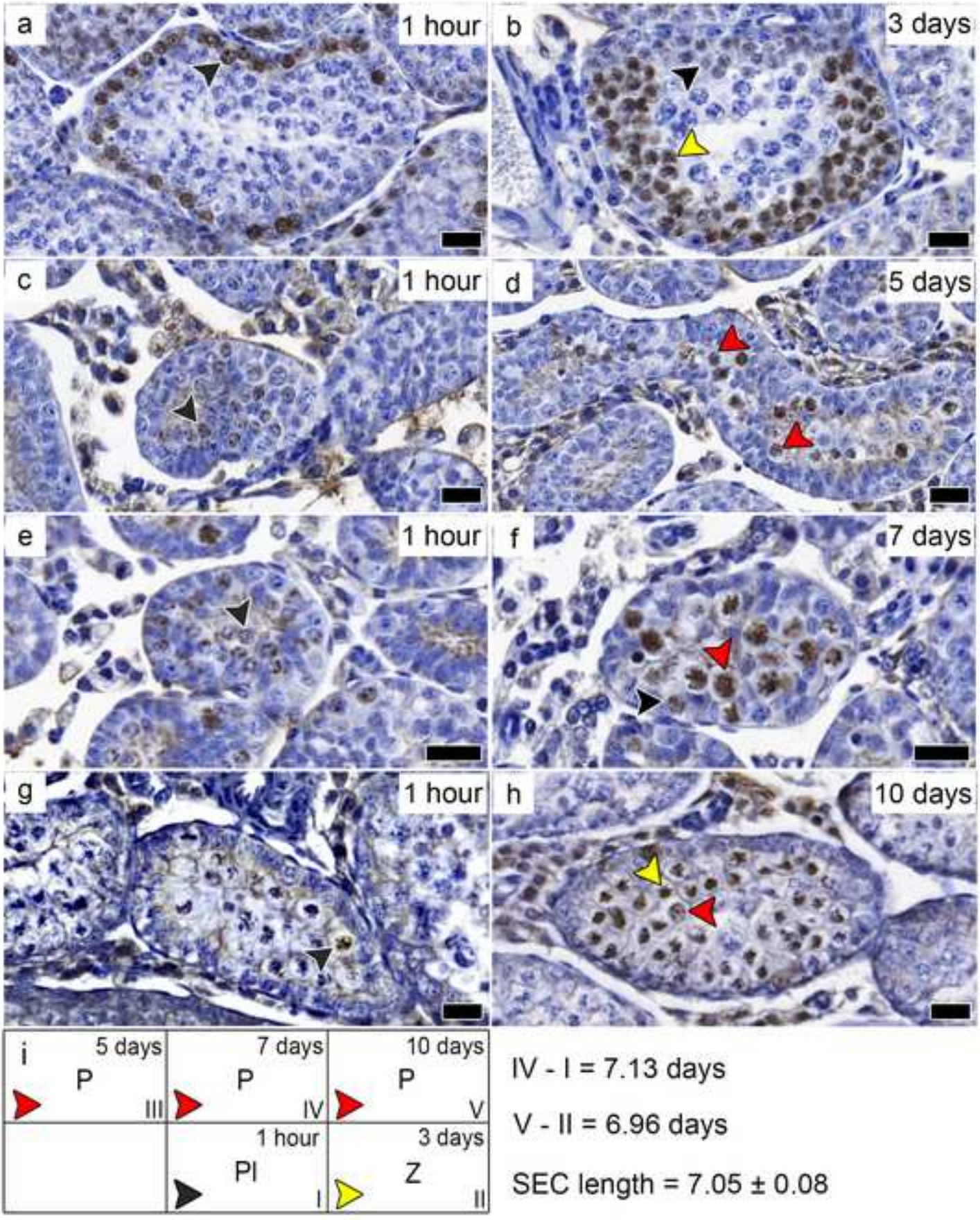

Figure 2

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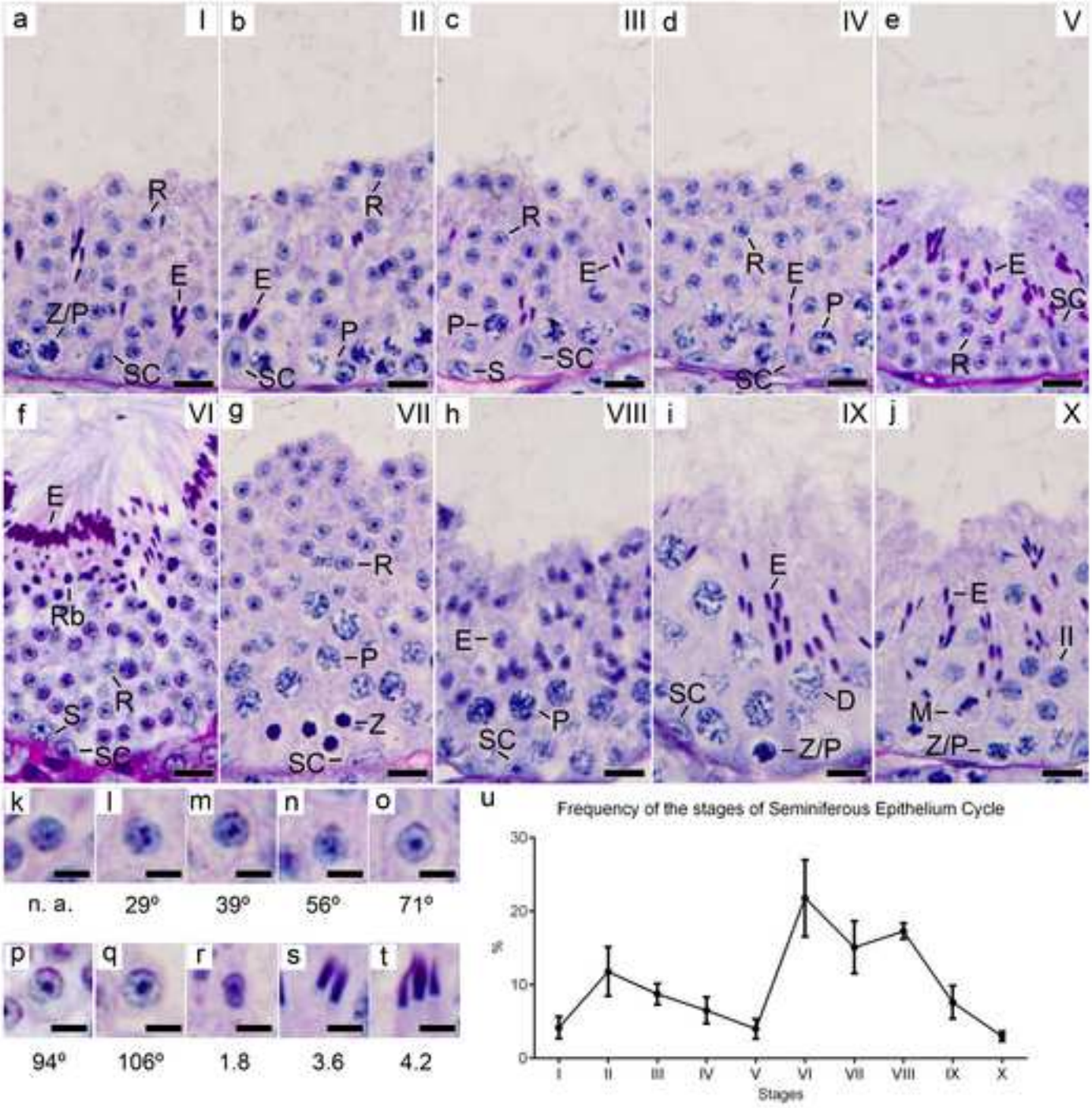

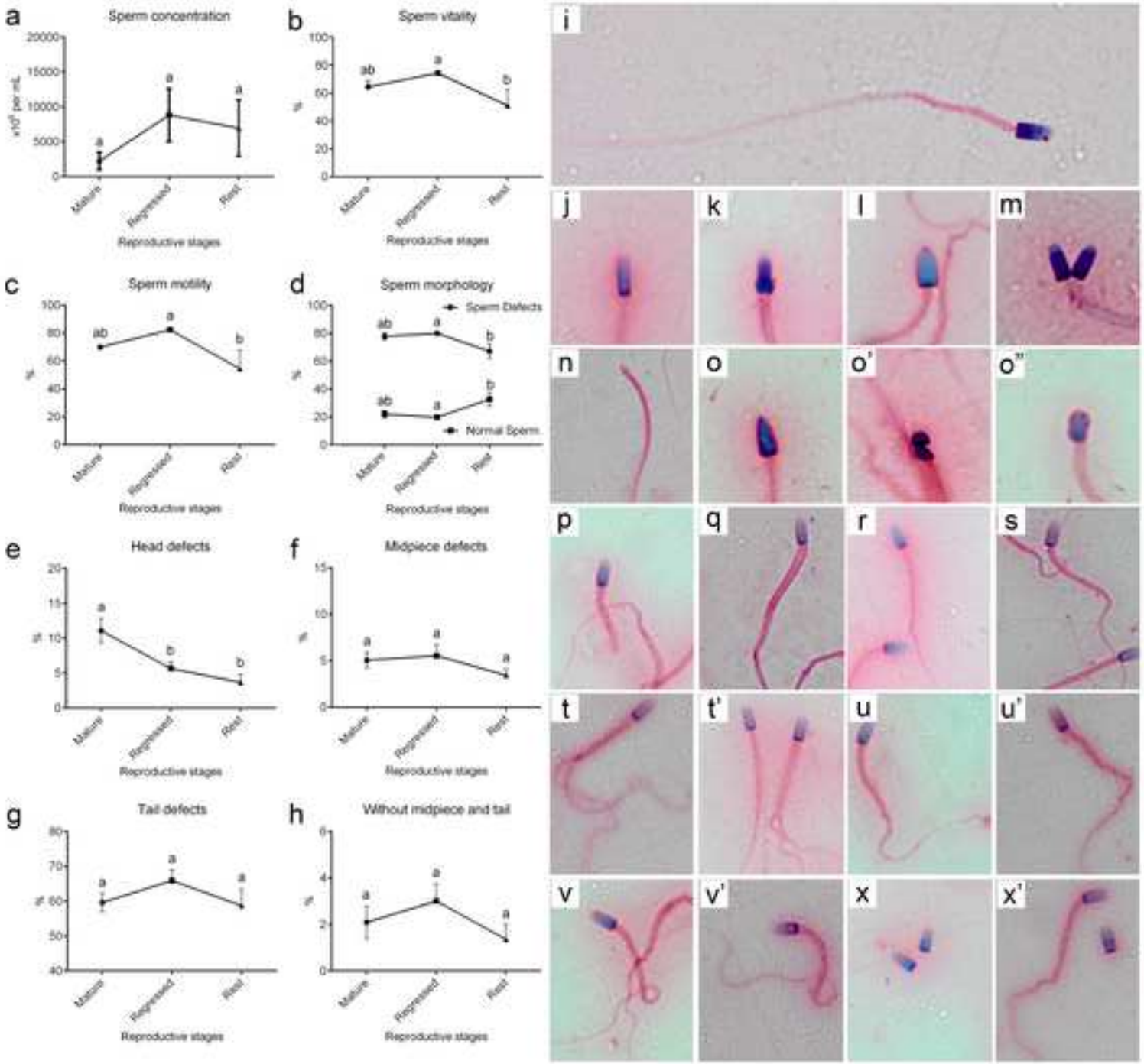

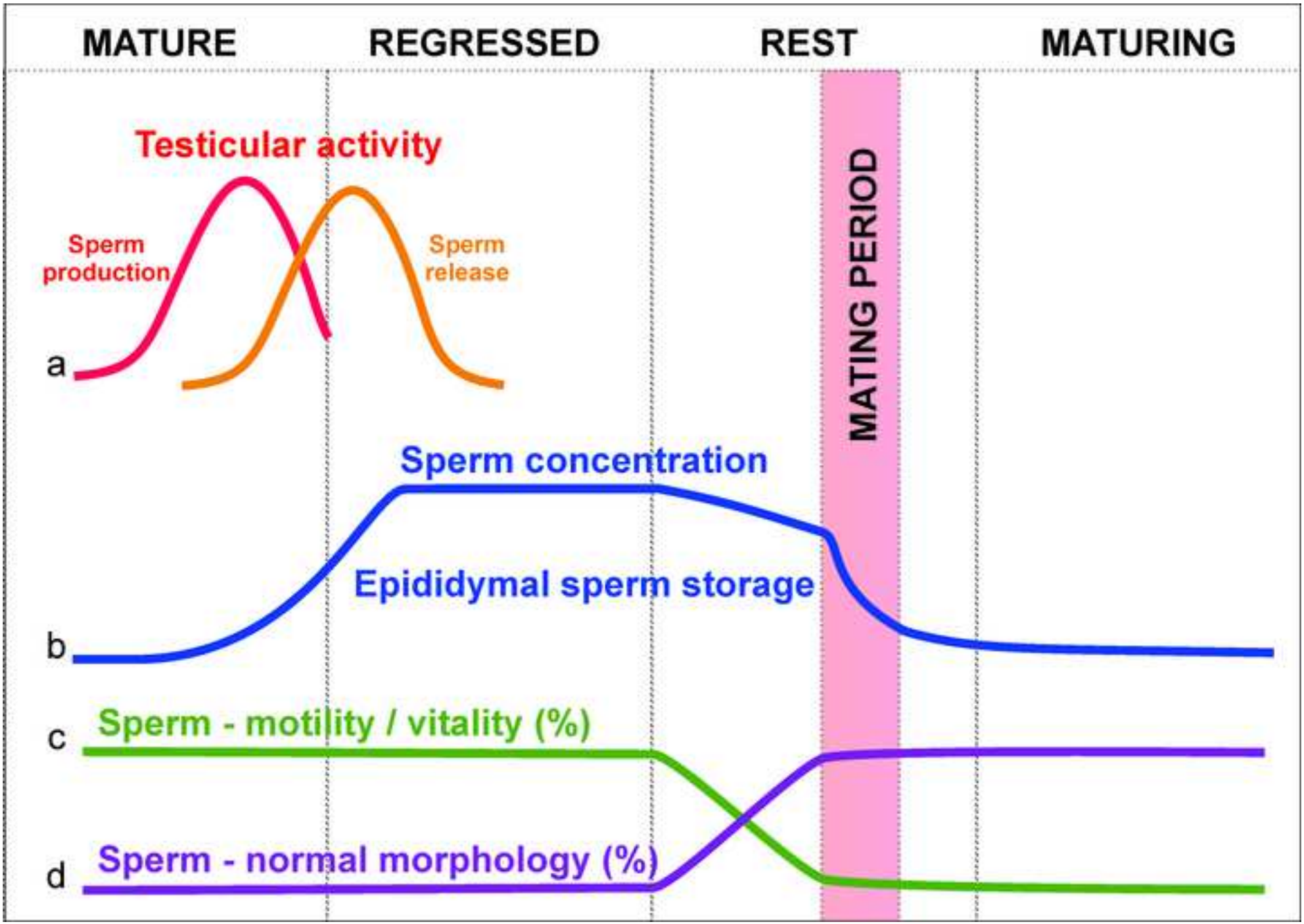

Theriogenology

Sperm production and seminal analyses of a Neotropical sperm-storing vespertilionid bat yellowish myotis (*Myotis levis*)

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Supplementary tables

Supplementary Table 1. Mean (± SEM) values (in g) of body mass (BM), testis mass (TM), and epididymis mass (EM) of yellowish myotis in the reproductive stages.

Reproductive stage	BM	TM	EM
Rest (n=5)	5.20 ± 0.13	0.0035 ± 0.0006[a]	0.0046 ± 0.0012[ab]
Maturing (n=29)	5.59 ± 0.09	0.0115 ± 0.0033[a]	0.0032 ± 0.0004[a]
Mature (n=11)	5.53 ± 0.14	0.0546 ± 0.0066[b]	0.0097 ± 0.0015[b]
Regressed (n=6)	5.58 ± 0.26	0.0137 ± 0.0023[ab]	0.0107 ± 0.0021[b]
ANOVA *(F)*	0.8908	27.09*	26.16*
P value	0.4529	< 0.0001	< 0.0001

Different column superscript letters show statistically significant differences, $p < 0.05$. *Kruskal-Wallis test.

To the Editor-in-Chief
Theriogenology
Belo Horizonte, Brazil, 13th of April, 2021

Dear Editor,

I confirm that all authors agreed to be listed and have approved the manuscript and its submission.

Sincerely yours,

Dr. Guilherme Mattos Jardim Costa

3.3 Artigo 3

Testis tissue and brown adipose tissue xenograft from the Neotropical sperm-storing vespertilionid bat yellowish myotis (*Myotis levis*)

Submitted for Reproduction - Bioscientifica

Reproduction

Testis and brown adipose tissue xenografts from yellowish myotis (Myotis levis)

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Keywords:	Testosterone, Germ cells, Leydig cells, Seasonality, Spermatogenesis

Testis and brown adipose tissue xenografts from yellowish myotis (*Myotis levis*)

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Short title: Bat testis and brown adipose tissue xenografts

Key-words: Testosterone, Germ cells, Leydig cells, Seasonality, Spermatogenesis

Abstract

Yellowish myotis present a seasonal reproduction, influenced by rainfall distribution, in which the testis mass, germ cell composition, and brown adipose tissue mass changed along the reproductive stages. Aiming to investigate, for the first time, the spermatogenesis development without the influence of environmental factors and the possible androgenic role of brown adipose tissue, xenografts were performed in immunodeficient mice. Forty-one adult male bats were captured in the Santuário do Caraça, Minas Gerais, Brazil. The gonads and brown adipose tissue were collected, weighed, and grafted under the back skin of mice. Mice biometric and hormonal data were evaluated after grafting, and the testis grafts and mice gonads were fixed for histological and immunohistochemical analyses. As a result, testis grafts presented a continuous germ cell development in all reproductive phases, indicating a constant undifferentiated spermatogonia differentiation. The germ cells differentiated until the round spermatid step in all testis tissues. These data indicated that yellowish myotis spermatogenesis can be continued in a stable endocrine milieu, as found in mice. The consumption of brown adipose tissue grafts by mice promoted increase of seminal vesicle mass and testosterone serum levels. To sum up, the brown adipose tissue is related to testosterone synthesis that may be stimulating the spermatogonia differentiation in yellowish myotis.

1. Introduction

Bats present a high diversity with more than 1400 species worldwide (Wilson & Mittermeier 2019) and are providers of essential ecosystem services (Jones *et al*. 2009, Kunz *et al*. 2011, Maine & Boiles 2015, Puig-Montserrat *et al*. 2015, Russo & Jones 2015, Russo *et al*. 2018, Rodríguez-San Pedro *et al*. 2020). Bats adapt well to different reproductive strategies (Racey & Entwistle 2000) but present a low reproductive rate (Barclay *et al*. 2004, Jones *et al*. 2009). This feature makes them more vulnerable to different threats, such as urbanization, climate change (e.g., severe weather), changes in water quality, loss of roost sites, deforestation, hunting, diseases, and exposure to environment contaminants (pesticides and heavy metals) (Jones *et al*. 2009, Zukal *et al*. 2015, Voigt & Kingston 2016, Frick *et al*. 2020).

Tissue xenograft, the biotechnology used in this study, has been used as a functional and powerful technique to investigate reproductive organ physiology in an ex-situ manner (Paris *et al*. 2004, Rodriguez-Sosa & Dobrinski 2009, Santos *et al*. 2010, Arregui & Dobrinski 2014). This technique was studied in wild mammals aiming to preserve the genetic material of endangered mammal species (Honaramooz *et al*. 2004, Arregui *et al*. 2008a, Abbasi & Honaramooz 2011, 2012, Gourdon & Travis 2011, Arregui *et al*. 2014, Campos-Junior *et al*. 2014, Pothana *et al*. 2015). Testis xenograft proved to be successful in some wild species, including seasonal species (Abbasi & Honaramooz 2012), generating viable sperm and offspring (Honaramooz *et al*. 2004, Campos-Junior *et al*. 2014, Liu *et al*. 2016). Until now, there have been no studies using tissues from bats.

The Neotropical vespertilionid yellowish myotis bat (*Myotis levis*), the investigated species of this study, present a seasonal reproduction in which the development of spermatogenesis is linked to the distribution of rainfall (Farias *et al*. 2020). Our research group demonstrated that the testis parenchyma of yellowish myotis shows a remarkable variation in the germ cell population, allowing the identification of four reproductive stages known as Rest, Maturing, Mature, and Regressed (Farias *et al*. 2020). The Rest stage is characterized by the presence of the spermatogonial phase only. Primary spermatocytes are observed in the Maturing and Mature stages, coinciding with the peak of the distribution of rainfall. Sperm formation occurs in the Mature and Regressed stages only, during which the spermiogenic phase is observed. After the conclusion of spermiation, long-term sperm storage in epididymis cauda (~8 months) begins in the Regressed stage (Farias *et al*. 2020). An exciting aspect is related to the cyclic and synchronic fluctuation of the testis and epididymis mass along the reproductive stages. During the reproductive cycle, the maximum testis size (Mature stage) is followed by the highest epididymis volume

in the next reproductive phase (Regressed stage). The investigation of the impacts of abiotic factors in testis physiology is needed to avoid valuable bat species loss, and testis tissue xenograft is a promising tool for this scientific study.

Another interesting observation is that the brown adipose tissue mass varies in the same pattern as the accessory sex gland mass throughout the reproductive stages, indicating a possible role of this organ in the yellowish myotis male reproductive cycle (Farias *et al*. 2020). Furthermore, comparing the Rest stage to the Mature stage, the brown adipose tissue mass negatively correlates with serum testosterone levels (Farias *et al*. 2020). These findings suggest that the brown adipose tissue was possibly being used for androgenic purposes in the yellowish myotis (Krutzsch & Wells 1960). Current data demonstrate that adipose tissue may contain the steroidogenic machinery necessary to initiate steroid biosynthesis *de novo* from cholesterol (Li *et al*. 2015). Once again, the xenograft can represent a valuable instrument to evaluate a possible steroidogenic stimulus of the brown adipose tissue.

Therefore, the present study aimed to use testis tissue xenograft from adult yellowish myotis to investigate the spermatogenesis development in the different reproductive stages, without the influence of environmental factors; and to use the brown adipose tissue xenograft from adult yellowish myotis to evaluate its possible androgenic role in bats for the first time.

2. Material and Methods

2.1. Study area and capture of bats

The yellowish myotis colony lives in Santuário do Caraça, a preserved area in Serra do Caraça, southeastern Brazil (20°04’30’’S, 43°24’28’’W), which belongs to the Iron Quadrangle geomorphological domain (Moreira & Pereira 2004, Abreu & Palú 2008). The reserve has a great diversity of fauna and flora because it is located in a transition region of Atlantic Forest and Cerrado biomes (Giulietti *et al*. 1997, Moreira & Pereira 2004, Abreu & Palú 2008). A seasonal climate characterizes this region with a rainy summer (rainy season - October to March) and a dry winter (dry season - April to September), in which the precipitation occurs mainly during the rainy season (81.5% of the annual average of 1.373 mm) and the remaining percentage of precipitation occurs in the dry season (Sá *et al*. 2012).

Bats were captured from August 2017 to November 2020 using mist-nets installed in the attic of Santuário do Caraça church, from 18:00h to 00:00h. Forty-one male bats were collected along the

reproductive stages Rest, Maturing, Mature and Regressed (Farias *et al*. 2020) for xenograft experiments (Table 1). The forearm length, body mass, age class and sex of each individual were recorded. Only adult males were used in the study, which was differentiated from subadults by the presence of ossified finger epiphyseal cartilages in the metacarpus (Anthony 1988), and complete testicular descent (Duarte & Talamoni 2010). Bats were euthanized through intraperitoneal injection of ketamine (240 mg/kg body weight) and xylazine (30 mg/kg body weight), and the gonads, epididymis and brown adipose tissue (BAT) from the interscapular region were collected.

All specimens were deposited in the reference collection of the Pontifical Catholic University of Minas Gerais. Captures were performed under license (#28120-4) granted by the Brazilian Chico Mendes Institute for Biodiversity Conservation and access to animal genetic legacy was granted by license nº A8CA63C of the Genetic Legacy Management Council by the Brazilian Ministry of Environment (SISGen). The Ethics Committee on Animal Use from the Federal University of Minas Gerais (CEUA document 386/2017) approved the study procedures.

2.2 Testis and brown adipose tissue xenograft

Each testis was sectioned into four fragments (3x3x5 mm), and half of the brown adipose tissue was used in each xenograft. After that, the tissues were maintained on Dulbecco's modified eagle's medium (#12500-062; DMEM/F12 - Gibco, Grand Island, NY) before the xenograft procedure in the sexually mature immunodeficient recipient mice.

Testis fragments (from each reproductive stage) of yellowish myotis were grafted under the back skin of fifteen castrated NSG mice (6 grafts per mouse), resulting in four experimental groups. The animals were positioned in ventral decubitus position, and three incisions of approximately 0.5 cm were performed bilaterally to the dorsal line. The skin was dissected, forming pockets in which the fragments were placed. After receiving the fragment, the incisions were sutured with 5.0 silk threads (Biosut, Brazil).

The brown adipose tissue of the Maturing stage was grafted under the back skin of eight non-castrated NUDE mice (2 grafts per mouse). The Nude mice (without fur) were used to observe more easily the BAT tissue mass reduction along the grafting time. The Maturing stage was chosen because it is the phase in which the brown adipose tissue is consumed by yellowish myotis. Additionally, five NUDE mice were used as control group. Anesthesia and surgery procedures were the same as above, but only one incision

was performed bilaterally to the dorsal line. During surgery, the animals were kept on a heated surface (37º C) to prevent hypothermia and facilitate recovery.

2.3 Biometric data and histological evaluation

The body and graft masses were evaluated at 5 and 2 months after grafting for testis tissue and brown adipose tissue, respectively. For testis xenografting, we waited for five months based on the meantime observed in the literature. BAT xenografting was discontinued (after two months) due to the severe reduction of this tissue under the back skin of NUDE mice. The seminal vesicle mass was used as an indicator of bioactive testosterone for the testis tissue and brown adipose tissue xenograft experiments (Arregui et al. 2008a, 2014). For recovery of the tissue xenografts, the mice were euthanized through intraperitoneal injection of ketamine (240 mg/kg body weight) and xylazine (30 mg/kg body weight).

The epididymis and one testis tissue fragment per bat were not used for grafting, allowing the observation of the original metabolic status of the organs. These organs and the brown adipose tissue were fixed in Bouin solution, routinely prepared and embedded in Paraplast® for histological analysis (Fig. 1A). Moreover, these histological images were used as controls for animal age, demonstrating that they were adult animals (spermatozoa identification) (Fig. 1B to E).

After five months of grafting, the testis tissue fragments were fixed in Bouin solution and Glutaraldehyde 4% for histological analysis. Each germ cell type (undifferentiated spermatogonia, differentiated spermatogonia, spermatocytes and round spermatids) was counted in twenty seminiferous tubules per reproductive stage. This quantification was performed to determine the spermatogenesis progression after testis tissue xenograft. Concerning the brown adipose tissue experiments, the NUDE mice gonads were fixed in Bouin solution, routinely processed and embedded in Paraplast® for histological analysis.

2.4 Hormonal analyses

Blood samples of mice were collected by cardiac puncture after anesthesia induction. Plasma was separated through centrifugation (2.000 rpm, for 10 min, at 4 ºC) and stored at −20 °C for subsequent hormone evaluation. The samples were analyzed in the automated Cobas e411 (Roche Diagnostics Inc., Indianapolis, IN, USA) platform for direct assessment of testosterone. Serum testosterone levels were measured using commercial kits (Roche Diagnostics Inc., Indianapolis, IN, USA) through

electrochemiluminescence method (sensitivity of 2.5 ng/dL). Testosterone intra- and inter-assay coefficients of variation (CV) were 1.1% and 1.5%, respectively. The procedures were performed by a Licensed Laboratory specialized in Animal Health (TECSA(R) Laboratory, Belo Horizonte, Brazil).

2.5 Immunostaining analyses

For immunohistochemical analysis, deparaffinized sections were dehydrated, and the endogenous peroxidase activity was blocked by incubating the sections in a 3% hydrogen peroxide solution (Sigma, St. Louis, MO, USA). After that, the antigens were exposed to heating in buffered sodium citrate (pH 6.0) at 96 °C for 10 min, and the protein was blocked using 10% normal rabbit serum (Sigma, St. Louis, MO, USA, #R9133) in PBS for 30 min. The slides were incubated overnight (4 ºC) with a specific primary antibody against the steroidogenic enzyme 3-Beta-HSD (1:100, Santa Cruz Biotechnology, USA, goat polyclonal antibody, sc-30820). Considering that the antibody was raised against the human protein, the protein homology between human and *Myotis* species was tested through *in silico* analysis (Basic Local Alignment Search Tool), showing 76.7 % of homology. The reaction was developed using rabbit pAb to goat secondary IgG antibody (ab6740; Abcam Inc., USA). Diaminobenzidine (DAB) was used as chromogen, and the negative control had the primary antibody omitted. For the evaluation of steroidogenic enzyme 3-Beta-HSD expression, protein labelling by immunostaining was quantified. In this analysis, three random images (30 cells) were captured from the testicular parenchyma of mice using an Olympus BX60 microscope with a coupled camera. The images were treated to convert into greyscale in Photoshop CS6 v13.0, and pixel intensity was measured from the labelled cells, normalized by the pixel intensity obtained from the background of the image (lumen of seminiferous tubules or blood vessels).

2.6 Statistical analysis

All quantitative data were tested for normality and homoscedasticity of variances by the D'Agostino & Pearson tests. The data obtained were expressed as the mean ± standard error of the mean (SEM). Statistical analyses were performed through the program GraphPad Prism 6 (GraphPad Software, Inc). The level of significance considered was $p < 0.05$.

For testis tissue xenograft, mice body mass, seminal vesicle mass, and serum levels of testosterone presented a normal distribution and were submitted to analysis of variance (One-way ANOVA), and the

Newman-Keuls test compared the means of the reproductive stages. The testis tissue graft mass in Mature and Maturing stages presented normal distribution and were submitted to Student's t-test. Testis tissue graft mass in Rest and Regressed stages presented a non-parametric distribution and were submitted to the Kolmogorov-Smirnov test. The percentages of seminiferous tubules with germ cells also showed a non-parametric distribution but were submitted to Kruskal-Wallis and Dunn's test to compare the means of the reproductive stages.

For brown adipose tissue xenograft, mice body mass, seminal vesicle mass, serum levels of testosterone and 3-Beta-HSD pixel intensity presented normal distribution and were submitted to Student's t-test. The brown adipose tissue graft mass presented a non-parametric distribution and was submitted to the Kolmogorov-Smirnov test.

3. Results

3.1 Mice seminal vesicle mass and serum testosterone levels did not change among the groups of testis tissue xenograft

The body mass and seminal vesicle masses of mice that received testis tissue xenografts showed no significant differences among the reproductive stages (Fig. 2A and B, Table 2). The absolute values of serum levels of testosterone increased from the Rest to the Mature stage and decreased in the Regressed stage. However, it is crucial to mention that no significant variation was observed among the reproductive stages (Fig. 2D, Table 2).

3.2 Testis tissue fragments from the Rest stage presented the highest growth index

The testis fragments from the Rest stage demonstrated an expressive and significant volume increase after grafting (prior grafting = 0.0012 ± 0.0002 g and after grafting = 0.0088 ± 0.0013 g) (*Kolmogorov-Smirnov test*, *KS* = 0.9667, P = 0.0002) (Fig. 2C). Although in a lower index, the volume of testis fragments from the Maturing stage also significantly increased after grafting (prior grafting = 0.0028 ± 0.0003 g and after grafting = 0.0054 ± 0.0006 g) (*t-test*, $t = 2.618$, d.f. = 22, P = 0.0157) (Fig. 2C). On the other hand, the testis fragments from the Mature stage presented a significant volume decrease (prior grafting = 0.0150 ± 0.0015 g and after grafting = 0.0091 ± 0.0014 g) (*t-test*, $t = 2.549$, d.f. = 16, P = 0.0215) (Fig. 2C), and testis fragment size did not differ in the Regressed stage after grafting (prior grafting = 0.0064 ± 0.0007 g and after grafting =0.0161 ± 0.0089 g) (*Kolmogorov-Smirnov test*, *KS* =

241 0.2778, P = 0.8782) (Fig. 2C).

242

243 **3.3 Xenografting in the Rest stage promoted an excellent development of the spermiogenic phase**

244 The yellowish myotis testis parenchyma presented only Sertoli and undifferentiated spermatogonial
245 cells in the Rest stage (*in situ*) (Fig. 3A). Surprisingly, the testis fragments (xenografting) in this phase
246 resulted in an expressive development of the seminiferous epithelium, displaying germ cells from the
247 three phases of spermatogenesis (Fig. 3A' and A", 4A). Undifferentiated and differentiated
248 spermatogonial cells were evident (>95%) in the basal compartment (Fig. 3A" and 4A) of the
249 seminiferous tubule cross-sections (Fig. 4E and F, Table 2). Primary spermatocytes, pre-leptotene,
250 zygotene and pachytene cells were readily observed (Fig. 3A" and 4A) in 75-85 % of the seminiferous
251 tubule cross-sections (Fig. 4C and D, Table 2). Regarding the third phase of spermatogenesis, round
252 spermatids were the most advanced germ cell type identified (Fig. 3A" and 4A), showing the highest
253 percentage among the reproductive stages (Fig. 4B, Table 2).

254

255 **3.3.1. Spermatogenesis progressed very well until the spermatocytary phase after xenografting**
256 **in the Maturing stage**

257 The differentiation of spermatogonia into primary spermatocytes characterizes the yellowish myotis
258 Maturing stage (*in situ*) (Fig. 3B). Similarly, the testis grafts presented differentiated spermatogonial cells
259 and primary spermatocytes (Fig. 3B' and B", 4A) in the majority of the seminiferous tubule cross-sections
260 (85% and 50-70%, respectively) (Fig. 4C to F, Table 2). Although in a lower frequency, it should be
261 mentioned that round spermatids were observed in the seminiferous epithelium (Fig. 3B", 4A and B,
262 Table 2).

263

264 **3.3.2 Reduced spermatogenic activity was observed in testis fragments xenografted in the**
265 **Mature stage**

266 In the yellowish myotis Mature stage (*in situ*), a natural gap was observed between undifferentiated
267 spermatogonial cells and primary spermatocytes. Furthermore, in this phase, more advanced germ cells,
268 including round and elongated spermatids, were identified (Fig. 3C). After grafting in this phase, a small
269 percentage of seminiferous tubule cross-sections showed germ cells beyond the undifferentiated
270 spermatogonial cells (Fig. 4A to F, Table 2). Although in a reduced number, differentiated

271 spermatogonia, spermatocytes and round spermatids were also identified (Fig. 3C' and C", 4A to E, Table
272 2).
273
274 **3.3.3 Reduced activity of meiotic and spermiogenic phases was observed after xenografting in**
275 **the Regressed stage**
276 The yellowish myotis testis in the Regressed stage (*in situ*) presents unique characteristics, such as
277 seminiferous tubules with a wide lumen and a vast gap between undifferentiated spermatogonial cells and
278 elongated spermatids (Fig. 3D). After grafting, approximately half of the seminiferous tubule cross-
279 sections (>52.5%) displayed undifferentiated and differentiated spermatogonia (Fig. 4E and F). Although
280 in a lower frequency, primary spermatocytes (pre-leptotene, leptotene, zygotene and pachytene) and
281 round spermatids (Fig. 3D", 4A-D, and Table 2) were also observed.
282
283 **3.4 Mast cells were frequently observed in the most advanced testis tissue fragments**
284 Although the germ cell composition was quite different among the reproductive phases, all testis
285 xenografts led to the formation of round spermatids (Fig. 3 and 4). Interestingly, mast cells were
286 frequently observed in the xenograft interstitial compartment, especially in those fragments that presented
287 the highest development (Rest and Maturing stages) (Fig. 3A" and B"). Several histopathological
288 alterations were observed in the xenografts of the Maturing, Mature, and Regressed stages (Fig. 3B', C'
289 and D').
290
291 **3.5 The brown adipose tissue graft mass decreased promoting androgenic stimuli**
292 After two months of grafting, no significant variation was observed in mice weight (Table 3).
293 Differently, the seminal vesicle mass presented a significant increase (Fig. 5A, Table 3). In an opposite
294 pattern, the brown adipose tissue graft mass decreased significantly (*Kolmogorov-Smirnov test*, *KS* =
295 0.6875, P = 0.0010) (Fig. 5B). Moreover, testosterone serum levels increased more than sixfold in the
296 grafted mice (Fig. 5C, Table 3). The different 3-Beta-HSD immunolabeling patterns in Leydig cell
297 cytoplasm from control mice (Fig. 5E) and grafted mice (Fig. 5F) and the significant increased 3-Beta-
298 HSD pixel intensity evaluation (Fig. 5D) indicated that these cells are being stimulated by the brown
299 adipose tissue consumption.
300

4. Discussion

Yellowish myotis presents a seasonal reproduction linked to rainfall distribution (Farias *et al*. 2020). For the first time, we strongly suggest that the brown adipose tissue plays a pivotal function in the physiology of the seasonal yellowish myotis testis. The cyclic fluctuation of the weight is coincident with the high serum testosterone levels. This feature indicates that the brown adipose tissue may stimulate gonad steroidogenic activity, inducing spermatogonia differentiation and spermatogenesis progression. Herein, we opted to graft the organs in immunodeficient mice to observe the gonad and brown adipose tissue physiology in a stable endocrine milieu. In general, the recipient mice allowed a constant testosterone production and promoted continuous spermatogenesis in testis tissues from all reproductive stages. Additionally, the steroidogenic function of brown adipose tissue was confirmed, reinforcing that this organ is crucial for yellowish myotis reproduction (Fig. 6).

In our study, testis tissue xenografts of an adult Neotropical bat were successfully performed for the first time. The testosterone serum levels of grafted mice did not show significant differences among the reproductive stages, suggesting that a stable LH stimulus in mice regulated the hormonal synthesis. Additionally, the germ cells progressed until the round spermatid step in testis grafts of all reproductive stages. This finding indicates that testosterone would be vital in promoting undifferentiated spermatogonial differentiation, as observed in other wild mammalian species (collared peccary) (Campos-Junior *et al*. 2012). Interestingly, the Rest stage fragments were more successful considering the spermatogenic progression, probably due to a low state of spermatogenesis (Arregui *et al*. 2008b). One can say that, in the Rest stage, more undifferentiated spermatogonial cells were able to differentiate compared to the Maturing, Mature and Regressed stages.

The success of testis xenografts is highly variable in adult wild mammal species (Table 4). Using a seasonal and adult animal (Djungarian hamster), Schlatt and colleagues showed that most testis tissues degenerated after grafting (Schlatt *et al*. 2002); however, spermatocytes were found in photoregressed testis tissues seven weeks post-grafting (Schlatt *et al*. 2002). Unlike the good results achieved in yellowish myotis, testis grafts from adult Linx (*Lynx pardinus*) and older monkeys (*Macaca mulatta*; 11-12 years-old) degenerated after grafting (Arregui *et al*. 2008b, 2014). Grafts from subadult monkeys (6 years-old) presented a discrete advance of spermatogenesis, and spermatocytes were observed in 0.3% of the seminiferous tubules cross-sections (Arregui *et al*. 2008b). Grafts from younger subadult monkeys (3 years-old) showed higher percentages of seminiferous tubules with spermatocytes (64.1%) after 24 weeks

of transplantation. However, it should be mentioned that elongated spermatids were identified in few seminiferous tubules cross-sections (1.1%) (Arregui *et al*. 2008b).

The testis tissue xenografts from immature donors usually show a better development than tissue grafts from sexually mature donors (Arregui *et al*. 2008b, Arregui & Dobrinski 2014). Several factors could be favoring juvenile graft development, such as a lower metabolism of spermatogenesis, higher resistance to ischemic conditions and intense somatic cell proliferation (Schlatt *et al*. 2002, Arregui *et al*. 2008b, Arregui & Dobrinski 2014). As previously mentioned, most grafted tissues from adult donors usually degenerate (Schlatt *et al*. 2002, Arregui *et al*. 2008b, 2014). The first report of complete spermatogenesis resulting in viable and functional sperm occurred in testis tissue xenografts from immature mice, pigs and goats (Honaramooz *et al*. 2002). This technique was successfully applied on juvenile wild animals, such as bison calves, white-tailed deer, collared peccary, ferret, Djungarian hamster and rhesus monkey, resulting in sperm production (Schlatt *et al*. 2002, Honaramooz *et al*. 2004, Abbasi & Honaramooz 2011, 2012, Gourdon & Travis 2011, Campos-Junior *et al*. 2014). In the endangered immature Cuvier's gazelle (Arregui *et al*. 2014), a similar spermatocyte percentage was found in testis parenchyma compared to yellowish myotis grafting (Rest stage). Interestingly, the spermatogenic development in testis grafts from adult yellowish myotis was better than some testis xenografting of immature wild animals. Among them, bison, white-tailed deer, banteng and Iberian lynx can be cited (Honaramooz *et al*. 2005, Abbasi & Honaramooz 2011, 2012, Aguerri *et al*. 2014).

The brown adipose tissue of yellowish myotis showed a weight fluctuation along the reproductive stages (Farias *et al*. 2020). The consumption of the brown adipose tissue by yellowish myotis coincided with the better progression of spermatogenesis and production of testosterone. It is thought that the latter allowed the differentiation of undifferentiated spermatogonia (Farias *et al*. 2020). The androgenic activity of brown adipose tissue was previously explored in hibernating bat *Myotis lucifugus* (Krutzsch & Wells 1960). In this study, non-castrated rats were treated with a fraction (nonsaponifiable) of the interscapular brown adipose tissue of *M. lucifugus*. This treatment promoted an evident seminal vesicle hypertrophy. Furthermore, the authors suggested that 1 gram of this fraction corresponded to 676 µg of testosterone, indicating a high androgenic activity of the brown adipose tissue (Krutzsch & Wells 1960).

To observe if the yellowish myotis brown adipose tissue had an influence on testosterone production, we performed a xenograft with this tissue for the first time. The recipient mice consumed the brown adipose tissue during the two months of grafting. Consequently, there was an increase in the mice seminal

vesicle weight (two times higher) and serum testosterone levels (six times higher). These data confirmed the androgenic role of the yellowish myotis brown adipose tissue. Furthermore, the consumption of brown adipose tissue by yellowish myotis, coinciding with the spermatogonia differentiation, suggests that this organ is linked to germ cell development. Future molecular studies must be conducted to investigate if the Regressed and Rest stages promote undifferentiated spermatogonia expansion before the serum testosterone peak. According to the 3-Beta-HSD immunostaining and pixel intensity, the brown adipose tissue, directly or indirectly, stimulates the Leydig cell steroidogenic activity. The more robust 3-Beta-HSD immunolabeling pattern was previously demonstrated in yellowish myotis (*in situ*) during brown adipose tissue consumption (Farias *et al*. 2020). While brown adipose tissue contributes to heat production during the arousal from hibernation in temperate zone bats (Smalley & Dryer 1963, Hayward & Ball 1966, Lyman 1970), it is linked to reproduction in Neotropical yellowish myotis, possessing an essential androgenic function. Presently, we will further investigate the androgenic stimuli promoted by the brown adipose tissue products in detail to demonstrate the specific mechanisms of interaction in the hypothalamic-pituitary-gonadal axis (indirect action) or testis parenchyma (direct action).

5. Declaration of interest, funding, contributions and Acknowledgements

5.1 Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

5.2 Funding

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5.3 Author contribution statement

T.O.F., S.A.T and G.M.J.C. planned the experiments. T.O.F and G.M.J.C captured the animals, performed the experiments and wrote the paper. A.F.A.F and N.T.W did the mouse IHC analyzes. A.F.A.F, S.A.T. and N.T.W. did a critical revision of the manuscript. T.O.F. and G.M.J.C. approved the final version of the paper.

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571 **7. Figure caption**

572 **Fig. 1 Brown adipose tissue and epididymides from yellowish myotis donors.** Yellowish myotis
573 brown adipose tissue is located in the interscapular region and presented several lipid droplets (A). The
574 sperm (*) found in yellowish myotis epididymides (in all reproductive stages) confirmed that all animals
575 were adults. Sperm cells were found in all animals captured in the Rest (B), Maturing (C), Mature (D) and
576 Regressed (E) stages. Bars: a to d = 20µm.
577
578 **Fig. 2 Mean (± SEM) values of biometric and hormonal data of mice grafted with testis tissue**
579 **fragments in yellowish myotis reproductive stages**. The body mass (A) and seminal vesicle mass (B)
580 do not differ among the reproductive stages, while the testis graft mass increased significantly after
581 grafting in the Rest stage (C). No significant differences were observed for the serum testosterone levels
582 (D). Different letters indicate statistically significant differences, $p < 0.05$.
583
584 **Fig. 3 Spermatogenesis development in yellowish myotis testis tissue xenograft.** The testis
585 parenchyma of yellowish myotis in the reproductive stages *in situ* showed a huge variation in germ cell
586 composition (A to D). The testis fragments of all the reproductive stages showed the three phases of
587 spermatogenesis, with the seminiferous tubules (ST) presenting round spermatids as the most advanced
588 germ cell type in the seminiferous epithelium (SE) (A' to D' and A" to D"). The presence of mast cells in
589 the intertubular compartment (IC) was frequently observed in the Rest and Maturing phases (A" and B").
590 Except for the Rest phase fragments, histopathological alterations (HA) were observed in the other
591 phases' fragments (B' to D'). Arrowheads: white (Sertoli cell), black (undifferentiated spermatogonia),
592 grey (differentiated spermatogonia), blue (pre-leptotene spermatocyte), green (zygotene spermatocyte),
593 yellow (pachytene spermatocyte), orange (round spermatid), brown (elongating spermatid), red
594 (elongated spermatid), pink (mast cells). Bars: A to D, A' to D', and A" to D" = 20µm; A" to D" inserts =
595 4 µm.
596
597 **Fig. 4 Germ cell composition and quantification in the yellowish myotis testis tissue xenograft.** The
598 seminiferous epithelium of the testis grafts presented undifferentiated spermatogonia (UND),
599 differentiated spermatogonia (DIFF), pre-leptotene spermatocyte (Pl), leptotene spermatocyte (L),
600 zygotene spermatocyte (Z), pachytene spermatocyte (P) and round spermatid (R) in all the reproductive

stages of yellowish myotis (A). However, the testis grafts in the Rest stage presented a significantly higher percentage of round spermatids. In an opposite pattern, the testis grafts in the Mature stage showed fewer germ cells in the seminiferous tubules (B to F). Different letters show statistically significant differences, $p < 0.05$. Bars: 4 µm.

Fig. 5 Biometric, hormonal and immunohistochemical parameters of mice grafted with brown adipose tissue grafts. The seminal vesicle mass increased significantly in the grafted group (A). The brown adipose tissue graft mass decreased significantly after grafting (B), while the serum levels of testosterone significantly increased more than six times in the grafted mice (C). Moreover, there is a difference in the 3-Beta-HSD immunolabelling pattern between the control (E) and grafted group (F), demonstrated by the significant increase in the enzyme expression (D). Insert shows the negative control. Bars: 21 µm.* show statistically significant differences, $p < 0.05$.

Fig. 6 Physiological behavior of testis and brown adipose tissue in situ and grafted in mice. Increased testosterone serum levels promoted spermatogonial differentiation in Yellowish myotis (in situ). The stability of testosterone serum levels in recipient mice allowed the spermatogonial differentiation in testis tissue xenograft from all reproductive stages. The consumption of the brown adipose tissue in yellowish myotis (in situ) coincided with the higher testosterone production. The brown adipose tissue xenograft promoted massive testosterone production in immunodeficient mice, confirming its androgenic function.

8. Tables

Table 1. Yellowish myotis sampling from August 2017 to November 2020.

Reproductive stages	Months	Number of animals	Analyses
Rest	May - June	6	Testis xenograft
	July - August	6	Testis xenograft
Maturing	November - December	14	Testis and brown adipose tissue xenograft
	January - February	3	Brown adipose tissue xenograft
Mature	March	6	Testis xenograft
Regressed	April	6	Testis xenograft

Table 2. Mean (± SEM) values of mouse and testis fragment parameters after grafting.

Parameters	Rest	Maturing	Mature	Regressed	ANOVA *(F)*	P value
BM (g)	29.20 ± 1.02	32.00 ± 2.00	27.40 ± 2.40	29.33 ± 1.33	1.253	0.3473
SVM (g)	0.4664 ± 0.0594	0.4807 ± 0.1247	0.4084 ± 0.0045	0.4427 ± 0.0916	0.1040	0.9556
TSL (ng/dL)	97.13 ± 19.50	193.70 ± 92.03	307.00 ± 5.00	193.20 ± 56.13	1.725	0.2484
UND (%)	95.00 ± 3.44^{a}	87.50 ± 6.15^{a}	45.00 ± 8.03^{b}	57.50 ± 8.33^{b}	27.97*	<0.0001
DIFF (%)	97.50 ± 2.50^{a}	85.00 ± 6.40^{ab}	12.50 ± 7.14^{c}	52.50 ± 10.60^{b}	39.89*	<0.0001
Pl-Z (%)	75.00 ± 7.70^{a}	70.00 ± 6.70^{a}	12.50 ± 6.15^{b}	25.00 ± 8.51^{b}	34.10*	<0.0001
P (%)	85.00 ± 5.26^{a}	50.00 ± 8.11^{ab}	10.00 ± 5.85^{c}	37.50 ± 8.80^{bc}	34.12*	<0.0001
R (%)	37.50 ± 7.14^{a}	5.00 ± 3.44^{b}	5.00 ± 5.00^{b}	7.50 ± 4.10^{b}	24.81*	<0.0001

Different line superscript letters show statistically significant differences, $p < 0.05$. * Kruskal-Wallis test (H)

BM = Body mass, SVM = seminal vesicle mass, TSL = testosterone serum levels, UND = undifferentiated spermatogonia, DIFF = differentiated spermatogonia, Pl-Z = pre-leptotene to zygotene spermatocytes, P = pachytene spermatocytes, R = round spermatids.

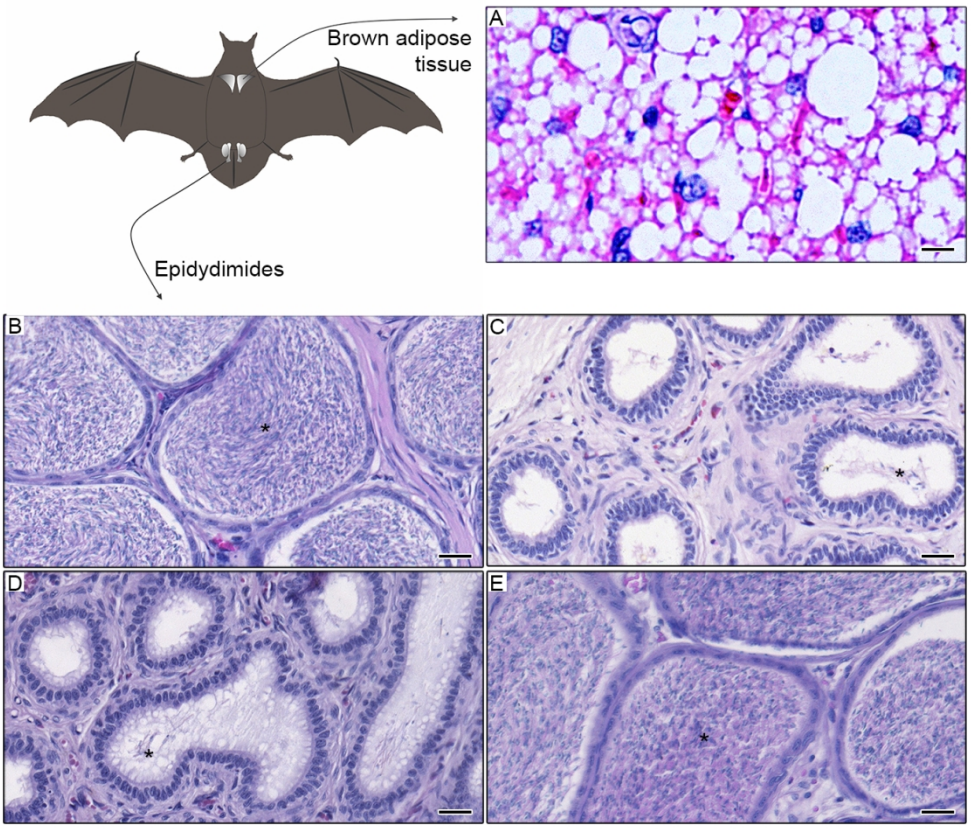
Table 3. Mean (± SEM) values (in g, except for TSL: ng/Dl, and PI) of body mass (BM), seminal vesicle mass (SVM), testosterone serum levels (TSL) and 3-Beta-HSD pixel intensity (PI) of control mice and mice that received brown adipose tissue (BAT) xenografts of yellowish myotis.

Experimental groups	BM	SVM	TSL	PI
Control	25.10 ± 1.57	0.1617 ± 0.0326[a]	283.6 ± 101.9[a]	115.3 ± 3.8[a]
BAT grafts	24.05 ± 1.69	0.3158 ± 0.0425[b]	1857.0 ± 274.9[b]	130.2 ± 4.3[b]
Student's t-test *(t)*	0.4237	2.564	4.343	2.523
P value	0.6800	0.0263	0.0012	0.0326

Different column superscript letters show statistically significant differences, $p < 0.05$.

Table 4. Morphological aspects of testis tissue xenografting in mature wild mammal species.

Xenograft development	Species and age	Reference
Degeneration	Iberian lynx (2 years old), Rhesus monkey (11 and 12 years old)	Arregui et al. 2014, 2008b
Sertoli cell only	Rhesus monkey (11 and 12 years old)	Arregui et al. 2008b
Spermatocytes	Djungarian hamster, Rhesus monkey (6 years old)	Schlatt et al. 2002, Arregui et al. 2008b
Round spermatids	Yellowish myotis	Present study
Elongated spermatids	Rhesus monkey (3 years old)	Arregui et al. 2008b



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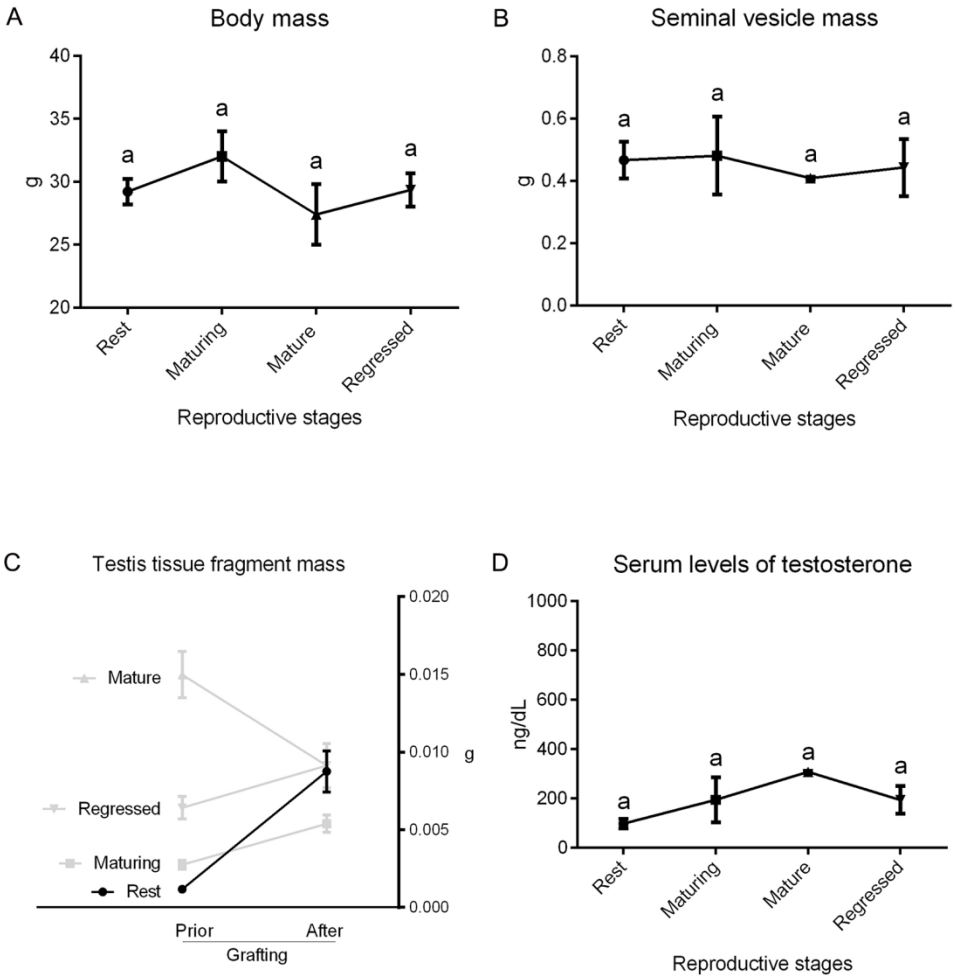

Fig. 2 Mean (± SEM) values of biometric and hormonal data of mice grafted with testis tissue fragments in yellowish myotis reproductive stages. The body mass (A) and seminal vesicle mass (B) do not differ among the reproductive stages, while the testis graft mass increased significantly after grafting in the Rest stage (C). No significant differences were observed for the serum testosterone levels (D). Different letters indicate statistically significant differences, $p < 0.05$.

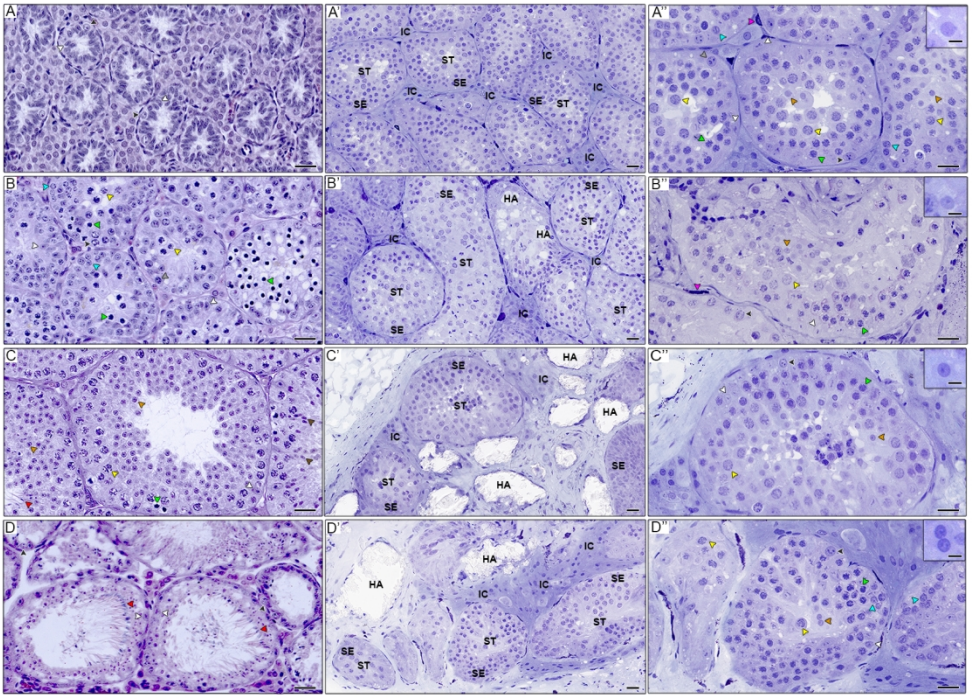

Fig. 3 Spermatogenesis development in yellowish myotis testis tissue xenograft. The testis parenchyma of yellowish myotis in the reproductive stages in situ showed a huge variation in germ cell composition (A to D). The testis fragments of all the reproductive stages showed the three phases of spermatogenesis, with the seminiferous tubules (ST) presenting round spermatids as the most advanced germ cell type in the seminiferous epithelium (SE) (A' to D' and A'' to D''). The presence of mast cells in the intertubular compartment (IC) was frequently observed in the Rest and Maturing phases (A'' and B''). Except for the Rest phase fragments, histopathological alterations (HA) were observed in the other phases' fragments (B' to D'). Arrowheads: white (Sertoli cell), black (undifferentiated spermatogonia), grey (differentiated spermatogonia), blue (pre-leptotene spermatocyte), green (zygotene spermatocyte), yellow (pachytene spermatocyte), orange (round spermatid), brown (elongating spermatid), red (elongated spermatid), pink (mast cells). Bars: A to D, A' to D', and A'' to D'' = 20μm; A'' to D'' inserts = 4 μm.

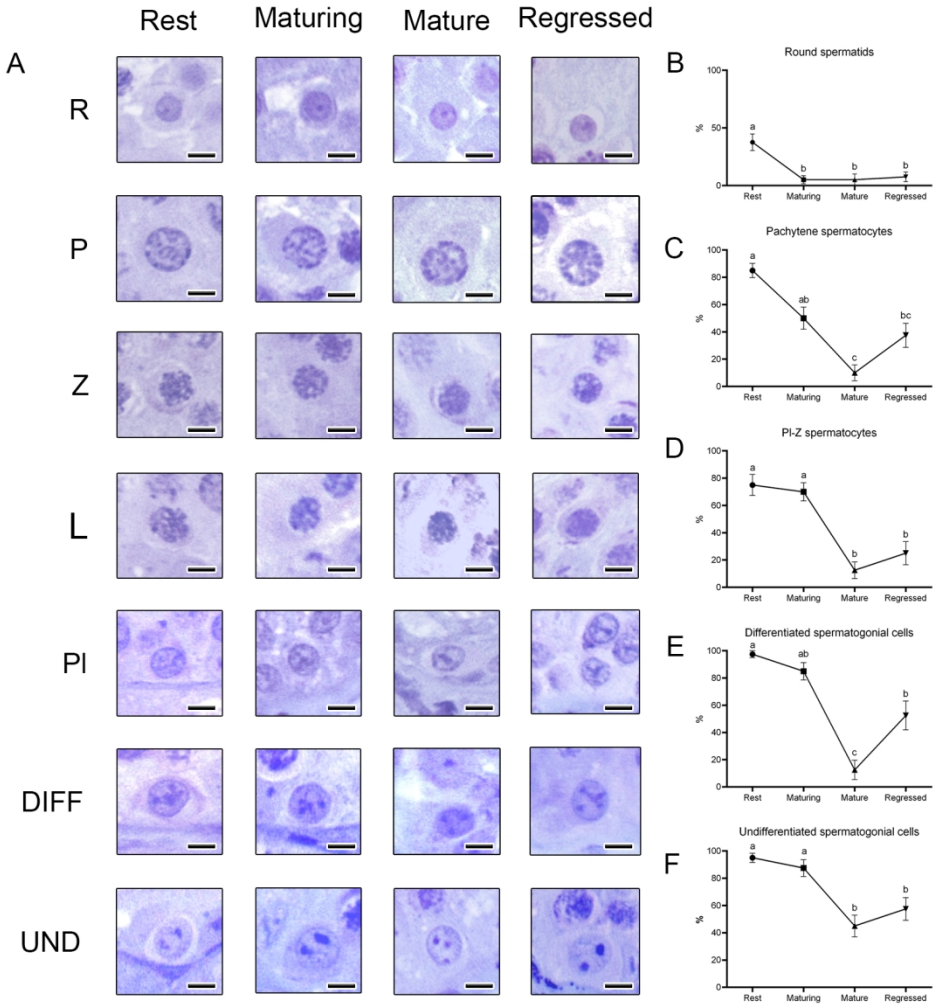

Fig. 4 Germ cell composition and quantification in the yellowish myotis testis tissue xenograft. The seminiferous epithelium of the testis grafts presented undifferentiated spermatogonia (UND), differentiated spermatogonia (DIFF), pre-leptotene spermatocyte (Pl), leptotene spermatocyte (L), zygotene spermatocyte (Z), pachytene spermatocyte (P) and round spermatid (R) in all the reproductive stages of yellowish myotis (A). However, the testis grafts in the Rest stage presented a significantly higher percentage of round spermatids. In an opposite pattern, the testis grafts in the Mature stage showed fewer germ cells in the seminiferous tubules (B to F). Different letters show statistically significant differences, $p < 0.05$. Bars: 4 µm.

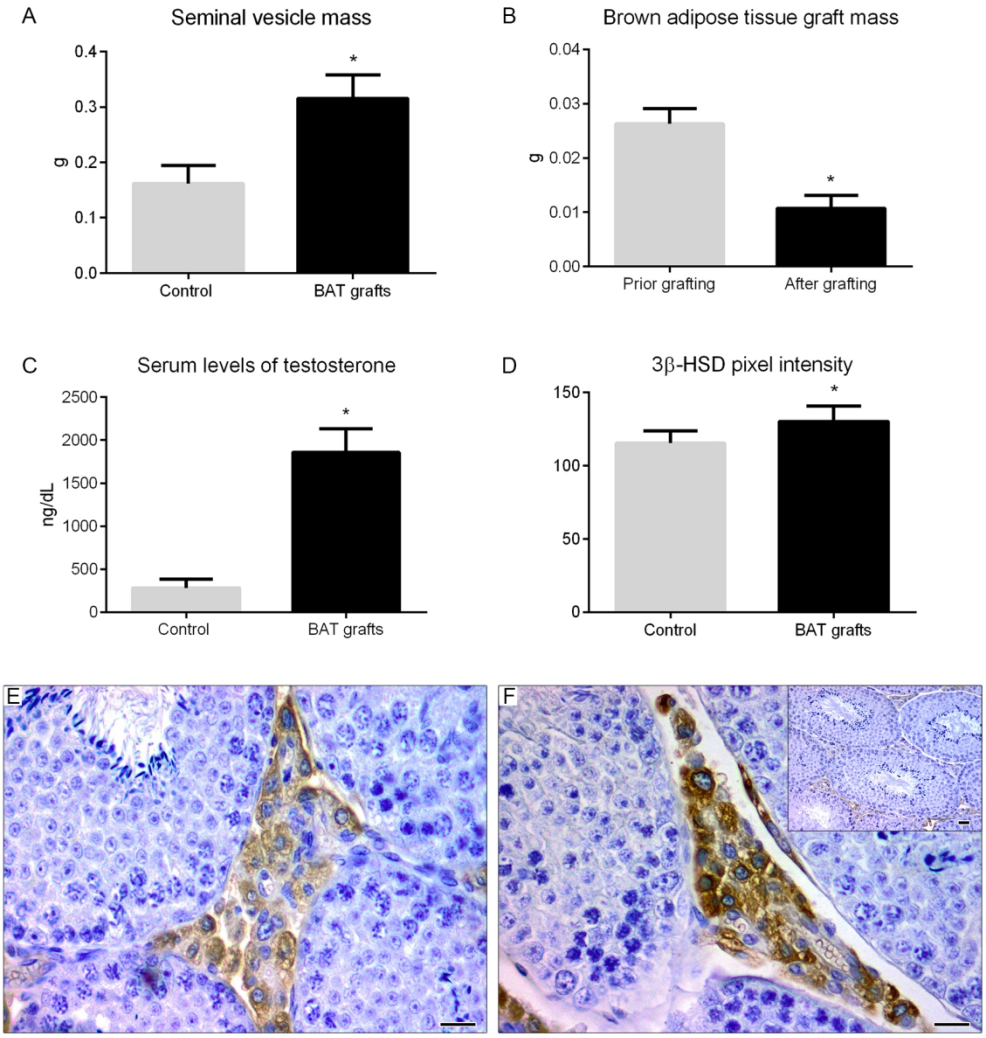

Fig. 5 Biometric, hormonal and immunohistochemical parameters of mice grafted with brown adipose tissue grafts. The seminal vesicle mass increased significantly in the grafted group (A). The brown adipose tissue graft mass decreased significantly after grafting (B), while the serum levels of testosterone significantly increased more than six times in the grafted mice (C). Moreover, there is a difference in the 3-Beta-HSD immunolabelling pattern between the control (E) and grafted group (F), demonstrated by the significant increase in the enzyme expression (D). Insert shows the negative control. Bars: 21 μm.* show statistically significant differences, $p < 0.05$.

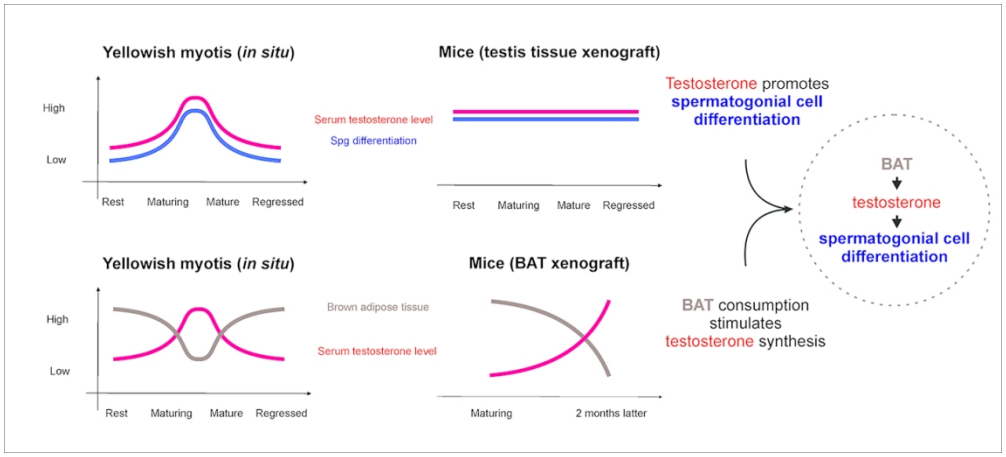

Fig. 6 Physiological behavior of testis and brown adipose tissue in situ and grafted in mice. Increased testosterone serum levels promoted spermatogonial differentiation in Yellowish myotis (in situ). The stability of testosterone serum levels in recipient mice allowed the spermatogonial differentiation in testis tissue xenograft from all reproductive stages. The consumption of the brown adipose tissue in yellowish myotis (in situ) coincided with the higher testosterone production. The brown adipose tissue xenograft promoted massive testosterone production in immunodeficient mice, confirming its androgenic function.

4. DISCUSSÃO

Myotis levis é um vespertilionídeo neotropical que apresenta padrão reprodutivo sazonal caracterizado por distintos estágios de atividade reprodutiva, em relação a morfofisiologia dos órgãos reprodutivos, correlacionados com a distribuição da precipitação.

As gônadas persistem no estágio de Repouso por seis meses, mas ocorre proliferação das células de Sertoli, detectada e confirmada pelos marcadores Ki-67 e GATA-4, respectivamente. Os epidídimos armazenam os espermatozoides do ciclo reprodutivo anterior, onde o número de espermatozoides morfologicamente normais apresenta um aumento significativo nesse estágio, coincidindo com o período de acasalamento (Araújo *et al*., 2013). Interessantemente, fragmentos testiculares em Repouso enxertados em um meio hormonal estável (camundongos imunodeficientes) e sem influência de fatores ambientais apresentam maior taxa de crescimento e excelente desenvolvimento da fase espermiogênica. Acredita-se que a baixa atividade metabólica, a proliferação de células somáticas e os níveis adequados de testosterona sejam os principais fatores responsáveis (Arregui & Dobrinski, 2014).

A reativação das gônadas ocorre no estágio de Maturação, em que a atividade proliferativa das células germinativas é expressiva e coincide com a máxima produção de testosterona após pico de precipitação e possivelmente devido a maior disponibilidade de recursos alimentares (Janzen & Schoener, 1968; Rautenbach *et al*., 1988). Após o período de acasalamento, poucos espermatozoides vivos ainda estão presentes na cauda do epidídimo, ressaltando a capacidade do epidídimo de armazenar e manter os espermatozoides por longos períodos. Nesse estágio, o xenoenxerto de fragmentos testiculares apresenta boa progressão até a fase espermatocitária, sendo a fase espermiogênica pouco evidente. Contudo, cabe ser mencionado que foram produzidas espermátides arredondadas em baixa frequência.

O desenvolvimento do processo espermiogênico ocorre no estágio Maduro, ainda que nem todas as células germinativas estejam presentes no epitélio seminífero. A produção espermática de *M. levis* ocorre em um restrito período do ano, sendo que a fase espermiogênica é concluída no estágio de Regressão. O ciclo do epitélio seminífero pode ser dividido em dez estádios e o epitélio seminífero apresenta um grande número de células de Sertoli. Foi visto que a espermatogênese apresenta curta duração, resultando em uma alta produção espermática diária. Nessa fase, elevados níveis séricos

de testosterona estão correlacionados positivamente com a maior expressão da enzima esteroidogênica 3-Beta-HSD no citoplasma das células de Leydig. O xenoenxerto testicular nesse estágio apresentou os piores índices de desenvolvimento, provavelmente devido ao maior metabolismo testicular, e a presença de poucas espermatogônias indiferenciadas comprometidas com a diferenciação e de células somáticas mais diferenciadas no parênquima testicular.

O estágio de Regressão é caracterizado pela finalização da liberação dos espermatozoides e armazenamento dos mesmos na cauda do epidídimo. Nas gônadas, ocorre uma marcante atividade apoptótica, indicada pela expressão da enzima Caspase-3 em espermátides alongadas no epitélio seminífero e em espermatozoides no lúmen testicular. No epidídimo, os parâmetros de concentração, vitalidade e motilidade espermática apresentam um pico, apesar de uma alta concentração de espermatozoides anormais. O xenoenxerto de fragmentos testiculares em regressão apresentou baixa eficiência nas fases espermatocitária e espermiogênica. Dessa forma, observa-se que as fases produtoras e liberadoras de espermatozoides apresentaram menor progressão da espermatogênese no xenoenxerto de fragmentos testiculares.

O tecido adiposo marrom apresenta uma diminuição de sua massa ao longo dos estágios de Maturação e Maduro, sendo consumido coincidentemente com o aumento dos níveis séricos de testosterona. Dessa forma, através do xenoenxerto de tecido adiposo marrom foi possível comprovar o envolvimento desse tecido na síntese de andrógenos, ressaltando a sua importância na fisiologia testicular de *M. levis*. Portanto, é necessário investigar os mecanismos e fatores envolvidos na função androgênica do tecido adiposo marrom de *M. levis*. Essa investigação poderia ser útil, inclusive, para o desenvolvimento de terapias para tratamento de hipogonadismo masculino.

De modo geral, os resultados do presente estudo fornecem novas informações sobre diferentes aspectos da reprodução de *M. levis*. A utilização de xenoenxertos de tecidos comprovou a influência da sazonalidade sobre o processo espermatogênico, ampliando a nossa compreensão sobre a biologia reprodutiva dos vespertilionídeos neotropicais. Ainda, pode-se vislumbrar o xenoenxerto testicular como uma potencial ferramenta para a produção de gametas e preservação do genótipo de morcegos. Em outra vertente, a avaliação da relação entre o tecido adiposo marrom e os órgãos genitais de *M. levis* foi muito importante para compreender os aspectos relacionados a síntese de andrógenos, que podem ser fundamentais para a manutenção e maturação dos gametas.

5. CONCLUSÃO

Após a investigação dos aspectos celulares, moleculares e endócrinos da reprodução de machos adultos do morcego insetívoro neotropical *Myotis levis*, conclui-se que:

- Os estágios reprodutivos de *M. levis* são fortemente correlacionados aos níveis de precipitação, em que os órgãos reprodutivos e tecido adiposo marrom apresentam uma flutuação cíclica em sua atividade.
- O testículo e as células germinativas alcançam máximo desenvolvimento no estágio Maduro, enquanto o epidídimo possui maior massa no estágio de Regressão e armazena espermatozoides por até oito meses.
- As alterações morfológicas e funcionais das células de Leydig estão altamente correlacionadas com os níveis séricos de testosterona.
- A dinâmica de morte e proliferação das células testiculares é expressiva nos estágios de Regressão e Maturação, respectivamente.
- O processo espermatogênico de *M. levis* é caracterizado por curta duração e elevada produção espermática.
- Os parâmetros de concentração, vitalidade e motilidade espermática apresentam os maiores valores no estágio de Regressão, porém a maior porcentagem de espermatozoides normais ocorre no estágio de Repouso.
- O epidídimo é capaz de eliminar os espermatozoides anormais antes do período de acasalamento.
- A espermatogênese de *M. levis* é capaz de progredir até a fase espermiogênica em todos os estágios reprodutivos desde que exista em um ambiente hormonal estável.
- A função androgênica do tecido adiposo marrom foi comprovada, sugerindo sua forte participação na reprodução de *M. levis*.

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7. ANEXOS

7.1 Artigos publicados

- Farias, T. O., Talamoni, S. A., Godinho, H. P. (2018). Reproductive dynamics of the nectarivorous Geoffroy's tailless bat *Anoura geoffroyi* (Glossophaginae) in a highland Neotropical area of Brazil, with evidence of a mating period. *Acta Chiropterologica*, 20, 251-261.

- Viana, P. I., Farias, T. O., Talamoni, S. A., Godinho, H. P. (2018). Sertoli cell efficiency of the Neotropical bats *Anoura geoffroyi*, *Artibeus lituratus* and *Myotis levis* (Mammalia: Chiroptera). Acta Chiropterologica, 20, 493-501.

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Reproductive dynamics of the nectarivorous Geoffroy's tailless bat *Anoura geoffroyi* (Glossophaginae) in a highland Neotropical area of Brazil, with evidence of a mating period

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This study investigated the reproductive dynamics of a colony of Geoffroy's tailless bat (*Anoura geoffroyi*), an important pollinating bat, in a highland area of Southeastern Brazil. The colony was monitored each month from November 2014 to December 2015 with 154 males and 117 females captured. Testicular and ovarian histological data from 31 adult males and 22 adult females were obtained. Body condition index of male and female, and gonadosomatic index, epididymis-somatic index and Sertoli cell efficiency were analyzed. Females with spermatozoa in uterine crypts, embryos in oviducts, gravid uteri, and pregnant females were registered during the rainy season (November–March) and into the beginning of the following dry season (April), indicating asynchrony of births. The ovaries showed all types of ovarian follicles, and a polarized ovary cortex was found, differing from the organization pattern of most mammals. The testes showed continuous spermatic activity, but testicular parameters analyzed showed significantly higher values during the rainy season. A shorter mating period during the beginning of this season was detected, when values of the gonadosomatic and epididymis-somatic indexes, and Sertoli cell efficiency showed more pronounced differences in relation to the remaining period of study. Unlike polyestry known for some Neotropical phyllostomids, the reproductive cycle of *A. geoffroyi* was characterized as seasonal monoestrous, with the main reproductive events occurring during the rainy season, as the best time for females to reproduce, but with offspring recruitment occurring predominantly during the dry season, a period with fewer food resources.

Key words: *Anoura*, asynchrony of births, Chiroptera, mating period, monoestry, seasonal reproduction

Introduction

Geoffroy's tailless bat (*Anoura geoffroyi* Gray, 1838) is a glossophagine with morphological characteristics that evolved as specializations for nectarivory, such as elongated snout, short ears and leaf nose, lower jaw extending beyond the upper jaw, lower incisors absent, and upper incisors reduced and displaced laterally. The lower lip has a deep, medially located groove which facilitates the passage of a long, narrow and highly extensible tongue during nectar feeding (Winter and von Helversen, 2003). Therefore, this bat plays an essential role in the maintenance of ecosystems through pollination (Fleming *et al*., 2009; Kunz *et al*., 2011).

Anoura geoffroyi has a wide range in the Neotropics, occurring from Mexico to Peru, Bolivia and Brazil (Simmons, 2005). In Brazil, it occurs in areas of Cerrado (Brazilian grassland savanna) and Atlantic Forest (Marinho-Filho and Sazima, 1998), where it mainly roosts in caves (Guimarães and Ferreira, 2014). Based on external reproduction traits, differences occur in relation to the reproductive period recorded for this species along its distribution area. Pregnant females were found at the end of the dry season (August, September) to the middle of the rainy season (December) in an area of the Brazilian Cerrado (Willig, 1985) and in Trinidad (Heideman *et al.*, 1992). In another area of the Cerrado in Central Brazil, pregnant females were found at the end of the rainy season (March) and early dry season (April) (Baumgarten and Vieira, 1994). In the Brazilian Semiarid Caatinga, pregnant females were found only in the rainy months of November and January (Willig, 1985). In other South American localities, pregnant females were predominantly recorded in dry months (March to June) in Costa Rica (Mares and Wilson, 1971), (June) in Bolivia (Anderson, 1997) and (May to August) in Peru (Graham, 1987). Besides this conflicting information,

the process of spermatogenesis and folliculogenesis remains unknown for this species. Knowledge on these reproductive processes is essential for understanding the reproductive cycle since reproduction involves costs of energy and body condition (Clutton-Brock *et al.*, 1989) that are dependent on environment conditions.

There is a strong relationship between body condition and sexual development (Speakman and Racey, 1986; Crichton and Krutzsch, 2000). Body condition is dependent on the availability of food resources in the past and present, because nectar-feeding bats do not store a lot of fat (Voigt and Speakman, 2007), which thereby influences decisions about when to reproduce (Thompson, 1992). So, environmental variation typical of terrestrial habitats affects reproduction such that reproductive success is greater at a particular time and place, creating a selective advantage for the use of environmental cues for regulating reproduction (Heideman, 2000). Other decisions involve the social context, the future reproductive potential, and the advantages of synchronizing offspring birth with peaks of food resource availability (Racey and Entwistle, 2000).

The best time for females to reproduce determines the period of reproduction (Heideman, 2000), and there are two types of reproductive strategies that females employ: (i) when the individuals try to reproduce whenever the conditions are favorable, characterizing simple opportunism or (ii) when individuals use a predictive environmental cue (photoperiod, temperature and food resources) that indicates a future period of favorable conditions for reproduction (Bronson and Heideman, 1994; Heideman, 2000).

In Neotropical chiropterans, opportunism may be rare because of their relatively long gestation period (Weir and Rowlands, 1973; Tuttle and Stevenson, 1982), i.e. 3.5 to four months in *Artibeus jamaicensis* (Fleming, 1971) and *Carollia perspicillata* (Kleiman and Davis, 1979), about four months in *Glossophaga soricina*, *Sturnira lilium* and *Uroderma bilobatum* (Fleming *et al*., 1972), five months in *Phyllostomus hastatus* (James, 1977), and five to eight months in *Desmodus rotundus* (Wimsatt and Trapido, 1952). It is unlikely that females can develop follicles, ovulate, mate and reach a condition suitable for the large energy demand of lactation before favorable conditions have passed. Therefore, bats having seasonal reproduction react to environmental cues that indicate a future period when the climate, food availability and other environmental conditions are favorable for successful reproduction (Bronson, 1985; Racey and Entwistle, 2000; Heideman, 2000).

Studies have shown a seasonal polyestrous reproductive cycle for some Neotropical bat species, which have a main cycle during the rainy season (Fleming *et al*., 1972; Chaverri and Kunz, 2006), when the chance for survival of offspring is greater because of the increased availability of fruits (Kunz *et al*., 1998). In nectarivorous bats, lactation is synchronized with flowering (Baumgarten and Vieira, 1994; Racey and Entwistle, 2000), demonstrating the importance of nutrition and food resources availability in determining the reproductive period in bat species.

Aiming to understand the dynamics of the reproductive activity of Geoffroy's tailless bat in the study area, we analyzed reproductive parameters of males and females during one seasonal cycle of rainfall related to: (i) structure of a colony regarding age classes and sex ratio; (ii) individual biometric parameters; (iii) histomorphology of ovaries and testes and (iv) body condition index, gonadosomatic index, epididymis-somatic index, and Sertoli cell efficiency.

Materials and Methods

We studied a colony of Geoffroy's tailless bats that lives in the ferruginous Piedade Cave (19°49'20"S, 43°40'33"W; 1,414 m above sea level, a.s.l.). The cave is situated in the Piedade Hills, State of Minas Gerais, Brazil, in the Iron Quadrangle geomorphological domain (Bueno, 1992). This region is characterized by the presence of ferruginous and canga caves, a type of iron armour that protects against erosion and which contains crevices and small cavities (Bueno, 1992). This ecosystem is under highly demanding mining activity (Jacobi *et al*., 2007).

Piedade Hills possesses a great fauna and flora diversity with typical Atlantic Forest phytophysiognomy in the foothills, and rupestrian fields at higher elevations (Bueno, 1992). The climate of the region is classified as Köppen's Cwa, altitudinal subtropical (Bueno, 1992), with the month of June being the coldest (16.6ºC) and the month of January the warmest (23.5ºC). The rainy season (October to March) accounts for 81.5% of the annual average rainfall of 1,373 mm, with the remaining occurring during the dry season (April to September). The elevations of Piedade Hills range from 500 m in the uneven slope of the foothills to the highest point of 1,746 m a.s.l. (Bueno, 1992).

Capture, Processing of Bats and Data Analyses

Bats were monitored for two days each month between November 2014 and October 2015, using mist-nets set inside the cave from 08:00h to 12:00h. They were captured and released without marking. Forearm length, body mass, age class, sex and reproductive stage were recorded from all captured individuals. Adults were distinguished from subadults by the presence of respectively ossified or cartilaginous plates in

the metacarpus (Anthony, 1988). The reproductive stage was determined by examination of external reproductive characteristics, i.e. fully developed scrotal testes in adults, underdeveloped testes in subadults, and not apparent or not well apparent testes in juveniles. Pregnancy was detected by distension of the lower abdomen and if a fetus was palpable (Racey, 1988), lactation was determined by the presence of milk-filled teats and flaccid and dark teats in post-lactating females. Individuals lacking these characteristics were considered non-reproductive. Body condition index (BCI) of both sexes was determined by calculating BCI = body mass/forearm length (Reichard and Kunz, 2009).

Histological Procedures and Analyses

During colony monitoring, 31 adult males (16 males during the rainy season and 15 males during the dry season) and 22 adult females (10 females during the rainy season and 12 females during the dry season) were randomly removed from the cave and taken to the laboratory for gametogenic analyses. Captured pregnant and lactating females were immediately released after their biometric parameters were taken. The adults assigned to morphometric and histological studies were weighted and euthanized with an intraperitoneal injection of sodium thiopental (0.9 mg/g body mass), following manufacturer instructions.

Testes, epididymides, ovaries and uteri were weighted and fragments of each organ were removed and fixed in Bouin's solution for 12 hours at room temperature. The fragments were dehydrated in a graded series of ethanol, embedded in paraffin, sectioned at five µm of thickness and stained with hematoxylin-eosin (HE). Prior to histological procedures, organs were weighed and the following parameters calculated: mean right testis mass (RTM), mean left testis mass (LTM), mean right epididymis mass (REM), mean left epididymis mass (LEM), mean ovary and uteri mass, gonadosomatic index (GSI = mass of testes x 100/body mass) and epididymis-somatic index (ESI = mass of epididymides × 100/body mass).

Histological sections of the gonads of males and females, randomly chosen, were digitally photographed with an Olympus BX50 light microscope coupled to an Olympus SC-30 camera. A total of 840 cross-sections of right and left ovaries and uteri were observed under light microscopy and the ovarian follicles were categorized according to their developmental stage (Junqueira and Carneiro, 2008). A total of 1421 cross-sections of seminiferous tubules from the left testis were randomly selected under light microscopy for measuring the seminiferous tubules diameter (STD), the seminiferous epithelium height (SHE) and the nuclear diameter of Leydig cells (LD) of each individual. Micrometric measurements of male gonads were performed using ImageJ software (Rasband, 2014). Additionally, round spermatids and Sertoli cells present in seminiferous tubule cross-sections in stages 1 and 5 of the seminiferous epithelium cycle (SEC), following the tubular morphology method of classification (Clermont, 1972; Berndtson, 1977), were counted. The counted round spermatids were those recently formed in the previous stage 4 and remained essentially round until the stage 1 of the next SEC, and which could be readily counted before the elongation process in the following stage 2 took place (Amann and Almquist, 1962; Berndtson, 1977). Also, counting round spermatids in those two stages would allow the evaluation of eventual cell losses during the SEC development. The counts were made on the nuclei of round spermatid and nucleoli of Sertoli cell in 10 seminiferous tubule cross-sections for each male, totaling 290 cross-sections in stage 1 and 260 in stage 5 of the SEC. We used the respective numbers to determine the ratios of Sertoli cell to round spermatid in each stage, corresponding to Sertoli cell efficiency (Leal and França, 2009). The counts were adjusted to the thickness of the histological cross-sections using Abercrombie's formula (Abercrombie, 1946) modified by Amann and Almquist (1962): Corrected number = obtained count × section thickness/section thickness $+\sqrt{}$ (mean nuclear or nucleolar diameter/2)^2 - (mean nuclear or nucleolar diameter/4)^2.

The cauda epididymis was digitally photographed and checked for the presence of spermatozoa. Student's *t*-test and Mann-Whitney test (*U*-test) were used to evaluate possible seasonal variations and between different reproductive stages in the values of evaluated parameters. Variation on body condition index (BCI) between individuals was evaluated through analysis of variance (ANOVA), applying the Tukey test for a posteriori comparison when necessary. Significance was set at $P < 0.05$.

Captures were performed under license (#45686-3) granted by the Brazilian Chico Mendes Institute for Biodiversity Conservation. The procedures used in this study were previously approved (#024/2015) by the Ethics Committee on the Use of Animals of Pontifícia Universidade Católica de Minas Gerais. All specimens studied are deposited in the reference collection of the Pontifícia Universidade Católica de Minas Gerais.

Results

A total of 154 males and 117 females (1.3:1), including young recruits of the year, were captured. The sex ratio favored males, but when considering only adults, the ratio was 1:1 (83 ♂♂, 83 ♀♀). The mean (± 1 SD) body mass of adult males was 16.1 ± 1.7 g, and of adult females was 16.3 ± 2.0 g, showing no significant difference between sexes (Mann-Whitney *U*-test, $U = 3379.00$, $P = 0.42$). The mean (± SD) forearm length of these individuals also did not show significant difference between sexes (♂♂ = 41.0 ± 1.5 mm; ♀♀ = 41.0 ± 1. 4 mm — Student's *t*-test, $t = -0.07$, $d.f. = 82$, $P = 0.47$).

For females, BCI varied significantly, being bigger in pregnant females (0.453 ± 0.050, $n = 20$) when compared with females without reproductive external traits (0.381 ± 0.041, $n = 28$) and with post-lactating females (0.379 ± 0.011, $n = 3$) (ANOVA $F = 23.28$, $d.f. = 2$, $P < 0.001$). For males, BCI did not vary among different ages and reproductive stage (reproductive adults: 0.393 ± 0.040, $n = 83$; non-reproductive subadults: 0.400 ± 0.043, $n = 29$; juveniles: 0.386 ± 0.032, $n = 42$ — ANOVA $F = 1.17$, $d.f. = 2$, $P = 0.31$). Ovary and uteri masses did not differ seasonally (rainy season, mean = 0.030 ± 0.048 g, $n = 10$; dry season, mean = 0.025 ± 0.014 g, $n = 12$ — *U*-test, $U = 42.00$, $P = 0.12$).

Six ovarian follicle types were identified: primordial (Fig. 1A), unilaminar and multilaminar

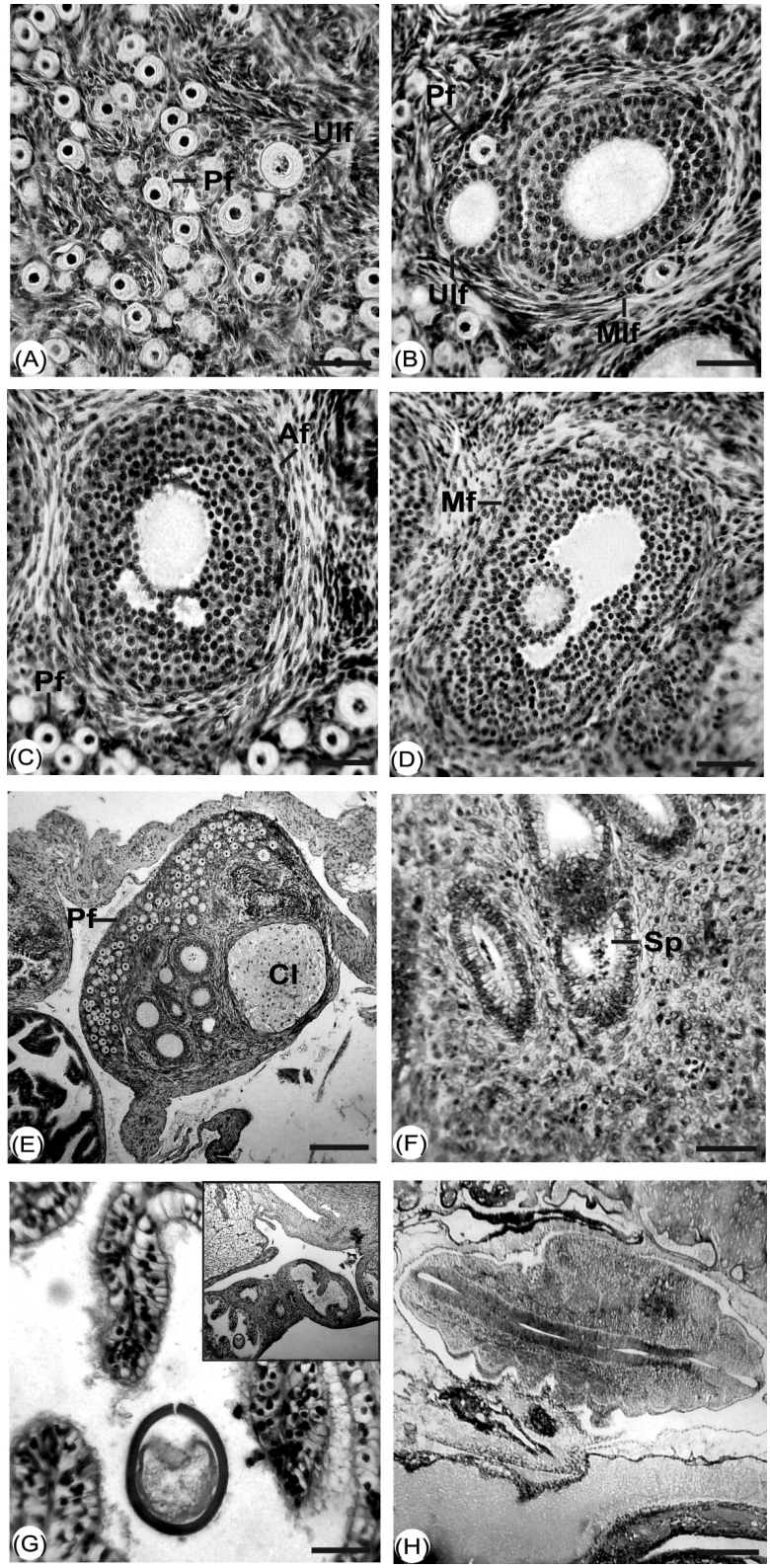

FIG. 1. Histological sections of ovaries, uteri and uterine tubes of the nectarivorous bat *A. geoffroyi*. A) primordial follicle (Pf) and unilaminar primary follicle (Ulf). B) primordial follicle (Pf), unilaminar primary follicle (Ulf) and multilaminar primary follicle (Mlf). C) primordial follicle (Pf) and antral follicle (Af). D) mature follicle (Mf), scale bar: 22 µm. E) polarized ovary presenting primordial follicles (Pf) located in the antimesometrial cortex area and corpus luteum (Cl), scale bar: 88 µm. F) spermatozoa (Sp) housed in uterine crypts, scale bar: 45 µm. G) uterine tube containing an embryo in initial stage of development (identified with the help of P. H. Krutzsch, University of Arizona; Tucson, Arizona, EUA), scale bar: 42 µm. H) gravidic uterus showing an embryo in initial stage of development with neural tube evident, scale bar: 25 µm. All sections H–E stained

(Fig. 1B), antral (Fig. 1C), mature (Fig. 1D) and atretic (not shown in Fig. 1). Mature follicles were observed only in September 2015 whereas the remaining follicle types were found in all months, except March and April when only pregnant females were captured. The primordial ovarian follicles were restricted to the antimesometrial cortex area of the ovary (Fig. 1E). Corpus albicans was also found (not shown in Fig. 1). The presence of corpora lutea (Fig. 1E) was recorded from November 2014 to February 2015. Three females captured in November 2014 (42.9%), one female captured in January 2015 (14.2%), and three females captured in September 2015 (42.9%) had spermatozoa housed in their uterine crypts (Fig. 1F).

The earliest signs of pregnancy were indicated by the presence of an embryo in an uterine tube in November 2014 (Fig. 1G), and an embryo in initial stage of development with an evident neural tube in December 2014 (Fig. 1H). In July 2015, only three post-lactating females were recorded. During the period of November 2014 to April 2015, 24 pregnant females were captured, all in advanced stage of pregnancy: one (4.2%) in January, five (20.8%) in February, 13 (54.2%) in March, and five (20.8%) in April 2015.

Since the latest pregnant females in advanced stage of development were captured in April, parturition may probably have occurred from November to May 2015. Seventy-six juveniles and 29 subadults not sexed were recorded from April to October 2015. The bats remained in the cave throughout the year, but non-pregnant and pregnant females were not recorded from May to the end of July, but were again recorded in August and September with no signs of breeding. These data allowed us to establish the reproductive phenology of *A. geoffroyi* in the study area as bearing a seasonal breeding period (September to March) and a principal mating period occurring mainly from September to January (Table 1).

Body mass of males did not vary significantly between seasons (Table 2), nor between mating period (September to January) and non-mating period (remaining months) (Table 3); similarly, the BCI (Fig. 2) of adult males showed no significant variation between rainy ($\bar{x}$ ± SD = 0.382 ± 0.037, n = 16) and dry (0.384 ± 0.055, n = 15) seasons (t-test, t = -0.165, $d.f.$ = 29, P = 0.44), nor between mating (0.382 ± 0.041, n = 19) and non-mating (0.384 ± 0.055, n = 12) periods (U-test, U = 105.00, P = 0.36).

Throughout the year, adult males exhibited active spermatogenesis and had the epididymis packed with spermatozoa (Fig. 3). However, testis and epididymis parameters differed significantly between rainy and dry seasons, except for the left epididymis mass and presented bigger values in rainy season (Table 2). Similarly, testis and epididymis parameters differed significantly between mating and non-mating period and presented higher values in mating period (Table 3). The GSI of males varied seasonally (Table 2 and Fig. 4A) and between mating and non-mating periods (Table 3 and Fig. 4B), reaching higher values in rainy season and mating period. The identical pattern as in the GSI was also observed in the ESI of males (Tables 2, 3, and Fig. 4C–D).

TABLE 1. Reproductive phenology based on histomorphological characteristics of *A. geoffroyi* females captured in the Piedade Cave, from November 2014 to September 2015. The months of October to March correspond to the rainy season and the months of April to September correspond to the dry season. Estimated periods are hatched. During March and April 2015 only pregnant females were captured which were not used in histological analyses

Reproductive phenology	Months												
	Sep	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep
Primordial follicle			●	●	●	●				●		●	●
Unilaminar primary follicle			●	●	●	●				●		●	●
Multilaminar primary follicle			●	●	●	●				●		●	●
Antral follicle			●			●						●	●
Mature follicle													●
Atresic follicle			●	●		●						●	●
Corpus luteum			●	●	●	●							●
Corpus albicans			●	●	●	●				●		●	●
Mating	○	○	●	●	●								○
Spermatozoa housed in uterine crypts			●		●								●
Pregnancy	○	○	●	●	●	●	●	●					
Parturition			○	○	○	●	●	●	○				
Lactation				○	○	●	●	●	●	●	●		
Dispersion (recruitment)							○	●	●	●	●	●	●

TABLE 2. Mean (± 1 SD) values (in g except for GSI and ESI) of body mass (BM), right testis mass (RTM), left testis mass (LTM), right epididymis mass (REM), left epididymis mass (LEM), gonadosomatic index (GSI) and epididymis-somatic index (ESI) of *A. geoffroyi* in rainy and dry seasons; [a] — Mann-Whitney *U*-test

Season	BM	RTM	LTM	REM	LEM	GSI	ESI
Rainy (n = 16)	15.5 ± 1.60	0.058 ± 0.03	0.061 ± 0.03	0.013 ± 0.00	0.011 ± 0.00	0.785 ± 0.41	0.155 ± 0.05
Dry (n = 15)	15.8 ± 2.30	0.042 ± 0.02	0.042 ± 0.02	0.008 ± 0.00	0.009 ± 0.00	0.545 ± 0.28	0.104 ± 0.04
Student's t-test	-0.38	1.75	2.24	2.76	81.00[a]	1.88	2.77
P-value	0.35	0.045	0.016	0.004	0.061	0.035	0.004

No seasonal significant differences were found for seminiferous tubules diameter, seminiferous epithelium height and Leydig cell nuclear diameter of adult males (Table 4), but significant differences were detected for these parameters when comparing mating and non-mating periods (Table 4). Likewise, Sertoli cell efficiency in stage 1 and stage 5 of the SEC did not differ significantly between seasons (Table 4 and Fig. 4E) but differed between mating and non-mating periods (Table 4 and Fig. 4F). All of these parameters had larger values during the rainy season and mating period.

Comparisons between Sertoli cell efficiencies at stages 1 and 5 of the SEC (Table 4) did not show differences in rainy season (*U*-test, U = 69.00, n = 14 and 12, P = 0.22), nor in dry season (U = 99.50, n = 15 and 14, P = 0.41), and during the mating period (t-test, t = 1.181, $d.f.$ = 33, P = 0.13), or non-mating period (t = 0.457, $d.f.$ = 18, P = 0.33).

DISCUSSION

The capture and release of *A. geoffroyi* during the period of study indicated the species inhabits the Piedade Cave throughout the year. The absence of adults and pregnant females during the initial months of the dry season suggests they probably migrate to some non-identified maternity roost, and the females ready for breeding were taking care of their puppies in that roost. The sex ratio of the colony varied during the study and was skewed towards males due to the absence of females in captures during this specific period.

Movement of individuals was also observed in a maternity colony in Mexico where, contrary to this study, the males evade the colony during birth and lactation periods (Galindo-Galindo *et al*., 2000). Male movement from maternity roosts, resulting in a decrease in their numbers during gestation and lactation, was also observed in Trinidad, French Guiana and Brazil (Goodwin and Greenhall, 1961; Brosset and Charles-Dominique, 1990; Baumgarten and Vieira, 1994). Sexually segregated colony of Geoffroy's tailless bat during certain periods of the year was suggested by Goodwin and Greenhall (1961), and Alvarez and Ramirez-Pulido (1972) in Mexico, and in a Brazilian Savannah area (Baumgarten and Vieira, 1994).

Morphometric Testis Parameters

Testes in full spermatogenesis and the occurrence of spermatozoa in the cauda epididymis throughout the year indicate a continuous spermatogenic activity during the year (Wimsatt and Trapido, 1952; Krutzsch, 1979). Nevertheless, the mass of testes and epididymides varied seasonally and between mating and non-mating periods, with larger masses occurring during the rainy season/mating period. While the BCI did not change in males, GSI, ESI, Leydig nuclear diameter and Sertoli cell efficiency had values peaking in the mating period, indicating a possible peak in spermatogenesis. Such a peak generally occurs when there is a need to synchronize spermatic production with female reproductive cycle (Heideman, 2000). Similar result was

TABLE 3. Mean (± SD) values (in g except for GSI and ESI) of body mass (BM), right testis mass (RTM), left testis mass (LTM), right epididymis mass (REM), left epididymis mass (LEM), gonadosomatic index (GSI) and epididymis-somatic index (ESI) of *A.geoffroy*i in mating and non-mating period; [a] — Mann-Whitney *U*-test

Reproductive period	BM	RTM	LTM	REM	LEM	GSI	ESI
Mating (n = 19)	15.4 ± 1.60	0.064 ± 0.02	0.065 ± 0.02	0.013 ± 0.00	0.012 ± 0.00	0.856 ± 0.33	0.157 ± 0.05
Non-mating (n = 12)	16.0 ± 2.30	0.028 ± 0.01	0.030 ± 0.01	0.007 ± 0.00	0.007 ± 0.00	0.374 ± 0.19	0.089 ± 0.02
Student's t-test	-0.77	22.00[a]	4.38	4.66	5.02	4.54	4.79
P-value	0.22	< 0.000	< 0.000	< 0.000	< 0.000	< 0.000	< 0.000

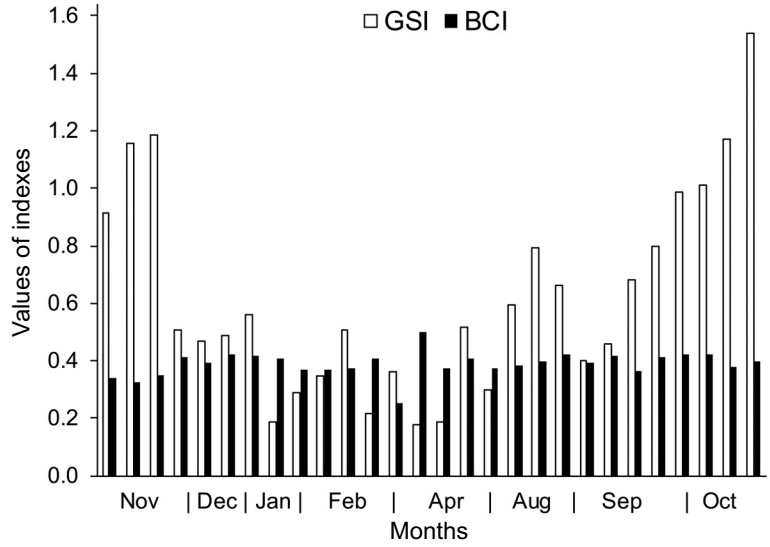

FIG. 2. Individual gonadosomatic index (GSI) and body condition index (BCI) of *A. geoffroyi* males collected in Piedade Cave. The months of October to March correspond to the rainy season and the months April to September correspond to the dry season. Note: missing months indicate absence of males in the cave

found for the phyllostomid *Artibeus planirostris*, which exhibited two peaks of sperm production corresponding to two peaks of GSI, and coinciding with the bimodal polyestrous reproductive cycle of females (Beguelini *et al*., 2013). Sertoli cells play important roles in the spermatogenic process like support, nutrition and immunological protection of developing germ cells and release of mature spermatids into seminiferous tubule lumen. While Leydig cells are responsible for secretion of steroid hormones, such as testosterone and estrogen, which act in the activation of sperm production and maturation of the secondary sexual organs (Neuweiler, 2000; Junqueira and Carneiro, 2008).

Morphometric Ovary Parameters

The polarized ovary of *A. geoffroyi* was found to possess a region with developing follicles (medulla) and a region with primordial follicles (cortex), differing from the organization pattern of most mammals, including chiropterans (Rasweiler and Badwaik, 2000). Similarly, the phyllostomids *G. soricina* (Komar *et al*., 2007; Antonio-Rubio *et al*., 2013), *A. jamaicensis* and *S. lilium* (Antonio-Rubio *et al*., 2013) have polarized ovaries, but not *Carollia* sp. (Bonilla and Rasweiler, 1974) and *D. rotundus* (Wimsatt and Trapido, 1952).

Histological data of the female reproductive tract showed the breeding period possibly begins in the transition of dry to rainy seasons and extends during the rainy season. This is based on observations of mature follicles found in September 2015, spermatozoa in uterine crypts in November 2014, January 2015 and September 2015, and occurrence of embryos in initial stages of development in December 2014 (present study). The presence of corpora lutea in the period of November 2014 to February 2015 is another strong evidence of initial stage of reproduction in these females, mainly during the mating period. The presence of pregnant

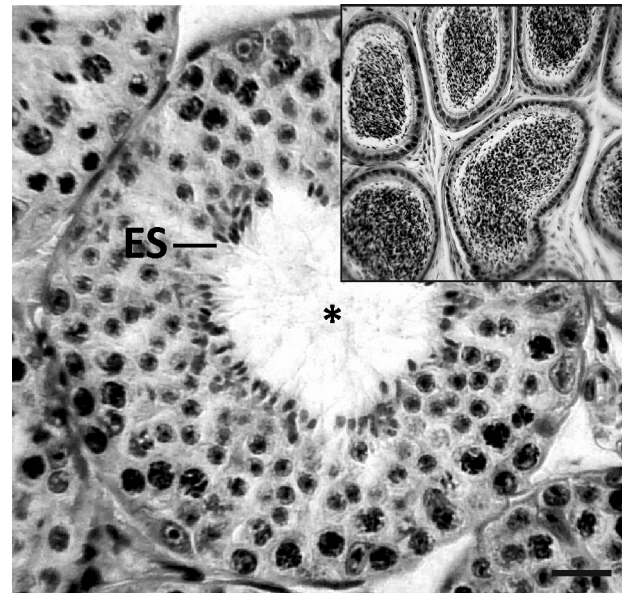

FIG. 3. Cross-section of seminiferous tubule of *Anoura geoffroyi* in reproductive activity. Note elongated spermatids (ES) and abundant spermatozoa in the lumen (*). Insert shows cross-section of cauda epididymis packed with spermatozoa, scale bar: 22 µm. HE stain

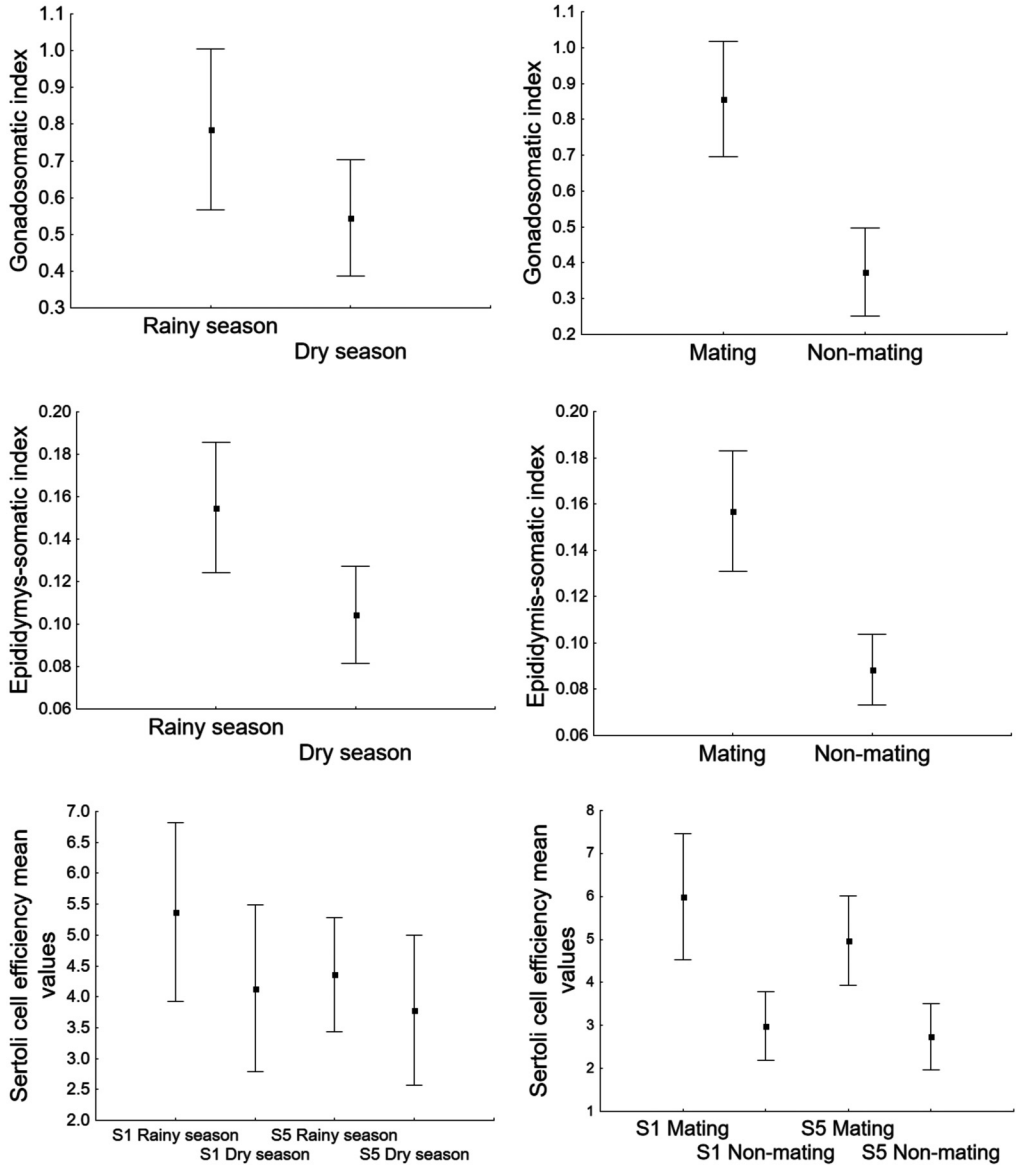

FIG. 4. Mean values and 95% confidence intervals related to gonadosomatic index, epididymis-somatic index and Sertoli cell efficiency obtained during rainy and dry seasons and mating and non-mating periods of *A. geoffroyi*. Confidence intervals overlap indicates non-significant difference between the estimated values

females in the rainy season, post-lactating females in July (dry season), and recruitment of juveniles during the dry season indicate an extensive reproductive cycle, with mating occurring from September to January, when a possible peak in spermatic production was observed, matched by highest GSI, ESI, Leydig nuclear diameter and Sertoli cell efficiency. These reproductive events suggest Geoffroy's tailless bat, in the study area, as having a single reproductive event during the year, characterizing it as seasonally monoestrous.

Birth of offspring was asynchronous during the rainy season, as indicated by the extended period of occurrence of pregnant females, but there were more births in the beginning of the dry season (April), because of the occurrence of many females in advanced pregnancy captured in March. Six months have elapsed between the first females captured with unequivocal signs of reproductive activity and the last pregnant females recorded in colony captured in April at an advanced stage of pregnancy when juveniles began to be captured. Thus, we can infer that at least the six months left in the cycle were needed for gestation and lactation.

The gestation of Neotropical bats lasts from three to eight months (Wimsat and Trapido, 1952;

TABLE 4. Mean (± 1 SD) values (in µm except for SCE) of seminiferous tubule diameter (STD), seminiferous epithelium height (SEH), Sertoli cell efficiency (SCE) and Leydig cell nucleus diameter (LCD) of *A geoffroyi*. Mean comparisons (*t*-test) between rainy and dry seasons and between mating and non-mating periods. Sample sizes in italics

Histometric parameters	Season		*P*-value	Reproductive period		*P*-value
	Rainy	Dry		Mating	Non-mating	
STD	149.4 ± 29.8, *16*	144.4 ± 28.8, *15*	0.317	161.9 ± 21.3, *19*	123.3 ± 23.5, *12*	< 0.000
SEH	48.8 ± 8.3, *16*	48.7 ± 9.1, *15*	0.497	53.2 ± 6.0, *19*	41.8 ± 7.5, *12*	< 0.000
SCE-Stage 1	4.7 ± 2.4, *14*	3.5 ± 2.3, *15*	0.188	5.0 ± 2.5, *18*	2.5 ± 1.0, *11*	0.003
SCE-Stage 5	3.8 ± 1.3, *12*	3.5 ± 2.0, *14*	0.409	4.2 ± 1.7, *17*	2.3 ± 0.8, *9*	0.005
LCD	4.5 ± 0.6, *15*	4.4 ± 0.5, *15*	0.102	4.6 ± 0.6, *18*	4.3 ± 0.4, *12*	0.040

Fleming *et al*., 1972; James, 1977; Kleiman and Davis, 1979). For Geoffroy's tailless bat from Central Brazil (Baumgarten and Vieira, 1994; Zortéa, 2003) and from Mexico (Galindo-Galindo *et al*., 2000), it is estimated to be three months. So, for this colony, it can be inferred that lactation might have occurred over a period of three months.

Therefore, considering that six months are needed for gestation and lactation (Baumgarten and Vieira, 1994; Galindo-Galindo *et al*., 2000; Zórtea, 2003; present study), we can infer an adjustment on reproductive events of Geoffroy's tailless bat to climate seasonality (Happold and Happold, 1990; Bernard and Cumming, 1997; Racey and Entwistle, 2000), i.e. gestation, lactation and a peak in spermatic production occurring during the rainy season, when there is greater availability of food resources in the region, particularly insects and fruits (Sazima *et al*., 1999; Morellato *et al*., 2000). The capture of juveniles and subadult individuals occurred during the dry season, when supposedly these animals face a shortage of food resources; however, in the next rainy season they would find the resources necessary for gonadal maturation.

Although *A. geoffroyi* is a species widely adapted to nectarivory (Winter and von Helversen, 2003), it can consume large proportions of insects at certain times of the year (Gardner, 1977), as well as greater proportion of fruits and insects than nectar and pollen throughout the year (Zortéa, 2003). For the congeneric *A. caudifer*, in a population studied in the south of Brazil, there was a seasonal variation in the diet of the species, which showed high consumption of nectar and pollen throughout the year and high consumption of insects and fruits during spring and summer (Barros *et al*., 2013), seasons that occur in the months corresponding to the rainy season in the area of the present study. In the study of Zortéa (2003), a higher consumption of fruit pulp and insects was also observed during the lactation period observed for that studied population. Although *A. geoffroyi* is considered an important pollinating agent of several plant species in the Atlantic Forest (Sazima *et al*., 1999), it may depend on the availability of different food items to determine the best time for reproductive activities that favors a larger investment in the production of offspring and gonadal maturation when different food resources are more abundant.

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Sertoli Cell Efficiency of the Neotropical Bats Anoura geoffroyi, Artibeus lituratus and Myotis levis (Mammalia: Chiroptera)

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Sertoli cell efficiency of the Neotropical bats *Anoura geoffroyi*, *Artibeus lituratus* and *Myotis levis* (Mammalia: Chiroptera)

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Sertoli cells play an essential role in spermatogenesis, being determinant of male reproduction capability. In this study we determined and compared the Sertoli cell efficiency (SCE), i.e. the ratio of the number of round spermatids to the number of Sertoli cells, of three species of Neotropical chiropterans, *Anoura geoffroyi*, *Artibeus lituratus* and *Myotis levis*, and additionally we tested for correlations between SCE, the combined mass of the testes and epididymides (CMTE), and the body condition index (BCI), considering that both BCI and testis mass can influence gonadal function of males. For SCE determination, the number of round spermatids and Sertoli cells present in stage 1 of the cycle of the seminiferous epithelium were counted. The Sertoli cell efficiency (mean ± 1 SD) was 4 ± 2.4 for *A. geoffroyi*, 4 ± 1.0 for *A. lituratus* and 6 ± 2.0 for *M. levis*. No significant variation was observed in SCE among the species, but *A. lituratus* exhibited a statistically significant correlation between CMTE and BCI. *Anoura geoffroyi* and *M. levis* exhibited significant positive correlations between SCE and CMTE, but not between SCE and BCI. Previous studies have shown that, unlike *A. lituratus*, *A. geoffroyi* and *M. levis* are subject to strong mating pressures, leading us to hypothesize that these findings may be related to differential mating pressures between species.

Key words: body condition index, chiropteran reproduction, epididymis mass, testicular mass

INTRODUCTION

Mammals have diverse strategies that ensure their success throughout reproductive events (Bronson, 1989). This is particularly true for bats, which exhibit varied reproductive patterns including polyestry, seasonal polyestry and seasonal monoestry (Jerrett, 1979). The spermatogenic process of vertebrates occurs continuously or seasonally, according to the reproductive pattern of each particular species (Hess and Franca, 2007). The reproductive pattern of a species generally results from an adjustment to control factors, for example, studies on reproduction of bats reveal variation in gonadal regression, gonadal recrudescence, and acceleration of the spermatogenic processes according photoperiod variation (Beasley and Zucker, 1984; Heideman, 2000; Haldar and Alipreeta, 2001), variation in ovulation time according temperature variation (Oxberry, 1979), and influence of the individual's body condition on spermatogenesis in adult males (Entwistle *et al*., 1998).

In addition to several other functions, Sertoli cells play a central role creating an immunoregulatory environment where immune protection is provided to the developing germ cells (Kaur *et al*., 2014; França *et al*., 2016), further being responsible for supporting germ cells (Griswold, 1998; Hess and França, 2005; França *et al.,* 2016). The number of Sertoli cells is a determinant of sperm production and testis size in the adult, since each individual Sertoli cell is in morphological and functional contact with a defined number of sperm, which is variable between the species (Nieschlag *et al*., 2010). In an experimental study, Orth *et al*. (1988) showed significant variation in the production of round spermatids associated to the number of Sertoli cells present in seminiferous epithelium. In adult mammals, the number of Sertoli cells is constant and their ability to sustain germ cell populations is limited and species-specific (Hess and Franca, 2007).

Determination of spermatic production encompasses different morphometric and histological analyses, such as daily sperm production and the

ratio of number of germ cells per Sertoli cell — known as Sertoli cell efficiency. The Sertoli cell efficiency (SCE) is one of the parameters used to evaluate spermatogenic activity in mammals (França and Godinho, 2003; Costa *et al*., 2008; Leal and França, 2009; Cordeiro-Júnior *et al*., 2010; Morais *et al*., 2014). Recent studies on spermatogenesis of Neotropical bats have broadened the understanding of their reproductive biology, regarding several morphological and morphometric parameters of the testes (Beguelini *et al*., 2009, 2013*a,* 2013*b*; Duarte and Talamoni, 2010; Morais *et al*., 2013*a*, 2013*b*; Notini *et al*., 2015), and seasonal regression of the seminiferous epithelium (Araújo *et al*., 2013; Farias *et al*., 2015). However, SCE has only been determined for three species of bats: *Molossus molossus* (Morais *et al*., 2013*b*), *Sturnira lilium* (Morais *et al*., 2014) and *Desmodus rotundus* (Morais *et al*., 2017). A positive correlation between body mass and testis mass of adult males of 10 bat families was reported (Wilkinson and McCracken, 2003). Thus, considering that both testis mass (Wilkinson and McCracken, 2003) and body condition of individuals (Speakman and Racey, 1986) can influence gonadal function of males, the present study aimed to evaluate SCE of three species of Neotropical bats as related to body condition index and to combined mass of testes and epididymides. The studied species were the Phyllostomidae *Anoura geoffroyi* Gray, 1838 and *Artibeus lituratus* (Olfers, 1818), both species with males exhibiting continuous spermatogenesis (Farias, 2016; Duarte and Talamoni, 2010); the third species was the vespertilionid *Myotis levis* (I. Geoffroy, 1824) with males exhibiting seasonal spermatogenesis (Farias *et al*., 2015). We have chosen to study these species because they have their annual reproductive cycles known (Duarte and Talamoni, 2010; Araújo *et al*., 2013; Farias, 2016).

Material and Methods

Animals and Capture Areas

Twenty-five adult males *A. geoffroyi* (mean body mass ± 1 SD = 16.0 ± 1.7 g) were captured from October 2014 to December 2015 in the State Sanctuary Reserve of Piedade located in the municipality of Caeté, State of Minas Gerais, Brazil (19°49’20”S, 43°40’33”W). The site is diverse in its vegetation, being composed of semideciduous forest, altitudinal grasslands, rupestrian grasslands and patches of cerrado-savannah-like grassland. Local temperatures reach below 18ºC in the cooler months and 22ºC in the warmer months (Sá Júnior *et al*., 2012).

Twenty adult males *A. lituratus* (mean body mass ± 1 SD = 66.7 ± 1 g) were captured from December 2001 to May 2003 in the Special Protected Area of Fechos located in the municipality of Nova Lima, State of Minas Gerais, Brazil (20°04’S, 43°57”W). The local vegetation is predominantly characterized by semideciduous forest. The mean maximum temperature is 23.3°C, and the mean minimum temperature is 17.6°C (https://pt.climate-data.org; accessed in 24 February 2016).

Five adult males *M. levis* (mean body mass ± 1 SD = 5.5 ± 0.5 g) were collected from March 2010 to May 2011 in a Private Reserve Area of the Caraça Sanctuary located in the municipalities of Catas Altas and Santa Bárbara, State of Minas Gerais, Brazil (20°04’30”S, 43°24’28”W). The reserve area possesses a mosaic of vegetation in which semideciduous forest, riparian forest, rupestrian grasslands and cerrado predominate. The maximum local temperature is 25°C and the minimum temperature is 17°C (Araújo *et al*., 2013).

Capture, Collection of Biological Samples and Data Analyses

The bats were captured using mist nets (12 × 3 m), followed by data collection for each individual, which included forearm length (mm), sex, body mass (g), age and reproductive condition. Only reproductive adult individuals were analysed, which were differentiated from subadults by possessing complete ossification of the epiphyseal plates of the metacarpus (Kunz and Anthony, 1982) and scrotal testes. The animals were collected both during the dry season (April to September) and the rainy season (October to March), with the exception of *M. levis*, that were only collected in the rainy season since this is the season during which the species exhibits active spermatogenesis (Araújo *et al*., 2013).

After capture, the animals were euthanized with an intraperitoneal injection of sodium thiopental, following manufacturer instructions. Testes and epididymides were removed, weighed, fixed in Bouin’s solution for 12 h at room temperature, dehydrated in a graded series of ethanol and embedded in paraffin. The tissues were cut at 5 μm of thickness and stained with hematoxylin-eosin (HE). Six to ten cross-sections were used per animal, obtained from the 50 bats of all three species. The cross-sections were randomly selected and digitally photographed with an Olympus BX50 light microscope coupled to an Olympus SC-30 camera, and each image was categorized into one of the eight stages (stages 1–8) of the seminiferous epithelium cycle (SEC). Stage classification is based on the types of germ cells present in each cross-section and their positioning in the seminiferous epithelium, according to the tubular morphology method (Clermont, 1972; Berndtson, 1977; França and Godinho, 2003).

In general, studies on Sertoli cell efficiency (SCE) are associated to several other quantitative spermatogenic analyses. In these cases, the authors may count all different cell types in a particular stage of the seminiferous epithelium cycle, and usually indicate the SCE as related to the total number of germ cells, or present specific index values for each type of germ cell (França and Godinho, 2003; Costa *et al*., 2008; Leal and França, 2009; Cordeiro-Júnior *et al*., 2010). In this study, the Sertoli cell efficiency (SCE) was estimated as the number of round spermatids per Sertoli cell by counting the Sertoli cell nucleoli and the round spermatid nuclei present in seminiferous tubule cross-sections at the stage 1 of the SEC, this index being considered very effective in representing the functional efficiency of these cells in a given species (Russel and Peterson, 1984). Cell counting was performed with the aid of the ImageJ program (Rasband, 2014).

The counts were corrected according to the histological section thickness, nuclear diameter of the round spermatids and the nucleolar diameter of the Sertoli cell, according to the formula of Abercrombie (1946), modified by Amann and Almquist (1962). The mean nuclear diameter of round spermatids and nucleolar diameter of the Sertoli cells were obtained from 30rounded transverse sections of seminiferous tubules for each species. Sertoli cell efficiency (SCE = corrected number of round spermatids/corrected number of Sertoli cell nucleoli) was then calculated.

For each species, SCE values were correlated with combined mass of testes and epididymides (CMTE) and the body condition index (BCI). The CMTE was obtained from the sum of the masses of the testes and epididymides (right and left) while the BCI was calculated as body mass/forearm length (Speakman and Racey, 1986) for each bat. The data from each species were submitted to analysis of variance (ANOVA). Student's *t*-test and the non-parametric Mann-Whitney a posteriori test were used when necessary. Initially, the means of SCE, CMTE and BCI were submitted to Lilliefors normality test and then correlated with each other by the Pearson correlation test or the non-parametric Spearman test. The level of significance adopted was $P < 0.05$.

Animal handling procedures followed the standards of the Conselho Nacional de Controle de Experimentação Animal (CONCEA), which are in line with international standards. Captures were performed under license (# 206/2001, #28120-4, #45686-3) granted by the Brazilian Chico Mendes Institute for Biodiversity Conservation. All specimens studied are deposited in the reference collection of the Pontifícia Universidade Católica de Minas Gerais.

Results

Cell Components of Stage 1 of the Seminiferous Epithelium Cycle

The germ cell association present in the seminiferous epithelium (SE) allowed us to categorize eight stages of the seminiferous epithelium cycle (stages 1 to 8) in each of the species studied, but here only the cellular components of stage 1 are presented. Stage 1 was characterized by the presence of type A spermatogonia on the basement membrane of the epithelium, two generations of primary spermatocytes, one in pre-leptotene and the other in pachytene, occupying the middle third of the epithelium, and a generation of round spermatids dispersed in the upper or luminal third of the seminiferous epithelium. The Sertoli cell nuclei were found resting on the basement membrane of the seminiferous tubule (Fig. 1A–C). Primary spermatocytes in zygotene were registered in stage 1 of the seminiferous epithelium cycle from *A. geoffroyi* (Fig. 1A).

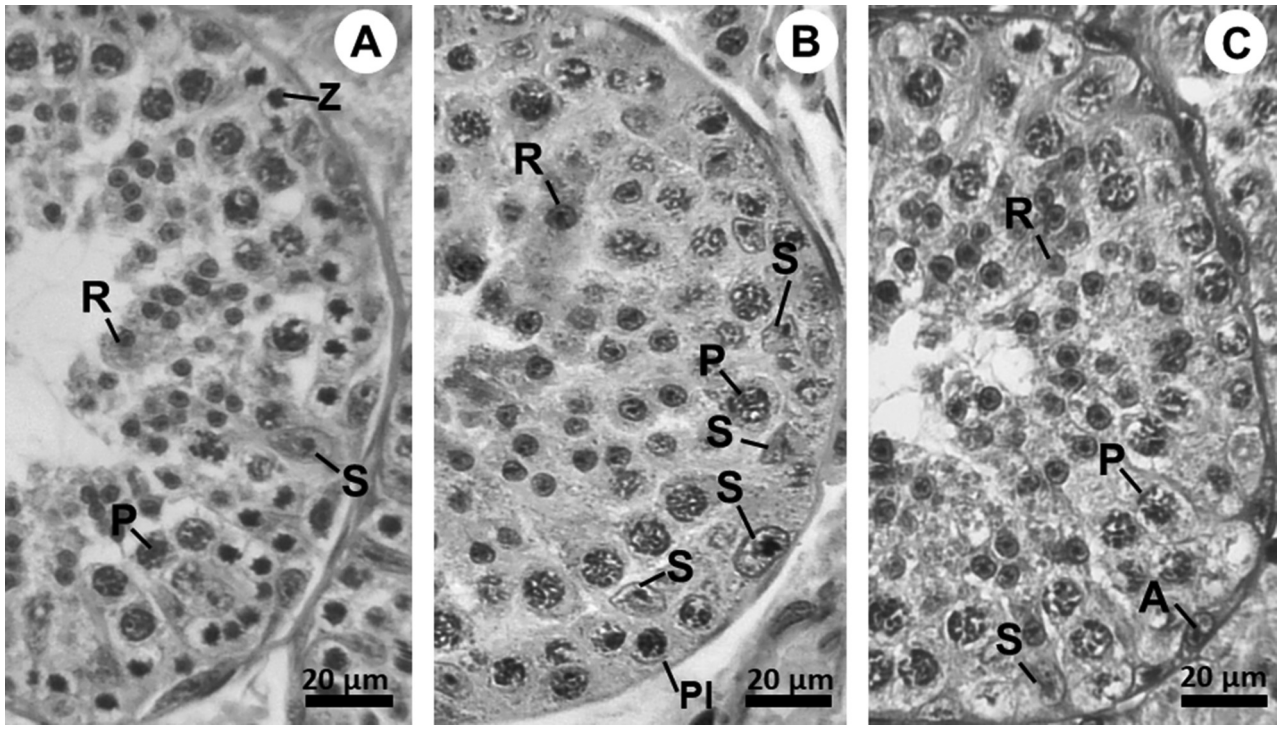

Fig. 1. Cell composition of stage 1 seminiferous epithelium cycle of: A — *A. geoffroyi*, B — *A. lituratus*, and C — *M. levis* according to the tubular morphology method. Legend: S = Sertoli cell; A = type A spermatogonia; Pl = primary spermatocyte in pre-leptotene, P = primary spermatocyte in pachytene; Z = primary spermatocyte in zygotene and R = rounded spermatid. Staining with H–E in (A and C), blue toluidine staining in (B), bar =10 µm

Sertoli Cell Efficiency (SCE)

The SCE ($\bar{x} \pm 1$ SD) was slightly lower for *A. geoffroyi* (4 ± 2.4) and *A. lituratus* (4 ± 1.1) than for *M. levis* (6 ± 1.9), however, the differences were not statistically significant ($F_{2, 47} = 1.55$, $P = 0.22$ — Fig. 2).

Combined Mass of Testes and Epididymides (CMTE) and Body Condition Index (BCI)

The mean (± 1 SD) CMTE measured for *A. geoffroyi*, *A. lituratus* and *M. levis* was 0.126 ± 0.06 g, 0.281 ± 0.12 g, and 0.124 ± 0.05 g, respectively. The mean body condition index measured for the same three species was 0.388 ± 0.04, 0.972 ± 0.07 and 0.138 ± 0.02, respectively.

The correlation between CMTE and BCI was not statistically significant for *A. geoffroyi* (Spearman rank, $r_s = -0.003$, *d.f.* = 23, $P = 0.99$), positive and statistically significant for *A. lituratus* (Pearson correlation, $r_p = 0.591$, *d.f.* = 15, $P = 0.012$) and not statistically significant for *M. levis* (Pearson correlation, $r_p = -0.703$, *d.f.* = 3, $P = 0.18$), although a negative trend had been observed (Fig. 3).

Sertoli Cell Efficiency (SCE) and Combined Mass of Testes and Epididymides (CMTE)

The correlation between SCE and CMTE was positive and statistically significant for *A. geoffroyi* (Spearman rank, $r_s = 0.533$, *d.f.* = 23, $P < 0.01$) and *M. levis* (Pearson correlation, $r_p = 0.897$, *d.f.* = 3, $P < 0.05$), but not statistically significant for *A. lituratus* (Pearson correlation, $r_p = 0.217$, *d.f.* = 17, $P = 0.37$ — Fig. 3).

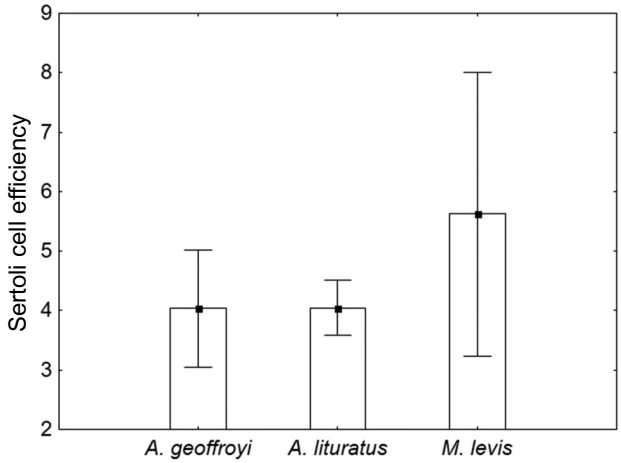

FIG. 2. Mean (dot) and 95% confidence interval (bar) of Sertoli cell efficiency in males of *A. geoffroyi*, *A. lituratus* and *M. levis*

Sertoli Cell Efficiency (SCE) and Body Condition Index (BCI)

The correlation between SCE and BCI was not statistically significant for *A. geoffroyi* (Spearman rank, $r_s = -0.090$, *d.f.* = 23, $P = 0.67$), *A. lituratus* (Pearson correlation, $r_p = 0.132$, *d.f.* =15, $P = 0.61$) and *M. levis* (Pearson correlation, $r_p = -0.500$, *d.f.* = 3, $P = 0.39$ — Fig. 3).

DISCUSSION

The cell association present in stage 1 of SEC of our species followed the pattern previously described for the Neotropical phyllostomids: *A. lituratus*, *A. planirostris*, *Carollia perspicillata*, and *Platyrrhinus lineatus* (Beguelini *et al*., 2009). The presence of primary spermatocyte in zygotene in stage 1 of SEC of *A. geoffroyi* has been previously described for the phyllostomid *S. lilium* and the molossid *M. molossus* (Morais *et al*., 2012, 2013*a*).

In addition to bats, other species of mammals have similar cell composition in stage 1 of the SEC. Among Rodentia, such species include *Hydrochoerus hydrochaeris* (Paula *et al*., 1999), *Chinchilla lanigera* (Leal and França, 2009), *Dasyprocta leporina* and *Cuniculus paca* (Costa *et al*., 2010*a*). Primates (*Callithrix penicillata* — Leal and França, 2006), and Perissodactyla (*Equus asinus* and *E. mulus mulus* — Neves *et al*., 2002) also exhibit similar cell composition. Among Carnivora, *Leopardus tigrinus* (Balarini *et al*., 2012) has similarities with the studied bats, but *Felis catus* (França and Godinho, 2003) shows different cell composition due to the presence of primary spermatocytes in leptotene in stage 1 of the SEC. The Artiodactyla *Capra hircus* (França *et al*., 1999) and *Tayassu tajacu* (Costa *et al*., 2010*b*) exhibit divergence in cell composition as these species have type B spermatogonia in stage 1. Thus, although there is a wide convergence in cell associations of stage 1 among the previously studied mammals, the exceptions point to the need to broaden the list of the studied species so that patterns of cell associations can be securely established and possible differences between species interpreted.

The SCE exhibits great variation among the mammals already investigated. Table 1 shows the SCE values obtained for several species, which were calculated as the ratio of round spermatid:Sertoli cell, according to data provided by the authors. For the order Chiroptera, *M. molossus* and *S. lilium* (Morais *et al*., 2013*b*, 2014) have SCEs like that of

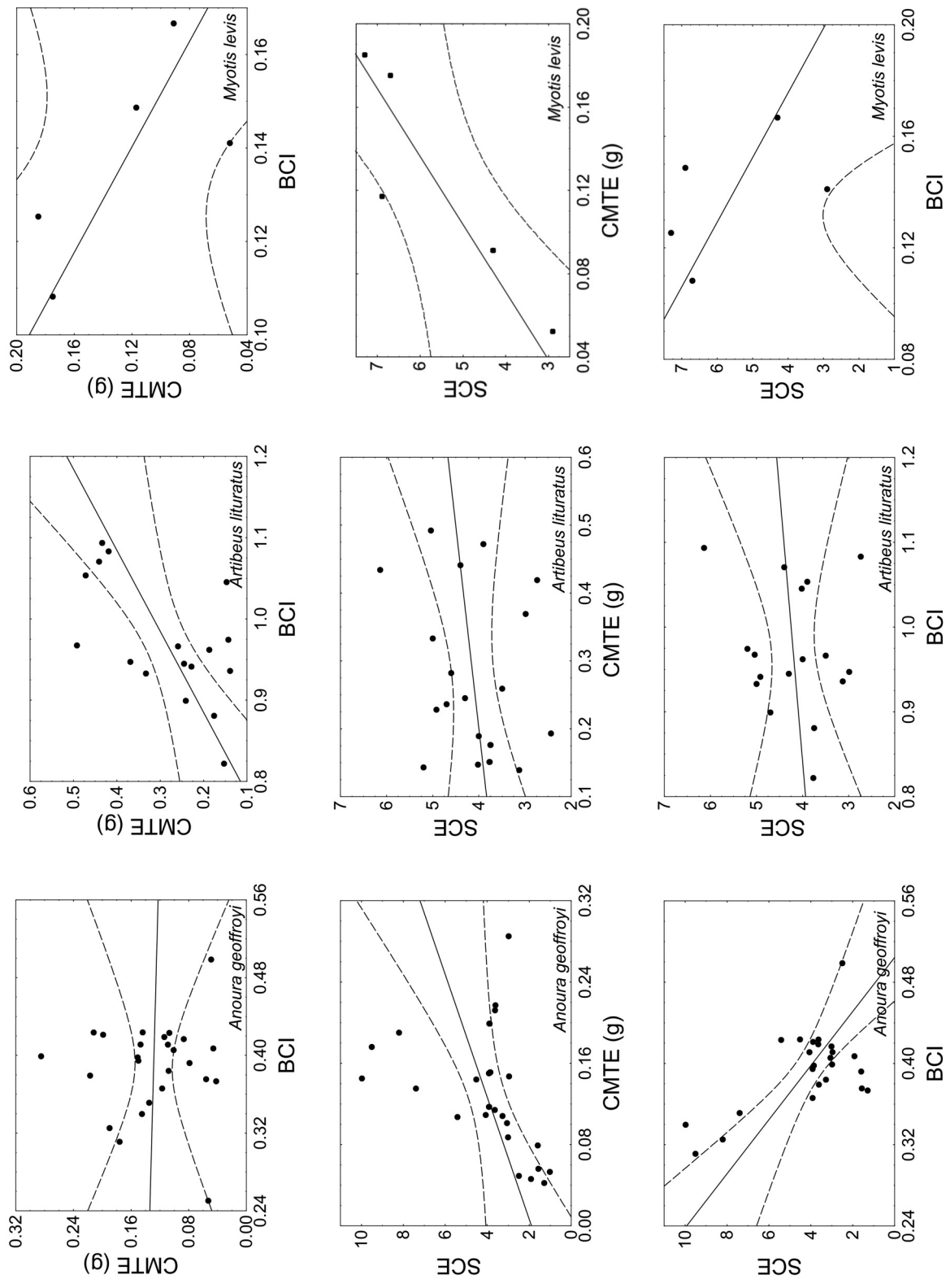

FIG. 3. Correlation between combined mass of the testes and epididymides (CMTE) and body condition index (BCI), between Sertoli cell efficiency (SCE) and combined mass of testes and epididymides (CMTE), and between Sertoli cell efficiency (SCE) and body condition index (BCI) for *A. geoffroyi*, *A. lituratus* and *M. levis*. The 95% confidence interval is represented by the dashed line

A. geoffroyi and *A. lituratus*, although lower than that of *M. levis*. The SCE of *D. rotundus* (Morais *et al*., 2017) was the highest among Chiroptera so far studied.

Species of the orders Rodentia, Perissodactyla, Artiodactyla and Lagomorpha possess SCEs from 51% to 58% higher than those of the bats of the present study (Table 1). In general, species of Carnivora possess SCEs close to those of Chiroptera, except for *M. levis*, which had a higher SCE than *L. pardalis* (Silva *et al*., 2010) and *F. catus* (França and Godinho, 2003). The mammals with lowest and highest known SCEs are, respectively, humans, with about three round spermatids/Sertoli cell (Sinha Hikim *et al*., 1985), and the goat, with 15.4 round spermatids/Sertoli cell (Leal and França, 2014).

SCE correlates significantly with CMTE in *A. geoffroyi* and *M. levis*. Analysis between SCE and BCI, in turn, showed no statistically significant correlations among the studied species. A particularity of *M. levis* (Araújo *et al*., 2013; Farias *et al*., 2015) and *A. geoffroyi* (Farias, 2016), which may be related to our results, is the occurrence of monoestrous cycles and short periods of mating. *Myotis levis* is seasonally monoestrous with sperm production limited a to brief period, followed by testis regression. The epididymal sperm storage seen in this species is due to the lack of synchrony between sperm production and maturation of females (Araújo *et al*., 2013; Farias *et al*., 2015). It exhibits a short mid dry-season mating period (Araújo *et al*., 2013), thus, the statistically significant correlations between SCE and CMTE in this species may be an adaptive response to its short mating periods. Males would need to adjust their reproductive activity to the reproductive activity of the females, investing,

TABLE 1. Sertoli cell efficiency (SCE, calculated as the ratio of round spermatid:Sertoli cell), recorded for species of different orders of mammals. Some values marked with asterisks (*) were calculated from author data

Order / Species	Common name	SCE	Reference
Chiroptera			
Anoura geoffroyi	Geoffroy's tailless bat	4.0	Present study
Artibeus lituratus	Great fruit-eating bat	4.0	Present study
Desmodus rotundus	Common vampire bat	11.4	Morais *et al*. (2017)*
Molossus molossus	Pallas's mastiff bat	4.3	Morais *et al*. (2013*b*)*
Myotis levis	Yellowish myotis	6.0	Present study
Sturnira lilium	Little yellow-shouldered bat	4.2	Morais *et al*. (2014)*
Primates			
Callithrix penicillata	Black-tufted-ear marmoset	8.0	Leal and França (2006)
Homo sapiens	Human	2.9	Sinha Hikim *et al*. (1985)
Macaca fascicularis	Crab-eating macaque	12.4	Zhengwei *et al*. (1997)
Carnivora			
Felis catus	Domestic cat	5.1	França and Godinho (2003)
Leopardus pardalis	Ocelot	4.5	Silva *et al*. (2010)
Panthera leo	African lion	7.9	Barros *et al*. (2007)
Panthera onca	Jaguar	7.9	Costa *et al*. (2008)
Perissodactyla			
Equus asinus	Donkeys	15.1	Neves *et al*. (2014)
Artiodactyla			
Capra hircus	Goat	15.4	Leal and França (2014)
Bos taurus	Domestic cattle	8.0	Berndtson *et al*. (1987)
Pecari tajacu	Collared peccary	11.1	Costa *et al*. (2010*b*)
Tayassu pecari	White-lipped peccary	10.9	Costa *et al*. (2007)
Sus domesticus	Domestic pig	12.4	Costa *et al*. (2011)
Sus scrofa scrofa	Wild boar	6.6	Costa *et al*. (2011)
Rodentia			
Chinchilla lanigera	Long-tailed chinchilla	13.0	Leal and França (2009)
Cuniculus paca	Spotted paca	10.9	Costa *et al*. (2010*a*)
Dasyprocta leporina	Red-rumped agouti	9.2	Costa *et al*. (2010*a*)
Meriones unguiculatus	Mongolian gerbil	12.6	Segatelli *et al*. (2004)
Mus musculus molossinus	Japanese house mouse	7.9	Costa *et al*. (2017)
Trinomys moojeni	Moojen's Atlantic spiny rat	14.7	Cordeiro-Júnior *et al*. (2010)
Lagomorpha			
Oryctolagus cuniculus	European rabbit	10.9	Thompson and Berndtson (1993)

therefore, in greater gonadal production, as well as in elevated SCE.

Anoura geoffroyi, in turn, is monoestrous (Baumgarten and Vieira, 1994; Zortéa, 2003; Farias, 2016). The males exhibit continuous spermatogenesis, and this species has a longer period of repro-ductive activity. However, a more intense mating period is detected during the months of September to January (Farias, 2016). The males of the present study that exhibited higher SCE were collected from September to November. Thus, we hypothesize that, for species with reduced mating periods, it may be advantageous to invest in higher SCEs during these periods than in body mass. This would explain the lack of a correlation between SCE and BCI for this species.

A positive correlation was found between CMTE and BCI for *A. lituratus*, that is, individuals with better body condition tend to have greater gonadal mass (Entwistle *et al*., 1998). However, for this species it has been observed that positive relation does not imply in an increase in SCE. *Artibeus lituratus* males possess continuous spermatogenesis (Duarte and Talamoni, 2010; Notini *et al*., 2015), whereas the females are polyestrous (Fleming *et al*., 1972; Reis, 1989), and gravid females may occur throughout the year (Tamsitt and Valdivieso, 1963; Duarte and Talamoni, 2010), suggesting that, for this species, there is apparently no pressure for mating during a restricted period, which could imply in the need for greater SCE.

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7.2 Divulgação científica

- Matéria intitulada “Pesquisa no Caraça: Morcegos do sótão”. Jornal Voz do Caraça, p.10, 2017.

Abril de 2017

Pesquisa no Caraça: Morcegos do sótão

Durante o ano de 2017, a equipe do projeto “Ecologia reprodutiva de quirópteros brasileiros em uma área do sudeste do Brasil: Ciclos reprodutivos e mecanismos adaptativos” realizará expedições à RPPN Santuário do Caraça para a coleta de morcegos da espécie *Myotis levis*.

O projeto está investigando aspectos da reprodução de machos do morcego *Myotis levis*, cuja colônia é encontrada na RPPN Santuário do Caraça. Os animais dessa espécie se alimentam de insetos e são bioindicadores e provedores de importantes serviços ecossistêmicos, atuando como agentes de controle de pragas agrícolas e de vetores de doenças. Destaca-se, portanto, a importância de sua conservação.

A equipe é composta pela discente de doutorado Talita Farias (UFMG), pelos professores Guilherme Costa (UFMG), Sônia Talamoni (PUC Minas), pesquisadores e alunos de graduação e pós-graduação da Universidade Federal de Minas Gerais (UFMG) e Pontifícia Universidade Católica de Minas Gerais (PUC Minas).

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