REGIANA LUCIA DE OLIVEIRA

INVESTIGAÇÃO DE ALTERAÇÕES NOS ELEMENTOS DO SISTEMA RESPONSIVO A ESTRÓGENOS E SUAS POSSÍVEIS CORRELAÇÕES FUNCIONAIS NAS VIAS GENITAIS MASCULINAS DO MORCEGO-DAS-FRUTAS *Artibeus lituratus*, DURANTE O CICLO REPRODUTIVO ANUAL

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

Área de concentração: Biologia Celular

Instituto de Ciências Biológicas

Universidade Federal de Minas Gerais

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Esta tese foi realizada no laboratório de Biologia da Reprodução do Departamento de Morfologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, sob a orientação da Profa. Dra. Cleida Aparecida de Oliveira, e contou com auxílio financeiro da Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG) e do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

Universidade Federal de Minas Gerais - Instituto de Ciências Biológicas
Programa de Pós-Graduação em Biologia Celular

ATA DA DEFESA DE TESE DE DOUTORADO DE

REGIANA LÚCIA DE OLIVEIRA

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Às **nove horas** do dia **27 de fevereiro de 2013**, reuniu-se, no Instituto de Ciências Biológicas da UFMG, a Comissão Examinadora da Tese, indicada pelo Colegiado de Programa, para julgar, em exame final, o trabalho final intitulado: **"INVESTIGAÇÃO DE ALTERAÇÕES NOS ELEMENTOS DO SISTEMA RESPONSIVO A ESTRÓGENOS E SUAS POSSÍVEIS CORRELAÇÕES FUNCIONAIS NAS VIAS GENITAIS MASCULINAS DO MORCEGO-DAS-FRUTAS ARTIBEUS LITURATUS, DURANTE O CICLO REPRODUTIVO ANUAL"**, requisito final para obtenção do grau de Doutora em Biologia Celular, área de concentração: **Biologia Celular.** Abrindo a sessão, a Presidente da Comissão, **Dra. Cleida Aparecida de Oliveira**, 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**
Dra. Cleida Aparecida de Oliveira	UFMG	Oliveira/aprovada
Dr. Nilo Bazzoli	PUC Minas	aprovada
Dr. Ralph Gruppi Thomé	UFJF	APROVADA
Dra. Sônia Talamoni	PUC Minas	Aprovada
Dra. Vanessa Pinho da Silva	UFMG	Aprovada

Pelas indicações, a candidata foi considerada: Aprovada

O resultado final foi comunicado publicamente à candidata pela Presidente da Comissão. Nada mais havendo a tratar, a Presidente encerrou a reunião e lavrou a presente ATA, que será assinada por todos os membros participantes da Comissão Examinadora. **Belo Horizonte, 27 de fevereiro de 2013.**

Dra. Cleida Aparecida de Oliveira (Orientadora) Oliveira

Dr. Nilo Bazzoli

Dr. Ralph Gruppi Thomé Ralph G. Thomé

Dra. Sônia Talamoni Sônia M. Talamoni

Dra. Vanessa Pinho da Silva Vanessa Pinho da Silva

Oliveira
Prof. Cleida Aparecida de Oliveira
COORDENADORA DO PROGRAMA DE PÓS GRADUAÇÃO
EM BIOLOGIA CELULAR ICB/UFMG

Obs: Este documento não terá validade sem a assinatura e carimbo do Coordenador

À minha orientadora, Profa. Dra.

Cleida A. Oliveira, modelo de ética e dedicação.

À minha família, especialmente à minha mãe

pelo apoio incondicional em todos os momentos.

Ao meu marido Pedro pelo companheirismo

em todos os momentos dessa minha jornada.

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...porque não há felicidade quando nos encontramos, seja onde for sem a presença daqueles que sempre compartilham conosco as alegrias e dificuldades (Manoel P. Miranda).

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SUMÁRIO

RESUMO

Os dúctulos eferentes (ED) e o epidídimo (EP) exercem funções essenciais que garantem a viabilidade espermática e consequentemente a fertilidade masculina. Entre as funções exercidas por esses órgãos está o transporte transepitelial de água e solutos, o qual envolve a participação das aquaporinas (AQPs). Essa função reabsortiva pode ser modulada por estrógenos, os quais atuam via receptores ERα e ERβ. Sabe-se que os receptores de estrógenos participam da regulação da homeostase tecidual, sendo ERα considerado um fator indutor de proliferação celular e ERβ um fator pró-apoptótico. Os ED são os principais alvos de estrógenos, sendo que altas concentrações de ERα foram detectadas em todas espécies estudadas até o momento. Por outro lado, no EP a presença de ERα é controversa, assim como a presença de aromatase, a enzima que converte andrógenos em estrógenos. Estudos anteriores mostraram que o morcego-das-frutas *Artibeus lituratus* apresenta variação sazonal nos componentes do sistema responsivo a estrógenos e andrógenos nos testículos, tornando-o um bom modelo para estudo do papel desses esteroides. Assim, o presente estudo teve como objetivo investigar, nas vias genitais de *A.lituratus*, a expressão dos componentes do sistema responsivo a estrógenos (aromatase, receptores ERα e ERβ e níveis teciduais de estradiol), bem como suas possíveis correlações funcionais com eventos de apoptose, proliferação celular e reabsorção de fluido através das AQP1 e AQP9. Para comparação, foram estudados também os níveis de testosterona e seus receptores (AR). Os resultados revelaram que ao longo do ciclo reprodutivo anual, ERα apresenta variação, com níveis mais altos durante a regressão sexual. Esse aumento ocorre concomitante ao aumento do índice de proliferação celular, corroborando a função proliferativa de ERα. A enzima aromatase foi detectada nos ED e EP sem variação ao longo do ciclo reprodutivo da espécie, assim como observado para ERβ e AR. As aquaporinas AQP1 e AQP9 tiveram expressão célula- e região-especifica, com expressão constitutiva para AQP1, que se restringe aos ED e espermátides, e variação sazonal para AQP9, expressa apenas nos EP. Não foi encontrada relação entre a expressão de AQP9 com os níveis de estrógenos ou andrógenos, mas sim com a concentração espermática, crescente em direção à região da cauda. Esses dados são indicativos de que o sistema responsivo a estrógenos, via ERα, pode exercer papel na modulação da homeostase tecidual, preparando as vias genitais para a recrudescência sexual, mas não foi evidenciado seu envolvimento com a regulação de AQP1 e AQP9.

Palavras chave: Receptores de estrógenos, aromatase, aquaporinas, apoptose, proliferação celular, dúctulos eferentes, epidídimo, *Artibeus lituratus*, sazonalidade.

ABSTRACT

The efferent ductules (ED) and epididymis (EP) play essential roles which guarantee sperm viability and, thus, male fertility. Among the roles played by these organs, is the transepithelial water and solute transport, which involves aquaporins (AQPs). The reabsorptive function can be modulated by estrogens, which act via receptors ERα and ERβ. Furthermore, it is known that estrogens via ERα and ERβ, participate in the regulation of proliferation and apoptosis, ERα being considered a proliferative factor and ERβ considered a pro-apoptotic factor. The ED are the main targets for estrogens, being ERα detected in high levels in all species studied to date. On the other hand, presence of ERα in the EP is controversial, as it is the presence of aromatase, the enzyme responsible for the conversion of androgens into estrogens. Previous studies show that the big fruit-eating bat *Artibeus lituratus* present seasonal variation in the testis estrogen- and androgen-responsive system, making it a good model for steroid function studies. Therefore, the present study aimed to investigate the expression of the components of the estrogen-responsive system (aromatase, ERα, ERβ and tissue estradiol levels) in the genital tract of *A. lituratus* during regressive and reproductive periods, as well as their functional correlation with apoptosis, cell proliferation and fluid reabsorption through AQP1 and AQP9. For comparison, testosterone levels and its receptors (AR) have also been studied. Results showed that ERα levels vary during the annual reproductive cycle, being higher at the regressive period. This increase parallels that of cell proliferation index, corroborating the proliferative role of ERα. The aromatase was detected in ED and EP throughout the reproductive cycle of the species, without seasonal variation as observed for ERβ and AR. AQP1 and AQP9 showed cell- and region-specific expression. AQP1 is constitutively expressed in the ED and spermatids, whereas AQP9 expression showed seasonal variation, being expressed only in the EP. No correlation was found between AQP9 expression and androgen or estrogen levels, although a crescent correlation with sperm concentration was observed towards the cauda epididymidis. Based on these data, we conclude that the estrogen-responsive system, acting via ERα, may play a role in tissue homeostasis, preparing the genital tract for sexual recrudescence, although its involvement with AQP1 and AQP9 regulation has not been evidenced.

Keywords: Estrogen receptors, aromatase, aquaporins, apoptosis, cellular proliferation, efferent ductules, epididymis, *Artibeus lituratus*, seasonality.

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I – INTRODUÇÃO E REVISÃO DE LITERATURA

1. Sistema genital masculino

O sistema genital masculino é responsável pela produção, nutrição, proteção e estocagem de espermatozoides. Os órgãos que compõem esse sistema incluem os testículos, as vias genitais (rede testicular, dúctulos eferentes, ducto epididimário, ducto deferente), glândulas genitais acessórias e o órgão copulador (Setchell & Breed, 2006). Dentre as vias genitais, os dúctulos eferentes e o epidídimo desempenham papéis cruciais que garantem a viabilidade dos espermatozoides e consequentemente, a fertilidade masculina. Esses segmentos serão os principais alvos do presente estudo.

2. Dúctulos eferentes

Os dúctulos eferentes consistem em uma série de delgados e convolutos túbulos localizados entre a rede testicular e o epidídimo (Ilio & Hess, 1994; Hess, 2002; Hess et al., 2011; Joseph et al., 2011). Seu número varia de acordo com a espécie ou em diferentes indivíduos de uma mesma espécie, sendo cerca de 6 no rato, 8 a 16 no macaco, 6 a 7 no camelo, 6 a 8 nos pequenos morcegos europeus *Pisistrellus savii* e *Vesperugo piccolo*, 14 a 16 no javali, 16 a 19 no bode, 19 a 25 no carneiro, 12 a 15 no homem (Ramos & Dym, 1977; Hemeida et al., 1978; Singh & Bharadwaj, 1980; Azzali et al., 1983; Jones & Jurd, 1987; Ilio & Hess, 1994).

Cada dúctulo parte individualmente da rede testicular e seguem paralelamente uns aos outros na região proximal. Na sua porção medial, formam o cone vasculoso, onde tornam-se mais convolutos, e em seguida, na sua porção distal, unem-se ao ducto epididimário (Hemeida et al., 1978; Jones & Jurd, 1987; Yeung et al., 1991; Guttroff et al., 1992). A conexão dos dúctulos eferentes ao epidídimo pode ocorrer de duas maneiras distintas. Na primeira (Fig. 1 A), os dúctulos eferentes se unem na porção distal formando um único ducto que desemboca no epidídimo, como ocorre em ratos, camundongos e porquinhos-da-índia (Cooper & Jackson, 1972; Guttroff et al., 1992; Ilio & Hess, 1994; Hess, 2002). Na segunda (Fig. 1B), os dúctulos eferentes podem ou não se unir uns aos outros, formando dúctulos terminais que se unem ao epidídimo através de múltiplas entradas. Esse padrão é observado em grandes mamíferos e primatas, incluindo o homem (Goyal & Dhingra, 1975c; Hemeida et al., 1978; Saitoh et al., 1990). Em algumas espécies de grandes mamíferos e no homem, os dúctulos eferentes formam a maior parte da cabeça do epidídimo (Yeung et al., 1991; Hess, 2002).

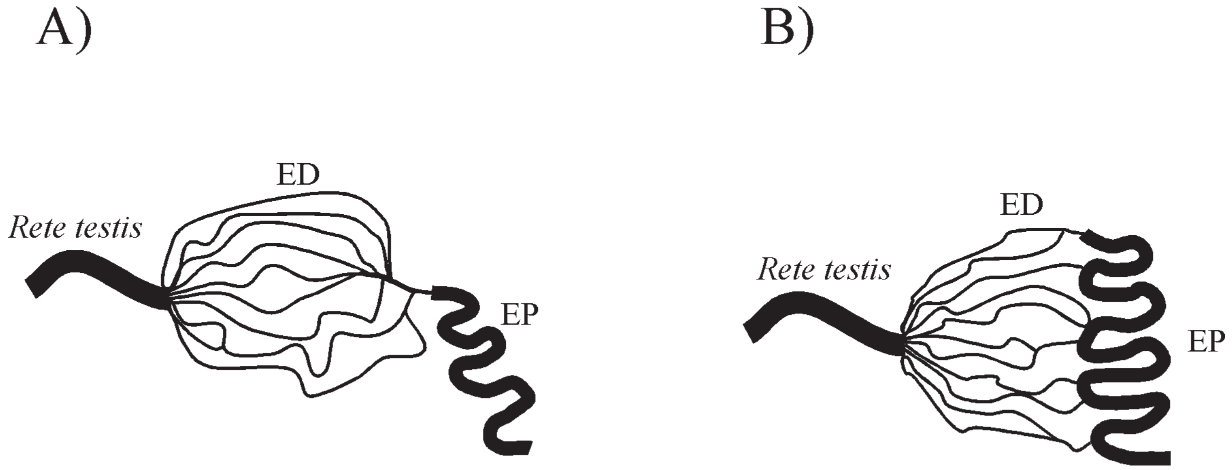

Fig. 1 – Padrões de conexão entre os dúctulos eferentes e o epidídimo. No padrão de conexão (A), os dúctulos eferentes partem da rede testicular (*rete testis*) e se unem ao epidídimo através de um único dúctulo. No padrão (B) os dúctulos eferentes conectam-se ao epidídimo através de múltiplas entradas. Adaptado de Hess, 2002.

O revestimento dos dúctulos eferentes é feito por epitélio colunar simples, o qual é constituído por dois tipos celulares: as células não-ciliadas e as células ciliadas (Ilio & Hess, 1994; Stoffel & Friess, 1994).

As células não-ciliadas, em muitas espécies, são as células mais abundantes ao longo do epitélio (Lohiya & Mathur, 1983; Ilio & Hess, 1994). Seu núcleo é oval, eucromático e localiza-se na porção basal da célula (Flickinger et al., 1978; Hoffer & Greenberg, 1978; Ilio & Hess, 1994). Essas células possuem abundantes microvilosidades apicais e aparelho endocítico bem desenvolvido, localizado na porção supranuclear, favorecendo sua função reabsortiva (Yokoyama & Chang, 1971; Hoffer & Greenberg, 1978; Ilio & Hess, 1994). Além disso, o citoplasma apresenta numerosos grânulos, vacúolos citoplasmáticos e lisossomos de tamanhos variados localizados especialmente na região apical das células (Yokoyama & Chang, 1971; Hemeida et al., 1978; Hoffer & Greenberg, 1978; Ilio & Hess, 1994).

As células ciliadas possuem núcleo oval localizado na região apical do citoplasma. Apresentam longos cílios e poucas microvilosidades na borda apical. Seu aparelho endocítico é pouco desenvolvido, apresentando apenas alguns pequenos lisossomas (Yokoyama & Chang, 1971; Hoffer & Greenberg, 1978; Ilio & Hess, 1994; Stoffel & Friess, 1994).

A visualização de espermatozoides no lume dos dúctulos eferentes não é realizada com frequência em cortes histológicos, especialmente na região proximal dos dúctulos, próximo à rede testicular, devido ao alto volume de fluido testicular presente no local.

Contudo, na região distal, próximo ao epidídimo, onde a maior parte do fluido testicular já foi reabsorvida, os espermatozoides são mais abundantes (Talo, 1981).

Externamente, os dúctulos eferentes são circundados por células musculares lisas em arranjo concêntrico, formando uma ou mais camadas. O tecido conjuntivo circundante abriga vasos sanguíneos e linfáticos além de células linfocitárias e células mononucleares (Hemeida et al., 1978; Ilio & Hess, 1994; Hess, 2002).

A principal função atribuída aos dúctulos eferentes é a reabsorção de mais de 90% do fluido testicular, aumentando a concentração espermática no lúmen epidídimário, onde os espermatozoides completam o processo de maturação e são estocados (Clulow et al., 1994; Ilio & Hess, 1994). Além disso, os dúctulos eferentes realizam endocitose, secreção de proteínas e condução de espermatozoides até o epidídimo (Hermo et al., 1992; Clulow et al., 1994; Ilio & Hess, 1994; Hess, 2002).

O processo de reabsorção de fluido testicular pelos dúctulos eferentes ocorre através do transporte ativo de íons sódio gerado pela bomba de sódio e potássio (Na^+, K^+- ATPase), localizada na membrana basolateral do epitélio. Ao mesmo tempo, a captação luminal de sódio ocorre através de trocadores de sódio/hidrogênio (NHE), localizados na membrana apical das células não-ciliadas. A isoforma de NHE, denominada NHE3 está presente na membrana apical das células não ciliadas e é a única isoforma funcional para a reabsorção de NA^+ na região (Clulow et al., 1998; Leung et al., 2001; Zhou et al., 2001).

Adicionalmente ao transporte de íons, ocorre a reabsorção transepitelial passiva de água pelo epitélio dos dúctulos eferentes através de proteínas denominadas aquaporinas, as quais serão descritas em sessão própria (Brown et al., 1993; Pastor-Soler et al., 2001; Hermo et al., 2004).

3. Epidídimo

3.1. Morfologia

O epidídimo é formado por um único ducto longo, altamente convoluto, também denominado ducto epididimário. Este ducto localiza-se entre os dúctulos eferentes e o ducto deferente e desempenha importante papel para a manutenção da viabilidade dos espermatozoides (Serre & Robaire, 1998; Cooper, 1999; Hermo & Robaire, 2002).

Em mamíferos eutérios, o epidídimo é anatomicamente dividido em segmento inicial e cabeça, que se une ao testículo pelos dúctulos eferentes; corpo, geralmente uma região mais alongada e estreita; e cauda, a região distal geralmente mais alargada (Goyal & Dhingra, 1975a; Cooper, 1999; Robaire et al., 2006).

Em toda sua extensão, o epidídimo é revestido por epitélio pseudo-estratificado cilíndrico, apoiado sobre uma membrana basal. Do segmento inicial até a cauda é possível notar uma gradativa diminuição na altura do epitélio e um aumento no diâmetro luminal e na concentração de espermatozoides (Ramos & Dym, 1977; Goyal & Williams, 1991; Palacios et al., 1993; Cooper, 1999).

O epitélio epididimário é formado por seis tipos celulares que contribuem para a realização das funções epididimárias. São elas as células principais, basais, estreitas, apicais, claras e halo (Fig. 2).

As células principais são as células mais numerosas em toda a extensão do epidídimo, compreendendo cerca de 70-85% das células epididimárias dependendo da espécie. Apesar de estarem presentes em todo epidídimo estas células mostram variação estrutural e funcional ao longo do ducto (Ramos & Dym, 1977; Goyal & Williams, 1991; Robaire et al., 2006). As células principais são células cilíndricas, com longas microvilosidades apicais. Seu núcleo é geralmente redondo, eucromático e localizado na região basal da célula. O citoplasma é rico em organelas e apresenta aparelho endocítico desenvolvido e numerosos grânulos apicais, vesículas pinocitóticas, lisossomos, vesículas cobertas e gotas lipídicas (Goyal & Dhingra, 1975c; Ramos & Dym, 1977; Goyal & Williams, 1991; Palacios et al., 1993; Yeung et al., 1991).

As funções desempenhadas pelas células principais incluem: (1) síntese de proteínas que podem tanto ter ações intracelulares quanto serem secretadas para o lume; (2) transporte de íons, água e solutos orgânicos; (3) endocitose de proteínas luminais; (4) reabsorção de parte do fluido testicular, principalmente na sua porção proximal; (5) fagocitose de resquícios de gotas citoplasmáticas e espermatozoides degenerados; (6) secreção de fluido em resposta a condições locais (Cooper, 1999; Hermo & Robaire, 2002; Robaire et al., 2006).

As células basais, assim como as células principais, estão presentes ao longo de todo o epidídimo. Seu formato pode ser arredondado ou triangular. Possuem núcleo grande arredondado ou oval e pouca heterocromatina. Seu citoplasma é escasso e possui poucas organelas, sendo as mitocôndrias visualizadas com maior freqüência (Goyal & Dhingra, 1975c; Ramos & Dym, 1977; Hoffer & Greenberg, 1978; Goyal & Williams, 1991; Palacios et al., 1993; Yeung et al., 1994; Holschbach & Cooper, 2002). Em algumas espécies, as células basais representam cerca de 15% das células do epitélio epididimário, mas em outras, elas podem ser mais numerosas (Goyal & Williams, 1991). Estão localizadas abaixo das células principais, sem comunicação com o lume. Contudo, há evidências de que algumas células basais podem emitir finas projeções citoplasmáticas que atingem o lume (Goyal & Williams, 1991; Cooper, 1999; Shum et al., 2008).

As funções desempenhadas pelas células basais incluem: (1) reciclagem de lipídios, através da apolipoproteína E, envolvida no transporte de esterol; (2) detoxificação através da ação das enzimas glutationa S-transferase e superoxido dismutase, expressas em altos níveis em seu citoplasma e (3) regulação indireta do transporte de água e eletrólitos pelas células principais (Cooper, 1999; Leung et al., 2004; Robaire et al., 2006). Essas células também induzem secreção de prótons pelas células claras, contribuindo indiretamente para a acidificação luminal, importante para a maturação e estocagem espermática (Shum et al., 2008; Shum et al., 2011). A expressão de antígenos de macrófagos já foi detectada nas células basais, sugerindo para um papel de proteção imunológica para os espermatozoides. Inicialmente foi sugerido que as células basais funcionariam como células-tronco do epidídimo (Martan, 1969). Essa hipótese foi provada ser infundada, pois figuras de mitoses são observadas tanto nas células basais como nas células principais. Além disso, as células basais são visualizadas apenas depois do nascimento, quando já existe uma população de células principais no epitélio epididimário (Holschbach & Cooper, 2002).

As células apicais geralmente estão presentes no segmento inicial e cabeça do epidídimo, mas podem ser ocasionalmente visualizadas em outras regiões, como por exemplo, em humanos, onde representam cerca de 2% das células epiteliais presentes no corpo epididimário (Palacios et al., 1993; Robaire et al., 2006). Sua característica morfológica mais marcante é a ausência de contato com a membrana basal. Na sua região apical são observadas apenas poucas e curtas microvilosidades (Cooper, 1999; Ramos & Dym, 1977). Seu núcleo é arredondado e localizado no citoplasma apical. O citoplasma possui numerosas mitocôndrias localizadas principalmente na região apical. Alguns poucos vacúolos e lisossomos de vários tamanhos também estão presentes na região citoplasmática apical. As células apicais não possuem vesículas cobertas ou pinocítica (Ramos & Dym, 1977; Goyal & Williams, 1991; Palacios et al., 1993; Robaire et al., 2006).

Até o momento, pouco se sabe sobre as funções das células apicais (Robaire et al., 2006). Entretanto, a presença da anidrase carbônica, sugere que essas células estejam envolvidas na acidificação luminal (Cooper, 1999). Além disso, a presença da cathepsina D e β-hexosaminidase sugerem a participação das células apicais na degradação de proteínas (Adamali & Hermo, 1996; Hermo & Robaire, 2002).

As células estreitas assim como as células apicais, são geralmente visualizadas no segmento inicial e cabeça do epidídimo. São células alongadas e estreitas e ao contrário das células apicais, fazem contato com a membrana basal (Hermo & Robaire, 2002). Possuem núcleo pequeno e alongado localizado na porção apical do citoplasma. Seu citoplasma apresenta numerosas mitocôndrias, endossomas e lisossomas. Suas funções incluem endocitose, secreção de íons hidrogênio (H^+) para o lume e secreção de proteínas, como a anidrase carbônica II e glutationa S-transferase (GST), cuja secreção pode variar de acordo com a região onde se localizam (Adamali & Hermo, 1996; Robaire et al., 2006).

As células claras estão presentes na cabeça, corpo e cauda do epidídimo (Cooper, 1999; Robaire et al., 2006). Seu núcleo geralmente está localizado na porção basal do citoplasma. O citoplasma possui numerosas vesículas, endossomos, lisossomos, corpos multivesiculares na região apical e gotas lipídicas na região basal (Robaire et al., 2006). As células claras também possuem grandes vacúolos no seu citoplasma, cujo tamanho depende da concentração luminal de Na^+. As principais funções desempenhadas pelas células claras incluem endocitose de proteínas e remoção de restos citoplasmáticos dos espermatozoides. No rato, as células claras contem anidrase carbônica e próton-ATPase envolvidos na acidificação luminal (Cooper, 1999; Shum et al., 2011; Belleannee et al., 2010; Robaire et al., 2006). Apesar de sua importância e abundância descrita para algumas espécies, as células claras estão ausentes no porquinho-da–índia, cabra, cão, touro e macaco (Goyal & Dhingra, 1975b; Ramos & Dym, 1977; Hoffer & Greenberg, 1978; Goyal & Williams, 1991; Schimming et al., 1997).

As células halo são ocasionalmente observadas ao longo do epidídimo, localizadas principalmente na base do epitélio (Goyal & Williams, 1991; Palacios et al., 1993; Robaire et al., 2006). Em animais jovens as células halo consistem em linfócito T, linfócito T citotóxico e monócitos (Hermo & Robaire, 2002). Essas células são caracterizadas por seu pequeno tamanho e núcleo arredondado e heterocromático. O citoplasma possui poucas organelas e número variável de grânulos densos. Devido a seu citoplasma pouco corado pelas colorações de rotina, formam um halo ao redor do núcleo arredondado, responsável por sua nomenclatura (Hoffer & Greenberg, 1978; Goyal & Williams, 1991; Palacios et al., 1993). No rato as células halo estão presentes no epitélio epididimário antes da formação da barreira hemato-epididimária, sugerindo que a função dessas células seja de proteger o epitélio epididimário (Hermo & Robaire, 2002; Robaire et al., 2006).

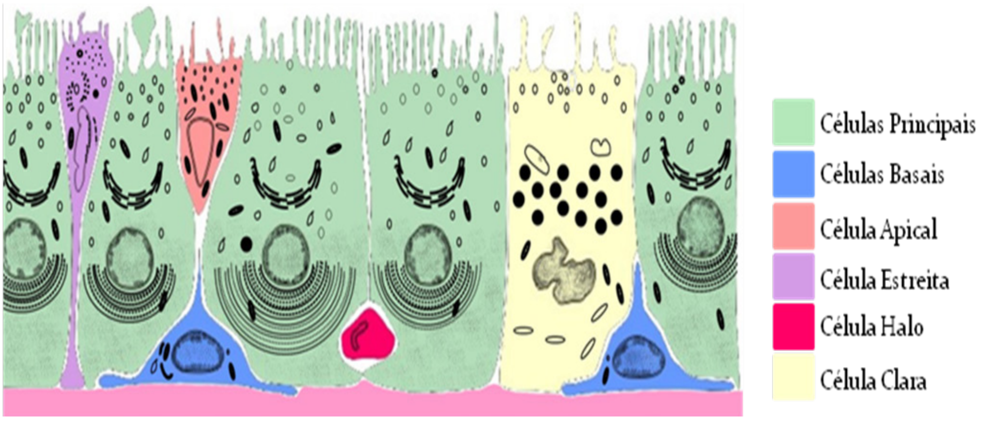

Fig. 2 – Representação esquemática das células presentes no epitélio epididimário. Adaptado de Hermo & Robaire, 2002.

Externamente, o ducto epididimário é envolvido por células musculares lisas dispostas concentricamente em camadas que aumentam da cabeça até a cauda (Ramos & Dym, 1977; Goyal & Williams, 1991; Palacios et al., 1993). A camada muscular é responsável pelas contrações que movem os espermatozoides, ao longo do ducto epididimário. No tecido conjuntivo adjacente, encontram-se, fibras colágenas e elásticas, vasos sanguíneos e linfáticos, fibroblastos, macrófagos, mastócitos e fibras nervosas (Ramos & Dym, 1977; Oke et al., 1988).

3.2. Funções do epidídimo

3.2.1 Proteção

Durante o trânsito ao longo do epidídimo, os espermatozoides são mantidos em condições especiais, com pH ideal (7,2 no segmento inicial e 5,5-6,0 na cauda) e acesso a oxigênio, íons e nutrientes necessários para o processo de maturação (Levine & Kelly, 1978; Cooper, 1999; Cyr et al., 2002; Turner, 2002). Nesse ambiente altamente regulado, os espermatozoides são protegidos do contato com células do sistema imune, através da barreira hemato-epididimária, a qual restringe a passagem de íons, solutos, macromoléculas e células através do epitélio epididimário (Hoffer & Hinton, 1984; Cooper, 1999; Robaire et al., 2006). A barreira hemato-epididimária é considerada uma das mais desenvolvidas em mamíferos eutérios, sendo formada por extensas junções de oclusão presentes entre células principais adjacentes ao logo de todo o epidídimo (Suzuki & Nagano, 1978; Cyr et al., 2007).

Além da proteção fornecida pela barreira hemato-epididimária, o epidídimo utiliza outros mecanismos de proteção dos espermatozoides, tais como secreção de proteínas antimicrobianas, das quais fazem parte as defensinas (Robaire et al., 2006), e proteínas antioxidantes, como superoxido-dismutase, glutationa peroxidases, glutationa transferase (Vernet et al., 2004).

3.2.2. Secreção de outras proteínas

O fluido que envolve os espermatozoides no epidídimo é considerado um dos mais complexos em termos de constituição química (Dacheux & Dacheux, 2002). Sua complexidade se deve a alterações em sua composição de forma contínua e progressiva ao longo do ducto epididimário (Brooks, 1983; Cornwall, 2009; Dacheux & Dacheux, 2002). Dentre todas as espécies estudadas, algumas proteínas são ativamente secretadas, dentre essas proteínas estão a clusterina-α, clusterina- β, proteína epididimária ligadora à ácido retinóico (EP-RABP), proteína secretora rica em cisteína (CRISP-1α e CRISP-1β), lactoferrina, glutationa peroxidase, prostaglandina D2 sintetase, ocitocina, imobilina, entre outras (Tung & Fritz, 1985; Syntin et al., 1996; Fouchecourt et al., 2000; Dacheux & Dacheux, 2002).

3.2.3. Transporte de espermatozoides

Na maioria das espécies, o tempo médio do trânsito dos espermatozoides pelo epidídimo é de 1 ou 2 semanas em roedores e entre 2 e 4 dias no homem (Cooper, 1999; Robaire et al., 2006). Ao entrar no epidídimo, os espermatozoides possuem pouca mobilidade, de forma que seu transporte se dá por atividade peristáltica do ducto (Cooper, 1999). Do segmento inicial até o corpo, as contrações das células musculares lisas são reguladas por hormônios como andrógenos, estrógenos, vasopressina e ocitocina, além de citocinas como as prostaglandinas (Meistrich et al., 1975; Filippi et al., 2002; Robaire et al., 2006). A função contrátil realizada pela ocitocina sofre influencia de estrógeno (Filippi et al., 2002; Filippi et al., 2005), o qual tem sido descrito como um acelerador da contração epididimária (Meistrich et al., 1975).

As fibras nervosas do tipo adrenérgicas, colinérgicas, purinérgicas e peptidergicas, associadas a elementos musculares e vasculares do epidídimo também regulam a função contrátil epididimária (Setchell, 2002). Assim, o aumento das camadas de células musculares lisas na região da cauda, é acompanhado pelo aumento da inervação autônoma epididimária (Laitinen & Talo, 1981; Robaire et al., 2006; Jaakkola, 1983).

3.2.4. Maturação espermática

Durante o trânsito pelo epidídimo, os espermatozoides passam por um complexo processo de maturação. Esse processo envolve alterações morfológicas, fisiológicas e bioquímicas que permitem aos espermatozoides a capacidade de fertilização (Orgebin-Crist, 1969). Quando chegam à cauda, os espermatozoides já são em sua maioria maduros, apresentando maior capacidade de mobilidade quando comparado aos situados na cabeça epididimaria (Robaire et al., 2006). Os lipídios presentes na membrana dos espermatozoides também sofrem alterações e a gota citoplasmática é deslocada gradualmente até seu desprendimento do gameta (Aveldano et al., 1992; Jones, 2002; Olson et al., 2003). O processo de maturação espermática envolve a perda de algumas proteínas de membrana, enquanto a quantidade de pontes de dissulfeto e protaminas é aumentada (Rifkin & Olson, 1985; Su et al., 2005; Bedford, 2004). Também são adquiridas durante o trânsito pelo epidídimo, algumas funções espermáticas necessárias para a fertilização como, alteração do estado de condensação da cromatina, metilação, motilidade, capacidade de se ligar a zona pelúcida e fundir com o ovócito, e habilidade de formar o pró-núcleo masculino (Cooper, 1999; Robaire et al., 2006).

3.2.5. Manutenção dos espermatozoides quiescentes

A cauda epididimária armazena espermatozoides em estado quiescente, por um período semelhante ao que os gametas levam para cruzar o epidídimo (Cooper, 1999; Robaire et al., 2006). Entretanto, em animais sazonais esse tempo é variável, podendo chegar a dez meses na espécie de morcego *Rhinolophus capensis,* o qual ultrapassa o tempo de estocagem observada em outros mamíferos (Bernard, 1984).

Para que possam ser estocados, os espermatozoides maduros entram em estado quiescente, evitando assim a reação acrossômica prematura (Navarro et al., 2007). A quiescência espermática é induzida pela diminuição das concentrações de HCO_3^- e consequentemente aumento no pH intraluminal que envolve a participação de proteínas como as trocadoras de sódio/hodrogênio (NHEs) e as imobilinas (Cornwall, 2009). As NHEs contribuem para a acidificação do meio extracelular mediando a troca de Na^+ extracelular por H^+ intracelular, através da membrana plasmática (Bagnis et al., 2001), enquanto as imobilinas mantem os espermatozoides imóveis mecanicamente através de sua propriedade visco elástica (Usselman et al., 1985).

4. Regulação hormonal dos dúctulos eferentes e epidídimo

A regulação hormonal dos dúctulos eferentes inclui a participação tanto de andrógenos quanto de estrógenos (Ilio & Hess, 1994; Hess, 2002). Contudo, diversos estudos têm mostrado que os estrógenos são os principais hormônios reguladores da função dos dúctulos eferentes (Hess et al., 1997a; Oliveira et al., 2001; Oliveira et al., 2002; Hess et al., 2011). De fato, a inativação química ou genética de ERα revelou que os estrógenos participam da manutenção da citoarquitetura epitelial dos dúctulos eferentes e da função reabsortiva desses dúctulos, sendo que distúrbios nesta função levam secundariamente à atrofia testicular e infertilidade (Hess et al., 1997a; Zhou et al., 2001; Oliveira et al., 2001; Oliveira et al., 2002; Oliveira et al., 2005 Hess et al., 2011).

4.1. Os andrógenos e seus receptores

Os andrógenos são considerados os principais hormônios reguladores da função epididimária. Tanto a testosterona quanto a 5α-diidrotestosterona (DHT) estão presentes no fluido epididimário, sendo que os níveis de DHT são mais altos que os da testosterona (Vreeburg, 1975). A principal fonte de andrógenos epididimários são os testículos. A testosterona produzida pelas células de Leydig é transportada dos testículos até o epidídimo pela proteína carreadora de andrógenos ABP. No epidídimo, a testosterona é convertida à DHT pelas enzimas 5α-redutase (Inano et al., 1969; Viger & Robaire, 1994; Robaire & Hamzeh, 2011). Esses hormônios desempenham papéis fundamentais para a manutenção da citoarquitetura do epitélio epididimário (Hoffman et al., 1976; Smithwick & Young, 2001) e são capazes de regular a expressão de genes específicos em cada região do epidídimo (Chauvin & Griswold, 2004). Para exercer suas funções, os andrógenos se ligam aos receptores de andrógenos (AR), os quais são expressos nos dúctulos eferentes e ducto epididimário de todas as espécies estudadas até o momento (Ruizeveld de Winter et al., 1991; Nie et al., 2002; Zhou et al., 2002; Pearl et al., 2006; Robaire et al., 2006; Robaire et al., 2007).

Os receptores de andrógenos (AR) são fatores transcricionais ligante-dependentes, pertencentes à superfamília dos receptores nucleares, à qual também pertencem os receptores de progesterona (PR), glicocorticóides (GR), mineralocorticóides (RM), vitamina D3 (VDR) e receptores de estrógenos (ER), além do grupo de receptores órfãos, para os quais os ligantes são desconhecidos (Tsai & O'Malley, 1994; MacLean et al., 1997).

A estrutura molecular do AR inclui seis regiões morfofuncionais denominadas de A a F, onde se destacam três domínios funcionais, sendo os domínios N-terminal (NTD), domínio de ligação ao DNA (DBD) e domínio de ligação ao ligante (LBD) (Fig. 3). Esses domínios estão localizados nas regiões A/B, C e E, respectivamente (Hiipakka & Liao, 1998; Claessens et al., 2008).

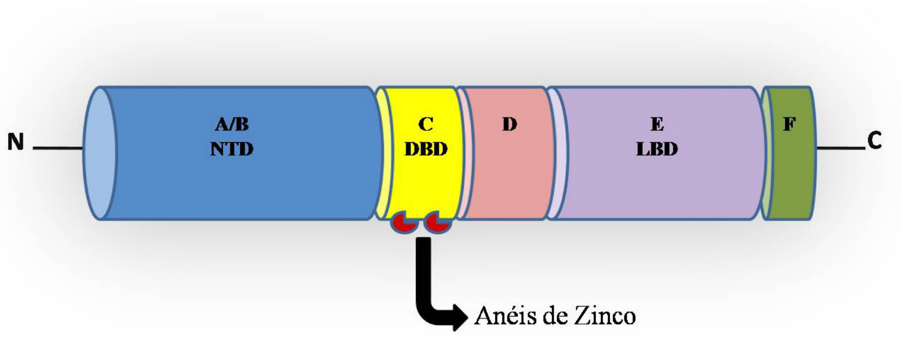

Fig. 3 – Representação esquemática da estrutura molecular dos receptores de andrógenos. Adaptado de Beato & Klug, 2000.

O domínio NTD é o menos conservado entre os diferentes membros da família dos receptores nucleares (Lavery & McEwan, 2005). É responsável por modular a ativação transcricional através da interação com as proteínas envolvidas na síntese celular basal. Contém uma sequência de aminoácidos importante que o permite se comunicar com LBD estabilizando a ligação ao hormônio, regulando a expressão gênica e evitando possíveis alterações como mutações que podem comprometer a atividade de AR. Possui também a função ativadora AF1, a qual pode ser subdividida em unidades de ativação transcricional, TAU-1 e TAU-5. AF1 é um domínio estruturalmente flexível, se tornando mais estável na presença de proteínas como o fator transcricional TFIIF (Reid et al., 2001; Betney & McEwan, 2003; Lavery & McEwan, 2005; Claessens et al., 2008).

O domínio DBD é responsável pelo reconhecimento de regiões específicas do DNA, denominadas elemento responsivo a andrógeno (ARE). É o mais conservado entre os domínios dos receptores nucleares e está localizado entre o NTD e o LBD. Possui nove resíduos de cisteína, dos quais oito estão envolvidos na formação de dois anéis de zinco. O primeiro anel, situado mais próximo de NTD, determina o reconhecimento da sequência correta do DNA e o segundo é responsável por estabilizar a ligação do receptor ao DNA, estando envolvidos com a dimerização do receptor (Verrijdt et al., 2006; Claessens et al., 2008).

O domínio LBD é o responsável pela ligação ao ligante. Após ligar a um andrógeno, este domínio induz a mudança conformacional do receptor e aumenta sua afinidade pelo DNA após sua dissociação do complexo de chaperonas. Além disso, o LBD estabiliza a homodimerização e coordena a interação do receptor com outros co-reguladores. Possui também a função ativadora AF-2, responsável por mediar a interação molecular entre LBD e NTD, necessária para a ativação transcricional de AR (Hiipakka & Liao, 1998; Berrevoets et al., 1998; Claessens et al., 2008).

Na ausência do ligante, AR é encontrado no citoplasma ligado ao complexo multiprotéico de chaperonas, como a Hsp90, Hsp70 e Hsp56. Após a ativação pelo ligante, AR se dissocia do complexo, formando um homodímero que é fosforilado e translocado para o núcleo, onde se liga aos elementos responsivos a andrógenos na região promotora de genes alvo. Posteriormente, AR inicia a transcrição gênica pelo recrutamento de co-reguladores e outros fatores da maquinaria transcricional, agindo na modulação da expressão de genes alvo (MacLean et al., 1997; Hiipakka & Liao, 1998; Beato & Klug, 2000).

As ações transcricionais mediadas por andrógenos/AR são fundamentais para manter as funções epididimárias. Contudo, especialmente para os dúctulos eferentes, outros fatores como estrógenos são necessários para o normal funcionamento do órgão.

4.2. Os estrógenos e seus receptores

Os estrógenos são hormônios de grande relevância na fisiologia do sistema genital masculino, o qual é encontrado em altas concentrações no fluido testicular (250 pg/ml no rato) e sêmen de diversas espécies de mamíferos (Free & Jaffe, 1979; Hess, 2003). Nos machos, a principal fonte de estrógenos são os testículos, embora seu principal alvo seja os dúctulos eferentes, onde o epitélio expressa níveis muito altos de RNAm de receptores de estrógenos, os quais atingem aproximadamente 3,5 vezes as concentrações encontradas no útero, que é um clássico alvo de estrógeno (Hess et al., 1997b; Joseph et al., 2011).

Nos dúctulos eferentes os estrógenos são responsáveis por manter a citoarquitetura epitelial dos dúctulos e regular sua função reabsortiva (Hess et al., 2000; Lee et al., 2000; Oliveira et al., 2001; Oliveira et al., 2002; Joseph et al., 2011). Além disso, os estrógenos participam diretamente da regulação de proteínas-chave para a manutenção da função dos dúctulos. Dentre estas proteínas estão a aquaporina 9 e o trocador de sódio/hidrogênio NHE3 (Hess et al., 2000; Hansen et al., 1999; Zhou et al., 2001; Oliveira et al., 2002; Oliveira et al., 2005; Hermo & Smith, 2011; Joseph et al., 2011).

O papel dos estrógenos na regulação do epidídimo, por outro lado, é mais controverso. Há inconsistência nos dados sobre a distribuição de seus receptores, sendo descrita presença ou ausência de ER dependendo da espécie e do segmento analisado, e até mesmo da metodologia utilizada nas análises (Hess, 2003; Oliveira et al., 2005; Hess et al., 2011; Joseph et al., 2011). Além disso, as alterações morfológicas vistas no epidídimo de animais cujos ERs foram inativados são negligíveis, quando comparadas com aquelas dos dúctulos eferentes e testículos (Hess et al., 2000; Cho et al., 2003). Porém, há evidências de que estrógenos participam na fisiologia epididimária, especialmente regulando o trânsito espermático (Meistrich et al., 1975; Filippi et al., 2005; Vignozzi et al., 2008).

Apesar do papel dos estrógenos na função epididimária ainda não estar claro, a presença de estrógeno sulfotransferase e sulfatase, enzimas que atuam como fator protetor do epitélio contra o excesso de estrógenos e aumento das concentrações de estrógenos livre respectivamente, também indica a participação dos estrógenos na regulação da função epididimária (Lemazurier & Seralini, 2002; Hoffmann et al., 2010). Há indícios ainda, de que os estrógenos auxiliam o trânsito de espermatozoides atuando na contração da musculatura lisa do epidídimo (Meistrich et al., 1975; Filippi et al., 2005; Fibbi et al., 2009). Para que os estrógenos possam exercer suas funções biológicas é necessária a ligação a receptores específicos, denominados receptores de estrógenos.

Assim como os receptores de andrógenos, os receptores de estrógenos são fatores transcricionais dependentes de ligante, pertencentes à superfamília dos receptores nucleares. Até o momento foram descritos dois tipos de receptores de estrógenos, os ERα e ERβ (também denominados ESR1 e ESR2), que apesar da semelhança estrutural, são produtos de genes distintos localizados em diferentes cromossomos (Enmark & Gustafsson, 1999; Heldring et al., 2007; Hess et al., 2011). Os receptores de estrógenos também possuem os três domínios funcionais característicos: NTD, DBD e LBD (Fig. 4), os quais desempenham as mesmas funções básicas descritas para AR (Parker et al., 1993; Joseph et al., 2011).

O receptor ERα é uma proteína de aproximadamente 595 aminoácidos e peso molecular de cerca de 66 kDa (Enmark et al., 1997). O gene que a codifica se localiza no braço longo do cromossomo 6 em humanos e cromossomo 10 em camundongos (Enmark & Gustafsson, 1999). Já ERβ possui aproximadamente 485 aminoácidos, pois sua região N-terminal é mais curta. Seu peso molecular de aproximadamente 54,2 kDa e é codificado pelo cromossomo 14, em humanos e cromossomo 12 em camundongos (Walter et al., 1985; Enmark et al., 1997; Tremblay et al., 1997; Enmark & Gustafsson, 1999; O'Donnell et al., 2001). Apesar da grande homologia entre ERα e ERβ no domínio de ligação ao DNA (cerca de 95%), o domínio de ligação ao ligante apresenta apenas cerca de 55% de homologia (Mosselman et al., 1996; Kuiper et al., 1997). As diferenças observadas no domínio de ligação ao ligante podem justificar algumas alterações nas atividades transcricionais tanto de ERα quanto de ERβ. Entretanto, a maioria dos ligantes se liga a ambos os receptores com similar afinidade, exceto por alguns fitoestrógenos, como genisteína e cumestrol, que se liga aos ERβ com maior afinidade que aos ERα (Kuiper et al., 1998).

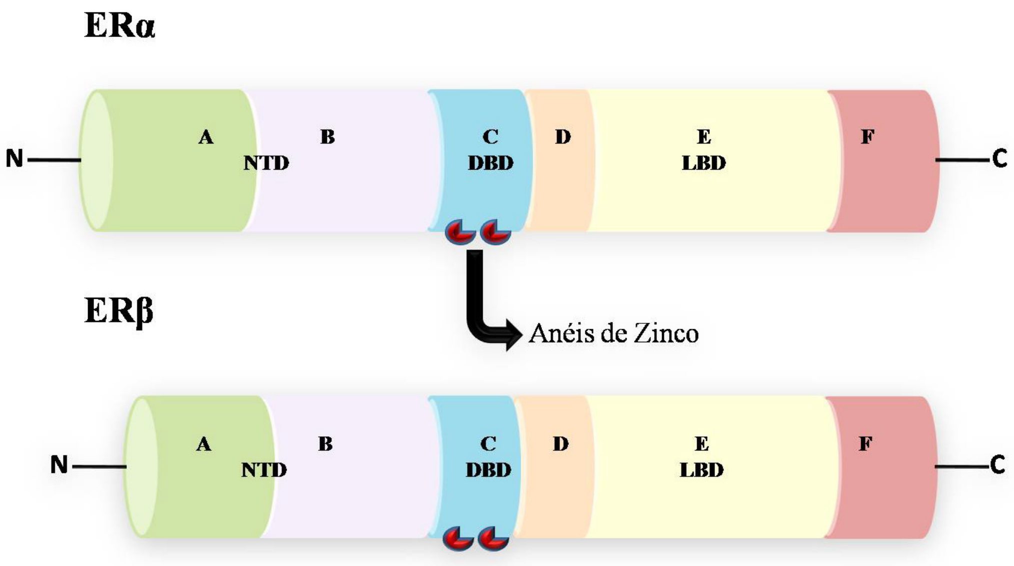

Fig. 4 – Representação esquemática da estrutura molecular dos receptores de estrógenos ERα e ERβ. Adaptação de Akingbemi, 2005.

4.3. Localização dos receptores de estrógenos nas vias genitais masculinas

Ambos os receptores de estrógenos ERα e ERβ estão presentes ao longo das vias genitais masculinas, mas apresentam distribuição distinta e espécie-específica. De maneira geral, os ERα possuem localização restrita ao epitélio dos dúctulos eferentes de todas as espécies estudadas até o momento, como camundongos, hamster, ratos, cães, gatos, porco, cabrito, carneiro, veado, e primatas, incluindo o homem, além do leão-marinho, galo, lagarto e tartaruga (Fisher et al., 1997; Goyal et al., 1997; Hess et al., 1997a; Kwon et al., 1997; Pelletier & El-Alfy, 2000; Sar & Welsch, 2000; Saunders et al., 2001; Nie et al., 2002; Zhou et al., 2002; Tian et al., 2004; Shapiro et al., 2005; Gist et al., 2007; Pearl et al., 2007; Schon & Blottner, 2008; Colegrove et al., 2009; Joseph et al., 2011; Oliveira et al., 2011; Verderame et al., 2012). No epidídimo, a presença de ERα no epididimo é controversa. O receptor está presente em algumas espécies como camundongo (Zhou et al., 2002; Joseph et al., 2011), hamster (Joseph et al., 2011), cães, gatos, (Nie et al., 2002), suínos, cavalo (Hejmej et al., 2005; Parlevliet et al., 2006; Pearl et al., 2006) e tartaruga (Gist et al., 2007). Entretanto, está ausente em outras espécies como rato (Fisher et al., 1997), cabrito (Goyal et al., 1997), macaco (Joseph et al., 2011) e homem (Saunders et al., 2001). Os receptores ERβ estão amplamente distribuídos ao longo das vias genitais, sendo detectados tanto nos dúctulos eferentes, quanto no epidídimo (Hess et al., 1997a; Saunders et al., 2001; Nie et al., 2002; Zhou et al., 2002; Parlevliet et al., 2006; Hejmej et al., 2005). Apesar de sua ampla distribuição, o papel fisiológico dos ERβ nas vias genitais masculinas não está bem estabelecido. No entanto, há evidências de que os estrógenos, via ERα e ERβ, participam da regulação da proliferação e morte celular por apoptose, sendo os ERα mais envolvidos em função proliferativa, enquanto os ERβ se relacionam mais com funções pró-apoptóticas e anti-proliferativas, como evidenciado especialmente no tecido prostático (Weihua et al., 2001; Zhou et al., 2004; Imamov et al., 2004). Nas vias excurrentes, até o momento não se sabe se os ER participam similarmente na regulação do balanço entre proliferação e morte celular.

Em conjunto, os dados existentes sobre o papel de estrógenos nos machos indicam que aromatização de andrógenos para estrógenos é uma via alternativa para a regulação de funções reprodutivas masculinas, indicando que a fisiologia do sistema genital masculino não pode mais ser entendida baseada somente na ação de andrógenos.

4.4. A enzima aromatase

A enzima P450 aromatase (P450arom) é um complexo enzimático responsável pela conversão irreversível de andrógenos a estrógenos. O substrato androgênico utilizado pela enzima pode ser tanto a testosterona quanto a androstenediona, os quais originam o estradiol (E_2) e a estrona (E_1), respectivamente (Thompson & Siiteri, 1974; Simpson et al., 1994). A aromatase é composta por duas proteínas: uma é a ubíqua flavoproteína NADPH-citocromo P450 redutase e a segunda, a glicoproteína citocromo P450 aromatase, a qual contém o grupo heme e a região de ligação de esteróide, específico para a biossíntese de estrógenos. A citocromo P450arom é responsável por ligar e catalisar a modificação do substrato esteroide (Simpson et al., 1994; Carreau et al., 2003), enquanto a NADPH-citocromo P450 redutase é responsável pela transferência de elétrons do NADPH para o citocromo P450 (Fig. 5) (Simpson et al., 1997; O'Donnell et al., 2001; Carreau et al., 2003).

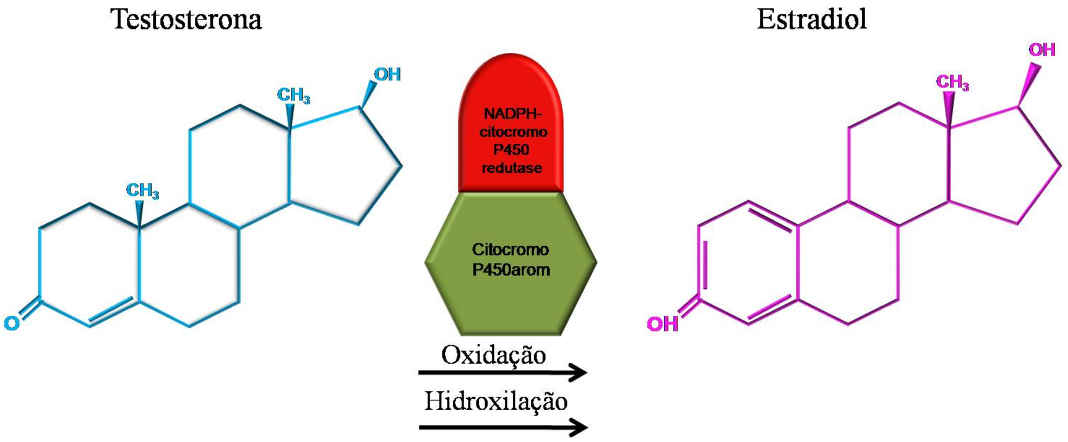

Fig. 5 – Representação esquemática da enzima P450 aromatase formada por duas proteínas: a NADPH-citocromo P450 redutase e a citocromo P450 aromatase, responsáveis pela conversão de andrógenos a estrógenos.

A P450arom é o produto de um único gene chamado *CYP19*, o qual pertence à superfamília do gene *P450* que contém mais de 600 membros, pertencentes a cerca de 100 famílias da qual a P450arom é o único membro da família 19 (O'Donnell et al., 2001; Carreau et al., 2003). Em humanos, o gene *CYP19* se localiza na região q21.1 do cromossomo 15. O gene alcança o comprimento de mais de 100 kb e é composto por dezessete exons dos quais nove são traduzidos. A expressão do gene é regulada por promotores tecido-específico pelo uso alternativo de múltiplos exons, mas a proteína traduzida é a mesma em todos os tecidos (O'Donnell et al., 2001; Carreau et al., 2007). Entretanto, em alguns animais a aromatase pode apresentar diferentes isoformas, como por exemplo, o porco que apresenta três isoformas e o *zebrafish* que possui duas isoformas distintas codificadas por genes específicos (Graddy et al., 2000; Kishida & Callard, 2001)

A proteína traduzida pelo gene *CYP19* é composta de 503 aminoácidos e sua massa molecular é de aproximadamente 55 kDa (Carreau et al., 2002). A aromatase apresenta localização citoplasmática, especificamente no retículo endoplasmático liso e já foi detectada em diversos tecidos como placenta, gônadas masculinas e femininas, cérebro, ossos, fígado fetal e tecido adiposo (Simpson et al., 1997; Graddy et al., 2000; O'Donnell et al., 2001; Carreau et al., 2002).

No sistema genital masculino, a aromatase desempenha importante papel na diferenciação sexual durante o desenvolvimento embrionário, na reprodução e também no comportamento sexual (Arnold & Gorski, 1984; Gustafson & Donahoe, 1994; Honda et al., 1998; Toda et al., 2001; Hughes et al., 1999). Nas espécies estudadas até o momento, a aromatase está presente principalmente nos testículos, embora haja informação sobre expressão também nas vias genitais e na próstata (Nitta et al., 1993; Carpino et al., 2004; Hejmej et al., 2005; Gist et al., 2007; Pereyra-Martinez et al., 2001). Contudo, sua expressão é espécie-específica e pode variar com a idade (Papadopoulos et al., 1986; Carreau et al., 1999).

No testículo de animais roedores jovens a aromatase é detectada principalmente nas células de Sertoli (Papadopoulos et al., 1986; Carreau et al., 1999; Carreau et al., 2002). Em roedores adultos, a aromatase localiza-se nas células de Leydig e células espermáticas, incluindo espermatócitos em paquíteno e espermátides arredondadas e alongadas (Nitta et al., 1993; Levallet et al., 1998; Janulis et al., 1998). No cavalo e em humanos, as células de Leydig foram detectadas como a única fonte testicular de estrógenos (Inkster et al., 1995; Hejmej et al., 2005). Em espermatozoides em trânsito pelas vias genitais a presença da aromatase também foi detectada (Aquila et al., 2002; Nitta et al., 1993; Janulis et al., 1996; Janulis et al., 1998; Lambard et al., 2004).

A presença da aromatase nas vias genitais até o momento tem sido pouco estudada e controversa. Nos dúctulos eferentes do homem a detecção da enzima foi descrita tanto nas células ciliadas, quanto nas células não-ciliadas (Carpino et al., 2004). No epidídimo, inicialmente pensava-se que a única fonte de aromatase era proveniente dos espermatozoides em trânsito pelo órgão. Entretanto, o epitélio dos dúctulos eferentes (células ciliadas e não ciliadas) e do epidídimo (células principais e basais) tem sido demonstrado como fonte adicional de estrógenos (Kwon et al., 1995; Carpino et al., 2004; Wiszniewska, 2002; Hejmej et al., 2005).

A sazonalidade é um importante fator que influencia na expressão da aromatase. No roedor *bank vole*, um animal cuja reprodução é sazonal, a P450 aromatase é muito mais expressa nos espermatócitos em paquíteno e espermátides de animais expostos a ciclos de dias longos do que em animais expostos a ciclo de dias curtos (Bilinska et al., 2000).

No esquilo (*Citellus dauricus brandt*), no guaxinim (*Nyctereutes procynoides*) e no urso negro (*Ursus americanus*), durante o período reprodutivo, a aromatase foi detectada nas células somáticas e espermatogênicas do testículo. Entretanto, durante o período de regressão, a enzima está ausente (Tsubota et al., 1997; Qiang et al., 2003; Zhang et al., 2010). No urso tibetano (*Ursus thibetanus japonicus*), os níveis de aromatase diminuem durante o período de regressão, mas não se tornam indetectáveis, similar ao observado no urso-negro (Komatsu et al., 1997). Ao contrário do observado na maioria dos animais estudados, na tartaruga *Trachemys scripta* os mais altos níveis de aromatase foram detectados no testículo durante o período não-reprodutivo (Gist et al., 2007).

A importância da enzima aromatase para a fertilidade masculina foi comprovada através da inativação gênica da enzima, onde os machos são inférteis, como consequência do impedimento da espermiogênese associado com o aumento no número de células espermatogênicas em apoptose, anormalidades no desenvolvimento inicial do acrossoma e diminuição da motilidade espermática, hiperplasia e hipertrofia das células de Leydig e inabilidade de fertilização (Robertson et al., 1999; Toda et al., 2001). Além disso, a infertilidade também é característica de homens com mutação natural da enzima (Simpson et al., 1997; Simpson, 1998). A inativação da aromatase também provoca alterações no comportamento sexual onde os machos apresentam prolongada latência ao comportamento de monta e redução no número de monta em resposta ao comportamento receptivo das fêmeas, sugerindo um importante papel da aromatase no comportamento sexual (Honda et al., 1998; Robertson et al., 2001; Toda et al., 2001; Lambard et al., 2005). Esses dados demonstram que os estrógenos são ativamente participativos na regulação do sistema genital masculino e são essenciais para a manutenção da fertilidade masculina.

5. Aquaporinas

As aquaporinas (AQPs) compreendem uma família de pequenas proteínas integrais de membrana, amplamente expressas nos diversos sistemas fisiológicos animal, responsáveis pelo transporte de água através da membrana plasmática. Essas proteínas possuem cerca de 30 kDa em estado não-glicosilado e estão diretamente relacionadas ao transporte de água e pequenos solutos não carregados (Verkman, 2005; Agre et al., 1995). São proteínas integrais de membrana, contendo seis domínios transmembrânicos e cinco alças de conexão, sendo os domínios C-terminal e N-terminal citoplasmáticos (Fig. 2 A). Cada proteína se une a outras três, formando homotetrâmeros na membrana plasmática, mas cada subunidade atua como um poro independente (Verkman, 2005; Agre et al., 1995). O diâmetro do poro de cada aquaporina é de cerca de 3Å, como na AQP1, por exemplo, mas pode variar de acordo com a especificidade de transporte da proteína (Murata et al., 2000). O pequeno diâmetro do poro permite que apenas uma molécula de água se acomode ao longo do eixo do poro, fazendo com que as moléculas de água sejam rapidamente transportadas em fila única (Fig. 2 B) (de Groot et al., 2001; Tajkhorshid et al., 2002).

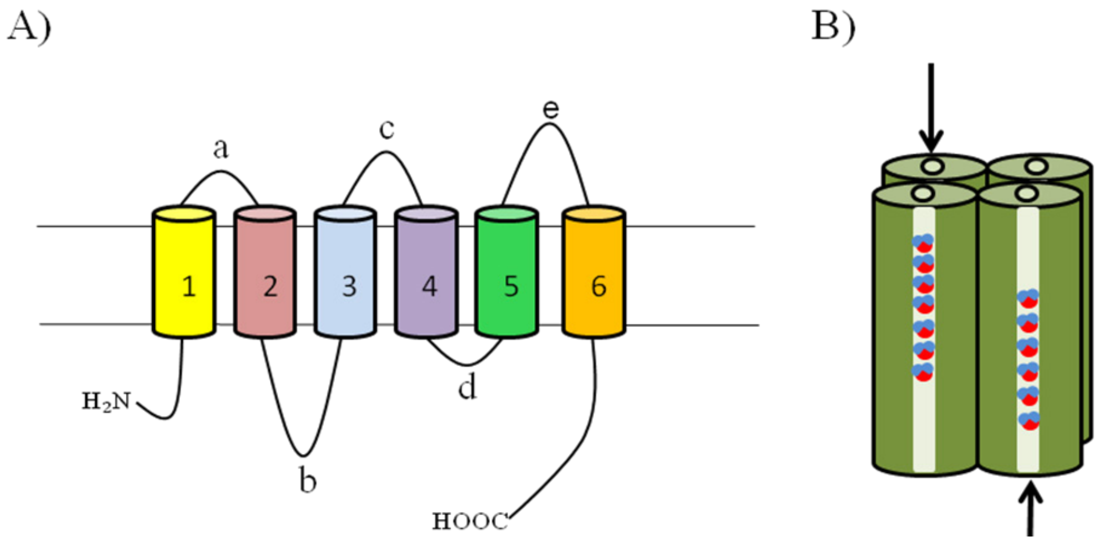

Fig. 6 – Representação esquemática das aquaporinas quanto à estrutura molecular (A) e a formação de homotetrâmeros que atuam como poros individuais para o transporte bidirecional de água (setas em B). 1 - 6 = domínios transmembrânicos; a – e = alças de conexão. Adaptado de Agre et al, 1995 e Verkman, 2005.

Atualmente são descritas 13 aquaporinas (AQP0-AQP12), localizadas em diversos tipos celulares de mamíferos, como epitélios e endotélios, bem como em células de tecidos não especializados no transporte de fluidos, como pele, tecido adiposo e bexiga urinária (Verkman, 2005). As aquaporinas podem ser classificadas de acordo com o tipo de substâncias as quais são permeáveis. Assim as AQP0, AQP1, AQP2, AQP4, AQP5 e AQP8, que atuam especialmente como canais seletivos para o transporte de água, são consideradas como aquaporinas clássicas (Borgnia et al., 1999; Agre et al., 2002; Verkman, 2005; Cerda & Finn, 2010). Outras, como as AQP3, AQP7, AQP9 e AQP10, realizam o transporte de glicerol, uréia e amônia, além de água, sendo classificadas como aquagliceroporinas (Agre et al., 2002; Verkman, 2005; Cerda & Finn, 2010). As AQP6, AQP11 e AQP12 são aquaporinas cujas funções ainda não estão bem definidas.

Com exceção da AQP12, todas as demais já foram investigadas no sistema genital masculino e mostraram expressão órgão-, célula- e espécie-dependente. Dentre todas as aquaporinas estudadas, as AQP1 e AQP9 têm sido amplamente estudadas no trato genital masculino, especialmente em órgãos com alta taxa reabsortiva como os dúctulos eferentes e epidídimo.

Nos dúctulos eferentes, as AQP1 e AQP9 são expressas nas células epiteliais, contribuindo para a reabsorção de fluido testicular. As AQP1 estão localizadas especialmente na borda apical das células não-ciliadas dos dúctulos eferentes (Brown et al., 1993; Fisher et al., 1998; Badran & Hermo, 2002; Oliveira et al., 2005; Da Silva et al., 2006a; Domeniconi et al., 2008; Lu et al., 2008; Arrighi et al., 2010b). No epidídimo, AQP1 não é expressa no epitélio, mas sim nas células endoteliais dos vasos sanguíneos presentes no tecido conjuntivo circundante tanto dos dúctulos eferentes quanto do epidídimo (Badran & Hermo, 2002; Oliveira et al., 2005; Da Silva et al., 2006b; Domeniconi et al., 2008; Arrighi et al., 2010b). A expressão da AQP1 nos dúctulos eferentes é considerada constitutiva, uma vez que sua expressão não é alterada por ligação dos dúctulos eferentes, castração ou tratamento com o anti-estrógeno ICI 182,780 (Zhou et al., 2001; Badran & Hermo, 2002; Oliveira et al., 2005).

A AQP9 também foi descrita nas células não-ciliadas dos dúctulos eferentes de todas as espécies estudadas e células principais do epidídimo (Elkjaer et al., 2000; Pastor-Soler et al., 2001; Badran & Hermo; Zaniboni et al., 2004; Domeniconi et al., 2007; Hermo et al., 2008; Arrighi et al., 2010b; Hashem, 2010). Ao contrário da AQP1 que é constitutivamente expressa no trato genital masculino, a expressão de AQP9 tem mostrado ser regulada por andrógenos e estrógenos. Nos dúctulos eferentes, a castração, ligação, o uso do antagonista dos receptores de estrógenos ICI 182, 780, e deleção gênica (*knockouts*) do receptor de estrógeno ERα, resultou em drástica redução na expressão de AQP9 (Oliveira et al., 2005; Ruz et al., 2006). Entretanto, a reposição hormonal com estrógeno, diidrotestosterona (DHT) ou 3β-diol (um metabólito da DHT que se liga aos receptores de estrógenos), mas não testosterona, restaura os níveis de AQP9, indicando que nos dúctulos eferentes de roedores a expressão da AQP9 é regulada tanto por estrógenos quanto por andrógenos (Oliveira et al., 2005; Picciarelli-Lima et al., 2006).

No epidídimo, a expressão da AQP9 também foi detectada em todas as regiões do órgão (Pastor-Soler et al., 2001; Badran & Hermo, 2002; Domeniconi et al., 2007; Hermo et al., 2008). Contudo, castração, ligação dos dúctulos eferentes ou tratamento com flutamida (um composto anti-androgênico), reduz a expressão da AQP9 nas regiões do segmento inicial e cauda (Badran & Hermo, 2002; Oliveira et al., 2005). Ao contrário do observado nos dúctulos eferentes, estradiol não é capaz de reverter a expressão de AQP9 no epidídimo (Oliveira et al., 2005). Entretanto, a reposição hormonal com DHT restaura a expressão da AQP9 para níveis similares aos dos animais controle (Oliveira et al., 2005). Por outro lado, a reposição da testosterona pode ou não restaurar a expressão da AQP9 (Badran & Hermo, 2002; Pastor-Soler et al., 2002). Apesar de haver dados que apontem os fatores de regulação da AQP9 nas vias genitais masculinas, sua função no ducto epididimário ainda não está determinada. Por se tratar de uma aquagliceroporina, a presença da AQP9 no epidídimo pode estar diretamente relacionada com o transporte de glicerol, além de água. Entretanto, mais estudos são necessários para elucidar o verdadeiro papel da AQP9 no epidídimo. Nesse sentido, o mapeamento dessa proteína em outras espécies pode contribuir com novos dados sobre o papel das aquaporinas nas vias genitais masculinas, especialmente com uso de um modelo como o morcego *Artibeus lituratus*, o qual mostrou variação natural dos receptores de andrógenos e estrógenos (Oliveira et al., 2009).

6. Proliferação e morte celular

O equilíbrio entre proliferação e morte celular é fundamental para a manutenção da homeostase dos organismos, sendo que qualquer desequilíbrio em um desses processos pode levar a consequências drásticas envolvendo diversas patologias como doenças autoimunes, e neurodegeneração, quando ocorre um aumento desenfreado na ocorrência de apoptoses (Degterev et al., 2003). Já o aumento de ocorrências de proliferação celular está relacionado com as neoplasias e câncer (Bologna-Molina et al., 2012).

6.1. Proliferação celular

O ciclo celular é o processo pelo qual uma célula somática duplica seu material genético, dividindo-o igualmente entre suas células-filhas. Durante o ciclo ocorre a duplicação do DNA e divisão nuclear (mitose), dando origem a uma nova célula. Didaticamente o ciclo celular pode ser dividido em duas fases principais: intérfase e mitose. A interfase, intervalo entre duas mitoses, pode ser dividida nas fases G1, S e G2, sendo suas principais características a duplicação do DNA e a preparação para a fase seguinte: a mitose, (Norbury & Nurse, 1992). Durante a fase G1, a célula se prepara para a síntese do DNA que ocorre na fase S (síntese). Na fase G2, a célula se prepara para a mitose. Durante a mitose ocorre a divisão celular, considerada um processo crucial para o crescimento e diferenciação dos organismos multicelulares, onde as células passam pelas fases denominadas prófase, metáfase, anáfase e telófase (Vermeulen et al., 2003).

Para que o ciclo celular se inicie, a célula que se encontra em repouso (fase G0) precisa ser estimulada por fatores de crescimento como fator de crescimento derivado das plaquetas (PDGF) ou fator de crescimento epidérmico (EGF), insulina, hormônios ou citocinas produzidas pela própria célula ou por células vizinhas. Esses fatores desencadeiam cascatas de reações químicas, culminando com a ativação de fatores de transcrição (c-myc, c-jun, c-fos), responsáveis pela síntese de RNA de diversas enzimas que atuarão na síntese do DNA, como por exemplo, o diidrofolato redutase, DNA polimerase e topoisomerases. Essa cascata de eventos químicos e morfológicos ocorre de forma sucessiva e ordenada, fazendo com que a célula avance da fase G0 para as fases G1-S e G2 e para a mitose (Malumbres & Barbacid, 2001).

Durante a mitose dos organismos eucariotos, a replicação do DNA é um processo complexo que requer ações coordenadas e altamente reguladas, envolvendo uma extensa maquinaria molecular. A célula pode escolher centenas, senão milhares de origem de replicação, onde posteriormente, diversas proteínas ou complexos serão recrutados para esse local (Kelly & Brown, 2000). Para que a duplicação do DNA tenha início, dois fatores são essenciais: a proteína iniciadora e o replicador. A proteína iniciadora é o fator chave que seleciona o local de início da replicação, responsável por reconhecer uma sequência especifica do DNA. O replicador é o elemento responsável pelo início da replicação, sendo também denominado como sequência de replicação autônoma (ARS - *autonomous replicating sequences*), o qual tem sido muito bem caracterizado na levedura *Saccharomyces cerevisae*, onde apresenta pequenas sequências, de aproximadamente 150 pares de bases, essencial para o início da replicação desse organismo. Entretanto, em outras espécies o número de pares de bases presentes no replicador é variável (Bell et al., 1995; Bell & Dutta, 2002; Chang et al., 2011).

Em eucariotos, uma grande proteína iniciadora do complexo de reconhecimento de origens denominada ORC (*origin recognition complex*), é responsável por determinar o local onde a replicação do DNA se iniciará. Essa proteína possui seis subunidades (Orc_{1-6}) e diversos sítios de ligação para proteínas auxiliares que contribuem para atrair ORC para o local de replicação (Dutta & Bell, 1997; Bell & Dutta, 2002; Chang et al., 2011). Quando ORC interage com o DNA, na fase G1, ocorre o recrutamento de diversos fatores de replicação, incluindo um complexo multiproteico denominado complexo MCM (***M**ini-**C**hromosome **M**aintenance*). Na fase S, ocorre a ativação do complexo MCM, dando início à replicação do DNA (Chang et al., 2011). Dessa forma, em todas as células eucariotas, o recrutamento do complexo MCM é fundamental para o início da replicação e para a correta progressão da fase S (Pasion & Forsburg, 1999; Diffley, 2001; Chang et al., 2011).

O complexo MCM é formado por seis proteínas (MCM-2/BM28, MCM-3/P1, MCM-4/Cdc21, MCM-5/Cdc46, MCM-6/Mis5 e MCM-7/Cdc47) altamente conservadas entre os eucariotos (Tye, 1999; Bell & Dutta, 2002). Essas proteínas formam um heterohexâmero composto por cada um de seus membros, onde cada proteína desempenha uma função distinta, mas para uma atividade comum (Pasion & Forsburg, 1999). Aparentemente, o hexâmero é formado por um subcomplexo constituído por uma forte associação entre MCM4, MCM6 e MCM7 que se associa fracamente à MCM2 e posteriormente e também fracamente, ocorre a associação ao heterodímero formado por MCM3 e MCM5 (Fig.7) (Burkhart et al., 1995; Kubota et al., 1997; Thommes et al., 1997). O heterohexâmero MCM2-7 possui massa molecular de 560 kDa e 27nm de diâmetro (Tye, 1999). As proteínas que compõem o complexo MCM estão localizadas no núcleo ao longo do ciclo celular na maioria dos organismos, com exceção da levedura *Saccharomyces cerevisiae*, onde a maioria das proteínas MCM está presentes no núcleo apenas nas fases G1 e S (Hennessy et al., 1990; Yan et al., 1993; Dalton & Whitbread, 1995; Pasion & Forsburg, 1999).

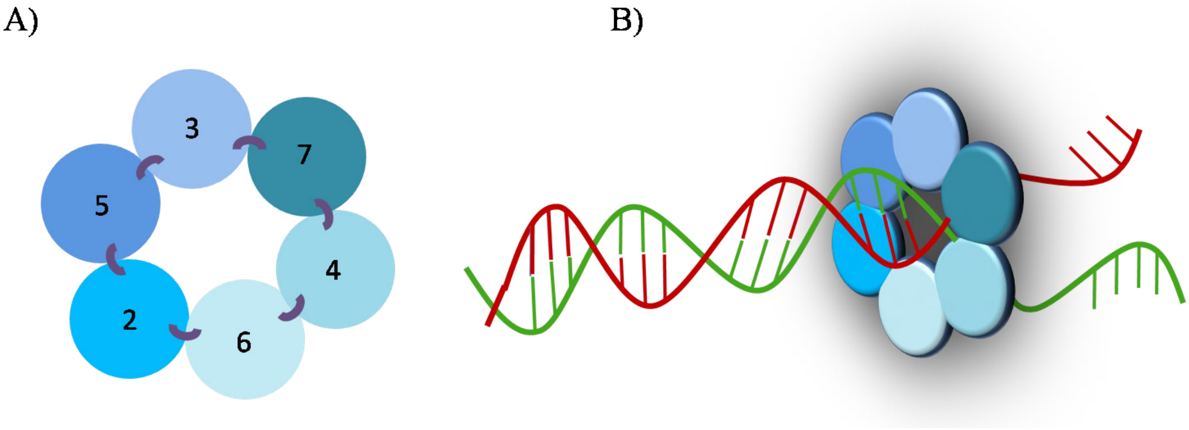

Fig. 7 - Organização estrutural das proteínas formadoras do complexo MCM (A). O complexo MCM atua como helicase abrindo a fita de DNA (B). Adaptado de Lei & Tye, 2001 e Aparício et al, 2006.

Durante a replicação, o complexo MCM atua tanto como helicase ATP-dependente, separando as fitas de DNA, como na progressão da forquilha de replicação. O complexo atua como heterohexâmero simples, mas em alguns organismos pode atuar como heterohexâmeros duplos, onde permanece associado ao DNA até o final da fase S (Adamali & Hermo, 1996; Jagannathan et al., 2012; Brewster & Chen, 2010). O recrutamento do hexâmero MCM é um passo extremamente importante. Prova disso é que a deleção de qualquer componente do complexo é letal para o organismo (Gibson et al., 1990; Dalton & Whitbread, 1995; Kelly & Brown, 2000; Bell & Dutta, 2002).

Para a avaliação do índice de proliferação celular através de imunohistoquímica, diversos marcadores têm sido utilizados, como por exemplo, o PCNA e o Ki-67, entretanto, os resultados obtidos com o uso desses marcadores têm sido bastante controversos (Raymond et al., 1988; Gallee et al., 1989; Visakorpi, 1992; McLoughlin et al., 1993; Vesalainen et al., 1994; Cher et al., 1995; Coetzee et al., 1997; Stoeber et al., 2001). O PCNA é uma ciclina e uma proteína auxiliar da DNA polimerase δ, essencial para a replicação do DNA, mas também participa do processo de reparo celular (Nichols & Sancar, 1992; Shivji et al., 1992; Takasaki et al., 2001). O Ki-67 é uma proteína não-histona expressa durante todas as fases do ciclo celular, com exceção da fase G0, mas sua função ainda não está bem elucidada (Bologna-Molina et al., 2012; Tumuluri et al., 2002; Verheijen et al., 1989).

Recentemente, as proteínas do complexo MCM têm sido extensivamente utilizadas como marcadores de células em proliferação (Freeman et al., 1999; Hiraiwa et al., 1997; Stoeber et al., 2002). As proteínas do complexo são detectadas em células durante a replicação, mas não em células quiescentes, em diferenciação ou senescentes, sugerindo que elas podem ser um marcador confiável da proliferação (Hiraiwa et al., 1997; Williams et al., 1998; Stoeber et al.; Stoeber et al., 2001). A expressão de MCM7 tem sido detectada restritamente em regiões de proliferação da epiderme, intestino e tecido linfóide (Hiraiwa et al., 1997; Todorov et al., 1998). O aumento da expressão desse complexo foi também detectado em tumores e estado proliferativo pré-malígnos (Hiraiwa et al., 1997; Williams et al., 1998). Dessa forma, o uso do MCM7 como marcador de proliferação celular tem sido considerado um método bastante confiável. Além disso, a correlação entre a proliferação celular e a abundância do complexo MCM pode ser utilizada como indicador de neoplasticidade e pode ser considerada uma efetiva ferramenta no diagnóstico de câncer (Hiraiwa et al., 1997; Hiraiwa et al., 1998).

6.2. Apoptose

A apoptose, em associação com outros processos como proliferação e diferenciação celular, é um processo fundamental para a manutenção da homeostase dos organismos. Qualquer desequilíbrio no processo apoptótico pode levar a consequências drásticas envolvendo diversas patologias, como doenças autoimunes, cânceres e neurodegeneração (Degterev et al., 2003). No sistema genital masculino a perda de numerosas células germinativas por apoptose durante a espermatogênese é um evento fisiológico, mas no epitélio das vias genitais e glândulas sexuais anexas, a identificação de células apoptóticas é rara em condições normais (Billig et al., 1996; Fan & Robaire, 1998; Jara et al., 2004; Carballada et al., 2007).

A apoptose é um evento codificado geneticamente, que faz com que as células entrem em um complexo processo de morte, envolvendo diversos fatores reguladores (Degterev et al., 2003). Durante esse processo, as células sofrem alterações morfológicas características, como retração celular, perda de aderência com a matriz extracelular e células vizinhas, condensação da cromatina na periferia do envoltório nuclear, fragmentação internucleossômica do DNA pela endonuclease CAD (gerando fragmentos de DNA de aproximadamente 180 pares de base), translocação da fosfatidilserina para membrana externa e fragmentação da célula em corpos apoptóticos que são posteriormente, fagocitados por macrófagos e/ou outras células circunvizinhas (Hengartner, 2000; Ura et al., 2001; Degterev et al., 2003).

O processo de morte celular pode ocorrer de duas formas: pela disfunção de organelas, onde as mitocôndrias exercem papel crucial para o processo de morte celular programada, ou via caspases (Gross et al., 1999). No segundo caso, e alvo de nosso estudo, a apoptose ocorre através da grande família de proteases cisteínas, denominadas caspases, caracterizadas pela presença de uma cisteína no sítio ativo, capaz de reconhecer e clivar substratos que possuam resíduos de aspartato (Cryns & Yuan, 1998; Degterev et al., 2003).

Até o momento 14 caspases foram descritas em mamíferos e 11 em humanos, sendo seis participantes do processo apoptótico (caspases-2, -3, -6 -7, -8, -9 e -10) e as caspases-1, -4, -5, -11, -12, -13 e -14 são ativadoras de citocinas, estando envolvidas na mediação de respostas imunes, mas eventualmete participam do processo apoptótico (Earnshaw et al., 1999; Denault & Salvesen, 2002; Pistritto et al., 2002).

As caspases envolvidas diretamente na apoptose são divididas em dois grupos: caspases iniciadoras (caspase-2, 8, 9 e 10) e caspases efetoras (caspase-3, -6, e -7) (Denault & Salvesen, 2002; Boatright & Salvesen, 2003). A família das caspases compartilha características comuns como, por exemplo, o fato de todas serem sintetizadas como zimogênios (pró-enzimas) contendo três domínios: um pró-domínio N-terminal, seguido por uma subunidade maior p20 e uma subunidade menor p10 (Fig. 8 A) (Hengartner, 2000; Degterev et al., 2003; Li & Yuan, 2008). Contudo, a principal característica é a especificidade de clivagem que ocorre sempre após um resíduo de ácido aspártico (Hengartner, 2000; Degterev et al., 2003; Boatright & Salvesen, 2003). A ativação da pró-enzima é mediada por uma serie de clivagens que separam a subunidade maior e menor, seguida pela remoção do pró-domínio (Fig. 8 B). As clivagens de ativação ocorrem após o ácido aspártico, o mesmo utilizado para clivar os substratos das caspases, sugerindo uma possível ativação autocatalítica pelas caspases (Thornberry et al., 1997; Hengartner, 2000; Degterev et al., 2003; Li & Yuan, 2008).

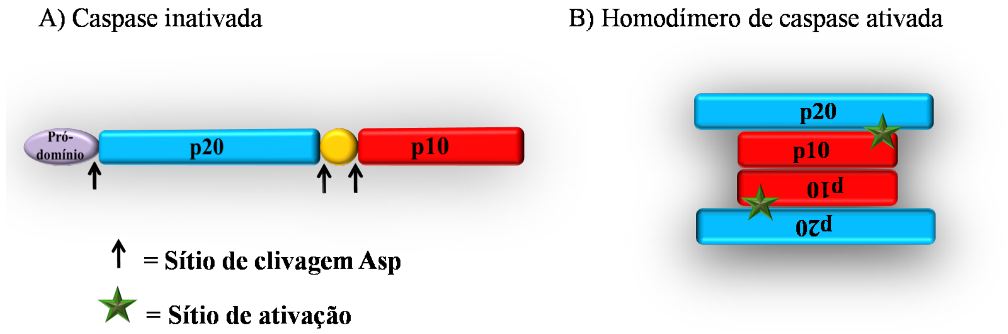

Fig. 8- Organização estrutural das caspases. Adaptado de Gruter, 2000.

Os pró-domínios das caspases possuem características marcantes que permitem classificá-los de acordo com sua função. Assim, caspases iniciadoras possuem pró-domínio maior, contendo a sequência de interação proteína-proteína: o domínio efetor de morte (DED -*Death Effector Domain*), responsável pela interação com as proteínas adaptadoras. As caspases efetoras possuem o domínio de recrutamento e ativação de caspases (CARD - *Caspase Recruitment Domain*), responsável por promover a interação dessas caspases com outras caspases e proteínas adaptadoras (Degterev et al., 2003; Earnshaw et al., 1999).

As caspases iniciadoras são as primeiras a serem ativadas após o primeiro sinal do início da apoptose. Ao serem ativadas formam homodímeros, contendo dois sítios ativos, onde cada monômero é formado por uma subunidade maior e outra menor. Esta topologia é diferente da caspase-9, por exemplo, onde apenas um centro ativo é formado (Earnshaw et al., 1999; Hengartner, 2000; Renatus et al., 2001). As caspases efetoras, por sua vez, são expressas como dímeros inativos, tornando-se ativos após serem clivados pelas caspases iniciadoras (Denault & Salvesen, 2002; Boatright et al., 2003).

Em seu estado ativado, as caspases provocam a morte da célula rapidamente. Por isso as células precisam de um rigoroso controle para evitar a ativação das caspases numa célula saudável. Para isso, algumas medidas são tomadas, como a síntese das caspases como pró-enzimas inativas e vias altamente complexas que controlam a ativação e a disponibilidade de inibidores endógenos que mantém as enzimas ativas sob controle constante (Degterev et al., 2003).

A morte celular via caspases pode ser dividida em duas categorias: a via intrínseca e a via extrínseca. A via extrínseca é ativada em resposta a sinais extracelulares que indicam que a existência da célula já não é necessária ao bem-estar do organismo. Essa via é iniciada pela ativação de um receptor de morte transmembrânico dos quais fazem parte CD95/Fas/Apo1, TNFR1, TNFR2, DR3/Wsl-1/Tramp, DR4/TRAIL-R1, DR5/TRAIL-R2/TRICK2/Killer e

DR6. No caso do Fas, após a interação com seu ligante, o receptor de morte forma microagregados, permitindo o recrutamento do adaptador FADD (proteína associada ao domínio de morte de Fas). Por sua vez, FADD recruta as caspases iniciadoras (caspase-8 ou -10), que então recrutam as caspases efetoras, dando início às alterações que culminarão com a morte da célula (Hengartner, 2000; Degterev et al., 2003; Boatright & Salvesen, 2003).

Na via intrínseca, diferentes mecanismos podem ser usados para ativar a caspase-9. Nesse caso, após um estímulo, por exemplo, lesões do DNA por agentes químicos, radiação, etc, ocorre recrutamento de membros pró-apoptóticos da família de proteínas Bcl-2 (Bax, Bak e Bid). Esses interagem com a mitocôndria, ocasionando a despolarização da membrana mitocondrial, e consequentemente, a liberação do citocromo-c, que ao acoplar à proteína Apaf-1 (Fator-1 de ativação de protease apoptótica) ativará a pró-caspase-9. A pró-caspase-9 em conjunto com a Apaf-1 e o citocromo c, forma o complexo proteico denominado apoptossomo, responsável pela ativação das pró-caspases-3 e -7 (Hengartner, 2000; Degterev et al., 2003; Boatright & Salvesen, 2003).

Para a identificação da apoptose, diversos marcadores podem ser utilizados. Entretanto, por se tratar de um evento que obedece a uma sequência cronológica e hierárquica, o uso de mais de um marcador para um mesmo experimento é comum, sendo os anticorpos anti-caspase-3 ativada e a técnica de TUNEL, os métodos mais utilizados (Mirkes et al., 2001; Jara et al., 2004; Hurst et al., 2006).

A técnica de TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) é utilizada para a detecção *in situ* de células apoptóticas pela marcação do DNA fragmentado em regiões internucleossomicas específicas, gerado pela atividade endógena da DNase. Esse método utiliza a enzima transferase deoxinucleotidil terminal (TdT) que catalisa e transfere um nucleotídeo dUTP para a região livre do grupo 3´hidroxil, presente em fitas fragmentadas de DNA (Huppertz et al., 1999).

A ativação das caspases é considerado o evento mais precoce da apoptose, iniciando-se cerca de 30 minutos após a indução do estímulo de morte. Assim o uso de imunohistoquímica com anticorpos específicos para as caspases efetoras são amplamente utilizadas para a detecção da apoptose (Porter & Janicke, 1999; Huppertz et al., 1999; Men et al., 2003). Dentre as caspases executoras, a caspase-3 tem sido a mais estudada entre os mamíferos em termos de especificidade e papel na apoptose (Porter & Janicke, 1999). Além disso, a caspase-3 pode ser ativada tanto pela via intrínseca como pela via extrínseca nos diversos tipos celulares investigados. Assim, o uso de anticorpos anti- caspase-3 ativada é considerada um importante marcador do processo apoptótico independente da via de ativação

(Porter & Janicke, 1999; Snigdha et al., 2012). Assim, o uso desses dois marcadores apoptóticos, são utilizados de forma complementar, uma vez a caspase-3 marca um período mais inicial do evento apoptótico (cerca de 30 minutos após o ínicio) e o TUNEL o DNA fragmentado (cerca de 12 horas após o início) (Duan et al., 2003; Singh et al., 2009).

6.3 Proliferação e morte celular nas vias genitais

Estudos sobre os índices de proliferação e morte celular nas vias genitais são escassos (Nagy & Edmonds, 1975; Fan & Robaire, 1998). No epidídimo, a maioria dos estudos foi realizado durante os períodos fetal e neonatal (Dyche, 1979; Sun & Flickinger, 1982). Em animais adultos, o epidídimo é considerado um órgão estático com baixos índices de proliferação e morte em todos os tipos celulares presentes no epitélio, com raras ocorrências de mitose nas células principais e basais (Clermont & Flannery, 1970; Nagy & Edmonds, 1975; Majumder & Turkington, 1976; Robaire et al., 2006). Dessa forma, estudar os índices de proliferação e morte celular em uma espécie sazonal pode contribuir com novos dados sobre a dinâmica e plasticidade das células que compõe o epitélio das vias genitais em indivíduos adultos.

7. Quirópteros

A ordem Quiróptera é a segunda maior entre os mamíferos e a mais diversificada. Possuem dezoito famílias, 202 gêneros e 1120 espécies, que totalizam cerca de 22% das espécies de mamíferos (Reis et al., 2007). Os quirópteros dividem-se em duas subordens: Megachiroptera, formada por uma única família, Pteropodidae, com representantes na África, Ásia e Oceania, e Microchiroptera constituída por 17 famílias com distribuição cosmopolita (Wang et al., 2004). No Brasil, são descritas nove famílias, 64 gêneros e 167 espécies de morcegos. A família Phyllostomidae é a mais representativa, com 40 gêneros e 92 espécies (Reis et al., 2007) e inclui a espécie *Desmodus rotundus*, considerada a principal espécie transmissora de raiva no país (Aguiar, 2007).

Em relação à reprodução sabe-se que os machos de espécies hibernantes apresentam inúmeras particularidades que os permite conciliar o período reprodutivo com o período de hibernação. Dentre estas particularidades estão regressão testicular após completar a espermatogênese, retenção prolongada de espermatozoides viáveis no epidídimo durante o período de hibernação, assincronia entre a espermatogênese e o acasalamento, assincronia entre a regressão dos testículos e das glândulas genitais acessórias e ainda assincronia entre a regressão das células de Leydig e o acasalamento, que ocorre quando os níveis de hormônios sexuais circulantes são baixos (Krutzsch, 1975; Gustafson, 1979; Hosken et al., 1998). As particularidades acima descritas estão estabelecidas para algumas espécies de morcegos de regiões temperadas, mais ainda são escassas as informações disponíveis sobre a biologia reprodutiva dos morcegos de regiões Tropicais.

8. O morcego-das-frutas *Artibeus lituratus*

A espécie *Artibeus lituratus*, popularmente conhecida como morcego-das-frutas, pertence à família Phyllostomidae e é muito comum no Brasil (Grelle et al., 1997; Baptista & Mello, 2001). Eles habitam áreas preservadas da Mata Atlântica, mas se adaptaram bem aos centros urbanos (Zortéa & Chiarello, 1994; Passos & Passamani, 2003). Em Belo Horizonte representam mais de 50% dos quirópteros capturados, sendo considerada a espécie mais abundante da região, podendo viver solitários ou em colônias de 5 a 16 indivíduos (De Knegt et al., 2005; Reis et al., 2007).

Fig. 9 – O morcego-das-frutas *Artibeus lituratus*. As principais características da espécie são duas listas faciais mais claras e uma "folha nasal" membranosa na extremidade do focinho.

As principais características da espécie são duas listas faciais mais claras e uma "folha nasal" membranosa na extremidade do focinho (Fig. 9). São animais primariamente frugívoros, mas complementam sua dieta com insetos. Dessa forma, atuam no controle populacional de insetos e como dispersores de sementes, o que os tornam importantes para o controle e manutenção da diversidade das florestas tropicais e para a sucessão secundária (Zortéa & Chiarello, 1994; Passos & Graciolli, 2004).

Estudos sobre os aspectos reprodutivos desta espécie descrevem para as fêmeas, padrão poliéstrico bimodal (Fleming et al., 1972; Fleming, 1973). Para os machos foi descrito padrão de reprodução contínua ao longo do ano (Tamsitt & Valdivieso, 1963). Entretanto, resultados prévios de nosso Laboratório mostraram que os machos apresentam um ciclo reprodutivo anual bem definido, com o período reprodutivo entre agosto e dezembro, regressão sexual entre dezembro e abril e um período de recrudescência entre abril e julho (Oliveira et al, 2009) (Anexo). Além disso, durante a regressão sexual, foi descrito nos testículos de *A. lituratus*, uma considerável variação nos níveis dos receptores de andrógenos e estrógenos ao longo do seu ciclo anual (Oliveira et al., 2009). Durante o período de regressão testicular, os níveis de AR e ERβ estão aumentados, sugerindo que o ciclo reprodutivo anual da espécie seja regulado pelo balanço entre os níveis de andrógenos e estrógenos (Oliveira et al., 2009). Dessa forma, é importante expandir esses conhecimentos para as vias genitais da espécie, considerando que os dúctulos eferentes e o epidídimo são importantes alvos de estrógenos e andrógenos, respectivamente. Assim, uma investigação mais aprofundada do padrão de expressão desses receptores e de outros elementos do sistema responsivo a estrógenos envolvidos na função reprodutiva desta espécie poderá trazer novos dados e contribuir para elucidar o papel dos estrógenos nos machos.

II - JUSTIFICATIVA E OBJETIVOS

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1. Justificativa

Em quirópteros, o primeiro estudo sobre o mapeamento dos receptores de estrógenos foi realizado em nosso laboratório, na espécie *A. lituratus,* mas este estudo se limitou aos testículos (Oliveira et al., 2009). Dessa forma, as possíveis alterações no padrão de expressão desses receptores nas vias genitais, bem como os mecanismos de regulação dos mesmos e do balanço entre andrógenos e estrógenos nesses animais permanecem desconhecidas. Aprofundar esses estudos em um modelo animal sazonal, com variação natural na expressão de ER, como o *A. lituratus* é promissor, pois evitaria os inconvenientes da falta dos componentes do sistema responsivo a estrógenos durante todo período de desenvolvimento, como visto nos animais modificados geneticamente (*knockout)*, e também evitaria os complicadores de modelos com alterações patológicas, castrados ou sujeitos a tratamentos com drogas, que podem interferir em outros sistemas fisiológicos.

Sabe-se que em roedores, os estrógenos modulam a expressão de proteínas como as aquaporinas, que são essenciais para a reabsorção de solutos e água pelos dúctulos eferentes e epidídimos (Oliveira et al., 2005). Em morcegos, o mecanismo de regulação do transporte de fluido torna-se ainda mais crítico, uma vez que a viabilidade dos espermatozoides armazenados no epidídimo é mantida pelo estabelecimento de um ambiente hipermolar que desidrata os espermatozoides, reduzindo sua taxa metabólica, induzindo quiescência (Crichton et al., 1994). Pretendemos contribuir com esses conhecimentos, investigando as proteínas AQP1 e AQP9 nas vias genitais de *A. lituratus*, ao longo do ciclo reprodutivo anual e uma possível participação dos estrógenos na regulação dessas proteínas.

O equilíbrio entre proliferação e morte celular tem um papel essencial no desenvolvimento e manutenção da homeostase tecidual no sistema genital masculino (Billig et al., 1996; Carballada et al., 2007). No testículo de *A. lituratus*, durante o período de regressão, foi observado um aumento marcante no número de células apoptóticas, as quais comumente mostraram-se positivas para ERβ (Oliveira et al., 2009). Por outro lado, dados preliminares apontam para um aumento na expressão de ERα no epitélio epididimário, durante o período de regressão. Considerando que os ERα apresentam ação proliferativa, enquanto os ERβ atuem de forma anti-proliferativa e pró-apoptótica em diversos órgãos, hipotetizamos que os estrógenos, via ERα e ERβ atuem na regulação desses processos nas vias genitais durante o ciclo reprodutivo anual da espécie.

2. OBJETIVOS

2.1. Objetivo geral

O objetivo geral desse estudo é investigar as possíveis alterações no sistema responsivo a estrógenos, incluindo a fonte, os níveis teciduais, os alvos e possíveis funções desse hormônio na regulação da expressão das aquaporinas AQP1 e AQP9, bem como no balanço entre proliferação e morte celular nas vias genitais de *Artibeus lituratus* durante seu ciclo reprodutivo anual.

2.2. Objetivos específicos

Sempre comparando espécimes de *A. lituratus* em atividade e regressão reprodutiva, pretende-se:

- Descrever a morfologia macroscópica e microscópica dos dúctulos eferentes e epidídimos;
- Investigar a ocorrência e distribuição celular da enzima aromatase nos testículos e vias genitais, visando determinar a fonte de estrógenos;
- Determinar os níveis teciduais de testosterona e estradiol durante o ciclo reprodutivo anual;
- Mapear a distribuição de receptores de estrógenos ERα e ERβ e receptores de andrógenos (AR) nos dúctulos eferentes e epidídimos, e investigar possíveis alterações ocorridas na expressão dos mesmos;
- Determinar a ocorrência e distribuição celular e subcelular de AQP1 e AQP9 nos testículos, dúctulos eferentes e epidídimos, correlacionando sua expressão com os níveis teciduais de testosterona e estradiol e/ou seus receptores;
- Avaliar o índice de apoptose e proliferação celular nos dúctulos eferentes e epidídimos, durante os dois períodos reprodutivos, correlacionando com os níveis teciduais de testosterona e estradiol e/ou seus receptores.

III - RESULTADOS

CAPÍTULO DE LIVRO

R.L. Oliveira, C.A. Oliveira, Reproductive biology of male bats: anatomy, physiology and endocrinology. In: J.L. Zupan, S.L. Mlakar, (Eds.), **Bats: biology, bahavior and conservation**, Nova Science Publishers, New York, 2011, pp. 135-175.

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Chapter 3

Reproductive Biology of Male Bats: Anatomy, Physiology and Endocrinology

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Abstract

The chiropterans are the only mammals capable of true flight. They represent one of the largest and most diversified orders of mammals, inhabiting every continent, except Antarctica. This diversity is reflected in the anatomy and physiology of the reproductive organs that have been adapted for flight and geographic distribution, as seen in hibernating species inhabiting temperate zones and non-hibernating species generally inhabiting the tropics. Here we summarize the current knowledge of male bat reproductive strategies and genital organs morphophysiology. Similar to other mammals, the male genital system of Chiroptera is composed of the testis, efferent ductules, epididymis, vas deferens, urethra, penis and accessory sex glands, however a variety of peculiar features are observed in the morphophysiology of these organs. The bat testis presents a large variation in position, according to the presence or absence of scrotum, being scrotal, subcutaneous or abdominal. The testicular position may be permanent or transitory depending on the species considered. According to the acrossomic system method, ten to eleven stages of the cycle of seminiferous epithelium are recognized in bat seminiferous tubules. During regression, seminiferous tubules shelter only Sertoli cells and spermatogonia. Curiously, the Sertoli cells are located above the spermatogonia, forming a protective barrier for these cells that are responsible for re-colonization of the seminiferous tubules. The efferent ductules connect the rete testis to the epididymis. A description of *Artibeus lituratus* revealed 12 to 15 flexuous ductules arising separately from the *rete testis* and running parallel towards the epididymis, then anastomozing and forming multiple entries into the epididymal duct. This pattern of disposition is similar to that found for large eutherian mammals and men. The epididymis of bats may be anatomically divided into initial segment, caput, corpus and cauda. As shown in *A. lituratus*, the epididymal epithelium is composed of principal, basal, apical, narrow, clear and halo cells, with different distribution along the duct. In several hibernating species, the epididymis is responsible for storing viable sperm during long periods. Information about the male accessory sex glands of bats is scarce. The prostate and bulbourethral glands have been described in all species studied, whereas the ampullary gland and seminal vesicles are present in some species and absent in others. The bat penis is prominent and pendulous, presenting a well-developed glans. In some species, the penis presents a baculum (*os penis*), extending throughout the glans. In seasonal animals, environmental factors as photoperiod play an important role in gonadal activity. In these animals, the exposure to short days leads to decreased hypothalamic GnRH and pituitary gonadotropins (FSH and LH) releases, leading to a decrease in gonadal activity. Testosterone levels vary along the reproductive cycle of hibernating and non-hibernating bats. Besides androgens, it is now recognized that estrogens also play an important role in male reproduction. However, information about estrogen in male bats has been limited to one report about *A. lituratus*, a seasonal non-hibernating Neotropical species, in which both estrogen receptors ERα and ERβ were detected in the testis. During testicular regression, levels of estrogen receptors, especially ERβ, are increased, indicating that estrogens may be directly involved in the regulation of the bat male reproductive cycle.

I. INTRODUCTION

The chiropterans or bats are the only mammals capable of true flight. They represent the second largest and most diversified order of mammals in terms of geographical distribution as well as number of species, losing only for rodents. Bats are found in all continents, except Antarctica, and in some oceanic islands, they constitute the only native mammal species (Brunet-Rossinni and Austad, 2004). The order Chiroptera is comprised of two suborders, the Megachiroptera and Microchiroptera, which are distributed in 18 families, 202 genera and 1120 species, comprising approximately 22% of the total species of mammals (Singh and Krishna, 1996; Reis et al., 2007). The diversity of Chiroptera are reflected in the anatomy and physiology of the reproductive organs that have been adapted for flight and diverse geographical distribution, as seen in hibernating species inhabiting temperate zones and non-hibernating species mostly inhabiting the Tropics.

Despite the numerous reproductive peculiarities, very little is known about the anatomy and physiology of the genital system of male bats. The available information is often incomplete and fragmented, especially when dealing with species that inhabit tropical regions. Here, we summarize the current knowledge of male bat genital tract morphophysiology and reproductive strategies, in addition to some of our own findings on the tropical non-hibernating, big fruit-eating bat *Artibeus lituratus* (Olfers, 1818).

II. REPRODUCTIVE SYSTEM

The reproductive system of bats consists of paired testes, efferent ductules, epididymides, vas deferens and bulbourethral or Cowper's glands, unpaired urethra and penis, as well as variable accessory glands surrounding the proximal urethra, such as prostate, ampullary gland and seminal vesicle.

1. TESTES

The testes are pair organs, symmetrical and responsible for sperm production and sex steroid synthesis, both essential for reproductive functions. The bat testes are variable in shape depending on species and reproductive phase. In some species, the gonad is ovoid or elliptical (Matthews, 1941 - *Hipposideros caffer*; Krutzsch et al., 1976 - *Macrotus waterhousii*; Krutzsch and Crichton, 1986 - *Pipistrellus subflavus*;

Oliveira et al., 2009 - *Artibeus lituratus*), whereas in others, the testis is globular (Matthews, 1941 - *Nycteris luteola* and *N. hispida*; Vamburkar, 1958 - *Cynopterus sphinx*), becoming elliptical during spermatogenic activity (Vamburkar, 1958). Testis size also undergoes remarkable seasonal variation, as the greatest size is achieved at maximal spermatogenic activity. Therefore, even though the timing of spermatogenesis varies between species, the testis weight has been a reliable indicator of the organ activity (Hosken et al., 1998).

It is noteworthy that the bat testis presents large variations in position among species, according to the absence or presence of scrotum, being permanently abdominal, partially abdominal, subcutaneous or scrotal. Moreover, in some species, the testes may undergo migration into the abdominal cavity at certain periods of the year.

The testes lie permanently in the abdominal cavity without migration through the inguinal canal in members of Rhinopomatidae (e.g. *Rhinopoma kinneari)* and Pteropodidae (e.g. *Cynopterus sphinx gangeticus)* families (Vamburkar, 1958; Anand Kumar, 1965; Krutzsch, 1979; Singwi and Lall, 1983). In these species, the scrotum is absent and the spermatogenesis occurs only when the bats enter into winter torpor, possibly because at this period, the metabolism, including body temperature, is reduced (Anand Kumar, 1965).

Migration of the testes between the scrotum and abdominal cavity via the inguinal canal occurs in several species of the Emballonuridae (e.g. *Taphozous sp* and *Thapozous georgianus*) (Matthews, 1941; Jolly and Blackshaw, 1988) and Pteropodidae families, in which the testis are scrotal during the breeding season (usually in summer) and intra-abdominal at other seasons (Krutzsch, 1979). In *Taphozous georgianus*, the testicular descent is closely influenced by high temperature but not daylength (Jolly and Blackshaw, 1988). The fact that in this species there is no pampiniform plexus and the testicular arteries are not coiled is interesting (Jolly and Blackshaw, 1988). Differing from testis, in *T. georgianus,* the cauda epididymis is permanently in the scrotum, thus providing cooler temperatures adequate for sperm storage. In some members of the Molossidae family, as *Mormopterus planiceps* and *Tadarida (=Chaerephon) hindei*, the testes are inguinal in position and undergo migration into the abdominal cavity when spermatogenesis is ceased (Krutzsch, 1979; Krutzsch and Crichton, 1987).

The testes are subcutaneous and do not undergo migration into the abdominal cavity in some Hipposideridae (Matthews, 1941 - *Hipposideros caffer, Triaenops afer*), Vespertilionidae (Krutzsch, 1975 - *Pipistrellus hesperus*), and Phyllostomidae (Krutzsch et al., 1976 - *Macrotus waterhousii*; Beguelini et al., 2009 - *Artibeus lituratus, Artibeus planirostris, Carollia perspicillata, Platyrrhinus lineatus* and *Sturnira lilium*; Oliveira et al., 2009 - *Artibeus lituratus*). These species present the testes located in a subcutaneous pouch at a parapenial position, without forming a true scrotum (Krutzsch, 1975; Krutzsch et al., 1976;

Beguelini et al., 2009; Oliveira et al., 2009). The pouch is non-pigmented and richly vascular at the breeding season (Krutzsch et al., 1976).

Testes located in a true scrotum, without migration to the abdominal cavity, are observed in several species of the families Nycteridae, Megadermidae, Emballonuridae and Vespertilionidae (Matthews, 1941 - *Nycteris luteola, N. hispida, Cardioderma cor*; *Miniopterus minor, M. dasythrix*; Krutzsch and Crichton, 1986 - *Pipistrellus subflavus*; Beguelini et al., 2009 - *Myotis nigricans*). In *Myotis nigricans*, the testis is maintained into the scrotum by a suspensory ligament that attaches the cauda epididymis to the tail skeleton (Beguelini et al., 2009). Pigmentation of the scrotum is frequent in Vespertilionidae bats that store sperm (Krutzsch and Crichton, 1986; Krutzsch and Crichton, 1987).

1.1. Testicular Structure

The structural and functional characteristics of bat testes are similar to other mammals. They are surrounded by a thick albuginea, which emits septa of connective tissue in the mediastinum direction, dividing the parenchyma in lobules (Oliveira et al., 2009). The testicular parenchyma is formed by the seminiferous tubules interspersed by the interstitium, thus characterizing the tubular and intertubular compartments, respectively.

1.1.1. Intertubular Compartment

The intertubular compartment or interstitium is distributed between the seminiferous tubules and contains Leydig cells, fibroblasts, macrophages, mast cells, as well as blood and lymphatic vessels (Gustafson, 1987; Oliveira et al., 2009). The lymphatic vessels present in the interstitium are more prominent during the spermatogenic period (Gustafson, 1979; Gustafson, 1987). In hibernating bats, macrophages are frequently found in the interstitium at hibernation and periarousal periods, usually in close association with Leydig cells. Macrophages appear involved in endocytosis of residues of involuting Leydig cells. As a result of the endocytotic activity, these macrophages accumulate numerous cytoplasmic lipofuscin granules (Gustafson, 1987).

Leydig cells responsible for androgen production are usually organized in small clusters, close to blood vessels or to lymphatic spaces, lined by a thin endothelium, as seen in *Artibeus lituratus* (Oliveira et al., 2009). The Leydig cells are usually rounded or polyhedral (Richardson, 1977). The abundance of Leydig cells in bat testis is variable, as in some species, they are abundant whereas in others, they are scarce (Racey, 1974; Richardson, 1977; Oliveira et al., 2009). The number of cells also varies depending on the reproductive period considered, as Leydig cells appear more abundant in involuted testis, possibly due to the decreasing in seminiferous tubules size, thus providing more interstitial space (Krutzsch, 1975; Krutzsch et al., 1976; Richardson, 1977; Krutzsch and Crichton, 1987; Oliveira, nonpublished data).

The cytoplasm of active Leydig cells is characterized by the presence of numerous lipid droplets and well-developed smooth endoplasmic reticulum which can form concentric layers around the lipid droplets (Gustafson, 1979; Bernard, 1986; Jolly and Blackshaw, 1989; Bernard et al., 1991; Aoki, 1997). Mitochondria are numerous and possess primarily tubular cristae, even though mitochondria with lamellar cristae may be rarely found. Lysosomes, sparse free ribosomes and profiles of rough endoplasmic reticulum and Golgi apparatus are also observed (Gustafson, 1979; Jolly and Blackshaw, 1989; Krutzsch and Crichton, 1990). The nucleus of Leydig cells is usually euchromatic, central, spherical or round with one or two prominent nucleoli (Richardson, 1977; Jolly and Blackshaw, 1989; Krutzsch and Crichton, 1990; Beguelini et al., 2009). The cell surface is characterized by the presence of intricate interdigitations (Jolly and Blackshaw, 1989; Aoki, 1997).

In the seasonal species, during the testicular regression, Leydig cells undergo atrophy characterized by the smaller size, decreased nuclear diameter, heterochromatic nuclei and scarce cytoplasm, as observed in *Pipistrellus hesperus* (Krutzsch, 1975), *Pipistrellus subflavus* (Krutzsch and Crichton, 1986), *Rhinolophus capensis* (Bernard, 1986), *Mormopterus planiceps* (Krutzsch and Crichton, 1987), *Myotis lucifugus* (Gustafson, 1979; Gustafson, 1987), *Miniopterus schreibersii* (Krutzsch and Crichton, 1990; Bernard et al., 1991), *Tadarida brasiliensis* (Aoki, 1997), *Nyctophyllus geoffroyi* (Hosken et al., 1998) and *Artibeus lituratus* (Oliveira et al., 2009). In *Artibeus lituratus*, Leydig cell apoptosis can be observed during testicular regression (Figure 1) (Oliveira et al., 2009). The regressed cells possess few lipid droplets, little or no smooth endoplasmic reticulum, mitochondria with predominantly lamellar cristae, many free rybosomes or polysomes and some lipofuscin granules and dense bodies (probably lysosomes) (Gustafson, 1979; Bernard, 1986; Krutzsch and Crichton, 1987; Krutzsch and Crichton , 1990; Bernard et al., 1991; Aoki, 1997). These cells can undergo seasonal changes in volume according to the period of the reproductive cycle (Gustafson, 1987; Krutzsch and Crichton, 1990; Hosken et al., 1998), even though in some species such as *Nyctalus noctula, Pipistrellus pipistrellus* and *Taphozous georgianus*, no apparent change in the volume of Leydig cells is observed during the seasonal reproductive cycle (Racey, 1974; Racey and Tam, 1974; Jolly and Blackshaw, 1987).

The existence of Leydig cell functional phases in hibernating bat species is still a matter of debate, as ultrastructural features, histochemistry detection of spermatogenic enzymes and endocrine evaluations has been controversial (Gustafson, 1979; Bernard, 1986; Hosken et al., 1998). Nevertheless, in every species studied to date, these cells are active during spermatogenesis, when high levels of androgens are required. During mating and hibernation, the Leydig cells remain active in some species, whereas in others, they can be less active or regressed at all (Gustafson, 1979; Bernard, 1986; Gustafson, 1987; Hosken et al., 1998).

1.1.2. Tubular Compartment

The tubular compartment of the testis is comprised of the seminiferous tubules, formed by the seminiferous epithelium, composed by the Sertoli cells and spermatogenic cells, which are surrounded by a thin tunica propria, containing the peritubular myoid cells.

Sertoli Cells

Sertoli cells are present in the seminiferous epithelium of bats, resting on the basement membrane and extending to the lumen (Beguelini et al., 2009). These cells involve the developing germ cells, being responsible for essential functions such as nutrition and physical support of spermatogenic cells, as well as phagocytosis of residual cytoplasm released during spermiogenesis. As in other mammals, adjacent Sertoli cells of bats are joined by tight junctions and desmosomes, which in conjunction with the tunica propria, form the blood-testis barrier (Bernard and Hodgson, 1989; Crichton et al., 1993; Beguelini et al., 2009). This barrier creates a specialized and protective environment for the spermatogenic cell development. The tight junctions are formed by many parallel or anastomosing strands of ridges and complimentary grooves that cover large areas of the basal Sertoli cell membranes (Crichton et al., 1993). These specialized tight junctions between Sertoli cells demarcate two intraepithelial compartments, denominated basal and adluminal compartments (Bernard and Hodgson, 1989; Russell et al., 1990).

Despite the recognized importance of Sertoli cells, few studies have been found for bat species. The most complete study of Sertoli cell structures were performed in *Rhinolophus capensis* and *Miniopterus schreibersii* (Bernard and Hodgson, 1989), in which species the description below is based. The Sertoli cells are columnar in shape and present an irregular surface, where the spermatogenic cells are located. The nucleus is oval or elongated, highly euchromatic, with well-defined nucleolus (Bernard and Hodgson, 1989; Beguelini et al., 2009). The cytoplasm is rich in mitochondria, however with morphological differences depending on the apicobasal position, as mitochondria are usually round to oval in the basal cytoplasm and elongated or tubular when located at the apical cytoplasm. Morphology of the smooth endoplasmic reticulum is also variable depending on the germ cell to which they are associated. In this sense, the endoplasmic reticulum is sparse and forms platelike cisternae, when associated with spermatogonia, spermatocytes and early spermatids at the basal and middle portion of Sertoli cells, respectively. Conversely, the apical portion of the Sertoli cells, which contacts the late spermatids, presents tubular, smooth endoplasmic reticulum. Besides the nucleus, the basal cytoplasm contains lysosomes, lipofuscin granules, multivesicular bodies, Golgi apparatus, glycogen granules, as well as microtubules. Lipid droplets may be found in the cytoplasm of active Sertoli cells, however they are more conspicuous in the regressed cells.

During testicular regression, the diameter of the inactive seminiferous tubules is considerably reduced (Kurohmaru et al., 2002; Oliveira et al., 2009). The regressive Sertoli cells are characterized by the presence of pyknotic, ovoid to oblong nuclei (Krutzsch et al., 1976), and large lipid droplets distributed throughout the cytoplasm (Bernard and Hodgson, 1989). Curiously, the Sertoli cells cover the spermatogonial cells, thus protecting these testicular cells responsible for tubular re-colonization (Figure 1) (Racey, 1974; Racey and Tam, 1974; Gustafson, 1979; Gustafson, 1987; Krutzsch and Crichton, 1987; Bernard and Hodgson, 1989; Hosken et al., 1998; Kurohmaru et al., 2002; Oliveira et al., 2009).

Spermatogenic Cells

The seminiferous epithelium is composed by Sertoli cells in association with the spermatogenic cells, namely spermatogonia, spermatocytes and spermatids. The spermatogonia are cells restricted to the basal compartment (Bernard and Hodgson, 1989), which undergoes several mitoses and originates a large population of spermatocytes. The spermatocytes undergo meiotic division originating spermatids which differentiate into sperm. The spermatocytes and spermatids are located in the adluminal compartment closest to the lumen, where the sperm are delivered.

Bat species show three classes of spermatogonia, namely A, intermediary and B types, identified based on the shape, size and nuclear morphology (Singwi and Lall, 1983 - *Rhinopoma kinneari*; Saidapur and Patil, 1992 - *Rousettus leschenaultia*; Morigaki et al., 2001 - *Pteropus vampyrus* and *Rhinolophus cornutus*; Oliveira et al., 2009 - *Artibeus lituratus*). The A spermatogonia have large ovoid nuclei containing homogenously distributed chromatin and one or two nucleoli. According to the chromatin condensation, these cells may be subdivided in A1, A2 and A3 (Saidapur and Patil, 1992) or dark A spermatogonia and pale

A spermatogonia (Beguelini et al., 2009 - *Artibeus lituratus, Artibeus planirostris, Carollia perspicillata, Platyrrhinus lineatus* and *Sturnira lilium*). The chromatin is homogenous and diffuses in A1 spermatogonia, but gradually become more compact, being darkest at A3 stage. Intermediate spermatogonia are smaller than A type and present round and darker nuclei with heterochromatin disposed below the nuclear envelope (Saidapur and Patil, 1992; Oliveira et al., 2009). The B spermatogonia are round in shape and present dark nuclei with one or two nucleoli (Saidapur and Patil, 1992; Beguelini et al., 2009; Oliveira et al., 2009).

The primary spermatocytes in leptotene and zygotene may be found in the base of the seminiferous epithelium close to the basement membrane or at the first layer of the adluminal compartment. The pachytene presents a more condensed chromatin and occupies the second and third layers of cells in the seminiferous epithelium (Singwi and Lall, 1983; Beguelini et al., 2009; Oliveira et al., 2009). They represent the largest of all spermatogenic cells (Saidapur and Patil, 1992). The diplotene nuclei have condensed chromatin and are placed in superior layers, where they originate the round spermatids (Bernard and Hodgson, 1989; Beguelini et al., 2009). The primary and secondary spermatocytes are in close association with the Sertoli cells (Bernard and Hodgson, 1989).

Round spermatids are located adjacent to the seminiferous tubules lumen. Their nuclei are round, with evident nucleoli and homogenous chromatin. As the spermatids differentiate, the nuclei become elongated and the chromatin more compact, characterizing the elongated spermatids (Saidapur and Patil, 1992; Beguelini et al., 2009). Developing spermatids are associated with Sertoli cells by junctional complexes restricted to the region in contact with the developing acrosome. At this portion of the Sertoli cell cytoplasm, there are several cisterns of smooth endoplasmic reticulum placed in parallel to the cellular membrane of spermatids. Patches of microfilaments connect the Sertoli cell plasma membrane and the first cisternae of the smooth endoplasmic reticulum (Bernard and Hodgson, 1989). Late spermatids and Sertoli cells present justaposed membranes. At this point, the Sertoli cell cytoplasm presents bundles of microfilaments disposed at right angles to the head of the spermatids as seen in *Rhinolophus capensis* (Bernard and Hodgson, 1989). In other species, such as *Miniopterus schreibersii*, the microfilaments form a layer around the head of late spermatids, instead of perpendicular bundles. Subjacent to the microfilaments, there are one or two cisternae of smooth endoplasmic reticulum, whereas the remaining cytoplasm around the head of spermatid is filled with tubular smooth endoplasmic reticulum (Bernard and Hodgson, 1989).

At spermiation, the association between Sertoli cells and spermatids are changed, as the Sertoli cell separates from the spermatid heads forming a cavity, where the head of spermatids are located. Mature sperm are then released into the lumen of the seminiferous tubules and the residual cytoplasm of spermatids is phagocytosed by Sertoli cells (Bernard and Hodgson, 1989). Sperm are dimorphic with conical or blunt heads in *Rhinopoma kinneari* (Singwi and Lall, 1983), whereas the heads of sperm are scoop-like in the *Pteropus vampyrus* and spatula-like in the *Rhinolophus cornutus* (Morigaki et al., 2001). In *Myotis lucifugus*, the sperm head is oval in the frontal section and triangular in the sagittal section. The acrosome covers the anterior two-thirds of the nucleus. A pronounced constriction is located at the connecting piece segment. Posterior to this constriction, the tail thickens abruptly with the appearance of the mitochondrial sheath of the midpiece region. At the junction between the midpiece and principal piece, the annulus appears as a triangular wedge of electrondense material (Hoffman et al., 1987).

The arrangement of spermatogenic cells and Sertoli cells within the tubule vary depending on the spermatogenic activity (Bernard and Hodgson, 1989). The seminiferous tubules of seasonal bats have two distinct phases characterizing the testicular activity and testicular regression. During the testicular activity, the tubular pattern follows that of other mammals, with more immature spermatogonial cells positioned in the basal epithelium. The spermatocyte cells are found in the middle position and the more mature spermatids are located near the lumen. During the regression period, the seminiferous epithelium contains only inactive Sertoli cells and basally-located spermatogonia (Racey, 1974; Racey and Tam, 1974; Gustafson, 1979; Gustafson, 1987; Krutzsch and Crichton, 1987; Bernard and Hodgson, 1989; Hosken et al., 1998; Kurohmaru et al., 2002; Oliveira et al., 2009). The spermatogonia are characterized by voluminous nuclei and prominent nucleolus found in close contact to the basement membrane (Racey, 1974; Oliveira et al., 2009). Degenerating spermatocytes and spermatids are rarely observed in the regressive epithelium. There are no sperm in the regressed seminiferous tubules.

1.2. Spermatogenesis

Spermatogenesis is a cyclic process that occurs in seminiferous tubules, in which spermatogonia undergo sequential transformations culminating in the formation of sperm (Russell et al., 1990; França and Russell, 1998; Hess and Franca, 2007). This phenomenon is species-specific, as each species presents distinct durations of spermatogenesis, type, size, shape and population of germ cells, number of cells and associated mechanism for renewal of stem cells. Within the seminiferous epithelium, the differentiating cells do not have random distribution, but specific cell types are associated, forming the stages. The sequences of events that occur in a particular area of the seminiferous epithelium between two successive appearances of the same pattern of cell association or stage define the cycle of the seminiferous epithelium (Leblond and Clermont, 1952). The different stages of the seminiferous tubules have been determined by two methodologies: the acrosomic system and tubular morphology method. The method of the acrosomic system is based in the identification of the stages according to the acrosomic development. This method determines a variable number of stages for each species. The methodology of tubular morphology utilizes the meiosis occurrence, alterations in spermatid nuclei and the position of spermatogenic cells in the seminiferous epithelium. This method determines eight stages for all mammalian species (Berndtson, 1977).

According to the acrossomic system method, ten to eleven stages of the cycle of seminiferous epithelium are recognized in bat seminiferous tubules. Eleven stages of the seminiferous epithelial cycle are identified in *Rhinopoma kinneari* (Singwi and Lall, 1983) and *Pteropus vampyrus* (Morigaki et al., 2001), whereas *Rhinolophus cornutus* (Morigaki et al., 2001) and *Rousettus leschenaultia* (Saidapur and Patil, 1992) have a cycle of ten stages. Concerning the spermiogenic process, the developing spermatids present 13 (*Pteropus vampyrus* and *Rhinolophus cornutus*), 14 (*Rousettus leschenaulti*) or 16 (*Rhinopoma kinneari*) distinct steps of acrosomal system formation, including Golgi, cap, acrosome and maturation phases, culminating in the formation of sperm (Singwi and Lall, 1983; Saidapur and Patil, 1992; Morigaki et al., 2001).

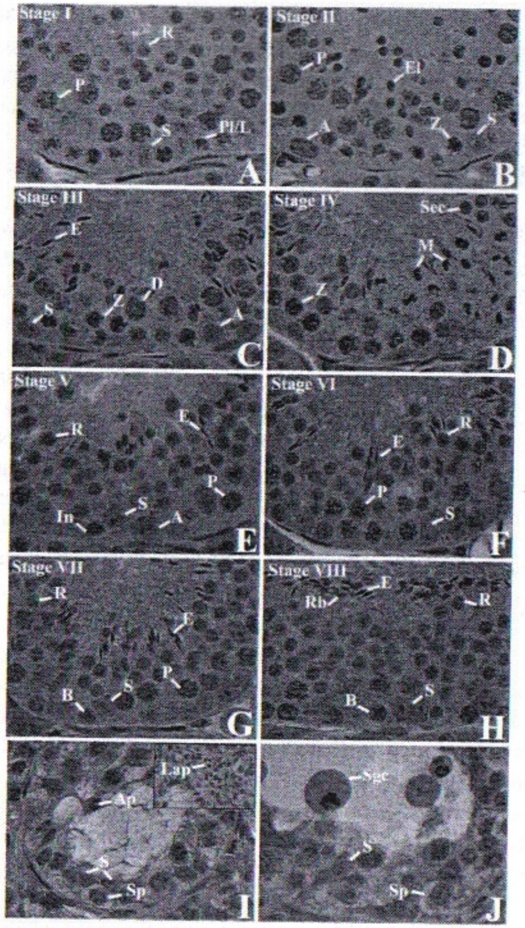

Figure 1. Morphology of the testis of *Artibeus lituratus* during reproductive and regressive periods. (A-H) Seminiferous epithelium at different stages of the spermatogenic cycle classified according to the tubular morphology method. Stage I - characterized by the presence of several layers of round spermatids close to the lume. Stage II - The spermatid nuclei begin to elongate. Stage III - The elongated spermatids are arranged in bundles deeply embedded in the seminiferous epithelium. Stage IV - The occurrence of metaphase and anaphase of meiosis in primary and secondary spermatocytes can be visualized in this stage. Stage V - At this stage, there are both round and elongated spermatids. Stage VI - bundles of elongated spermatids are found in the middle of the epithelium. Stage VII - The elongated spermatids are closer to the luminal surface. The B spermagonia are present in the base of the epithelium. Stage VIII - The elongated spermatids are located at the luminal surface ready for spermiation. The residual bodies are large and numerous. (I-J) During the regressive period, the seminiferous tubules are atrophic, formed mostly by Sertoli cells whose nuclei are located above the nuclei of spermatogonia cells. Insert in I = Leydig cells in apoptosis. Sloughed germ cells are common into the lumen (Sgc) (J). A = spermatogonia A; Ap = apoptotic cells; B = spermatogonia B; D = diplotene; E = elongated spermatid; El = elongating spermatid; In = intermediate spermatogonia; L = leptotene; Lap = Leydig cells in apoptosis; M = metaphase; P = paquitene; Pl = prelelptotene; R = round spermatid; Rb = residual bodies; S = Sertoli cells; Sec = secondary spermatocyte; Sp = spermatogonia; Z = zigotene. Bar in (A) = 30 μm.

Based on the tubular morphology method, eight stages of the seminiferous tubule cycle were identified in *Artibeus lituratus* (Figure 1) (Oliveira et al., 2009) and several other species of Neotropical Phyllostomidae bats (Beguelini et al., 2009). As seen in most mammals, each seminiferous tubule section of the Phyllostomidae species analyzed presents a single stage of the cycle (Beguelini et al., 2009; Oliveira et al., 2009). In contrast, there is overlapping of stages of seminiferous tubule of the Tropical Vespertilionid *Myotis nigricans* (Beguelini et al., 2009).

During the annual reproductive cycle, the testis undergoes remarkable changes in the equilibrium between cell proliferation and cell death or apoptosis, at the recrudescence and involution phases, respectively. Cell proliferation determines testicular growth, whereas apoptosis is an important event leading to testicular regression. It is known that oxidative stress is a key factor stimulating apoptosis in the testis and the occurrence of antioxidant scavenging systems is crucial for preventing apoptosis. Therefore, the balance between reactive oxygen species (ROS) generation and ROS scavenging is essential to determine the rate of cell death and growth. The testicular mechanism of antioxidant defense includes the activity of several enzymes, such as superoxide dismutase (SOD), catalase and glutathione peroxidase (GPX). There is evidence that in the Vespertilionidae *Corynorhinus mexicanus*, specific activity of these scavenger enzymes changes during the annual reproductive cycle in a manner closely related to the testis activity, as they peak during or slightly before the regressive period and decrease at the period of testis growth (Arenas-Rios et al., 2007).

2. EXCURRENT DUCTS

The bat excurrent ducts is comprised of the *rete testis*, efferent ductules, epididymal ducts, vas deferens and urethra.

2.1. Rete Testis

The seminiferous tubules converge to the mediastinum of the testis, where they gradually lose the germ cells, persisting only Sertoli cells, which modifies in a simple cubic epithelium lining the straight tubules. In the transition zone from seminiferous tubules to straight tubules, myoid cells forms one or two continuous layers below the epithelium (Oliveira et al., 2009). Following the straight tubules, the intratesticular *rete testis* is lined by a simple cubic epithelium, sustained by the connective tissue of the mediastinum.

2.2. Efferent Ductules

The efferent ductules consist of several tiny tubules that connect the *rete testis* to the epididymal duct (Ilio and Hess, 1994; Hess, 2002). Studies in several mammal species indicate that the efferent ductules are responsible for the reabsorption of more than 90% of the testicular fluid released from the testes, thus increasing the concentration of sperm in the epididymis and facilitating their maturation and storage (Clulow et al., 1994; Ilio and Hess, 1994). Due to the small size and difficulty in distinguishing this segment, the efferent ductules have been largely neglected in most studies concerning the male genital tract (Ilio and Hess, 1994). Therefore, information about efferent ductules was found only for the Italian species Vesperugo (=Pipistrellus) savii and *Vesperugo piccolo* (Azzali, 1983) and the Brazilian *Artibeus lituratus* (Oliveira, non-published data).

In *Artibeus lituratus,* the efferent ductules are composed by 12 to 15 flexuous ductules, which arise separately from the *rete testis* and run parallel towards the epididymis, then anastomosing and forming about six terminal ductules that enter the epididymal duct separately (Figure 2). This pattern of disposition is similar to that found for man and large eutherian mammals (Ilio and Hess, 1994). Also similar to humans (Yeung et al., 1991), the efferent ductules of *Artibeus lituratus* are composed of most of the head of the epididymis.

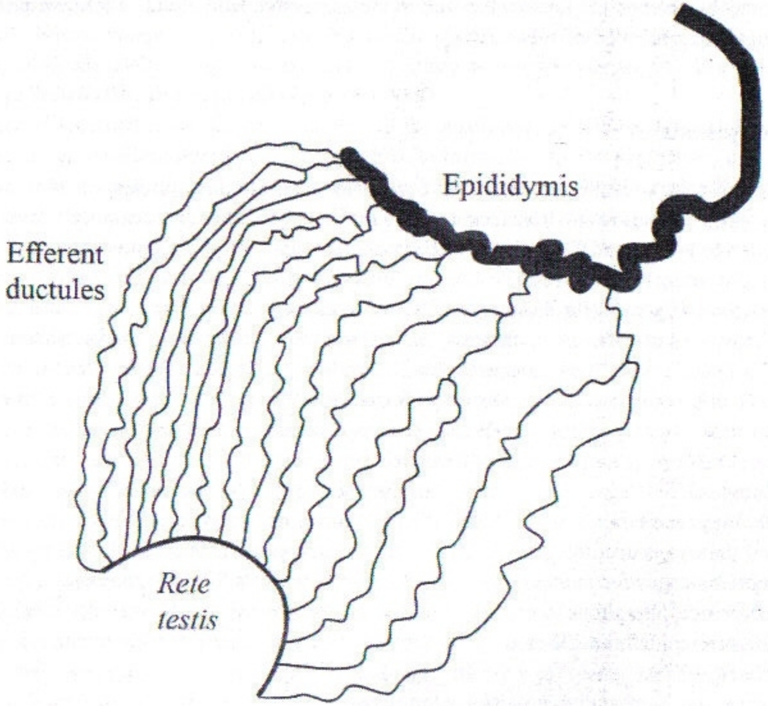

Figure 2. Squematic drawing showing the anatomical disposition of the efferent ductules of *Artibeus lituratus*.

The efferent ductules are lined by a columnar epithelium, composed of ciliated and non-ciliated cells randomly interspersed (Figure 3) (Azzali, 1983); Oliveira, non-published data). The non-ciliated cells are characterized by the presence of abundant microvillus in the apical surface, an oval nucleus placed in the basal cytoplasm and numerous PAS-positive granules. The PAS-positive granules are primarily located in the apical cytoplasm, even though they may also be found in the perinuclear region. The ciliated cells are apparently less numerous than non-ciliated cells. They are goblet-like and present numerous and long cilia. The nucleus of the ciliated cells is oval, but unlike the non-ciliated cells, it is more apical in location. Eventually, the cytoplasm of these cells presents few PAS-positive granules.

Externally, the efferent ductules are surrounded by one or two layers of smooth muscle cells. The tubular lumen is filled with fluid, but sperm are rarely seen, except in the terminal ductules. The intertubular connective tissue contains blood and lymphatic vessels as well as numerous mast cells.

During testicular regression, the efferent ductules present several morphological changes, including significant decrease in epithelial height and tubular diameter (Figure 3). The PAS-positive granules are decreased in non-ciliated cells and barely detectable in the ciliated cells.

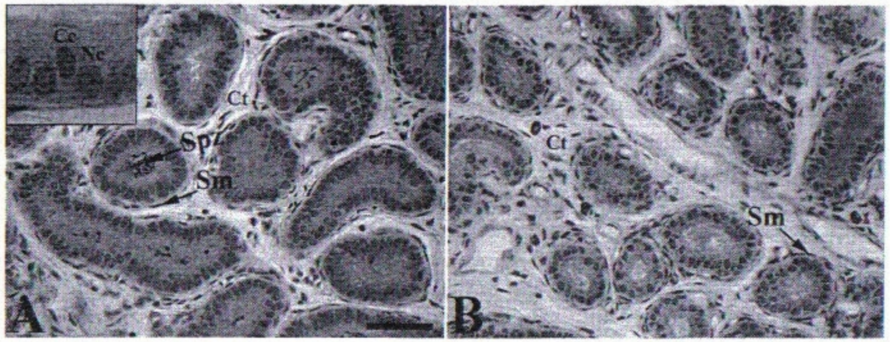

Figure 3. Efferent ductules of *Artibeus lituratus* during the reproductive (A) and regressive (B) periods. The epithelium of efferent ductules is composed of ciliated (Cc) and non-ciliated cells (Nc) (insert). Externally, the efferent ductules are surrounded by two or three layers of smooth muscle cells (Sm). Sperm (Spz) may be seen in the lumen of terminal ductules. During the regressive period, there is an expressive reduction in the epithelial height and tubular diameter. The intertubular connective tissue (Ct) became more conspicuous. Bar in A = B = 50µm.

2.3. Epididymis

The epididymis of bats consists of a single flexuous duct positioned laterally to the testis, continuing as the vas deferens on each side (Vamburkar, 1958). Anatomically the epididymis may be divided into caput, corpus and cauda (Matthews, 1941; Vamburkar, 1958; Azzali, 1983), even though an initial segment has also been easily distinguished in the epididymis of *Artibeus lituratus* (Oliveira, non-published data). In some species, the head and body of the epididymis are closely applied to the testis but the cauda lies caudal to the testis and is not closely applied to it (Matthews, 1941 - *Cardioderma cor*). Variation in the proportion of each epididymal region has been described for several bat species. In *Taphozous sp, Miniopterus minor* and *M. dasythrix,* the caput of epididymis is small and the cauda is very long, which usually projects caudally, beyond the posterior border of the testis (Matthews, 1941). The caput and cauda epididymis have the same size and the convolution of the duct is not well-defined in *Cynopterus sphinx gangeticus* (Vamburkar, 1958). In the *Nycteris luteola* and *N. hispida,* the caput, corpus and cauda are all well-developed, whereas *Triaenops afer* has a slender body connecting the moderately expanded head to a very large tail (Matthews, 1941). In *Hiposideros caffer,* the epididymis is small when compared with many other bat species and the cauda is not very extended caudally, suggesting that this segment is not involved in the reservoir of sperm (Matthews, 1941).

The epididymal duct is lined by a pseudostratified columnar epithelium (Krutzsch et al., 1976), which in some species, changes from columnar to cuboidal from the caput to the cauda (Richardson, 1977). As shown in *Artibeus lituratus*, the epididymal epithelium is composed by principal, basal, apical, narrow, clear and halo cells, which presents different distribution along the duct (Figure 4) (Oliveira, non-published data).

The principal cells are present in all segments of the epididymal duct. These cells are usually very tall columnar in the initial segment and become low columnar or cubic in the cauda region. The principal cell nucleus is round or oval, located in the basal cytoplasm and presenting one to three evident nucleoli (Oliveira, nonpublished data). These cells are characterized by the presence of many long microvilli (stereocilia) in the luminal surface (Krutzsch, 1975; Crichton et al., 1993). The cytoplasm of principal cells shows numerous PAS-positive granules whose distribution, amount and size vary along the epididymis. Usually, fewer clear PAS-positive granules are scattered in the supranuclear cytoplasm of the principal cells at the initial segment. The number and staining intensity of the granules increases in the caput region. In the corpus, the number of PAS-positive granules is even higher and they occupy both supranuclear and subnuclear regions (Oliveira, nonpublished data). In the cauda epididymis, the epithelium is lower and the microvillus are not as evident as in the caput (Krutzsch, 1975); Oliveira, nonpublished data}. Scarce PAS-positive granules are detected in the principal cells (Oliveira, nonpublished data).

Ultrastructural data concerning bat epididymis is scarce. One study in *Antrozous pallidus* revealed that the lateral plasma membranes of adjoining principal cells present junctional complexes at the cell apices, whereas basally, the lateral plasma membranes are characterized by the presence of many folds and interdigitation (Crichton et al., 1993). Tight junctions at caput and corpus epididymis appear as a series of branching interlacing strands, approximately 12 in number, whose horizontal meshwork run parallel to the lumen. These tight junctions form the blood-epididymis barrier to maintain a specialized luminal microenvironment for the maturation and maintenance of the sperm in transit. In the cauda epididymis, tight junction architecture is essentially similar to that of caput and corpus epididymis, during early sperm storage prior to winter torpor (named "ordinary" tight junctions). As the sperm storage season progresses, an additional tight junction type appears in the cauda. These tight junctions are composed of multiple, long parallel rows of strands, resembling those of the testis Sertoli cells. The strands often exceed 70 in number and mostly run tangential to the long axis of the cell. The presence of this highly specialized "Sertoli cell-like" tight junction may explain the ability of the cauda epididymis to withstand the excessively hyperosmolar epididymal luminal environment that is essential for long-term sperm storage in the hibernating bats (Crichton et al., 1993).

The basal cells are also present in all segments of the epididymis. These cells are small but numerous, and located at the base of the epithelium, apparently, without communication with the tubular lumen (Oliveira, nonpublished data). They present reduced cytoplasm without granules or vacuoles. The nucleus is small, round, ovoid or elongated, with one or two evident nucleoli.

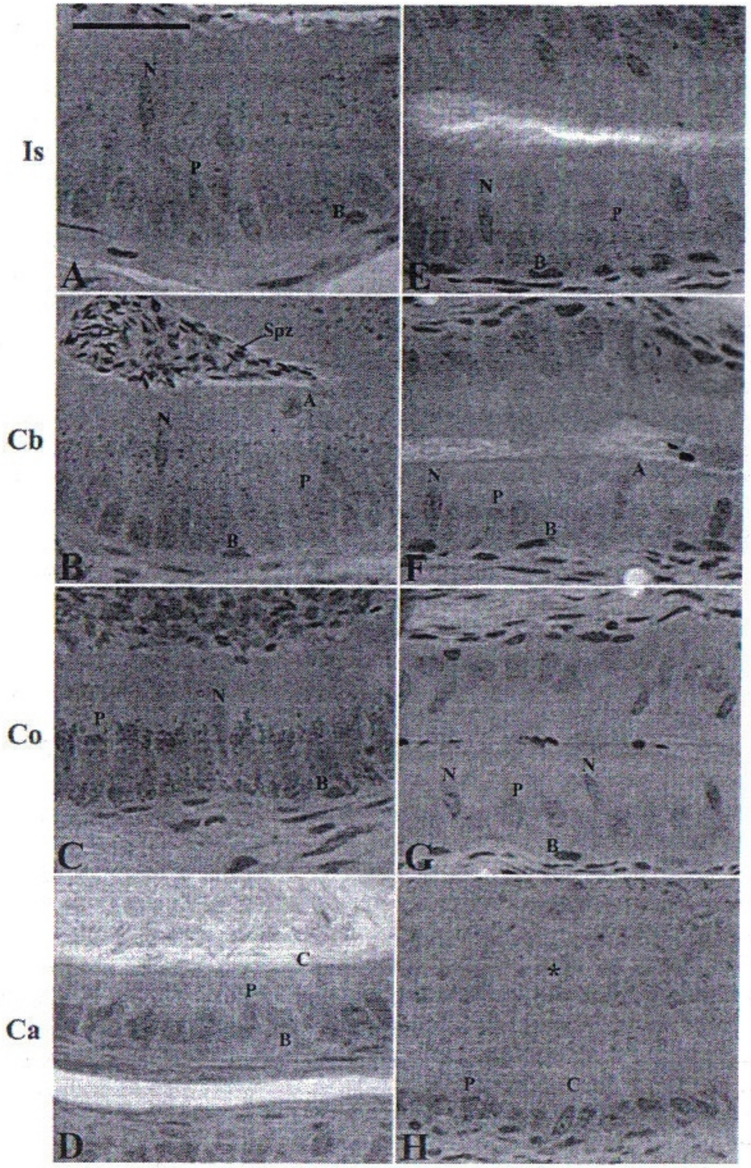

Figure 4. The epididymis epithelium of *Artibeus lituratus* in the reproductive (A-D) and regressive (E-H) periods. The epithelium presents principal (P), basal (B), narrow (N), apical (A) and clear (C) cells, evident in both reproductive and regressive phases. During the reproductive period, the epithelium presents PAS-positive granules that increase gradually from initial segment (IS) to caput (Ca) and corpus (Co). In cauda (Ca), the PAS-positive granules are nearly absent, as well as in all segments during the regressive period. During the regressive period, the epithelial height is greatly reduced compared with active period. Spz = luminal sperm; * = luminal degenerating cells; Bar in (A) = 30μm.

The apical and narrow cells are present in the initial segment, caput and corpus regions. The apical cells are located between the principal cells and present a round or ovoid nucleus at the apical cytoplasm. Narrow cells are intercalated with the principal and/or apical cells. The nucleus of the narrow cells is vertically elongated and positioned in the medial to apical cytoplasm (Oliveira, non-published data). Scarce clear cells are found restricted to the cauda region. The cytoplasm has no evident vesicles or granules (Oliveira, non-published data). The halo cells are present in all regions of the epididymis. Located at the base of the epithelium near the basal cells, the halo cells are hardly visualized. Halo cells are characterized by the small spherical and intensely basophilic nucleus. The cytoplasm does not stain with routinely used stains, thus forming a lighter halo around the nucleus (Oliveira, non-published data).

Externally, the epididymal duct presents three to four layers of peritubular smooth muscle cells in initial segment, caput and corpus regions. In the caudal region, the number of layers of smooth muscle cells is increased. The intertubular connective tissue is scant, containing evident fibroblasts, mast cells, blood and lymphatic vessels.

2.3.1. Seazonal Variation

The annual reproductive cycle of bats is marked by relevant morphological and physiological changes in the epididymis of both hibernating and non-hibernating seasonal species. During the regression period of *Artibeus lituratus*, a tropical, non-hibernating species, all segments of the epididymis show a significant reduction in epithelial height (Figure 4) and tubular diameter (Oliveira, non-published data). The tubular diameter is reduced by approximately 50% and become free of sperm, thus differing from most hibernating species. In contrast, numerous sloughed cells are found in the epididymal lumen (Figure 4). Even with considerable atrophy of the epididymal duct, it is possible to distinguish all of the cell types found in the epithelium during reproductive activity. Regarding principal cells, the amount and size of PAS-positive granules decrease in the regressive epididymis. Externally, there is an evident increase in the thickness of muscle layer, especially in the cauda epididymis.

Reduction in tubular diameter and absence of luminal sperm have also been described for other species (Krutzsch, 1975 - *Pipistrellus hesperus*; Krutzsch et al., 1976 - *Macrotus waterhousii*; Krutzsch and Crichton, 1987 - *Mormopterus planiceps*; Crichton et al., 1993 - *Antrozous pallidus*). During the reproductive inactivity, the epithelium becomes cubic and microvilli are more evident with the gradual reduction in the amount of luminal sperm. When a new spermatogenic cycle starts, this situation is reversed (Krutzsch, 1975). Upon involution, the intertubular connective tissue is more conspicuous (Krutzsch et al., 1976; Oliveira, non-published data). In addition, a large amount of adipose tissue lay down around the testis and epididymis during the winter, as seen in *Miniopterus australis* and *Miniopterus schreibersii* (Richardson, 1977).

In some hibernating species, the head and cauda of epididymis reach a maximum weight and epithelial height in the course of the summer associated with theengorgement with sperm. Thereafter, involution takes place and the tubules regress in the head epididymis, whereas the cauda remains distended and filled with sperm throughout the winter until middle spring (Racey and Tam, 1974 - *Pipistrellus pipistrellus*; Krutzsch and Crichton, 1986 - *Pipistrellus subflavus*). At the end of the sperm storage period, the cauda epididymis presents principal cells with irregular-shaped basal nucleus and prominent supra-nuclear cytoplasm. Conspicuous Golgi apparatus, mitochondria, rough endoplasmic reticulum, free ribosomes, and vacuoles of varying sizes, including apical coated pits, as well as lysosomes are observed. The cell surface facing the lumen presents many microvilli (Crichton et al., 1993).

2.3.2. Physiology

In several hibernating and some non-hibernating bats, the cauda epididymis is responsible for storing viable sperm during long periods. Therefore, studies on epididymis histology and physiology have been mostly focused on the cauda region.

Stored sperm form an extensive, densely packed mass within the caudal lumen, showing no specific orientation with respect to the epithelial lining (Hosken et al., 1998). The storage of sperm in the cauda epididymis usually occurs after ceasing spermatogenesis and may occur for a short period for species that mate before and during hibernation, or can extend over several months as observed in species that mate after hibernation (Anand Kumar, 1965; Racey, 1974; Bernard, 1984; Krutzsch and Crichton, 1986; Gustafson, 1987; Jolly and Blackshaw, 1987; Krutzsch and Crichton, 1987; Jolly and Blackshaw, 1988; Encarnação et al., 2004). In the last case, the time to store viable sperm in the epididymis exceeding the period observed in other mammals, epididymal storage may last about six months (Van der Merwe and Rautenbach, 1990 - South African *Pipistrellus rusticus*), seven months (Racey, 1974 - *Nyctalus Noctula*), eight months (Hosken et al., 1998 - Australian *Nyctophylus geoffroyi, N. major* and *Vespadelus regulus*) or ten months (Bernard, 1984 - African *Rhinolophus capensis*). In African and Australian Vespertilionidae of the genus *Miniopterus*, epididymal storage of sperm is retained for several months after the end of spermatogenesis, and the tip of the cauda filled with sperm is macroscopically enlarged projecting caudally from the testis limit (Matthews, 1941 - *Miniopterus minor* and *Miniopterus schreibersii*; (Richardson, 1977 - *Miniopterus australis* and *Miniopterus schreibersii*). It is not uncommon that aggregated sperm are recognized in the cauda epididymis throughout the year, even though the spermatogenesis is restricted to a short period of time (usually in the summer and early fall) (Anand Kumar, 1965).

The storage of sperm for long periods as observed in bats can be a natural consequence of hypothermia produced by hibernation. This ability is an important adaptation that allows the asynchronous reproductive cycle of males and females, important to reconcile the reproductive period with hibernation (Jolly and Blackshaw, 1987). The storage of sperm in the epididymis appears restricted to species of temperate zone belonging to three families of bats: *Emballonuridae,* Vespertilionidae and Rhinopomatidae (Gustafson, 1987; Jolly and Blackshaw, 1988; Van der Merwe and Rautenbach, 1990; Setchell et al., 1993; Hosken et al., 1998). However, it is now known that some species of non-hibernating tropical bats also store sperm in the epididymis (Cervantes et al, 2008). Prolonged sperm storage throughout the year is described for the Tropical Australian Emballonuridae *Taphozous georgianus* (Jolly and Blackshaw, 1987), the Tropical Indian Hipposideridae *Hipposideros speori* (Gopalakrishna and Bhatia, 1980) and Rhinopomatidae *Rhinopoma hardwickei* (Karim and Banerjee, 1985), as well as the Japanese *Rhinolophus cornutus*, which belongs to the Rhinolophidae family (Kurohmaru et al., 2002).

Nevertheless, it is unknown whether the prolonged survival of sperm in the epididymal cauda is possible due to characteristics of the luminal fluid, intrinsic factors of the sperm or both (Setchell et al., 1993). It is interesting that bat sperm do not differ from other mammals in terms of plasma membrane stability that could justify their resistance to long-term storage (Crichton et al., 1993). Long-term epididymal sperm preservation appears to be controlled by change in osmolality of the cauda milieu, as seen in male hibernating vespertilionid bats - *Eptesicus fuscus*, *Myotis lucifugus* and *M. sptentrionalis* (Crichton et al., 1994). Osmolalities of epididymal fluid in hibernating species of bats rise during sperm storage periods to values as high as 1,523 mmol/kg H_2O (5 times that of plasma). Seasonal establishment of hyperosmotic conditions driven by the epithelial cells dehydrate the sperm and thereby minimize their metabolic needs, which conserves energy by the imposition of quiescence. Protection from cold-induced membrane damage is afforded by the reduced activation energy of water at elevated osmolality. Reduction of osmolality (to 500-600 mmol/kg H_2O) induces swelling of sperm and allows the re-initiation of motility and increased metabolic rate (Crichton et al., 1994).

The fate of excess sperm stored in the bat cauda epididymis is still a matter of debate. In African *Rhinolophus capensis*, the sperm cells that remain in the epididymis after copulation are phagocytosed by luminal macrophages (Bernard, 1984). Similar occurrence is described for *Myotis lucifugus* and *M. velifer* (Krutzsch et al., 1982). Little or no evidence of spermiophagy by the epididymal epithelial cells is observed (Krutzsch et al., 1982; Bernard, 1984). Destruction of luminal sperm by macrophages is not a common finding in mammals at natural conditions, thus revealing another particularity of bat reproductive physiology.

Frequently, cytoplasmic droplets are seen on sperm at the cauda epididymis; thus indicating that, besides storage and spermiophagy, the cauda may also be involved in the final maturation of the sperm, during the storage period (Hoffman et al., 1987; Cervantes et al., 2008). Sequential induction of capacitation and acrosome reaction in sperm obtained from different epididymal regions (caput, corpus, cauda) throughout the annual reproductive cycle of *Corynorhinus mexicanus*, also reinforce the involvement of the cauda epididymis in sperm maturation during the storage period (Cervantes et al., 2008).

2.4. Ductus Deferens

The cauda epididymis is continuous with the ductus or vas deferens, which enters the abdominal cavity through the vaginal canal as part of the spermatic cord. Within the abdominal cavity, the vas deferens leaves the spermatic cord, passes medially and loops dorsally to the ureter. In most bat species, the vas deferens dilates terminally forming the ampullary gland (ampulla of Henle) (Matthews, 1941; Krutzsch and Crichton, 1986; Oliveira, non-published data). In some species, the vas deferens does not open directly into the urethra; they enter the seminal vesicle and opens into the vesicle collecting duct, which opens into the urethra. In other species, the vas deferens and the seminal vesicle duct join to each other forming a common ejaculatory duct that opens into the urethra (Matthews, 1941; Vamburkar, 1958). In species without a seminal vesicle, the distal part of the ampulla becomes narrower close to the urethra, forming the ejaculatory duct (Matthews, 1941).

In some hibernating species, the storage of sperm is not restricted to the cauda epididymis, but may also occur in the ductus deferens, primarily at its distal dilation, the ampullary gland (Anand Kumar, 1965 - *Rhinopoma kinneari*; Krutzsch and Crichton, 1986 - *Pipistrellus subflavus*).

2.5. Urethra

The urethra emerges from the neck of the urinary bladder as a uniform tube, cylindrical in shape, which is surrounded, partially or completely, by the prostate, and in some species, also by the seminal vesicle (Vamburkar, 1958; Krutzsch et al., 1976). The urethra extends from the neck of the bladder to the tip of the penis. On this course, the urethra is called a prostatic urethra while passing through the prostate, penile urethra while passing through the penis or membranous urethra in the portion located between the prostatic and penile urethra. The length of all urethral segments is variable. Accordingly, the prostatic plus membranous urethra are about 5.0 to 6.0mm in *Thaphozous sp*, 7.0 mm in *Cardioderma cor*, 8.0 to 9.0mm in *Triaenops afer* (Matthews, 1941) and 10mm long during the breeding season in *Cynopterus sphinx gangeticus* (Vamburkar, 1958). The prostatic and membranous urethra are lined with a transitional epithelium, thinner than in the penile urethra (Krutzsch et al., 1976). In some species, the lamina propria of the membranous urethra encloses numerous small simple alveolar mucous glands (Matthews, 1941; Krutzsch, 1975; Krutzsch et al., 1976). Externally, the prostatic urethra is surrounded by a thick muscular sheath (*musculi compressor urethrae*) (Krutzsch et al., 1976).

The prostatic urethra presents a well-defined median crista urethralis, in which numerous excretory ducts of the prostate glands open (Matthews, 1941; Vamburkar, 1958; Krutzsch, 1975). The ejaculatory ducts, when present, also enter the urethra at the colliculus seminalis in the apice of the crista urethralis (Krutzsch, 1975; Krutzsch and Crichton, 1987). A conspicuous utriculus prostaticus has been described in several species of bats (*Cardioderma cor; Hipposideros caffer, Nycteris luteola* and *Taphozous sp* - Matthews, 1941), but it is not present in others (*Cynopterus sphinx, Miniopterus minor, Miniopterus schreibersii, Nycteris hispida* and *Triaenops afer,* - Matthews, 1941; *gangeticus* - Vamburkar, 1958; *Mormopterus planiceps* - Krutzsch and Crichton, 1987). When present, the utriculus prostaticus opens on the crista urethralis distal to the ejaculatory ducts. It consists of a tubular evagination with a very small lumen, lined by a stratified cuboid epithelium (Matthews, 1941; Krutzsch and Crichton, 1987).

The caudal end of the membranous urethra widens to form the bulb on the base of the penis, where it is enclosed in paired, thick bulbocavernosus muscles (Krutzsch, 1975). The bulbous urethra receives the ducts of the Cowper's glands (Krutzsch, 1975; Krutzsch et al., 1976). It continues in the penile urethra, which extends from the base of the penis to the urethral orifice (Vamburkar, 1958). The penile urethra dilates distally into a *sinus navicularis*, before the opening to the exterior through the external urethral meatus (Krutzsch et al., 1976). The external urethral meatus is subterminal and may open on the ventral *(Cynopterus sphinx gangeticus, Hipposideros caffer, Macrotus waterhousii, Miniopterus minor, Nycteris hispida, Taphozous sp* and several species of Molossidae) or dorsal (*Cardioderma cor; Miniopterus schreibersii*) surface of the glans, whereas in some species, it is terminal (*Nycteris luteola; Triaenops afer*) (Matthews, 1941; Vamburkar, 1958; Krutzsch et al., 1976; Ryan, 1991a).

The penile urethra is lined by a transitional (Krutzsch et al., 1976; Ryan, 1991b) or stratified columnar epithelium two to six- layers thick (Krutzsch, 1975). Distally, the urethral epithelium gradually changes to stratified squamous epithelium which lines the sinus and external meatus (Krutzsch, 1975; Krutzsch et al., 1976). This epithelium is continuous with the external epithelium, covering the glans and the internal surface of the prepuce (Krutzsch, 1975). In some species, urethral glands lie in the connective tissue of the mucosa, whereas in others, the penile urethra is devoid of intramural glands (Krutzsch, 1975; Krutzsch et al., 1976).

3. PENIS

The remarkable interspecific variation in the morphology of the mammalian penis has usually served as an important phylogenetic tool in several orders, such as marsupials, rodents and primates. Lesser is known about interspecific diversity of the bat penis, therefore it is still premature to use phallic morphological variation as a systematic character for Chiroptera. The bat penis is prominent, highly variable in size, structure and position.

3.1. Anatomy

For descriptive purposes, the bat penis may be divided into three regions: the root or crura, the body or shaft, and the glans. The chiropterans penis is similar to the cavernosum penis of most mammals. It is formed by two symmetrical corpora cavernosa and the corpus spongiosum. The basis of these erectile structures in conjunction with tissues that surround them form the root of the penis. At the root, the membranous urethra expands to form the erectile bulb and the corpora cavernosa form the crura, which are attached to the isquiopubic bones. The bulb is surrounded by thick bulbospongiosum muscle. The corpora cavernosa form the bulk of the penis shaft. The glans of bats penile is defined as the part of the penis distal to the origin of the prepuce (Ryan, 1991a). The glans is well-developed in most bats and, in some species, a baculum (*os penis*) is found extending throughout the glans. The form of the glans and the baculum differs considerably among bats.

The bat penis may be pendulous (*Miniopterus minor* - Matthews, 1941; *Cynopterus sphinx gangeticus* - Vamburkar, 1958; *Mormopterus planiceps* - Krutzsch and Crichton, 1987), semipendulous (*Pipistrellus hesperus* - Krutzsch, 1975) or not pendulous (*Macrotus waterhousii* - Krutzsch et al., 1976; *Artibeus lituratus* - Oliveira, non-published data). The pendulous penis is directed caudally, whereas the non-pendulous penis is rostrally directed (Krutzsch et al, 1976). The organ is usually cylindrical and elongated (Matthews, 1941; Vamburkar, 1958), but varies in diameter (Matthews, 1941). The length of the penis is variable among species (4.5 mm in *Taphozous sp*; 5.0 to 7.0 mm in *Triaenops afer*; 5.5 to 7.5 mm in *Pipistrellus hesperus;* 6.0 mm in *Hipposideros caffer*; *Cardioderma cor;* 6.5 to 7.5 mm in *Macrotus waterhousii*; 7.0 mm in *Miniopterus minor;* 9.4 mm in *Mormopterus planiceps;* 14 mm in *Cynopterus sphinx gangeticus* - and 18 mm in *Artibeus lituratus*) (Matthews, 1941; Vamburkar, 1958; Krutzsch, 1975; Krutzsch et al., 1976; Krutzsch and Crichton, 1987); Oliveira, nonpublished data). Proportionally, the bat penis correspond to 7-10% of the body length in most species, except *Artibeus lituratus* in which the penis reaches up to 16% of the body length.

3.2. Shaft Structure

Similar to other mammals, the bat penis possesses three cylindrical columns of erectile tissue: two corpora cavernosa and the corpus spongiosum. The bulk of the penis shaft is composed by paired erectile, bilaterally symmetrical, corpora cavernosa, which lie above the urethra and are enclosed by a very thick tunica albuginea (Matthews, 1941; Vamburkar, 1958; Krutzsch, 1975; Krutzsch and Crichton, 1987; Ryan, 1991b). The corpora cavernosa are constituted by trabeculae of fibroeslastic and muscular tissues, which traverse the interior of the corpora, separating numerous vascular sinuses or lacunae that fill with blood during erection (Ryan, 1991b).

The corpora cavernosa originate in the form of two lateral crura, which are connected to the rami of the pubic arc (Vamburkar, 1958). The two corpora cavernosa are apparently fused in some species but remain internally separated by a median fibrous septum of tunica albuginea (Vamburkar, 1958; Krutzsch, 1975; Ryan, 1991a; Ryan, 1991b), which may be complete or incomplete in some species (Vamburkar, 1958; Ryan, 1991b). In other species, the corpora cavernosa is completely fused throughout the length of the penis to form a single erectile body (Matthews, 1941).

Hipposideros caffer, *Miniopterus minor*, *Miniopterus schreibersii*, *Nycteris hispida*, *Nycteris luteola* and *Taphozous sp*). In *Triaenops afer*, the corpora cavernosa are fused in the shaft of the penis but become separated in the base of the glans, where the tunica albuginea remain joined (Matthews, 1941). In most bats, the bat corpora cavernosa is not restricted to the shaft of the penis but extends well into the glans (Ryan, 1991a).

The corpus spongiosum of bat penis is usually poorly developed. It lies ventral to the corpora cavernosa and surrounds the urethra from the bulb to the base of the glans, as a very thin cylinder of erectile tissue consisting of a small venous plexus (Matthews, 1941 - *Cardioderma cor, Miniopterus minor, Miniopterus schreibersii, Nycteris hispida, Taphozous sp* and *Triaenops afer*). The tunica albuginea is thinner and more elastic than that of the corpora cavernosum (Ryan, 1991a; Ryan, 1991b). Usually, the corpus spongiosum does not extend into the glans (Matthews, 1941; Ryan, 1991a; Ryan, 1991b). In *Cynopterus sphinx* and *Nycteris luteola,* the corpus spongiosum is considerably more developed than in other species of bats, and extends at a short distance into the glans (Matthews, 1941; Vamburkar, 1958). Corpus spongiosum is also present in the proximal glans of some Molossidae species (Ryan, 1991a). Conversely, in the Hipposideridae *Hipposideros caffer,* a minute corpus spongiosum surrounds the urethra as far as the middle of the penis, distal to which it is absent (Matthews, 1941).

In addition to the corpora cavernosum and spongiosum, a specialized accessory erectile body, the accessory cavernosus tissue, is present in some chiropterans (Matthews, 1941; Ryan, 1991a; Ryan, 1991b), but absent in others (Matthews, 1941; Krutzsch et al., 1976; Krutzsch and Crichton, 1987). The accessory cavernosous tissue contains large trabecular sinus, rich in elastic fibers, which is not enclosed by the albuginea tunica, but by the penile deep fascia (Ryan, 1991b). This tissue may be restricted to the distal half of the glans in Molossidae, surrounding the corpus spongiosum and *os penis* (Ryan, 1991a; Ryan, 1991b). In *Molossus ater,* some accessory erectile tissue is found dorsal to the corpora cavernosa in the mid-glans and also in the bacular mound (Ryan, 1991a). They are more conspicuous in *Cardioderma cor* and *Nycteris luteola* (Matthews, 1941). The accessory cavernous body when engorged, forms a large, bulbous, hyperemic distention. These specializations may be important in the reproductive process of species whose solitary roosting habits presumably provide fewer copulatory opportunities (Krutzsch, 1975).

The tunica albuginea of the erectile corpora cavernosum and corpus spongiosum are surrounded by the deep penile fascia, followed by the superficial fascia or subcutaneous tissue and, externally, the skin (Ryan, 1991b). The penile skin is lightly pigmented, containing sparsely distributed hair, including the prepuce (Krutzsch, 1975; Krutzsch et al., 1976; Ryan, 1991b). In some species, the penis skin presents thin short hairs amongst which project a number of longer (2-3 mm) stiff hairs (Matthews, 1941).

3.3. Glans Structure

The glans is well-developed in most bat species, but can be minute in others (Table 1) (Matthews, 1941; Krutzsch and Crichton, 1987). In *A. lituratus* ,the glans represents about 15% of the penis length, in *Macrotus waterhousii* 20% (Krutzsch et al., 1976) and 40% in *Pipistrellus hesperus* (Krutzsch, 1975). On the other hand, a very well-developed glans, comprising 90% of the penis length, is found in *Mormopterus planiceps* (Krutzsch and Crichton, 1987). In this species, the glans presents two parts: a long, dorsal primary portion and a short ventral secondary portion. The primary portion contains a well-developed baculum and the urethra, which emerges from the ventral surface (Krutzsch and Crichton, 1987).

The form of the bat glans has been described as conical, ovoid, cylindrical or club-shaped (Matthews, 1941; Vamburkar, 1958; Ryan, 1991b). The glans of chiropterans presents several specializations that appears species-specific. In this sense, the glans of *Pipistrellus hesperus* terminates in a pair of blunt, rounded processes conspicuous upon erection as bright, red, rounded knobs. The surface of flaccid glans in this species is wrinkled, presenting a number of deep longitudinal furrows and folds that permit the glans to expand upon erection (Krutzsch, 1975). In *Cynopterus sphinx gangeticus,* the glans surface is covered with minute denticles, which point cranially and are arranged in longitudinal rows (Vamburkar, 1958). The glans of *Cardioderma cor* is mostly covered with minute spiniform tubercles (Matthews, 1941). Most Molossidae species present epidermal spines in the glans, which are proximally directed (Ryan, 1991a; Ryan, 1991b). The keratinized spines are large in *Mormopterus jugularis* (75-100µm), but varies from 25-50µm in *M. planirostris* and *M. teminckii* to 15-30µm in *Nyctinomops laticaudatus* and *N. luteola* (Ryan, 1991b). In *Tadarida brasiliensis* and *T. aegyptiaca,* the glans surface is covered by several rows of blunt epithelial papillae instead of spines (Ryan, 1991b). Also in *Mormopterus planiceps*, the surface of the glans is covered by keratinized spines, which project backward and originate from nests of epithelial basal cells (Krutzsch and Crichton, 1987).

Differing from other mammal glans that are mainly filled by spongiosum tissue (e.g. stallion, human), the bat glans may contain corpora cavernosa, corpus spongiosum and/or accessory cavernosus tissue as the main erectile tissue. Accordingly, the glans of several Molossidae and some Vespertilionidae species contains mostly corpora cavernosa (Matthews, 1941; Krutzsch, 1975; Ryan, 1991a; Ryan, 1991b). In the Pteropodidae *Cynopterus sphinx gangeticus,* the corpora cavernosa end at the proximal part of the *os penis*, but the corpus spongiosum that surrounds the urethra, enters the glans, where they became thicker and practically fill the glans space beyond the corpora cavernosa (Vamburkar, 1958). In the

Emballonuridae *Taphozous sp,* the corpora cavernosa also terminates at the proximal end of the *os penis*, however the bulk of the tissue within the glans is formed by accessory corpora cavernosa (Matthews, 1941). A similar situation is found in *Molossops planirostris* and *M. temminckii* (Ryan, 1991b). In the Nycteridae *Nycteris hispida,* there is practically no erectile tissue within the glans, so the urethra and the *os penis* fill most of the glans (Matthews, 1941).

The *os penis* is characteristic of a wide range of genera representing virtually all bat families, except Phyllostomidae and Noctilionidae (Table 1) (Brown et al., 1971; Krutzsch, 1975; Hosken et al., 2001). When present, the *os penis* lies above the urethra and is contained into a prominent bacular mound, which is found distal to the urinary meatus (Ryan, 1991a; Herdina et al., 2010). The form and size of the *os penis* is greatly variable. It can be minute and restrict to the proximal half of the glans (*Taphozous sp*), distal half of the glans *(Cynopterus sphinx gangeticus, Hipposideros caffer,* Mollosidae species), well-developed extending for the entire glans (*Mormopterus planiceps*, *Triaenops afer*) or even from the glans into the shaft of the penis *(Nycteris luteola)* (Matthews, 1941; Vamburkar, 1958; Krutzsch and Crichton, 1987; Ryan, 1991a; Ryan, 1991b). The length of the *os penis* may be greatly variable depending upon species: 0.2 mm in *Thaphozous sp*, 0.76 mm in *Pipistrellus subflavus*, 1.5 mm in *Nycteris hispida,* 2.0 mm in *Cardioderma cor,* 2.5 mm in *Cynopterus sphinx gangeticus*, 5.0 mm in *Nycteris luteola* and 7.9 mm in *Mormopterus planiceps* (Matthews, 1941; Vamburkar, 1958; Krutzsch and Crichton, 1986; Krutzsch and Crichton , 1987). The smallest baculum is found in members of the families, Emballonuridae (0.07 mm in *Balantiopterix io*) and Molossidae, whereas the greatest is found in Pteropodidae (12.9 mm in *Pteropus neohibernicus*) (Hosken et al., 2001). The large variation in baculum size across bats does not appear to be associated with sexual selection or risk of sperm competition (Hosken et al., 2001).

Concerning the baculum shape, it may be cylindrical *(Taphozous sp, Nycteris hispida* - Matthews, 1941), Y-shaped (Krutzsch and Crichton, 1986 - *Pipistrellus subflavus*; Herdina et al., 2010 - *Plecotus austriacus*), disc-shaped (Nwoha, 2000 - *Eidolon helvum*), elongated and strongly concave ventrally (Krutzsch, 1975 - *Pipistrellus hesperus*) or curved with a ventral convexity and a dorsal concavity (Vamburkar, 1958 - *Cynopterus sphinx gangeticus*). The baculum periosteum is continuous with the dense connective tissue of the thick tunica albuginea of the corpora cavernosa (Krutzsch, 1975; Krutzsch and Crichton, 1987).

The functional significance of this ossicle is not clear. In species presenting well-developed baculum, it has been proposed that it may support and stiffen the penis (Krutzsch, 1975). The elongated baculum and spiny surface of the glans, as seen in *Mormopterus planiceps*, are considered specializations important for penetration and retention of the penis into the vagina at copulation (Krutzsch and Crichton, 1987). However, according to Nwoha, (2000), in the Nigerian *Eidolon helvum*, the *os penis* may function to support erection of the penis more for micturition than for intromission. A recent study on *Plecotus austriacus* (Herdina et al., 2010) suggested that the microanatomy of baculum implies a mechanical function, as it forms a functional unit with the corpora cavernosa. The collagen fibers of the tunica albuginea of both corpora cavernosa insert via fibrocartilage into the woven bone of the baculum branches. This fibrocartilage may represent alternating shearing forces on this joint-like interface during erection and copulation. In addition, a function in keeping the orifice of the urethra opened during copulation has also been proposed for the concave surface of the distal tip of the baculum (Herdina et al., 2010).

Table 1. Characteristics of the glans penis of bats.

Suborder/ Families/ Species	Glans Form	Glans Length (mm)	Baculum	Urethral meatus	References
MEGACHIROPTERA					
Pteropodidae					
Cynopterus sphinx	conical	4	Present	Subterminal	Vamburkar, 1958
Emballonuridae					
MICROCHIROPTERA					
Taphozous sp	Conical	0.8	Present	Subterminal	Matthews, 1941
Megadermatidae					
Cardioderma cor	Ovoid	4.0	Present	Subterminal	Matthews, 1941
Molossidae					
Eumops auripendulus	Oval	ND	Present	Subterminal	Ryan, 1991a
Eumops bonariensis	Oval	1.5-2.5	Present	Subterminal	Ryan, 1991a
Eumops perotis	ND	ND	Absent	ND	Ryan, 1991a
Mormopterus jugularis	Oval	3.5	Present	Subterminal	Ryan, 1991b
Molossus ater	Cone-shaped	5.6	Present	Subterminal	Ryan, 1991a
Molossus bondae	ND	5.0	Present	ND	Ryan, 1991a
Molossus coibensis	ND	4.5	Present	ND	Ryan, 1991a
Molossus molossus	ND	3.4	Present	ND	Ryan, 1991a
Molossus sinaloae	ND	3.0	Present	ND	Ryan, 1991a
Molossops temminckii	Oval	2.0	Absent	Terminal	Ryan, 1991b
Molossops planirostris	Oval	1.2	Absent	Subterminal	Ryan, 1991b
Nyctinomops (=Tadarida) macrotis	Cylindrical	ND	Present	Subterminal	Ryan, 1991b
Nyctinomops laticaudatus	Cylindrical	2.0	Present	Subterminal	Ryan, 1991b
Tadarida brasiliensis	Cylindrical	2.5-3.0	Present	Subterminal	Ryan, 1991b
Promops centralis	Long	4.0	Absent	ND	Ryan, 1991a
Nycteridae					
Nycteris luteola	club-shaped	ND	Present	Terminal	Matthews, 1941
Nycteris hispida	ND	0.8	Present	Subterminal	Matthews, 1941
Phyllostomidae					
Macrotus waterhousii	ND	1.7 2.0	Absent	ND	Krutzsch et al, 1976
Rhinolophidae					
Hipposideros caffer	Long and narrow	3.0	Present	Subterminal	Matthews, 1941
Triaenops afer	large and flattened dorso-ventrally	2.5	Present	Terminal	Matthews, 1941
Vespertilionidae					
Miniopterus minor	conical	0.5	Absent	Subterminal	Matthews, 1941
Miniopterus schreibersii	conical	0.5	Absent	Subterminal	Matthews, 1941
Pipistrellus hesperus	elongated and broad	2.3 - 3.5	Present	ND	Krutzsch, 1975

ND = Not detected.

In most species of bats, the glans is covered by a thin prepuce, readily retractable (Matthews, 1941; Vamburkar, 1958; Krutzsch and Crichton, 1987; Ryan, 1991a). In some species, the prepuce is thick, but still retractable, as seen in *Pipistrellus hesperus* and *Macrotus waterhousii* (Krutzsch, 1975; Krutzsch et al., 1976). In *Miniopterus schreibersii,* the glans is covered by a long prepuce which extends about 1.0 mm beyond its end (Matthews, 1941). Also, in *Cynopterus sphinx gangeticus, Macrotus waterhousii* and *Pipistrellus Hesperus,* the prepuce extends beyond the glans tip (Vamburkar, 1958; Krutzsch, 1975; Krutzsch et al., 1976). The prepuce is lightly pigmented and presents sparse hairs (Krutzsch and Crichton, 1987). Short hairs also cover the outer sheath of the prepuce in other Molossidae species (Ryan, 1991a). In these species, there is also a tuft of long stiff hairs along the dorsal margin of the prepuce. Preputial glands may be present in some species but absent in others (Krutzsch, 1975).

4. ACCESSORY SEX GLANDS

Information about the accessory sex glands of bats is scarce and mostly restricted to gross morphology. The available information revealed that bat accessory sex glands show great diversity and remarkable differences even when closely related species are considered (Mokkapati and Dominic, 1977). The complex of accessory gland of bats comprises the prostate, seminal vesicles, ampullary glands, bulbourethral glands (Cowper's glands) and urethral glands (Figure 5). The prostate and bulbourethral glands have been described in all species studied to date, whereas seminal vesicles, ampullary glands and urethral glands are present in some species but absent in others.

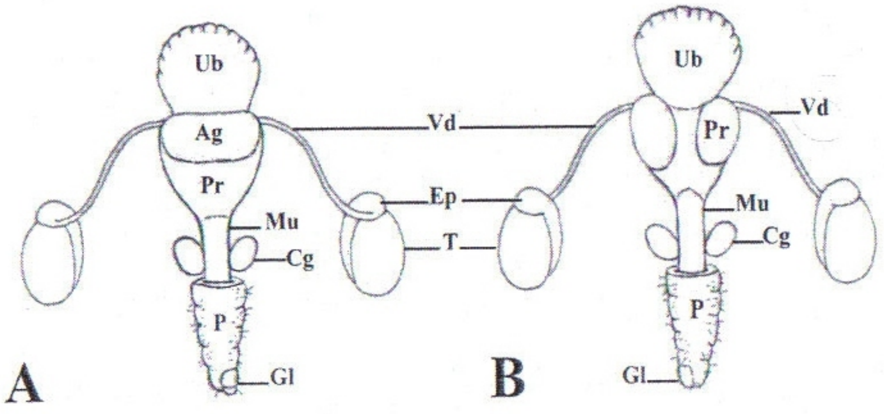

Figure 5. The accessory sex glands in *Artibeus lituratus*. (A) Dorsal and (B) ventral view. The accessory sex gland is comprised of the ampullary gland (Ag), prostate (Pr) and the Cowper's glands (Cg). Ub = urinary bladder; Mu = membranous urethra; P = penis; Gl = glans, T = testis; EP = epididymis; Vd = vas deferens.

4.1. Prostate

The prostate is an unpaired gland present in all bat species (Figure 5 and Table 2). It is located below the neck of the urinary bladder, however its position related to the prostatic urethra exhibit variation among species, being ventral to the urethra (Mokkapati and Dominic, 1977 - *Cynopterus sphinx, Pipistrellus mimus*), encircling dorsolaterally the urethra (Matthews, 1941 - *Nycteris luteola*; Krutzsch, 1975 - *Pipistrellus Hesperus*; Krutzsch, 1975 - *Macrotus waterhousii*; Mokkapati and Dominic, 1977 - *Scotophilus heathi, Scotophilus temmincki*; Krutzsch and Crichton, 1986 - *Pipistrellus subflavus;* Oliveira, non-published data - *A. lituratus*) or completely surrounding the urethra (Matthews, 1941 - *Taphozous sp; Hipposideros caffer, Triaenops afer, Miniopterus minor, M. schreibersii*; Mokkapati and Dominic, 1977 –

Rhinopoma hardwickei, Taphozous longimanus). Even in species that present the urethra surrounded by the prostate, the gland is larger in the dorsal portion than the ventral (Matthews, 1941).

The prostate is enclosed by a thick, connective tissue and smooth muscle capsule (Krutzsch, 1975; Krutzsch et al., 1976). The gland has no lobulations, except in *Pipistrellus hesperus* where the gland is described as ventrally bilobed (Krutzsch et al., 1976). The prostate consists of branched tubuloalveolar glands (Krutzsch, 1975; Mokkapati and Dominic, 1977). As seen in *Cynopterus sphinx*, it is composed of alveoli with 40 to 200 µm of diameter, which is lined by a tall simple columnar epithelium (Mokkapati and Dominic, 1977). However, the epithelium may vary from columnar to squamous, depending on the amount of secretory product accumulated in the alveoli lumen (Krutzsch, 1975; Krutzsch and Crichton, 1987). The nuclei of epithelial cells are located basally (Mokkapati and Dominic, 1977).

The prostate contains eosinophilic, PAS-positive, Alcian blue negative, diastase resistant secretion, suggesting a glycoprotein (Racey and Tam, 1974 - *Pipistrellus pipistrellus*; Krutzsch, 1975 - *Pipistrellus hesperus*; Bernard, 1986 - *Rhinolophus capensis*; Krutzsch and Crichton, 1987 - *Mormopterus planiceps*). The majority of the secretion is aggregated into clumps of ovoid to multi-sided globules into the lumen (Krutzsch and Crichton, 1990). This globular secretion is the result of a major breakdown of the glandular epithelium. Prostates secrete fructose in *Scotophilus beatbi, Taphozous longimanus, Macrotus waterhousii* and *Pipistrellus subflavus,* especially during breeding activity (Krutzsch et al., 1976; Mokkapati and Dominic, 1976; Krutzsch and Crichton, 1986). A great amount of citric acid is also produced by the prostate (Mokkapati and Dominic, 1976). The prostate secretion flows via multiple excretory ducts that open in the prostatic urethra in the neighborhood of the *crista urethralis* (Matthews, 1941; Krutzsch and Crichton, 1990).

During the inactive period, the gland alveoli are collapsed and interspersed by a large amount of connective tissue (Richardson, 1977; Krutzsch and Crichton, 1990).

4.2. Seminal Vesicles

The seminal vesicle consists of a pair of glands, usually pear-shaped, located laterally or proximal to the prostate on the outer border of the ampullary gland, when present (Matthews, 1941; Mokkapati and Dominic, 1976; Mokkapati and Dominic, 1977). The seminal vesicle is present in few species of bats (Figure 5 and Table 2) (Matthews, 1941; Krutzsch, 1975; Mokkapati and Dominic, 1977; Krutzsch and Crichton, 1990). As the structure of the bat seminal vesicles are similar to that of ampullary gland, it is possible that they were confounded in some studies. It is noteworthy that in all species examined to date, at least one of these glands is always present in bats. The occurrence of both ampullary glands and seminal vesicles is not a common finding (Matthews, 1941).

Mokkapati and Dominic (1977) described the accessory sex glands of seven species of Indian bats and found seminal vesicles in three species - *Cynopterus sphinx, Pipistrellus mimus* and *Taphozous longimanus*. The seminal vesicles of *Cynopterus sphinx* are large and coiled tubular structures, consisting of a rounded distal portion and a tubular proximal end opening into the urethra. The diverticula of the seminal vesicle are lined by low columnar epithelium (Matthews, 1941) or squamous secretory epithelium (Mokkapati and Dominic, 1976; Mokkapati and Dominic, 1977), as the height of the epithelium may vary depending on the amount of luminal secretion (Krutzsch, 1975).

The secretory product of the seminal vesicle is more amorphous, homogenous and mucinous in appearance than the prostate (Richardson, 1977 - *Miniopterus australis e Miniopterus schreibersii*; Krutzsch and Crichton, 1990 - *Miniopterus schreibersii*). Seminal vesicle secretion contains fructose and citric acid (Mokkapati and Dominic, 1976).

4.3. Ampullary Glands

The ampullary glands are present in most bat species (Figure 5 and Table 2). They consist of pear-shaped or elongated oval terminal enlargements of the distal extremity of the vas deferens, which become highly folded and undergo differentiation into a specialized secretory gland (Mokkapati and Dominic, 1977; Krutzsch and Crichton, 1990). The ampullary glands lie between the seminal vesicles when these are present. At the distal end of the ampulla, the ejaculatory ducts emerge, which pass through the prostatic tissue to join the urethra on its posterior surface, opening on the *crista urethralis* (Matthews, 1941). In some species, the vas deferens lies embedded in the center of the gland (Racey and Tam, 1974 - *Pipistrellus pipistrellus*; Mokkapati and Dominic, 1977 - *Rhinopoma hardwickei*; Krutzsch and Crichton, 1987 - *Mormopterus planiceps*). The vas deferens can be differentiated from the glandular diverticula because of the large lumen and the thicker muscular layer (Matthews, 1941).

The entire ampullary gland is ensheathed by a connective tissue and muscular capsule (Racey and Tam, 1974; Mokkapati and Dominic, 1977; Richardson, 1977; Krutzsch and Crichton, 1987). They consist of a mass of tubular diverticula, 30-60 µm in diameter, lined by a simple unfolded cuboidal secretory epithelium (Matthews, 1941; Racey and Tam, 1974; Bernard, 1986). The gland presents luminal secretions along the year, but the structure of the epithelium changes along the annual reproductive cycle. In the inactive condition, the epithelial cells present few and short microvilli, few secretory granules, but large autolytic vacuoles. Conversely, in the active gland, the microvilli are longer and abundant and the apical cytoplasm is filled with secretory granules (Bernard, 1986).

The ampullary gland produces fructose and citric acid in *Taphozous longimanus* but only fructose in *Scotophilus heathi* (Mokkapati and Dominic, 1976). Sperm are abundant in the lumen of ampullary gland collected in the breeding season (Richardson, 1977; Krutzsch and Crichton, 1987; Krutzsch and Crichton , 1990) or throughout the year (Anand Kumar, 1965).

Table 2. Occurrence of accessory sex glands in bat species.

Suborder/ Families/ Species	Prostate	Seminal vesicle	Ampullary gland	Cowper gland	Urethral glands	References
MEGACHIROPTERA						
Pteropodidae						
Cynopterus sphinx	Present	Present	Absent	Present	Present	Mokkapati and Dominic, 1977 Vamburkar, 1958
MICROCHIROPTERA						
Emballonuridae						
Taphozous georgianus	Present	Present	Present	ND	ND	Jolly and Blackshaw, 1987
Taphozous longimanus	Present	Present	Present	Present	Absent	Mokkapati and Dominic, 1977
Thaphozous sp	Present	Present	Present	Present	Present	Matthews, 1941
Megadermatidae						
Cardioderma cor	Present	Absent	Present	Present	Present	Matthews, 1941
Molossidae						
Mormopterus planiceps	Present	Present	Present	Present	Present	Krutzsch and Crichton, 1987
Nycteridae						
Nycteris luteola	Present	Absent	Present	Present	Absent	Matthews, 1941
Nycteris hispida	Present	Absent	Present	Present	Absent	Matthews, 1941
Phyllostomidae						
Artibeus lituratus	Present	ND	Present	Present	Present	Oliveira, nonpublished data
Macrotus waterhousii	Present	Present	Present	Present	Absent	Krutzsch et al 1976
Rhinolophidae						
Hipposideros speoris	Present	Absent	Present	Present	Present	Mokkapati and Dominic, 1977
Hipposideros caffer	Present	Absent	Present	Present	Present	Matthews, 1941
Rhinolophus capensis	Present	Absent	Present	Absent	Present	Bernard, 1986
Triaenops afer	Present	Present	Absent	Present	Present	Matthews, 1941
Rhinopomatidae						
Rhinopoma hardwickei	Present	Absent	Present	Present	Absent	Mokkapati and Dominic, 1977
Rhinopoma kinneari	Present	Absent	Present	Present	Absent	Anand Kumar, 1965
Vespertilionidae						
Miniopterus australis	Present	Present	Present	Present	ND	Richardson, 1977
Miniopterus minor	Present	Absent	Present	Present	Present	Matthews, 1941
Miniopterus schreibersii	Present	Present	Present	Present	Absent	Krutzsch and Crichton, 1990 Richardson, 1977
Myotis lucifugus	Present	Present	Present	Present	ND	Hunter et al, 1971
Nyctalus noctula	Present	Absent	Present	Present	Absent	Racey, 1974
Pipistrellus hesperus	Present	Present	Present	Present	Absent	Krutzsch, 1975
Pipistrellus mimus	Present	Present	Present	Present	Present	Mokkapati and Dominic, 1977
Pipistrellus pipistrellus	Present	Absent	Present	Present	Present	Racey and Tam, 1974
Pipistrellus subflavus	Present	Absent	Present	Present	Absent	Krutzsch and Crichton, 1986
Scotophilus heathi	Present	Absent	Present	Present	Present	Mokkapati and Dominic, 1977
Scotophilus temmincki	Present	Absent	Present	Present	Present	Mokkapati and Dominic, 1977

ND = Not detected.

4.4. Bulbourethral or Cowper's Glands

The bulbourethral glands are small, paired, bilaterally symmetrical glands present in all bat species (Figure 5 and Table 2). The gland size is variable depending on the species considered (0.6 mm in diameter in *Miniopterus minor*; 0.8 mm in *Nycteris luteola;* 1 mm in *Taphozous sp, Triaenops afer*; 1.2 mm in *Miniopterus schreibersii;* 1,7 mm in *A. lituratus*) (Matthews, 1941; Oliveira, non-published data). They are bean-shaped, spherical or ovoid in shape, lying cephalic to the bulbocavernosus muscles and opening into the penile urethra, cephalic to the bulb (Krutzsch, 1975; Mokkapati and Dominic, 1977; Richardson, 1977).

Histologically, they consist of compound tubuloalveolar glands, whose acini are lined by cubical cells (Krutzsch, 1975 - *Pipistrellus Hesperus*; Mokkapati and Dominic, 1976; Mokkapati and Dominic, 1977 - *Cynopterus sphinx*) or tall columnar cells, which produces a PAS-positive secretion (Richardson, 1977 - *Miniopterus schreibersii e Miniopterus australis*; Krutzsch and Crichton, 1987 - *Mormopterus planiceps*; Krutzsch and Crichton, 1990 - *Miniopterus schreibersii*). The cytoplasm of the epithelial cells is filled with secretory granules that flatten the nucleus in the basal cytoplasm (Richardson, 1977; Krutzsch and Crichton, 1987; Krutzsch and Crichton , 1990). The Cowper's glands are surrounded by a connective tissue capsule, which is covered by a thick tunica of striated muscle (*bulboglandularis*) (Krutzsch, 1975; Mokkapati and Dominic, 1977). Contraction of this muscle at ejaculation rapidly empties the gland secretion (Krutzsch, 1975). The glandular parenchyma is separated into lobules by thin septa of connective tissue originating from the capsule (Krutzsch et al., 1976).

4.5. Urethral Glands

The urethral glands are present in several species of bats, but is absent in others (Table 2). They consist of prominent tubuloalveolar glands embedded in the connective tissue surrounding the urethra (Matthews, 1941; Mokkapati and Dominic, 1977; Krutzsch and Crichton, 1987; Krutzsch and Crichton , 1990). The tubuloalveoli are lined with very tall, simple columnar epithelium, with nuclei at the base of the cells and cytoplasm filled with fine granules (Matthews, 1941; Krutzsch and Crichton, 1987). The lumen contains homogenous, fine, granular eosinophilic secretion (Krutzsch and Crichton, 1987).

The large urethral gland present in some species is apparently peculiar to chiropterans. Its presence is possibly correlated with the formation of a large vaginal plug in the female at insemination; however this function has not been proven (Matthews, 1941).

4.6. Seasonal Variation

The accessory complex gland of bats undergoes seasonal variation, which may be synchronous or asynchronous in relation to the testicular cycle. The accessory glands of *Macrotus waterhousii, Scotophilus heathi* and *Artibeus lituratus* undergo annual cycles in synchrony with the testis (Krutzsch, 1975; Krishna and Singh, 1998; Oliveira, non-published data). Also, the prostate, ampullary and Cowper's glands show seasonal variation, according to the testicular cycle of *Rhinopoma kinneari*, as they hypertrophy when Leydig cells are active in hormonal secretion, and become regressed when the testis are quiescent (Anand Kumar, 1965). Differing from other hibernating vespertilionid species, the accessory glands of Australian *Miniopterus schreibersii* regress during winter in synchrony with testis involution (Richardson, 1977; Krutzsch and Crichton, 1990).

However, in most hibernating species, there is asynchrony between testis and accessory gland activity. In this case, the glands remain maximally developed and secretorally active during mating and hibernation periods (through winter and early spring), after spermatogenesis has terminated, and Leydig cells are involuted (this occurs in later summer/early fall).

The glands regress during arousal (at spring) reaching minimum weight in late spring/early summer, when spermatogenesis occurs.

The gland weights are recovered in August/September reaching a maximum at autumnal copulation. (Racey, 1974 - *Nyctalus noctula*; Racey and Tam, 1974 - *Pipistrellus pipistrellus;* Gustafson, 1979; Richardson, 1977 - *Miniopterus australis*; Hosken et al., 1998 - *Nyctophylus geoffroyi; N. major* and *Vespadelus regulus*). The inactive glands are distinguished by the reduced size, empty alveoli and more conspicuous stroma, contrasting with active glands, which presents alveoli filled with secretion and the stroma is inconspicuous (Racey and Tam, 1974; Krutzsch, 1975; Krutzsch and Crichton, 1987; Krutzsch and Crichton , 1990).

The mechanism regulating the asynchrony between primary and secondary reproductive functions is intriguing and still not solved. There is evidence that a peak on the plasm testosterone levels occurs in the spermatogenic period which is the highest among mammal species (Gustafson and Shemesh, 1976). Therefore, even the lowered level of androgen occurring at hibernation would be enough for the maintenance of the accessory sex gland (Gustafson, 1979). It has also been proposed that the body metabolic reduction at hibernation may contribute for retarding the gland's physiological regression (Gustafson, 1979).

5. HORMONAL REGULATIONS

Chiroptera is an order of mammals with many unusual reproductive strategies, including some unique peculiarities, which depends fundamentally on endocrine regulation. Despite this fact, studies about the regulation of male reproductive function are scarce, outdated and mainly concentrated in hibernating species belonging to the Vespertilionidae and Rhinolophidae families. However, there are numerous critical pathways of hormonal regulation that are still not explored even in these animals. On the other hand, there are interesting reproductive strategies for non-hibernating bats that inhabit tropical regions, whose hormonal regulation remains unknown.

Concerning reproductive function, the hypothalamic-pituitary-gonadal axis is crucial in controlling each step of this complex process. The hypothalamus produces several neuropeptides, including the gonadotropin releasing hormone (GnRH), which stimulates the hypophysis to secrete gonadotropins that acts in the testis, modulating both spermatogenic and steroidogenic functions.

5.1. GnRH

The hypothalamic GnRH is a key regulator of reproductive function in all mammalian groups, including bats. This hormone is responsible for stimulating the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from gonadotropes located in the *pars distalis* of the adenohypophysis. By acting through the GnRH receptors on the plasma membrane of gonadotropes, GnRH stimulates a cascade of intracellular events, such as an increase of intracellular calcium and activation of protein kinase C, responsible for the synthesis and secretion of the gonadotropins. This primaryhormonal control is required for the initiation and maintenance of spermatogenesis, which involves the action of FSH and testosterone acting in concert on Sertoli cells, whereas LH modulates steroidogenesis in Leydig cells (Gnessi et al., 1997).

In several Vespertilionidae species, the hypothalamic GnRH neurons are fusiform or bipolar, mostly located in the medial basal hypothalamus, especially in the arcuate nucleus, a feature that resembles primates, including humans (King et al., 1984; (Oelschlager and Northcutt, 1992; Kawamoto, 2003). Conversely, GnRH neurons in hibernating Rhinolophidae bats are concentrated in the medial preoptic area and medial basal hypothalamus (Kawamoto et al., 1998; Kawamoto, 2003). It is noteworthy that GnRH neurons of the medial basal region vary seasonally in number and immunoreactivity in both Vespertilionidae and Rhinolophidae hibernating bats, thus indicating that these neurons play a key role in the seasonal variation of gonadotropins secretion in these bats (Kawamoto, 2003).

Seasonal changes in the release of gonadotropin required for gametogenesis and gonadal steroidogenesis is observed in hibernating bats (Kawamoto et al., 2000). The percentages of LH- or FSH-secreting cells and the secretory activity of gonadotropin, as well as the responsiveness of gonadotropes to GnRH increase in the spermatogenic period (summer), as seen in *Rhinolophus ferrumequinum* (Kawamoto et al., 2000). During the hibernation, the secretory activity of gonadotropes decreases markedly, but the stimulatory effect of GnRH on the secretion of gonadotropins is relatively persistent. This data suggests that GnRH receptors and the post-receptor events that mediate GnRH action on basal gonadotropin secretion do not disappear under heterothermic conditions of bats (Kawamoto et al., 2000).

5.2. Gonadotropins

The testicular functions are dependent on the gonadotropins FSH and LH secreted from the *pars distalis* of the adenohypophysis, under stimulation of GnRH. Most individual gondotropes of bats produce both FSH and LH, even though some cells contain only one of these hormones within each cell (Richardson, 1979; Mikami et al., 1988; Bernard et al., 1991). The male gonadotropes are large, oval, rounded or irregular in shape, exhibiting cytoplasmic processes which surround other secretory cells of the gland (Anthony and Gustafson, 1984; Mikami et al., 1988).

In bats, the secretion of FSH in response to GnRH is increased during the spermatogenic period compared to hibernation (Hayashi et al., 2002; Kawamoto, 2003). FSH indirectly influences spermatogenesis by modulating the functions of Sertoli cells (Richardson, 1979; Francis et al., 1981). The response of Sertoli cells to FSH is mediated by cyclic adenosine 3', 5'-monophosphate (cAMP) and/or possibly Ca^{2+} as second messengers.

The testicular gonadotropin receptors are crucial for the gonadal responsiveness to gonadotropin. Information about LH receptors in the bat testis was not found in the current literature. The receptors for FSH are expressed in Sertoli cells, as observed in rhinolophid bats (Hayashi et al., 2002). The concentration of FSH receptors in the testis varies in accordance with the reproductive cycle. During the breeding season, high concentrations of a FSH receptor is detected in the Sertoli cells, whereas during hibernation, the receptor concentration is low (Hayashi et al., 2002). These seasonal fluctuations in the number of FSH receptors in the testes are accompanied by corresponding changes in the responsiveness of the testes to FSH stimulation. Accordingly, the production of cAMP in response to FSH is far less in the hibernation period than in the spermatogenic period. These findings suggest that during hibernation, the number of FSH-binding sites per Sertoli cell in the bat testis decrease significantly, parallel to a decrease in the activity of the adenylate cyclase system stimulated by the FSH-receptor complex (Hayashi et al., 2002).

LH is responsible for stimulating the synthesis of testosterone by Leydigs cells, thus indirectly regulating spermatogenesis by increasing intratesticular testosterone. In hibernating Vespertilionid and Rinolophid species, the LH level is closely associated with the reproductive cycle and the testosterone levels. Secretion of LH is higher during spermatogenic and mating periods, whereas its level decreases significantly during hibernation and post-arousal, when the testosterone levels are lower (Gustafson, 1979; Kawamoto, 2003). Similar patterns of synchrony between LH secretion and Leydig cell and seminiferous epithelium activity is described for seasonal non-hibernating species (O'Brien et al., 2003 - *Pteropus poliocephalus*). Contrasting with activity, gonadotropes producing LH is detected in the *pars distalis* of male bats throughout the year apparently with constant size and shape (Anthony and Gustafson, 1984; Mikami et al., 1988).

It is interesting that in some species of hibernating vespertilionid, gonadotropes reactive to LH and FSH are found in the *pars tuberalis* besides the *pars distalis* of the adenohypophysis (Anthony and Gustafson, 1984; Mikami et al., 1988; Azzali et al., 2003; Kawamoto, 2003). The LHβ-positive cells are present during recrudescence, spermatogenic and hibernation periods, but are not detected in the mating period, thus indicating that this gonadotropin secretion is increased in autumn (Kawamoto, 2003). The functional activity of the bat *pars tuberalis* gonadotropins is presently unknown.

5.3. Androgens

Androgens are steroid hormones essential for the maintenance of male reproductive functions, whose actions are mediated by the androgen receptors (AR). These receptors are widely distributed in the male genital system and have been described in several species of wild and domestic animals. Nevertheless, only recently AR were mapped in the testis of *Artibeus lituratus*, which is a tropical bat presenting marked reproductive seasonality, with a period of testicular activity from August to December and regression from December to April (Oliveira et al., 2009). During the reproductive period, the AR are restricted to Sertoli cells and their expression is stage-specific, being more prominent during stages VII-VIII, in which spermatogenic cells associated with Sertoli cells are in a more differentiated phase. In the regressive testis, the AR expression remains restricted to Sertoli cells, but immunostaining is intense in all seminiferous tubules, despite the low levels of plasma testosterone (Oliveira et al., 2009).

The main androgen produced by Leydig cells under LH stimulation is testosterone, which is responsible for the initiation and maintenance of spermatogenesis. Besides control of spermatogenesis, testosterone is also involved in the regulation of gonadotropin release, both directly or indirectly via aromatization to estrogens (Hayes et al., 2001). In all bat species analyzed to date, the testosterone levels are directly related to the spermatogenic function, being higher at this period (Gustafson and Shemesh, 1976; Gustafson, 1979; Gustafson and Damassa, 1985; Bernard, 1986; Singh and Krishna, 2000), except for the Tropical *Taphozous georgianus* in which spermatogenesis occurs in summer/ autumn but the peak of testosterone level is found during winter (Jolly and Blackshaw, 1989). In hibernating bats (Vespertilionidae and Rhinolophidae), testosterone concentrations are elevated in the spermatogenic period but rapidly decrease in the mating period, reaching minimal values during hibernation (Hayashi et al., 2002). The androgen level at the spermatogenic period of the hibernating bat is the highest known for mammals (Table 3) (Gustafson and Shemesh, 1976; Gustafson, 1979). It is curious that in these species there is a marked asynchrony between spermatogenesis, that occurs under high testosterone levels in the summer, and the activity of sex accessory glands, that occurs during winter, when the testosterone level is low (Gustafson and Shemesh, 1976; Gustafson, 1979, 1987; Hayashi et al., 2002). However, there is evidence that even in hibernating species, steroidogenic enzymes can be detected in the regressed Leydig cells, as seen for the key enzyme 3β-HSD. According to Kurohmaru et al (2002), the Leydig cells of *Rhinolophus cornutus* are positive for 3β-HSD throughout the year, being more intense just prior to the beginning of spermatogenesis and weaker after spermatogenesis and during hibernation. *Nyctalus noctula* also show little variation in Leydig cell morphology and 3β-HSD expression throughout the year (Racey, 1974).

Table 3. Blood testosterone levels in bat species

Suborder/ Families/ Species	Spermatogenic period	Dormance	Hibernation	After hibernation/ dormance	References
MICROCHIROPTERA					
Emballonuridae					
Taphozous longimanus	22.46 ng/ml	6.91 ng/ml	No	ND	Singh and Krishna, 2000
Rhinolophidae					
Rhinolophus capensis	6.2 ng/ml	No	0.2/3.2 ng/ml	3.5 ng/ml	Bernard, 1986
Vespertilionidae					
Myotis lucifugus	60 ng/ml	No	2.5 ± 0.50/ 9.4 ± 3.4 ng/ml	2.8 ±0.4 ng/ml	Gustafson and Shemesh, 1976
Myotis lucifugus	105.4 ±19.3 ng/ml	No	2.0±0.2 ng/ml	2.0±0.2 ng/ml	Gustafson and Damassa, 1985
Nyctalus noctula	134 ng/ml	No	19 ng/ml	73 ng/ml	Racey, 1974*
Scotophilus heathi	19.32±1.14 ng/ml	5.4±0.94 ng/ml	No	4.31±0.19 ng/ml	Singh and Krishna 1996

ND = Not determined; * = animals in captivity.

Data about other androgens, such as dihydrotestosterone (DHT) and androstenedione, is scarce. Available information for the Australian Tropical common sheated-tail bat *Taphozous georgianus* shows that DHT and androstenedione levels are high compared to testosterone, but no significant seasonal changes is observed for these testosterone metabolites (Jolly and Blackshaw, 1989). Androstenedione peaks are parallel to testosterone, before the onset of spermatogenesis, and show stronger correlation with body mass than with size of the reproductive organs (Racey and Tam, 1974; Singh and Krishna, 1996; Singh and Krishna, 2000).

5.4. Estrogens

Estrogens are now recognized as a hormone of great relevance for maintenance of the male genital system morphophysiology and fertility. In males, the testis is the main source of estrogens and their biosynthesis occurs from androgens, in a reaction catalyzed by the P450 aromatase enzyme (Simpson et al., 1997; Carreau et al., 2002). The estrogen actions are mediated by the estrogen receptors ERα and ERβ, which are widely distributed in the male genital system of several mammalian species (Hess, 2003). In bats, estrogen level is very low (<10pg/ml) or indetectable in males of *M. lucifugus*, during the breeding season (Damassa and Gustafson, 1984). There is no information about local estrogen concentration in the male tract. Nevertheless, one recent study described the occurrence and distribution of ERα and ERβ in the testes of *Artibeus lituratus* (Oliveira et al., 2009). In this species, immunoreactivy for ERα is restricted to some nuclei of Leydig cells, during both reproductive and regressive periods. Conversely, ERβ are widely distributed in the somatic and spermatogenic cells of the testis. In the reproductive period, the ERβ distribution in the seminiferous tubules is stage-specific. In the regressive period, more intense immunostaining is observed for ERβ in both Sertoli and Leydig cells (Oliveira et al., 2009). Variations in the estrogen receptor levels during the annual reproductive cycle of *A. lituratus* indicates that estrogens may indeed play an important role in the hormonal regulation of the reproductive cycle of bats.

Interestingly, there is information that only in bats and primates, including humans, the steroid hormone binding globulin (SHBG) bind to estrogens with a higher affinity than androgens (Damassa et al., 1982; Damassa et al., 1985; Kwiecinski et al., 1987). This is in contrast with most mammals in which SHBG presents higher affinity for testosterone and dihydrotestosterone. SHBG is responsible for the regulation of steroid-free concentration, protecting them from degradation and excretion, therefore the higher affinity of this glycoprotein for estrogen may explain the low levels of circulating hormones in bats. This intriguing feature of SHBG was studied in some species of bats and a specific SHBG was identified in some Phyllostomid and Vespertilionid (Kwiecinski et al., 1987). However, most of these studies were discontinued, so the precise role of SHBG in bats and its relationship with estrogen activity has not been fully elucidated.

6. REPRODUCTIVE PATTERNS

The wide geographical distribution is in part a consequence of a high reproductive success of bats, even though most species produce just one litter per gestation. To ensure this reproductive success, bats adopt some interesting and often unique strategies only observed in this order. One strategy to adapt to different climates is the existence of hibernating and non-hibernating species usually inhabiting temperate and tropical regions, respectively. The difference in geographical distribution implies that some families or even genus of bats may present hibernating and non-hibernating representants, which may differ in the reproductive pattern.

Distinct reproductive patterns are important to reconcile the mating and the hibernation period with the reproductive cycle of females, ensuring that the litters are born in a period of greater availability of food, contributing to reproductive success (Gustafson, 1979; Oxberry, 1979; Kawamoto, 2003).The same diversity applies to non-hibernating species, which may be seasonal or non-seasonal in spite of the family or genus to which they belong.

The hibernating male bats belonging to Vespertilionidae and Rhinolophidae families present two different reproductive patterns, determined based on the duration of spermatogenesis in relation to mating and hibernating periods. In all patterns, the spermatogenesis occurs during summer, but the period of activity of the Leydig cells and male genital accessory gland is variable (Gustafson, 1979). In some species, the mating occurs shortly after spermatogenesis, before hibernation or torpor. These species usually store sperm in the female genital tract or delay the implantation. This pattern of reproduction, characterized by mating after spermatogenesis, is described in *Nyctalus noctula* (Racey, 1974), *Pipistrellus pipistrellus* and *P. hesperus* (Racey and Tam, 1974; Krutzsch, 1975), *Myotis lucifugus* (Gustafson, 1979; Gustafson, 1987), *Pipistrellus subflavus* (Krutzsch and Crichton, 1986), *Miniopterus schreibersii* (Richardson, 1977; Gustafson, 1979) and *Rhinolophus ferrumequim* (Hayashi et al., 2002). Other species mate after hibernation; therefore, they present prolonged sperm storage in the male epididymis, which will be used in mating occurring during or after arousal from hibernation, as seen in *Rhinolophus capensis* (Bernard, 1984; Bernard, 1986).

Although the bats belonging to the Rhinolophidae and Vespertilionidae families are mainly hibernating, they also present members that are non-hibernating with reproductive seasonal patterns like Rhinolophidae members: *Hipposideros caffer* (Nigeria), *H. commersoni* (Africa), *Rhinolophus landeri* (Nigeria) and *Triaenops afer* (East Africa); Vespertilionidae members: *Eptesicus furinalis* (Paraguai), *Lasiurus ega* (Paraguai), *Miniopterus australis* (Australia), *Myotis nigricans* (Panama), *Tylonycteris pachypus* (Malaysia) and *T. robustula* (Malaysia) (Richardson, 1977; Krutzsch, 1979).

Non-hibernating bats inhabiting regions where the seasons are poorly defined also present seasonality associated with various reproductive strategies. In these bats, the reproductive cycle is also directly linked to environmental conditions, especially in relation to rainfall (Fleming et al., 1972; Krutzsch, 1979). In some species of the family Pteropodidae (*Eonycteris spelaea, Epomorphorus labiatus, Myonyscteris torquata, Stenonycteris lanosus*), Nicteridae (*Nycteris hispida* and *N. luteola*) and Phyllostomidae (*Artibeus jamaicensis, Desmodus rotundus* and *Micronycteris megalotis*), the testis and accessory sex glands are

active throughout the year. However, it is not known whether all individuals maintain the reproductive activity during the year (Krutzsch, 1979).

Seasonality in non-hibernating bats is observed in members of Pteropodidae (Eidolon helvum, Pteropus eotinus, P. geddei, P. giganteus, P. paliocephalus, P. scapulatus), Megadermatidae (Macroderma gigas, Megaderma lyra and Megaderma spasma), Natalidae (Natalus stramineus - Krutzsch, 1979), Molossidae (Otomops martiensseni, Tadarida (=Mops) condylurus, Tadarida (=Mops) longica, Tadarida (=Mops) nanulus, Tadarida (=Chaerephon) hindei, Tadarida (=Chaerephon) pumila and Tadarida brasiliensis - Aoki, 1997; Krutzsch, 1979) and Phyllostomidae (Anoura geoffroyi, Choeronycteris Mexicana, Leptonycteris curasoae, Macrotus californicus and Pteronotus parnellii - Krutzsch, 1979; Artibeus lituratus - Oliveira et al., 2009). In these species, testis and accessory sex glands activities are synchronous. In contrast, in the non-hibernating Emballonuridae Taphozous georgianus there is asynchrony between spermatogenesis and accessory sex gland activity (Jolly and Blackshaw, 1987; Jolly and Blackshaw, 1989).

CONCLUSION

The male bats present many reproductive particularities and strategies that enable them to have high reproductive success even when inhabiting regions inaccessible for other mammals. The peculiarities include testicular regression after completion of spermatogenesis, prolonged retention of viable sperm in the epididymis, asynchrony between spermatogenesis, genital glands activity and mating period. These reproductive adaptations raise several questions concerning physiology and neuroendocrine regulation of the male genital organs. On the other hand, bats present several similarities with primates, including men, suggesting that they may provide a useful model for study of some aspect of the reproductive functions. Additionally, the species that reproduces seasonally represent excellent models for the study of several aspects of the male reproduction, without interference of surgical, chemical or genetical manipulations that could interfere with other physiological events. Together, these advantages indicate that studies on the male reproductive system of bats may bring new perspectives for the reproductive biology of mammals, thus justifying an effort of investigators worldwide to go deeper on the studies of this peculiar order of mammals and insist on the possibility of maintaining breeding colonies in controlled environments that could ensure a more experimental than descriptive design of investigations.

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ARTIGO 1

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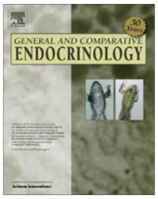

Seasonal variation in estrogen receptor ERα, but not ERβ, androgen receptor and aromatase, in the efferent ductules and epididymis of the big fruit-eating bat *Artibeus lituratus*

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ABSTRACT

The efferent ductules (ED) are a major target for estrogens, which act via the estrogen receptors ERα (ESR1) and ERβ (ESR2). ERα has been found in the ED of all species studied so far. However, in the epididymis (EP), the expression of ERα is controversial, as is data about the occurrence of aromatase in the epithelium lining the excurrent ducts. Therefore, to further investigate this estrogen-responsive system, we used a seasonal breeder, the Neotropical bat, *Artibeus lituratus*, in which testicular expression of androgen (AR) and estrogen (ER) receptors vary with reproductive phase. The localization of aromatase, ERα, ERβ and AR in the ED and EP of *A. lituratus* was investigated. The results showed that aromatase, AR and ERβ were distributed throughout the excurrent ducts and did not vary during the annual reproductive cycle. Conversely, ERα was detected primarily in the ED epithelium, had marked seasonal variation and was increased during regression, especially in the EP epithelium. The results suggest that ERα may be involved in preparing the male genital tract for recrudescence. Together, the data obtained under natural conditions emphasize that specific segments of the excurrent ducts downstream of the testis are the primary targets for estrogen action via ERα, which is similar to previous findings in animals lacking functional ERα.

1. Introduction

The aromatization of androgens to estrogens by cytochrome P450 aromatase is crucial for the maintenance of male fertility. In males, estrogen biosynthesis occurs mainly in the testis, where aromatase has been largely detected in the Leydig cells, as well as in germ cells and released sperm [revised by [7]. Aromatase expression in the genital tract has been controversial. Earlier studies described the expression and activity of aromatase as restricted to sperm traversing the excurrent ducts [24,25]. Others have found aromatase in the epithelial cells lining the efferent ductules and epididymis of a few species; however, some of these studies were performed only at the mRNA level [6,17,39,44]. The presence of aromatase mRNA does not necessarily correlate with the presence of the protein. For instance, it was previously demonstrated that the presence of aromatase mRNA in the germ cells of the testis can precede the protein and that the protein may remain in the tissue in the absence of the mRNA [19]. Therefore, to better elucidate the presence of aromatase in other species, it would be important to clarify whether the epididymis has the ability to produce estrogen.

Estrogens act via the nuclear receptors ERα (ESR1) and ERβ (ESR2), which are expressed in the male genital system in an organ- and species-specific manner [21,28]. ERβ has a widespread expression in the epithelium and connective tissue cells along the male genital tract. Concerning ERα, there is consensus in the literature that it is mainly confined to the efferent ductules [14,16,21,22,28,32,33,40]. In agreement with the protein data, the ERα mRNA concentration in this segment exceeds those of the classic estrogen targets such as the uterus [22]. ERα plays a major role in modulating local fluid reabsorption, an essential function for male fertility and genetic or chemical disruption causes severe abnormalities in the structure of the efferent ductules, thus affecting the reabsorption function [20,35,37]. As a consequence, there is local fluid accumulation that backs up to the testis, which leads to testicular atrophy and infertility. There is evidence that ERα modulates the expression of key proteins involved in fluid reabsorption, such as Na^+–H^+ exchanger 3 (NHE3) and aquaporin 9 [34,37,57].

In contrast to the efferent ductules, the expression of ERα in other segments of the male genital system is still a matter of debate, as it was detected in some mammalian species [17,32,58] but not in others [14,16,21,22,28,40]. These controversial data

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about the ERα expression have made it difficult to elucidate the functional role of estrogens in the epididymis. Moreover, there are no data about estrogen receptors in the male genital tract of Chiroptera.

Little is known about the physiology of the male reproductive system of bats that inhabit tropical regions, especially concerning their hormonal regulation. Recently, it was demonstrated that Neotropical bats from the *Artibeus lituratus* species present considerable variation in the testicular expression of androgen and estrogen receptors during the annual reproductive cycle [38]. However, information about the occurrence of estrogen receptors in the excurrent ducts of bats is still lacking. Additionally, there is no information about aromatase in the male genital system of Chiroptera. This enzyme is important in regulating the reproductive cycle in other seasonal animals [5,47,55]. Furthermore, there is evidence that bats, in addition to primates including man, are unique among mammals, as they present steroid hormone-binding globulin (SHBG) with a higher affinity for estrogens than androgens [9,30]. All these features make bats an appropriate model for studies on estrogen in male reproduction.

Therefore, as a seasonal breeder with natural variations in ER levels, *A. lituratus* is an interesting model for elucidating the role of estrogen in the male without interference from other physiological systems, as occurs in knockout or castrated and chemically treated models. Therefore, the purpose of this study was to investigate the expression and possible alteration in the level and cellular distribution pattern of the P450 aromatase enzyme, ERα and ERβ in the male genital tract of big fruit-eating bat *A. lituratus* by comparing the reproductive and non-reproductive periods. As a primary steroid hormone regulating the male system, the distribution of androgen receptors was also assessed.

2. Materials and methods

Adult male bats of the *A. lituratus* species were captured during the reproductive (August to beginning of December) and regressive (middle of December to early April) periods. The captures were carried out in Belo Horizonte (19° 55′S and 43° 56′W) in southeastern Brazil. The captures were performed using mist nets (3×12 m) that were placed on trails of flight pathways to intercept bats flying 1–2 m above the ground.

The captured males were considered adults when they possessed teeth wear and a complete ossification of the metacarpal epiphyseal. These parameters are routinely used for determining the age of bats [38]. The capture and all experimental procedures were authorized by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA, Brazil) and the Comitê de Ética em Experimentação Animal (CETEA) da Universidade Federal de Minas Gerais (UFMG).

2.1. Tissue preparation

The bats were weighed and anesthetized with 30 mg/kg pentobarbital and 20 mg/kg ketamine chloridrate (i.p.). The perfusion was performed transcardially via the left ventricle with 10% neutral buffered formalin (NBF) for the immunohistochemical assay or 2.5% glutaraldehyde in 0.1 M phosphate buffer for the histological studies. After fixation, the efferent ductules and epididymis were removed and weighed together.

2.2. Microdissection

Microdissection, in association with a procedure for the differentiation of epithelial derivatives [50], was performed to describe the number and anatomical organization of the efferent ductules. This procedure allows a better visualization of the epithelial tissues that are intensely stained by Ehrlich's acid hematoxylin, while the connective tissue is slightly stained, which facilitates the microdissection. For this purpose, NBF-fixed efferent ductules were macerated by denaturation in an aqueous 4% KOH solution under agitation until the tissues became translucent. After maceration, the tissues were immersed in an aqueous clearing solution containing glycerin (1 part) and glacial acetic acid (1.5 parts) for 12 h. Then, the efferent ductules were stained with Ehrlich's hematoxylin for approximately 2 h with two changes of the staining solution. After staining, the efferent ductules were rinsed in the clearing solution for 3 min and transferred to an aqueous solution of glycerin (1 part) for 4 h.

2.3. Histology

The efferent ductules and epididymis were dehydrated in graded ethanol solutions (50–100%), embedded in glycol methacrylate (Technovit 7100; Heraeus Kulzer, Germany), sectioned at 3 μm and placed in glass slides. The sections were stained with hematoxylin and eosin (HE), and toluidine blue/sodium borate (1%) for histological analysis. To evaluate the presence of glycoconjugates, such as those present in lysosomes [23], the sections were also stained by periodic acid-Schiff reactive (PAS) and counterstained with hematoxylin.

2.4. Morphometry

The epithelial height and external diameter of efferent ductules and the epididymal duct were measured on histological sections of 04 animals in the reproductive and regressive periods. For this purpose, digital images were obtained using a Nikon Eclipse E600 microscope (Nikon Co., Melville, NY) and analyzed with Image Tool software (University of Texas Health Sciences Center, San Antonio, TX). The epithelial height was measured from the basement membrane to the microvillus base in twenty-five cells of each animal in straight sections with evident nuclei [35]. The external tubular diameter was measured in five randomly chosen transversal profiles of efferent ductules and the epididymal duct from the initial segment, caput, corpus and cauda regions.

The number of ERα positive cells per area of efferent ductules (non-ciliated, ciliated, smooth muscle and connective cells) and in the epididymis (principal, basal, smooth muscle and connective cells) ($n = 4$ for each period) was estimated in four randomly selected sections of each segment. In each section, the positively stained nuclei were counted at 400× magnification in a total area of 0.25 mm^2 by using a grid divided into 100 squares, which was coupled to the eyepiece of the microscope. The results were converted to cells per mm^2 and statistically analyzed, according to Aherne and Dunnill [1].

2.5. Immunohistochemistry

Fragments of NBF-fixed efferent ductules and epididymis ($n = 5$) were dehydrated in graded ethanol solutions (50–100%), embedded in paraffin and sectioned at 5.0 μm. Immunohistochemistry was performed to detect the occurrence and cell distribution of aromatase, estrogen receptors (ERα and ERβ) and androgen receptors (AR). For this purpose, the sections were deparaffinized, hydrated and immersed in a 0.6% methanol hydrogen peroxide solution for blocking the endogenous peroxidase. After microwaving for antigen retrieval, the endogenous biotin activity was blocked with avidin and a biotin-blocking solution (avidin/biotin blocking kit; Vector Laboratories, Burlingame, USA). Prior to incubation with the primary antibodies, normal goat serum (10%) was used for blocking the nonspecific antibody binding. Then, the sections were incubated overnight at 4 °C with mouse anti-aromatase (AbD Serotec, Oxford, UK; diluted 1:50), mouse anti-human ERα (Novocastra Laboratories, Newcastle, UK; diluted 1:100), mouse anti-human ERβ (Novocastra Laboratories, Newcastle, UK) diluted 1:25 and rabbit anti-human AR (PG21; Upstate, Lake Place, NY; diluted 1:500) primary antibodies. The titre and specificity of the antibodies have previously been standardized for *A. lituratus* [38]. The negative control sections received phosphate- buffered saline (PBS) instead of the primary antibody. After washing in PBS, all sections were exposed for 1 h to biotinylated secondary antibodies (Dako, Carpinteria, USA), including goat anti-mouse for aromatase, ERα and ERβ and goat anti-rabbit for AR. The sections were incubated with the avidin–biotin complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, USA) for 30 min, and the immunoreaction was visualized using 3,3 diaminobenzidine containing 0.01% hydrogen peroxide in 0.05 M Tris–HCl buffer, pH 7.6. Mayer hematoxylin was used to counterstain the sections. The immunostaining was performed in triplicate to confirm the results.

2.6. Western blotting

Western blot assays were performed on the efferent ductules and epididymis (caput, corpus and cauda) to confirm the immunohistochemistry results. For this purpose, pooled tissues of six animals in the reproductive and regressive periods were used.

The efferent ductules and epididymis tissues were macerated with dry ice, and the total protein was extracted with a sucrose buffer (pH 7.4) containing 0.01 M EDTA and a protease inhibitor cocktail (Sigma–Aldrich, St. Louis, USA). The proteins were quantified by a Bradford protein assay using Proteoquant (Proteobras, Paulínia, BR). The proteins (20 μg/lane) were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), transferred to nitrocellulose membranes and blocked with 10% normal goat serum (NGS) for 1 h at room temperature. The membranes were incubated overnight with primary antibodies diluted 1:300 for mouse anti-human ERα (Novocastra, Newcastle, UK) and rabbit anti-human AR (Upstate, Temecula, EUA) or 1:150 for mouse anti-aromatase (AbD Serotec, Oxford, UK) and mouse anti-human ERβ (Novocastra, Newcastle, UK). Then, the membranes were washed with PBS-Tween 0.05% followed by incubation with the secondary antibodies (goat anti-mouse for aromatase, ERα and ERβ and goat anti-rabbit for AR; Dako, Carpinteria, CA) diluted 1:1000. After several washes in PBS-Tween 0.05%, the reaction was developed by the addition of 0.1% of 3,3 diaminobenzidine and 0.05% of chloronaphthol in PBS, 16.6% methanol and 0.04% H_2O_2. Deionized water was used to stop the reaction. All Western blots were replicated and the densities of the bands obtained estimated by Image-Tool software 3.00 (University of Texas Health Sciences Center, San Antonio, USA).

2.7. Statistical analysis

The values obtained from the organ weights and morphometrical studies were analyzed using the software Statistica 7.0 for Windows (StatSoft Inc., Tulsa, OK, USA). Initially, the data were subjected to the Shapiro–Wilk W normality test followed by a Student's *t*-test analysis. A normal distribution of the data was considered if $P \geqslant 0.05$, and differences between groups were considered statistically significant when $P \leqslant 0.05$.

3. Results

3.1. Efferent ductules

In common with large mammals and primates [23], the efferent ductules of *A. lituratus* emerged from the extra-testicular *rete testis* near the tunica albuginea that connects the testis to the epididymis through 12–15 flexuous ductules (Fig. 1A). The ductules run parallel to each other in the proximal region. They then anastomose,

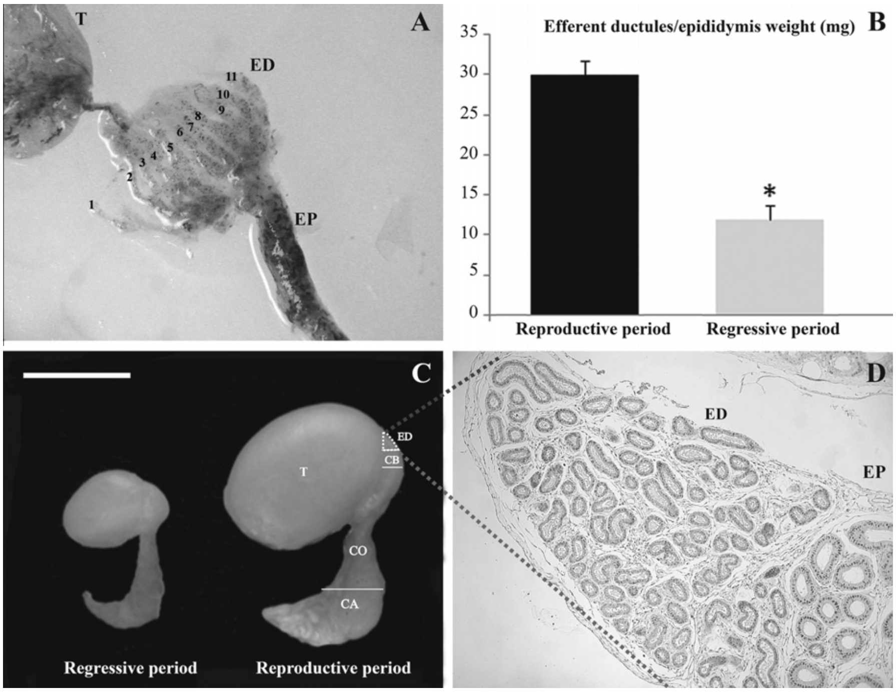

Fig. 1. Anatomical aspects of the efferent ductules (ED) and epididymis (EP) of *Artibeus lituratus*. (A) The ED (numbered 1-11) emerges from the *rete testis* and joins to the epididymis. (B) During the regressive period, there was a significant reduction in the efferent ductules and epididymis weight. $^*P \leqslant 0.05$; $n = 5$. (C) The epididymis was grossly divided into the caput (CB), corpus (CO) and cauda (CA) segments. The epididymis regression occurred simultaneously to the testis regression (T). (D) The efferent ductules compose mostly the epididymis caput. Scale bar in C = 0.5 cm.

Table 1
Epithelial height and external diameter of efferent ductules and epididymis of *Artibeus lituratus* during the reproductive and regressive periods.

Region	Epithelial height (μm)		External diameter (μm)	
	Reproductive period	Regressive period	Reproductive period	Regressive period
Efferent ductules				
Proximal	14.4^{a}	12.0^{*,a}	50.5^{a}	41.7^{*,a}
Distal	13.8^{b}	11.1^{*,b}	42.1^{b}	34.7^{*,b}
Epidydimis				
Initial segment	40.4^{c}	17.9*	125.9	61.4*
Caput	31.3^{d}	17.5*	110.1^{g}	60.5*
Corpus	29.6^{e}	22.0*	128.9	67.8*
Cauda	21.4^{f}	18.2*	133.8	93.7^{*,h}

$n = 4$; *Statistical difference between periods ($P \leqslant 0.05$).
^{a,b} Statistical difference between regions of efferent ductules.
^{c,d,e,f,g,h} Statistical difference between regions of Epididymis.

forming around six terminal ductules that open separately in the epididymal duct.

The efferent ductules were lined by a simple columnar epithelium containing ciliated and non-ciliated cells, with the latter being more abundant. The non-ciliated cells were characterized by the presence of oval nuclei with a basal location and numerous PAS-positive granules dispersed throughout the cytoplasm. The ciliated cells were usually goblet-like in shape and had nuclei that were located more apically than those of the non-ciliated cells. The epithelial height and the external diameter of the ductules were more pronounced in the proximal region than in the distal region (Table 1). Externally, the ductules were surrounded by one or two layers of smooth muscle cells that were concentrically arranged.

During the regressive period, there was a significant reduction in the epithelial height and external diameter of the efferent ductules (Table 1). In these ductules, the PAS-positive granules were scarce in the cytoplasm of the non-ciliated cells and absent in the ciliated cells. The connective tissue that is interspaced on the ductules became more prominent in this period.

3.2. Epididymis

The epididymis of *A. lituratus* weighed approximately 30 mg and presented well-defined anatomical caput, corpus and cauda regions (Fig. 1B and C). The caput of the epididymis was mostly composed of the efferent ductules (described above), which were followed by a short initial segment and the caput proper (Fig. 1C and D). The histological structure of the epididymal duct of *A. lituratus* was similar to that described in other mammals [18]. The epididymal epithelium was pseudostratified and composed of principal, basal, apical, narrow, halo and clear cells. These cells were randomly distributed throughout the epididymal duct. The epithelial height of the epididymal duct varied according to the region (Table 1) and from the initial segment toward the cauda, the epithelial height gradually decreased.

During the non-reproductive period, *A. lituratus* showed an epididymal regression in all segments and the epididymal weight was reduced by approximately 60% (Fig. 1B). The epithelium height was reduced by 55% on average in the initial segment, 44% in the caput, 25% in the corpus and 15% in the cauda (Table 1). The external tubular diameter was also significantly reduced in all regions: 50%, 45%, 47% and 30% for the initial segment, caput, corpus and cauda, respectively (Table 1). Even with the regression, it was possible to distinguish all epithelial cell types identified during the reproductive period. The lumen became reduced without sperm but presented some secretion and sloughed degenerating cells. A pronounced increase in the layers of smooth muscle cells and adjacent connective tissue, especially in the caudal region, was observed.

3.3. Pattern of aromatase distribution

In the efferent ductules, the cytoplasm of non-ciliated cells were positive for aromatase, whereas the ciliated cells were unreactive (Fig. 2A), and the cytoplasm of the principal cells and some basal cells of the epididymis were aromatase positive (Fig. 2C, E, G, I). Cells identified as apical, narrow and halo cells presented immunonegative or just slightly positive cytoplasm. The clear cells were not identifiable with the methods employed in this study. In the epididymal cauda (Fig. 2I) and ductus deferens (data not shown), the epithelial immunostaining for aromatase appeared weaker than in the caput and corpus. Aromatase was not immunodetected in the peritubular smooth muscle cells.

There were no detectable changes in the pattern and intensity of the aromatase reactivity when ducts at the reproductive and regressive periods were compared (Fig. 2).

3.4. Pattern of ERα distribution

During the reproductive period, the nuclei of non-ciliated cells of the efferent ductule epithelium presented an intense immunoreaction for ERα, whereas the ciliated cells were slightly positive for the receptor or negative (Fig. 3A). Rare connective tissue cells and smooth muscle cells surrounding the ductules were slightly positive for ERα (Fig. 3A). The staining of these cells was weak, which is in contrast with the cells of the blood vessels tunica media that were moderately to strongly stained for the receptor (data not shown).

In the regressive efferent ductules, the ERα staining intensity of the epithelial cells was similar to that observed in the reproductive period (Fig. 3B), although the number of positive ciliated cells was significantly increased (Fig. 4A). The number of ERα-positive cells was also increased in the periductal smooth muscle layer and connective tissue (Fig. 4A).

The epididymal duct in the reproductive period was negative for ERα, except for some basal epithelial cells and smooth muscle cells surrounding the ducts that were slightly to moderately positive for this receptor (Fig. 3C, E, G, I). Some connective tissue cells were strongly positive for ERα, especially in the cauda region.

During regression, the immunoreaction for ERα was more extensive in the epididymis compared to the reproductive period, especially in the initial segment (Fig. 3D, F, H, J). In the epithelium, in addition to the intense positivity of the basal cells along the duct, a moderate staining was detected in the principal cells of the initial segment, caput and corpus. The increase in ERα-positive cells in the epithelium of all these segments was statistically significant (Fig. 4B–E). The increase in the number of periductal smooth muscle and connective tissue cells in the regressive epididymis was also significant (Fig. 3D, F, H, J and 4B–E).

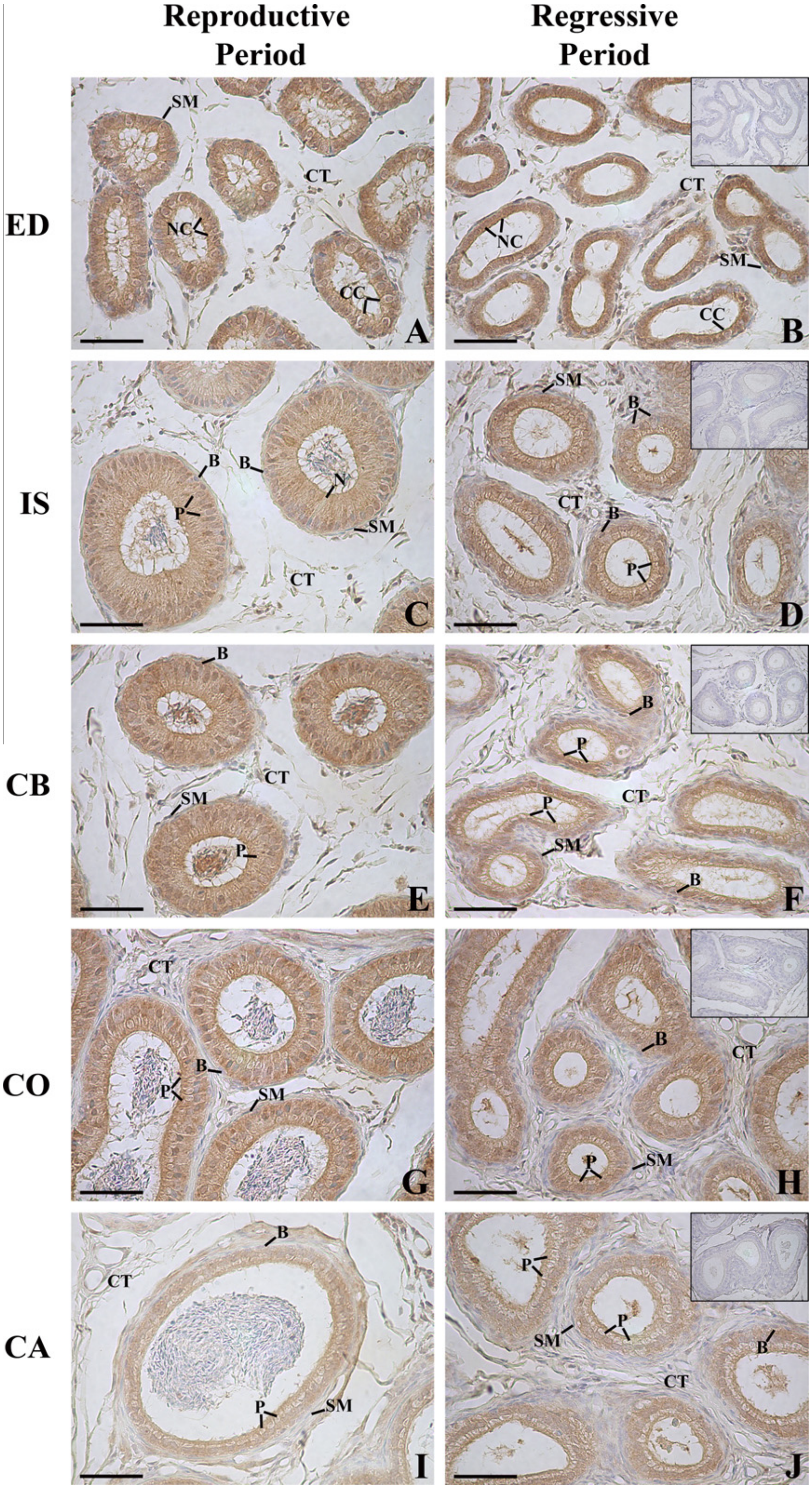

Fig. 2. Immunolocalization of P450 aromatase in the efferent ductules (ED) and epididymis of *Artibeus lituratus* during the reproductive and regressive periods. IS = initial segment; CB = caput; CO = corpus; CA = cauda; NC = non-ciliated cells; CC = ciliated cells; CT = connective tissue; SM = smooth muscle cells; P = principal cells; B = basal cells; and N = narrow cells. Inserts in (B, D, F, H and J) are negative controls. Scale bars in (A–J) = 50 μm.

Similar to the cauda epididymis, the epithelium of the ductus deferens was negative for ERα in both periods analyzed. Conversely, most smooth muscle cells were positive for the receptor. The intensity of the staining was higher in cells during the regressive period (data not shown).

3.5. Pattern of ERβ distribution

In the epithelium of the efferent ductules, both the ciliated and non-ciliated cells were positive for ERβ (Fig. 5A). The intensity of staining for these cells was similar. Along the epididymis (Fig. 5C,

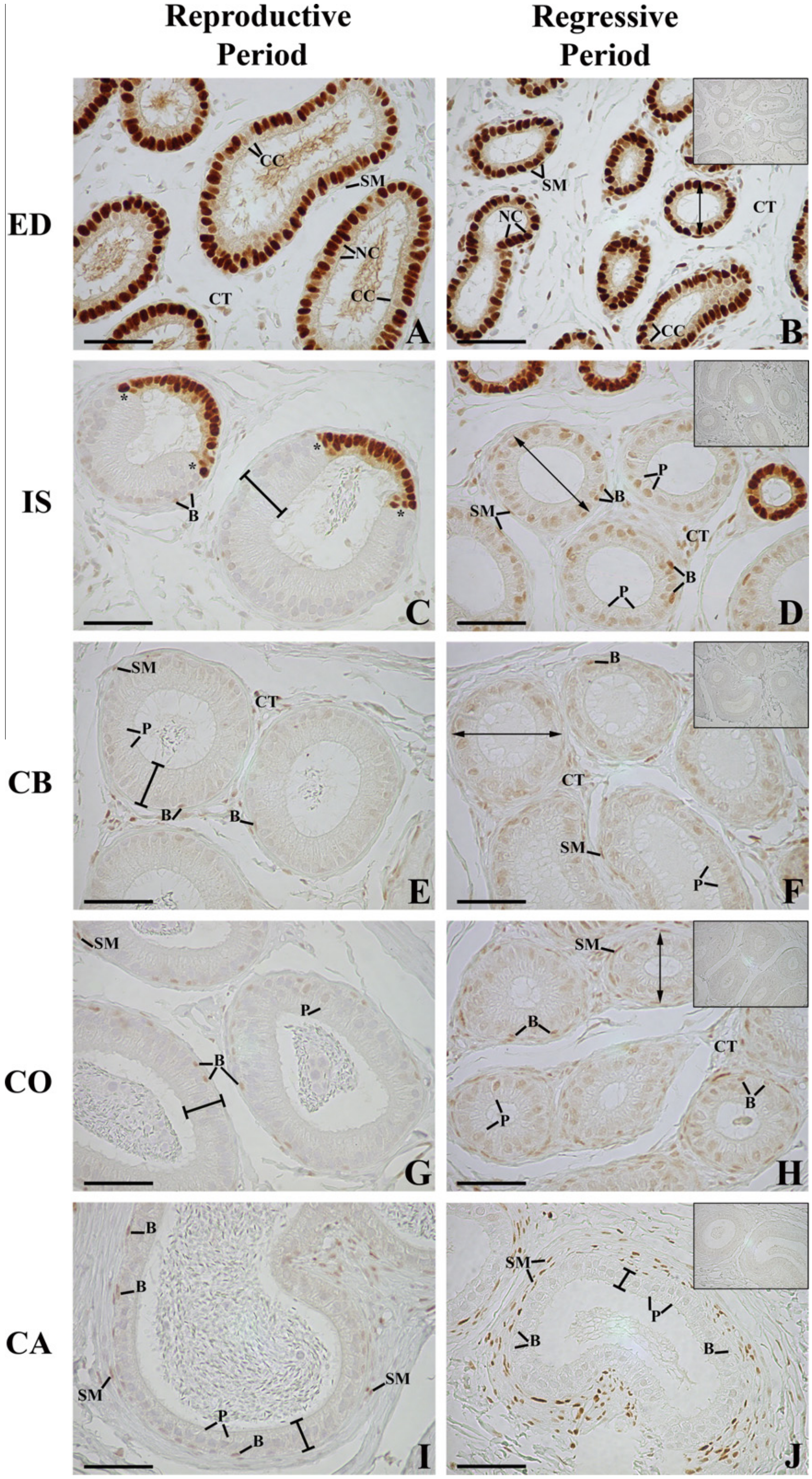

Fig. 3. Immunolocalization of ERα in the efferent ductules (ED) and epididymis of *Artibeus lituratus* during the reproductive and regressive periods. IS = initial segment; CB = caput; CO = corpus; CA = cauda; NC = non-ciliated cells; CC = ciliated cells; CT = connective tissue; SM = smooth muscle cells; P = principal cells; B = basal cells; bar = epithelial height; and double arrows = external diameter; ∗ = transition between efferent ductules and epididymis. Inserts in (B, D, F, H and J) are negative controls. Scale bars in (A–J) = 50 μm.

E, G, I) and ductus deferens (data not shown), all epithelial cells showed a strong positivity for ERβ. Some basal cells were more intensely stained than the other epithelial cells in both segments.

The epithelial ERβ immunoreaction was similar in the efferent ductules, epididymis (Fig. 5) and ductus deferens (data not shown) when the reproductive and regressive periods were compared.

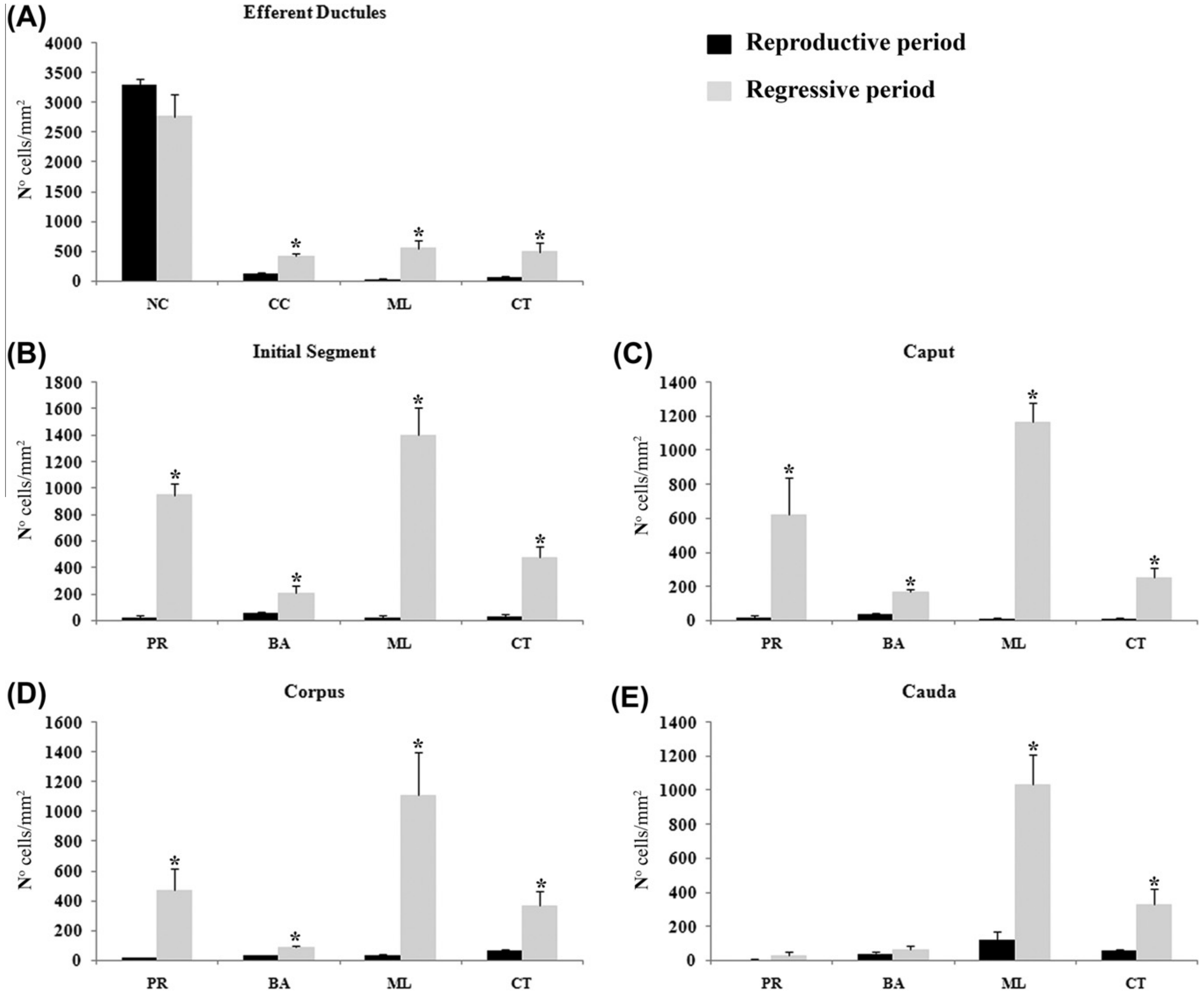

Fig. 4. Statistical analysis of the ERα expression in the efferent ductules and epididymis of *Artibeus lituratus* during the reproductive and regressive periods. NC = non-ciliated cells; CC = ciliated cells; CT = connective tissue; SM = smooth muscle cells; PR = principal cells; and BA = basal cells. $^*P \leqslant 0.05$.

Additionally, most smooth muscle cells surrounding the ducts and the connective tissue cells from the efferent ductules to the ductus deferens were positive for ERβ in both periods analyzed.

3.6. Pattern of AR distribution

During the reproductive period, the nuclei of non-ciliated cells of the efferent ductules epithelium were moderately positive for AR, whereas the ciliated cells were unreactive or weakly stained (Fig. 6A). In the epididymal epithelium, the principal cells presented a more intense reaction for AR, which is in contrast with the basal, apical, narrow and clear cells that were just slightly stained (Fig. 6C, E, G, I). Some basal cells were barely stained for AR. An intense positivity for AR was also detected in the epithelium of the ductus deferens. An intermittent staining for AR was detected in the periductal smooth muscle cells and some cells of the connective tissue along the excurrent ducts (data not shown).

The pattern of AR staining remained similar in the regressive efferent ductules, epididymis (Fig. 6B, D, F, H, J) and ductus deferens (data not shown), compared to those of the reproductive period (Fig. 6A, C, E, G, I).

The results of immunohistochemistry assays for P450 aromatase, ERα, ERβ and AR are summarized in Table 2 and Fig. 7.

3.7. Western blotting assays

The results of the immunohistochemistry for aromatase, ERα, ERβ and AR were confirmed by Western blot assays in which specific bands of 55, 68, 54 and 110 KDa, respectively, were detected (Fig. 8A–D). The expression of ERα was higher in the efferent ductules, especially in the regression. Along the epididymis, the ERα level was more pronounced during regression. The blots revealed similar expressions of aromatase, ERβ and AR in the efferent ductules and diverse segments of the epididymis during both the reproductive and regressive periods.

4. Discussion

This study describes for the first time the occurrence and differential distribution of ERα, ERβ, aromatase and the androgen receptor along the excurrent ducts of a seasonal bat species, *A. lituratus*. It was interesting to find that ERα was the only protein to show variations in expression in the epithelial, peritubular and connective tissue cells during the annual reproductive cycle. This result was obtained under natural conditions, which provide reliable results without the usage of drug or genetic interference. Different

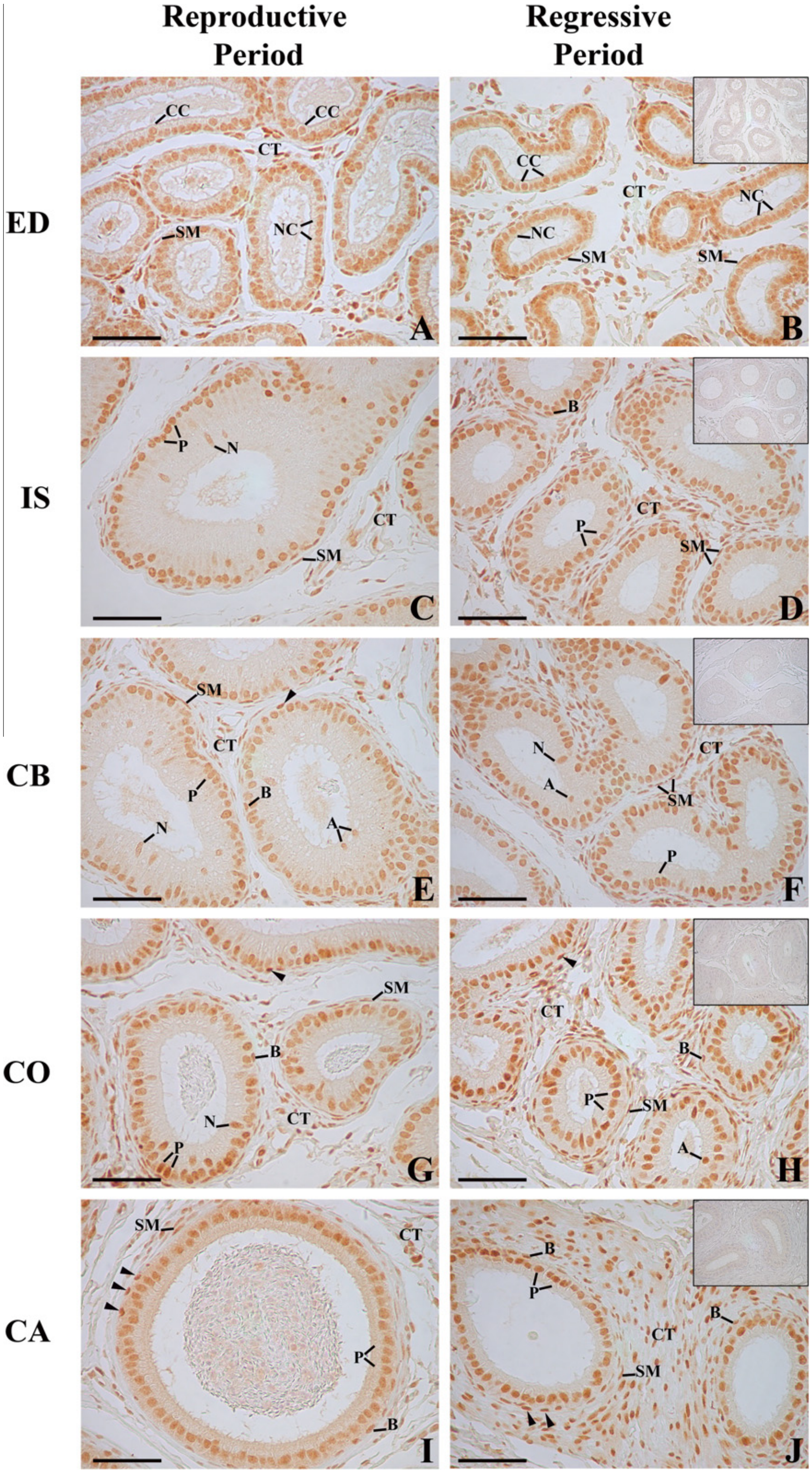

Fig. 5. Immunolocalization of ERβ in the efferent ductules (ED) and epididymis of *Artibeus lituratus* during the reproductive and regressive periods. IS = initial segment; CB = caput; CO = corpus; CA = cauda; NC = non-ciliated cells; CC = ciliated cells; CT = connective tissue; SM = smooth muscle cells; P = principal cells; B = basal cells; A = apical cells; and N = narrow cells. Arrowhead = stronger staining in some basal cells. Inserts in (B, D, F, H and J) = negative control. Scale bars in (A–J) = 50 μm.

from the excurrent ducts, a previous study on the testis of *A. lituratus* revealed that ERβ and AR, but not ERα, were the receptors that followed a seasonal variation [38]. Together, these data further emphasize that specific segments of the excurrent ducts downstream of the testis are the primary targets for estrogen action via ERα, as shown in other models [20,37].

The efferent ductules of *A. lituratus* compose most of the caput of the epididymis, a feature that is similar to those of humans [23,53]. Also similar to humans and large mammals, there are 12–15 efferent ductules that merged to form approximately 6 terminal ductules, which connect to the epididymal duct separately [21,23,53]. Information about the bat efferent ductules was found

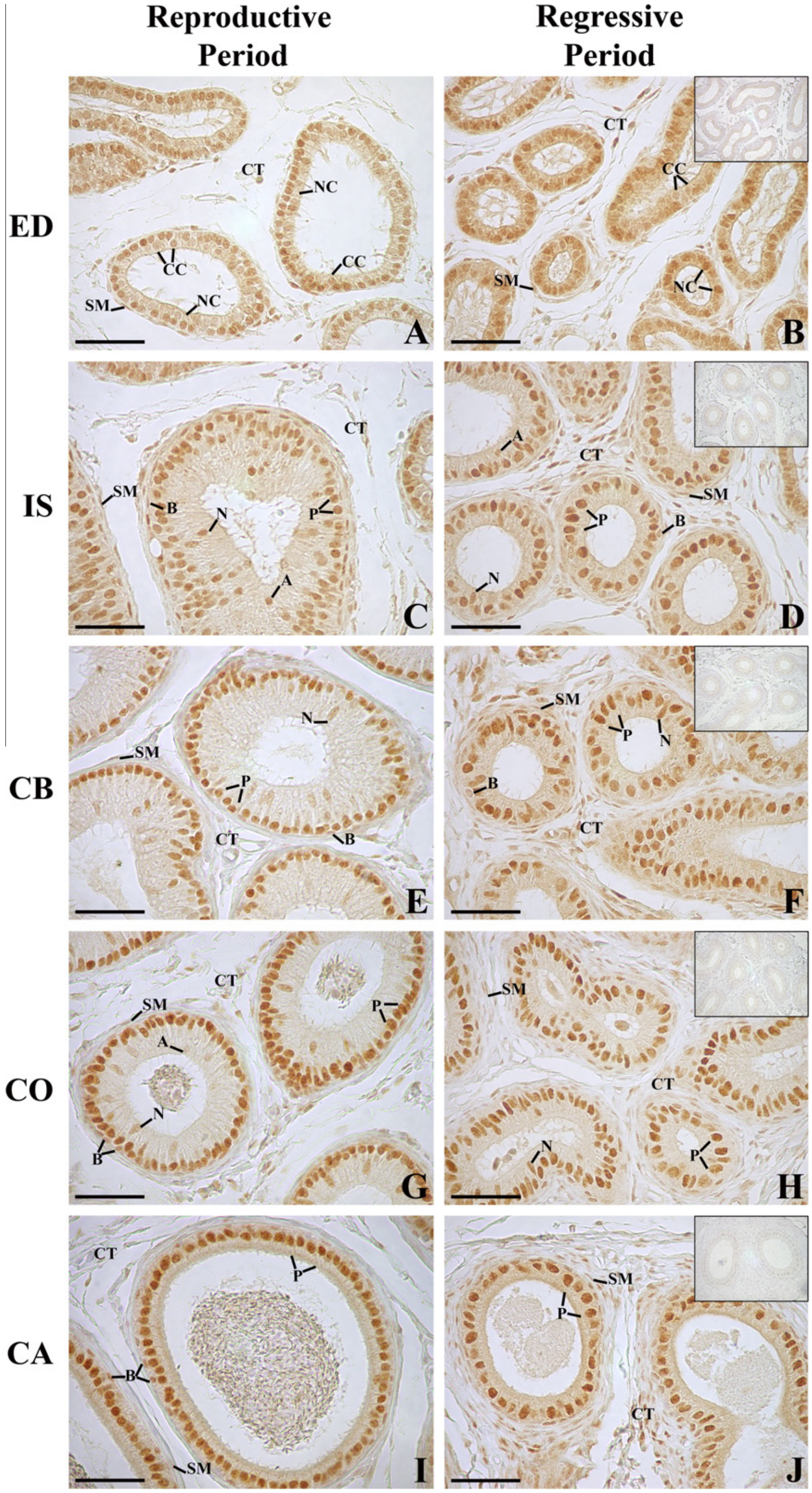

Fig. 6. Immunolocalization of AR in the efferent ductules (ED) and epididymis of *Artibeus lituratus* during the reproductive and regressive periods. IS = initial segment; CB = caput; CO = corpus; CA = cauda; NC = non-ciliated cells; CC = ciliated cells; CT = connective tissue; SM = smooth muscle cells; P = principal cells; B = basal cells; A = apical cells; and N = narrow cells. Inserts in (B, D, F, H and J) = negative control. Scale bars in (A–J) = 50 μm.

only for *Vesperugo (=Pipistrellus) savii* and *Vesperugo piccolo* [4] and fewer individual ductules are described (4–6) than those found in *A. lituratus*.

Data about the localization of aromatase in the efferent ductules and epididymis are scarce and in efferent ductules it has only been described in man [6]. In the epididymis, aromatase has been immunolocalized in the stallion epididymis [17], and there is also information that the epithelium may produce estrogen in the rhesus monkey and rat, as shown by *in vivo* and *in vitro* experiment, respectively [39,51]. Corroborating these data, immunoreaction

Table 2
Comparison of immunohistochemical staining for P450Aromatase, estrogen receptors (ERα, ERβ) and androgen receptor (AR) in excurrent ducts of *Artibeus lituratus* during reproductive and regressive periods.

Region	P450Arom		ERα		ERβ		AR	
	Reproductive period	Regressive period	Reproductive period	Regressive period	Reproductive period	Regressive period	Reproductive period	Regressive period
Efferent ductules								
NC	++	++	++++	++++	+++	+++	+++	+++
CC	–	–	–	++	+++	+++	+/–	+/–
SM	–	–	–	++	+++	+++	+/–	+/–
CT	–	–	–	++	+++	+++	+/–	+/–
Initial segment								
EP	++(P,B)	++(P,B)	–	++(P,B)	+++	+++	+++	+++
SM	–	–	–	+	+++	+++	+/–	+/–
CT	–	–	–	+	+++	+++	+/–	+/–
Caput								
EP	++(P,B)	++(P,B)	–	++(P,B)	+++	+++	+++	+++
SM	–	–	–	+	+++	+++	+/–	+/–
CT	–	–	–	+	+++	+++	+/–	+/–
Corpus								
EP	++(P,B)	++(P,B)	–	+(P,B)	+++	+++	+++	+++
SM	–	–	–	+	+++	+++	+/–	+/–
CT	–	–	–	+	+++	+++	+/–	+/–
Cauda								
EP	+(P,B)	+(P,B)	–	–	+++	+++	+++	+++
SM	–	–	–/+	++	+++	+++	+/–	+/–
CT	–	–	–/+	++	+++	+++	+/–	+/–

NC = nonciliated cells; CC = ciliated cells; SM = smooth muscle cells; CT = connective tissue; E = Epithelial cell of epididymis; P = principal cells; B = basal cell; – = negative; + = weak staining; +/– = intermittent staining; ++ = moderate staining; +++ or ++++ = strong staining.

for aromatase in *A. lituratus* was confined to some epithelial cells of efferent ductules and epididymis, confirming that the epithelium of both segments may produce estrogen to target the local ERα and/or ERβ. In contrast with the findings of a reduced aromatase concentration in the testis of seasonal animals in regression [5,47,55], the aromatase level in the regressed efferent ductules and epididymis of *A. lituratus* was unchanged. It is hypothesized that the maintenance of this enzyme at regression may guarantee a source of the estrogens to act at the increased epithelial ERα in the regressed epididymis.

Concerning the estrogen receptors, ERα was mostly restricted to the non-ciliated epithelial cells of the efferent ductules, as has been described for several other species [10,14,16,21,28,32, 33,40,41,46]. This result is in agreement with the main functional role that has been attributed to ERα, which is the modulation of fluid reabsorption by the non-ciliated cell [20,21,37,57].

Conversely, the epithelium of the epididymis of reproductive *A. lituratus* was mostly unreactive for ERα. An unreactive epididymis for ERα has been found in several species, including adult rats [3,14], hamsters [21], dogs [32], goats [16], roe deer [41], sheep [46], marmosets [14,21] and humans [10], although in developing animals there is a variable distribution of ER in the epididymal epithelium [2,3,14,17]. The higher ERα expression during development and in the regressive phase of the adult epididymis (present results) suggests a role for estrogen in determining the structure of the male genital tract. These data also reinforce the idea that in addition to species differences for ERα expression, it is important to take in account the age and reproductive phase of the animals to interpret the physiological role of local ER expression.

In the epididymal epithelium of *A. lituratus*, the strongest positivity for ERα, ERβ and aromatase was detected in some basal cells, which reinforced the postulate that the basal cells comprise different cellular populations and may play different roles in the epididymal epithelium [8,54]. ERα-positive basal cells were also found in the golden hamster, cat, roe deer, horse and *Macaca arctoides* [17,28,32,40,41]. Similarly, the basal cells that were positive for aromatase were described for stallions and humans [6,17]. Conversely, the basal cells of the *A. lituratus* epithelium were weakly reactive for AR, which corroborated a previous study that described little dependency on androgens for these cells [43]. The basal cells have multifunctional roles in the epididymis, such as endocytosis, detoxification, and immune protection [45,54]. An additional function of the basal cells is paracrine regulation of the principal and clear cells for electrolyte and water transport [31,45]. Direct regulation of fluid reabsorption has been described as a major role for estrogen/ERα in the efferent ductules [20,34,37]. Less is known about the modulation of ion/water movement by estrogen in other segments of the epididymis. Nevertheless, recent findings on ERα knockout mice (αERKO) revealed that the epididymal duct was unable to regulate the pH of the luminal fluid in the absence of this receptor, which thus interferes with the sperm maturation and fertility [27,29]. Given the importance of these findings, it would be of interest to further investigate the possible role of estrogen in regulating the luminal fluid homeostasis in the epididymis, possible through basal cell modulation.

As presently found, little expression of ERα in the interstitium of the efferent ductules has been described [22,32,58]. However, this receptor was more widely expressed during regression, and it was detected in most connective cells and smooth muscle cells surrounding the efferent ductules of *A. lituratus*. These data are in agreement with other studies that revealed interstitial components of efferent ductules as a molecular target for estrogen modulation [15,52]. A greater number of positive connective and muscle cells was also found in the regressive *rete testis* [38] and epididymis (present result) of *A. lituratus*. ERα has been consis-

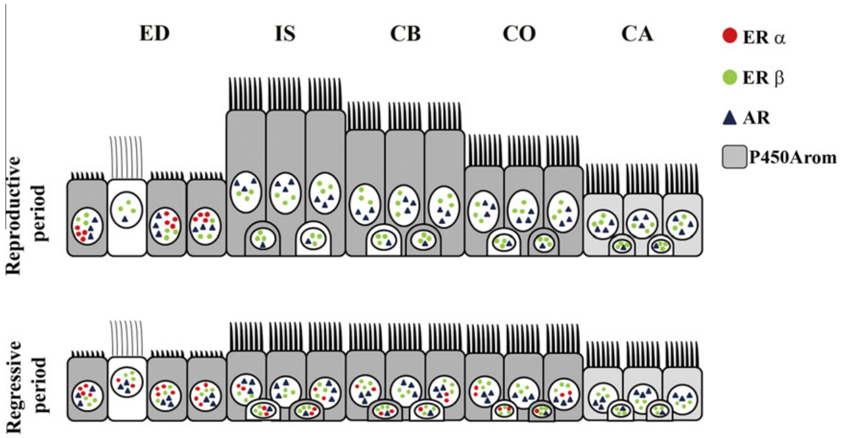

Fig. 7. Schematic representation of the expression of P450 aromatase, ERα, ERβ and AR in the efferent ductules (ED) and initial segment (IS), caput (CB), corpus (CO) and cauda (CA) of the *Artibeus lituratus* epididymis during the reproductive (A) and regressive (B) periods.

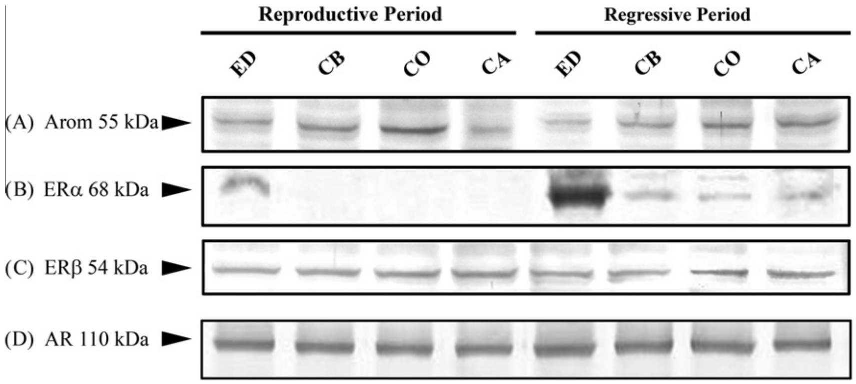

Fig. 8. Western blot assays for aromatase (A), ERα (B), ERβ (C) and AR (D) in the genital tract of *Artibeus lituratus*. The respective molecular weights are shown on the left. The blots shown are representative of three different assays. ED = efferent ductules; CB = caput; CO = corpus; and CA = cauda.

tently described in the peritubular smooth muscle cells of the epididymis and ductus deferens [32,58]. A functional role for estrogens acting via ERα in modulating the contractile activity of the epididymis smooth muscle cells has long been described [12,13]. However, a greater expression of ERα in the muscle layer of the male genital tract of *A. lituratus* was detected in the regressive period when the luminal sperm were absent. These data suggest that besides contractibility, ERα may be involved in other epididymal functions. Indeed a recent study revealed that ERα is essential for proliferation of smooth muscle cells in the rat prostate [56]. Additionally, there is a growing body of evidence that factors arising from the interstitium may be important in stimulating epithelial cells by a paracrine action in diverse organs [49,56].

In contrast with ERα, the epithelial staining for ERβ and AR was similar in all segments of the male tract examined during the reproductive and regressive periods. This lack of change suggests that these receptors are important for the maintenance of essential epithelial functions irrespective of the season. A constitutive expression of ERβ in the efferent ductules of rats was previously described [36]. Unaltered ERβ expression in other male genital organs submitted to different experimental designs has also been found [2,3,11,15,40,42,48]. During the annual reproductive cycle of hibernating bats, there is a drastic change in testosterone, but not dihydrotestosterone (DHT), and androstenedione levels [26]. The occurrence of another AR ligand, such as DHT, may explain the unchanged levels of AR in the *A. lituratus* epididymis during the regressive period, when it is possible that the testosterone levels are low.

5. Conclusions

In conclusion, the presence of aromatase in both the reproductive and regressive periods indicates an alternative source of estrogen when the testicular levels are reduced. The AR and ERβ receptors appeared to be constitutively expressed in the male genital tract of *A. lituratus* during the reproductive annual cycle, whereas ERα followed a remarkable seasonal variation by increasing during regression. These data suggest that ERα may be involved in important functions, such as preparing the male genital tract for the next reproductive period.

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ARTIGO 2

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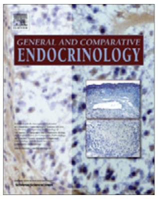

Differential expression and seasonal variation on aquaporins 1 and 9 in the male genital system of big fruit-eating bat *Artibeus lituratus*

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Chiroptera

ABSTRACT

Efferent ductules and epididymis are involved in water and solute transport, which is indispensable for storage and maintenance of the sperm viability. The reabsorption process involves proteins such as aquaporins (AQP), which has been described in the male genital system of limited species, including primate, rodents, cats and dogs. To contribute with information about AQPs in the male system, here we investigated the distribution of AQP1 and AQP9 in the tropical bat *Artibeus lituratus*, along the annual reproductive cycle. *A. lituratus* is a seasonal breeder with natural variation in components of the androgen and estrogen responsive system, thus being a good model for exploring the AQPs modulation. AQP1 was found restricted to differentiating spermatids, efferent ductules epithelium and venular endothelia along the male tract. AQP9 was detected throughout the epididymis being more abundant in the cauda and ductus deferens, but was not found in testis, rete testis and efferent ductules. Contrasting with AQP1 which appear to be constitutively expressed, there was seasonal variation in AQP9 expression, which was reduced in regressed epididymis. The AQP9 does not appear to be modulated by estradiol or androgens, but possibly by other factor related to luminal sperm. The establishment of specific function for aquaporins in the male tract remains undetermined; however, the cellular distribution presently found are compatible with the main function of AQP1, as a selective water channel, and AQP9, which is a conduct for water and a plethora of neutral solutes present in the epididymis milieu such as glycerol and urea.

1. Introduction

The efferent ductules and epididymis epithelia are involved in water and solute transport, which is indispensable for transport, maturation, storage and maintenance of the sperm viability in the epididymal lumen (Clulow et al., 1998; Hess, 2002; Joseph et al., 2011).The reabsorption process involves key proteins such as Na$^+$, K$^+$ ATPase, at the basolateral membrane, the apically located sodium/hydrogen exchanger (NHE3), and the cytoplasmic carbonic anhydrase II (CAII), that catalyzes the H$^+$ generation (Clulow et al., 1998; Hansen et al., 1999; Pastor-Soler et al., 2005). The transcellular fluid movement depends on aquaporins (AQP), which constitute a family of 13 integral membrane proteins named AQP0–AQP12 (Verkman, 2005). There are some aquaporins that selectively permeate water (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6, and AQP8), whereas other can transport water and other small polar molecules, mainly glycerol and urea, thus been denominated aquaglyceroporins (AQP3, AQP7, AQP9 and AQP10) (Agre et al., 1993; Cerda and Finn, 2010; Tsukaguchi et al., 1998).

Among aquaporins, the isoforms AQP1 and AQP9 have been described in the efferent ductules and/or epididymis (Arrighi et al., 2010a, b; Badran and Hermo, 2002; Da Silva et al., 2006b; Danyu et al., 2008; Domeniconi et al., 2007, 2008; Elkjaer et al., 2000; Fisher et al., 1998; Hashem, 2010; Lu et al., 2008; Oliveira et al., 2005; Pastor-Soler et al., 2001; Rojek et al., 2007). The pattern of expression of these aquaporins varies in a cell-, region-, and species-specific manner, as well as depending on developmental phase. There is evidence that AQP9 expression in the rat efferent ductules is modulated by estrogen and dihydrotestosterone (DHT), but only by DHT in the initial segment of the epididymis (Oliveira et al., 2005). Others have shown that AQP9 is testosterone-modulated in the cauda epididymis (Pastor-Soler et al., 2002, 2010). Conversely, AQP1 has been described restricted to the efferent ductules, where it appears to be constitutively expressed (Arrighi et al., 2010a; Badran and Hermo, 2002; Fisher et al., 1998; Oliveira et al., 2005).

Despite localizations of several AQPs in different regions of the male reproductive system of primates (marmoset and man), rodents (mice and rat) and some domestic animals (cat and dog), information about aquaporins in other mammals, including Chiroptera, has not been described in the male genital system. Several interesting reproductive features have been described for

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chiropteran, including prolonged storage of sperm in the epididymis for up to ten months (Bernard, 1984). The mechanism for maintaining the sperm viability for long periods of time includes the establishment of an excessively hyperosmolar environment, as seen in the hibernating bats or even in some species of non-hibernating tropical bats (Cervantes et al., 2008; Crichton et al., 1993, 1994; Oliveira and Oliveira, 2011). Osmolalities of bat epididymal fluid may reach values as high as 1000 mOsm/L, which is much higher than other mammals (300 mOsm/L) Crichton et al., 1994; Hinton et al., 1981; Johnson and Howards, 1977; Turner, 2002. The hyperosmolar lumen dehydrates the sperm, reducing their metabolic rate, inducing quiescence (Crichton et al., 1994). Despite this peculiarity, little is known about the molecular mechanism regulating the luminal microenvironment in the male genital tract of bats.

Therefore, in this study we aim to contribute with information about the cell distribution of AQP1 and AQP9 along the testis and the male genital tract of the tropical bat *Artibeus lituratus*, comparing the reproductive and regressive periods of the annual reproductive cycle. AQP1 and AQP9 are the subtypes more consistently described in the male genital system. Furthermore, besides their diverse functions as aquaporin or aquagliceroporin, respectively, they also appear to be differentially modulated in the male genital tract (Oliveira et al., 2005; Pastor-Soler et al., 2002, 2010; Picciarelli-Lima et al., 2006). *A. lituratus* has been shown to be a seasonal breeder with natural variation in components of the androgen and estrogen responsive system in the male reproductive organs. During the regressive period, *A. lituratus* showed increased expression of estrogen receptor ERβ and androgen receptor in the testis, as well as ERα in the male genital tract, indicating an important role for these steroids in regulation of the annual reproductive cycle of this species (Oliveira et al., 2009, 2012; Oliveira and Oliveira, 2011). This physiological variation in the sex steroids responses without interference in other physiological systems, as occurs in most experimental models, such as knockouts, castrated or chemically treated models, points out that this species may be a good experimental model for exploring the distribution and modulation of AQPs in the male reproductive system.

2. Materials and methods

Adult male bats of the *A. lituratus* species were captured during the reproductive (August to beginning of December) and regressive (middle of December to early April) periods. The captures were carried out in Belo Horizonte (19° 55′S and 43° 56′W) in southeastern Brazil. The captures were performed using mist nets (3 × 12 m) that were placed on trails of flight pathways to intercept bats flying 1–2 m above the ground.

The captured males were considered adults according to parameters routinely used for determining the age of bats, such as teeth wear and ossification of the metacarpal epiphyses (Oliveira et al., 2009). The captures were authorized by the Brazilian Institute of Natural Environment and Renewable Resources (IBAMA, Brazil). The experimental procedure was approved by the Experimental Animal Ethics Committee of the Federal University of Minas Gerais (CETEA/UFMG).

2.1. Tissue preparation

The bats were weighed and anesthetized with an association of 30 mg/kg pentobarbital and 20 mg/kg ketamine chloridrate (i.p.). The perfusion was performed transcardially via the left ventricle with 10% neutral buffered formalin (NBF) for the immunohistochemical assay. After fixation, the efferent ductules and epididymis were removed and weighed together.

2.2. Immunohistochemistry

Fragments of testis, efferent ductules, epididymis and ductus deferens NBF-fixed and embedded in paraffin ($n = 5$) were used to detect the occurrence and cell distribution of AQP1 and AQP9 by immunohistochemistry. For this purpose, the sections were deparaffinized, hydrated and immersed in a 0.6% methanol hydrogen peroxide solution for blocking the endogenous peroxidase. After microwaving for antigen retrieval, normal goat serum (10%) was used for blocking nonspecific antibody binding. Then, the sections were incubated overnight at 4 °C with AQP1 or AQP9 polyclonal rabbit anti-rat primary antibody (Alpha Diagnostic International, San Antonio, TX, USA), diluted 1:300 for AQP1 or 1:500 for AQP9. After, the sections were exposed for 1 h to biotinylated secondary goat anti-rabbit antibody (Dako, Carpinteria, USA). The sections were incubated with the avidin–biotin complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, USA) for 30 min, and the immunoreaction was visualized using 3,3 diaminobenzidine containing 0.01% hydrogen peroxide in 0.05 M Tris–HCl buffer, pH 7.6. Mayer hematoxylin was used to counterstain the sections. The immunostaining was performed in triplicate to confirm the results.

2.3. Morphometry

The immunostaining intensity for AQP1 and AQP9 was quantified by computer-assisted image analysis, based on previously reported protocols (Picciarelli-Lima et al., 2006). Pictures from 10 different areas of the efferent ductules epithelium (for AQP1 analysis) and epididymis (for AQP9 analysis) of five animals at each periods analyzed were taken by using an x40 objective lens of a Nikon Eclipse E600 microscope (Nikon Corp., Melville, USA). Digital images were processed with Adobe Photoshop CS3 (Adobe Systems, Mountain View, USA), converted to the grayscale mode and inverted. The images were then exported to Image-Tool software 3.00 (University of Texas Health Sciences Center, San Antonio, USA), for quantitative analysis. For this purpose, the stained apical areas of efferent ductules (for AQP1) and epididymis (for AQP9) were traced and measured by pixel intensity. Background intensity was determined by tracing an unlabeled area adjacent to the measured cells and subtracted from values detected in the labeled regions. Data were expressed as mean ± standard deviation.

2.4. Western blotting

Western blot assays were performed on the efferent ductules and epididymis (caput, corpus and cauda regions) to validate the use of the antibodies in bats and to confirm the immunohistochemistry results. For further confirmation of the specificity of the antibody produced in rats for use in bats, efferent ductules and epididymis of rats were previously run in parallel to the bat tissues. The assays revealed major bands of 28 and 31 kDa, in both

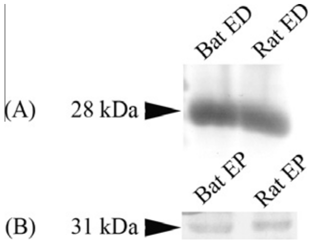

Fig. 1. Western blotting assays confirming the specificity of the antibodies for AQP1 (A) and AQP9 (B).The respective molecular weights are shown on the left. ED = efferent ductules; EP = epididymis.

tissues from bats and rats (Fig. 1). These molecular weights are in agreement with those previously found for AQP1 and AQP9, respectively, in other mammalian species (Da Silva et al., 2006b; Domeniconi et al., 2007, Elkjaer et al., 2000; Fisher et al., 1998; Lu et al., 2008; Pastor-Soler et al., 2001). These results indicate that the anti-rat AQP1 and AQP9 antibodies may cross-react with the bat proteins, thus corroborating previous findings that show that aquaporin sequences are highly conservative among mammals (Anthony et al., 2000; Higa et al., 2000; Jin et al., 2006; Kuriyama et al., 2002; Tsukaguchi et al., 1999; Wang et al., 2005; Wintour et al., 1998).

For the Western blot assays, pooled tissues of six animals in the reproductive and six animals in the regressive periods were used. The efferent ductules and epididymis tissues were macerated with dry ice, and submitted to total protein extraction and quantification by the Bradford methodology. The proteins (15 μg/lane) were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), transferred to nitrocellulose membranes and blocked with 10% normal goat serum (NGS) for 1 h at room temperature. The membranes were incubated overnight with primary antibodies diluted 1:500 for rabbit anti-rat AQP1 or AQP9 (Alpha Diagnostic International, San Antonio, TX, USA). Then, the membranes were washed with PBS-Tween 0.05% followed by incubation with the secondary antibodies goat anti-rabbit (Dako, Carpinteria, CA, USA) diluted 1:1000. After several washes in PBS-Tween 0.05%, the reaction was developed by the addition of 0.1% of 3,3 diaminobenzidine and 0.05% of chloronaphthol in PBS, 16.6% methanol and 0.04% H_2O_2. Deionized water was used to stop the reaction. All Western blots were replicated to confirm the results.

2.5. Enzime linked immune sorbent assay – ELISA

The dosage of estradiol, testosterone and DHT in the plasma, testis and epididymis (corpus and cauda) tissue was performed by using commercial ELISA kits. For this purpose, pooled frozen testes of four animals in the reproductive and four animals in the regressive periods were macerated in dry ice. Caput of bat epididymis, including efferent ductules, are very tiny segments, therefore they could not be included in the assay due to shortage of tissue available. The samples of tissue (150 mg) were suspended in 250 μl of PBS (pH 7.4) and homogenized. After this step, lipid extraction and enrichment was performed by using diethyl ether. For this purpose, 670 μl of diethyl ether was added and subsequently vortexed. Then, the samples were centrifuged and the ether supernatant was frozen in liquid nitrogen and decanted. Afterwards, the extracts were evaporated at room temperature to about 100 μl and sonicated for 10 min. The ether was evaporated to about 20 μl, and then 60 μl of PBS was added (Hany et al., 1999). The plasma samples were obtained after centrifugation of total blood (4000 rpm for 10 min) in heparin-coated tubes. ELISA measurements were performed using 25 μl of samples per well according to the manufacturer's instructions (DRG Instruments GmbH, Marburg, Germany). The sensitivity of the assays for estradiol, testosterone and DHT was 9.714, 0.083, and 6.0 pg/mL, respectively. All samples were measured in quadruplicate and repeated in two independent assays.

2.6. Statistical analysis

Quantitative data was statistically analyzed by using the Matlab® (MathWorks, Natick, MA, USA) software. Initially, the data was subjected to the Lilliefors test, a modification of the Kolmogorov–Smirnov test, to check normality of the datasets. After, the data were analyzed by the Student's *t*-test to compare the means of two populations or the ANOVA test if comparisons were made between more than two populations. The significance level used for all the tests was $P < 0.05$.

3. Results

3.1. Testis and rete testis

Positivity for AQP1 was found restricted to differentiating spermatids of the seminiferous tubules epithelium in a stage-specific manner during reproductive period (Fig. 2A–H; Table 1). At stage I, the round spermatids were unreactive, whereas at stage II, the elongating spermatids showed slight positivity for AQP1. The immunoreactions were more intense in elongated spermatids at stages III and IV. Thereafter, the immunostaining decreased in elongated spermatids, which became unreactive at stage VIII. The regressed testes were unreactive for AQP1 (Fig. 2I–J). The Sertoli cells, myoid cells and Leydig cells, as well as the rete testis, were unreactive for AQP1, whereas testicular microvessels endothelia were positive (Fig. 2I). AQP9 was not detected in the testis (Fig. 2K–L) and rete testis (not shown) of *A. lituratus*, neither in the reproductive nor in the regressive periods.

3.2. Efferent ductules

The efferent ductules of *A. lituratus* were reactive for AQP1 but not AQP9 at both periods analyzed (Fig. 3). AQP1 positivity was restricted to the apical membrane of the epithelial nonciliated cells as well as vein endothelia (Table 1). The epithelial ciliated cells, peritubular smooth muscle cells, connective tissue cells, as well as arterial endothelia were immunonegative for AQP1. The cell distribution and intensity of AQP1 staining along the efferent ductules remained similar when regressive and reproductive periods were compared (Figs. 3 and 4). AQP9 was not detectable in any cell type of the efferent ductules, even when higher concentration of the antibody was used (1:25; 1:50 and 1:100).

3.3. Epididymis and ductus deferens

The epididymis and ductus deferens were unreactive for AQP1, except for the endothelia of veins, which presented positivity for AQP1 throughout the male genital tract of *A. lituratus*, irrespective of the period analyzed (Fig. 3E–F; Table 1).

During the reproductive period, AQP9 was detected along the microvilli of the principal cells of all epididymal regions, with a crescent gradient of intensity from the initial segment to cauda region (Figs. 4 and 5). Apical, basal, narrow, clear and halo cells were immunonegative for AQP9 (Fig. 5A, C, E, G). The ductus deferens stained as intensely as the cauda epididymis (Fig. 5I–J). The same pattern of AQP9 expression was observed in the regressed epididymis and ductus deferens; nevertheless, there was a significant reduction in the staining intensity along the epididymis (Fig. 4B and Fig. 5B, D, F, and H; Table 1).

3.4. Western blotting

The immunohistochemistry results and the specificity of the antibodies used were confirmed by Western blotting assays.

The AQP1 was equally detected in the efferent ductules at the reproductive and regressive periods (Fig. 6A). In the epididymis, a weak band for AQP1 was also detected, probably corresponding to the endothelial expression.

The Western blotting assays confirmed that AQP9 was undetectable in the efferent ductules, but present throughout the entire length of the epididymis (Fig. 6B). The strongest reaction was

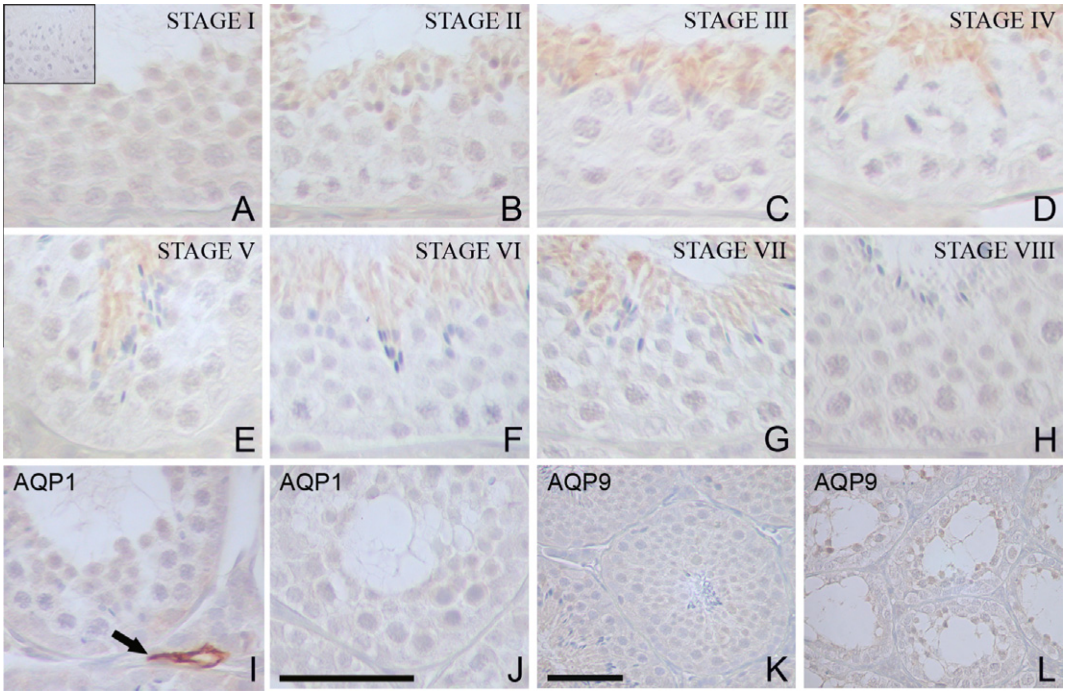

Fig. 2. Immunolocalization of AQP1 and AQP9 in the testis of *Artibeus lituratus* during the reproductive and regressive periods. (A–H) During reproductive period AQP1 was detected in differentiating spermatids of the seminiferous tubules epithelium in a stage-specific manner. (I–J) AQP1 was undetected in the regressed testis. (K–L) AQP9 was not detected in the testis in both periods analyzed. Arrow = blood vase endothelia; insert in (A) = negative control; scale bar in (J) = 50 μm, same to (A–I); scale bar in (K) = 50 μm, same to (L).

Table 1
Comparison of immunohistochemical staining for AQP1 and AQP9 in the male genital system of *Artibeus lituratus* during reproductive and regressive periods.

	AQP1		AQP9	
	Reproduction	Regression	Reproduction	Regression
Rete testis	–	–	–	–
Testis				
Leydig cells	–	–	–	–
Myoid cells	–	–	–	–
Sertoli cells	–	–	–	–
Spermatogonium	–	–	–	–
Spermatocytes	–	–	–	–
Spermatids	–/+/++	NP	–	NP
Efferent ductules				
Nonciliated cells	+++	+++	–	–
Ciliated cells	–	–	–	–
Vein endothelia	++	++	–	–
Initial Segment				
Epithelium	–	–	+	+*
Vein endothelia	++	++	–	–
Caput				
Epithelium	–	–	++	++*
Vein endothelia	++	++	––	–
Corpus				
Epithelium	–	–	+++	+++*
Vein endothelia	++	++	–	–
Cauda				
Epithelium	–	–	++++	+++*
Vein endothelia	++	++	–	–
Ductus deferens				
Epithelium	–	–	++++	+++*
Vein endothelia	++	++	–	–

– = Negative; + = weak staining; ++ = moderate staining; +/++ = weak to moderate; +++ = strong staining; ++++ = strongest staining; NP = cell not present

* Staining weaker than those at reproduction.

detected in the cauda epididymis, whereas the weakest was that of the initial segment. A significant reduction in the intensity of the AQP9 immunoreactive bands was more evident in the regressive period compared to the reproductive period.

3.5. Hormones measurement

The testosterone, DHT and estrogen levels are summarized in Table 2. The concentration of androgens was higher during the regressive period at all tissues analyzed. Higher levels of estradiol were found in the male genital system, compared to the plasma. The estradiol levels in the testis and epididymis were similar. There were no changes in the estradiol levels in both periods analyzed.

4. Discussion

This is the first description of the occurrence and distribution of aquaporins 1 and 9 in the male genital system of a bat species. *A. lituratus* showed a segment-specific distribution of AQP1 and AQP9 throughout the male reproductive system. Among the results, the occurrence of AQP1 in the testis and the absence of AQP9 in the efferent ductules have not been described for any species of mammals investigated to date. The AQP1 was found restricted to differentiating spermatids in the testis, nonciliated cells in the efferent ductules and venular endothelium along the male genital tract. On the other hand, AQP9 was detected throughout the epididymis and ductus deferens, but not in the testis, rete testis and efferent ductules. Contrasting with AQP1, which appears to be constitutively expressed, there was seasonal variation in the expression of AQP9. The differential distribution and regulation of both aquaporins may reflect different physiological significance for these proteins in the male genital tract.

In *A. lituratus*, AQP1 was found restricted to elongating and elongated spermatids from stage II to VII of the seminiferous epithelium cycle. These results differ from other species in which AQP1 has not been described in any cell of the testis (Fisher et al., 1998; Lu et al., 2008; Nicotina et al., 2005). Besides our

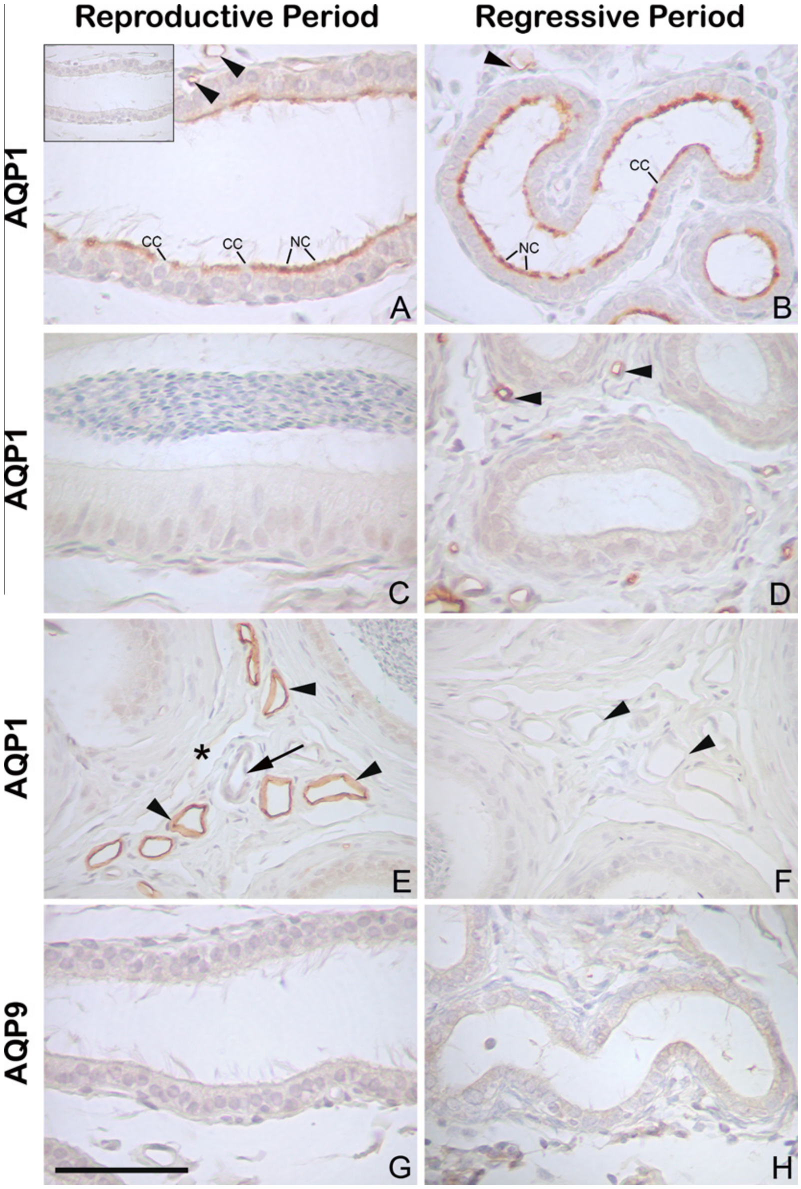

Fig. 3. Immunolocalization of AQP1 and AQP9 in the efferent ductules and epididymis of *Artibeus lituratus* during the reproductive and regressive periods. (A–B) AQP1 positivity was detected in apical membrane of the epithelial nonciliated cells in both reproductive and regressive periods. (C–D) In the epididymis, only endothelia of veins were positive for AQP1. (E–F) In the interstitium of the male tract the endothelia of veins (arrowheads), but not arteries (arrow) and lymphatic vases (∗), were positive for AQP1. (G–H) The efferent ductules were unreactive for AQP9 in both periods analyzed. NC = nonciliated cells; CC = ciliated cells; insert in (A) and Fig. F = negative controls; scale bar in (G) = 50 μm.

present result, spermatids positive for aquaporins have been described only for rats (AQP7 and AQP8) and humans (AQP7) Calamita et al., 2001; Ishibashi et al., 1997; Suzuki-Toyota et al., 1999; Yeung et al., 2010. The reason for the discrepancy between species is not clear; however it is not uncommon that diverse aquaporins have similar function despite species-specific distribution. Therefore, the detection of diverse aquaporins in the spermatids offers evidence that they may be important for spermatozoa differentiation, possibly allowing the efflux of water necessary for cytoplasmic condensation and reduction of cell size during the spermiogenic process.

AQP9 was undetectable in the testis of *A. lituratus*. In line with our findings, other studies also failed in detecting AQP9 in the dog, mice and human testis (Domeniconi et al., 2007; Hashem, 2010; Ko et al., 1999; Tsukaguchi et al., 1999). Conversely, AQP9 has been found exclusively in the Leydig cells of rat (Badran and Hermo, 2002; Elkjaer et al., 2000; Nicchia et al., 2001; Nihei et al., 2001). We do not have a clear explanation for these differences, but they highlight the necessity of further investigation of other aquaporin subtypes in the species already studied, as well as investigation of other mammal species, before any clear conclusion about the role of aquaporin in the testis could be made.

Our findings showing AQP1 at the apical membrane of nonciliated cells of the efferent ductules epithelium corroborate the evidences that AQP1 is the only aquaporin subtype consistently found across species to date (Arrighi et al., 2010a, b; Badran and Hermo, 2002; Brown et al., 1993; Domeniconi et al., 2007, 2008; Fisher et al., 1998; Lu et al., 2008; Oliveira et al., 2005; Ruz et al., 2006). The cell distribution of AQP1 is compatible with the reabsorptive function of the efferent ductules, which is responsible for reabsorption of more than 90% of the fluid coming from the testis (Clulow et al., 1998; Hess, 2002).

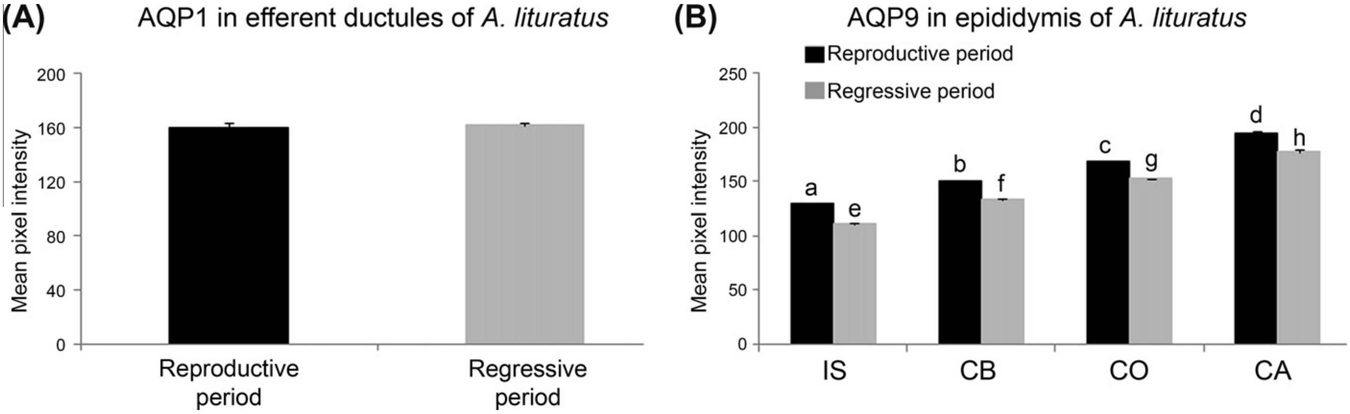

Fig. 4. Quantification of AQP1 and AQP9 staining intensity in the efferent ductules and epididymis of *Artibeus lituratus* during the reproductive and regressive periods. (A) Immunoexpression of AQP1 in efferent ductules was similar when reproductive and regressive periods were compared. (B) The immunostaining for AQP9 was significantly increased from initial segment to cauda in both periods analyzed. During regressive period occurred a significant reduction in staining at all epididymal segments. Mean values with different letters represent significant differences ($P < 0.05$), $n = 5$.

AQP1 levels were similar when *A. lituratus* efferent ductules at reproductive and regressive periods were compared, pointing out that this protein may have constitutive expression in this segment. In fact, evidence of constitutive expression of AQP1 has been a common finding for other species and experimental models. On this sense, AQP1 was unchanged after orchidectomy, efferent ductules ligation, administration of GnRH antagonist or testosterone and dihydrotestosterone replacement, as well as undernutrition, suggesting that AQP1 regulation is not affected by testicular factors, including androgens (Arrighi et al., 2010a; Badran and Hermo, 2002; Fisher et al., 1998; Oliveira et al., 2005). Concerning estrogens, treatment with the antiestrogen ICI 182,780 or estradiol replacement after castration also did not alter AQP1 staining in the efferent ductules epithelium (Oliveira et al., 2005). Conversely, the AQP1 levels were diminished after neonatal exposition to diethylstilbestrol or genetic inactivation of estrogen receptor ERα (αERKO); however, these alterations appear to be secondary to alterations in the epithelial cytoarchitecture (Fisher et al., 1998; Ruz et al., 2006).

The presence of AQP1 in the venular endothelia throughout the male genital system of *A. lituratus* is in agreement with previous studies (Arrighi et al., 2010a, b; Badran and Hermo, 2002; Nicotina et al., 2005; Oliveira et al., 2005), even though the identification of the stained vessels was frequently omitted. The functional role attributed to the endothelial AQP1 has been removal of water reabsorbed from the epithelium and maintenance of the water equilibrium in the interstitial tissue (Arrighi et al., 2010a, b; Badran and Hermo, 2002; Da Silva et al., 2006a; Nicotina et al., 2005). Differing from our results, others have found AQP1 in aorta endothelium, where they appear to be involved in nitric oxide transportation besides water (Herrera and Garvin, 2007). The expression of AQP1 in venular but not arterial or lymphatic endothelia of the male genital tract further emphasizes the heterogeneity in endothelial properties between organs and vessel types.

Concerning AQP9, contrasting with previous descriptions for rat, mice, dog and cat (Arrighi et al., 2010a, b; Badran and Hermo, 2002; Domeniconi et al., 2007; Oliveira et al., 2005; Ruz et al., 2006), the efferent ductules of *A. lituratus* were unreactive for AQP9. It is well established that, despite the fact that AQP9 presents similar permselectivity in different species, there are distinct patterns of tissue distribution, possibly due to the different requirements of metabolites among the species (Tsukaguchi et al., 1999). On this sense, rats and bats differ in the structure of the efferent ductules, as in bats the efferent ductules are more numerous (12–15) and enter the epididymis separately through about six terminal ductules (Oliveira et al., 2012; Oliveira and Oliveira, 2011), whereas in rats all 6–8 ductules converge into one common duct that continues directly into the epididymal duct (Ilio and Hess, 1994). It is known that, as the sperm progress from proximal to distal efferent ductules there is substantial fluid reabsorption, concentrating the luminal spermatozoa (Ilio and Hess, 1994). Therefore, the anatomical difference may reflect in higher compaction of spermatozoa into the rat ductules, thus justifying the requirement of additional AQP, such as AQP9, to better establish the adequate luminal milieu for maintenance of the spermatozoa. To corroborate this point of view, information about AQP9 distribution in other species with efferent ductule morphophysiology similar to that of bats, such as humans, would be helpful. On the other side, one cannot rule out the possibility that other aquaglyceroporins, equivalent in function to AQP9, are expressed in the bat ductules.

As presently found, epididymis epithelium strongly positive for AQP9 but unreactive for AQP1 has been consensual in the literature (Badran and Hermo, 2002; Domeniconi et al., 2007, 2008; Oliveira et al., 2005). The pattern of AQP9 immunoreaction showed a gradual increase from the initial segment to the cauda epididymis of *A. lituratus*. This pattern of expression paralleled the increase in luminal osmolality from caput to cauda, which is considered an important mechanism for sperm maturation (Cooper and Yeung, 2003). Moreover, in hibernating bats, the hyperosmolar environment of cauda epididymis prolongs the survival and guarantees the viability of spermatozoa for long periods of time (Crichton et al., 1994). Thus, considering that spermatozoa concentration is gradually increased from caput to cauda, the higher levels of AQP9 may play a role in the process of maintenance of the hyperosmolar environment necessary to keep the sperm quiescence. On the other hand, it is well known that the caudal region of the epididymis is responsible for the storage and protection of spermatozoa (Hermo and Smith, 2011). As member of the aquaglyceroporin group, the AQP9 presents high permeability to glycerol, urea and other small noncharged solutes, besides water (Tsukaguchi et al., 1998), thus indicating that this protein may participate in other physiological processes beyond water homeostasis. On this sense, a possible role for AQP9 in the epididymis may be transport of glycerol, which is a known component of the luminal fluid, serving as an energy substrate for the stored sperm (Cooper and Brooks, 1981). Corroborating this point of view, functional assays have demonstrated that the epididymis is permeable to glycerol in a manner dependent of AQP9, which is in turn regulated by cAMP and bradykinin (Belleannee et al., 2009; Pietrement et al., 2008).

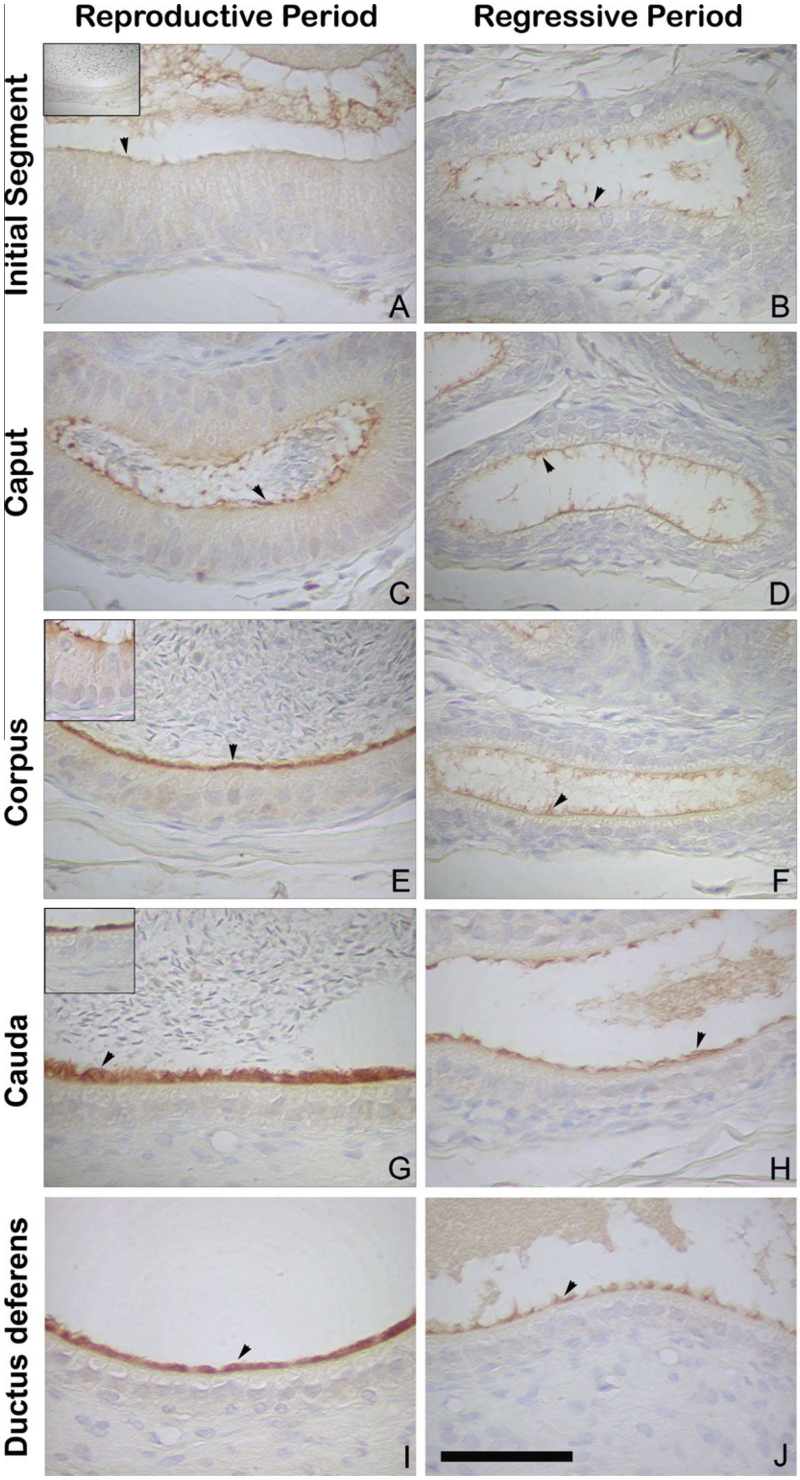

Fig. 5. Immunolocalization of AQP9 in the epididymis and ductus deferens of *Artibeus lituratus* during the reproductive and regressive periods. (A, C, E and G) During the reproductive period AQP9 was detected in the microvilli of the principal cells along the epididymis with a gradient of intensity increasing from initial segment to cauda. (B, D, F and H) During the regressive period the same pattern of AQP9 staining was observed, but in lower levels. (I–J) The ductus deferens presents similar staining intensity as the cauda. Arrowheads = positivity for AQP9; insert in (A) = negative control; Insert in (E) = negative apical and narrow cells; insert in (G) = negative clear cell; scale bar in (J) = 50 μm.

Indeed, the expression of AQP9 paralleled the luminal concentration of sperm along the *A. lituratus* epididymis (Oliveira et al., 2012). As the AQP7 has been described in sperm of several species (Calamita et al., 2001; Saito et al., 2004; Yeung et al., 2009), this aquaglyceroporin may allow the influx of glycerol coming from the luminal milieu.

Considering the broad spectrum of molecules that may permeate AQP9, it is reasonable to expect multiple functional roles for this protein. In line with this point of view, as high levels of urea have been described in cauda epididymis (Turner and Cesarini, 1983; Turner et al., 1979), another possibility is that AQP9 may be involved in urea transportation from the epididymis, thus

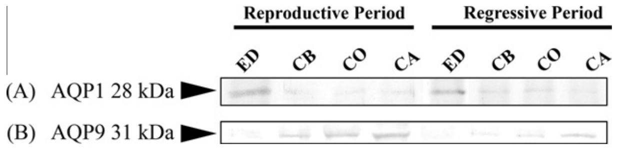

Fig. 6. Western blotting assays for AQP1 in efferent ductules (A) and AQP9 in the epididymis (B) of *Artibeus lituratus*. The respective molecular weights are shown on the left. ED = efferent ductules; CB = caput; CO = corpus; CA = cauda.

Table 2
Levels of testosterone, DHT and estradiol in the plasma, testis and epididymis of *Artibeus lituratus* during the reproductive and regressive periods.

	Reproduction	Regression
Plasma		
Testosterone (ng/mL)	0.3 ± 0.1	1.7 ± 0.5*
DHT (pg/mL)	77.0 ± 55.8	233.0 ± 171.3*
Estradiol (pg/mL)	24.0 ± 3.9	19.0 ± 2.0
Testis		
Testosterone (ng/mL)	9.0 ± 0.4	26.0 ± 0.8*
DHT (pg/mL)	2686.0 ± 308.0	4538.0 ± 786.0*
Estradiol (pg/mL)	32.0 ± 1.9	38.0 ± 7.9
Corpus epididymis		
Testosterone (ng/mL)	1.0 ± 0.1	4.1 ± 0.5*
DHT (pg/mL)	ND	ND
Estradiol (pg/mL)	33.5 ± 2.0	36.0 ± 0.1
Cauda epididymis		
Testosterone (ng/mL)	0.8 ± 0.1	1.4 ± 0.5*
DHT (pg/mL)	ND	ND
Estradiol (pg/mL)	36.2 ± 0.2	36.0 ± 0.9

DHT = Dihydrotestosterone; ND = not determined.
* $P < 0.05$.

presenting a role in detoxification of the luminal environment. Corroborating this hypothesis, there is evidence that, in hepatocytes, AQP9 indeed facilitates glycerol influx and urea efflux, being more permeable to urea and glycerol than water (Carbrey et al., 2003). Recent findings have also attributed a role for urea uptake in improving skin barrier and antimicrobial defense (Grether-Beck et al., 2012). Considering that both barrier and antimicrobial defense are important roles attributed to the cauda epididymis, it would be of interest to further investigate whether local urea uptake by AQP9 may also contribute to these local functions.

During the regressive period, AQP9 presented the same pattern of distribution in the epididymis as that observed during the reproductive period of *A. lituratus*, although with significant reduction in the protein levels. Androgens have been a factor described as a modulator of epididymal AQP9 (Oliveira et al., 2005; Pastor-Soler et al., 2002, 2010). However, here we show that AQP9 expression is reduced in the regressed epididymis, even with high levels of local, testicular and plasmatic androgens (present results), as well as androgen receptor expression (Oliveira et al., 2012). These findings corroborate previous suggestion that AQP9 is modulated by other luminal factors than androgens (Badran and Hermo, 2002). Estradiol does not appear to be a factor modulating AQP9 in the epididymis of *A. lituratus*, as the concentration of this steroid is not changed along the annual cycle, neither locally nor systemically. Lack of estradiol modulation of AQP9 in the epididymis has also been described for rat (Oliveira et al., 2005). Corroborating these data, there is evidence that the aromatase levels in the epididymis of *A. lituratus* do not vary during the annual reproductive cycle (Oliveira et al., 2012). Together, these findings add to previous suggestion that the mechanism of regulation of AQP9 in the male genital tract may be more complex than previously anticipated (Badran and Hermo, 2002; Belleannee et al., 2009; Oliveira et al., 2005; Picciarelli-Lima et al., 2006; Pietrement et al., 2008).

5. Conclusion

In summary, we have characterized for the first time the expression of AQP1 and AQP9 in the male genital system of a bat species and demonstrated that these proteins present cell- and region-specific distribution and differential seasonal variation, as AQP1 was constitutively expressed restricted to differentiating spermatids in the testis, efferent ductules nonciliated cells and venous endothelia, whereas seasonally-modulated AQP9 was detected throughout the epididymis epithelium being more abundant in the cauda and ductus deferens. The AQP9 does not appear to be modulated by estradiol or androgens, but possibly by other factors related to luminal sperm. The establishment of specific functions for aquaporins in the male genital tract remains to be elucidated; however, the cellular distribution presently found is compatible with the main function of AQP1, as a selective water channel, important for the spermiogenic process as well as for fluid reabsorption in the efferent ductules, and AQP9, which is a conduct for water and a plethora of neutral solutes present in the luminal content of the epididymis, such as glycerol and urea, among others, which may be required for the maintenance of an adequate milieu for the spermatozoa. Some differences between species were presently highlighted, indicating that further studies in diverse species will be necessary in order to allow a better interpretation of the role of aquaporins in each segment of the male genital tract.

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ARTIGO 3 – em preparação

Oliveira, R.L; Nogueira J.C; Mahecha G. A. B; Oliveira C.A. Avaliação dos índices de proliferação e morte celular nos dúctulos eferentes e epidídimo do morcego-das-frutas *Artibeus lituratus* durante o ciclo reprodutivo anual.

Avaliação dos índices de proliferação e morte celular nos dúctulos eferentes e epidídimo do morcego-das-frutas *Artibeus lituratus* durante o ciclo reprodutivo anual

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1. Introdução

O equilíbrio entre proliferação e morte celular é um passo importante para a manutenção da homeostase fisiológica do sistema genital masculino (Billig et al., 1996). Contudo, alguns fatores são capazes de interferir nesse equilíbrio tais como, fotoperíodo, aumento da temperatura ou variação nos níveis hormonais (Billig et al., 1996, Jara et al., 2002, Carballada et al., 2007). A testosterona é o principal hormônio produzido pelos testículos, que participa na homeostase tecidual do sistema genital masculino, diretamente, ou indiretamente, via seus metabólitos diidrotestosterona (DHT) e estradiol (Hess & Franca, 2007). Os andrógenos testosterona e DHT exercem suas funções biológicas via receptores específicos (AR), enquanto os estrógenos agem via receptores ERα e ERβ.

O morcego-das-frutas *Artibeus lituratus* é uma espécie de morcego tropical que apresenta um período marcante de regressão sexual com aumento de células somáticas e espermatogênicas em apoptose, as quais apresentam-se intensamente positivas para ERβ (Oliveira et al., 2009). Concomitante ao aumento de células apoptóticas e dos níveis dos receptores ERβ, o qual é considerado fator pró-apoptótico e anti-proliferativo (Weihua et al., 2001, Cheng et al., 2004, Imamov et al., 2004), os níveis de receptores ERα, considerados fatores pró-proliferação (Attia & Ederveen, 2012), mantiveram-se inalterados. Ao contrário do observado nos testículos, nas vias genitais de *A. lituratus* os níveis de ERα aumentaram durante a regressão sexual, enquanto os níveis de ERβ não variaram (Oliveira et al., 2012). Dessa forma se torna interessante investigar de forma mais aprofundada se existe relação entre o padrão de expressão dos elementos do sistema responsivo a estrógenos com a manutenção da homeostase tecidual nos diferentes órgãos que compõem as vias genitais.

Nas vias genitais masculinas, estudos sobre a regulação dos processos de morte e proliferação celular em situações não-patológicas são escassos. Considerando especificamente os dúctulos eferentes, informações sobre índices de proliferação do epitélio foram limitadas a uma investigação (Nagy & Edmonds, 1975), enquanto em relação à morte celular não foram encontradas informações na literatura consultada. O epidídimo de indivíduos adultos é considerado um órgão estático, devido aos baixos índices de proliferação e morte celular encontrados sob condições normais (Fan & Robaire, 1998, Robaire et al., 2006). Ao contrário, altos índices de proliferação epididimária são vistos durante os períodos fetal e neonatal (Dyche, 1979, Sun & Flickinger, 1982).

Em face da escassez de informações sobre proliferação e morte celular nas vias genitais masculinas em condições normais, julgamos que por se tratar de uma espécie sazonal, com variação natural dos níveis de receptores de estrógenos, sem interferência em outros sistemas fisiológicos, como ocorre em animais *Knockouts*, castrados ou quimicamente tratados, *A. lituratus* poderia servir como modelo adequado para os estudos que visam contribuir para o conhecimento sobre o papel dos estrógenos nos machos. Assim, o objetivo do presente estudo foi investigar os índices de proliferação e morte celular nos dúctulos eferentes e epidídimo de *A. lituratus*, durante o ciclo reprodutivo anual, fazendo uma correlação dos resultados obtidos com os níveis de estrógenos e seus receptores (ERα e ERβ) no local.

2. Materiais e Métodos

2.1. Capturas

As capturas foram realizadas em Belo Horizonte com o uso de redes de neblina (3 m X 12 m), dispostas estrategicamente em trilhas para interceptar morcegos durante o vôo. Machos adultos da espécie *Artibeus lituratus* foram capturados durante o período reprodutivo (entre Agosto a início de dezembro) e durante o período de regressão (entre segunda quinzena de dezembro a início de abril). Os animais capturados foram classificados como adultos através das análises do desgaste dos dentes, pelagem e grau de ossificação das epífises das falanges das asas vistas por transparência. Essas características são comumente utilizadas em morcegos para determinação da faixa etária (Dinerstein, 1986 2005, De Knegt et al., 2005). Tanto as capturas quanto os experimentos foram realizados mediante a autorização do IBAMA (processo número 12017-2) e do Comitê de Ética em Experimentação Animal da Universidade Federal de Minas Gerais.

2.2. Processamento dos tecidos

Os animais capturados foram anestesiados com injeção de pentobarbital sódico 30mg/Kg em associação com cloridrato de Quetamina 20mg/Kg via intraperitoneal. A seguir, foram fixados, por perfusão transcardíaca, com solução de formalina neutra tamponada (NBF) a 10% para os ensaios imunohistoquímicos e de TUNEL. Após fixação, os órgãos genitais foram dissecados, pesados e imersos no mesmo fixador, onde foram estocados a 4^{o}C. Para os ensaios de Western blotting, após a perfusão com salina, os tecidos foram congelados em nitrogênio liquido e estocados em freezer -80^{o}C.

2.3. Imunohistoquímica

Os ensaios imunohistoquímicos foram realizados em fragmentos de dúctulos eferentes e epidídimos (n = 5 animais de cada período), incluídos em parafina e posteriormente seccionados a 5µm. Os tecidos foram desparafinizados em xilol, hidratados em série decrescente de etanol e incubados em solução de metanol/peróxido de hidrogênio 0,6% para bloqueio da peroxidase endógena. Após a recuperação antigênica realizada em solução de tampão citrato 0,1M pH 6,0, em forno microondas, os cortes foram submetidos ao bloqueio da avidina e biotina endógena pelo avidin/biotin blocking kit (Vector Laboratories, Burlingame, USA). Após lavagens em tampão fosfato (PBS), foi realizado o bloqueio de ligações inespecíficas com soro normal de cabra 10% por uma hora, antes da incubação do anticorpo primário. Em seguida, os cortes foram incubados *overnight* com anticorpo anti-CDC47/MCM7 (Thermo scientific, Fremont, CA, USA), diluído 1:300, ou anti-Caspase-3 ativada (Asp 175; Cell signaling technology, Beverly, MA, USA), diluído 1:500. Os controles negativos foram incubados com tampão fosfato em substituição aos anticorpos. Após lavagens em PBS, todos os cortes foram expostos por uma hora ao anticorpo secundário biotinilado (Dako, Carpinteria, USA), diluídos 1:100. Para amplificação dos resultados, os cortes foram submetidos à incubação com o complexo avidina-biotina (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, USA) por 30 minutos e em seguida a imunoreação foi visualizada com a adição do substrato contendo 3,3 diaminobenzidina. Para a confirmação dos resultados, foram realizados três ensaios distintos para cada anticorpo.

2.4. TUNEL (*TdT-mediated dUTP Nick End Labeling*)

A técnica visa a identificação de células apoptóticas pela marcação enzimática das extremidades 3'OH livres geradas pela fragmentação do DNA, característica de células apoptóticas (Dang & Kim, 2009). Os ensaios foram realizados a partir do kit ApopTag® Plus Peroxidase *In Situ* Apoptosis Detection (Millipore Corporation, EUA). Para isso, fragmentos de dúctulos eferentes e epidídimos fixados em NBF e incluídos em parafina foram seccionados a 5 µm, desparafinizados e hidratados. Após a recuperação antigênica com proteinase K (IHC SELECT® - Millipore Corporation, EUA), diluída 1:30 em tampão Tris –HCI 0,1%, pH8, os tecidos foram incubados solução contendo H2O2 3% diluída em Tris-HCL salino (TBS) por 30 minutos. Após lavagem em tampão TBS, os tecidos foram incubados por 30 minutos em tampão de equilíbrio fornecido pelo fabricante. Em seguida, os cortes foram incubados com enzima TdT diluídos em 30% de tampão de reação, por uma hora, a 37ºC, enquanto os controles negativos foram incubados em tampão de equilíbrio em substituição a enzima TdT. Essa enzima reconhece as extremidades 3'OH livres e acrescenta nucleotídeos marcados com moléculas de digoxigenina. Após a parada da reação, os tecidos foram incubados com anticorpo anti-digoxigenina conjugado a peroxidase por 30 minutos. Em seguida a visualização da reação foi realizada pela exposição dos tecidos a solução contendo 3,3 diaminobenzidina. Posteriormente, os cortes foram contracorados com hematoxilina, desidratados e montados. Os ensaios foram realizados em triplicatas para a confirmação dos resultados.

2.5. Western blotting

Para a confirmação dos resultados imunohistoquímicos e para validar o uso dos anticorpos em tecido de morcego, ensaios de Western blotting foram realizados em *pools* de dúctulos eferentes e epidídimo (cabeça, corpo e cauda) de cinco animais de cada período. Após a extração de proteínas totais com tampão de sacarose (pH 7.4) contendo 0,01 M de EDTA, e *cocktail* de inibidor de proteases (Sigma-Aldrich, St. Louis, USA), as proteínas foram quantificadas pelo método de Bradford. Em seguida, as proteínas foram separadas por eletroforese contínua (20 µg/por canaleta para MCM7 e 25 µg/por canaleta para Caspase-3) em géis de poliacrilamida (SDS-PAGE) 12% para MCM7 ou 15% para Caspase-3. Após transferências das proteínas para membrana de nitrocelulose, foi realizado o bloqueio de ligações inespecíficas com soro de cabra 10%. Posteriormente, as membranas foram incubadas com anticorpos primários anti-CDC47/MCM7 (Thermo scientific, Fremont, CA, USA), ou anti-caspase-3 ativada (Asp 175; Cell signaling technology, Beverly, MA, USA), diluídos 1:500. Em seguida, as membranas foram incubadas com anticorpos secundários (Dako, Carpinteria, USA), diluídos 1:1000. A revelação foi realizada por adição do substrato contendo diaminobenzidina e cloronaftol. Os ensaios de Western blotting foram realizados em triplicatas para a confirmação dos resultados.

2.6. Morfometria

O número de células positivas para MCM7 e Caspase-3 ativada por área foi estimado em quatro regiões randomicamente selecionadas de cada segmento (dúctulos eferentes, segmento inicial, cabeça, corpo e cauda do epidídimo) em cinco animais de cada período. Em cada região, núcleos positivos foram contados com amplificação de 400x, utilizando uma gradícula (com área total de 0,06 mm^2) contendo 100 subdivisões, acoplada à ocular do microscópio. O resultado foi convertido para células por mm^2 e analisado estatisticamente de acordo com Aherne & Dunnill (1982).

2.7. Análise estatística

Os valores obtidos a partir das análises morfométricas foram analisados através do software Matlab® (MathWorks, Natick, MA, USA). Os dados foram submetidos ao teste de normalidade Lilliefors test, uma modificação do teste Kolmogorov-Smirnov. Quando a normalidade foi confirmada, os dados foram analisados pelo teste t de Student para comparar a media entre duas populações ou ANOVA para comparar mais de duas populações. Quando a normalidade não foi confirmada, o teste Wilcoxon rank-sum e Kruskal-Wallis foi utilizado respectivamente para comparações entre duas ou mais populações. O nível de significância utilizado para todos os testes foi $P<0,05$. Os resultados foram representados como média ± erro padrão da média.

3. Resultados

3.1. Proliferação celular

Durante o período reprodutivo, poucas células mostraram-se imunopositivas para o marcador de proliferação celular MCM7 ao longo dos dúctulos eferentes e epidídimo (Fig. 1A-D e 2A). A maioria das imunomarcações apresentou padrão de divisão celular colateral, perpendicular à membrana basal, e mais raramente divisões paralelas à membrana basal, em todas as regiões analisadas (Fig. 1). Eventualmente foram ainda observados pequenos grupos de células imunomarcadas. No epitélio dos dúctulos eferentes, as células imunopositivas foram identificadas como células não-ciliadas, enquanto no epidídimo a maioria das células MCM7-positivas foi identificada como células principais. Ocasionalmente células basais e outras em posição mais apical no epitélio, que podem ser células apicais, estreitas ou halo, também mostraram-se positivas para MCM7 (Fig. 1B e C). Quantitativamente, o número de células imunomarcadas em todas as regiões do epidídimo foi semelhante ao observado nos dúctulos eferentes (Fig. 2A).

Durante o período de regressão sexual houve um aumento significativo no número de células positivas para MCM7 tanto no epitélio dos dúctulos eferentes quanto em todas as regiões do epidídimo, quando comparado com o período reprodutivo (Fig. 1 E-H e 2A). Na regressão, foi possível observar diversos agrupamentos de células

MCM7-positivas ao longo das vias genitais. Similar ao período reprodutivo, as células não-ciliadas são as células imunopositivas para MCM7 nos dúctulos eferentes, enquanto as células principais representaram a maioria das células positivas no epidídimo, sendo os demais tipos celulares de difícil identificação (Fig. 1). Comparativamente, o segmento inicial foi a região com maior número de células imunopositivas comparado com todos os demais segmentos (Fig. 2A).

Ressalta-se que não foi incomum a presença de células morfologicamente apoptóticas próximas às células proliferativas MCM7-positivas (Fig.1 inserto em B).

3.2. Apoptose

Durante o período reprodutivo, o epitélio dos dúctulos eferentes apresentou raras células positivas para caspase-3 ativada (Fig. 2B e 3 A-D). No epidídimo, embora as células positivas tenham sido proporcionalmente maiores que nos dúctulos eferentes (8,5 x no segmento inicial, 12,4x na cabeça, 25,5x no corpo e 14,2x na cauda), essa diferença não foi estatisticamente significativa (Fig. 2 B).

Durante o período de regressão, o perfil apoptótico das vias genitais seguiu padrão similar ao período reprodutivo (Fig. 3 E-H). Apesar de ter havido aumento do número de células positivas no epitélio dos dúctulos eferentes e epidídimo em regressão, esse aumento não foi significativo (Fig 2 B). Ressalta-se que o desvio padrão das amostras analisadas foi muito alto, devido à presença de valores extremos dentro das amostras, onde havia animais com células imunopositivas para caspase-3 e animais sem imunomarcação (Fig. 2 B). Essa grande variabilidade na distribuição das células caspase-3 positivas pode justificar a falta de significância estatística.

Durante os dois períodos analisados, foram observadas células morfologicamente apoptóticas, mas imunonegativas para a caspase-3 ativada (Fig. 3 F e inserto em H).

Para a confirmação dos resultados obtidos pela técnica de imunohistoquímica para a caspase-3 ativada, foram realizados ensaios de TUNEL. Os resultados obtidos foram semelhantes aos observados para caspase-3 nos dois períodos analisados, inclusive com a presença de células morfologicamente apoptóticas sem qualquer marcação pelo TUNEL (Fig. 3, insertos em C, G e H).

3.3. Western blotting

Os ensaios de Western blotting para MCM7 e caspase-3 ativada detectaram bandas específicas com peso molecular de 80 kDa e 17 kDa, respectivamente (Fig. 4). Estes resultados estão de acordo com o peso molecular descrito para as proteínas analisadas (Kwong et al., 1999, Ren et al., 2006). Os ensaios para MCM7 detectaram bandas de menor intensidade ao longo dos dúctulos eferentes e epidídimos durante o período reprodutivo, comparado com o período de regressão (Fig. 4A).

Durante o período reprodutivo, foram detectadas bandas de menor intensidade para caspase-3 ativada, nos dúctulos eferentes e cabeça do epidídimo, comparadas com as regiões do corpo e cauda, em ambos os períodos analisados, confirmando os dados imunohistoquímicos (Fig. 4B).

4. Discussão

Este estudo mostrou pela primeira vez dados sobre os índices de proliferação celular e apoptose nas vias genitais de uma espécie de quirópteros durante o ciclo reprodutivo anual. Os resultados mostraram que durante o período de atividade sexual, poucas células apresentaram atividade proliferativa ou entraram em apoptose, indicando uma estabilidade na homeostase tecidual. Contudo, durante o período de regressão, observou-se a atividade proliferativa significativamente aumentada. Situação similar ocorreu em relação à morte celular, que também foi mais expressiva na regressão tecidual, possivelmente como estratégia para continuar mantendo a homeostasia tecidual nessa fase funcional.

Durante o período reprodutivo, baixos índices de proliferação foram detectados ao longo do trato genital da espécie, como observado anteriormente nos dúctulos eferentes e epidídimo de outras espécies (Nagy & Edmonds, 1975, Robaire et al., 2006). O epidídimo é conhecido por ser um órgão com baixas taxas de proliferação, mesmo sob estimulo androgênico, ao contrário de outros órgãos genitais, como a próstata e vesícula seminal (Robaire et al., 2006). Corroborando esse fato, sabe-se que o fator de regulação transcricional B-myc, o qual é conhecido por inibir a atividade proliferativa, é expresso em altos níveis no epitélio epididimário (Gregory et al., 2000). Os dúctulos eferentes também mostraram baixas taxas de proliferação celular, mas a escassez de informação sobre o assunto inviabiliza uma discussão mais elaborada. Em suma, os dados obtidos sobre proliferação celular durante a atividade sexual de *A. lituratus* estão de acordo com a característica pouco mitogênica das vias genitais.

Durante a regressão sexual, o aumento do número de células em proliferação nos dúctulos eferentes e epidídimo de *A. lituratus* foi semelhante ao observado em roedores durante o desenvolvimento (Sun & Flickinger, 1979, Clermont & Flannery, 1970). Curiosamente, esse aumento ocorreu simultaneamente ao aumento dos níveis de ERα, tanto no epitélio quanto no estroma das vias genitais de *A. lituratus* (Oliveira et al., 2012). ERα está reconhecidamente relacionado com estímulo de proliferação celular em diversos órgãos (Lucas et al., 2008, Zhou et al., 2011, Attia & Ederveen, 2012). Prova disso, é que altos níveis do receptor são detectados no epidídimo de animais neonatos (Jefferson et al., 2000, Atanassova et al., 2001). Ao contrário, em animais adultos, os níveis de ERα são baixos ou indetectáveis (Fisher et al., 1997, Pelletier & El-Alfy, 2000, Nie et al., 2002, Hess et al., 2011). Dessa forma, o aumento de células em proliferação concomitante ao aumento de ERα nas vias genitais de *A. lituratus* durante a regressão sexual, suporta a hipótese de participação de ERα na preparação das vias genitais para um novo período reprodutivo, como sugerido anteriormente (Oliveira et al., 2012).

Outro fato de destaque foi a ocorrência de células proliferando mais no sentido colateral que paralelo a membrana basal dos dúctulos eferentes e epidídimo. A adição de células no sentido colateral indica aumento no diâmetro tubular, enquanto que a divisão no sentido horizontal indica aumento no comprimento tubular (Hinton et al., 2011). Esse dado corrobora prévios resultados morfométricos que indicam que durante a regressão sexual há redução de 30 a 50% no diâmetro tubular do epidídimo, dependendo da região considerada (Oliveira et al, 2012).

Em relação à morte celular, raras células apoptóticas foram detectadas ao longo das vias genitais durante o período reprodutivo, especialmente nos dúctulos eferentes. Dados sobre morte celular nos dúctulos eferentes em mamíferos adultos sob condições normais são inexistentes. Contudo, no epidídimo, baixos índices de morte celular são comumente detectados, o qual pode ser alterado na ausência de fatores luminais, diminuição dos níveis de testosterona ou aumento da temperatura (Turner & Riley, 1999, Jara et al., 2002). Durante o período de regressão, a proporção de células apoptóticas aumentou ao longo do ciclo reprodutivo anual de *A. lituratus*, apesar de não ter sido significativo, devido à alta variabilidade das amostras analisadas. Esse aumento pode estar associado ao equilíbrio entre proliferação e morte celular, também na fase de regressão, uma vez que essas células apoptóticas foram comumente detectadas próximas às células MCM7-positivas.

Ao longo de todo o epidídimo, especialmente no período de regressão, foram detectadas células morfologicamente apoptóticas sem marcação para caspase-3. Sabe-se que as vias envolvidas na execução da apoptose são altamente complexas e podem ocorrer independentemente de caspases efetoras (Gross et al., 1999, Desagher & Martinou, 2000). Por isso, utilizamos a técnica de TUNEL para a confirmação dos resultados, a qual também mostrou diversas células morfologicamente apoptóticas, mas TUNEL-negativas. É fato que o padrão de fragmentação do DNA pode interferir na detecção de apoptose pelo TUNEL, pois quando ocorre a clivagem escalonada do DNA, os fragmentos de DNA se tornam inacessíveis para a adição de nucleotídeos pela enzima TdT (Didenko & Hornsby, 1996). Uma vez que os dois marcadores clássicos de apoptose utilizados no presente estudo não foram suficientes para a detecção da população total de células apoptóticas, acreditamos que uma análise mais detalhada, com o auxílio de ferramentas morfológicas associada a outros marcadores, poderá contribuir para a obtenção de resultados mais precisos sobre o complexo processo de morte celular.

Em resumo, os baixos índices de proliferação e morte celular detectados durante o período de atividade sexual, estão de acordo com o observado para outros mamíferos. O aumento de células em proliferação durante o período de regressão sexual, concomitante com o aumento na expressão de ERα, corrobora com a hipótese sobre a participação do sistema responsivo a estrógenos na preparação das vias genitais para a recrudescência sexual. Os dados sobre apoptose não foram conclusivos, apontando que os marcadores utilizados são passíveis de falhas, sugerindo a necessidade de utilização de outras ferramentas de investigação.

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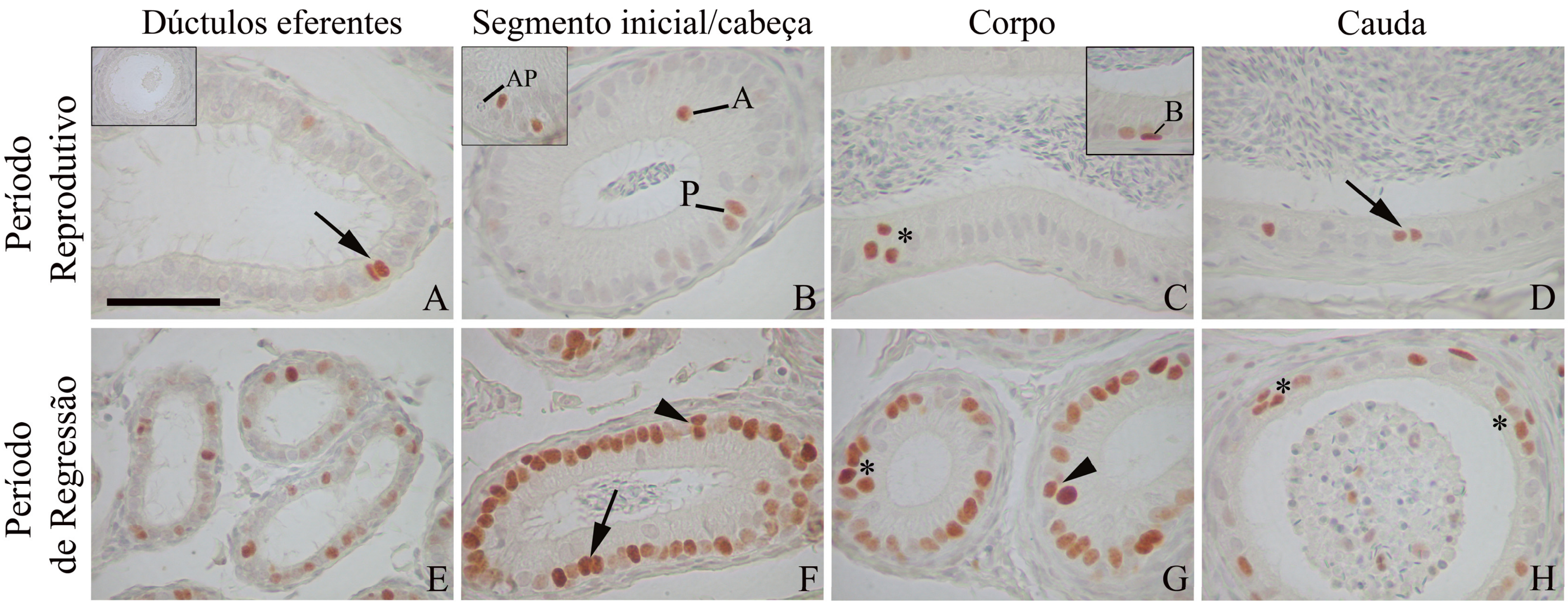


Fig . 1 – Imunolocalização de MCM7 nos dúctulos eferentes do morcego *Artibeus lituratus* durante os períodos reprodutivo (A-D) e de regressão (E-H). (P) = célula principal; (B) = célula basal; (A) = célula apical; (AP) = célula apoptótica não marcada; (seta) = divisão células colateral; (cabeça de seta) = divisão celular perpendicular; * = agrupamentos de células imunopositivas; inserto em (A) = controle negativo. Barra em (A) = 50µm.

Figuras

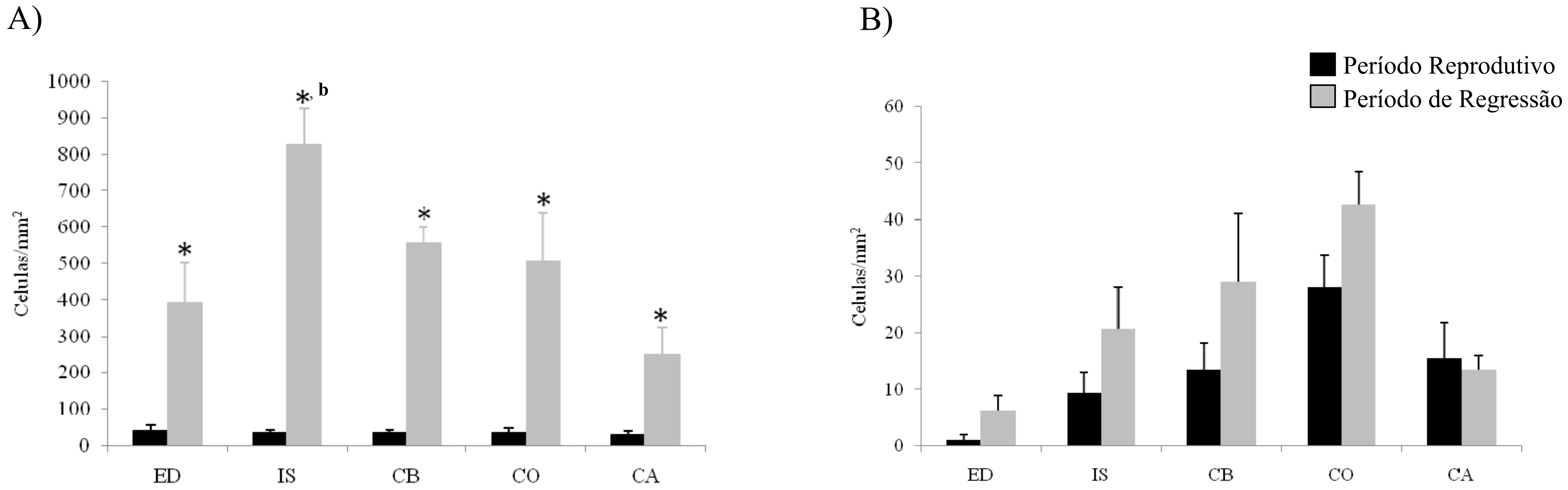


Fig. 2 – Análise quantitativa da expressão de MCM7 (A) e caspase-3 ativada (B) no epitélio dos dúctulos eferentes e epidídimo de *Artibeus lituratus* durante os períodos reprodutivo e de regressão. ED = dúctulos eferentes; IS = segmento inicial; CB = cabeça; CO = corpo; CA = cauda. * = diferença entre os dois períodos (P<0,05); b = diferença entre os segmentos (P<0,05).

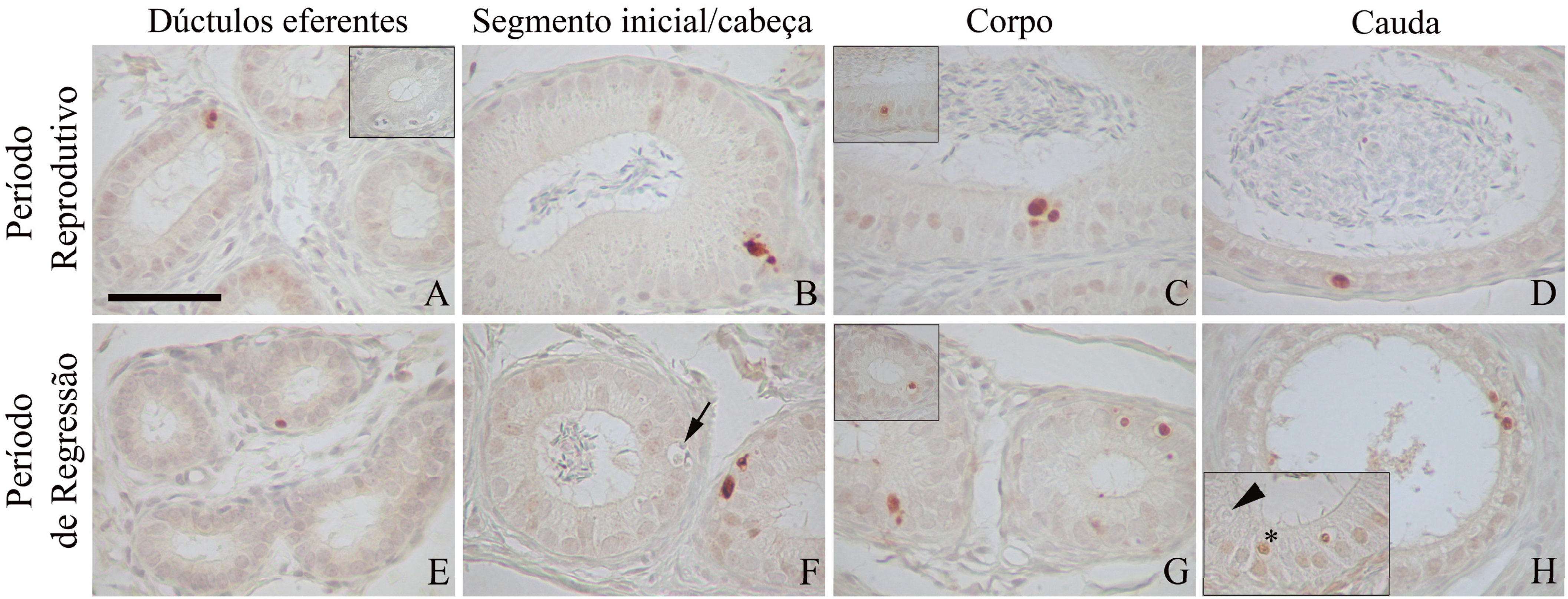
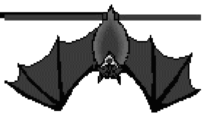


Fig. 3 – Imunolocalização de caspase-3 ativada nos dúctulos eferentes e epidídimo do morcego *Artibeus lituratus* durante os períodos reprodutivo (A-D) e de regressão (E-H). Seta = célula apoptótica negativa para caspase-3; inserto em (A) = controle negativo para caspase-3; Insertos em (C, G e H) = células apoptóticas TUNEL-positivas detectadas durante os períodos de atividade e regressão sexual, respectivamente, onde se observam célula apoptótica TUNEL-negativa (cabeça de seta) e célula não apoptótica TUNEL-positiva (*). Barra em (A) = 50µm.

Fig. 4 – Ensaios de Western blotting para MCM7 (A) e caspase-3 ativada (B) nos dúctulos eferentes e epidídimo de *Artibeus lituratus* durante os períodos reprodutivo e de regressão. Os respectivos pesos moleculares são indicados à esquerda. ED = dúctulos eferentes; CB = cabeça; CO = corpo; CA = cauda.



IV - DISCUSSÃO E CONCLUSÃO

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O presente estudo descreveu pela primeira vez o padrão de expressão dos receptores de estrógenos (ERs) nas vias genitais de uma espécie de morcego, bem como um possível papel desses receptores na regulação das aquaporinas e dos processos de proliferação e morte celular nas vias genitais durante o ciclo reprodutivo anual. Nosso objetivo foi expandir os conhecimentos prévios sobre a distribuição e variação sazonal dos receptores de estrógenos para as vias genitais, bem como avaliar a expressão de proteínas conhecidamente reguladas por estrógenos, as aquaporinas, durante o ciclo reprodutivo anual da espécie. Ainda, avaliamos a existência de uma possível correlação entre alterações nos níveis dos ERs com a proliferação e morte celular, uma vez que o aumento nos níveis testiculares de ERβ (considerado um fator pró-apoptótico) concomitante ao aumento de células apoptóticas foi observado durante a regressão sexual.

Devido à ausência de informações sobre a anatomia das vias genitais de *A. lituratus*, iniciamos esse trabalho com uma descrição anatômica das vias genitais dessa espécie. Nossos resultados mostraram que *A. lituratus* apresenta entre 12 e 15 dúctulos eferentes, que na sua porção distal, une-se ao epididímo através de múltiplas entradas, como observado anteriormente em grandes mamíferos e no homem (Yeung et al., 1991; Ilio & Hess, 1994). O epidídimo de *A. lituratus* apresenta as mesmas características descritas para outros mamíferos (Robaire et al., 2006). O conjunto epidídimo e dúctulos eferentes pesam cerca de 30 mg e apresentam três regiões distintas: cabeça, corpo e cauda, sendo a maior parte da cabeça epididimária composta pelos dúctulos eferentes, como anteriormente descrito em humanos (Yeung et al., 1991; Ilio & Hess, 1994).

Ao contrário do observado em morcegos de regiões temperadas (Gustafson, 1979; Oliveira & Oliveira, 2011), a regressão das vias genitais em *A. lituratus* ocorre entre dezembro e abril, mesmo período da regressão testicular. Nesse período observou-se marcante regressão acompanhada por uma significativa redução no peso dos dúctulos eferentes e epidídimos. A análise histológica mostrou uma significativa redução na altura do epitélio e diâmetro dos dúctulos eferentes e ducto epididimário, além da ausência de espermatozoides luminais.

Em relação aos receptores de estrógenos, os resultados mostraram que, durante o período reprodutivo, ERα e ERβ apresentam um padrão de expressão nas vias genitais semelhante ao descrito para outras espécies de mamíferos eutérios, sendo ERα restrito ao epitélio dos dúctulos eferentes e ERβ amplamente distribuído tanto no epitélio quanto no tecido conjuntivo circunjacente (Ergun et al., 1997; Fisher et al., 1997; Goyal et al., 1997; Hess et al., 2011; Joseph et al., 2011; Nie et al., 2002; Nielsen et al., 2001; Saunders et al., 2001; Schon & Blottner, 2008; Tian et al., 2004). Entretanto, apenas ERα apresentou variação sazonal com aumento de seus níveis durante o período de regressão sexual, tanto no epitélio quanto no tecido conjuntivo e células musculares lisas dos dúctulos eferentes e epidídimo. No epidídimo, o segmento inicial foi a região que apresentou níveis mais altos de ERα durante a regressão. Curiosamente, esse é o segmento do epidídimo que mais depende de estrógenos, uma vez que é a primeira região do epidídimo a expressar ERα durante o desenvolvimento, além de ser a região do epidídimo que mais sofre alterações na ausência desse receptor (Cooke et al., 1991; Hess et al., 2000).

Durante o período de regressão, concomitante ao aumento da expressão de ERα, houve um aumento significativo para o marcador de proliferação nas células epiteliais dos dúctulos eferentes e epidídimo. Os ERα são conhecidos fatores pró-proliferação e esses dados corroboram com a função proliferativa de ERα, uma vez que já foi observado que esse é um fator fundamental para a proliferação da musculatura lisa prostática de ratos (Zhou et al., 2011). Além disso, há evidências de que células epiteliais são constantemente estimuladas por fatores parácrinos oriundos do tecido conjuntivo adjacente (Pu et al., 2007; Turner et al., 2003; Zhou et al., 2011). Dessa forma, o aumento de ERα pode ser importante para assegurar a indução da proliferação celular necessária para o restabelecimento das funções reprodutivas.

A presença de células em proliferação, principalmente no plano colateral indica maior incremento no diâmetro que no comprimento tubular (Hinton et al, 2011). Esse dado é compatível com os resultados morfométricos que indicam que durante a regressão há redução de 30 a 50% no diâmetro tubular do epidídimo, dependendo da região considerada (Oliveira et al., 2012). O aumento identificado na proporção de células apoptóticas, apesar de não ter sido estatisticamente significativo, pode ser um fator importante para manter a homeostasia tecidual, uma vez que frequentemente células com morfologia de apoptose foram identificadas próximas às células em proliferação. Entretanto, um estudo mais detalhado desse evento, com auxílio de outras ferramentas, poderá contribuir para a obtenção de resultados mais conclusivos, como por exemplo, as vias envolvidas na apoptose.

Ao contrário do observado para ERα, não houve alterações nos níveis de ERβ ou de AR ao longo do ciclo reprodutivo de *A. lituratus*. Isso sugere que esses receptores sejam importantes para a manutenção de funções essenciais das vias genitais, independente do período reprodutivo. Além disso, a expressão constitutiva de ERβ já foi previamente descrita, mostrando-se inalterada mesmo quando submetida a diferentes situações experimentais (Atanassova et al., 2001; Saunders et al., 2001; Turner et al., 2001; Oliveira et al., 2003; Oliveira et al., 2004; Albrecht et al., 2004; Shapiro et al., 2005; Schon et al., 2009; Fernandes et al., 2011; Gomes et al., 2011). Em relação a AR, sua expressão constitutiva pode ser importante para as vias genitais, especialmente para o epidídimo que é considerado um órgão regulado principalmente por andrógenos (Robaire et al., 2006). Contudo, um fato intrigante foi o aumento significativo de testosterona e diidrotestosterona durante a regressão sexual, ao contrário do observado em morcegos hibernantes (Racey, 1974; Gustafson & Shemesh, 1976; Bernard, 1986; Oliveira & Oliveira, 2011). Esse fato, apesar de inusitado, já foi anteriormente observado em outra espécie de morcego sazonal de região tropical (Jolly & Blackshaw, 1989). Entretanto, mais estudos são necessários para maiores esclarecimentos, já que dados relacionados à endocrinologia de morcegos de regiões tropicais são escassos.

Um dado importante obtido pelo nosso estudo foi a localização da enzima aromatase nas vias genitais, já que em mamíferos eutérios, a detecção da enzima havia sido realizada apenas nos dúctulos eferentes de humanos (Carpino et al., 2004) e no epidídimo de cavalos (Hejmej et al., 2005). Nesse sentido, nossos resultados contribuíram para mostrar em mais uma espécie, a presença da aromatase nas vias genitais, demonstrando ainda, a localização específica da aromatase nas células não-ciliadas dos dúctulos eferentes e células principais e algumas basais do epidídimo em ambos os períodos analisados. Esse foi um dado importante do nosso trabalho e indica que as vias genitais são uma importante fonte de estrógenos para os receptores de estrógenos presentes no local e que o padrão de localização da aromatase é semelhante à observada em outros mamíferos (Carpino et al., 2004; Hejmej et al., 2005). Além disso, os níveis inalterados de aromatase durante o período de regressão estão de acordo com os níveis de estrógenos plasmáticos e testiculares que também são inalterados. Assim, a presença da aromatase durante o período de regressão pode ser importante para garantir a presença do ligante para os receptores de estrógenos durante todo o ciclo anual da espécie, contribuindo para o restabelecimento das funções reprodutivas das vias genitais e preparando-o para o próximo período reprodutivo.

Em relação às aquaporinas, AQP1 e AQP9 mostraram uma distribuição célula e órgão-dependente ao longo dos testículos e vias genitais, além de variação sazonal.

Além disso, a presença da AQP1 nos testículos e a ausência de AQP9 nos dúctulos eferentes são resultados não descritos para outras espécies de mamíferos investigadas até o momento. Essa distribuição diferenciada, bem como a variação sazonal da AQP9 sugere que essas proteínas exerçam papéis fisiológicos distintos ao longo do sistema genital masculino.

A presença da AQP1 nas espermátides dos testículos em *A. lituratus* difere do observado, para outras espécies, onde AQP1 não foi detectada em qualquer célula somática ou germinativa dos testículos (Fisher et al., 1998; Lu et al., 2008; Nicotina et al., 2005). Entretanto, a presença de outras aquaporinas foi anteriormente detectada nas espermátides de ratos (AQP7 e AQP8) e de humanos (AQP7) (Calamita et al., 2001; Ishibashi et al., 1997; Suzuki-Toyota et al., 1999; Yeung et al., 2010). A razão de diferentes aquaporinas, aparentemente exercerem funções semelhantes em diferentes espécies não é conhecida. Entretanto, a presença das aquaporinas nas espermátides, sugere a participação dessas proteínas na diferenciação dessas células, possivelmente facilitando o efluxo de água necessário para a condensação citoplasmática e redução do volume celular, importante para o processo espermiogênico.

A AQP9 não foi detectada nos testículos de *A. lituratus*, assim como previamente observado no camundongo, no cão e no homem (Domeniconi et al., 2007; Hashem, 2010; Ko et al., 1999; Tsukaguchi et al., 1999). Por outro lado, a AQP9 foi detectada nas células de Leydig do rato (Elkjaer et al., 2000; Nicchia et al., 2001; Nihei et al., 2001; Badran & Hermo, 2002). O motivo da incompatibilidade desses resultados ainda não foi determinado. Entretanto, a realização de mais investigações sobre a presença das aquaporinas em espécies ainda não investigadas e de outras aquaporinas nos testículos das espécies até então estudadas, poderão contribuir para determinação do exato papel das aquaporinas nos testículos.

A presença da AQP1 na membrana apical das células não ciliadas dos dúctulos eferentes está de acordo com o descrito para outras espécies de mamíferos (Brown et al., 1993; Fisher et al., 1998; Badran & Hermo, 2002; Domeniconi et al., 2007; Domeniconi et al., 2008; Oliveira et al., 2005; Ruz et al., 2006; Lu et al., 2008; Arrighi et al., 2010a; Arrighi et al., 2010b). Esse dado está de acordo, por tanto, com a função reabsortiva dos dúctulos eferentes, responsável pela reabsorção de mais de 90% do fluido testicular que chega às vias com os espermatozoides (Clulow et al., 1998; Hess, 2002). A expressão da AQP1 mostrou ser constitutiva em *A. lituratus*, ou seja, sem variação sazonal, assim como observado anteriormente em experimentos utilizando animais knockouts para

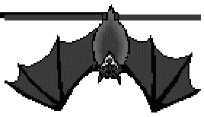
ERα, o anti-estrógeno ICI 182,780 castração, ligação dos dúctulos eferentes, administração de antagonista de GnRH ou testosterona e desnutrição, sugerindo que AQP1 não é regulada por fatores testiculares, incluindo andrógenos (Fisher et al., 1998; Badran & Hermo, 2002; Oliveira et al., 2005; Arrighi et al., 2010a). Em relação a estrógenos, foi observado que tratamentos com o anti-estrógeno ICI 182,780 ou reposição com estradiol após a castração também não alteram a expressão da AQP1 no epitélio dos dúctulos eferentes (Oliveira et al., 2005). Contudo, os níveis da AQP1 foram reduzidos após a exposição de animais neonatais ao dietilbestrol (um estrógeno sintético) e após inativação genética de ERα (αERKO). Entretanto, estas alterações foram consideradas secundárias já que esses animais apresentaram drásticas alterações na citoarquitetura epitelial (Fisher et al., 1998; Ruz et al., 2006).

Em relação à AQP9, os dúctulos eferentes de *A. lituratus* mostrou-se negativo, ao contrário do observado em camundongo, rato, cão e gato (Badran & Hermo, 2002; Domeniconi et al., 2007; Oliveira et al., 2005; Ruz et al., 2006; Arrighi et al., 2010a; Arrighi et al., 2010b). Nesse caso, já está bem estabelecido que, apesar da AQP9 apresentar uma seletividade similar em diferentes espécies (água, glicerol, uréia), existe um padrão distinto de distribuição para essa aquaporina em diferentes tecidos, possivelmente devido a uma necessidade específica de metabólitos entre diferentes espécies (Tsukaguchi et al., 1999). Nesse sentido, ratos e morcegos apresentam diferenças estruturais em relação aos dúctulos eferentes. Em *A. lituratus*, os dúctulos eferentes são mais numerosos (12-15) em relação aos ratos (6-8 dúctulos). Além disso, a conexão entre dúctulos eferentes e epidídimo é feita através de múltiplas entradas em morcegos (Oliveira & Oliveira, 2011; Oliveira et al., 2012) e através de uma única entrada no rato (Ilio & Hess, 1994). Essa diferença anatômica pode refletir em uma maior concentração de espermatozoides nos dúctulos dos ratos e, portanto, justifica-se a necessidade de várias aquaporinas nos dúctulos eferentes desses animais para garantir o estabelecimento de um ambiente luminal adequado os espermatozoides. Entretanto, não há dados sobre a expressão da AQP9 em outras espécies com morfofisiologia dos dúctulos eferentes similar ao de *A. lituratus*. Por outro lado, a ausência da AQP9 nos dúctulos eferentes de *A. lituratus* não exclui a possibilidade da presença de outras aquaporinas e/ou aquagliceroporinas nos dúctulos eferentes de *A. lituratus*.

No epitélio epididimário, AQP9 foi detectada, enquanto AQP1 não mostrou imunoreação, como anteriormente observado para outras espécies (Badran & Hermo, 2002; Oliveira et al., 2005; Domeniconi et al., 2007; Domeniconi et al., 2008). O padrão de expressão da AQP9 apresentou um aumento gradual do segmento inicial em direção à cauda epididimária. Esse padrão está de acordo com o aumento luminal da osmolalidade que é considerado um mecanismo importante para a maturação espermática (Cooper & Yeung, 2003). Em morcegos hibernantes, o ambiente hiperosmolar da cauda é responsável por prolongar a sobrevivência e garantir a viabilidade dos espermatozoides por longos períodos (Crichton et al., 1994). Assim, considerando que a concentração dos espermatozoides também aumenta do segmento inicial até a cauda, os altos níveis de AQP9 podem contribuir para a manutenção de um ambiente hiperosmolar necessário para a manutenção dos espermatozoides quiescentes. Além disso, por se tratar de uma aquagliceroporina permeável a glicerol, uréia e outros pequenos solutos, além de água (Tsukaguchi et al., 1998), a AQP9 pode participar de outros processos fisiológicos como, por exemplo, o transporte de glicerol que está presente no fluido luminal epididimário, o qual atua como fonte de energia para a estocagem dos espermatozoides na cauda (Cooper & Brooks, 1981). Corroborando com essa hipótese, estudos anteriores mostraram que o epidídimo é permeável a glicerol e esse processo é dependente da AQP9 e regulado por cAMP e bradicinina (Pietrement et al., 2008; Belleannee et al., 2009). Outra possibilidade, é que a AQP9 possa estar envolvida com o transporte de uréia presente em altas concentrações na cauda epididimária (Turner et al., 1979; Turner & Cesarini, 1983). Assim a AQP9 estaria contribuindo para a remoção da uréia presente no fluido luminal epididimário e assim, contribuindo para a desintoxicação do ambiente luminal. Nesse sentido, há estudos que mostram em hapatócitos a AQP9 facilita o efluxo de uréia e glicerol, sendo mais permeáveis a esses dois solutos que à água (Carbrey et al., 2003). Além disso, recentemente foi demonstrado que a captação de uréia pela pele auxilia na barreira da pele e contribui para a defesa antimicrobiana (Grether-Beck et al., 2012). Considerando que o epidídimo seja responsável por proteger os espermatozoides de agentes externos ao ducto, a AQP9 poderia contribuir com essa função epididimária. Entretanto, mais estudos são necessários para confirmar se AQP9 de fato contribuem para essas funções.

Durante o período de regressão, *A. lituratus* apresentou o mesmo padrão de distribuição da AQP9 no epidídimo do observado durante o período reprodutivo, porém com níveis significativamente mais baixos. No epidídimo, os andrógenos têm sido considerados os fatores moduladores da AQP9 (Pastor-Soler et al., 2002; Oliveira et al., 2005; Pastor-Soler et al., 2010). Entretanto, nossos dados mostram uma reduzida expressão de AQP9 no epidídimo mesmo com altos níveis local, plasmático e testicular de andrógenos (presente estudo), bem como quando os níveis de receptores de andrógenos também estão altos (Oliveira et al., 2012). Esses achados corroboram com sugestões prévias de que AQP9 pode ser modulada por outros fatores luminais (Badran & Hermo, 2002). Estradiol também não parece ser um fator modulador da AQP9 no epidídimo de *A. lituratus*, já que as concentrações desse esteróide não variam no local ou sistemicamente ao longo do ciclo reprodutivo anual da espécie. Além disso, há evidências de que a enzima aromatase também não varia no epitélio epididimário durante seu ciclo reprodutivo anual (Oliveira et al., 2012). Dessa forma, nossos dados estão de acordo com o previamente descrito de que os mecanismos de regulação da AQP9 nas vias genitais masculinas podem ser mais complexos do que sugerido previamente (Badran & Hermo, 2002; Oliveira et al., 2005; Picciarelli-Lima et al., 2006; Pietrement et al., 2008; Belleannee et al., 2009).

Assim concluímos que os receptores de estrógenos ERα foram os únicos a apresentarem variação sazonal, enquanto os níveis de ERβ e AR mantiveram-se inalterados. O aumento dos níveis de ERα e de células em proliferação nas vias genitais de *A. lituratus* durante a regressão sexual sugere que os estrógenos, através de ERα, atuam na recuperação das funções reprodutivas, preparando os machos da espécie para um novo período reprodutivo. A presença da enzima aromatase nas vias genitais indica que o epitélio dos dúctulos eferentes e epidídimo atuam como uma fonte local de estrógenos, especialmente durante a regressão sexual quando os espermatozoides, considerados importante fonte de estrógenos para as vias genitais, estão ausentes. O aumento do número de células apoptóticas durante a regressão sexual, mesmo não sendo significativo, indica um equilíbrio entre proliferação e morte celular, uma vez que frequentemente, células morfologicamente apoptóticas são visualizadas ao lado de células em proliferação. Em relação às aquaporinas, as AQP1 e AQP9 mostraram expressão célula- e região-especifica, sendo as AQP1 detectadas nas espermátides testiculares e epitélio dos dúctulos eferentes e as AQP9 expressas no epitélio epididimário. AQP1 apresentou expressão constitutiva, enquanto AQP9 apresentou variação sazonal. A sazonalidade de AQP9 aparentemente não está relacionada à variação dos níveis de testosterona, diidrotestosterona ou estradiol, mas sim possivelmente a fatores luminais do epidídimo.



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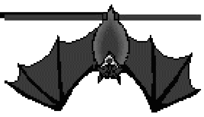
VI - ANEXO



ARTIGO 4

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Distribution of estrogen receptors (ERα and ERβ) and androgen receptor in the testis of big fruit-eating bat *Artibeus lituratus* is cell- and stage-specific and increases during gonadal regression

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ABSTRACT

The testis is a classical target for androgens, especially testosterone, acting via androgen receptor (AR). Alternatively, androgens can be aromatized to produce estrogens which act via specific receptors ERα and ERβ. Although estrogen action is essential for maintenance of male fertility, studies regarding the expression of ERα and ERβ in testis are restricted to a few species of rodent and domestic animals, but rarely in wild species. To our knowledge, there are no studies in Chiroptera species. Chiroptera represent one of the largest and most diversified orders of mammals, which possess several interesting reproductive features, including higher affinity of SHBG for estrogens than androgens. Therefore, we thought that bats would constitute a good model for investigation of the role of estrogens in the male. In this study, the distribution of ERα, ERβ and AR were evaluated in the testis of the big fruit-eating bat *Artibeus lituratus* and their levels were compared during reproductive and regressive periods. The results showed that ERα and AR were restricted to the somatic cells of the testis, whereas ERβ was widely distributed in both somatic and spermatogenic cells in a cellular and stage-specific fashion. We demonstrated for the first time by immunohistochemistry, and confirmed by Western blotting, that ERβ and AR increased during regression. The localization of ERα, ERβ and AR in a seasonal, cell and stage-specific fashion in the testis of *A. lituratus* suggests that these receptors may play important roles in testis function during reproductive and non-reproductive periods.

1. Introduction

The testis is a known target for androgen action, especially testosterone, acting via androgen receptors (AR), which are usually found restricted to Sertoli, Leydig and myoid cells. However, testosterone may also be locally metabolized to estrogen, which acts via specific estrogen receptors ERα and ERβ (Hess, 2003). Among the estrogen receptors, ERβ is more widely distributed in the testis, being expressed in both spermatogenic and somatic cells (Saunders et al., 2001; Nie et al., 2002; Zhou et al., 2002). The testicular expression of ERα has been controversial, as it is present in some species but absent in others (Fisher et al., 1997; Goyal et al., 1997; Saunders et al., 2001; Nie et al., 2002; Zhou et al., 2002; Gaskell et al., 2003; Berensztein et al., 2006). The relevance of estrogen for the maintenance of male fertility has been demonstrated by several animal models, including genetic or chemical inactivation of aromatase (ArKO), ERα (αERKO), ERβ (βERKO), or both ERα and ERβ (αβERKO), whose males are infertile (Hess et al., 1997; Robertson et al., 1999; Dupont et al., 2000; Oliveira et al., 2001, 2002; Antal et al., 2008). Besides these animal models, testis abnormalities have also been found in men possessing natural inactivating mutation of aromatase or ERα gene (Smith et al., 1994; Morishima et al., 1995). A possible role played by estrogens in the higher incidence of reproductive disorders in human and other animal species, such as cryptorchidism, hypospadia, and testicular and prostate cancer, has also received major attention in the last decade (Yoshida et al., 2005).

Despite the importance of estrogen and its receptors for keeping normal testis physiology, studies on estrogen receptors (ER) distribution in the mammalian testis have been limited to some species of rodents (rat and mouse), domestic animals (cat, dog, boar, goat, horse) and primates (*Macaca mulata, M. fascicularis*, *M. arctoides* and *Callithrix jacchus*), including human (Fisher et al., 1997; Goyal et al., 1997; Pelletier et al., 1999; McKinnell et al., 2001; Saunders et al., 2001, 2002; Nie et al., 2002; Zhou et al., 2002; Gaskell et al., 2003; Hejmej et al., 2005; Mutembei et al., 2005; Shapiro et al., 2005; Berensztein et al., 2006; Ramesh et al., 2007). In non-mammalian vertebrates similar studies were made in turtle *Trachemys scripta* (Gist et al., 2007) and three species of fishes (*Oncorhynchus mykiss*, *Anguilla japonica* and *Dicentrarchus labrax*) (Miura et al., 1999; Bouma and Nagler, 2001; Vinas and Piferrer, 2008). Therefore, the occurrence and distribution of ER in the testis of wild species are largely unknown.

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The Chiroptera represent one of the largest and most diversified orders of mammals, with 18 families, 202 genus and 1120 species (Reis et al., 2007), inhabiting every continent except Antarctica (Brunet-Rossinni and Austad, 2004). Several interesting reproductive features have been described for bat species from Temperate zone, including prolonged storage of sperm in male genital system, temporal asynchrony between spermatogenesis and mating, as well as asynchrony between testis and accessory glands activity (Gustafson, 1979). However, little information is available on reproduction of Neotropical Chiroptera. Concerning the distribution of androgen and estrogen receptors, no information is available on the male genital system of bats. It is interesting that in bats the steroid hormone binding globulin (SHBG) has higher affinity for estrogens than androgens (Renoir et al., 1980). This feature of Chiroptera is shared by men and other primates, but differs from other mammalian species (Renoir et al., 1980; Damassa et al., 1985; Kwiecinski et al., 1987). Several emerging roles for the association between SHBG and estrogens have been described (Caldwell et al., 2006; Selva and Hammond, 2006), leading us to consider the possibility that bats may constitute a good model for investigation of the role of estrogens in the males.

Therefore, in the present study we aim to investigate the pattern of cellular localization of ERα, ERβ and AR within the testes of big fruit-eating bat *Artibeus lituratus* and to compare the levels of these receptors in testes at both reproductive and non-reproductive periods. *Artibeus lituratus* is a frugivorous bat that belongs to the suborder Microchiroptera and family Phyllostomidae. This species is common in preserved areas and urban centers and is widely distributed throughout Tropical America (Zortéa and Chiarello, 1994).

2. Materials and methods

2.1. Animals and tissue preparation

Testes were obtained from adult male *A. lituratus*, captured in different month of the year, in the urban perimeter of Belo Horizonte county (19°55′S and 43°56′W), South-eastern Brazil. The climate on this region is characterized by a rainy season (October–March) coinciding with the hottest period and a dry season (April–September) coinciding with the coldest period (Zortéa, 2003). The capture was performed by using a mist-nets (3 m × 12 m) set to intercept bats flying 1–2 m above the ground. The animal captures were carried with the permission of the Brazilian Institute of Natural Environment and Renewable Resources (IBAMA, Brazil). The Ethics Committee in Animal Experimentation of the Federal University of Minas Gerais (CETEA-UFMG) approved the experimental procedures.

The bats were aged as adult based on the body weight, complete ossification of the metacarpal epiphyses and wear of the teeth (De Knegt et al., 2005). The reproductive status (activity or regression) was determined on the base of testicular size and occurrence of complete spermatogenesis or frequency of apoptosis in the testis, as confirmed by histological analysis.

2.2. Histology

After capture, the bats were weighed, anesthetized (i.p. pentobarbital 30 mg/kg and ketamine chloridrate 20 mg/kg) and perfused via left ventricle with 2.5% glutaraldehyde in 0.1 M phosphate buffer or 10% neutral buffered formalin (NBF), for histological and immunohistochemical studies, respectively. After perfusion, the testes were dissected and weighed. Fragments of testis were dehydrated through graded ethanol solutions, embedded in glycolmethacrylate (Technovit 7100; Heraeus Kulzer, Germany), sectioned at 3 μm and stained with toluidine blue, hematoxylin and eosin (HE) or periodic acid-Schiff (PAS) followed by hematoxylin counterstaining, for histological analysis.

2.3. Testis morphometry

The seminiferous tubule diameter was measured in 20 transversal tubular profiles randomly chosen, and measured in three animals in reproductive or regressive periods. For these measurement, images of histological sections were obtained using a Nikon Eclipse E600 microscope (Nikon Co., Melville, NY) and analyzed morphometrically using Image Tool software (University of Texas Health Sciences Center, San Antonio, TX).

2.4. Immunohistochemistry

The occurrence and distribution of ERα, ERβ and AR in the testis of bats during reproductive and regressive periods were investigated by immunohistochemistry ($n = 5$ for each period). For this study, NBF fixed fragments of testis were dehydrated through graded ethanol solutions, embedded in paraffin and sectioned at 5 μm. After microwaving for antigen retrieval, endogenous biotin activity was blocked by using avidin and biotin blocking solution (Avidin/biotin blocking kit – Vector Laboratories, Burlingame, USA). For blocking non-specific antibody binding, the sections were treated with 10% normal goat serum prior to incubation with the primary antibodies mouse anti-human ERα (Novocastra Laboratories, Newcastle, UK) diluted 1:100, mouse anti-human ERβ (Novocastra Laboratories, Newcastle, UK) diluted 1:25 and rabbit anti-human AR (PG21, Upstate, Lake Place, NY) diluted 1:500, at 4 °C. For negative controls, the sections received phosphate buffer saline (PBS) in place of the primary antibody. After washing in PBS, the sections were exposed for 1 h to a biotinylated secondary antibody goat anti-mouse (for ERα and ERβ) or goat anti-rabbit (for AR) (Dako, Carpinteria, USA), used at 1:50 (ERβ) or 1:100 (ERα and AR) dilution. After this step the sections were incubated with the avidin–biotin complex (Vectastain Elite ABC kit – Vector Laboratories, Burlingame, USA) for 30 min and the immunoreaction was visualized using diaminobenzidine containing 0.01% H_2O_2 in 0.05 M Tris–HCl buffer, pH 7.6. Sections were slightly counterstained with Delafield's hematoxylin. To confirm the results, immunostaining were performed in triplicate sets.

2.5. Western blotting

Western blotting assays were performed in order to determine the specificity of the antibodies used and to compare the expression of the AR, ERα and ERβ in the testis of *A. lituratus* during the reproductive and non-reproductive periods ($n = 4$ for each period). Following dissection, one of the two testes was frozen in liquid nitrogen for the Western blotting and the contralateral testis was processed for histology, in order to identify the testicular phase of reproduction. After maceration using dry ice, tissue were thawed and total protein was extracted by addition of 1% sodium dodecyl sulfate, 30 mM Tris–HCl pH 6.8, 2-mercaptoethanol, 12% (v/v) glycerol and bromophenol blue. Proteins were then subjected to continuous electrophoresis using 10% SDS–PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 10% normal goat serum (NGS) for 1 h at room temperature and incubated with mouse anti-human ERα (1:300), mouse anti-human ERβ (1:150) (Novocastra, Newcastle, UK) or rabbit anti-human AR (1:300) (Upstate, Temecula, EUA) antibodies for 1 h. After washing with PBS–Tween 0.05% (PBST), the blots were incubated in secondary goat anti-mouse (for ERα and ERβ) and goat anti-rabbit antibodies (for AR) (Dako, Carpinteria, CA), diluted 1:1000. After several washes in PBST, the reaction was developed by the addition of 3,3 diaminobenzidine and chloronaphthol. The reaction was stopped with deionized water. All protein assays were replicated and the density of the bands obtained was estimated by using Scion Image software (http://www.scioncorp.com).

2.6. Statistical analysis

Data were first subjected to the Shapiro–Wilk's W normality test and then analyzed by the Student's *t*-test. Normal distribution of the data was considered if $P \geqslant 0.05$ whereas differences between groups were significant at $P \leqslant 0.05$.

3. Results

3.1. Testicular structure

The adult bats presented similar mean body weight and length, irrespective of the reproductive period (Fig. 1A–C). The testes of *A. lituratus* were localized on either side at the base of the penis, into a subcutaneous pouch, without forming a true scrotum (Fig. 1A). Testes were found in reproductive period from August to December, coinciding with the rainy season in South-eastern Brazil. From December to the beginning of April the testes were mostly regressive, whereas testicular recrudescence was observed from April to July. Some overlapping of active/regressive and regressive/recrudescent periods was observed in December and April, respectively.

The testes were elliptic in shape and weighted 113 ± 0.04 and 50 ± 0.02 mg during the reproductive and regressive periods, respectively (Fig. 1D and E). They were surrounded by a thick albuginea, which emitted septa of connective tissue in the mediastinum direction, dividing the parenchyma in lobules. The parenchyma was formed by the seminiferous tubules interspersed by the interstitium, containing abundant Leydig cells (Figs. 2 and 3). The Leydig cells were found in clusters located in close proximity to numerous lymphatic spaces, lined by a thin endothe-

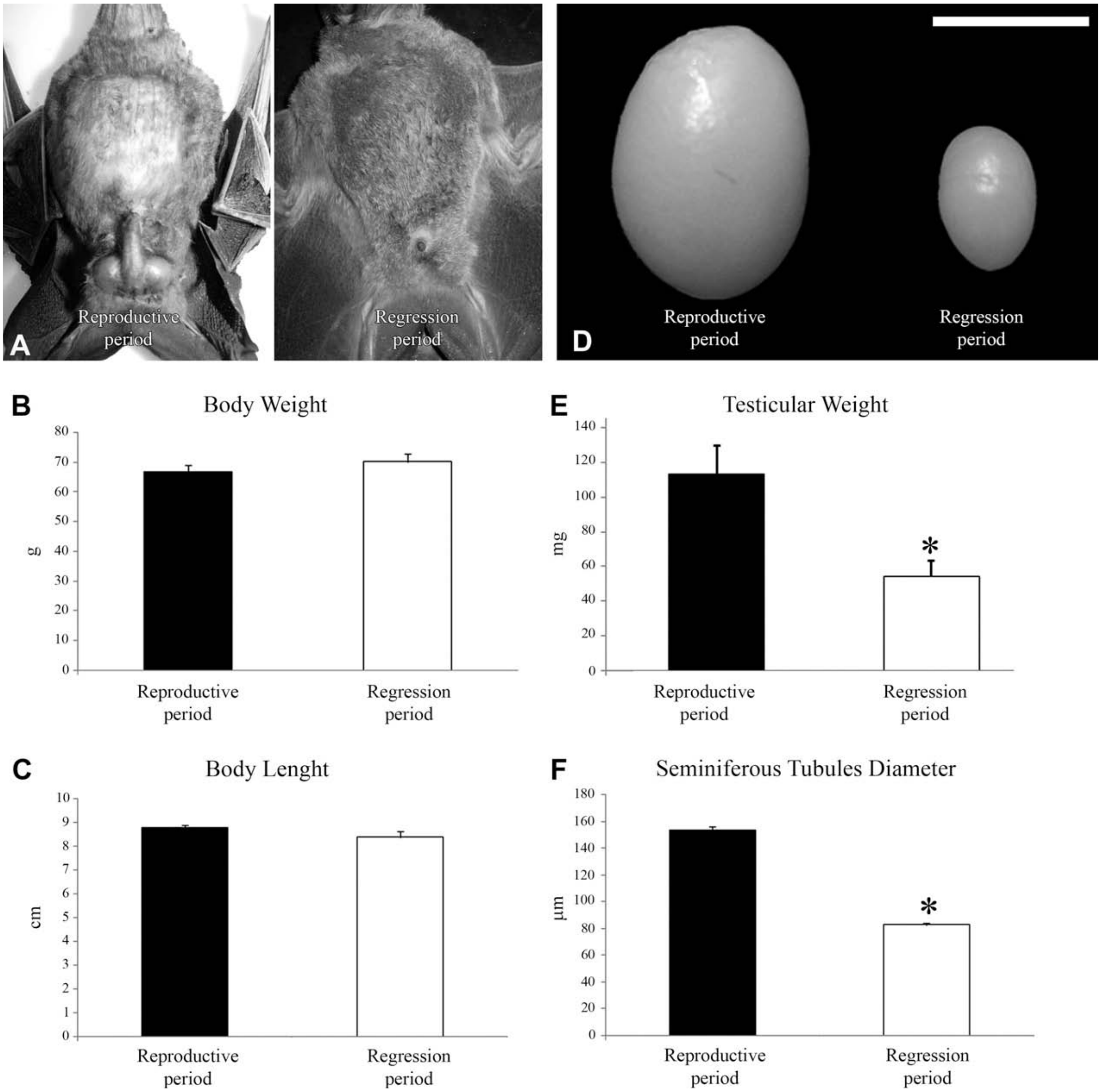

Fig. 1. General and testicular aspects of *Artibeus lituratus* during reproductive and regressive periods. No differences in body weight and length were found between both periods analyzed (A–C). On the other hand, the testis of animals in regressive period presented a marked reduction in size (D), weight (E), and in seminiferous tubules diameter (F). $^{*}P \leqslant 0.05$; $n = 5$. Bar = 0.5 cm.

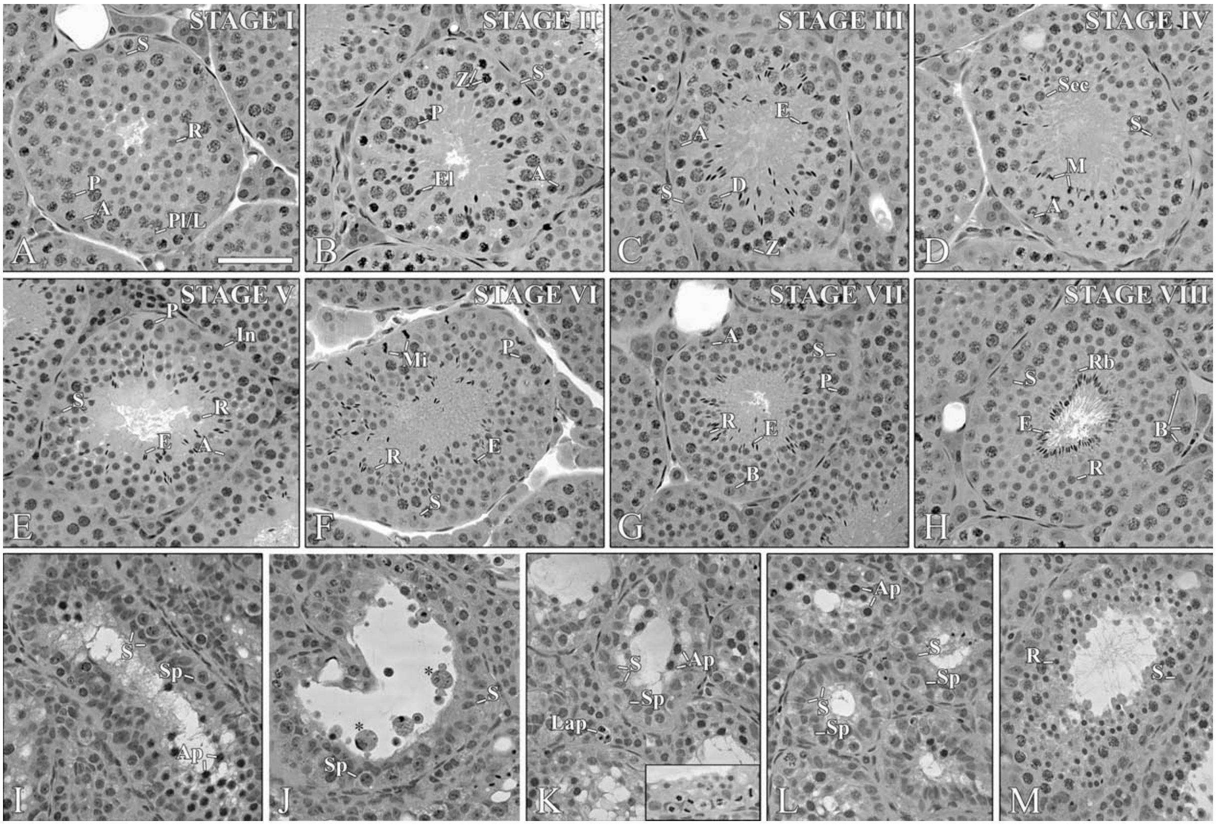

Fig. 2. Morphology of the testis of *Artibeus lituratus* during reproductive (A–H) and regressive periods (I–M). In testis at reproductive period, eight stages of the seminiferous epithelial cycle was identify based on the tubular morphology (A–H). Conversely, in the regressing testis, the seminiferous tubules were atrophic, presenting several apoptotic cells (Ap) (I), sloughed germ cells (∗) (J) and frequent Leydig cell apoptosis (Lap) (K). The insert in (K) highlights spermatogonia in mitosis. Some tubules presented only Sertoli cells and spermatogonia (L), whereas more differentiated germ cells were rarely observed (M). A, spermatogonia A; B, spermatogonia B; In, intermediate spermatogonia; Pl, prelelptotene; L, leptotene; Z, zigotene; P, paquitene; D, diplotene; El, elongating spermatid; E, elongated spermatid; R, round spermatid; Sec, secondary spermatocyte; M, metaphase; S, Sertoli cells; Mi, mitosis; Rb, residual bodies; Sp, spermatogonia. Bar in (A) = 50 μm.

lium. Blood vessels and rare macrophages and mast cells were seen in the interstitium.

The seminiferous tubules were surrounded by a thin tunica propria, containing the peritubular myoid cells, and the seminiferous epithelium, containing the Sertoli cells and spermatogenic cells. The mean diameter of the tubules during the reproductive period was 154 ± 19.6 μm (Fig. 1F). Based on the method of tubular morphology, which takes into account the localization and shape of spermatid nuclei, presence of meiotic divisions and overall seminiferous epithelium composition (Berndtson, 1977), eight stages of the spermatogenic cycle were identified in *A. lituratus* (Fig. 2):

- Stage I – Characterized by the presence of about four layers of round spermatids surrounding the lumen of the seminiferous tubules. Below them, there were approximately two layers of pachytene spermatocytes as well as preleptotene/leptotene and zygotene spermatocytes near the base of the epithelium. Type A spermatogonia were seen close to the basement membrane, among Sertoli cells.
- Stage II – The spermatids nuclei were elongating. Pachytene spermatocytes predominate in the middle part of the epithelium, whereas leptotene and zygotene spermatocytes were closer to the base. Nuclei of A spermatogonia were in line with those of the Sertoli cells at the base of the tubules.
- Stage III – Elongated spermatids were arranged in bundles deeply embedded in the seminiferous epithelium. Most spermatocytes were in diplotene phase. Pachytene and zygotene cells, as well as A spermatogonia were also present.
- Stage IV – Distinguished by the occurrence of metaphase and anaphase configurations of meiotic primary and secondary spermatocytes. Secondary spermatocytes and round spermatids were the cell population found nearest the tubule lumen. Primary spermatocytes were in diplotene, zygotene or pachytene phase. Type A spermatogonia and Sertoli cells formed the basal layer of the epithelium.
- Stage V – At this stage there were both generations of round and elongated spermatids. The round spermatids formed 3–4 layers of cells close to the lumen, whereas the elongated spermatids formed bundles that were located deeper into the epithelium. Pachytene was the predominant generation of spermatocytes, although zygotenes were also found. The population of A spermatogonia, characterized by the large and ovoid nuclei containing homogenously distributed chromatin, appeared more abundant than previously. Another population of spermatogonia presenting smaller and round nuclei was also observed. In these cells, recognized as intermediate spermatogonia, the nuclei was darker and the chromatin was frequently disposed below the nuclear envelop.
- Stage VI – The bundles of elongated spermatids were found in the middle of the epithelium. Other epithelial components were similar to previous stage. Another feature of this stage was the occurrence of spermatogonial cells mitosis near to the base of the tubular epithelium.
- Stage VII – Elongated spermatids were distributed in the luminal surface of the epithelium. A few residual bodies were evidenced. Deeper, there was a layer of round spermatids, followed by

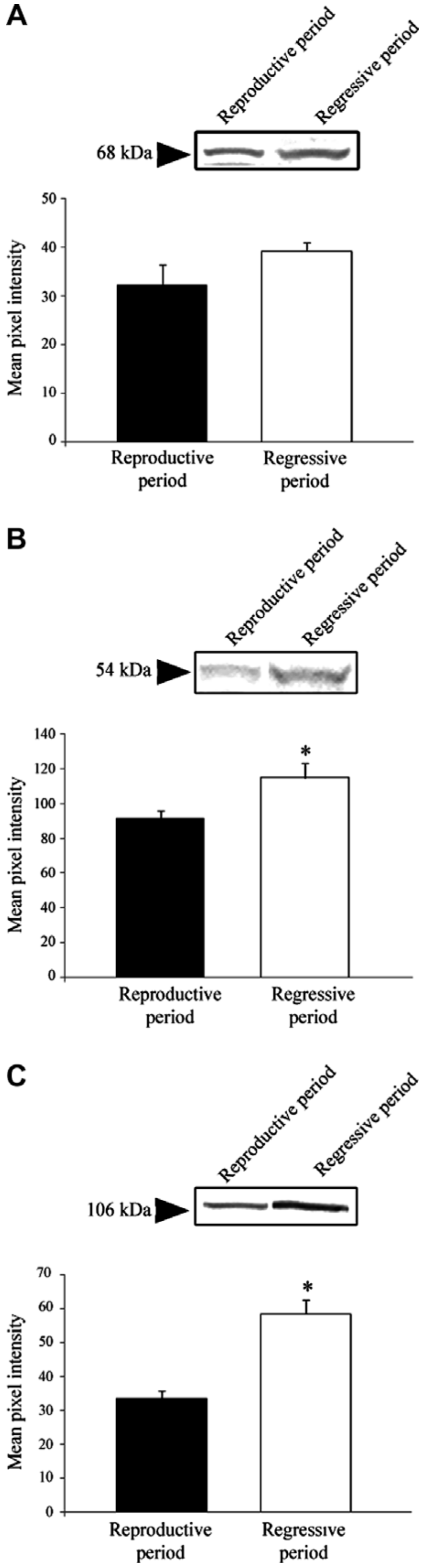

Fig. 3. Representative Western blotting and respective image analysis of the assays for detection of ERα (A), ERβ (B) and AR (C) expression in the testis of *Artibeus lituratus* during reproductive and regressive periods. The expression of ERα was similar in both phases analyzed (A). Compared to reproductively active animals, the expression of ERβ and AR were significantly increased in regressive testis (B and C). The molecular weight is shown in the left. $^*P \leqslant 0.05$; $n = 4$.

pachytene and preleptotene/leptotene spermatocytes. Numerous A and B spermagonia as well as mitoses were still distinguishable in the base of the epithelium.

- Stage VIII – Characterized by the arrangement of elongated spermatids on the luminal surface, as well as the occurrence of numerous and large residual bodies. Other cell populations were similar to the previous stage.

The seminiferous tubules converged to the mediastinum, where they gradually lost the germ cells, persisting only Sertoli cells, which modified in a simple cubic epithelium lining the straight tubules. In this transition zone, myoid cells formed one or two continuous layers below the epithelium. Leydig cells were abundant in the transition zone of the seminiferous to the straight tubules. Following the straight tubules, the intratesticular *rete testis* was lined by a simple cubic epithelium, sustained by the connective tissue of the mediastinum.

In the regressing testis most seminiferous tubules were atrophic, measuring 83 ± 9.9 μm in diameter (Fig. 1F) and presenting only Sertoli cells and spermatogonia in the epithelium (Fig. 2I–L). Rare tubules still presented spermatocytes and even spermatids (Fig. 2M). Apoptosis in the seminiferous tubules were abundant. The spermatogonia were characterized by voluminous nuclei found in close contact to the basement membrane. Spermatogonial cells in mitosis were common (insert in Fig. 2K). Usually, the Sertoli cell nuclei formed a layer located above the spermatogonial cells, in a manner resembling a bistratified epithelium (Fig. 2I). In other cases, a group of 3–5 Sertoli cells were found surrounding the spermatogonia (Fig. 2I–L). In the interstitium, the Leydig cells were characterized by the smaller size, dense nuclei and scarce cytoplasm, usually without vacuolization. Leydig cells apoptosis were frequently found (Fig. 2K).

3.2. Western blotting analysis

The specificity of the antibodies used was confirmed by Western blotting assays. The assays revealed major ERα, ERβ and AR immunoreactive bands of, respectively, 68, 54 and 106 kDa, in extracts of *A. lituratus* testis (Fig. 3). These molecular weights are in agreement with those previously found for other mammalian species (Nie et al., 2002; Zhou et al., 2002). Quantitative analyze of the blotting revealed that ERα levels were similar in both periods (Fig. 3A) but there was a significant increase in testis ERβ and AR during regressive period (Fig. 3B and C).

3.3. Immune reactivity for ERα

In the testis of *A. lituratus,* during reproductive period, ERα positivity was restricted to nuclei of some Leydig cells in the interstitium, even though the intensity of staining was low (Fig. 4A). Intermittent immunostaining was also detected in the nuclei of myoid cells as well as endothelial cells lining the lymphatic spaces. ERα was not detected in the spermatogenic cells, Sertoli cells and blood vessels. In the regressing testes, the Leydig cells ERα positivity was more intense compared with those of reproductive period (Fig. 4B). High intensity of ERα staining was also detected in some myoid cells and lymphatic endothelial cells.

The epithelium of the straight tubules and *rete testis* was negative or slightly positive for ERα, in animals at reproductive period (Fig. 4C). Few cells in the subjacent connective tissue were positive for this receptor. During regression, the epithelium was still weakly stained for ERα but a greater number of positive connective cells was found in the *rete testis* (Fig. 4D).

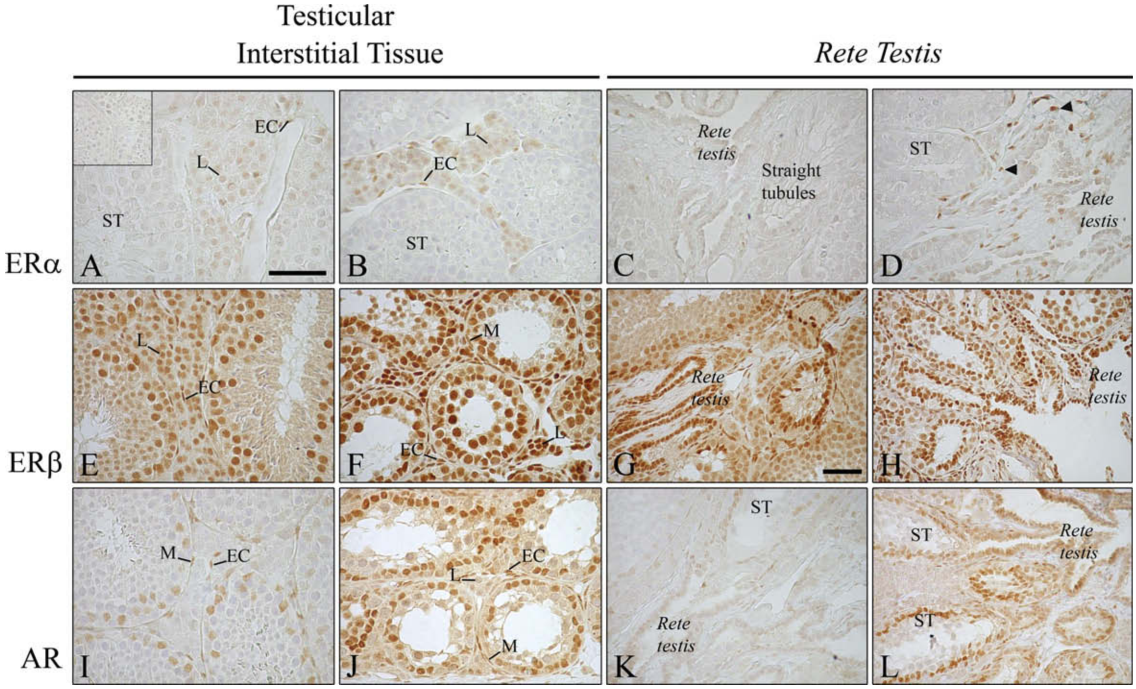

Fig. 4. Expression of ERα (A–D), ERβ (E–H) and AR (I–L) in the testicular interstitial tissue and *rete testis* of *Artibeus lituratus* in reproductive and regression periods. ERα staining was intermittent and slight in some Leydig (L) and endothelial cells (EC) of the lymphatic spaces during the reproductive period (A). Conversely, in regressive testis, ERα positivity was more intense in these cells (B). In *rete testis*, the epithelium was negative or slightly positive in reproductive (C) and regression periods (D). Several ERα positive cells (arrowheads) were seen in the *rete testis* connective tissue at regression. Insert in (A) show the negative control. ERβ was detected in Leydig cells (L), myoid cells (M) and endothelium (EC) of the lymphatic spaces in both reproductive (E) and regression (F) periods, however the staining was more intense during regression. *Rete testis* epithelial and connective tissue cells were strongly positives for ERβ in reproductive (G) and regression periods (H). AR was expressed in myoid cells (M) and endothelium (EC) of lymphatic spaces and some Leydig cells (L) during reproduction (I) and regression (J). In *rete testis* the epithelium showed slight to negative staining and few cells in connective tissue showed positivity for AR in both periods (K, reproduction; L, regression). ST, seminiferous tubules. Bar in (A) = 50 μm, same to (B)–(F), (I) and (J); bar in (G) = 50 μm, same to (H), (K), (L).

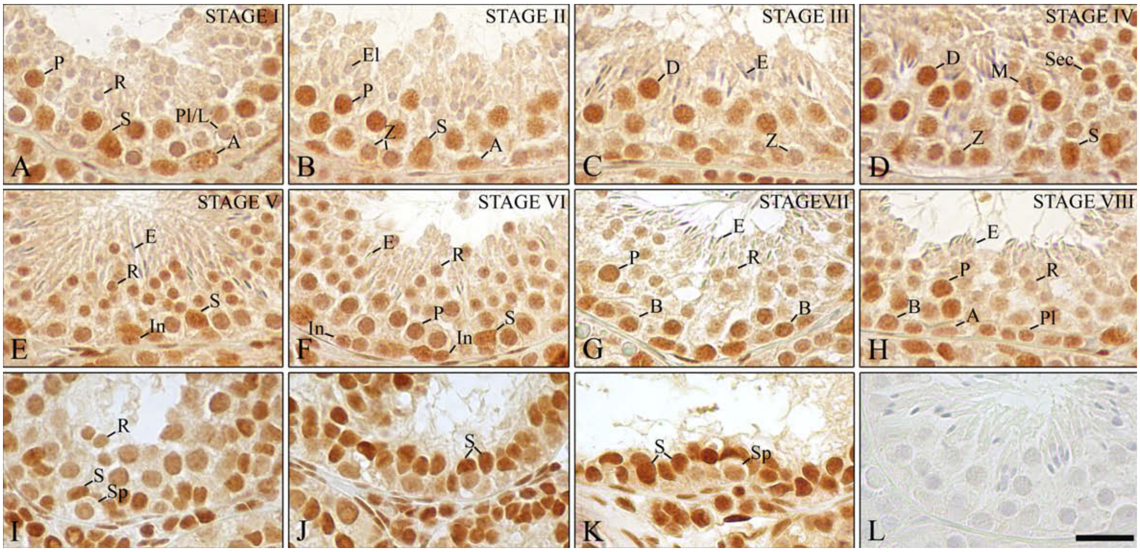

Fig. 5. Expression of ERβ in the seminiferous tubules of *Artibeus lituratus* during reproductive (A–H) and regression periods (I–K). In reproductively active testis the expression of ERβ was stage-specific (A–H, stage I–VIII, respectively). During regression (I–K) the nuclei of Sertoli cells were intensely stained contrasting with spermatogonia, which were moderately stained. The negative control is shown in (L). A, spermatogonia A; B, spermatogonia B; In, intermediate spermatogonia; Pl, preleptotene; L, leptotene; Z, zigotene; P, paquitene; D, diplotene; El, elongating spermatid; E, elongated spermatid; R, round spermatid; Sec, secondary spermatocytes; M, metaphase; S, Sertoli cell; Sp, spermatogonias. Bar in (L) = 30 μm.

3.4. Immune reactivity for ERβ

ERβ was detected in the nuclei of somatic cells and most spermatogenic cells in the testis of *A. lituratus* at both periods analyzed. Most Leydig cells were strongly positive for ERβ, whereas others were slightly reactive (Fig. 4E). Strong immunopositivity for ERβ was also seen in the myoid cells and Sertoli cells, irrespective of the seminiferous tubule stage (Fig. 4E). On the other hand, the positivity of the spermatogenic cells was stage-specific (Fig. 5A–H).

- Stage I – The spermatogonia were moderately positive, the pachytenes spermatocytes were strongly positive, whereas the preleptotene/leptotene and zygotene were negative or weakly positive for ERβ. Round spermatids were negative for this receptor.
- Stage II – The spermatogonia and primary spermatocytes followed the same pattern of staining seen in stage I, whereas elongating spermatids were devoid of ERβ.
- Stage III – The most intense staining was found in the diplotene spermatocytes. Spermatogonia showed moderate staining; the zygotenes were negative or weakly positive, whereas elongated spermatids were negative for ERβ.
- Stage IV – With exception of the elongated spermatids which were negative for ERβ, all spermatogenic cells found in tubules at this stage were intensely positive. Cytoplasmic staining was detected in spermatocytes in late meiosis, specially those presenting metaphasic plaques.
- Stage V – Spermatogonia, pachytene spermatocytes and round spermatids were positive for ERβ, whereas elongated spermatids were negative.
- Stage VI – The spermatogonia were more intensely positive for ERβ than in previous stages. Pachytene spermatocytes and round spermatids were positive for ERβ, whereas elongated spermatids were negative.
- Stage VII – Spermatogonia and pachytene spermatocytes were strongly positive for ERβ. The round spermatids were slightly positive and elongated spermatids were negative for ERβ.
- Stage VIII – Similar to the previous stage, the spermatogonia and pachytene spermatocytes were strongly positive for ERβ. The round spermatids were weakly stained and the elongated spermatids were negative for ERβ.

During regression, Leydig and Sertoli cell nuclei appeared more intensely stained than in reproductive period (Fig. 5I–K). Myoid cells were similarly stained in both periods. Considering the spermatogenic cells, the spermatogonia were moderately stained for ERβ, contrasting with the Sertoli cells intensely stained. Other spermatogenic cells still present in the regressing tubules showed moderate to strong staining. When present, the elongated spermatids were negative for ERβ. Occasionally, some sloughing cells strongly positive for ERβ were seen in the tubular lumen (Fig. 4F).

In both periods analyzed, intense ERβ staining was found in the endothelium of the lymphatic spaces, as well as in the endothelium and smooth muscle cells of the blood vessels (Fig. 4E and F).

In the transition zone to straight tubules the immunoreaction of Sertoli cells for ERβ was intense (Fig. 4G and H). The straight tubules and *rete testis* epithelium as well as some connective tissue cells were positive for ERβ, in both periods analyzed.

3.5. Immune reactivity for AR

AR positivity was found restricted to the nuclei of somatic cells in the testis of *A. lituratus* at both periods analyzed. In bats sexually active, the intensity of Sertoli cells AR staining was stage-dependent (Fig. 6A–H). Nuclei of Sertoli cells were moderately stained in stage I, and slightly positive to negative in stages II–IV. The staining intensity increased to moderate in stage V and reached the strongest positivity in stages VI–VIII. Myoid cells showed strong but intermittent staining in tubules of different stages. During regression, both Sertoli and myoid cells showed intense staining in all seminiferous tubules, contrasting with the spermatogonia and rares spermatocytes and spermatids still present in the epithelium, which were negative for AR (Fig. 6I–L). The contrast of this

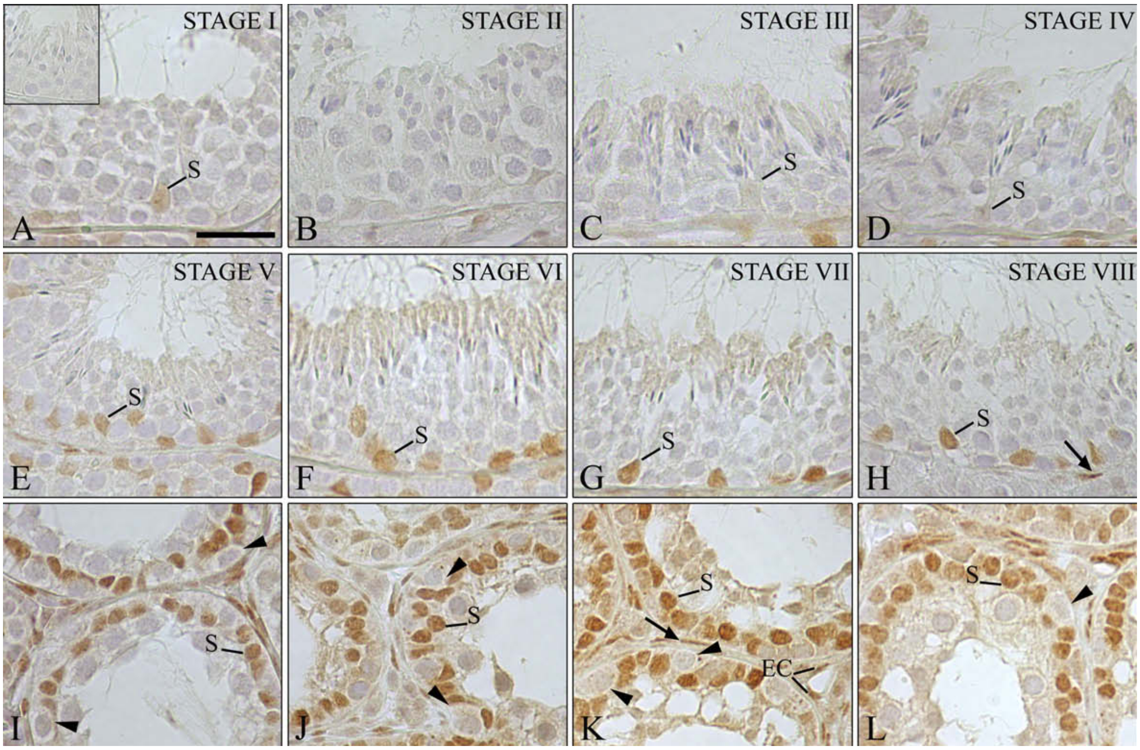

Fig. 6. Expression of androgen receptor (AR) in the seminiferous tubules of *Artibeus lituratus* during reproductive (A–H) and regression periods (I–L). In both periods AR expression was restricted to the nuclei of Sertoli cells (S), myoid cells (arrows) and endothelial cells (EC). In seminiferous tubule of bats sexually active AR staining was stage-dependent: the nuclei of Sertoli cells were moderately stained in stage I (A), slightly positive to negative in stages II–IV (B–D), became moderate in stage V (E) and strongly positive in stages VI–VIII (F–H). During regression, the nuclei of Sertoli cells showed intense staining for AR (I–L) in all tubules. In both periods, AR was not detected in spermatogenic cells. Arrowheads, spermatogonias surrounded by Sertoli cells. Insert in (A) shows the negative control. Bar in (A) = 30 μm.

staining further revealed the position of Sertoli cells surrounding the spermatogonia.

In the interstitium, some Leydig cells were faintly stained for AR, whereas most of them appeared negative, irrespective of the period considered (Fig. 4I and J). Strong positivity was observed in the endothelium of lymphatic spaces, as well as in the endothelium and smooth muscle cells of the blood vessels.

In both period analyzed the epithelium of the straight tubules and *rete testis* was negative to slightly positive for AR (Fig. 2K and L). Few cells in the connective tissue showed positivity for this receptor. In the transition of the seminiferous tubules to the straight tubules there were a greater number of myoid cells, which were strongly positive for AR.

4. Discussion

We report the first study demonstrating that ERα, ERβ and AR are distributed in a cell- and stage-specific manner in the testis of a Tropical bat species. An interesting observation was that the immune reactivity of these receptors was pronounced during testicular regression. A systematic examination concerning ER distribution along the cycle of the seminiferous epithelium and reproductive annual cycle of a mammal, as presently shown, has not been carried out before. Owing to a total lack of information on ER and AR distribution on Chiroptera comparisons can only be made with other mammalian species.

The structure of testis of *A. lituratus* followed those of seasonal species of bats, showing a clear reproductive and non-reproductive period along the year (Bernard and Hodgson, 1989; Kurohmaru et al., 2002). It was noteworthy the pattern of association of Sertoli cell with the spermatogonia in the regressive testis, as the Sertoli cell nuclei were mostly distributed above or surrounding the spermatogonia. Similar feature has previously been described for little brown bats *Myotis lucifugus*, ground squirrel *Citellus lateralis* and Prairie dog *Cynomys ludovicianus* (Pudney, 1986; Gustafson, 1987; Foreman, 1997). This close association of Sertoli cells and spermatogonia of seasonal animals may be related to isolation of the stem germ cells from the adluminal compartment where intensive apoptosis is taking place. This isolation may be important to maintain the spermatogenic cell precursor viability necessary to repopulate the testis during the following reproductive period.

In the testis of *A. lituratus*, the ERα immune reactivity was weak and limited to interstitial cells, suggesting that ERα is not directly necessary for the development of testis germ cells, as previously demonstrated (Mahato et al., 2000). This restricted pattern of ERα localization is in agreement with those found for most mammals described to date (Fisher et al., 1997; Nie et al., 2002; Zhou et al., 2002), except goat, human and other primates, which are devoid of ERα (Goyal et al., 1997; Saunders et al., 2001; Berensztein et al., 2006). Although not detected by the Western blotting assay, during regression, the presence of ERα appeared more intense and in greater number of cells in both testis and *rete testis*. Higher ERα expression has also been described in testicular interstitial cells of neonate and infantile marmosets, contrasting with peripubertal and adult animals (Fisher et al., 1997; McKinnell et al., 2001). These data are suggestive that higher levels of ERα may be associated with lower testosterone concentration, as seen in young as well as regressive testis of bats (Gustafson, 1979; Gustafson and Damassa, 1984; Kawamoto, 2003). Accordingly, studies on mice lacking ERα (αERKO) provided evidences that ERα is involved in regulating the androgen biosynthesis, by inhibiting steroidogenesis (Akingbemi et al., 2003; Gould et al., 2007). This may also be the case in *A. lituratus*. This assumption associated with the fact that Leydig cells of *A. lituratus* are positive to both ER subtypes but that androgen receptors were barely detectable indicates that this cell population is more closely regulated by estrogen than androgen.

ERβ was more widely distributed in the testis of *A. lituratus* than ERα and AR, being detected in most somatic and germ cells, as previously described for other mammalian species (Saunders et al., 2001; Nie et al., 2002; Hejmej et al., 2005; Mutembei et al., 2005). Despite the vast distribution of ERβ, male mice lacking ERβ (βERKO) was first reported to be fertile (Krege et al., 1998; Dupont et al., 2000). Studies using knockout mice for ERα (αERKO) also indicated that ERβ was not sufficient for maintaining the testicular functions. However, a novel ERβ-null mutant mouse devoid of any transcript variants was recently generated, and the male resulted to be fully infertile (Antal et al., 2008). In addition, a detailed investigation of adult βERKO mice from a previous colony showed that inactivation of ERβ does affect cellular composition of the testis (Gould et al., 2007). Together, these findings point out that ERβ may indeed have a direct role in the spermatogenesis.

Our present findings revealed stage-dependent differences in immune reactivity of ERβ in the *A. lituratus* testis, especially concerning spermatogonia and round spermatids. This result is in agreement with a physiological role for ERβ in testicular function. Spermatogonial cells were positive for ERβ at all stages of the spermatogenic cycle; however they were strongly positive at stages VI–VIII. In keeping with these findings, Sertoli cell AR positivity was also stronger in tubules at stages VI–VIII. Coincidently, spermatogonial mitosis was frequently observed in tubules presenting more robust immune reactivity of AR as well as spermatogonial ERβ. These findings corroborate previous data indicating that ERβ may have a role in regulating negatively the proliferation of spermatogenic cell precursors, as seen in neonate or adult βERKO mice, which presented increased gonocytes or spermatogonial number per testis, respectively (Delbes et al., 2004; Gould et al., 2007). On the other hand, Sertoli cells are known by their role in guarantee spermatogenic cells proliferation and differentiation, by secreting a number of regulatory factors, under androgen stimulation (Skinner et al., 1991; Hill et al., 2004). Therefore, higher levels of AR in Sertoli cells and ERβ in spermatogonia at the same specific stages of the cycle may be considered a possible mechanism to determine a balance in cell proliferation.

Round spermatids also presented differences in ERβ immunostaining depending upon the seminiferous epithelium cycle stage, as they were strongly positive at stages IV–VI, became moderately stained at stage VII–VIII and were mostly negative at stage I. The spermatids completely loose the immunoreaction as they become elongated (stages I–III). Previous studies have indicated that by expressing ERβ round spermatids were preferential target for estrogen within the seminiferous tubules (Saunders et al., 2002). On this sense, male mice lacking aromatase (ArKO) were infertile and showed major defects in round spermatids, which presented disturbances in acrosome formation and failed to differentiate into mature elongated spermatids, usually undergoing apoptosis (Robertson et al., 1999). Treatment with high estrogen level showed similar effects on spermatids (Toyama et al., 2001; D'Souza et al., 2005). These results suggest that a balanced estrogen level acting via ERβ may be important for maintaining the spermatid differentiation and survival.

Another interesting observation concerning ERβ in the *A. lituratus* testis was that the immunoreaction increased as the meiosis advanced, being weaker in preleptotene/leptotene and zygote phases and reaching higher level in pachytene/diplotene and secondary spermatocytes. Changes in level of ERβ in different cell population may be indicative of specific role in the spermatogenesis. ERβ appears to be associated with events that regulate the progression of the first meiosis or that lead spermatocytes to an apoptotic pathway (Selva et al., 2004). Accordingly, previous results indicate that zygotene and pachytene, the stages with more intense immunoreactions for ERβ as presently shown, represents a check-point for apoptosis within the spermatogenic process (Morales and Cavicchia, 2002). Our results showing increased ERβ immune reactivity during testis regression, especially in sloughing spermatogenic cells, also corroborate these pro-apoptotic and/or anti-proliferative role of ERβ.

The pattern of AR distribution in the testis of *A. lituratus* during reproductive period followed those described for other mammalian species, being restricted to somatic cells (Bremner et al., 1994; Goyal et al., 1997; Suarez-Quian et al., 1999; Zhou et al., 2002). As expected, Sertoli cells were characterized by a stage-specific expression of AR, as stronger reactivity was found in stages VI–VIII, followed by a gradual decrease from stage I to IV. Contrasting with reproductive period, Sertoli cells of all regressive seminiferous tubules were positive for AR. Similar extensive expression of AR was previously described for marmosets at infancy (McKinnell et al., 2001), pointing out that the AR levels is not well correlated with the plasma testosterone concentration.

In conclusion, the results showed a cell and stage-specific distribution of ERα, ERβ and AR in the testis of *A. lituratus* during the reproductive phase, suggesting that these hormones may play different roles in spermatogenesis. Also, these receptors followed a seasonal pattern of immune reactivity in the testis, as more intense levels were observed in the regressive testis.

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