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NOVOS PARÂMETROS PARA AVALIAÇÃO ANDROLÓGICA EM MACACO-DE-CHEIRO (*Saimiri collinsi* Osgood, 1916): DISTÂNCIA ANOGENITAL, MICROMORFOLOGIA E PERFIL PROTEICO ESPERMÁTICO

BELÉM
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Área de Concentração: Produção Animal.
Orientador (a): Profª. Dra. Sheyla Farhayldes Souza Domingues.
Coorientador: Prof. Dr. Arlindo de Alencar Araripe Noronha Moura.

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Aprovado em setembro de 2019

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Aos meus pais, Jerceu e Ione, minha irmã Danuta, minha avó Benedita e a Pérola que sempre me apoiaram nessa jornada ♥

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“Em todas as páginas de um livro
A celulose de um ser vivo
Alerta da preservação”

(Papel - Falcão, P., 2018)

RESUMO

O macaco-de-cheiro (*Saimiri collinsi*), um primata neotropical endêmico da Amazônia brasileira, apresenta uma sazonalidade reprodutiva em vida livre marcante, contudo pouco estudada. Recentemente essa espécie foi adotada como modelo experimental biológico para pesquisas reprodutivas sobre o gênero *Saimiri,* contribuindo para conservação desses animais. Para melhor compreensão da fisiologia reprodutiva do gênero *Saimiri,* esse estudo teve como objetivos: (i) avaliar a correlação entre a distância anogenital com os parâmetros espermáticos e hormônios reprodutivos (estradiol e testosterona) em macacos-de-cheiro (*S. collinsi*); (ii) descrever as características micromorfologicas e ultraestruturais dos espermatozoides de macacos-de-cheiro (*S. collinsi*) por meio da microscopia eletrônica de varredura e transmissão; (iii) caracterizar o perfil proteico dos espermatozoides de macaco-de-cheiro (S. collinsi); (iv) associar as expressões de proteínas espermáticas e as características espermáticas de macaco-de-cheiro (*S. collinsi*). Quanto a análise da qualidade seminal, além do pH, não houve diferença na qualidade seminal entre as estações seca e chuvosa. No tocante as proteínas espermáticas, foram identificadas 2.343 proteínas presentes nas amostras de espermatozoides ao longo dessas duas estações. Das 79 proteínas expressas diferencialmente entre as duas estações do ano, 39 proteínas relacionadas à espermatogênese, motilidade espermática, capacitação, fecundação e sistemas de defesa contra o estresse oxidativo foram aumentadas na estação seca. A micromorfologia analisada por microscopia de varredura revelou que os espermatozóides normais de *Saimiri collinsi* medem 71,73 ± 0,71 μm com inserção lateral da cauda, cabeça achatada em forma de remo e acrossoma ocupando a maior parte da cabeça, e complementarmente, pela análise da ultraestrutura por microscopia de transmissão foi observado que a peça intermediária é composta por um axonema central com 9 pares de 9 + 2 microtúbulos rodeado por nove fibras densas, e as mitocôndrias justapostas formando a bainha mitocondrial. A distância anogenital foi correlacionada com o volume seminal, motilidade, vigor e integridade da membrana plasmática, mas não com o peso corporal, hormônios reprodutivos e volume testicular. Assim, nossos resultados ajudam a avançar na compreensão da fisiologia reprodutiva de *S. collinsi*, fornecendo informações valiosas para a melhoria dos protocolos utilizados nas técnicas de reprodução assistida para a conservação de espécies ameaçadas de extinção no gênero *Saimiri*.

Palavras-chaves: Primatas neotropicais, espermatozoides, proteômica, microscopia eletrônica, distância anogenital.

ABSTRACT

The squirrel monkey (*Saimiri collinsi*), a Neotropical primate endemic to the Amazon in Brazil, with a remarkable but little studied free-living reproductive seasonality. Recently this species was adopted as an experimental biological model for reproductive research on the genus *Saimiri*, contributing to the conservation of these animals. To better understand the reproductive physiology of the genus *Saimiri*, this study aimed to (i) to evaluate the correlation between anogenital distance with sperm parameters and reproductive hormones (estradiol and testosterone) in squirrel monkeys (*S. collinsi*); (ii) describe the micromorphological and ultrastructural characteristics of spermatozoa from squirrel monkeys (*S. collinsi*) through scanning and transmission electron microscopy; (iii) characterize the protein profile of squirrel monkeys sperm (*S. collinsi*); (iv) associate the expression of sperm proteins and the sperm characteristics of squirrel monkey (*S. collinsi*). The study approach based on bottom-up proteomics (Shotgun proteomics) allowed the identification of 2343 proteins present in the sperm samples throughout these two seasons. Of the 79 proteins that were differentially expressed between the two seasons, 39 proteins that were related to spermatogenesis, sperm motility, capacitation, fecundation, and defense systems against oxidative stress were upregulated in the dry season. The SEM electromyrographies revealed a normal *Saimiri collinsi*'s sperm measure 71.73±0.71 μm with lateral tail insertion, a paddle-shaped flattened head and a acrosome occupied most of the head, and completely, the TEM also showed that the middle piece is characterized by a central 9 + 2 microtubule axoneme surrounded by nine dense fibers, and the mitochondria were juxtaposed forming the mitochondrial sheath. Anogenital distance was correlated with seminal volume, sperm motility, vigour, and plasma membrane integrity, but not with body weight, reproductive hormones, and testicular volume. Thus, our results help to advance our understanding of the reproductive physiology of S. collinsi, providing valuable information for the improvement of protocols used in assisted reproduction techniques for the conservation of endangered Saimiri species.

Keywords: Neotropical primates, sperm, proteomics, electron microscopy, anogenital distance.

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LISTA DE ABREVIATURAS E SIGLAS

cm	Centímetro
dpi	Pontos por polegada
µg	Micrograma
µL	Microlitro
g	Gravidade
g	Grama
h	Hora
kDa	Quilodalton
kg	Quilograma
L	Litro
M	Molar
m	Metro
mg	Miligrama
mA	Miliamperagem
min	Minuto
mm	Milímetro
mM	Milimolar
mL	Mililitro
mOsm	Miliosmol
NaCl	Cloreto de sódio
nm	Nanômetro
ºC	Celsius
p	Probabilidade
pH	Potencial hidrogeniônico
TRIS	Tris (hidroximetil) aminometano
V	Volts
W	Walts

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1 CONTEXTUALIZAÇÃO

De acordo com o relatório divulgado pela União Internacional para a Conservação da Natureza – IUCN, duas espécies de primatas não-humanos (PNH) brasileiras: o macaco-caiarara (*Cebus kaapori*) e o bugio-marrom (*Alouatta guariba guariba)*, estão entre os 25 primatas mais ameaçados do mundo (SCHWITZER et al., 2017). Diante dessa realidade, faz-se prioritária a aplicação de alternativas para a conservação *ex-situ* de PNH, sobretudo o desenvolvimento de biotécnicas reprodutivas (DOMINGUES et al., 2011; SAMPAIO et al., 2017b).

Pensando nesse contexto, a espécie neotropical *Saimiri collinsi,* popularmente conhecida como macaco-de-cheiro, tem sido proposta como modelo experimental para o emprego de biotécnicas da reprodução em outras espécies do mesmo gênero (OLIVEIRA et al., 2015; 2016ab), como é o caso das espécies *S. oerstedii*, *S. vanzollini* (vulneráveis) e *S. ustus* (quase ameaçada), que já constam na lista vermelha de espécies ameaçadas de extinção (BOUBLI; RYLANDS, 2018).

Contudo, em *S. collinsi*, assim como em outras espécies de PNH, a ocorrência de sêmen coagulado é fator limitante para aplicação de biotécnicas da reprodução animal (VALLE et al., 2004), visto que após sua dissolução *in vitro*, os parâmetros espermáticos apresentam-se com valores diminuídos (LEÃO et al., 2015; LIMA et al., 2017; OLIVEIRA et al., 2011, 2015, 2016a, 2016b), com gametas imóveis (NAGLE; DENARI, 1983), e até mesmo sêmen azoospérmico (BENNETT, 1967), o que pode inviabilizar o uso deste material biológico em programas de reprodução assistida para esses indivíduos. Dessa forma, se faz necessário pesquisas mais detalhadas acerca das características reprodutivas de *S. collinsi*, tendo em vista sua representatividade como referência para outras espécies de primatas neotropicais.

Um outro fator peculiar às espécies do gênero *Saimiri,* é a ocorrência de sazonalidade reprodutiva (LINDBURG et al., 1987; STONE, 2014; WOLF et al., 1975). Desse modo, é possível que durante e após o período de sazonalidade reprodutiva, o sêmen desses animais apresente peculiaridades quanto a micromorfologia e ultraestrurua, assim nos componentes biológicos espermáticos, principalmente os proteicos. Porém, até o presente momento não existem relatos nesse primata Neotropical que demonstrem uma correlação entre tais componentes e a qualidade espermática.

Nesse contexto, apesar da espécie *S. collinsi*, ser utilizado em pesquisas voltadas a reprodução e conservação de espécies do gênero *Saimiri*, ainda existem limitações na seleção de machos para programas de reprodução assistida, principalmente quando se envolve técnicas de colheita de sêmen, em que os animais passam por um procedimento de sedação, o que pode ocasionar estresse durante captura e refletir na baixa qualidade seminal desses animais. Pensando nisso, esse trabalho também trouxe como proposta um novo método não invasivo, de fácil execução e barato que pode ser realizado tanto no criadouro quanto a campo ser requerer uma estrutura física complexa e nem equipamentos, para avaliação e seleção de machos aptos a reprodução ou para serem utilizados em programas de reprodução assistida. E para isso foi utilizada a distância anogenital, o qual foi proposto como um biomarcador para a fertilidade no homem, o qual é baseado na mensuração da distância do ânus à base posterior do escroto (AGD_{AS}) e do ânus à inserção cefálica do pênis (AGD_{AP}) (MENDIOLA et al., 2011).

Sabe-se que a distância do ânus aos órgãos genitais (distância anogenital; AGD) é considerado uma característica sexualmente dimórfica em algumas espécies de mamíferos, incluindo ratos e humanos. No homem, as concentrações aumentadas de andrógeno levam ao aumento da AGD, resultando em uma AGD quase duas vezes maior que as mulheres (MACLEOD et al., 2010; THANKAMONY et al., 2016; WELSH et al., 2008; WOLF et al., 1999). A AGD é determinada durante o desenvolvimento inicial, pela exposição fetal ao meio androgênico, o útero, durante a janela de programação da masculinização (EISENBERG et al., 2012a, 2012b; MENDIOLA et al., 2011; WELSH et al., 2008;).

Estudos em humanos também mostram que um tamanho menor de AGD foi associado a um tamanho menor de testículo (DEAN; SHARPE, 2013), como baixos níveis de testosterona e síndrome de disgenesia testicular, e, consequentemente, menor concentração espermática (LÓPEZ-ESPÍN et al., 2017; MENDIOLA et al., 2011, 2015). O *Saimiri collins* possui testículos simétricos e existe uma correlação positiva entre o volume testicular e a fração líquida do ejaculado (OLIVEIRA et al., 2016), no entanto, não sabemos se existe uma relação entre o volume testicular e os parâmetros espermáticos com o tamanho da AGD. Desta forma, a medição do AGD pode ser usada como parâmetro não invasivo auxiliar no exame andrológico para a seleção de primatas

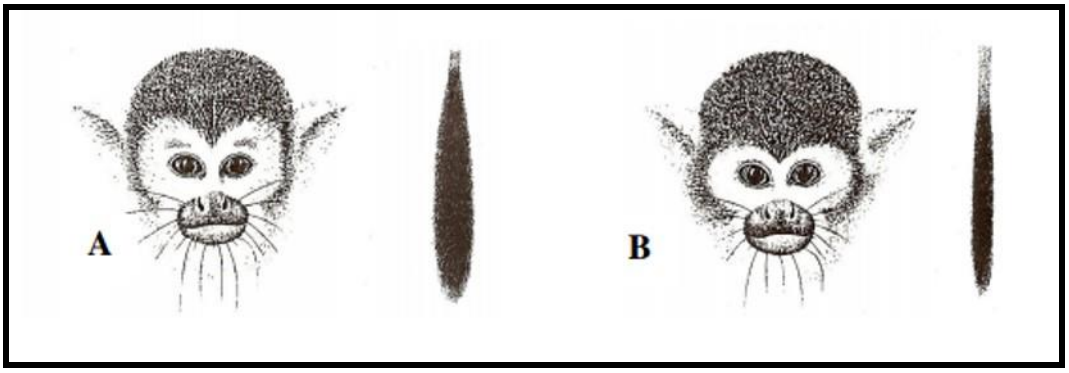
não-humanos em programas de biotecnologia para a conservação de espécies ameaçadas de extinção, bem como estudos em fisiologia reprodutiva.

1.1 Taxonomia e aspectos gerais do gênero Saimiri

Atualmente são reconhecidos no gênero *Saimiri* a existência de oito táxons: *S. sciureus*, *S. collinsi*, *S. oerstedi*, *S. cassiquiarensis*, *S. macrodon*, *S. ustus*, *S. boliviensis* e *S. vanzolinii* (MERCÊS et al., 2015; PAIM et al., 2013; RYLANDS et al., 2013). As espécies que compõem o gênero têm como características: pequeno porte, com coloração preta esverdeada que varia conforme a espécie e tons que vão do preto ao amarelo-alaranjado (GROVES, 2005). Ao redor dos olhos, possuem uma máscara de pelos branco, que formam um arco, cujo o padrão os divide em dois grupos: o gótico e o romano (Figura 1) (GROVES, 2005; INGBERMAN; STONE; CHEIDA, 2008).

São animais de hábitos diurnos (INGBERMANN; STONE; CHEIDA, 2008), arborícolas, que se locomovem predominantemente de forma quadrúpede (BOINSKI, 1989), e se alimentam de frutas, pequenos vertebrados, flores, goma e insetos (STONE, 2007).

Figura 1- Diferenças entre os arcos superciliares e o pincel caudal entre os grupos gótico (A) e romano (B).



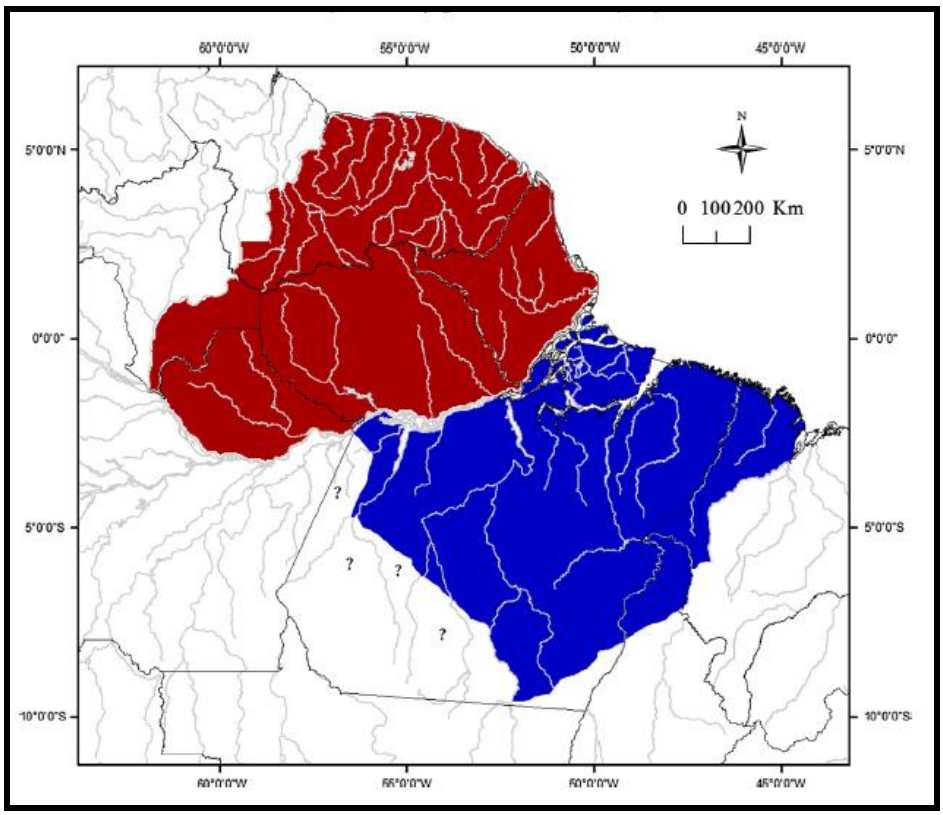
Fonte: HERSHKOVITZ (1984).

Vivem em grupos sociais compostos por vários machos e várias fêmeas (GOODALL; MITTERMEIER, 1999) cujo tamanho varia de dezenas a centenas de indivíduos (BALDWIN; BALDWIN, 1981). A longevidade conhecida em torno de 20 anos (MENDOZA, 1999), mas estima-se que possam chegar aos 30 anos (WILLIMS, 2008).

1.2 A espécie *Saimiri collinsi*

A espécie *Saimiri collinsi* (Osgood, 1916), é uma espécie endêmica brasileira que ocorre no arquipélago do Marajó (Estado do Pará), e também na região situada ao sul do rio Amazonas, entendendo-se até o Estado do Maranhão (Figura 2) nas matas de transição entre os biomas Amazônia e Cerrado. Contudo, o limite sul ainda é desconhecido e a porção oeste da distribuição de *S. collinsi* se estende até a margem esquerda do rio Tapajós (MERCÊS et al., 2015).

Figura 2 - Distribuição geográfica de *S. collinsi* (azul).



Fonte: MERCÊS et al. (2015).

Em vida livre vivem em grupos de múltiplos machos e múltiplas fêmeas (cerca de 45-50 indivíduos) (STONE et al., 2015). Em cativeiro, os machos adultos pesam cerca de 620–1.115g (OLIVEIRA et al., 2015, 2016ª, 2016b) (Figura 3). No tocantre a alimentação, ainda não está bem descrita a composição alimentar do *S. collinsi* em seu habitat natural. Entretanto, em cativeiro, são ofertadas frutas, legumes, leite, ração

industrializada, e larvas de besouro tenébrio gigante (*Zophobas morio*) (OLIVEIRA et al., 2015, 2016ª, 2016b).

Figura 3 - Exemplar macho da espécie *S. collinsi* oriundo do Centro Nacional de Primatas (Ananindeua/Pará/Brasil).

Fonte: A autora.

1.3 Aspectos reprodutivos de Saimiri sp.

Em *S. sciureus*, a maturidade sexual ocorre entre 2,5 a 3,5 anos de idade (RICHTER; LEHNER; HENDRICKSON, 1984). São animais poligâmicos (sistema multimachos-multifêmeas) (DIXON; ANDERSON, 2002; FORTMAN et al., 2002; e, como já mencionado, são sazonais com estações reprodutivas bem definidas (BALDWIN; BALDWIN, 1981; TAUB; ADAMS; AUERBACH, 1978).

A reprodução sazonal é a distribuição temporal de cópulas durante um período do ano para ser seguido por nascimentos em outro período. Em PNH há três tipos de reprodução: a sazonalidade discreta, estrita e a ausência de sazonalidade (LINDBURG, 1987).

As espécies do gênero *Saimiri* são consideradas sazonais estritas, visto que a cópulas só ocorrem em um determinado período do ano (época de acasalamento), logo, os nascimentos são agrupados no período seguinte. Na região amazônica, *S. sciureus*

reproduzem-se de julho a setembro, com os nascimentos e lactação acontecendo entre dezembro e fevereiro (DUKELOW, 1983). Stone (2007) define que para essa espécie a estação chuvosa corresponde ao nascimento e aleitamento dos filhotes, enquanto a estação seca corresponde ao acasalamento e gestação.

Durante a estação reprodutiva ocorrem alterações morfofisiológicas e comportamentais tanto nos machos quanto nas fêmeas do gênero *Saimiri* (CHEN et al., 1981). Dentre as alterações morfológicas, nos machos, ocorre um fenômeno conhecido como "*fatted*", que é o aumento da massa corporal (85 a 222g) por meio do acúmulo de gordura e água (DuMOND; HUTCHISON, 1967; STONE, 2004), principalmente na parte superior do corpo, que produz um aspecto de "gordo" (MENDONZA et al., 1978; STONE, 2004), essa condição de "*fatted*" tem início cerca de dois a três meses antes do início do acasalamento e é restrita à estação reprodutiva (BALDWIN, 1985). Contudo, em indivíduos *S. sciureusi* mantidos em cativeiro, a condição de "*fatted*" não foi necessária para manutenção da capacidade fecundante espermática *in vitro*, visto que ao longo de todo o ano (estação seca e chuvosa) foi possível obter sêmen com espermatozoides viáveis para a fecundação *in vitro*, com obtenção de zigoto (KUEHL; DUKELOW, 1979).

Não se sabe ao certo quais fatores ambientais teriam a maior influência sobre a sazonalidade reprodutiva dos macacos-de-cheiro. Acredita-se que este padrão temporal reprodutivo seja influenciado pelo índice pluviométrico, temperatura e fotoperíodo, entretanto o papel de cada uma dessas variáveis ainda não é completamente estabelecido (DUMOND, 1968; HARRISON; DUKELOW, 1973, HEARN, 1983).

Estudos realizados por Granados et al. (2014) relacionaram a variação da concentração espermática com as porções do ejaculado (líquido seminal, coágulo seminal e *plug* copulatório). Esses estudos ainda pautaram a emissão de coágulo seminal ao longo do ano em *Macaca arctoides*, demonstrando que a concentração espermática foi significativamente maior no coágulo seminal, a qual variou sazonalmente em relação ao fotoperíodo, visto que a concentração espermática encontrada foi maior quando o período de dia e noite apresentaram horas semelhantes, e diminuiu em que o período do dia foi maior.

Já em *Saimiri* sp. mantidos em semi-cativeiro, DuMond e Hutchinson (1967) revelaram uma espermatogênese sazonal em machos mantidos em grupos sociais compostos por ambos os sexos. Nessa pesquisa, foram realizadas biopsias testiculares

durante 13 meses, os quais revelaram que na estação de acasalamento em quase todos os túbulos seminíferos havia a presença de células germinativas em todas as fases, e fora da estação de acasalamento os túbulos regrediram, com uma parede reduzida à camada basal e com pouca indicação de atividade mitótica.

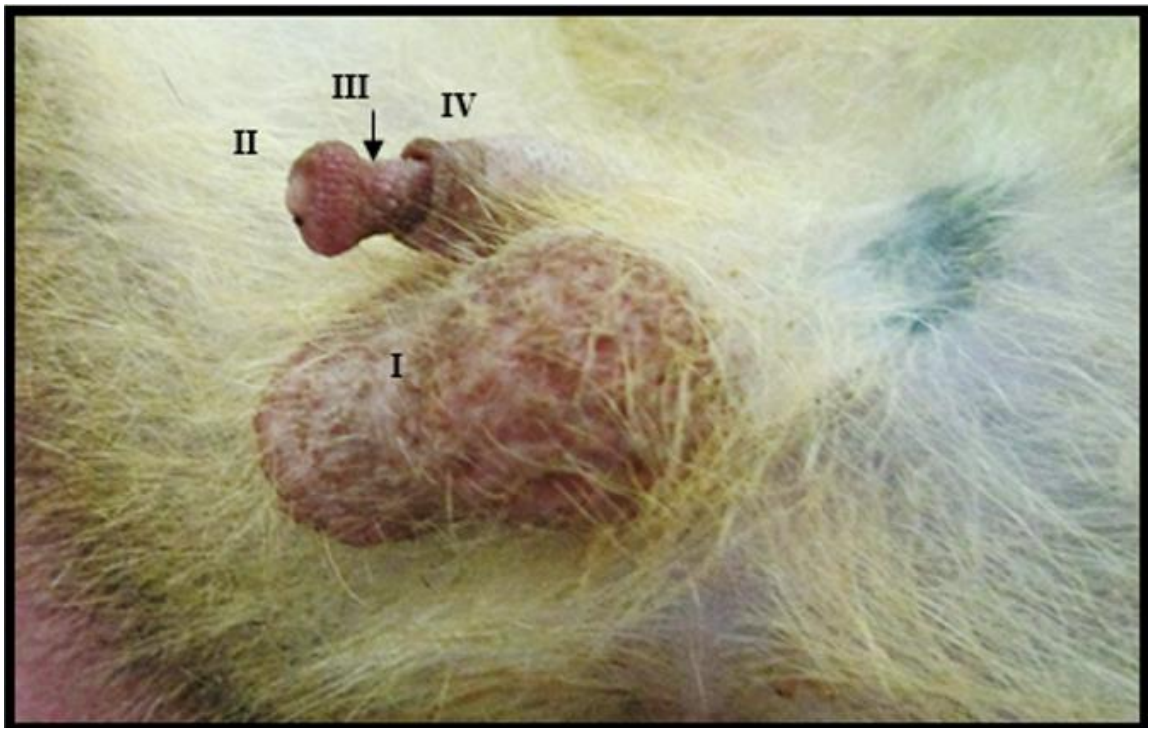
Entretanto, Chen et al. (1981) avaliaram a influência da sazonalidade na qualidade seminal em *S. sciureus* mantidos em cativeiro, e verificaram que não há diferença na concentração e motilidade espermática desses animais durante 13 meses. Contudo, durante a estação reprodutiva, há um aumento no volume do ejaculado, no peso corporal e nos níveis de testosterona circulante (CHEN et al., 1981).

Esses estudos em *Saimiri* sp. demonstraram que a sazonalidade reprodutiva pode ou não influenciar na produção espermática e qualidade seminal em animais de cativeiro, mas, pouco se sabe em relação aos componentes moleculares, a exemplo das proteínas que também podem ser influenciadas por esse processo. Vale ressaltar que em *S. collinsi*, assim como em outras espécies do gênero *Saimiri*, ainda não há estudos que correlacionem os diferentes parâmetros espermáticos e o proteoma, com a sazonalidade reprodutiva.

1.4 O sistema reprodutor do macho de Saimiri sp.

O pênis de *Saimiri* tem comprimento médio de 21 mm (Paulino, G. Dados não publicados), encoberto por prepúcio retrátil, osso peniano e algumas espículas queratinizadas vestigiais laterais ao seu corpo (STEINBERG et al., 2005). O escroto é semi pendulosa e assimétrica, proporcionalmente grande considerando-se o porte desses animais (STEINBERG et al., 2005) (Figura 4). O testículo e o epidídimo juntos formam uma massa relativamente pequena e globular. As glândulas seminais são tubulares e não ramificadas, e a próstata localiza-se na parte proximal da uretra, imediatamente distal à bexiga (HILL, 1960). Quanto a biometria testicular, em *S. collinsi,* foi descrita pela primeira vez por Oliveira et al. (2015), conforme dados da tabela 1.

Figura 4 - Genitália externa do macho de *S. collinsi*: I- escroto e testículo; II- glande; III- corpo do pênis; IV- Prepúcio. Seta: espículas laterais do corpo do pênis.



Fonte: A autora.

Tabela 1 - Biometria testicular de *S. collinsi* segundo Oliveira et al., 2015.

Testículo	Comprimento (cm)	Largura (cm)	Altura (cm)	Volume (cm^3)
Direito	1.86 ± 0,21	1,23 ± 0,11	1,16 ± 0,05	1,40 ± 0,26
Esquerdo	1,83 ± 0,24	1, 34 ± 0,14	1,18 ± 0,09	1,55 ± 0,42

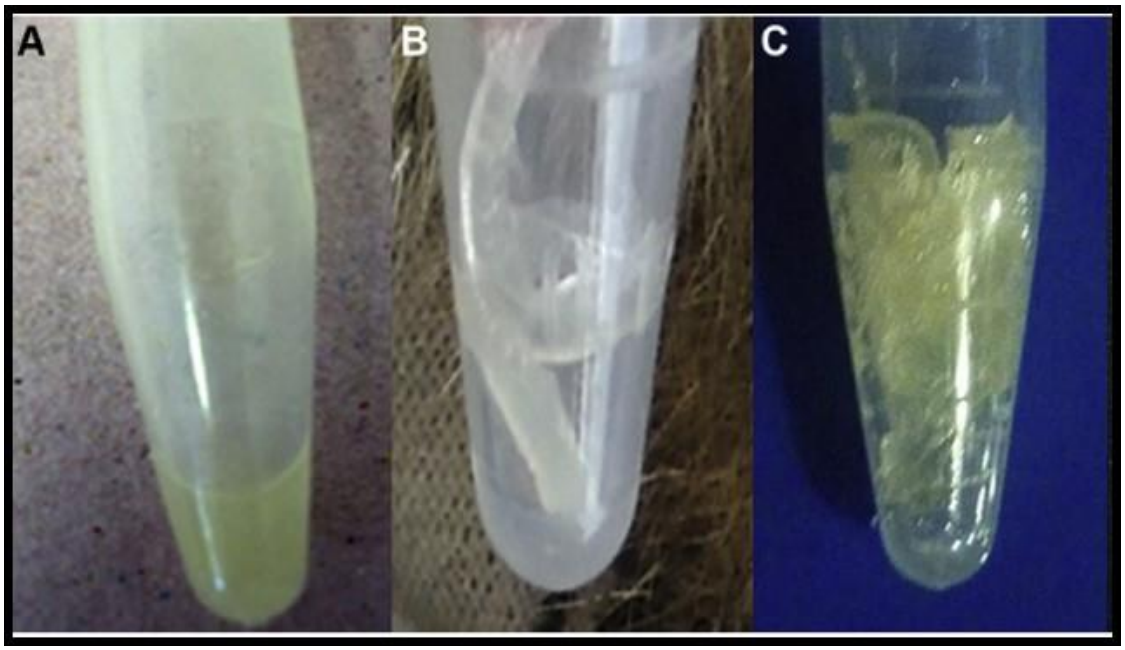
1.5 O sêmen de *Saimiri collinsi*

O sêmen de *Saimiri collinsi* é caracterizado por possuir duas frações, uma líquida e outra coagulada (Figura 5). Nesta espécie, a ejaculação quase sempre é iniciada pela fração líquida que é total ou parcialmente coagulada após 10 segundos no ambiente (OLIVEIRA et al., 2015).

A fração coagulada possui aparência filamentar ou amorfa, sendo que ambas as frações podem apresentar-se incolores, esbranquiçadas ou amareladas, transparentes ou opaca. O volume seminal obtido por eletroejaculação é de 51,8 ± 49,5 µL (5–200 µL) e

304 ± 283,6 µL (10–1100 µL), para a fração líquida e coagulada respectivamente. A concentração espermática do sêmen é cerca de 6,48 x10^6 espermatozoides/mL (OLIVEIRA et al., 2015).

Figura 5 - Sêmen de *S. collinsi* coletado por eletroejaculação: (A) fração líquida opaca, (B) coágulo filamentar e (C) coágulo amorfo.



Fonte: Oliveira et al. (2015).

1.6 O espermatozoide de Saimiri sp.

A espermatogênese dos macacos-de-cheiro dura cerca de 39 dias, sendo 30,5 dias da condição de espermatogônia até o estágio de espermátide, restando 8,5 dias para a diferenciação celular, culminando na formação dos espermatozoides (BARR, 1973).

A morfometria dos espermatozoides de macaco-de-cheiro foi primeiramente descrita por Bennett (1967), onde o comprimento da cabeça mediu 6 µm, o comprimento da peça intermediária 12 µm e o comprimento da cauda 60 µm, posteriormente outros pesquisadores também descreveram a morfometria dos espermatozoides, algumas delas estão demonstradas na tabela 2. Os espermatozoides de *Saimiri* sp., são caracterizados por apresentar cabeça achatada lateralmente com intumescimento apical, área superficial pequena e margem posterior do acrossomo com

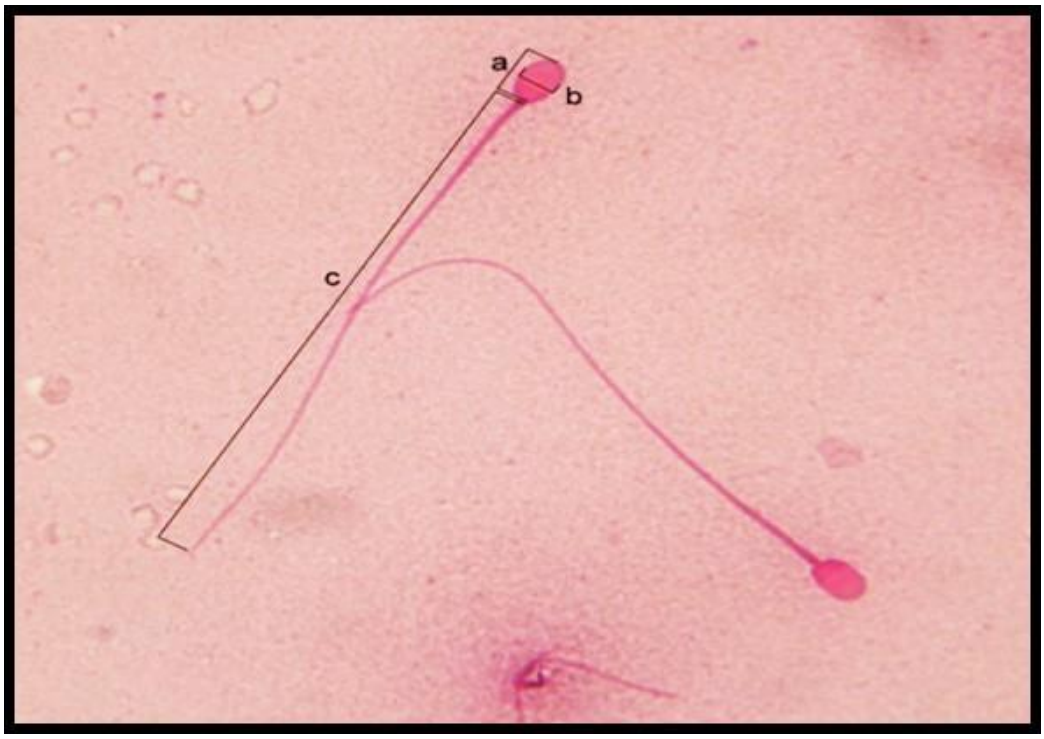
aparência serrilhada ou de microvilosidades, além da peça intermediária inserir-se de forma excêntrica na porção posterior da cabeça (Figura 6).

Tabela 2- Morfometria dos espermatozoides de espécies do gênero *Saimiri* sp.

	Dimensões lineares do espermatozoide (µm) - Média ± DP				
Espécie	Largura da cabeça	Comprimento da cabeça	Comprimento da PI*	Comprimento total da cauda	Comprimento total
	3,51±0,01[1]	5,11 ± 0,02[1]	9,03 ± 0,12[1]	55,34± 0,13[1]	69,24 ± 0,15[1]
S. sciureus	3,6 ± 0,3[2]	5,5 ± 0,03[2]	10 ± 0,5[2]	55,1± 2,7[2]	70,6 ± 2,8[2]
	-	5,1[3]	9,0[3]	55,3[3]	69,2[3]
S. boliviensis	3,76±0,45[4]	5,71 ± 0,45[4]	12,2 ± 0,45[4]	65,68± 0,45[4]	71,39 ± 0,45[4]
S. collinsi	4,3 ± 0,01[5]	6,2 ± 0,01[5]	-	70,5 ± 0,19[5]	76, 7 ± 0, 19[5]

*PI: Peça intermediária. [1]DUKELOW, 1983; [2] LAVERDE-CORREA; ROBLES-MEDINA, 2001; [3]GAGE; FRECKLETON, 2003; [4]STEINBERG ET AL., 2005; [5]SAMPAIO et al., 2017.

Figura 6 **-** Morfometria do espermatozoide de *S. collinsi* (a: comprimento da cabeça, b: largura da cabeça e c: comprimento da cauda) corados com eosina-nigrosina.

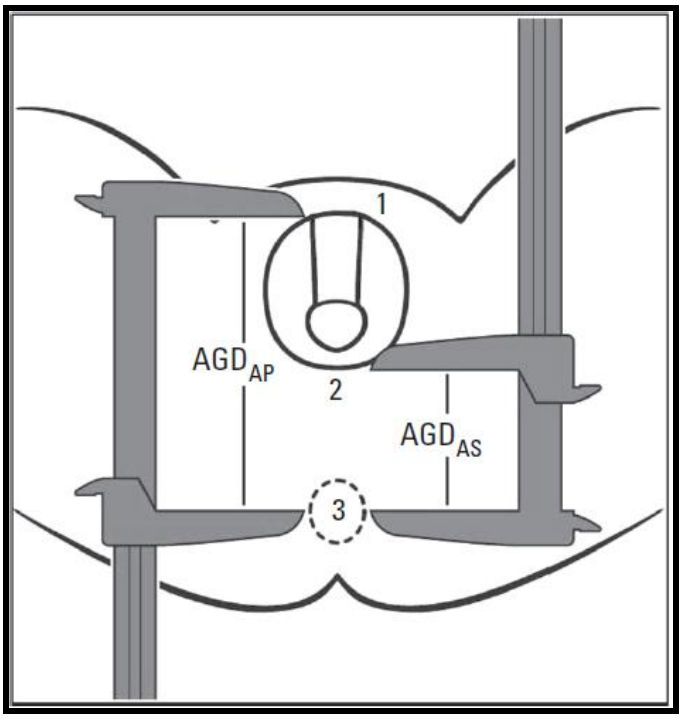


Fonte: SAMPAIO, W.V. (2014).

1.7 A distância anogenital

A distância do ânus aos órgãos genitais (distância anogenital; AGD) é considerada uma característica sexualmente dimórfica em mamíferos, uma vez que os machos têm uma AGD quase duas vezes maior que as fêmeas (MACLEOD et al., 2010; SWAN, 2008; THANKAMONY et al., 2009;). As variantes do AGD medidas com paquímetro universal, são: AGDap – que é a distância da inserção cefálica do pênis ao centro do ânus, e AGDas que é a distância da base posterior (primeira dobra) do escroto para o centro do ânus (Figura 7; MENDIOLA et al., 2011)

Figura 7 - Desenho esquemático das duas medidas de AGD: AGD_{AP}, da inserção cefálica do pênis até o centro do ânus (ponto 1 ao ponto 3); e AGD_{AS}, da base posterior (primeira dobra) do escroto ao centro do ânus (ponto 2 ao ponto 3).



Fonte: MENDIOLA et al. (2011).

Em ratos, as medidas de AGD foram associadas a um tamanho menor de pênis, testículos, próstata e vesículas seminais (MACLEOD et al., 2010; SCOTT et al., 2008; VAN DEN DRIESCHE et al., 2011). O tamanho da AGD é determinado pela quantidade de andrógeno à qual um feto masculino é exposto no desenvolvimento fetal durante a janela de programação da masculinização (WELSH et al., 2008). Da mesma forma, em humanos, foi relatado que a AGD é determinada no útero como em ratos, e

isso influencia o desenvolvimento genital e a função testicular do adulto, assim como a baixa qualidade seminal (MENDIOLA et al., 2011).

Nesse contexto, o AGD tem sido relatado como um biomarcador que determina a síndrome de disgenesia testicular (DEAN; SHARPE, 2013), como criptorquidia e hipospádia (GILBOA et al., 2017; JAIN; SINGAL, 2013), bem como um determinante de uma concentração alta ou baixa de espermatozóides (LÓPEZ-ESPÍN et al., 2017; MENDIOLA et al., 2011, 2015).

Embora existam estudos que avaliam a correlação AGD com uma qualidade seminal em humanos, essa medida pode auxiliar na determinação da etiologia dos homens com azoospermia (EISENBERG et al., 2012). Não se sabe se o tamanho da AGD também está associado à qualidade seminal e à biometria testicular em outras espécies de primatas como o *Saimiri collinsi*, pois, de acordo com a literatura, essa associação só foi estudada em homens (DEAN; SHARPE, 2013; EISENBERG et al., 2011, 2012; MENDIOLA et al., 2011; PARRA et al., 2016; ZHOU et al., 2015). Se confirmada, a medida de AGD pode substituir exames andrológicos invasivos durante a avaliação e seleção de machos *Saimiri collinsi* em programas de biotecnologia de reprodução animal para conservação de espécies ameaçadas.

1.8 O estudo da micromorfologia e ultraestrutura dos espermatozoides de primatas

Na reprodução animal, a análise seminal é uma importante ferramenta para avaliar o potencial de fertilidade do macho (PARIZ et al., 2014). Contudo, a análise espermática sob a microscopia óptica possui limitação no tocante à resolução, visto que não permite a identificação e análise de defeitos morfológicos que podem acometer as organelas dos espermatozoides, como defeitos da teca perinuclear, estruturas mitocondriais e dos axonemas, os quais são frequentemente associadas com patologias espermáticas como a teratozoospermia, a astenozoospermia e infertilidade idiopática, que influenciam negativamente na capacidade fecundante da célula espermática (MORETTI; COLODELL, 2012; PARIZ et al., 2014).

Nesse contexto, a microscopia eletrônica de varredura possibilita a avaliação tridimensional dos espermatozoides, e a de transmissão possui um alto poder de resolução em relação ao microscópio óptico, visando a analise de estruturas extra e

intracelulares, respectivamente, proporcionando uma visão de alto alcance, em dimensões nanométricas, que auxiliam à descrição celular e à detecção de possíveis anormalidades espermáticas (MORETTI; COLODELL, 2012). Essas técnicas já foram utilizadas em diversos mamíferos como touros (DUCHA et al., 2012; OLIVEIRA et al., 2015), cavalos (BLOTTNER et al., 2001; PESCH et al., 2006), carneiros (LÓPEZ-ARMENGOL et al., 2012), cães (SILVA et al., 2009), tatus (SOUSA et al., 2013), ursos (BRITO et al., 2010), capivara (BATALHA; OBA, 2006) e primatas não-humanos (OKADA et al., 2001; RUTLLANT; POMMER; MEYERS, 2003; SHABDULYA et al., 1982; STEINBERG et al., 2009).

Por meio da análise por microscopia eletrônica de varredura, Zaneveld et al. (1974) demonstraram diferenças entre os aspectos morfológicos superficiais do coágulo seminal de humanos e de macacos *Rherus* (*Macaca mulatta*), determinando a relação entre esses aspectos e a taxa de liquefação do coágulo. Nesse mesmo estudo, as eletromicrografias mostraram que os coágulos de *Macaca* sp. consistem de fibras desorganizadas de espessura variável, formando uma estrutura sólida, e que o coágulo humano se apresentou constituído por uma fina rede extensivamente organizada de longos e finos filamentos fibrosos que confinam os espermatozoides. Em ambas as espécies, essas fibras tornam-se desorganizadas e frouxas à medida que o coágulo se liquefaz, havendo a liberação gradativa dos espermatozoides. Um fato interessante em sêmen de *Macaca* sp. é que a maioria dos espermatozoides foi visualizada na superfície do coágulo, sendo possível a existência de alguma propriedade de adesão. Também, em macacos *Rhesus*, Sivashanmugam et al. (1997) verificaram, por meio da microscopia eletrônica de transmissão, alterações na condensação da cromatina e nos componentes da membrana plasmática de espermatozoides durante a maturação epididimária, e relataram uma reorganização dos lipídios da membrana plasmática durante o transito pelo epidídimo, no qual a restrição de domínios lipídicos sobre a região acrossomal pode ser importante para a capacitação e subsequente reação acrossômica.

Já em primatas neotropicais, Grabner (2016) avaliou por microscopia eletrônica de transmissão, a morfologia espermática de três espécies (*Leontopithecus chtysomelas*, *Alouatta caraya* e *A. guariba clamitans*), e verificou que os espermatozoides desses primatas não-humanos apresentaram os componentes básicos já descritos para o homem, entretanto existe variação na forma e no tamanho dos mesmos, sendo que na extensão do acrossomo, bem como a largura, comprimento e organização da peça

intermediária demonstram as diferenças mais marcantes da superfamília Hominoidea para o gênero *Homo*. Em os macacos-de-cheiro (*S. sciureus*), os estudos utilizando técnicas de microscopia eletrônica de varredura e de transmissão foram utilizados há décadas também com o objetivo de analisar a morfologia espermática (GOULD, 1980; MARTIN, GOULD, 1975). Steinberg et al. (2009) obtiveram, com auxílio de imagens captadas por microscopia eletrônica de varredura, dados morfométricos da cabeça dos espermatozoides (comprimento da cabeça: 4,93 ± 0,41 µm e largura da cabela: 3,83± 0,26 µm) como ferramenta para identificação taxonômica mais precisa da espécie *S. boliviensis*.

Nesse contexto, para um maior conhecimento sobre os aspectos mais intrínsecos, e ainda não relatados em sêmen de *S. collinsi*, faz-se necessária a descrição em detalhes nanométricos de componentes da matriz extracelular, de organelas celulares e das membranas dos espermatozoides, possíveis somente por análise em microscopia eletrônica de varredura e de transmissão.

1.9 O estudo das proteínas na reprodução

A proteômica busca fornecer informações a partir da análise sistemática de macromoléculas em um tecido ou célula (COX; MANN, 2007). Técnicas de proteômica têm sido utilizadas no intuito de identificar constituintes expressos no sistema reprodutor masculino em diferentes espécies, tais como bovinos (REGO et al., 2014; THEPPARAT et al., 2012), equinos (SWEGEN et al., 2015), caprinos (HE et al., 2015; VAN TILBURG et al., 2013), ovinos (RODRIGUES et al., 2013), suínos (GONZÁLEZ-CADAVID et al., 2014), catetos (SANTOS et al., 2014), PHN (LUNDWALL; OLSSON, 2001; SKERGET et al., 2013; VALTONEN-ANDRÉ et al., 2005) e no homem (FRAPSAUCE et al., 2014). Tais pesquisas trazem resultados importantes das interações entre proteínas do plasma seminal e espermatozoides. Atualmente, marcadores proteicos têm sido descritos no intuito de detectar propriedades biológicas relacionadas com a qualidade seminal (FRAZER et al., 2006; PARK et al., 2012; THEPPARAT et al., 2012), e assim, desenvolver novos critérios para o prognóstico e aumento na taxa de fertilidade dos machos (AGARWAL et al., 2016; LÉGARÉ et al., 2014; NARESH; ATREJA, 2015).

Além disso, em PNH, algumas pesquisas têm oferecido informações básicas acerca da composição proteica do plasma seminal relacionada ao processo de coagulação seminal como demonstrado em sagui-do-tufo-branco (*Callithrix jacchus*) (VALTONEN-ANDRÉ et al., 2005) e sagui-da-cabeça-de-algodão (*Saguinus oedipus*) (LUNDWALL; OLSSON, 2001), assim como para a caracterização das proteínas espermáticas do gênero *Macaca* (KAWASE; CAO; XUAN, 2015; SKERGET et al. 2013; ZHOU et al., 2015).

1.10 Proteínas espermáticas

Durante a espermatogênese, os espermatozoides perdem a capacidade de sintetizar e secretar proteínas (SHABANOWITZ; KILLIAN, 1987), e ao final deste processo estão funcionalmente imaturos e imóveis (YOSHIDA et al., 2008). Mas, durante o trânsito epididimário (ou espermiação), os espermatozoides passam pelo processo de maturação em que ocorre uma série de mudanças, incluindo aquisição de motilidade, remodelação da membrana plasmática, alterações no pH intra-espermático, e fosforilação de tirosina (DACHEUX et al., 2009). Durante a ejaculação, o gameta masculino entra em contato com as secreções das glândulas sexuais acessórias resultando na aderência de outras proteínas à membrana plasmática (VARRICCHIO et al., 1996), dando início a mudanças bioquímicas e estruturais relacionadas a hipermotilidade, capacitação e reação acrossômica, que tornam os espermatozoides aptos à fecundação (MOREAU et al., 1999).

Essa série de alterações funcionais dependem integralmente de mudanças na composição proteica dos espermatozoides, que inclui a remoção e adição de proteínas, além de uma complexa matriz de modificações pós-translacionais, as quais são responsáveis por conduzir alterações estruturais e funcionais dos espermatozoides em função da maturação e capacitação espermática (BAKER et al., 2012; DACHEUX; DACHEUX, 2014). Além disso, as proteínas espermáticas possuem papel importante na integridade, morfologia e funcionalidade da célula espermática ao qual incluem a motilidade, capacitação, fecundação, ativação oocitária e desenvolvimento embrionário (PARISI et al., 2014).

No tocante a motilidade espermática, sabe-se que as proteínas associadas a esse parâmetro são classificadas em (i) proteínas enzimáticas relacionadas com a energia por

vias mitocondriais e glicolíticas; (ii) proteínas estruturais, tais como a fibras densas externas e proteínas que ancoram quinase A (AKAPs) nos flagelos; e (iii) proteínas de ativação como as proteínas quinase A transdutoras de sinal (PKA) e fosfatases de serina-treonina-tirosina-quinase (MURATORI et al., 2009). Em ratos, a proteína das fibras densas externas 2 (ODF2) foi identificada como um componente estrutural da cauda espermática, estando envolvida na regulação da motilidade dos espermatozoides (TARNASKY et al., 2010).

Em espermatozoides epididimários humanos foram identificadas 35 proteínas com massa molecular entre 15 e 160 kDa. Parte dessas proteínas está localizada na região subcelular desses gametas, e foram associadas a diferentes estruturas como o citoesqueleto, citoplasma, núcleo e mitocôndrias, sendo correlacionadas com diferentes funções biológicas (sinalização, transporte e ciclo celular, apoptose e resposta ao estresse, síntese proteica e metabolismo) (PILATZ et al., 2014). Algumas proteínas foram previamente reportadas apresentando relação com a capacitação espermática (SECCIANI et al., 2009) e também com a infertilidade (HOSSEINIFAR et al., 2013).

Na membrana plasmática espermática humana foi identificado, na região que abrange o acrossoma, um componente proteico denominado de 5'- nucleotidase (5 – NT; 125 kDa). Esta enzima, a priori, quando purificada e adicionada em um sistema de incubação *in vitro* para espermatozoides não teve nenhum efeito sobre a motilidade. No entanto, quando inibidores da 5'-nucleotidase foram incubados nesse mesmo sistema, foi observada uma inibição clara da motilidade dos espermatozoides, de um modo dependente da dose. Este resultado é interpretado como uma indicação de que a 5'-nucleotidase possui um papel significativo na regulação da motilidade espermática (AUMULLER et al., 1997).

Em espermatozoides epididimários de macaco-japonês (*Macaca fuscata*), foram identificadas 154 proteínas por meio da eletroforese bidimensional seguida da espectrometria de massa. Estas foram agrupadas em proteínas de membrana plasmática e mitocondrial, citoplasmáticas, de matriz mitocondrial e núcleo, e estão envolvidas na sinalização celular, motilidade, transporte espermático através do trato reprodutor da fêmea, hiperativação e reação acrossômica, eventos estes de suma importância para que ocorra a fecundação (KAWASE; CAO; XUAN, 2015). Zhou et al. (2015) avaliaram a composição proteica dos espermatozoides de primatas do gênero *Macaca*, por meio da cromatografia líquida acoplada à espectrometria de massas, e identificaram 2.044

proteínas espermáticas das quais 1.948 são ortólogas ao homem, indicando assim que esses PNH possuem a composição proteica similar comparada ao espermatozoide humano.

1.10.1 Métodos para estudo das proteínas espermáticas

O recente progresso metodológico no estudo do proteoma tem aberto novos caminhos para obtenção de informações descritivas e quantitativas acerca dos processos biológicos que ocorrem no organismo animal e humano (BARBOSA et al., 2012). Sabe-se que as metodologias empregadas na proteômica podem ser classificadas em *bottom-up, middle-down* e *top-down.* A proteômica tipo *bottom-up é* caracterizada por um processo de separação por cromatografia líquida dos peptídeos obtidos após digestão enzimática, seguida da análise por espectrometria de massas (MS do inglês *Mass Spectrometry*). Essa técnica também é conhecida por proteômica *Shotgun*, contudo nesse processo as misturas proteicas são fracionadas por cromatografia líquida livre de gel. Já na proteômica tipo *middle-down* as proteínas são submetidas à digestão enzimática, entretanto, geram fragmentos de peptídeos maiores quando comparado a técnica *bottom-up*, que passará em seguida pela análise por MS, e por fim proteômica tipo *top-down,* no qual as proteínas (e não os peptídeos) são submetidas à análise por espectrometria de massa, essa estratégia de análise foca na caracterização completa da proteína intacta e de suas modificações pós-traducionais (ARMIROTTI; DAMONTE, 2010; AHRENS et al., 2010; WU eta l., 2012; ZHANG et al., 2013). A combinação dessas abordagens com outros processos, como fracionamento subcelular ou precipitação de proteínas, tem sido utilizado como uma alternativa efetiva no enriquecimento de amostras compostas de baixas concentrações proteicas (KOSAKO et al., 2011).

Contudo, para que se tenha sucesso nesse processo, inicialmente é de suma importância a extração adequada das proteínas, o qual deve se levar em consideração o tipo e origem das amostras biológicas. Logo, o procedimento de extração necessita de otimização individual, mas em geral, as proteínas precisam ser solubilizadas, desagregadas, desnaturadas e submetidas a tratamento com agentes redutores de pontes dissulfeto (DE MARQUI et al., 2006).

Técnicas de eletroforese uni (1D) e bidimensional (2D) têm sido comumente utilizadas com o objetivo de separação proteica. Na eletroforese 1D ocorre a separação das proteínas de acordo com o peso molecular, em que há a formação de bandas, enquanto que na eletroforese 2D elas são separadas em duas etapas, em que primeiramente há a separação de proteínas utilizando focalização isoelétrica (etapa I), seguida por uma eletroforese em gel de poliacrilamida na presença de SDS (SDS-PAGE do inglês *Sodium dodecyl sulfate polyacrylamide gel electrophoresis*) visando a separação numa segunda dimensão, ou seja, de acordo com o peso molecular (etapa II), onde há a formação dos *spots* (HACHEY; CHAURAND, 2004). Posteriormente, para tornar visíveis as bandas ou *spots* proteicos (eletroforese 1D e 2D, respectivamente), os géis são comumente corados com Azul de *Coomassie*, nitrato de prata ou outros corantes comerciais (DOWSEY et al., 2003; GHARAHDAGHI et al., 1999; SHEVCHENKO et al., 1996;). Em seguida há a excisão das bandas ou *spots* de proteínas individuais do gel e digestão com uma protease (ex. tripsina), seguindo-se de uma identificação por espectrometria de massas (DELAHUNTY; YATES, 2005).

A eletroforese 1D e 2D já foi utilizada na análise de proteínas em espermatozoides de mamíferos tais como: homem (JOHNSTON et al., 2005; PILCH; MANN, 2006), touros (PARK et al., 2012), búfalos (ASADPOUR et al. 2007), carneiros (YUE et al., 2009), suínos (CORCINI et al., 2012), catetos (SANTOS et al., 2014), PNH (KAWASE; CAO; XUAN, 2015) e cães (AQUINO-CORTEZ et al., 2017).

Neste cenário, a MS têm sido comumente empregada nos últimos anos devido a possibilidade de identificação direta dos constituintes individuais de complexos proteicos envolvidos em uma ampla gama de funções fisiológicas, sendo assim, tornou-se uma plataforma padrão na proteômica (PLATT et al., 2008; SCHILLER et al., 2000; SWEGEN et al., 2015; ZERBINATI et al., 2017). MS é uma ferramenta analítica que identifica proteínas ou peptídeos medindo as massas de moléculas convertidas em íons por meio da sua relação massa sobre carga (*m/z*). Entretanto, essa técnica depende ou não da digestão proteolítica de proteínas em peptídeos antes da introdução no espectrômetro de massa (PLESSIS et al., 2011).

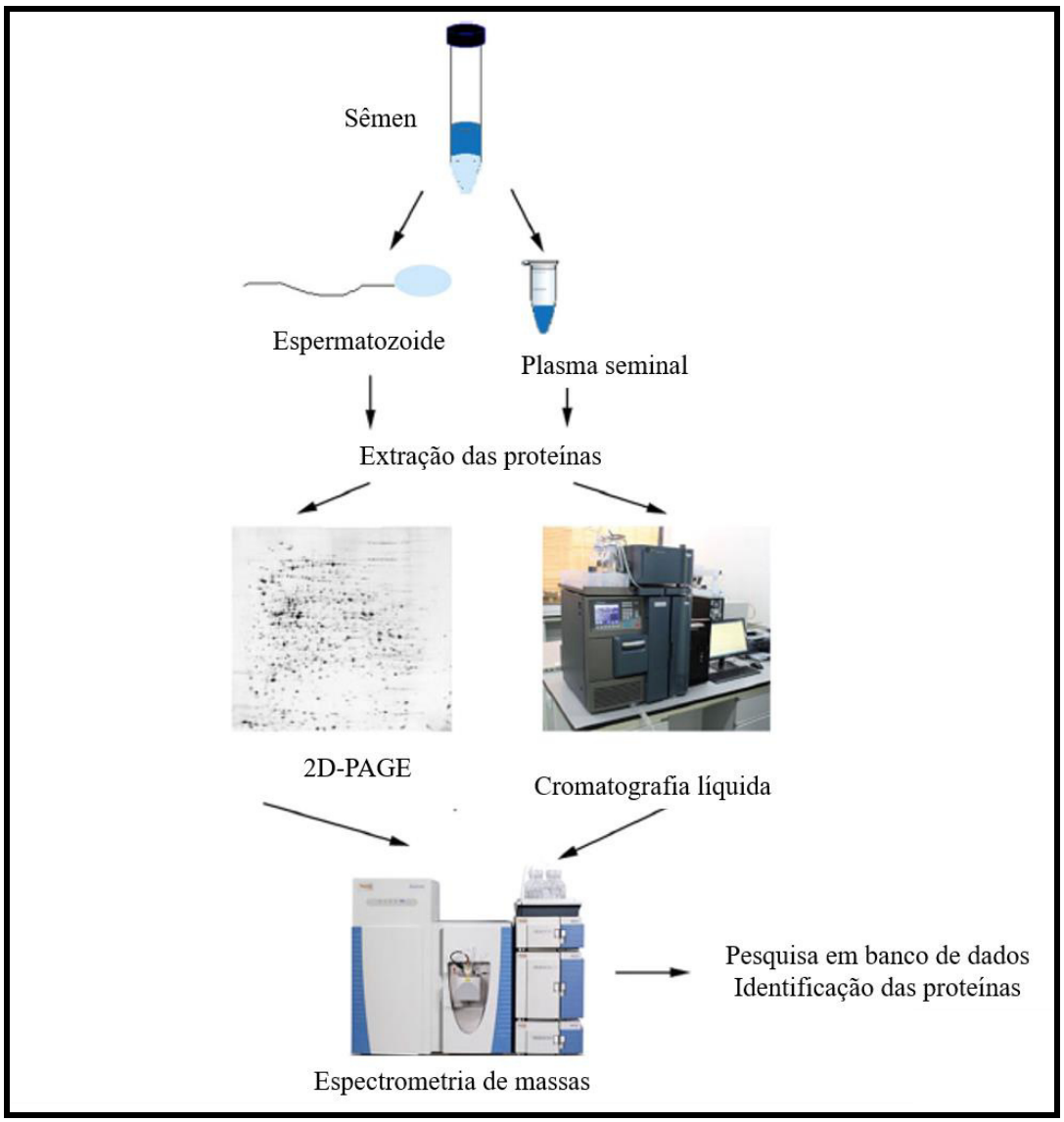
No estudo das proteínas espermáticas, duas das técnicas MS tem sido mais comumente empregadas para caracterizar *spots* proteicos de um gel de eletroforese previamente executado, que são: a ionização e dessorção a laser assistida por matriz – analisador do tipo tempo de voo, conhecida como MALDI-TOF (do inglês *Matrix*

Associated Laser Desorption-Ionization - Time of Flight), e a cromatografia líquida com um espectrômetro de massa em tandem (LC-MS/MS do inglês *Liquid Chromatography–Mass Spectrometry*). Os dados espectrais obtidos a partir da MS proporcionam a relação *m/z* da proteína e sua intensidade associada, o que ajuda a determinar a expressão diferencial proteica e suas modificações (MITULOVIC; MECHTLER, 2006; PLESSIS et al., 2011).

Na análise MALDI-TOF, a determinar da relação *m/z* de uma proteína é realizada após a digestão das bandas de proteínas do gel com proteases (PLESSIS et al., 2011). A digestão de proteínas utilizando enzimas resultam em fragmentos peptídicos que são facilmente ionizados no espectrômetro de massa (MCLUCKEY, 1992). Depois que as massas peptídicas foram registradas, elas são combinadas com conjuntos de massas de referência de proteína teoricamente digeridas a partir de um banco de dados para identificação proteica (mapeamento de massa de peptídeo).

Por outro lado, o LC-MS/MS, é uma técnica analítica altamente sensível e específica que combina a separação física de peptídeos gerados pela digestão enzimática de um *spo*t por cromatografia líquida de alta eficiência (do inglês *High performance liquid chromatography* - HPLC) seguida da determinação da massa e subsequente sequenciamento em MS/MS (MITULOVIC; MECHTLER, 2006) (Figura 8).

Figura 8- Diagrama esquemático mostrando a análise proteômica do plasma seminal e da célula espermática.



Fonte: Adaptado de Cao et al., (2018).

Contudo, nos últimos anos, a proteômica *Shotgun* tornou-se o método de escolha para identificar e quantificar proteínas em larga escala (ALVES et al., 2007; WASHBURN; WOLTERS; YATES, 2001), já sendo utilizada na identificação proteica de espermatozoides de camundongos (BAKER et al., 2008), humano (NIXON et al., 2011), bovinos (CANIO et al., 2014), primatas não-humanos (ZHOU et al., 2015). Esta estratégia baseia-se na digestão de proteínas em peptídeos (sem termos previamente a etapa de separação proteica em gel de eletroforese), seguido do sequenciamento usando espectrometria de massa em tandem (LC-MS/MS) e pesquisa automatizada de banco de dados (WASHBURN; WOLTERS; YATES, 2001). Em comparação com os métodos

identificação baseada em MS, como os géis 1D e 2D, na proteômica *Shotgun* há uma melhor sensibilidade à detecção de proteínas e com isso uma maior taxa de transferência de dados (GORG; WEISS; DUNN, 2004).

1.11 Hipóteses

- A distância anogenital pode ser utilizada como um biomarcado no exame andrológico para a seleção de machos de S. collinsi.
- A micromorfologia e ultraestrutura dos espermatozoides de *S. collinsi* demonstrada por eletromicroscopia de varredura e transmissão, é semelhante do que foi descrito em outros primatas do gênero Saimiri.
- As proteínas associadas com a qualidade seminal em macacos-de-cheiro (*S. collinsi*) são comuns para outros primatas.

1.12 Objetivos

1.12.1 Geral

- Estudar a morfofisiologia reprodutiva de macaco-de-cheiro (*S. collinsi*).

1.12.2 Específicos

- Associar a distância anogenital com os parâmetros espermáticos e hormônios reprodutivos em macacos-de-cheiro (*S. collinsi*).
- Descrever as características micromorfologicas e ultraestruturais dos espermatozoides de macacos-de-cheiro (*S. collinsi*) por meio da microscopia eletrônica de varredura e transmissão.
- Caracterizar o perfil proteico dos espermatozoides de macaco-de-cheiro (*S. collinsi*).
- Associar as expressões de proteínas espermáticas e as características espermáticas de macaco-de-cheiro (*S. collinsi*).

REFERÊNCIA BIBLIOGRÁFICA

ANDERSON, T. F. Techniques for the preservation of three-dimensional structure in preparing specimens for the electron microscope. **Transactions of the New York Academy of Sciences.** v.13, p.130, 1951.

AGARWAL, A.; BERTOLLA, R. P.; SAMANTA, L. Sperm proteomics: potential impact on male infertility treatment. **Expert Review of Proteomics**, v.13, p.285-96, 2016.

ALVES, P.; ARNOLD, R. J.; NOVOTNY, M. V.; RADIVOJAC, P.; REILLY, J. P.; TANG, H. Advancement in protein inference from shotgun proteomics using peptide detectability. **Pacific Symposium on Biocomputing**, v.12, p.409-420, 2007.

AMARAL, A.; CASTILLO, J.; RAMALHO-SANTOS, J.; OLIVA, R. The combined human sperm proteome: cellular pathways and implications for basic and clinical science. **Human Reproduction Update**, v. 20, p. 40–62, 2014.

ANDERSON, T. F. Techniques for the preservation of three-dimensional structure in preparing specimens for the electron microscope. **Transaction of the New York Academy of Science**, v.13, 1951.

AQUINO-CORTEZ, A.; PINHEIRO, B. Q.; LIMA, D. B. C.; SILVA, H. V. R.; MOTA FILHO, A. C.; MARTINS, J.A.M.; RODRIGUEZ-VILLAMIL, P.; Moura, A.A.; SILVA, L. D. M. Proteomic characterization of canine seminal plasma. **Theriogenology**, v. 95, p. 178-186, 2017.

ASADPOURA, R.; ALAVI-SHOUSHTARIA, S. M.; ASRI REZAIIB, S. A.; ANSARIC, M. H. K. H. SDS-polyacrylamide gel electrophoresis of buffalo bulls seminal plasma proteins and their relation with semen freezability. **Animal Reproduction Science**, v.102, p.308–313, 2007.

AUMÜLLER, G.; RENNEBERG, H.; SCHIEMANN, P. J.; WILHELM, B.; SEITZ, J.; KONRAD, L.; WENNEMUTH, G. The role of apocrine released proteins in the post-testicular regulation of human sperm function. **Advances in Experimental Medicine and Biology**, v.424, p.193-219, 1997.

BAKER, M. A.; HETHERINGTON, L.; WEINBERG, A.; NAUMOVSKI, N.; VELKOV, T.; PELZING, M.; DOLMAN, S.; CONDINA, M. R.; AITKEN, R. J. Analysis of phosphopeptide changes as spermatozoa acquire functional competence in the epididymis demonstrates changes in the posttranslationalmodification of Izumo1. **Journal of Proteome Research**, v. 11, p. 5252–5264, 2012.

BAKER, M. A.; HETHERINGTON, L.; REEVES, G. M.; AITKEN, R. J. The mouse sperm proteome characterized via IPG strip prefractionation and LC-MS/MS identification. **Proteomics**, v. 8, p. 1720–1730, 2008.

BALDWIN, J. D. The Behavior of Squirrel Monkeys (*Saimiri*) in Natural Environments *In*: ROSENBLUM, L. A.; COE, C. L. Handbook of Squirrel Monkey Research. **New York: Plenum Press**, p. 35-53, 1985.

BALDWIN, J. D.; BALDWIN, J. I. Primates. The squirrel monkeys, genus *Saimiri*. *In*: COIMBRA-FILHO A. F.; MITTERMEIER, R. A. **Ecology and Behavior Neotropical**. Academia Brasileira de Ciências, Rio de Janeiro, p. 277-330, 1981.

BARAZANI, Y.; AGARWAL, A.; SABANEGH JR, E. S. Functional Sperm Testing and the Role of Proteomics in the Evaluation of Male Infertility. **Urology**, v. 84 (2), p.255-6, 2014.

BARBOSA, E. B.; VIDOTTO, A.; POLACHINI, G. M.; HENRIQUE, T.; MARQUI, A. B. T.; TAJARA, E.H. Proteômica: metodologias e aplicações no estudo de doenças humanas. **Revista da Associação Médica Brasileira**, v.58, p.366-375, 2012.

BARR, A. B. Timing of spermatogenesis in four nonhuman primate species. **Fertility and Sterility**, v. 24, n. 5, p. 381-389, 1973.

BARROS, T. B.; GUIMARÃES, D. B.; ARAÚJO, L. R. S.; CANTANHÊDE, L. F.; DIAS, A. V.; QUEVEDO FILHO, I. B.; MOURA, A. A. A.; TONIOLLI, R. O estudo proteômico e o seu potencial de avaliação da fertilidade em machos de diferentes espécies domésticas. **Revista Brasileira de Reprodução Animal**, v.37, p.266-271, 2013.

BATALHA, L. M.; OBA, E. Caracterização morfométrica e morfológica de célula espermática de capivara (*Hydrochoerus hydrochaeris*). **Archives of Veterinary Science**, v. 11, p. 66-72, 2006.

BENNETT, J. P. Semen Collection in the squirrel monkey. **Journal of Reproduction and Fertility**, v.13, p.353-355, 1967.

BLOTTNER, S.; WARNKE, C.; TUCHSCHERER, A.; HEINEN, V.; TORNER, H. Morphological and functional changes of stallion spermatozoa after cryopreservation during breeding and non-breeding season. **Animal Reproduction Science**, v. 65, p. 75–88, 2001.

BOINSKI, S. The positional behavior and substrate use of squirrel monkeys: ecological implications. **Journal of Human Evolution**, v.18, p. 659-677, 1989.

BRITO, L. F. C.; SERTICH, P. L.; STULL, G. B.; RIVES, W.; KNOBBE, M. Sperm ultrastructure, morphometry, and abnormal morphology in American black bears (*Ursus americanus*).**Theriogenology,** v. 74, n. 8, p. 1403-1413, 2010.

BRUESCHKE, E. E.; ZANEVELD, L. J. D.; RODZEN, R.; BERNS, D. Development of a reversible vas deferens occluding device. III. Morphology of the human and dog vas deferens: a study with the scanning electron microscope. **Fertility and Sterility**, (in press) 1974.

BOUBLI, J. P., RYLANDS, A. B., 2008. *Samiri*. *In*: IUCN. IUCN Red List of Threatened Species, Version 2011.2. Diponivel em: www.iucnredlist.org. Acessado em: 20 nov. 2018.

CAO, X.; CUI, Y.; ZHANG, X.; LOU, J.; ZHOU, J.; BEI, H.; WEI, R. Proteomic profile of human spermatozoa in healthy and asthenozoospermic individuals. **Reproductive Biology and Endocrinology**, v. 16, n.1, p. 1-8, 2018.

CHEN, J. J.; SMITH, E. R.; GRAY, G. D.; DAVIDSON, J. M. Seasonal Changes in Plasma Testosterone and Ejaculatory Capacity in Squirrel Monkeys (*Saimiri sciureus*). **Primates**, v.22, p.253-260, 1981.

CHIU, W. W. C.; ERIKSON, E. K. L.; SOLE, C. A.; SHELLING, A. N.; CHAMLEY, L. W. SPRASA, a novel sperm protein involved in immunemediated infertility**.** **Human Reproduction**, v.19 (2), p.243-249, 2004.

CORCINI, C. D.; VARELA JR., A. S.; PIGOZZO, R.; RAMBO, G.; GOULARTE, K. L.; CALDERAM, K.; LEON, P. M. M.; BONGALHARDO, D. C.; LUCIA JR., T. Pre-freezing and post-thawing quality of boar sperm for distinct portions of the ejaculate and as a function of protein bands present in seminal plasma. **Livestock Science**, v. 145, p.28–33, 2012.

COX, J.; MANN, M. Is proteomics the new genomics?. **Cell**, v. 130, p.395–398, 2007.

CUI, Z.; SHARMA, R.; AGARWAL, A. Proteomic analysis of mature and immature ejaculated spermatozoa from fertile men. **Asian Journal of Andrology**, v.18, p. 1–12, 2016.

DACHEUX, J. L.; DACHEUX, F. New insights into epididymal function in relation to sperm maturation. **Reproduction**, v. 147, p R27–R42, 2014.

DACHEUXA, J. L.; BELLEANNÉE, C.; JONES, R.; LABAS, V.; BELGHAZI, M.; GUYONNET, B.; DRUART, X.; GATTI, J. L.; DACHEUX, F. Mammalian epididymal proteome. **Molecular and Cellular Endocrinology**, v.306, p.45–50, 2009.

DEAN, A.; SHARPE, R. M. Anogenital distance or digit length ratio as measures of fetal androgen exposure: relationship to male reproductive development and its disorders. **Journal of Clinical Endocrinology & Metabolism**, v. 98 (6), p. 2230–2238, 2013.

DELAHUNTY, C.; YATES III, J.R. Protein identification using 2D-LC-MS/MS. **Methods**, v.35, p.248–255, 2005.

DENIS, L. T.; POINDEXTER, A. N.; RITTER, M. B.; SEAGER, S. W. J.; DETER, R. L. Freeze preservation of squirrel monkey sperm for use in timed fertilization studies. **Fertility and Sterility**, v. 27, n. 6, p. 723-726, 1976.

DIXSON, A. F.; ANDERSON, M. J. Sexual selection, Seminal coagulation and Copulatory plug formation in Primates. **Folia Primatologica**, v.73, p.63-69, 2002.

DOMINGUES, S. F. S; LIMA, J. S.; OLIVEIRA, K. G.; SANTOS, R. R. Biotecnologias de reprodução como uma estratégia complementar à conservação *in situ* de primatas neotropicais ameaçados de extinção: perspectivas e desafios. **Revista Brasileira de Reprodução Animal**, Belo Horizonte, v.35, n.2, p.124-129, 2011.

DOWSEY, A. W.; DUNN, M. J.; YANG, G. Z. The role of bioinformatics in two-dimensional gel electrophoresis. **Proteomics**, v.3, p.1567-1596, 2003.

DUCHA, N.; SUSILAWATI, T.; AULANNI, A. M.; WAHYUNINGSIH, S.R.I.; PANGESTU, M. Ultrastructure and Fertilizing Ability of Limousin Bull Sperm after Storage in Cep-2 Extender with and Without Egg Yolk. **Pakistan Journal of Biological Science**, v. 15, p. 979-985, 2012.

DUKELOW, W.R. The Squirrel Monkey (*Saimiri sciureus*). In: HEARN, J.P. **Reproduction in New World Primates: New Models in Medical Science. Lancaster**: MTP Press Limited, p. 149-180, 1983.

DUMOND, F. V.; HUTCHISON, T. C. Squirrel monkey reproduction: The "fatted" male phenomenon and seasonal spermatogenesis. **Science**, v.58, p.1067–1070, 1967.

DuMOND, F. V. The Squirrel moneky in a seminatural environment. *In*: ROSENBLUM, L. A.; COOPER, R. W. The Squirrel Monkey. **New York/London: Academic Press**, p. 87-145, 1968.

EISENBERG, M. L.; JENSEN, T. K.; WALTERS, R. C.; SKAKKEBAEK, N. E.; LIPSHULTZ, L. I. The relationship between anogenital distance and reproductive hormone levels in adult men. **Journal of Urology**, v.187 (2), p.594–598, 2012a.

EISENBERG, M. L.; SHY, M.; WALTERS, R. C, LIPSHULTZ, L. I. The relationship between anogenital distance and azoospermia in adult men. **International Journal of Andrology**, v.35 (5), p.726–730, 2012b.

FORTMAN, J. D.; HEWETT, T. A.; BENNETT, B. T. The laboratory nonhuman primate. *In*: FOX, J.; COHEN, B.; LOEW F. (Eds.). **Library of congress cataloging-in-publication data**. Washington, D. C.: CRC Press, 2002.

FRAPSAUCE, C.; PIONNEAU, C.; BOULEY, J.; DELAROUZIERE, V.; BERTHAUT, I.; RAVEL, C.; ANTOINE, J. M.; SOUBRIER, F.; MANDELBAUM, J. Proteomic identification of target proteins in normal but nonfertilizing sperm. **Fertility and Sterility**, v.102 (2), p. 372-380, 2014.

FRASER, L.; WYSOCKI, P.; CIERESZKO, A.; PŁUCIENNICZAK, G.; KOTŁOWSKA, M.; KORDAN, W.; WOJTCZAK, M.; DIETRICH, G.; STRZEŻEK, J. Application of biochemical markers for identification of biological properties of animal semen. **Reproductive Biology**, v.6, p.5-20, 2006.

GHARAHDAGHI, F.; WEINBERG, C. R.; MEAGHER, D. A.; IMAI, B. S.; MISCHE, S. M. Mass spectrometric identification of proteins from silver-stained polyacrylamide gel: A method for the removal of silver ions to enhance sensitivity. **Electrophoresis**, v. 20, p.601-605, 1999.

GILBOA, Y.; PERLMAN. S.; KIVILEVITCH, Z.; MESSING, B.; ACHIRON, R. Prenatal anogenital distance is shorter in fetuses with hypospadias. **Journal of Ultrasound in Medicine**, v.36, p.175–182, 2017.

GONZÁLEZ-CADAVID, V.; MARTINS, J. A. M.; MORENO, F. B. M.; ANDRADE, T. S.; SANTOS, A. C. L.; MONTEIRO-MOREIRA, A. C. O.; MOREIRA, R. A. M.; MOURA, A. M. Seminal plasma proteins of adult boars and correlations with sperm parameters. **Theriogenology**, v. 82, p.697–707, 2014.

GOODALL, J.; MITTERMEIER, R. The pictorial guide to the living primates. **Hong Kong: Pogonias Press**, p. 263, 1999.

GORG, A.; WEISS, W.; DUNN, M. J. Current two-dimensional electrophoresis technology for proteomics. **Proteomics**, v.4, p. 3665–3685, 2004.

GOULD, K. G. Scanning electron microscopy of the primate sperm. **International Review of Cytology**, v. 63, p. 323-354, 1980.

GRABNER, A. P. Análise comparativa dos aspectos da ultraestrutura do espermatozoide de mico-leão-de-cara-dourada (*Leontopithecus chrysomelas*) e bugio (*Alouatta caraya* e *Alouatta guariba guariba*). Dissertação (Mestrado) – Universidade de São Paulo. Faculdade de Medicina Veterinária e Zootecnia. Departamento de Reprodução Animal, 2016.

GRANADOS, M. D. G.; HERNÁNDEZ-LÓPEZ, L. E.; AGUILAR, A. C.; MOLINA, A. L. C.; PÉREZ-RAMÍREZ, O.; MONDRAGÓN-CEBALLOS, R. Effect of photoperiod on characteristics of semen obtained by electroejaculation in stump-tailed macaques (*Macaca arctoides*). **Primates**, v.55, p.393–401, 2014.

GROVES, C. The taxonomy of primates in the laboratory context. In: WOLF-COOLE, HACHEY, D.L.; CHAURAND, P. Proteomics in reproductive medicine: the technology for separation and identification of protein. **Journal of Reproductive Immunology**, v.63, p. 61–73, 2004.

HARRISON, R. M.; DUKELOW, W. R. Seasonal adaptation of laboratory maintained squirrel monkeys (*Saimiri sciureus*). **Journal of Medical Primatology**, v.2, p.277-283, 1973.

HE, A. Y.; WANG, B. K.; ZHAO, A. X.; ZHANG, A. Y.; MA, C. Y.; HU, J. Differential proteome association study of freeze-thaw damage in ram sperm. **Cryobiology**, p.1-9, 2015.

HERSHKOVITZ, P. Taxonomy of Squirrel Monkeys genus Saimiri (Cebidae, Platyrrhini): A Preliminary Report With Description of a Hitherto Unnamed Form. **American Journal of Primatology**, v.7, p.155-210, 1984.

HILL, W. C. O. Genus *Saimiri* Voigt, 1831. *In*: **Primates comparative anatomy and taxonomy, Cebidae, part A**. Edinburgh: R & R Clark, Ltda, v.4, p. 251-319, 1960.

HOSSEINIFAR, H.; GOURABI, H.; SALEKDEH, G. H.; ALIKHANI, M.; MIRSHAHVALADI, S.; SABBAGHIAN, M.; MODARRESI, T.; GILANI, M. A. Study of sperm protein profile in men with and without varicocele using two-dimensional gel electrophoresis. **Urology**, v.81, p. 293–300, 2013.

INGBERMANN, B.; STONE, A. I.; CHEIDA, C. C. Gênero *Saimiri* (VOIGT, 1831). *In*: REVIS, N. R.; PEROECH, A. L; ANDRADE, F. R. (orgs). Primatas do Brasil. **Londrina: Technical Book Editora**, p. 41-46, 2008.

JAIN, V. G.; SINGAL, A. K. Shorter anogenital distance correlates with undescended testis: A detailed genital anthropometric analysis in human newborns. **Human Reproduction**, v. 28, p.2343–2349, 2013.

JOHNSTON, D. S.; WOOTERS, J.; KOPF, G. S.; QIU, Y.; ROBERTS, K. P. Analysis of the human sperm proteome. **Annals of the New York Academy of Sciences**, v.1061, p.190-202, 2005.

JUNQUEIRA, L. C. U.; CARNEIRO, J. **Biologia celular e molecular**. 7.ed. Rio de Janeiro: Guanabara Koogan, 338p, 2000.

KAWASE, O.; CAO, S.; XUAN, X. Sperm membrane proteome in wild Japanese macaque (*Macaca fuscata*) and Sika deer (*Cervus nippon*). **Theriogenology**, v.83, p. 95–102, 2015.

KUEHL, T. J.; DUKELOW, W. R. Maturation and in vitro fertilization of follicular oocytes of the squirrel monkey (*Saimiri sciureus*). **Biology of Reproduction**, v.21, p.545-56, 1979.

LEÃO, D .L.; MIRANDA, A. S.; BRITO, A. B.; LIMA, J. S.; SANTOS, R. R,; DOMINGUES, S. F. S. Efficacious long-term cooling and freezing of *Sapajus apella* semen in ACP-118®. **Animal Reproduction Science**, v.159, 118–123, 2015.

LÉGARÉ, C.; DROIT, A.; FOURNIER, F.; BOURASSA, S.; FORCE, A.; CLOUTIER, F.; TREMBLAY, R; SULLIVAN, R. Investigation of male infertility using quantitative comparative proteomics. **Journal of Proteome Research**, v.13 (12), p. 5403–5414, 2014.

LIMA, J. S.; LEÃO, D. L.; OLIVEIRA, K. G.; BRITO, A. B.; SAMPAIO, W. V.; SANTOS, R. R.; QUEIROZ, H. L.; DOMINGUES, S. F. Seminal coagulation and sperm quality in different social contexts in captive tufted capuchin monkeys (*Sapajus apella*). **American Journal Primatology**, v.79, p. 1-8, 2017.

LINDBURG, D. G. Seasonality of reproduction in primates. **Comparative Primate Biology**,v. 2B, p. 167–218, 1987.

LÓPEZ ARMENGOL, M. F.; JURADO, S. B.; PELUFO, V.; AISEN, E. G. A quantitative ultramorphological approach for systematic assessment of sperm head regions: an example in rams. **Cryobiology**, v. 64, p.223–234, 2012.

LÓPEZ-ESPÍN, J. J.; PÉREZ-PALAZÓN, C.; MALDONADO-CÁRCELES, A. B.; ROMÁN-ARIAS, J. D.; MENDIOLA, J.; TORRES-CANTERO, A. M. Anogenital distance and variability in semen parameters. **Systems Biology in Reproductive Medicine**, v.64 (1), p. 1–9, 2017.

LUNDWALL, A.; OLSSON, A. Y. M. Semenogelin II gene is replaced by a truncated line1 repeat in the cotton-top tamarin. **Biology of Reproduction**, v. 65, p. 420–425, 2001.

MACLEOD, D. J.; SHARPE, R. M.; WELSH, M.; FISKEN, M.; SCOTT, H.M.; HUTCHISON, G. R.; DRAKE, A. J.; VAN DEN DRIESCHE, S. Androgen action in the masculinization programming window and development of male reproductive organs. **International Journal of Andrology**, v.33 (2), p. 279–287, 2010.

MARTIN, D. E.; GOULD, K. G. Comparative morphology of primate spermatozoa using scanning electron microscopy. i. Families Hominidae, Pongidae, Cercopithecidae and Cebidae. **Journal of Human Evolution**, v.4, p.287-292, 1975.

MCCORMACK, A. L.; SCHIELTZ, D. M.; GOODE, B.; YANG, S.; BARNES, G.; DRUBIN, D.; YATES, J. R. Direct analysis and identification of proteins in mixtures by LC/MS/MS and database searching at the low-femtomole level. **Analytical Chemistry**, v. 67, p.767-776, 1997.

MCLUCKEY, S. A. Principles of collisional activation in analytical mass spectrometry. **Journal of the American Society for Mass Spectrometry**, v.3, p.599–614, 1992.

MCNUTT, T. L.; JOHNSON, L. A. Electrophoretic gel analysis of hoechst 33342 stained and flow cytometrically sorted bovine sperm membrane proteins. **Reproduction in Domestic Animals**, v.31, p.703-709, 1996.

MENDIOLA, J.; MELGAREJO, M.; MOÑINO-GARCÍA, M.; CUTILLAS-TOLÍN, A.; NOGUERA-VELASCO, J. A.; TORRES-CANTERO, A. M. Is anogenital distance associated with semen quality in male partners of subfertile couples?. **Andrology**, v. 3 (4), p. 672–676, 2015.

MENDIOLA, J.; STAHLHUT, R. W.; JØRGENSEN, N.; LIU, F.; SWAN, S. H. Shorter anogenital distance predicts poorer semen quality in young men in rochester, New York. **Environmental Health Perspectives**, v.119 (7), p.958–963, 2011.

MENDOZA, S. P. Squirrel monkey *In*. POOLE, T. (Eds.) **The ufaw handbook on the care and management of laboratory animals**. 7th Ed. Terrestrial vertebrades. Oxford Black. Sci., v. 1, p. 591-600, 1999.

MENDOZA, S. P.; LOWE, E. L.; RESKO, J. A.; LEVINE, S. Seasonal variations in gonadal hormones and social behavior in squirrel monkeys. **Physiology and Behavior**, v.20, p. 515-522, 1978.

MERCÊS, M. P.; ALFARO, J. W. L.; FERREIRA, W. A.; HARADA, M. L.; JÚNIOR, J. S. S. Morphology and mitochondrial phylogenetics reveal that the Amazon River separates two eastern squirrel monkey species: *Saimiri sciureus* and *S. collinsi*. **Molecular Phylogenetics and Evolution**, v. 82, p. 426-435, 2015.

MITULOVIC, G.; MECHTLER, K. HPLC techniques for proteomics analysis–a short overview of latest developments. **Briefings in Functional Genomics & Proteomics**, v.5, p.249-260, 2006.

MOREAU, R.; FRANK, P. G.; PERREAULT, C.; MARCEL, Y. L.; MANJUNATH, P. Seminal plasma choline phospholipid-binding proteins stimulate cellular cholesterol and phospholipid efflux**. Biochimica et Biophysica Acta**, v. 1438, p.38–46, 1999.

MORETTI, E.; COLLODEL, G. Electron microscopy in the study of human sperm pathologies. **Translocations**, v. 22, p. 343-352, 2012.

MURATORI, M.; LUCONI, M.; MARCHIANI, S.; FORTI, G.; BALDI, E. Molecular markers of human sperm functions. **International Journal of Andrology**. 2009; 32: 25-45.

NAGLE, C. A.; DENARI, J.H. The Cebus Monkey (*Cebus apella*). *In*: HEARN, John P. **Reproduction of New World Primates**. Lancaster: MTP Press, 1983, p. 39-67.

NARESH, S.; ATREJA, S. K. The protein tyrosine phosphorylation during *in vitro* capacitation and cryopreservation of mammalian spermatozoa. **Cryobiology**, v. 70, p.211–216, 2015.

NIXON, B.; MITCHELL, L. A.; ANDERSON, A. L.; MCLAUGHLIN, E. A.; O'BRYAN, M. K.; AITKEN, R. J. Proteomic and functional analysis of human sperm detergent resistant membranes. **Journal of Cellular Physiology**, v. 226, p.2651–2665, 2011.

OKADA, A.; IGARASHI, H.; KURODA, M.; TERAO, K.; YOSHIKAWA, Y.; SANKAI, T. Cryopreservation-induced acrosomal vesiculation in live spermatozoa from cynomolgus monkeys (*Macaca fascicularis*). **Human Reproduction**, v. 16, p.2139-2147, 2001.

OLIVEIR, K. G.; LEÃO, D. L.; ALMEIDA, D. V. C.; SANTOS, R. R.; DOMINGUES, S. F. S. Seminal characteristics and cryopreservation of sperm from the squirrel monkey, *Saimiri collinsi*. **Theriogenology**, v.84, p.743-749, 2015.

OLIVEIRA, K. G.; SANTOS, R. R.; LEÃO, D. L.; BRITO, A. B.; LIMA, J. S.; SAMPAIO, W. V.; DOMINGUES, S. F. S. Cooling and freezing of sperm from captive, free-living and endangered squirrel monkey species. **Cryobiology**, v. 72, p.283-9, 2016b.

OLIVEIRA, K. G.; MIRANDA, S. A.; LEÃO, D. L.; BRITO, A. B.; SANTOS, R. R.; DOMINGUES, S. F. Semen coagulum liquefaction, sperm activation and cryopreservation of capuchin monkey (Cebus apella) semen in coconut water solution (CWS) and TES–TRIS. **Animal Reproduction Science**, v.123, p.75–80, 2011.

OLIVEIRA, K. G.; SANTOS, R. R..; LEÃO, D. L.; QUEIROZ, H. L.; PAIM, F. P.; VIANEZ-JÚNIOR, J. L. S. G.; DOMINGUES, S. F. S. Similarities in testicular and seminal aspects in four squirrel monkeys' species. **Theriogenology**, v.86, p. 879–87, 2016a.

OLIVEIRA, L. Z.; LIMA, V. F. H.; LEVENHAGEN, M. A.; SANTOS, R. M.; ASSUMPÇÃO, T. I.; JACOMINI, J. O.; ANDRADE, A. F. C.; ARRUDA, R. P.; BELETTI, M. E. Transmission electron microscopy for characterization of acrosomal damage after Percoll gradient centrifugation of cryopreserved bovine spermatozoa**. Journal of Veterinary Science**, v.12, p.267-272, 2001.

PARRA, M. D.; MENDIOLA, J.; JØRGENSEN, N.; SWAN, S. H.; TORRES-CANTERO, A. M. Anogenital distance and reproductive parameters in young men. **Andrologia**, v. 48, p.3–10, 2016.

PAIM, F.P.; SILVA, J.S.J.; VALSECCHI, J.; HARADA, M.L.; QUEIROZ, H.L. Diversity, geographic distribution and conservation of squirrel monkeys, Saimiri (Primates, Cebidae), in the floodplain forests of Central Amazon. **International Journal of Primatology**, v.34, p.1055–1076, 2013.

PARIZ, J. R.; CHEMES, H. E.; MONTEIRO, R. A. C.; CAMPOS PIERI, P. C.; JORGE HALLAK, J. Aplicação da avaliação ultraestrutural de espermatozoides na rotina da andrologia. **Reprodução & Climatério**, v.29, p.37-40, 2014.

PARK, Y. J.; KWON, W. S.; OH, S. A.; PANG, M. G. Fertility-related proteomic profiling bull spermatozoa separated by percoll. **Journal of Proteome Research**, v.11, p.4162-4168, 2012.

PESCH, S.; BOSTEDT, H.; FAILING, K.; BERGMANN, M. Advanced fertility diagnosis in stallion semen using transmission electron microscopy. **Animal Reproduction Science**, v. 91, p.285–298, 2006.

PILATZ, A.; LOCHNIT, G.; KARNATI, S.; PARADOWSKA-DOGAN, A.; LANG, T.; SCHULTHEISS, D.; SCHUPPE, H. C.; HOSSAIN, H.; BAUMGART-VOGT, E.; WEIDNER, W.; WAGENLEHNER, F. Acute epididymitis induces alterations in sperm protein composition. **Fertility and Sterilit**, v. 101, p.1609–1617, 2014.

PILCH, B.; MANN, M. Large-scale and high-confidence proteomic analysis of human seminal plasma. **Genome Biology**, v.7, p. 1-10, 2006.

PLATT, M. D.; SALICIONI, A. M.; HUNT, D. F.; VISCONTI, P. E. Use of differential isotopic labeling and mass spectrometry to analyze capacitation-associated changes in the phosphorylation status of mouse sperm proteins. **Journal of Proteome Research**, v.8 (3), p.1431-40, 2009.

REGOA, J. P. A.; CRISP, J. M.; MOURA, A. A.; NOUWENS, A. S.; LI, Y.; VENUSE, B.; CORBETF, N. J.; CORBET, D. H.; BURNS, B. M.; BOE-HANSEN, G. B.;

MCGOWAN, M. R. Seminal plasma proteome of electroejaculated *Bos indicus bulls*. **Animal Reproduction Science,** v.148, p.1–17, 2014.

RICHTE, C. B.; LEHNER, N. D. M.; HENDRICKSON, R. V. Primates. *In*: FOX, J; Coehen, B.; Loew, F. (Eds.) Laboratory Animal Medicine. San Diego: Academic Press, 1984.

RODRIGUES, M. A. M.; SOUZA, C. E. A.; MARTINS, J. A. M.; REGOA, J. P. A.; OLIVEIRA, J. T. A.; DOMONTC, G.; NOGUEIRA, F. C. S.; MOURA, A. A. Seminal plasma proteins and their relationship with sperm motility in Santa Ines rams. **Small Ruminant Research**, v.109, p.94– 100, 2013.

RUTLLANT, J.; POMMER, A. C.; MEYERS, S. A. Osmotic tolerance limits and properties of Rhesus monkey (*Macaca mulatta*) spermatozoa. **Journal of Andrology**, v. 24, p. 534-541, 2003.

RYLANDS, A. B.; MITTERMEIER, A. R.; SILVA-JÚNIOR., J. S. Neotropical primates: taxonomy and recently described species and subspecies. International Zoo Yearbook. The Zoological Society of London, v. 46, p. 1-14, 2013.

SAMPAIO, W. V. **Morfometria e morfologia espermática de *Saimiri collinsi***. Trabalho de conclusão de curso apresentado a Faculdade de Medicina Veterinária. UENF, Campos dos Goytacazes-RJ, 2014.

SAMPAIO, W. V.; OLIVEIRA, K. G.; LEÃO, D. L.; CALDAS-BUSSIERE, M. C.; QUEIROZ, H. L.; PAIM, F. P.; DOMINGUES, S. F. S. Morphologic analysis of sperm from two neotropical primate species: Comparisons between the squirrel monkeys *Saimiri collinsi* and *Saimiri vanzolinii*. **Zygote**, v. 16, p.1-8, 2017a.

SAMPAIO, W. V.; LEAO, D. L.; SOUSA, P. C.; DOMINGUES, S. F. S. Contribuições da biotecnologia da reprodução sobre os desafios na manipulação do sêmen de primatas do gênero Saimiri. **Revista Brasileira de Reprodução Animal**, v. 41, p. 222-230, 2017b.

SANTOS, E. A. A.; SOUSA, P. C.; MARTINS, J. A. M.; MOREIRA, R. A.; MONTEIRO-MOREIRA, A. C. O.; MORENO, F. B. M. B.; OLIVEIRA, M. F.; MOURA, A. A.; SILVA, A. R. Protein profile of the seminal plasma of collared peccaries (Pecari tajacu Linnaeus, 1758). **Reproduction**, v. 147, p.753–764, 2014.

SCHILLER, J.; ARNHOLD J.;, GLANDER, H.J.; ARNOLD, K. Lipid analysis of human spermatozoa and seminal plasma by MALDI-TOF mass spectrometry and NMR spectroscopy — effects of freezing and thawing. **Chemistry and Physics of Lipids**, v.106, p.145–156, 2000.

SCHWITZER, C.; MITTERMEIER, R. A.; RYLANDS, A. B.; CHIOZZA, F.; WILLIAMSON, E. A.; MACFIE, E. J.; WALLIS, J.; COTTON, A. (eds.). 2017. Primates in Peril: The World's 25 Most Endangered Primates 2016–2018. IUCN SSC Primate Specialist Group (PSG), International Primatological Society (IPS), Conservatio International (CI), and Bristol Zoological Society, Arlington, VA. 99 pp.

SCOTT, H. M.; HUTCHISON, G. R.; JOBLING, M. S.; MCKINNELL, C.; DRAKE, A. J.; SHARPE, R. M. Relationship between androgen action in the 'male programming window,' fetal sertoli cell number, and adult testis size in the rat. **Endocrinology**, v.149, p.5280–5287, 2008.

SECCIANI, F.; BIANCHI, L.; ERMINI, L.; CIANTI, R,; ARMINI, A.; LA SALA, G. B.; FOCARELLI, R.; BINI, L.; ROSATI, F. Protein profile of capacitated versus ejaculated human sperm. **Journal of Proteome Research**, v.8, p.3377-89, 2009.

SHABANOWITZ, R. B.; KILLIAN, G .J. Two-dimensional electrophoresis of proteins in principal cells, spermatozoa, and fluid associated with the rat epididymis. **Biology of Reproduction**, v.36, p.753-768, 1987.

SHANDILYA, L.; CLARKSON, T. B.; ADAMS, M. R.; LEWIS, J. C. Effects of gossypol on reproductive and endocrine functions of male cynomolgus monkeys (*Macaca fascicularis*). **Biology of Reproduction**, v.27, p.241-52, 1982.

SHEVCHENKO, A.; WILM, M.; VORM, O.; MANN, M. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. **Analytical Chemistry**, v. 68, 850-858, 1996.

SILVA, A. R.; FONTENELE-NETO, J. D.; CARDOSO, R. C. S.; SILVA, L. D. M.; CHIRINÉA, V. H.; LOPES, M. D. Description of ultrastructural damages in frozen-thawed canine spermatozoa. **Ciência Animal Brasileira**, v. 10, p. 595-601, 2009.

SIVA, A. B.; KAMESHWARI, D. B.; SINGH, V.; PAVANI, K.; SUNDARAM, C. S.; RANGARAJ, N.; DEENADAYAL, M.; SHIVAJI, S. Proetomics-based study on astenozoospermia: differential expression of proteasome alpha complex. **Molecular Human Reproduction**, v.16, p.452-462, 2010.

SIVASHANMUGAM, P.; RAJALAKSHMI, M. Sperm maturation in rhesus monkey: Changes in ultrastructure, chromatin condensation, and organization of lipid bilayer. **The anatomical record**, v. 247, p. 25–32, 1997.

SKERGET, S.; ROSENOW, M.; POLPITIYA, A.; PETRITIS, K.; DORUS, S.; KARR, T. L. The rhesus macaque (*Macaca mulatta*) sperm proteome**. Molecular & Cellular Proteomics**, v.12, p.52-67, 2013.

SOUSA, P. C.; SANTOS, E. A. A.; BEZERRA, J. A. B.; LIMA, G. L.; CASTELO, T. S.; FONTENELE-NETO, J. D.; SILVA, A. R. Morphology, morphometry and ultrastructure of captive six-banded armadillo (*Euphractus sexcinctus*) sperm. **Animal Reproduction Science**, v.140, n.3-4, p. 279-285, 2013.

SOUZA, C. E. A.; ARAÚJO, A. A.; OLIVEIRA, J. T. A.; LIMA-SOUZA, A. C. B.; NEIVA, J. N. M.; MOURA, A. A. Reproductive development of Santa Inês rams during the first year of life: body and testis growth, testosterone concentrations, sperm parameters, age at puberty and seminal plasma proteins. **Reproduction in Domestic Animals**, v.45, p.644–53, 2010.

SOUZA, C. E. A.; REGO, J. P.; LOBO, C. H.; OLIVEIRA, J. T.; NOGUEIRA, F. C.; DOMONT, G. B.; FIORAMONTE, M.; GOZZO, F. C.; MORENO, F. B.; MONTEIRO-MOREIRA, A. C.; FIGUEIREDO, J. R.; MOURA, A. A. Proteomic analysis of the reproductive tract fluids from tropically-adapted Santa Ines rams. **Journal of Proteomics**, v.75, p.4436–4456, 2012.

STEINBERG, E. R.; NIEVES, M.; ASCUNCE, M. S.; PALERMO, A. M.; MUDRY, M. D. Morphological and genetic characterization of *Saimiri boliviensis*. **International Journal of Primatology**, v.30, p. 29–41, 2009.

STONE, A. I. Responses of squirrel monkeys to seasonal changes in food availability in an Eastern Amazonian rainforest. **American Journal of Primatology**, v.69, p.142–157, 2007.

STONE, A. **Juvenile feeding ecology and life history in a neotropical primate, the squirrel monkey (*Saimiri sciureus*)**. Ph.D. thesis, University of Illinois at Urbana-Champaign, 2004.

STONE, A.I. 2014. Is fatter sexier? Reproductive strategies of male squirrel monkeys (*Saimiri sciureus*). **International Journal of Primatology**, v.35, p.1-15, 2014.

STONE, A. I.; CASTRO, P. H. G.; MONTEIRO, F. O. B.; RUIVO, L. P.; SILVA JÚNIOR, J. S. A novel method for capturing and monitoring a small neotropical primate, the squirrel monkey (*Saimiri collinsi*). **American Journal of Primatology**, v.77, p.239–245, 2015.

STRZEZEK, J.; WYSOCKI, P.; KORDAN, W.; KUKLINSKA, M.; MOGIELNICKA, M.; SOLIWODA, D.; FRASER, L. Proteomics of boar seminal plasma – current studies possibility of their application in biotechnology of animal reproduction. **Reproductive Biology,** v.5, p.279-290, 2005.

SWAN, S. H.; MAIN, K. M.; LIU, F.; STEWART. S. L.; KRUSE, R. L.; CALAFAT, A. M.; MAO, C. S.; REDMON, J. B.; TERNAND, C. L.; SULLIVAN, S.; TEAGUE, J. L. Decrease in anogenital distance among male infants with prenatal phthalate exposure. **Environmental Health Perspectives**, v.113, p.1056–1061, 2005.

SWEGEN, A.; CURRY, B. J.; GIBB, Z.; LAMBOURNE, S. R.; SMITH, N. D.; AITKEN, R. J. Investigation of the stallion sperm proteome by mass spectrometry. **Reproduction,** v.149, p.235-244, 2015.

TARNASKY, H.; CHENG, M.; OU, Y.; THUNDATHIL, J. C.; OKO, R.; VAN DER HOORN, F. A. Gene trap mutation of murine outer dense fiber protein-2 gene can result in sperm tail abnormalities in mice with high percentage chimaerism. **BMC Developmental Biology**, v.10, p.67-76, 2010.

TAUB, O. M.; ADAMS, M. R.; AUERBACH, K. G. Reproductive performance in a breeding colony of Brazilian squirrel monkeys (*Saimiri sciureus*). **Laboratory Animal Science**, v. 28, n.5, p. 562-566, 1978.

THANKAMONY, A.; PASTERSKI, V.; ONG, K .K.; ACERINI, C. L.; HUGHES, I. A.; Anogenital distance as a marker of androgen exposure in humans. **Andrology**, v. 4 (4), p. 616–625, 2016.

THEPPARAT, T.; KATAWATIN, S.; VONGPRALUB, T.; DUANGJINDA, M.; THAMMASIRIRAK, S.; UTH, A. Separation of bovine spermatozoa proteins using 2D-PAGE revealed the relationship between tektin-4 expression patterns and spermatozoa motility. **Theriogenology**, v.77, p.1816–1821, 2012.

VALLE, R. R.; GUIMARÃES, M. A. B. V.; MUNIZ, J. A. P. C.; BARNABE, R. C.; VALE, W. G. Collection and evaluation of semen from captive howler monkeys (*Alouatta caraya*). **Theriogenology**, v.62, 131–138, 2004.

VALTONEN-ANDRÉ, C.; OLSSON, A. Y.; NAYUDU, P. L.; LUNDWALL, K. ejaculates from the common marmoset (*Callithrix jacchus*) contain semenogelin and beta-microseminoprotein but not prostate-specific antigen. **Molecular Reproduction and Development**, v. 71, p.247–255, 2005.

VAN DEN DRIESCHE, S.; SCOTT, H. M.; MACLEOD, D. J.; FISKEN, M.; WALKER, M.; SHARPE, R. M. Relative importance of prenatal and postnatal androgen action in determining growth of the penis and anogenital distance in the rat before, during and after puberty. **International Journal of Andrology**, v.34, p. e578-586, 2011.

VAN TILBURG, M. F.; RODRIGUES, M. A .M.; MOREIRA, R. A.; MORENO, F. B.; MONTEIRO-MOREIRA, A. C. O.; CÂNDIDO, M. J. D.; MOURA, A. A. Membrane-associated proteins of ejaculated sperm from morada nova rams. **Theriogenology**, v.79, p.1247–1261, 2013.

VARRICCHIO, E.; LANGELLA, M.; MAHARAJAN, V.; PAINO, G. Struture and function of the mammalian epididymis. **Acta os Medicine Veterinaty**, v. 42, p. 221-234, 1996.

VIANA, C.F. **Características do sêmen, perfil da concentração de testosterona no extrato fecal, variação da massa corporal e volume testicular de micos-de-cheiro (Saimiri sciureus, Linnaeus, 1758) mantidos em cativeiro sob condições ambientais controladas**. Dissertação de mestrado apresentada ao programa de Pós Graduação em Ciência Animal, UENF, Campos dos Goytacazes-RJ, 2012.

WASHBURN, M. P., WOLTERS, D., YATES, J. R. Large-scale analysis of the yeast proteome by multidimensional protein identification technology. **Nature Biotechnology**, v.19, p. 242–247, 2001.

WELSH, M., SAUNDERS, P. T. K., FISKEN, M., SCOTT, H. M.; HUTCHISON, G. R.; SMITH, L. B.; SHARPE, R.M. Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. **Journal of Clinical Investigation**, v.118 (4), p.1479–1490, 2008.

WILLIMS, L. Aging Cebidae. *In*: ATSALIS, S.; S.W. MAGULIS, S. W. **Primate Reproductive Aging: cross-taxon perspectives**. Switzerland: Ed Karger v. 36, p.49-61, 2008.

WOLF, C.; LAMBRIGHT, C.; MANN, P.; PRICE, M.; COOPER, R. L.; OSTBY, J.; GRAY, L. E. Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p,p′-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differen. **Toxicology and Industrial Health**, v. 15 (1-2), p. 94–118, 1999.

WU, C.; TRAN, J.C.; ZAMDBORG, L.; DURBIN, K.R.; LI, M.; AHLF, D.R.; EARLY, B.P.; THOMAS, P.M.; SWEEDLER, J.V.; KELLEHER, N.L. A protease for 'middle-down' proteomics. **Nature Methods**, v.9, p. 822–824, 2012.

YEOMAN, R. R.; RICKER, R. B.; WILLIAMS, L. E.; SONKSEN, J.; ABEE, C. R. Vibratory stimultion of ejaculatory yields increased motile spermatozoa, compared with electroejaculation, in squirrel monkeys (*Saimiri boliviensis*). **Contemporary Topics in Laboratory Animal Science**, v. 36, n. 1, p. 62-64, 1997.

YOSHIDA, M.; KAWANO, N.; YOSHIDA K. Control of sperm motility and fertility: diverse factors and common mechanisms. **Cellular and Molecular Life Sciences**, v. 65, p.3446-57, 2008.

YUE, W.;, SHI, L.;, BAI, Z.; REN, Y.; ZHAO, Y. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of ram seminal plasma proteins and their correlation with semen characteristics. **Animal Reproduction Science**, v. 116, p.386–391, 2009.

ZANEVELD, L. J. D.; TAUBER, P. F.; PORT, C.; PROPPING, D.; SCHUMACHER, G. F. B. Scanning electron microscopy of the human, guinea-pig and rhesus monkey seminal coagulum. **Journal of Reproduction and Fertility**. v.40, p.223-225, 1974.

ZHANG, Z.; FONSLOW, B. R.; SHAN, B.; BAEK, M. C.; YATES, J. R. Protein analysis by Shotgun/Bottom-up proteomics. **Chemical Reviews**, v.113, p. 2343–2394, 2013.

ZERBINATI, C.; CAPONECCHIA, L.; PUCA, R.; CIACCIARELLI, M.; SALACONE, P.; SEBASTIANELLI, A.; PASTORE, A.; PALLESCHI, G.; PETROZZA, V.; PORTA, N.; RAGO, R.; CARBONE, A.; IULIANO, L. Mass spectrometry profiling of oxysterols in human sperm identifies 25- hydroxycholesterol as a marker of sperm function. **Redox Biology**, v.11, p.111–117, 2017.

ZHOU, T.; WANG, G.; CHEN, M.; ZHANG, M.; GUO, Y.; YU, C.; ZHOU, Z.; SI, W.; SHAL, J.; GUO, X. Comparative analysis of macaque and human sperm proteomes: Insights into sperm competition. **Proteomics**, v.15, p. 1564–1573, 2015.

WOLF, R. H.; HARRISON, R.M.; MARTIN, T.W. A review of reproductive patterns of New World monkeys. **Laboratory animal Science**, v. 25, p. 814-821, 1997

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The use of anogenital distance as a noninvasive predictor of seminal quality in captive squirrel monkey (*Saimiri collinsi* Osgood, 1961)

Anogenital distance in squirrel monkey

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ABSTRACT

Background: Anogenital distance is considered a noninvasive measure to assess the development and functionality of sexual organs in different animal species. Hence, this measurement could potentially be used during the selection of non-human primates for reproductive biotechnology programs. The aim of this study was to assess the correlation between anogenital distance and reproductive parameters in captive *Saimiri collinsi*.

Methods: Eight mature *S. collinsi* males were evaluated. Body weight, reproductive hormone levels, testicular volume and seminal parameters were determined and their relationship with anogenital distance measurements was assessed.

Results: Anogenital distance was correlated with seminal volume, sperm motility, vigour, and plasma membrane integrity, but not with body weight, reproductive hormones, and testicular volume.

Conclusion: The determination of anogenital distance is a noninvasive method to predict seminal quality. This procedure has the advantage of providing andrologic information without a negative impact on animal welfare.

Keywords: anogenital distance, spermatozoa, neotropical primates, testes.

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1 INTRODUCTION

Anogenital distance (AGD) is an measure and has been used as a noninvasive method to evaluate the development and functionality of male sex organs in rats [1] and humans[1,2,3,4,5]. Deficient androgen exposure during a critical period of testis development defined as "masculinisation programming window - MPW" (8–14 weeks of gestation in humans) was associated with outcomes such as reduced penile length, testicular dysgenesis syndrome, and the reduction in AGD[6,7,8,9,10,11].

In adult human studies, AGD is measured in two different ways: in man, from the posterior base of the scrotum to the center of the anus (AGD_{AS}) and from the cephalad insertion of the penis to the center of the anus (AGD_{AP})[9,3,12] . In women, the measurement is made from the anterior clitoral surface to the center of the anus (AGD_{AC}) and from the posterior fourchette to the center of the anus (AGD_{AF})[13]. Since increased androgen concentrations lead to increased AGD in utero, males have AGDs almost twice as long those in females, and this is considered a sexually dimorphic characteristic in humans[14], rodents[10,15] and rhesus monkeys [16]. In adult rats, it has been reported that AGD may be mediated by modulation of local androgen/estrogen action.[17] However, only in men, studies have shown that a short AGD predicts small testes size and poor seminal quality.[9,8,18,3] In an Old World Primate, the Rwenzori Angolan colobus monkeys (*Colobus angolensis ruwenzorii*), the AGD_{AS} measurement was considered a noninvasive method to assess male competitive ability in the wild because it correlated with male dominance rank, but no information was given on seminal quality[19].

Saimiri collinsi, popularly known as squirrel monkey, is a Neotropical primate endemic to the Amazon in Brazil[20], and was proposed as an experimental model for reproductive biotechnology for the conservation of free-living species in the genus *Saimiri*.[21,22] Although, in squirrel monkeys there is documented a positive correlation between

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testicular volume and the liquid fraction of the ejaculate[23], nothing is known about relationship between testosterone- and estradiol levels, testicular volume and sperm quality and the AGD. Given the biological importance of this potential biomarker, our overall hypothesis is that *S. collinsi* testicular function is related to anogenital length, and could predict seminal quality in nonhuman primates. In this way, the AGD measurement could be used an auxiliary noninvasive parameter in andrologic examination for the selection of non-human primates in biotechnology programs for the conservation of endangered species, as well as studies on reproductive physiology. Thus, the objective of this study was to evaluate the correlation between AGD values and hormone levels (testosterone and estradiol) with body weight, testicular biometry, seminal volume and sperm parameters in *S. collinsi* kept in captivity.

2 MATERIALS AND METHODS

2.1 Ethical Committee and place of execution

This study was approved by the Ethical Committee in Animal Research (no. 02/2015/CEPAN/IEC/SVS/MS) and by the System of Authorization and Information in Biodiversity (SISBIO/ICMBio/MMA no.47051-2). All procedures were performed under the supervision of a veterinarian. Semen collections were carried out during 12 months at the National Primate Center (CENP, Ananindeua, Pará, Brazil).

2.2 Animals

Saimiri collinsi males maintained in captivity (indoors) at the CENP (1°22'58"S and 48°22'51"W), were used for collecting semen. We selected (N=8) sexually mature and healthy males. The animals were housed in mixed groups (males and females varied numbers) in collective cages of 4.74 m x1.45 m x 2.26 m (length, width and height, respectively), under a natural photoperiod (i.e. 12 h of light and 12 h of dark). The climate is humid and tropical,

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with an average annual temperature of 28 ºC. The diet consisted of fresh fruits, vegetables, commercial pellet chow specific for neotropical non-human primates (P18, 18% protein, 6.5% fibe, Megazoo®, Minas Gerais, Brazil) and cricket larvae (*Zophobas morio*). Vitamins, minerals and eggs were supplied once a week and water was available *ad libitum*.

For biometric measurements, blood and semen collection, the animals were first physically restrained by a trained animal caretaker wearing leather gloves. After this, the animals were anesthetized with ketamine hydrochloride (20 mg/kg; IM; Vetanarcol, König S.A., Avellaneda, Argentina) and xylazine hydrochloride (1 mg/kg; IM; Kensol, König S.A.), and monitored by a veterinarian. All data (biometric measurements, blood and semen collection) were collected once a month for 12 months.

2.3 Genital measurements

After total anesthetic effect, the animals were weighed using a digital weighing balance (Toledo Prix 3 Plus 30 kg Digital Balance, Toledo do Brasil, São Bernardo do Campo, SP, Brazil) and the testicular biometry (length, width, height and circumference) was measured using a universal caliper. The testicular volume was calculated by the ellipsoid formula (length x width x height x 0.524) according to Oliveira el al.[21]

Two variants of AGD were assessed as described for humans[9,4] using a universal caliper: the first was measured from the cephalad insertion of the penis to the center of the anus (AGD_{AP}), and the second was measured from the posterior base (first fold) of the scrotum to the center of the anus (AGD_{AS}) (Figure 1).

2.4 Hormonal assays

For the blood collection (1 mL), the femoral vein was punctured with hypodermic needles (20 mm × 0.55 mm; 24 G × ¾") coupled to a 3 mL syringe, and blood was transferred to tubes containing the anticoagulating EDTA. Samples were centrifuged (3000 rpm for 5 minutes) (Modelo Combate, Celm, Brazil) and the obtained serum was frozen (-80 °C) for

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hormonal dosage. The plasm levels of Testosterone (ng/mL) and 7β-estradiol (pg/mL) were measured by chemiluminescence assay using an immunoassay system (VITROS® ECiQ, Ortho Clinical Diagnostics, New Jersey, United States of America). All the protocols were applied according to the manufacturer.

2.5 Semen collection and analyses

The genital region was sanitized with a mild soap and distilled water (1:10) and gauze. The prepuce was retracted with the thumb and index finger for a more efficient cleaning of the penis with saline solution. Animals were stimulated with rectal electro-ejaculation (EEJ) procedure described in Oliveira et al. [21,22] In brief, an EEJ (Autojac-Neovet, Uber-aba, Brazil) rectal probe was smeared with a sterile lubricant jelly (KY™ Jelly, Johnson & Johnson Co., Arlington, TX, USA), introduced in the rectum (~2.5 cm deep) and electrical stimuli were delivered. The stimulation session consisted of three series (7–8 min), composed of 35 electrical stimuli (12.5 – 100 mA) within an interval of 30s between series. For each animal, the intervals between semen collections were 30 days. If a male did not ejaculate after the session, no further attempts were made to collect semen.

Ejaculates were collected into microtubes (1.5 mL) and placed in a water bath at 37 °C immediately after ejaculation. Seminal volume (liquid fraction) was evaluated in a graduated tube. Appearances were assessed subjectively, i.e., color (colorless, yellowish, or whitish) and opacity (opaque or transparent). Seminal pH was measured with a pH strip (Merk Pharmaceuticals, Darmstadt, Germany). Sperm concentration was determined in a Neubauer chamber, 1 µL semen was diluted in 99 µL formalin solution 10%. All evaluations were performed under a light microscope (Nikon E400, Japan) at a magnification of 100×. Sperm morphology was evaluated by a smear prepared by adding 5 µL of eosin-nigrosin stain (Vetec, Rio de Janeiro, Brazil) to 5 µL of semen on a pre-warmed (37 °C) glass slide.

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Morphologic defects detected in sperm were classified as major or minor. Major defects are those that affect fertility, and minor defects are of less importance.[22]

2.6 Statistical analyses

All data are expressed as the mean ± standard error (SE) and analyzed by the StatView 5.0 program (SAS Institute Inc., Cary, NC, USA). Data were checked for normality using the Kolmogorov-Smirnov test. Correlation coefficients (Spearman correlation) were used to determine the relationship in *S. collinsi* between AGD measurements (AGD_{AP} and AGD_{AS}) and the hormone values (testosterone and estradiol) along with total testicular volume, seminal volume, sperm concentration, motility, vigor, normal sperm, major and minor sperm defects. $P \leq 0.05$ was considered statistically significant.

3 RESULTS

The mean (± SE) values in adult male *S. collinsi* of body weight was 860 ± 882 (694-1,152 g min-max), AGD_{AP} was 3.3 ± 0.06 (2.5-4.1 cm min-max), AGD_{AS} was 2.3±0.04 (1.7-2.9 cm min-max), testosterone level was 19.2 ± 2.6 (467-50,900 ng/dl; min-max) and estradiol level was 40.6 ± 6.0 (80.7-98.9 pg/ml; min-max) (Table 1).

Seminal liquid fractions were colorless, whitish or yellowish, transparent or opaque and the pH (Mean ± SE) of the ejaculates was 7.5 ± 0.06. The mean value of seminal parameters are summarized in Table 1.

Correlation analyses for body weight, reproductive hormones (testosterone and estradiol), testicular biometry and semen parameters with AGD measurements are depicted in Table 2. There was no significant correlation between body weight, testosterone and estradiol levels with $AGD_{AP\ and\ AS}$. On the other hand, the AGD_{AP} was positively and significantly correlated with the sperm parameters of motility ($\rho = 0.56$; $Z= 2.57$; $P= 0.01$) and vigour ($\rho = 0.59$; $Z= 2.72$; $P= 0.006$). In addition, it was possible to verify the positive and significant correlation

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between the AGD_{AS} with seminal volume (ρ = 0.35; Z= 2.06; P = 0.03) and sperm plasma membrane integrity (ρ = 0.46; Z= 2.09; P = 0.03) (Table 2).

The hormonal analysis showed a negative and significant correlation between testosterone levels and the weight of the animals (ρ= -0.35 Z= -2.32 P= 0.02), however there was no significant correlation between the estradiol levels and the weight, testicular biometry and seminal parameters analyzed (Table 3).

4 DISCUSSION

In the present study, we examined two variants of AGD (AGD_{AS} and AGD_{AP}) and it was possible to show that AGD measurements might be an important noninvasive parameter to select *Saimiri* monkeys, with good fertility to participate in assisted reproduction programs.

Human male AGD can be measured in a few different ways[23], because the measurements depend on different landmarks on the soft tissue. It was reported that in men, the AGD_{AS} is effortless to measure due to the easy identification of soft tissue boundaries.[24] Additionally in adult men, AGD_{AS} correlates most strongly with fertility parameters.[9] In Rwenzori Angolan colobus, only AGD_{AS} was measured and found to be a predictor of male competitive ability.[19] However, there is no gold standard method for measuring AGD in adult squirrel monkeys, so we cannot establish whether one measure is better than the other, thus we verified the relationship of both measures with reproductive parameters. In *S. collinsi*, both measurements of AGD had a positive and significant correlation with seminal parameters. Thus, it is worth analyzing if the same occurs in other species of non-human primates.

Our results showed that AGD measurements were not correlated with body weight, reproductive hormones (testosterone and estradiol), nor testicular volume in *S. collinsi*. In men, AGD measurements are also not correlated with body weight[25], but significant positive

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correlations between body mass index and AGD_{AP} and $_{AS}$ was previously shown[5]. Body mass is influenced by the accumulation of fat in the anterior region of the pubic symphysis, an area that is included in the AGD_{AP} measurement, but not at AGD_{AS}.[9] AGD_{AS} is considered to be the most reliable and repeatable measurement, in men, because this variant is unaffected by obesity and age. [26,27,28] Male *Saimiri* sp. gain weight seasonally about three months before, as well as during the copulation season. This increase is a result of the deposition of fat and water especially in the thorax, arms, and shoulders[29,30], thus not influencing the measures $AGD_{AP\ and\ AS}$.

During sexual development, the immature genital precursors migrate ventrally via an androgen mediated pathway, and endocrine disruptors during the MPW (to 8–14 weeks of gestation in humans) and can permanently alter genital development, growth and function.[31] On the other hand, it was shown in rats that, when the endocrine action occurs in the postnatal period there is less damage in the genital development.[14,32,33] Although studies with rats and humans have shown the relationship between small AGD size with low serum testosterone level and testicular size[9,1,34], we did not find correlation between AGD and testicular volume or reproductive hormones (testosterone and estradiol serum level) in *S. collinsi*. Jain et al.[35] observed, in humans, a correlation of AGD with testosterone levels, however, with increasing gestational age (22 to 44 weeks), in utero, this correlation was lost. Therefore, the authors suggest that AGD is influenced by testosterone early in gestation during the MPW, but with advancing gestational age, AGD becomes independent of testosterone levels. Thus, the increase in AGD over time is related to the growth of the body and not to testosterone levels. Liu et al.[36] measured cord blood testosterone and estradiol using radioimmunoassay in human term neonates and found no correlation with AGD. However, in adults, associations of AGD with testosterone levels have been inconclusive, since studies have shown an association[7], while others have not[4].

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Previous studies have shown that AGD is related to fertility and sperm production in men.[9,37] The current report establishes that AGD measurements might also help determine genital development and function in squirrel monkeys. We showed a positive and significant correlation between AGD_{AP} with motility and vigour, and AGD_{AS} with seminal volume and sperm plasma membrane integrity in *S. collinsi,* thus showing that the increase in AGD measurements are indicative of better seminal quality. In humans, Mendiola et al.[9] evaluated men's sperm parameters in relation to AGD_{AP} and AGD_{AS}, and found a significant association only for AGD_{AS} with sperm parameters (sperm concentration, motility, morphology, total sperm count and total motile count). Rodent studies indicate that seminal parameters may be associated with AGD measurements, since seminal volume may reflect seminal vesicle and prostate function, and the motility may reflect germ cell number and testicular function.[14,32]

Parra et al.[4] and Zhou et al.[5] reported in men that both AGD_{AS} and AGD_{AP} were not associated with any semen parameters. The authors reported the possible reason for these conflicting results may be due to the fact that most of these studies in men were conducted on patients attending in andrology practice for infertility assessment.[8,37,3] Thus, infertile men may have infertility reasons that can cause testis dysfunction and changes in sperm parameters, so therefore, the AGD may also have changed as a consequence of infertility.[5] In the present study, all seminal parameters analyzed are in accordance with the values described for healthy *S. collinsi*[21], as expected since animals were selected without a history of reproductive problems.

Despite the variable results in humans, it is worth mentioning that the men included these in studies were all submitted to a period of sexual abstinence prior to semen collection in order to obtain optimal sperm quality.[7,3,37] Nevertheless, in our experiment, for the sake of best animal welfare in captivity, all monkeys were housed in mixed groups of males and females. Therefore, it was not possible to isolate the animals and prevent copulation or male

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masturbatory activity, which is part of the natural behavioral repertoire in nonhuman primate species.[38,39] These factors (copulation and masturbation) directly influence seminal quality and may explain our negative but not significant correlation results found between AGD_{AS} and sperm concentration, motility, vigour and major sperm defects.

Sperm quality has a direct effect on the fertilization and developmental competence of embryos in mammals[40], due to the paternal factors contributing to embryo quality. In humans, lower cleavage rates and blastocyst formation rates have been noted when morphologically abnormal sperm were used to fertilize oocytes in in vitro embryo production.[41]

Squirrel monkeys (*Samiri* sp.) are Neotropical primates that show strict reproductive seasonality.[42] In addition to weight gain as previously mentioned during the breeding season (fatting phenomenon), an increase of testosterone hormone levels during the breeding season in *Saimiri* sp. has been described [29,43] Chen et al.[44] evaluated seven adult Bolivian squirrel monkeys (*Sairniri sciureus*) kept in captivity for 13 months, and showed there were seasonal changes in body weight, plasma testosterone and seminal volume in these animals. However, the authors report that seasonal changes in seminal volume appear independent of seasonal testosterone changes. In captive *S. collinsi*, we showed a decrease in testosterone levels with the increase in the animals weight, and no correlations between testosterone and estradiol levels and testicular biometry and seminal parameters were found. Nevertheless, it is still necessary to determine the reproductive and fattening period in captivity of these animals, as well as the influence of a social hierarchy in this period and the relationship with the levels of reproductive hormones, testicular biometry and seminal quality.

In conclusion, our results indicate that AGD size predicts seminal quality in *S. collinsi*, and the measurement of AGD may provide a new metric for andrologic examination in assisted reproduction programs in nonhuman primates. Furthermore, this noninvasive and

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rapid approach can be considered as a refinement to support the field control of free-living monkeys that are sometimes exposed to environmental contaminants or other stress sources that may affect their reproductive function.

CONFLICT OF INTEREST STATEMENT

None of the authors have any conflict of interest to declare.

REFERENCE

1. Dean A, Sharpe RM. Anogenital distance or digit length ratio as measures of fetal androgen exposure: relationship to male reproductive development and its disorders. J *Clin Endocrinol Metab*. 2013;98(6):2230–2238.

2. Eisenberg ML, Hsieh, MH, Walters RC, Krasnow R, Lipshultz LI. The relationship between anogenital distance, fatherhood, and fertility in adult men. *PLoS One*. 2011;6 (5):e18973.

3. Mendiola J, Melgarejo M, Moñino-García M, Cutillas-Tolín A, Noguera-Velasco JA, Torres-Cantero AM. Is anogenital distance associated with semen quality in male partners of subfertile couples? *Andrology*. 2015;3(4):672–676.

4. Parra MD, Mendiola J, Jørgensen N, Swan SH, Torres-Cantero AM. Anogenital distance and reproductive parameters in young men. *Andrologia*. 2016;48(1):3–10.

5. Zhou N, Sun L, Yang H, Chen Q, Wang X, Yang H, Tan L, Chen H, Zhang G, Ling X, Huang L, Zou P, Peng K, Liu T, Liu J, Ao L, Zhou Z, Cui Z, Cao J. Anogenital distance is associated with serum reproductive hormones, but not with semen quality in young men. *Hum Reprod*. 2016;31(5):958–967.

6. Abbott AD, Colman RJ, Tiefenthaler R, Dumesic DA, Abbott DH. Early-to-mid gestation fetal testosterone increases right hand 2D:4D finger length ratio in polycystic ovary syndrome-like monkeys. *PLoS One*. 2012;7(8):e42372.

1 Este artigo foi submetido na Journal of Medical Primatology, seguindo suas normas de apresentação.

7. Eisenberg ML, Jensen TK, Walters RC, Skakkebaek NE, Lipshultz LI. The relationship between anogenital distance and reproductive hormone levels in adult men. *J Urol*. 2012a;187(2):594–598.

8. Eisenberg ML, Shy M, Chanc Walters R, Lipshultz LI. The relationship between anogenital distance and azoospermia in adult men. *Int J Androl*. 2012b;35(5):726–730.

9. Mendiola J, Stahlhut RW, Jørgensen N, Liu F, Swan SH. Shorter anogenital distance predicts poorer semen quality in young men in rochester, New York. *Environ Health Perspect*. 2011;119(7):958–963.

10. Welsh M, Saunders PTK, Fisken M, Scott HM, Hutchison GR, Smith LB, Sharpe RM. Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. *J Clin Invest*. 2008;118(4):1479–1490.

11. Welsh M, Saunders PTK, Fisken M, Scott FM, Hutchison GR, Smith LB, Sharpe RM. Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism. *J Clin Invest*. 2008;118(4):1479-1490.

12. Mendiola J, Oñate-Celdrán J, Samper-Mateo P, Arense-Gonzalo JJ, Torres-Roca M, Sánchez-Rodríguez C, García-Escudero D, Fontana-Compiano LO, Eisenberg ML, S Swan SH, Torres-Cantero AM. Comparability and reproducibility of adult male anogenital distance measurements for two different methods. *Andrology*. 2016;4(4):626-631.

13. Mendiola J, Roca M, Mínguez-Alarcón L, Mira-Escolano MP, López-Espín JJ, Barrett E, Swan SH, Torres-Cantero AM. Anogenital distance is related to ovarian follicular number in young Spanish women: a cross-sectional study. *Environ Health*. 2012:8;11(90):1-8.

14. Thankamony A, Pasterski V, Ong KK, Acerini CL, Hughes IA. Anogenital distance as a marker of androgen exposure in humans. *Andrology*. 2016;4(4):616–625.

15. MacLeod DJ, Sharpe RM, Welsh M, Fisken M, Scott HM, Hutchison GR, Drake AJ, Van Den Driesche S. Androgen action in the masculinization programming window and development of male reproductive organs. *Int J Androl*. 2010;33(2):279–287.

1 Este artigo foi submetido na Journal of Medical Primatology, seguindo suas normas de apresentação.

16. Goy RW, Robinson JA. Prenatal exposure of rhesus monkeys to patent androgens: Morphological, behavioral, and physiological consequences. *Banbury Report*. 1982;11:355–378.

17. Mitchell RT, Mungall W, McKinnell C, Sharpe RM, Cruickshanks L, Milne L, Smith LB. Anogenital distance plasticity in adulthood: implications for its use as a biomarker of fetal androgen action. *Endocrinology*. 2015;156(1):24–31.

18. López-Espín JJ, Pérez-Palazón C, Maldonado-Cárceles AB, Román-Arias JD, Mendiola J, Torres-Cantero AM. Anogenital distance and variability in semen parameters. *Syst Biol Reprod Med*. 2018;64(1):1–9.

19. Teichroeb JA, Stead SM, Edwards PD, Landry F, Palme R, Boonstra R. Anogenital distance as a measure of male competitive ability in Rwenzori Angolan colobus. *Am J Primatol*. 2020;82(3):e23111.

20. Mercês MP, Lynch-Alfaro JW, Ferreira WAS, Harada ML, Silva-Júnior JS. Morphology and mitochondrial phylogenetics reveal that the Amazon River separates two eastern squirrel monkey species: *Saimiri sciureus* and *S. collinsi*. *Mol Phylogenet Evol*. 2015;82 (Part B):426–435.

21. Oliveira KG, Leão DL, Almeida DVC, Santos RR, Domingues SFS. Seminal characteristics and cryopreservation of sperm from the squirrel monkey, *Saimiri collinsi*. *Theriogenology*. 2015;84(5):743–749.

22. Oliveira KG, Santos RR, Leão DL, Queiroz HL, Paim FP, Vianez-Júnior JLSG, Domingues SFS. Testicular biometry and semen characteristics in captive and wild squirrel monkey species (*Saimiri* sp.). *Theriogenology*. 2016;86(3):879–887.

23. Hsieh MH, Breyer BN, Eisenberg ML, Baskin LS. Associations among hypospadias, cryptorchidism, anogenital distance, and endocrine disruption. *Curr Urol Rep*. 2008;9(2):137-142.

24. Papadopoulou E, Vafeiadi M, Agramunt S, Basagana X, Mathianaki K, Karakosta P, Spanaki A, Koutis A, Chatzi L, Vrijheid M, Kogevinas M. Anogenital distances in newborns and children from Spain and Greece: predictors, tracking and reliability. *Paediatr Perinat Epidemiol*. 2013;27:89–99.

1 Este artigo foi submetido na Journal of Medical Primatology, seguindo suas normas de apresentação.

25. Foresta C, Valente U, Di Nisio A, Cacco N, Magagna S, Cosci I, Presciutti A, Garolla A. Anogenital distance is associated with genital measures and seminal parameters but not anthropometrics in a large cohort of young adult men. *Hum Reprod*. 2018;1:33(9):1628-1635.

26. Swan SH, Main KM, Liu F, Stewart SL, Kruse RL, Calafat AM, Mao CS, Redmon JB, Ternand CL, Sullivan S, Teague JL. Decrease in anogenital distance among male infants with prenatal phthalate exposure. *Environ Health Perspect*. 2005;113(8):1056-1027.

27. Thankamony A, Ong KK, Dunger DB, Acerini CL, Hughes IA. Anogenital distance from birth to 2 years: a population study. *Environ Health Perspect*. 2009;117:1786–1790.

28. Sathyanarayana S, Beard L, Zhou C, Grady R. Measurement and correlates of ano-genital distance in healthy, newborn infants. Int J Androl. 2010: 33:317–323.

29. Mendoza SP, Lowe EL, Davidson JM, Levine S. Annual cyclicity in the squirrel monkey (*Saimiri sciureus*): The relationship between testosterone, fatting and sexual behavior. *Horm. Behav*. 1978;20:295–303.

30. Stone AI. Is Fatter Sexier? Reproductive strategies of male squirrel monkeys (*Saimiri sciureus*). *Int J Primatol*. 2014;35:628–642.

31. Liu C, Xu X, Huo X. Anogenital distance and its application in environmental health research. *Environ Sci Pollut Res*. 2014;:21:5457–5464.

32. Welsh M, MacLeod DJ, Walker M, Smith LB, Sharpe RM. Critical androgen-sensitive periods of rat penis and clitoris development. *Int J Androl*. 2010;33:e144–52.

33. Mitchell RT, Mungall W, McKinnell C, Sharpe RM, Cruickshanks L, Milne L, Smith LB. Anogenital distance plasticity in adulthood: implications for its use as a biomarker of fetal androgen action. *Endocrinology*. 2015;156(1):24-31.

34. Foster EPMD. Mylchreest KW, Sar GM. Effects of phthalate esters on the developing reproductive tract of male rats. *Hum. Reprod. Update*. 2001;7(3):231-235.

35. Jain VG, Goyal VG, Chowdhary VC, Swarup N, Singh RJ, Singal A, Shekhawat P. Anogenital distance is determined during early gestation in humans. *Hum Reprod*. 2018;33(9):1619-1627.

1 Este artigo foi submetido na Journal of Medical Primatology, seguindo suas normas de apresentação.

36. Liu C, Xu X, Zhang Y, Li W, Huo X. Associations between maternal phenolic exposure and cord sex hormones in male newborns. *Hum Reprod*. 2016;31(3):648-656.

37. Eisenberg ML, Lipshultz LI. Anogenital distance as a measure of human male fertility. *J Assist Reprod Genet*. 2015;32(3):479–484.

38. Dubuc C, Coyne SP, Maestripieri D. effect of mating activity and dominance rank on male masturbation among free-ranging male rhesus macaques. *Ethology*. 2013;119 (11):1-13.

39. Thomsen R, Sommer V. Masturbation in non-human primates, in: The International Encyclopedia of Human Sexuality. First Edition. *Eds Whelehan P. & Bolin A*. Boston: Wiley-Blackwell Press;2015:1-2.

40. Lasiene K, Gedrimas V, Vitkus A, Glinskyte S, Lasys V, Valanciute A, Sienkiewicz W. Evaluation of morphological criteria of sperm quality before in vitro fertilization and intracytoplasmic sperm injection. *Pol J Vet Sci*. 2013;16(4):773-785.

41. Colaco S, Sakkas D. Paternal factors contributing to embryo quality. *J Assist Reprod Genet*. 2018;35(11):1953-1968.

42. Stone AI. Responses of squirrel monkeys to seasonal changes in food availability in an eastern Amazonian forest. *Am J Primatol*. 2007;69:142–157.

43. Wiebe, RH, Williams LE, Abee CR, Yeoman RR, Diamond EJ. Seasonal changes in serum dehydroepiandrosterone, androstenedione, and testosterone levelsin the squirrel monkey (*S. bolivienses bolivienses*). *Am J Primatol*. 1988;14:285-291.

44. Chen JJ, Smith ER, Gray GD, Davidson JM. Seasonal changes in plasma testosterone and ejaculatorycapacity in squirrel monkeys (*Saimiri sciureus*). *Primates*. 1981;22(2): 253-260.

1 Este artigo foi submetido na Journal of Medical Primatology, seguindo suas normas de apresentação.

FIGURE 1 Representative image showing the measurement of the anogenital distances: (A) AGD_{AP} - from the cephalad insertion of the penis to the center of the anus and (B) AGD_{AS} - from the posterior base of the scrotum to the center of the anus.

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TABLE 1 Mean ± standard error (SE) of body weight, hormone levels (testosterone and estradiol), anogenital distance (AGD_{AP} and AGD_{AS}) and seminal parameters in adult squirrel monkeys (*Saimiri collinsi*; N=8).

Variable	Mean ± SE	Minimum-Maximum
Body weight (g)	860 ± 882	694-1,152
Reproductive Hormones		
Testosterone (ng/dl)	19,2 ± 2,6	467-50,900
Estradiol (pg/ml)	40,6 ± 6,0	80,7-98,9
Anogenital distance and tescticular biometry		
AGD_{AP} (cm)	3.3 ± 0.06	2.5-4.1
AGDAS (cm)	2.3 ± 0.04	1.7-2.9
Testicular volume (cm^3)	2.3 ± 0.1	0.69-5.2
Semen parameters		
Seminal Volume (µL)	179 ± 18.9	10-600
Sperm concentration ($x10^{6/}$mL)	127± 164	25-45
Motility (%)	60.6 ± 4.0	0-100
Vigour (1-5)	3.0 ± 0.2	0-5
Plasm membrane integrity (%)	60.4 ± 3.5	0-100
Normal sperm (%)	72.1 ± 2.3	37-96
Major sperm defects (%)	11.5 ± 1.8	0-46
Minor sperm defects (%)	5.2 ± 1.0	0-40

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TABLE 2 Correlation of anogenital distance (AGD_{AP} and AGD_{AS}) with hormone levels, body weight and seminal parameters in adult squirrel monkeys (*Saimiri collinsi*; N=8).

Parameters	AGD_{AP}	AGD_{AS}
Testosterone	ρ = -0.07 Z= -0.15 P = 0.88	ρ = -0.07 Z= -0.15 P = 0.88
Estradiol	ρ = -0.57 Z= -1.15 P = 0.25	ρ = -0,57 Z= -1.15 P = 0.25
Body weight	ρ = 0.17 Z= 1.12 P = 0.26	ρ = -0.27 Z= -1.78 P = 0.07
Testicular volume	ρ = 0.21 Z= 1.38 P = 0.16	ρ = 0.10 Z= 0.64 P = 0.51
Seminal Volume	ρ = 0.11 Z= 0.67 P = 0.50	ρ = 0.35 Z= 2.06 P = 0.03
Sperm concentration	ρ = 0.12 Z= 0.57 P = 0.56	ρ = -0.29 Z= -1.3 P = 0.18
Motility	ρ = 0.56 Z= 2.57 P = 0.01	ρ = -0.07 Z= -0.33 P = 0.73
Vigour	ρ = 0.6 Z= 2.72 P = 0.006	ρ = -0.06 Z= -0.27 P = 0.78
Plasm membrane integrity	ρ = 0.22 Z= 1.01 P = 0.30	ρ = 0.46 Z= 2.09 P = 0.03
Normal sperm	ρ = 0.32 Z= 1.51 P = 0.13	ρ = 0.4 Z= 1.87 P = 0.06
Major sperm defects	ρ = -0.01 Z= -0.06 P = 0.95	ρ = -0.2 Z= -1.07 P = 0.28
Minor sperm defects	ρ = 0.07 Z= 0.41 P = 0.67	ρ = 0.06 Z= 0.34 P = 0.73

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TABLE 3 Correlation of testosterone and estradiol levels with body weight and seminal parameters in adult squirrel monkeys (*Saimiri collinsi*; N=8).

Parameters	Testosterone	Estradiol
Body weight	ρ = -0,35 Z= -2,32 P = 0,02	ρ = -0,05 Z= -0,36 P = 0,71
Testicular volume	ρ = -0,05 Z= -0,37 P = 0,70	ρ = -0,20 Z= -1,27 P = 0,20
Seminal Volume	ρ = -0,07 Z= -0,49 P = 0,62	ρ = -0,05 Z= -0,33 P = 0,73
Sperm concentration	ρ = -0,15 Z= -0,96 P = 0,33	ρ = 0,07 Z= 0,46 P = 0,63
Motility	ρ = -0,02 Z= -0,15 P = 0,87	ρ = 0,16 Z= 0,877 P = 0,38
Vigour	ρ = -0,02 Z= -0,12 P = 0,89	ρ = 0,14 Z= 0,80 P = 0,42
Plasm membrane integrity	ρ = -0,05 Z= -0,27 P = 0,78	ρ = -0,142 Z= -0,77 P = 0,43
Normal sperm	ρ = 0,11 Z= 0,71 P = 0,47	ρ = 0,04 Z= 0,26 P = 0,79
Major sperm defects	ρ = 0,05 Z= 0,32 P = 0,74	ρ = 0,04 Z= 0,24 P = 0,80
Minor sperm defects	ρ = 0,12 Z= 0,6 P = 0,50	ρ = -0,13 Z= -0,75 P = 0,44

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Micromorphological and ultrastructural description of squirrel monkeys (*Saimiri collinsi* Osgood, 1916) sperm

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Abstract

Saimiri collinsi is an animal model in biotechnology research for conservation of species from the genus Saimiri. However, the development of biotechnologies depends on a proper knowledge of the sperm morphology to understand the basic aspects of the sperm physiology, since the potential male fertility depends of different cellular sperm's structures. Whit this purpose, this study characterized the micro morphological and ultrastructural characteristics of squirrel monkeys (*Saimiri collinsi*) sperm using scanning- and transmission eléctron microscopy (SEM and TEM, respectively). The SEM electromyography revealed that a normal Saimiri collinsi sperm measures 71.7 ± 0.7 μm with lateral tail insertion, a paddle-shaped flattened head and an acrosome occupied most of the head. The TEM also showed that the middle piece is characterized by a central 9 + 2 microtubule axoneme surrounded by nine dense fibres, and the mitochondria were juxtaposed forming the mitochondrial sheath. In conclusion, we provide the first micro morphological and ultrastructure description of *S. collinsi* sperm.

Keywords: Sperm, electron microscopy, scanning, transmission, neotropical primates

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Introduction

The squirrel monkey *Saimiri collinsi*, a neotropical primate endemic from Amazon-Brazil, is an animal model for conservational and biotechnology studies for species from the genus *Saimiri* (Oliveira et al., 2015; 2016ab). Among them, *S. oerstedii* and *S. vanzollini* are listed as vulnerable, while the *S. ustus* is almost threatened to extinction (IUCN, 2019).

Despite the relevant studies conducted to investigate the male reproductive physiology in this non-human primates using classical semen analysis (Oliveira et al., 2015; 2016ab; Sampaio et al., 2017; Almeida et al., 2018), knowledge on the morphology of sperm from *S. collinsi* is still limited. The seminal analysis or spermiogram is an important tool to evaluate and determine the sperm's morphological aspects that are directly related to the potential male fertility (Visco et al., 2010) and is crucial for the processes of artificial insemination, in vitro fertilization, and embryo development (Ozkavukcu et al., 2008).

At a routine basis, sperm morphology from neotropical primates is examined in semen smears with the main criteria for normality relying on morphological parameters of the sperm head, middle-piece and flagellum (Arakaki et al., 2017, 2018; Sampaio et al., 2017; Swanson et al., 2016; Oliveira et al., 2015, 2016ab; Leão et al., 2015). In *S. collinsi*, the sperm morphology was only described using eosin–nigrosine stain by optical microscopy (Oliveira et al., 2015; 2016ab; Sampaio et al., 2017). However, sperm analysis using optical microscopy has low resolution, and does not allow the identification and analysis with more details than the micro-anatomical aspect that can affect the sperm and decrease the sperm capacity of fertilizing the oocyte (Visco et al., 2010).

In this context, the scanning- and transmission electron microscopy are technologies providing a high-range view, in nanometric dimensions, that can aid the morphological description and the detection of possible abnormalities in the sperm (Nussdorfer et al., 2018). These techniques have already been used in domestic animals as horses (Pesch et al., 2006),

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sheep (López-Armengol et al., 2012), and wild animals as six-banded armadillo (Sousa et al., 2013), collared peccaries (Bezerra et al., 2018), jaguar (Silva et al., 2019) and some non-human primates (Bedford & Nicander et al., 1971; Martin et al., 1975; Gould, 1980; Steinberg et al., 2009; Nakazato et al., 2015). Regarding these microscopy technologies in the genus *Saimiri*, only the scanning electron microscopy was used to characterize the sperm morphology in squirrel monkeys (*S. sciureus*) (Martin et al., 1975; Gould, 1980), or obtained morphometric sperm data as a tool for more accurate taxonomic identification of the *S. boliviensis* (Steinberg et al., 2009). In *S. collinsi* nothing is known about the sperm components in higher resolution. Thus, the aim of the present study is to describe the micro morphological and ultrastructural characteristics of squirrel monkeys (*S. collinsi*) sperm using scanning- and transmission electron microscopy.

Material and Methods

Ethical aspects and place of execution

This study was approved by the Ethical Committee in Animal Research (no. 02/2015/CEPAN/IEC/SVS/MS) and by the System of Authorization and Information in Biodiversity (SISBIO/ICMBio/MMA no.47051-2). All procedures were performed under the supervision of a veterinarian. Semen collections were carried out at the National Primate Center (CENP, Ananindeua, Pará, Brazil), the scanning electron microscopy was conducted at the Analytical Center of the Universidade Federal do Ceará (UFC, Fortaleza, Ceará, Brazil), and transmission electron microscopy was conducted at the Laboratory of Electron Microscopy of the Evandro Chagas Institute (IEC, Belém, Pará, Brazil).

Animals and semen collection

Saimiri collinsi males originated from the Marajó Archipelago (0°58'S and 49°34'W) and maintained in captivity at the CENP (1°22'58"S and 48°22'51"W) were used for collecting

semen. The experimental group (n=5 males, ~15 years old) was selected by their physical characteristics, and clinical parameters such as complete hemogram, hepatic and renal function.

Animals were collectively housed in mixed groups (males and females in a varied number of members) in cages of 4.74 m x 1.45 m x 2.26 m (length, width and height, respectively), under natural photoperiod (i.e. 12 h of light and 12 h of dark). The climate is humid and tropical, with an average annual temperature of 28°C. The diet consisted of fresh fruits, vegetables, commercial pellet chow specific for neotropical non-human primates (Megazoo®P18, 18% protein, 6.5% fibre, Brazil) and cricket larvae (Zophobas morio). Vitamins, minerals and eggs were supplied once a week, and water was available ad libitum.

Semen collection

Semen was collected at the same period of the day (in the morning before feeding). Physical restraint was performed by a trained animal caretaker wearing leather gloves. All animals were anesthetized with ketamine hydrochloride (20 mg/kg; IM; Vetanarcol, König S.A., Avellaneda, Argentina) and xylazine hydrochloride 1 mg/kg; IM; Kensol, König S.A.), and monitored by a veterinarian.

Achieved total anaesthetic effect, the animals were placed in lateral recumbency, genital region was then sanitized with a mild soap and distilled water (1:10) and gauze. The prepuce was retracted with the thumb and index fingers for a more efficient cleaning of the penis with saline solution. Animals were stimulated with rectal electroejaculation (EEJ) procedure described by Oliveira et al. (2015). Ejaculates (liquid and coagulated fractions) were collected into microtubes (1.5 mL), then, were placed in a water bath at 37°C. Scanning and transmission electron microscopy were only performed in the liquid fraction of the ejaculate.

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Sperm preparation

The sample (liquid fraction) were centrifuged at 500g/5 min, and the supernatant was discarded. The pellet formed was washed three times in sodium cacodylate and fixed in Karnovsky (4% paraformaldehyde, 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2).

Semen evaluation

Seminal volume (liquid fractions) was evaluated in a graduated tube, with the aid of a pipette. Appearance and consistency were assessed subjectively, i.e., colour (colourless, yellowish, or whitish) and opacity (opaque or transparent) (Oliveira et al., 2015). Sperm motility, vigour, and morphology were evaluated according to Oliveira et al. (2015; 2016ab). Sperm morphology and plasma membrane integrity were evaluated by a smear prepared adding 5 µL eosin 1% (Vetec) and 5 µL nigrosine 1% (Vetec) to 5 µL of semen on a pre-warmed (37°C) glass slide. Sperm concentration was determined in a Neubauer chamber after dilution of 1 µL semen in 99 µL formalin solution 10%. Morphologic defects detected in sperm were classified as primary or secondary according Bloom (1973). Plasma membrane functionality was assessed by hypoosmotic swelling test (HOST) after dilution of 5 µL of semen in 45 µL of hypoosmotic solution (0.73 g sodium citrate, 1.35 g fructose and 100 mL ultrapure water; pH 7.2 and 108 mOsm/L). After 45 min incubation in water bath (37 °C), number of sperm tail coiled was assessed by placing 10µL of this solution in a pre-warmed (37 °C) glass slide with cover slip, and at least 200 spermatozoa were counted. Spermatozoa with functional plasma membrane were those presenting coiled tail. All evaluations were performed under a light microscope (Nikon E400, Japan) at a magnification of 100×.

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Scanning electron microscopy

Sperm samples were placed in a coated coverslip with poly-D-lysine, air-dried it onto a filter and dehydrated in a series of acetone solution (50, 70, 90 and 100% x 3, 5 min each). Afterwards, the samples were dried by the critical point method (EMS 850, Quorum Technologies, Lewes, United Kingdom) and then attached to supports using aluminium stubs with carbon tape. The samples were covered with a thin (20 nm) layer of gold (Q150T ES, Quorum Technologies, Lewes, United Kingdom) and observed under scanning electron microscopy (Quanta 450-FEG, Thermo Fisher Scientific, Massachusetts, EUA). The description of S. collinsi sperm by SEM was conducted according to that previously reported for non-humans primates by Martin et al. (1975).

Transmission electron microscopy

For transmission electron microscopy, the sperm sample were washed in 0.05 M cacodylate buffer (40 min/3x), post-fixed with osmium tetroxide (OsO_4) for 60 min and washed in 0.05 M cacodylate buffer with 0.08% potassium ferrocyanide (40 min/5x). Subsequently, the contracting block was performed with 2.5% uranyl acetate in 50% acetone for 1h. Then, the sample were dehydrated in series of acetone solution (70, 80, 90 and 100%) for 10 min each, the solution of 100% acetone was carried out 3 times. After dehydration, the samples were infiltrated with epoxy resin (EponPolibed), using increasing series of resin in acetone (dilution 1:2; 1:1; 2:1), until 100% Epon + DMP-30 (2,4,6-Trisdimethylaminomethyl) phenol). Polymerization of the resin was performed at 60 °C/ 48h. Semi-thin cuts of approximately 70 nm thickness were obtained in ultramicrotome with glass cutters (Leica EM UC7, Leica Microsystems, Wetzlar, Germany) and contrasted with 5% uranyl acetate for 20 min in 60 °C. The observations of the sperm ultrastructure's were performed in transmission electron microscopy (EM 900, Carl Zeiss do Brasil Ltda, São Paulo, Brazil).

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178 **Results**

179 **Semen evaluation**

180 Mean (±SEM) of seminal volume was 339 ± 61 µL (15–500 µL; min–max). liquid
181 fractions were whitish or yellowish, and opaque. The sperm concentration was 1.718 ± 610 x
182 10^6 sperm/ml. In addition, the samples presented a mean of, 67 ± 11% motility, 3 ± 0.3
183 vigour, 38 ± 13% and 43 ± 8% functional and intact sperm membranes, respectively. The
184 evaluation of sperm morphology through light microscopy was 56 ± 5% normal sperm, 13 ± 4
185 bent tail, 27 ± 4 coiled tail and 3.6 ± 1.2 strongly coiled tail.

186 **Scanning electron microscopy**

187 The scanning electron microscopy (SEM) revealed that the head of *S. collinsi* sperm is
188 paddle-shaped flattened with a uniform thickness throughout; measuring 5.14 ± 0.08 µm in
189 length and 3.36 ± 0.03 µm in width, with an intact and uninterrupted surface, and a acrosome
190 occupied most of the head with length of 3.56 ± 0.06 µm (Fig. 1A). The insertion of the tail is
191 lateral (abaxial; Figure 1A), with a length of middle piece of 9.49 ± 0.38 µm. The squirrel
192 monkeys' sperm presents a total length of 71.7 ± 0.7 µm (Fig. 1B).

193 **Transmission electron microscopy**

194 The transmission electron microscopy (TEM) images showed the *S. collinsi* sperm
195 presents a flattened head in a longitudinal cut, containing a very electron-dense nucleus,
196 which is larger at the base, narrowing as it goes to the tip of the head. There is a diffuse small
197 electronic spot. The structures present in the sperm head are the nucleus, the acrosome, and
198 membranous envelopes (Fig. 3A-B). The large nucleus covered by the acrosome (ac), which
199 occupies one-half to two-thirds of the anterior portion of the head. The acrosome is thickest
200 around the anterior margin of the sperm head, and thinnest from the equator to the posterior
201 third of the nucleus where it terminates (Fig. 3B). The neck area (na), which is the region
202 located between the head and the middle piece, is characterized by the presence of a centriole,

the proximal centriole (oriented transversally to the cell; pc), and segmented columns (Figure 3B). The middle piece is characterized by a central 9 + 2 microtubule axoneme surrounded by nine dense fibres. This set is surrounded by a plasmalemma and a helical mitochondrial sheath (Fig. 3C-D), where was identified the presence of an intact axoneme as well as outer dense fibres (ODFs). The mitochondria were juxtaposed, thus forming the mitochondrial sheath, which was surrounded by the plasmalemma with some undulations (Figure 3E). It is possible to visualize about ~72 mitochondria.

Discussion

Our study represents a valuable basis for the comprehension of sperm physiology through the description of micro morphological and ultrastructural aspects in *S. collinsi*. Over the years, the evaluation of sperm morphology has become much more precise, and the rigorous analysis of these parameters is of pivotal importance in semen analysis, since it permits the detailed study of different sperm abnormalities that predict the fertile capacity of the male gamete (WHO, 2010).

In the genus *Saimiri*, the SEM was performed only in *S. sciureus* (Martin et al., 1975) and *S. boliviensis* (Stembeirg et al., 2009). In *S. collinsi*, the abaxial tail insertion was also observed by SEM, as previously described as a normal morphology characteristic in the morphometric analysis of sperm stained with eosin-nigrosine for *S. collinsi* and *S. vanzollini* (Sampaio et al., 2017). The average total length of the *S. collinsi* sperm was similar described for other squirrel monkeys species in the stained samples (Nakazato et al., 2015; Sampaio et al., 2017). The morphology of sperm head showed a thick, paddle-shaped flattened, which looks similar to other primates, including humans (Martin et al., 1975; Gould & Martin, 1978). The measurements of head morphology using SEM agree with values obtained previously via optical microscopy for *S. collinsi* (Sampaio et al., 2017).

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For the first time, the ultrastructure description of *S. collinsi* sperm by TEM was reported. Thus, the results presented here will serve as a parameter for future morphological studies involving the preparation and evaluation of the semen in nonhuman primates, as well as for studies using morphological data for the diagnosis of sperm alterations, including acquired damages post-thawing, which is a relevant factor for the development of reproduction (reproductive)biotechnology (Sousa et al., 2013). The TEM might be an additional diagnostic tool in the presence of asthenozoospermia or the absence of motility, which is important since the pattern of axoneme structure can be visualized in the longitudinal and cross sections with this technique. In humans, TEM have been used in the diagnosis of sperm alterations with a possible genetic origin, known as systematic defects (Baccetti et al., 2001). The observation of sperm head ultrastructure revealed a large and obvious nucleus. The acrosome is the only cytoplasmic element in the sperm head. Similar as described for human sperm, in *S. collinsi*, the acrosome is a relatively inconspicuous, which covers the anterior two-thirds to half of the head, caplike structure containing enzymes that are essential for fertilization (Bartoov et al., 1980). In Rhesus monkeys sperm, the acrosome is thickest around the anterior margin of the sperm head and thinnest from the equator to the posterior third of the nucleus where it terminates, and the thin portion of the acrosome is usually referred to as the equatorial segment. As was shown in human and Rhesus monkey (Zamboni et al., 1971), the plasma membrane surrounding the anterior portion of the head, is continuous and often undulated in *S. collinsi*.

The sperm head and tail are connected via the sperm neck, a region formed by the centriole and connecting piece (Bornens, 2012). In Rhesus macaque, middle piece sheath consists of 84 to 86 mitochondria (Zamboni et al., 1971). In our study, the exact number of mitochondria in *S. collinsi* could not be measured due to the absence of longitudinal sections showing the middle piece to its full extent. These organelles function to provide energy for

253 the movement of the flagella, since the midpiece accommodates more mitochondria to
254 produce more energy in order to fuel motility (Pesch et al., 2006), and generates greater
255 propulsion forces in the principal piece (Gomendio & Roldan, 1991). However, it was
256 demonstrated that the swimming speed is not dependent on the size of the tail, but on the head
257 size, which in return may retard the propulsion given by the sperm tail (Humphries et al.,
258 2008).

259 Regarding morphological defects in light microscopy, according to Bloom (1973)
260 classification, some secondary defects were identified in the *S. collinsi* sperm, including bent
261 tail, coiled tail and strongly coiled tail. Such defects were also verified in the ejaculates of
262 neotropical primates (Arakaki et al., 2017; 2018; Leão et al., 2015; Oliveira et al., 2015;
263 Swanson et al., 2016). In our study compared to others already performed for the *S. collinsi*
264 species (Oliveira et al., 2015; 2016ab; Sampaio et al., 2017), we observed a high percentage
265 of sperm pathologies, which for Sampaio et al. (2017) is characterized as a group of animals
266 with low seminal quality. On the other hand, it is noteworthy that we only worked with the
267 liquid fraction for sperm characterization, since the dilution process could imply changes in
268 sperm morphology. It is known that the liquid fraction is the fraction of lower seminal volume
269 and sperm concentration (Oliveira et al., 2015), which may also contain sperm with higher
270 percentage of sperm pathologies as compared with coagulated fraction.

271 *Saimiri collinsi* ejaculates present a normal range for sperm morphology similar to that
272 accepted for the genus *Saimiri.* Additionally, they present a general sperm structure, but with
273 peculiarities such as abaxial middle piece insertion showed by SEM.

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278 **Conflict of interest statement**

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

281

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289

290 **References**

291 **Almeida DVC, Lima JS, Leão DL, Oliveira KG, Santos RR, Domingues SFS and**
292 **Miranda MS** (2018) The effects of Trolox on the quality of sperm from captive
293 squirrel monkey during liquefaction in the extender ACP-118™. *Zygote* **26**, 333-35.

294 **Arakaki PR, Carvalho FM, Castro PHG, Muniz JAPC and Valle RDRD** (2017)
295 Collection, evaluation, and coagulum dissolution of semen from Goeldi's Monkey,
296 *Callimico goeldii*. *Folia Primatol* **88**, 334-43.

297 **Arakaki PR, Nichi M, Monteiro FOB, Muniz JAPC, Guimarães MABV and Valle**
298 **RDRD** (2019) Comparison of semen characteristics and sperm cryopreservation in
299 common marmoset (*Callithrix jacchus*) and black-tufted-ear marmoset (*Callithrix*
300 *penicillata*). *J Med Primatol* **48**, 32-42.

301 **Baccetti B, Capitani S, Collodel G, Di Cairano G, Gambera L, Moretti E and Piomboni**
302 **P** (2001) Genetic sperm defects and consanguinity. *Hum Reprod* **16**, 1365–71.

303 **Bartoov B, Eltes F, Weissenberg R and Lunenfeld B** (1980) Morphological
304 characterization of abnormal human spermatozoa using transmission electron
305 microscopy. *Arch Androl* **5**, 305-22.
306 **Bedford JM and Nicander L** (1971) Ultrastructural changes in the acrosome and sperm
307 membranes during maturation of spermatozoa in the testis and epididymis of the rabbit
308 and monkey. *J Anat* **108**, 527–43.
309 **Bezerra LGP, Souza ALP, Silva HVR, Vasconcelos FR, Moura AAA, Pereira AF,**
310 **Oliveira MF and Silva AR** (2018) Ultrastructural description of fresh and
311 frozen/thawed sperm derived from collared peccaries (*Pecari tajacu* Linnaeus, 1,758).
312 *Microscop Res Tech* **81**, 1–9.
313 **Bloom E** (1973) The ultrastructure of some characteristic sperm defects and a proposal for a
314 new classification o the bull spermogram. *Nord Vet* **25**, 83–91.
315 **Bornens M** (2012) The centrosome in cells and organisms. *Science* **335**, 422–26.
316 **Gomendio M and Roldan ER** (1991) Sperm competition influences sperm size in mammals.
317 *Proc Biol Sci* **243**,181-5.
318 **Gould KG and Martin DE** (1978) Comparative morphology of primate spermatozoa using
319 scanning electron microscopy. II. Families Cercopithecidae, Lorisidae, Lemuridae. *J*
320 *Hum Evol* **7**, 637-42.
321 **Gould KG** (1980) Scanning electron microscopy of the primate sperm. *Int Ver Cytol* **63**, 323-
322 55.
323 **Humphries S, Evans JP and Simmons LW** (2008) Sperm competition: linking form to
324 function. *BMC Evol Biol* **8**, 319.
325 **IUCN** (2019) *The IUCN Red List of Threatened Species. Version 2019-2*.
326 http://www.iucnredlist.org. Downloaded on 20 August 2019.

1 Este artigo foi aceito para publicação na Zygote, seguindo suas normas de apresentação.

Leão DL, Miranda AS, Brito AB, Lima JS, Santos RR and Domingues SFS (2015) Efficacious long-term cooling and freezing of Sapajus apella semen in ACP-118®. *Anim Reprod Sci* **159**, 118–23.

López-Armengol MF, Jurado SB, Pelufo V and Aisen EG (2012) A quantitative ultramorphological approach for systematic assessment of sperm head regions: an example in rams. *Cryobiology* **64**, 223–34.

Martin DE, Gould KG and Warner H (1975) Comparative morphology of primate spermatozoa using scanning electron microscopy. I. Families Hominidae, Pongidae, Cercopithecidae and Cebidae. *J Hum Evol* **4**, 287–92.

Nakazato C, Yoshizawa M, Isobe K, Kusakabe KT, Kuraishi T, Hattori S, Matsumoto H, Fukui E, Kuwahata A, Ochi M, Kiso Y and Kai C (2015) Morphological characterization of spermatozoa of the night monkey. *J Mamm Ova Res* **31**, 37–40.

Nussdorfer P, Cilenšek I, Zorn B and Petrovič D (2018) Adapted methods for scanning electron microscopy (SEM) in assessment of human sperm morphology. *Bosn J Basic Med Sci* **18**, 43-8.

Oliveira KG, Leão DL, Almeida DVC, Santos RR and Domingues SFS (2015) Seminal characteristics and cryopreservation of sperm from the squirrel monkey, Saimiri collinsi. *Theriogenology* **84**, 743–9.

Oliveira KG, Santos RR, Leão DL, Brito AB, Lima JS, Sampaio WV and Domingues SFS (2016a) Cooling and freezing of sperm from captive, free-living and endangered squirrel monkey species. *Cryobiology* **72**, 283–9.

Oliveira KG, Santos RR, Leão DL, Queiroz HL, Paim FP, Vianez-Júnior JLSG and Domingues SFS (2016b) Testicular biometry and semen characteristics in captive and wild squirrel monkey species (*Saimiri* sp.). *Theriogenology* **86**, 879–87.

1 Este artigo foi aceito para publicação na Zygote, seguindo suas normas de apresentação.

Ozkavukcu S, Erdemli E, Ayca Isik A, Oztuna D and Karahuseyinoglu S (2008) Effects of cryopreservation on sperm parameters and ultrastructural morphology of human Spermatozoa. *J Assist Reprod Genet* **25**, 403–11.

Pesch S, Bostedt H, Failing K and Bergmannc M (2006) Advanced fertility diagnosis in stallion sêmen using transmission electron microscopy. *Anim Reprod Sci* **91**, 285–98.

Sampaio WV, Oliveira KG, Leão DL, Caldas-Bussiere MC, Queiroz HL, Paim HP, Santos RR and Domingues SFS (2017) Morphologic analysis of sperm from two neotropical primate species: comparisons between the squirrel monkeys Saimiri collinsi and *Saimiri vanzolinii. Zygote* **25**, 1-8.

Silva HVR, Nunes TGP, Ribeiro LR, Freitas LA, Oliveira MF, Assis Neto AC, Silva AR and Silva LDM (2019) Morphology, morphometry, ultrastructure, and mitochondrial activity of jaguar (Panthera onca) sperm. *Anim Reprod Sci* **203**, 84-93.

Sousa PC, Santos EAA, Bezerra JAB, Lima GL, Castelo TS, Fontenele-Neto JD, Silva AR (2013) Morphology, morphometry and ultrastructure of captive six-banded armadillo (Euphractus sexcinctus) sperm. *Anim Reprod Sci* **140**, 279– 85.

Steinberg ER, Nieves M, Ascunce MS, Palermo AM and Mudry MD (2009) Morphological and genetic characterization of *Saimiri boliviensis. Int J Primatol* **30**, 29–41.

Swanson WF, Valle RR, Carvalho FM, Arakaki PR, Rodas-Martínez AZ, Muniz JAPC and García-Herreros M (2016) Sperm morphology assessment in captive neotropical primates. *Reprod Dom Anim* **51**, 623–7.

Visco V, Raffa S, Elia J, Delfino M, Imbrogno N, Torrisi MR and Mazzilli F (2010) Morphological sperm defects analyzed by light microscopy and transmission electron microscopy and their correlation with sperm motility. *Int J Urol* **17**, 259–66.

1 Este artigo foi aceito para publicação na Zygote, seguindo suas normas de apresentação.

WHO (2010) Laboratory Manual for the Examination and Processing of Human Semen, 5th ed. Geneva, Switzerland: WHO Press.

Zamboni L, Zemjanis R and Stefanini M (1971) The fine structure of monkey and human Spermatozoa. *Anat Rec* **169**, 129-53.

1 Este artigo foi aceito para publicação na Zygote, seguindo suas normas de apresentação.

400 **Figure captions**

401

402 **Fig. 1**: Scanning electron micrograph of Saimiri collinsi sperm: A - Sperm head with plasma
403 membrane integrity, acrossomal region (ac), serrated band (sb); post-acrosomal region (par),
404 neck area (na), lateral insertion of the middle piece (limp) and middle piece (md); B -Normal
405 sperm: head (hd), middle piece (md) and tail (t).

406

407 **Fig. 2**: Transmission electron micrograph of the squirrel monkey (Saimiri collinsi) sperm: (A)
408 hd: head; md: middle piece; an: annulus; t: tail. (B) oam: outer acrosome membrane; ac:
409 acrosome; pl: plasmalemma; nu: nucleus; nm: nuclear membrane; pas: postacrosomal sheath;
410 pc: principal centriole: na: neck area; md: middle piece. (C) pl: plasmalemma; nm: nuclear
411 membrane: hd: head; na: neck area; md: middle piece; ax: axonema; ofd: outer dense fibers;
412 ms: mitochondrial spiral. (D) pl: plasmalemma; ax: axonema; odf: outer dense fibers; ms:
413 mitochondrial spiral; *: longitudinal cut. (E) ofd: outer dense fibers; ax: axonema; pl:
414 plasmalemma, ms: mitochondrial spiral.

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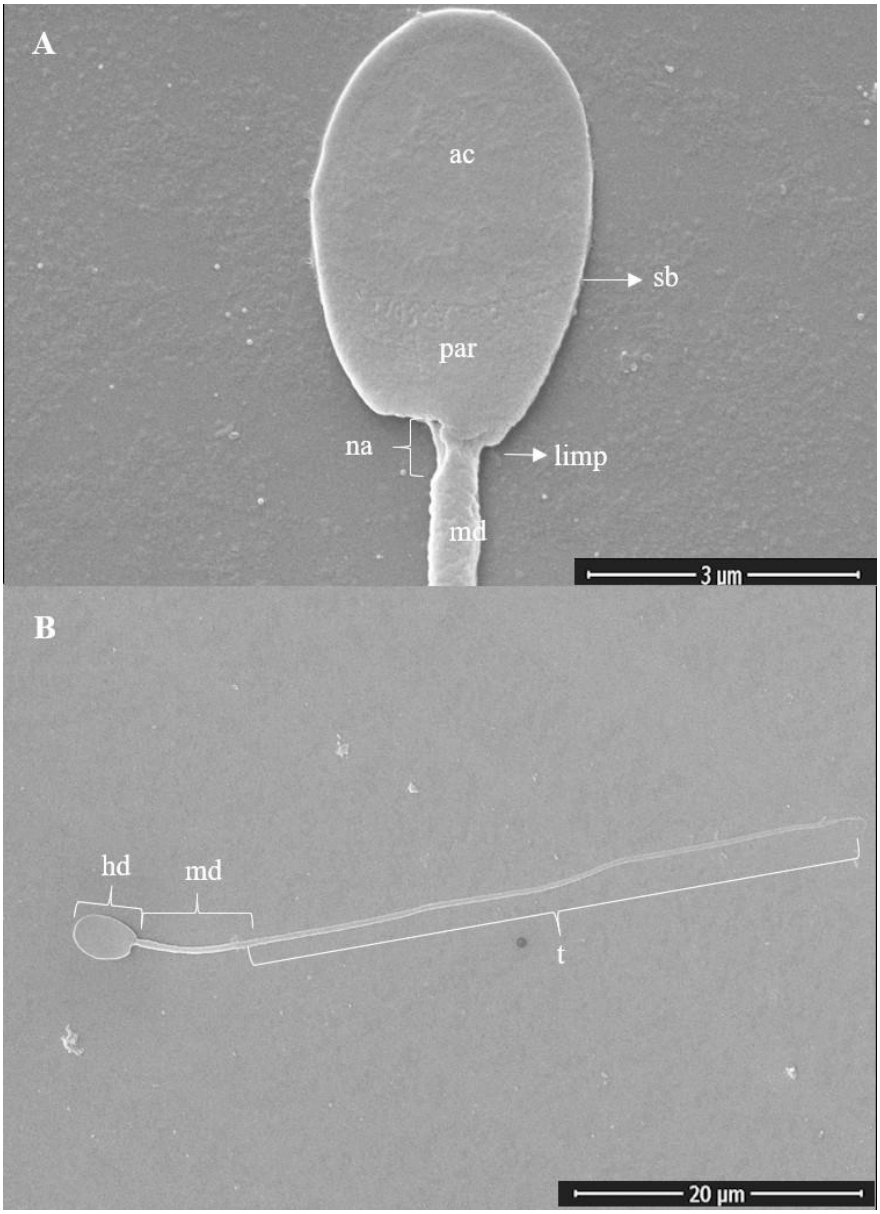

Fig. 1: Scanning electron micrograph of Saimiri collinsi sperm: A - Sperm head with plasma membrane integrity, acrossomal region (ac), serrated band (sb); post-acrosomal region (par), neck area (na), lateral insertion of the middle piece (limp) and middle piece (md); B -Normal sperm: head (hd), middle piece (md) and tail (t).

63x87mm (300 x 300 DPI)

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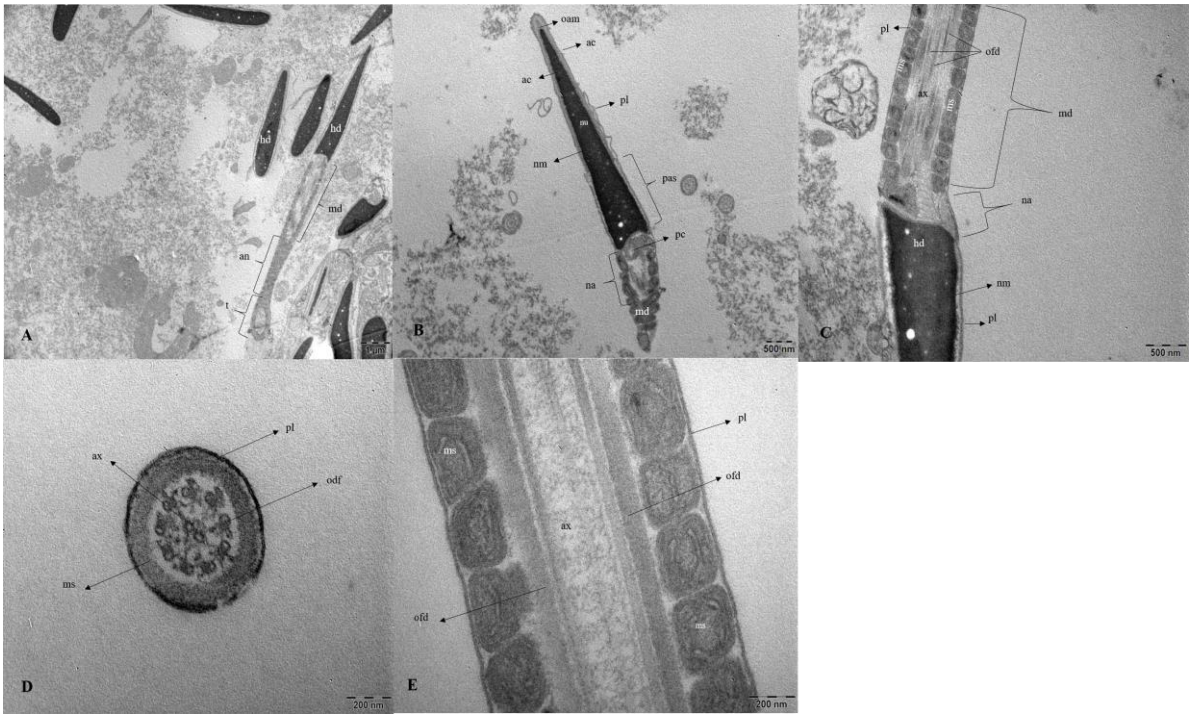

Fig. 2: Transmission electron micrograph of the squirrel monkey (Saimiri collinsi) sperm: (A) hd: head; md: middle piece; an: annulus; t: tail. (B) oam: outer acrosome membrane; ac: acrosome; pl: plasmalemma; nu: nucleus; nm: nuclear membrane; pas: postacrosomal sheath; pc: principal centriole: na: neck area; md: middle piece. (C) pl: plasmalemma; nm: nuclear membrane: hd: head; na: neck area; md: middle piece; ax: axonema; ofd: outer dense fibers; ms: mitochondrial spiral. (D) pl: plasmalemma; ax: axonema; odf: outer dense fibers; ms: mitochondrial spiral; *: longitudinal cut. (E) ofd: outer dense fibers; ax: axonema; pl: plasmalemma, ms: mitochondrial spiral.

174x93mm (300 x 300 DPI)

GLOBAL PROTEOMIC ANALYSIS OF SPERMATOZOA FROM CAPTIVE AMAZON SQUIRREL MONKEYS (*Saimiri collinsi* Osgood, 1916)

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Abstract

The aims of this study were to describe the global sperm proteomics in *Saimiri collinsi,* a Neotropical primate endemic to the Amazon in Brazil, which is used as a biological model for reproductive research on the genus *Saimiri*. The study approach based on bottom-up proteomics allowed the identification of 2,343 proteins present in the sperm samples. Of the 39 proteins that were related to spermatogenesis, sperm motility, capacitation, fecundation, and defense systems against oxidative stress were upregulated in the dry season. Knowledge on the sperm proteins provides crucial information for elucidating the underlying mechanisms associated with sperm functionality. Thus, our results help to advance our understanding of the reproductive physiology of *S. collinsi*, providing valuable information for the improvement of protocols used in assisted reproduction techniques for the conservation of endangered *Saimiri* species.

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1. Introduction

Mammalian male fertility depends on physiological events that begin with spermatogenesis and culminate with successful adhesion/signaling between the sperm membrane and the extracellular coat of the oocyte, followed by adhesion/fusion between the oocyte and sperm membranes during fertilization in the female reproductive tract [1, 2]. Proteins expressed by spermatozoa and those from the seminal plasma that bind to the sperm plasma membrane render the spermatozoa capable of fertilizing a mature oocyte [3, 4].

Studies in animals and humans have described sperm proteins that have significant associations with sperm motility (i.e., L-lactate dehydrogenase and dynein heavy chain 1 (DNAH1)) [5, 6], sperm capacitation (i.e., clusterin, spermadhesin, and mitochondrial peroxiredoxin-5) [7, 8], and fertility (i.e., enolase 1, ropporin- 1-like protein (ROPN1), and Izumo sperm–egg fusion 1 (IZUMO1)) [9, 10]. In non-human primates, sperm proteomics has been carried out only in Old World primates, in the genus *Macaca,* for characterization of the sperm protein profile [3, 11-15]. Knowledge about the absence, presence, underexpression, or overexpression of these sperm proteins could help to further our understanding of the mechanisms behind the reduction in the fertilization ability of sperm [4, 16].

The squirrel monkey (*Saimiri collinsi*), a Neotropical primate endemic to the Amazon in Brazil [17], was used as an experimental model for reproductive research on the genus *Saimiri* [18-20]. According to the International Union for Conservation of Nature's Red List of Threatened Species, two *Saimiri* species are ranked as vulnerable (*Saimiri oerstedii* and *Saimiri vanzolini*) and one species as almost threatened (*Saimiri ustus*) to extinction [21]. Defining the sperm protein profiles of *Saimiri collinsi* may provide us with a better understanding about the reproductive physiology of these animals, as well as whether the sperm cells could be used in assisted reproduction techniques throughout. Therefore, the aims of this study were to describe the global sperm proteomics in *S. collinsi* and evaluate the potential correlation between the expression of the sperm proteins and the seminal quality in *S. collinsi.*

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2. Material and methods

2.1 Study design

We conducted a global proteomic analysis of spermatozoa collected from adult squirrel monkeys (*S. collinsi*), in the Brazilian Amazon. The seminal coagulum was collected by electroejaculation and liquefied in a powdered coconut water extender (ACP-118; ACP Biotecnologia, Fortaleza, Ceará, Brazil). After 1 h in the ACP-118 extender, the viable sperm cells were separated on Percoll density gradient media and washed. Then, the sperm proteins were extracted and subjected to tryptic digestion, followed by liquid chromatography-tandem mass spectrometry. Computational biology was used for the identification of the proteins, categorization of the proteins, and *in silico* analysis of the protein network.

2.2 Animal ethics statement and study location

The animal study was approved by the Ethical Committee in Animal Research (Approval No. 02/2015/CEPAN/IEC/SVS/MS) and by the System of Authorization and Information in Biodiversity (SISBIO/ICMBio/MMA No. 47051-2), and carried the license of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES/IBAMA/Permit No. 17BR025045-DF). All procedures were performed under the supervision of a veterinarian.

Semen collections were performed at the National Primate Center (CENP, Ananindeua, Pará, Brazil) and the characterization of the protein profile was performed in the Faculty of Chemistry, Biotechnology and Food Science of Norwegian University of Life Sciences (NMBU, Ås, Norway).

2.3 Animals and housing conditions

S. collinsi males (n = 4) maintained in captivity at the Centro Nacional de Primatas, Brazil (1°22′58″S and 48°22′51″W) were used for the semen collection. The animals were housed collectively in cages (4.74 m × 1.45 m × 2.26 m), with 12 h of natural light each day. The mixed animal groups typically consisted of three males and three females and their

juvenile offspring. The animals were fed fresh fruits, vegetables, commercial pellet chow specific for Neotropical non-human primates (18% protein, 6.5% fiber; Megazoo, Minas Gerais, Brazil), and cricket larvae (*Zophobas morio*). Vitamins, minerals, and eggs were supplied once a week, and water was available *ad libitum*.

2.4 Body weight, testicular biometry, and semen collection

Semen was collected every morning before feeding, making up a total of 48 semen collections. For the semen collection, physical restraint of each animal was performed by a trained animal caretaker wearing leather gloves, and all animals were anesthetized with ketamine hydrochloride (20 mg/kg; intramuscularly (IM); Vetanarcol, König S.A., Avellaneda, Argentina) and xylazine hydrochloride (1 mg/kg; IM; Kensol, König S.A.) and monitored by a veterinarian. After anesthesia, the animals were weighed using a weight balance, and the testicular length, width, height, and circumference were measured using a universal caliper. The testicular volume was calculated according to the method described by Oliveira et al. [20]. After the animal had been placed in dorsal recumbency, the genital region was sanitized with a mild soap and distilled water (1:10) and the prepuce was retracted for a more efficient cleaning of the penis with saline solution. The animal was then stimulated according to the rectal electroejaculation procedure described by Oliveira et al. [18-20]. In brief, an electroejaculator (Autojac-Neovet, Uberaba, Brazil) rectal probe was smeared with a sterile lubricant gel (KY Jelly, Johnson & Johnson Co., Arlington, TX, USA) and introduced into the rectum (~2.5 cm deep) and electrical stimuli were then delivered. The stimulation session consisted of three series (7 and 8 min), composed of 35 electrical stimuli (12.5 and 100 mA), with an interval of 30 s between the series. The ejaculates (liquid and coagulated fractions) were collected into microtubes (1.5 mL).

2.5 Semen evaluation

The 1.5-mL conical microtubes containing the semen were placed in a water bath at 37°C immediately after collection for evaluation of the seminal volume, color, and viscosity. The semen appearance was assessed subjectively for color (colorless, yellowish, or whitish)

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143 and opacity (opaque or transparent) [18-20]. The sperm motility, vigor, and morphology were
144 evaluated according to the methods described by Oliveira et al. [18-20]. For evaluation of the
145 normal sperm morphology and plasma membrane integrity, a smear sample was prepared by
146 adding 5 µL of 1% eosin (Vetec, Rio de Janeiro, Brazil) and 5 µL of 1% nigrosine (Vetec, Rio
147 de Janeiro, Brazil) to 5 µL of semen on a prewarmed (37°C) glass slide. The plasma
148 membrane functionality was assessed with the hypoosmotic swelling test after the dilution of 5
149 µL of semen in 45 µL of hypoosmotic solution (0.73 g of sodium citrate, 1.35 g of fructose,
150 and 100 mL of ultrapure water; pH 7.2 and 108 mOsm/L). After a 45-min incubation in a
151 water bath (37°C), 10 µL of this solution was placed on a prewarmed (37°C) glass slide and
152 covered with a coverslip, and at least 200 spermatozoa were counted to determine the number
153 with coiled tails (indicative of spermatozoa with a functional plasma membrane). All
154 evaluations were performed under a light microscope (E400; Nikon, Tokyo, Japan) at a
155 magnification of 100×. The semen was assessed after dilution in ACP-118.
156
157 *2.6 Sperm separation and freeze-drying*
158 Owing to the occurrence of seminal coagulation in *S. collinsi*, the semen sample was
159 diluted 1:1 in ACP-118 (300 mOsm/kg and pH 6.42), incubated in a water bath (Biomatic,
160 Porto Alegre, Rio Grande do Sul, Brazil) at 37°C for 1 h, and then separated on 45%/90%
161 Percoll gradient media (centrifugation at 10,000 *g*, 15 min, 12°C). Thereafter, the samples
162 were washed in Tris-NaCl medium (centrifugation at 8000 *g*, 5 min, 12°C), and the separated
163 sperm fraction (pellet) was stored in microtubes, together with Tris-NaCl and a protease
164 inhibitor (1:1000; P8340 catalog, Sigma-Aldrich, St. Louis, MO, USA), in liquid nitrogen or a
165 –80°C freezer. For lyophilization, the frozen sperm samples were placed in a freeze dryer
166 (FreeZone 2.5 Liter Benchtop Freeze Dry System; Labconco, Kansas City, MO, USA) for 10 h
167 at a temperature of –55°C and vacuum pressure of 0.025 mbar.
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172 *2.7 Liquid chromatography-mass spectrometry*

173 Each individual dried sperm sample was resuspended in 50 µL of lysis buffer (0.1 M
174 Tris-Cl (pH 8.0), 4% sodium dodecyl sulfate, and 10 mM dithiothreitol) and centrifuged at
175 5000 *g* for 1 h at 4°C. The supernatant was reserved for the preparation of suspension samples
176 for bottom-up proteomic analysis with tryptic digestion, using the method established by
177 Zougman et al. [22]. The extracted peptides were analyzed on an UltiMate 3000 RSLCnano/Q-
178 Exactive system (Thermo Fisher Scientific, Bremen, Germany) that was set up with a
179 Nanospray Flex ion source. The tryptic peptides (~1 µg loaded) were separated on a 50 cm ×
180 75 µm (i.d.) column (Thermo Fisher Scientific) using a 120 min gradient of 12–45%
181 acetonitrile. The mass spectrometry (MS) and tandem mass spectrometry (MS/MS) data were
182 recorded using a standard data-dependent acquisition method, with the following conditions:
183 *m/z* range of 300–1600; Automatic Gain Control targets of 3×10^6 (MS) and 5×10^4 (MS/MS);
184 resolutions of 70 K (MS) and 35 K (MS/MS); dynamic exclusion set to 20 s, and normalized
185 collision energy set to 28. Xcalibur software (v. 3.1; Thermo Fisher Scientific) was used to
186 evaluate the raw data, which were converted to *mgf* format (for Mascot database searching)
187 using the MS convert module of ProteoWizard (v. 3.0.9016). The Mascot (v. 2.6) searches
188 were performed on an in-house server against an online *Saimiri boliviensis boliviensis*
189 (Bolivian squirrel monkey) database (National Center for Biotechnology Information,
190 Bethesda, MD, USA). MaxQuant software (v.1.6.1.0) [23] was used for the label-free
191 quantification.
192
193 *2.8 Protein categorization*

194 The protein information obtained by Mascot was analyzed using the STRuctural
195 Analysis Programs (STRAP) for searching annotations of proteins. STRAP automatically
196 obtains Gene Ontology (GO) terms associated with proteins in an identification list of results
197 based on homology search analysis using various freely accessible databases [24].
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201 *2.9 In silico protein network analysis*

202 Protein–protein networks were retrieved from the STRING database (v. 10.0), which
203 consists of known and predicted protein interactions collected from direct (physical) and
204 indirect (functional) associations. The database quantitatively integrates interaction data from
205 four sources: a genomic context, high-throughput experiments, co-expression data, and
206 previous knowledge from research publications [25]. The STRING program was set to show
207 no more than 10 interactions and medium confidence. Pathways not described for *S.*
208 *boliviensis boliviensis* were analyzed using those for other non-human primate species and
209 *Homo sapiens*.
210
211
212 **3. Results**
213 Mean (± SE) body weight was 853.7 ± 8.6 g (762 – 1,022 g; min–max), and testicular
214 width, length, height, and volume were 1.12± 0.1 (0.8 – 1.8 cm; min-max), 1.7 ±0.2 (0.8 – 2.2
215 cm, min-max), 1.2± 0.4 cm (0.7 – 2.2 cm, min-max) and 2.2 ± 0.1 (1.08 – 4.1 cm^3, min-max),
216 respectively, without significant differences between the left and right testis. Semen collection
217 was successful in 42 of the 48 attempts (88%); of these, 39 samples were used for the
218 experiments, because three ejaculates did not contain sperm. The highest percentage of
219 ejaculates in both the liquid and coagulated fractions was 59%. With regard to the semen color
220 and opacity, 10% of the samples were colorless, 33% were whitish, 57% were yellowish, 46%
221 were transparent, and 54% were opaque. The seminal parameters of *Saimiri collinisi* were
222 represented in Table 1.
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Table 1

	Mean (±SEM)
Motility	45±5.5[b]
Vigor	2±0.2[b]
PMF	65.4±4.1
PMI	71.1±3.4
Normal sperms	73.1±2.6

Mean (±SEM) values of sperm motility (%),, vigor, sperm plasma membrane functionality (PMF; %), sperm plasma membrane integrity (PMI; %), and normal sperm morphology (%) of diluted semen in *Saimiri collinsi.*

The approach based on bottom-up proteomics allowed the identification of 2,343 proteins in sperm samples after seminal coagulation dilution in ACP-118® extender (Supplemental Table 1). Of the total proteins identified, 223 were determined to participate in important reproductive events, such as spermatogenesis (67 proteins), sperm motility (42 proteins), capacitation/acrosome reaction (20 proteins), and fertilization (32 proteins) (Supplemental Table 2).

On the basis of the GO analysis, the proteins were grouped according to biological process, molecular function, and cellular component (i.e., localization) classes (Figure 1). In the cellular component class, most of the proteins identified were associated with the cytoplasm (12.3%), cytoskeleton (9.4%), and nucleus (8.9%) (Figure 1A). The most common biological processes associated with the proteins were cellular processes (41.6%), regulation (17.6%), and metabolic processes (11.4%) (Figure 1B). Binding (42.8%) and catalytic activity (42.9%) corresponded to the most frequent molecular functions for the proteins (Figure 1C).

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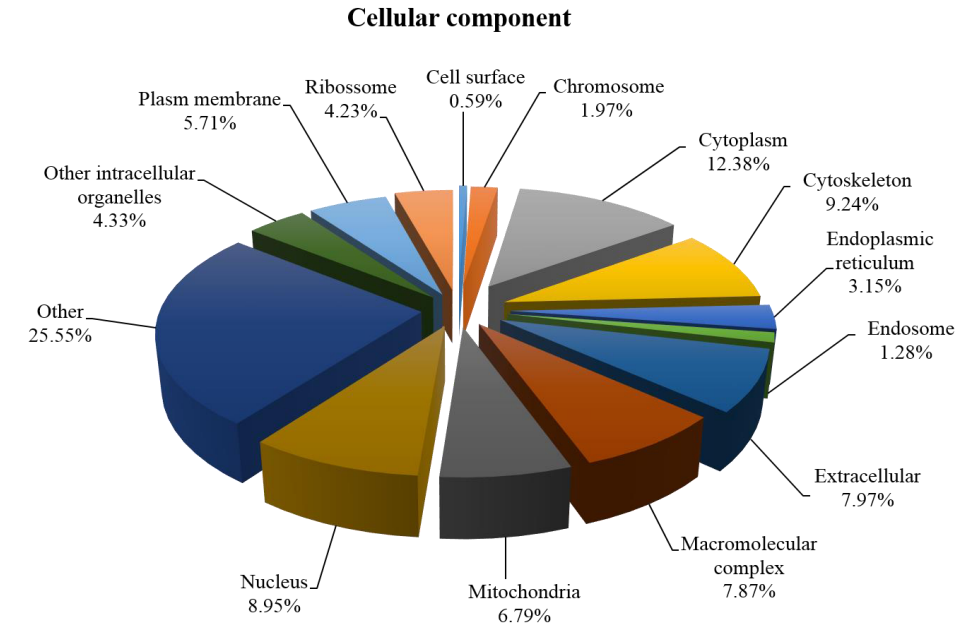
253 Figure 1



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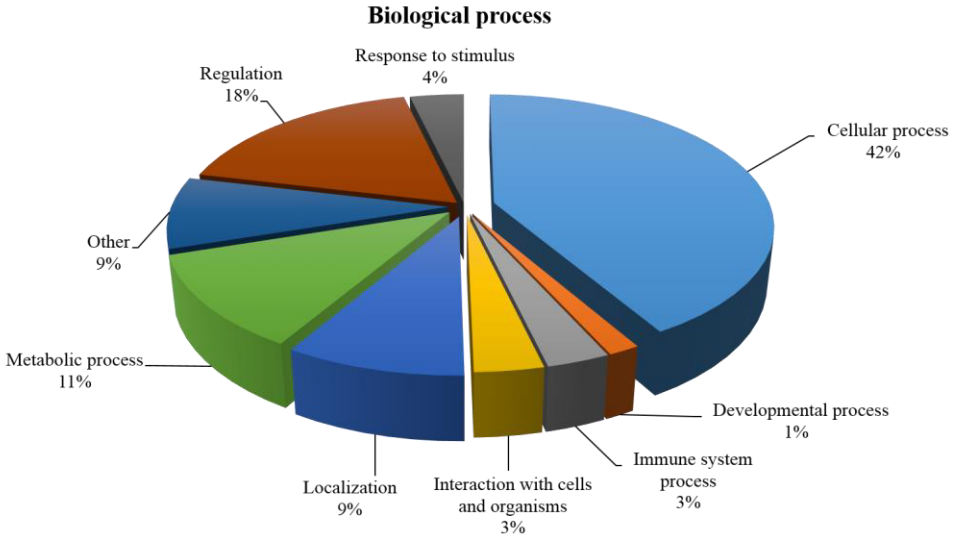

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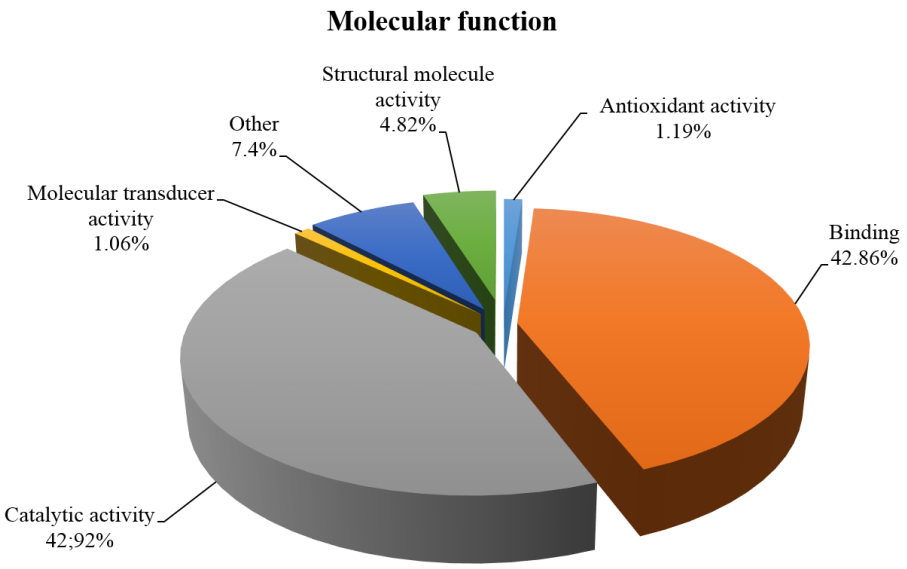

Cellular component, biological process and molecular function of identified proteins by nanoLC-QExactive spectrometry analyzed by STRAP. Gene ontology terms were obtained from the UniProtKB database.

In silico analysis indicated that semenogelin I, heat shock proteins members (60kDa, 70kDa and 90-alpha), sperm acrosome membrane-associated family member (3 and 4), and izumo sperm-egg fusion family members (1 and 4) interacted with other 10 proteins. Among these interactions, the heat shock proteins 60kDa and 70kDa proteins interacted with each other and heat shock proteins 90-alpha, as well as izumo sperm-egg fusion 1 proteins also interact with each and sperm acrosome membrane-associated protein 4. The sperm acrosome membrane-associated protein 4 interacts with both sperm acrosome membrane-associated protein 1 and 3 (Figure 02).

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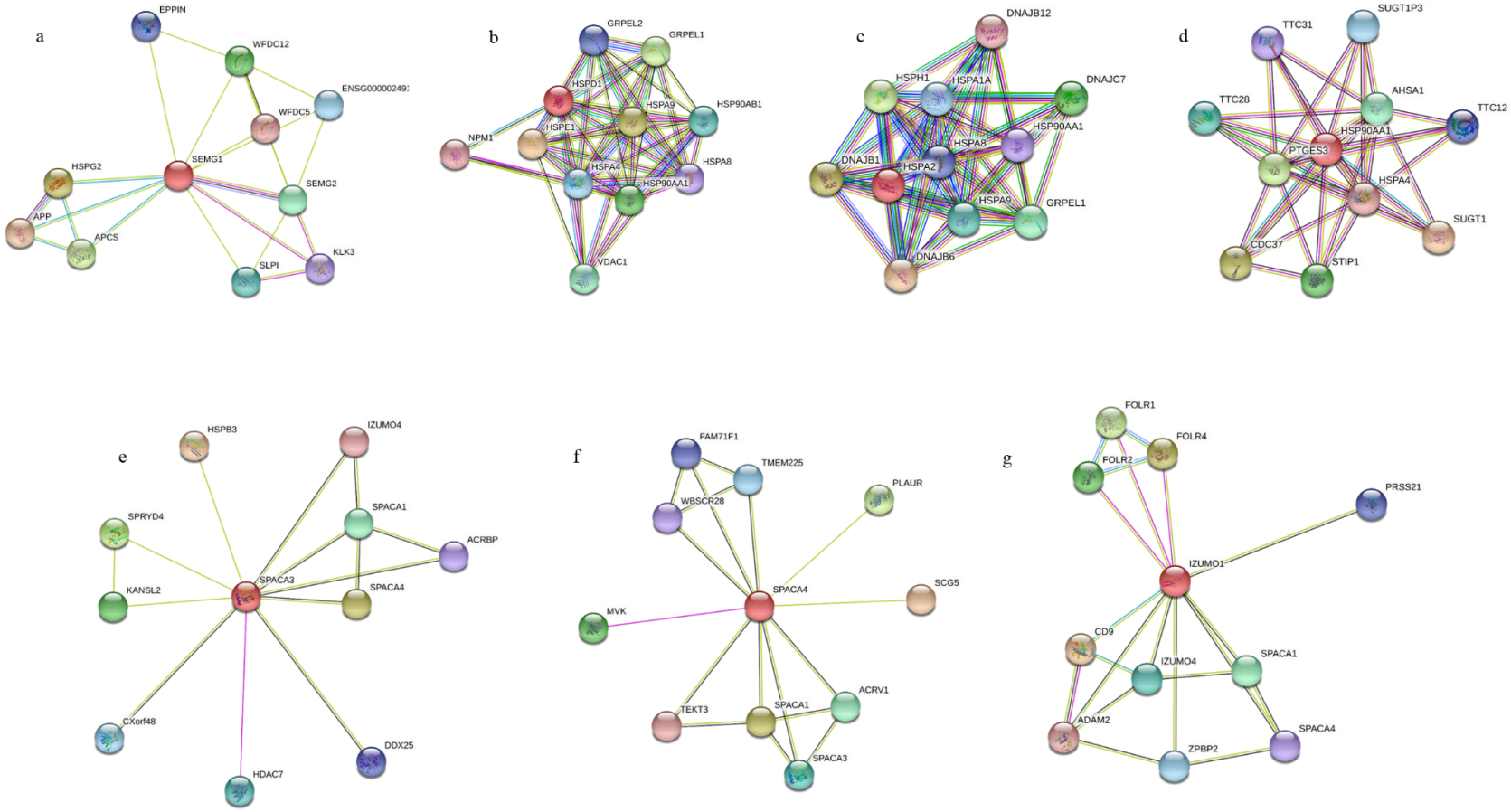
275 Figure 2



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Protein interaction analysis. Proteins were analyzed with the web-based STRING software. Analyzed proteins were: a- semenogelin I; b- 60kDa heat shock proteins; c- Heat shock proteins 70kDa protein 2; d- Heat shock proteins 90-alpha; e- Sperm acrosome membrane-associated 3; f- Sperm acrosome membrane-associated 4; g- Izumo sperm-egg fusion protein 1. Different line colors represent the types of evidence for the association. Yellow textming; black coexpression; blue databases; and pink experiments. APP Amyloid beta (A4) precursor protein; HSPG2 Heparan sulfate proteoglycan 2; APCS Amyloid P component, serum; WFDC12 WAP four-disulfide core domain 12; SEMG2 Semenogelin II; SLPI Secretory leukocyte peptidase inhibitor; ENSG00000249139 EPPIN-WFDC6 readthrough; EPPIN Epididymal peptidase inhibitor; KLK3 Kallikrein-related peptidase 3; WFDC5 WAP four-disulfide core domain 5; HSPE1 Heat shock 10kDa protein 1 (chaperonin 10); HSPA9 Heat shock 70kDa protein 9 (mortalin); GRPEL1 GrpE-like 1, mitochondrial (*E. coli*); HSP90AA1 Heat shock protein 90kDa alpha (cytosolic); VDAC1 Voltage-dependent anion channel 1; HSP90AB1 Heat shock protein 90kDa alpha (cytosolic), class B member 1; HSPA4 Heat shock 70kDa protein 4; GRPEL2 GrpE-like 2, mitochondrial (*E. coli*); HSPA8 Heat shock 70kDa protein 8; NPM1 Nucleophosmin (nucleolar phosphoprotein B23, numatrin); DNAJB6 DnaJ (Hsp40) homolog, subfamily B, member 6; DNAJB1 DnaJ (Hsp40) homolog, subfamily B, member 1; HSPH1 Heat shock 105kDa/110kDa protein 1; DNAJC7 DnaJ (Hsp40) homolog, subfamily C, member 7; HSPA1A Heat shock 70kDa protein 1A; DNAJB12 DnaJ (Hsp40) homolog, subfamily B, member 12; SUGT1 SGT1, suppressor of G2 allele of SKP1 (*S. cerevisiae*); CDC37 Cell division cycle 37 homolog (*S. cerevisiae*); PTGES3 Prostaglandin E synthase 3 (cytosolic); STIP1 Stress-induced-phosphoprotein 1; AHSA1 AHA1, activator of heat shock 90kDa protein ATPase homolog 1 (yeast); TTC28 Tetratricopeptide repeat domain 28; SUGT1P3 Suppressor of G2 allele of SKP1 (*S. cerevisiae*) pseudogene 3; TTC12 Tetratricopeptide repeat domain 12; TTC31 Tetratricopeptide repeat domain 31; HSPB3 Heat shock 27kDa protein 3; Inhibitor of actin polymerization; SPACA4 Sperm acrosome associated 4; SPRYD4 SPRY domain containing 4; KANSL2 KAT8 regulatory NSL complex subunit 2; SPACA1 Sperm acrosome associated 1; HDAC7 Histone deacetylase 7; CXorf48 Chromosome X open reading frame 48; DDX25 DEAD (Asp-Glu-Ala-Asp) box helicase 25; ACRBP Acrosin binding protein; IZUMO4 IZUMO family member 4; SCG5 Secretogranin V (7B2 protein); PLAUR Plasminogen activator, urokinase receptor; MVK Mevalonate kinase; ACRV1 Acrosomal vesicle protein 1; SPACA3 Sperm acrosome associated 3; TMEM225 Transmembrane protein 225; FAM71F1 Family with sequence similarity 71, member; WBSCR28 Williams-Beuren syndrome chromosome region 28;TEKT3 Tektin 3; Structural component of ciliary and flagellar microtubules; CD9 CD9 molecule; FOLR4 Folate receptor 4 (delta) homolog (mouse); FOLR1 Folate receptor 1 (adult); FOLR2 Folate receptor 2 (fetal); ZPBP2 Zona pellucida binding protein 2; PRSS21 Protease, serine, 21 (testisin); ADAM2 ADAM metallopeptidase domain 2.

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4. Discussion

In the present study, we used a liquid chromatography mass spectrometry approach (Shotgun proteomics) to characterize the protein profile of spermatozoa from the Amazon squirrel monkey (*Saimiri collinsi*) kept in captive. The seminal coagulum and the parameters, such as appearance, semen volume, sperm concentration, motility, vigor and sperm morphology in Amazon squirrel monkey were similar to those previously reported for sperm from coagulated fraction after dilution in ACP-118® extender [18-20].

This proteomic strategy allowed the identification of 2,343 proteins and represents a major contribution to the understanding of spermatozoa composition in *Saimiri collinsi* species. In *S. collinsi*, it was possible to verify important proteins that participated in spermatogenesis and sperm motility, such as ROPN1L, HSPA2, cat eye syndrome critical region protein 5, and phospholipid hydroperoxide glutathione. In mice, the loss of ROPN1L impairs sperm motility, cAMP-dependent protein kinase phosphorylation, and fibrous sheath integrity [26]. ROPN1L is a sperm flagellar protein that binds A-kinase anchoring protein (AKAP) 3 and 4, which are primary components of the sperm fibrous sheath. The fibrous sheath is a flagellar cytoskeletal structure unique to sperm that surrounds the outer dense fibers and axoneme [26, 27]. The degradation of AKAP3 and subsequent dephosphorylation of tyrosine result in sperm capacitation [28].

Heat shock proteins (HSPs) are chaperone proteins that are expressed in response to cell stress [29, 30]. Several HSP family members are expressed in the sperm, such as HSP 70 kDa (HSP70), which appears in the acrosome membranes. HSP 60 kDa (HSP60) is located primarily in the sperm midpiece, in association with the mitochondria, whereas HSP 90-alpha (HSP90AA1) is located in the sperm flagellum [31]. HSP60, HSP70, and HSP90AA1 are known components of sperm in different species, such as humans [32], rams [33], bulls, stallions, cats, and dogs [34]. The acrosomal HSP70 has a role in gamete interaction and fertilization [35], whereas HSP90AA1 expression has been correlated with the resistance of sperm to freezing [36, 37] since this protein is characterized as a ubiquitous molecular chaperone that provides protection and protein folding during thermal stress and resistance against cell oxidative stress [38].

HSPA2, which is a molecular chaperone that assists in the folding, transport, and assembly of proteins in the cytoplasm, mitochondria, and endoplasmic reticulum and is a testis-

352 specific member of the 70-kDa family [39], has been suggested to be crucially involved in
353 spermatogenesis and meiosis [40]. In humans, the downregulation of HSPA2 mRNA was
354 observed in testes with abnormal spermatogenesis, and the protein expression was high in normal
355 spermatogenesis and low in spermatogenesis arrest [41]. Human HSPA2 was shown to regulate
356 the expression of the sperm surface receptors involved in sperm-oocyte recognition [42], and its
357 depression in the testes was also associated with spermatogenic impairment and the fertilization
358 rate in men with azoospermia who were treated with intracytoplasmic sperm injections [43].
359 Sperm motility is driven mainly by the energy produced by the mitochondria present in
360 the intermediate piece of the male gamete [44]. However, the axoneme is another important
361 cellular component that is directly associated with sperm motility. The dynein heavy chains have
362 been annotated as subunits of the axonemal dynein complexes, which are multisubunit axonemal
363 ATPase complexes that generate the force for cilia motility and govern the beat frequency [45].
364 DNAH1 is related to spermatogenesis and cell proliferation [46]. In humans, mutations in
365 DNAH1 cause multiple morphologic abnormalities of the sperm flagella, leading to male
366 infertility [6]. The radial spoke proteins play a key role in regulating dynein activity and flagellar
367 motility [47, 48].
368 In this context, Imai et al. [49] showed that the failure to express phospholipid
369 hydroperoxide glutathione peroxidase (GPX4) caused human male infertility, with 30% of men
370 diagnosed with oligoasthenozoospermia showing a significant decrease in the level of the
371 enzyme. Those authors also found a significantly lower number of spermatozoa in the semen and
372 significantly lower motility of the spermatozoa than those seen in fertile men. GPX4 is an
373 intracellular antioxidant that directly reduces peroxidized phospholipids and is strongly expressed
374 in the mitochondria of the testis and spermatozoa. In bulls, GPX4 is considered a unique marker
375 for seminal quality analysis owing to the direct correlation between the selenoperoxidase and the
376 progressive motility of the sperm [50].
377 The acrosome, which is a membrane-bound exocytotic vesicle that is located over the
378 anterior portion of the nucleus, contains the hydrolytic enzymes that are required for the
379 acrosome reaction, binding of the zona pellucida (ZP), penetration through the ZP, and sperm–
380 egg membrane fusion, all of which are indispensable events during the fertilization process [51].
381 In the acrosome membrane (internal and external membranes), the sperm acrosome membrane-
382 associated family (i.e., SPACA3, SPACA1, and SPACA4) [52, 53] are sperm surface membrane

proteins that may be involved in the adhesion and fusion of the sperm to the egg prior to fertilization [54]. SPACA1 and SPACA3 are localized in the acrosomal matrix, including the principal segment and equatorial segment, and are proteins characterized as membrane antigens [55, 56, 57]. SPACA1 may be involved in sperm fusion with the oölemma, since treatment of human sperm with the anti-SPACA1 antibody prevented sperm penetration into zona-free hamster eggs [54]. Fujihara et al. [56] demonstrated that the SPACA1 protein was indispensable for the normal shaping of the sperm heads during spermiogenesis in mice. In humans, this protein was identified as a sperm membrane antigen, with a molecular mass ranging from 32 to 34 kDa [54].

Membrane fusion is a key event in the fertilization process that culminates in the merger of the male–female gamete membranes and cytoplasm and fusion of the genomes, thereby initiating embryonic development [57]. In humans, a change in the expression of the sperm proteins may be a major cause of subfertility in men with normozoospermia [58]. In this context, research has been focusing on the identification of the key molecular players and their functions, and several proteins in the egg or the spermatozoa have been found to be essential for fertilization.

Until now, IZUMO1 has been found to be the essential protein on the sperm side for the fusion process. As a testis-specific protein, IZUMO was discovered on the equatorial segment of the acrosome-reacted mouse spermatozoa through proteomic analysis of the antigen recognized by the monoclonal anti-mouse sperm antibody [590]. IZUMO is present in both mouse (~56 kDa protein) and human (~38 kDa protein) sperm [60]. In mice, immunization with the IZUMO protein caused a contraceptive effect in females, which was due to the significantly inhibited fusion of sperm to the zona-free mouse eggs with the anti-PrimeB antibody. However, no effect on sperm motility was observed [61]. IZUMO2, IZUMO3, and IZUMO4 have significant homology with the N-terminal domain of IZUMO1 [62]. Inoue et al. [9] showed the interaction between angiotensin-converting enzyme-3 located on the sperm acrosomal cap and IZUMO1 in the fertilization process. However, it was reported that angiotensin-converting enzyme-3 disappears from the membrane after the acrosome reaction. Nevertheless, the *in silico* protein interaction analysis of IZUMO1 revealed its association with the CD9 molecule, folate receptor 4 (delta) homolog (mouse), folate receptor 1 (adult), folate receptor 2 (fetal), SPACA1, SPACA4, IZUMO family member 4, zona pellucida binding protein 2, and metallopeptidase domain 2.

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After the acrosome reaction, the C-terminal calmodulin domain (20 kDa) of SPA17 (located on the external side of the sperm plasma membrane) is proteolytically cleaved to 17 kDa and then binds to the extracellular matrix of the oocyte. This C-terminus of SPA17 plays a role in cell–cell adhesion [63, 64].

5. Conclusions

The present study is a comprehensive overview of the sperm proteome in the Amazon squirrel monkey, and is the broadest inventory (investigation) of the sperm proteome in the genus *Saimiri* as well as in Neotropical primates thus far. The knowledge acquired about the sperm proteins is a significant step forward in helping toward our understanding of the reproductive biology of the genus *Saimiri*, as it provides crucial information for the elucidation of the underlying mechanisms associated with sperm function. In this way, our study amplifies the advances in biotechnological research on animal reproduction for the conservation of endangered species, and provides a reference for similar studies on other Neotropical primates.

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1 Este artigo está submetido na Theriogenology, seguindo suas normas de apresentação.

445 **6. References**

446

447 [1] Sutovsky P. Sperm-egg adhesion and fusion in mammals. Expert Rev Mol Med 2009;11:e11.
448 https://doi.org/10.1017/S1462399409001045.

449

450 [2] Ashrafzadeh A, Karsani SA, Nathan S. Mammalian sperm fertility related proteins. Int J Med
451 Sci 2013;10:1649-57. https://doi.org/10.7150/ijms.6395.
452 [3] Zhou T, Wang G, Chen M, Zhang M, Guo Y, Yu C, et al. Comparative analysis of macaque
453 and human sperm proteomes: Insights into sperm competition. Proteomics 2015;15:1564-73.
454 https://doi.org/10.1002/pmic.201400248.

455

456 [4] Agarwal A, Bertolla RP, Samanta L. Sperm proteomics: potential impact on male infertility
457 treatment. Expert Rev Proteomics 2016;13:285-96.
458 https://doi.org/10.1586/14789450.2016.1151357.

459

460 [5] Légaré C, Droit A, Fournier F, Bourassa S, Force A, Cloutier F, et al. Investigation of male
461 infertility using quantitative comparative proteomics. J Proteome Res 2014;13:5403-14.
462 https://doi.org/10.1021/pr501031x.

463

464 [6] Khelifa MB, Coutton C, Zouari R, Karaouzene T, Rendu J, Bidart M, et al. Mutations in
465 DNAH1, which encodes an inner arm heavy chain dynein, lead to male infertility from multiple
466 morphological abnormalities of the sperm flagella. Am J Hum Genet 2014;94:95-104.
467 https://doi.org/10.1016/j.ajhg.2013.11.017.

468

469 [7] Rahman MS, Kwon WS, Pang MG. Prediction of male fertility using capacitation-associated
470 proteins in spermatozoa. Mol Reprod Dev 2017;84:749-59. https://doi.org/10.1002/mrd.22810.

471

472 [8] Hernández-Silva G, López-Araiza JFE, López-Torres AS, Larrea F, Torres-Flores V, Chirinos
473 M. Proteomic characterization of human sperm plasma membrane associated proteins and their
474 role in capacitation. Andrology 2019;1-10. https://doi.org/10.1111/andr.12627.

475

1 Este artigo está submetido na Theriogenology, seguindo suas normas de apresentação.

476 [9] Inoue N, Kasahara T, Ikawa M, Okabe M. Identification and disruption of sperm-specific
477 angiotensin converting enzyme-3 (ACE3) in mouse. PLoS One. 2010;5:e10301. https://doi.org/
478 10.1371/journal.pone.0010301.
479
480 [10] Park YJ, Kwon WS, Oh SA, Pang MG. Fertility-related proteomic profiling bull
481 spermatozoa separated by Percoll. J Proteome Res. 2012;11:4162-68.
482 https://doi.org/10.1021/pr300248s.
483
484 [11] Srivastav A. Maturation-dependent glycoproteins containing both N- and O-linked
485 oligosaccharides in epididymal sperm plasma membrane of rhesus monkeys (*Macaca mulatta*). J
486 Reprod Fertil 2000;119:241-52. https://doi.org/10.1530/jrf.0.1190241.
487 [12] Skerget S, Rosenow M, Polpitiya A, Petritis K, Dorus S, Karr T. The Rhesus macaque
488 (*Macaca mulatta*) sperm proteome. Mol Cell Proteomics 2013;12:3052-67.
489 https://doi.org/10.1074/mcp.M112.026476.
490
491 [13] Kawase O, Cao S, Xuan X. Sperm membrane proteome in wild Japanese macaque (*Macaca*
492 *fuscata*) and Sika deer (*Cervus nippon*). Theriogenology 2015;83:95-102. https://doi.org/
493 10.1016/j.theriogenology.2014.08.010.
494
495 [14] Liu X, Jin SH, Liu XX, Wang WJ, Liu FJ. Proteome profiling of the sperm maturation
496 milieu in the rhesus monkey (*Macaca mulatta*) epididymis. Reprod Fertil Dev 2016;28:732-41.
497 https://doi.org/10.1071/RD14322.
498
499 [15] Zamboni L, Conaway CH, Van Pelt L. Seasonal changes in production of semen in free-
500 ranging rhesus monkey. Biol Reprod 1974;11:251-67.
501 https://doi.org/10.1095/biolreprod11.3.251.
502
503 [16] Jodar M, Soler-Ventura A, Oliva R. Semen proteomics and male infertility. J Proteom
504 2017;162:125-34. https://doi.org/10.1016/j.jprot.2016.08.018.
505

1 Este artigo está submetido na Theriogenology, seguindo suas normas de apresentação.

506 [17] Mercês MP, Paula WS, Júnior JSS. New records of *Saimiri collinsi* Osgood, 1916 (Cebidae,
507 Primates), with comments on habitat use and conservation. Mammalia 2017;85:1-5.
508 https://doi.org/10.1515/mammalia-2017-0050.
509
510 [18] Oliveira KG, Leão DL, Almeida DV, Santos RR, Domingues SF. Seminal characteristics
511 and cryopreservation of sperm from the squirrel monkey, *Saimiri collinsi*. Theriogenology
512 2015;84:743-49. https://doi.org/10.1016/j.theriogenology.2015.04.031.
513
514 [19] Oliveira KG, Santos RR, Leão DL, Brito AB, Lima JS, Sampaio WV, et al. Cooling and
515 freezing of sperm from captive, free-living and endangered squirrel monkey species. Cryobiology
516 2016a;72:283-89. https://doi.org/10.1016/j.cryobiol.2016.03.004.
517
518 [20] Oliveira KG, Santos RR, Leão DL, Queiroz HL, Paim FP, Vianez-Júnior JLSG, et al.
519 Testicular biometry and semen characteristics in captive and wild squirrel monkey species
520 (*Saimiri* sp.). Theriogenology: 2016b;86:879-87.
521 https://doi.org/10.1016/j.theriogenology.2016.03.009.
522
523 [21] Boubli, J.P., Rylands, A.B., 2008. *Samiri vanzolinii*. In IUCN 2011. IUCN Red List of
524 Threatened Species, Version 2011.2. Diponible in www.iucnredlist.org Acessed in 20/11/2015.
525
526 [22] Zougman A, Selby PJ, Banks RE. Suspension trapping (STrap) sample preparation method
527 for bottom-up proteomics analysis. Proteomics 2014;14:1006-10.
528 https://doi.org/10.1002/pmic.201300553.
529
530 [23] Tyanova S, Temu T, Carlson A, Sinitcyn P, Mann M, Cox, J. Visualization of LC-MS/MS
531 proteomics data in MaxQuant. Proteomics 2015;15:1453-56.
532 https://doi.org/10.1002/pmic.201400449.
533
534 [24] Bhatia VN, Perlman DH, Costello CE, McComb ME. Software tool for researching
535 annotations of Proteins (STRAP): Open-source protein annotation software with data
536 visualization. Anal Chem 2009;81:9819-23. https://doi.org/10.1021/ac901335x.

1 Este artigo está submetido na Theriogenology, seguindo suas normas de apresentação.

[25] Snel B, Lehmann G, Bork P, Huynen MA. STRING: a web-server to retrieve and display the repeatedly occurring neighbourhood of a gene. Nucleic Acids Res 2000;28:3442-44. https://doi.org/10.1093/nar/28.18.3442.

[26] Fiedler SE, Dudiki T, Vijayaraghavan S, Carr DW. Loss of R2D2 proteins ROPN1 and ROPN1L causes defects in murine sperm motility, phosphorylation, and fibrous sheath integrity. Biol Reprod 2013;88:1-10. https://doi.org/10.1095/biolreprod.112.105262.

[27] Eddy EM, Toshimori K, O'Brien DA. Fibrous sheath of mammalian spermatozoa. Microsc Res Tech 2003;61:103-15. https://doi.org/10.1002/jemt.10320.

[28] Vizel R, Hillman P, Ickowicz D, Breitbart H. AKAP3 degradation in sperm capacitation is regulated by its tyrosine phosphorylation. Biochim Biophys Acta 2015;1850:1912-20. https://doi.org/10.1016/j.bbagen.2015.06.005.

[29] Mayer MP, Bukau B. Hsp70 chaperones: cellular functions and molecular mechanism. Cell Mol Life Sci 2005;62:670-84. https://doi.org/10.1007/s00018-004-4464-6.

[30] Mortensen CJ, Choi YH, Ing NH, Kraemer DC, Vogelsang MM, Hinrichs K. Heat shock protein 70 gene expression in equine blastocysts after exposure of oocytes to high temperatures in vitro or in vivo after exercise of donor mares. Theriogenology 2010;74:374-83. https://doi.org/10.1016/j.theriogenology.2010.02.020.

[31] Naaby-Hansen S, Herr JC. Heat shock proteins on the human sperm surface. J Reprod Immunol 2010;84:32-40. https://doi.org/10.1016/j.jri.2009.09.006.

[32] Miller D, Brough S, Al-Harbi O. (1992). Characterization and cellular distribution of human spermatozoal heat shock proteins. Hum Reprod 1992;7:637-45. https://doi.org/10.1093/oxfordjournals.humrep.a137711.

1 Este artigo está submetido na Theriogenology, seguindo suas normas de apresentação.

568 [33] van Tilburg MF, Rodrigues MAM, Moreira RA, Moreno FB, Monteiro-Moreira ACO,
569 Cândido, MJ, et al. Membrane-associated proteins of ejaculated sperm from Morada Nova rams.
570 Theriogenology 2013;79:1247-61. https://doi.org/10.1016/j.theriogenology.2013.03.013.
571
572 [34] Volpe S, Galeati G, Bernardini C, Tamanini C, Mari G, Zambelli D, et al. Comparative
573 immunolocalization of heat shock proteins (HSP)-60, -70, -90 in boar, stallion, dog and cat
574 spermatozoa. Reprod Dom Anim 2008;43:385-92. https://doi.org/0.1111/j.1439-
575 0531.2007.00918.x.
576
577 [35] Spinaci M, Volpe S, Bernardini C, Ambrogi M, Tamanini C, Seren E, et al.
578 Immunolocalization of heat shock protein 70 (HSP 70) in boar spermatozoa and its role during
579 fertilization. Mol Reprod Dev 2005;72:534-41. https://doi.org/10.1002/mrd.20367.
580 [36] Casas I, Sancho S, Ballester J, Briz M, Pinart E, Bussalleu E, et al. The HSP90AA1 sperm
581 content and the prediction of the boar ejaculate freezability. Theriogenology 2010;74:940-50.
582 https://doi.org/10.1016/j.theriogenology.2010.04.021.
583
584 [37] Wang S, Wang W, Xu Y, Tang M, Fang J, Sun H, et al. Proteomic characteristics of human
585 sperm cryopreservation. Proteomics 2010;14:298-310. https://doi.org/10.1002/pmic.201300225.
586
587 [38] Fukuda A, Osawa T, Oda H, Tanaka T, Toyokuni S, Uchida K. Oxidative stress response in
588 iron-induced acute nephrotoxicity: enhanced expression of heat shock protein 90. Biochem
589 Biophys Res Commun 1996;219:76-81. https://doi.org/10.1006/bbrc.1996.0184.
590
591 [39] Huszar G, Stone K, Dix D, Vigue L. Putative creatine kinase Misoform in human sperm is
592 identifiedas the 70-kilodalton heat shock protein HspA2. Biol Reprod 2000;63:925-32.
593 https://doi.org/10.1095/biolreprod63.3.925.
594
595 [40] Zhu D, Dix DJ, Eddy EM. HSP70-2 is required for CDC2 kinase activity in meiosis I of
596 mouse spermatocytes. Development 1997;124:3007-14.
597

1 Este artigo está submetido na Theriogenology, seguindo suas normas de apresentação.

598 [41] Son WY, Han CT, Hwang SH, Lee JH, Kim S, Kim, YC. Repression of hspA2 messenger
599 RNA in human testes with abnormal spermatogenesis. Fertil Steril 2000;73:1138-44.
600 https://doi.org/10.1016/s0015-0282(00)00496-9.
601
602 [42] Redgrove KA, Anderson AL, McLaughlin EA, O'Bryan MK, Aitken RJ, Nixon B.
603 Investigation of the mechanisms by which the molecular chaperone HSPA2 regulates the
604 expression of sperm surface receptors involved in human sperm-oocyte recognition. Mol Hum
605 Reprod 2013; 19:120-35. https://doi.org/10.1093/molehr/gas064.
606
607 [43] Tian Y, Zhang F, Zhang X, Li L, Wang L, Shi B, et al. Depression of HspA2 in human testis
608 is associated with spermatogenic impairment and fertilization rate in ICSI treatment for
609 azoospermic individuals. J Assist Reprod Genet 2014;31:1687-93.
610 https://doi.org/10.1007/s10815-014-0360-7.
611 [44] Piomboni P, Focarelli R, Stendardi A, Ferramosca A, Zara V. The role of mitochondria in
612 energy production for human sperm motility. Int J Androl 2012;35:109-24.
613 https://doi.org/10.1111/j.1365-2605.2011.01218.x.
614
615 [45] Castleman VH, Romio L, Chodhari R, Hirst RA, Castro SC, Parker KA, et al. Mutations in
616 radial spoke head protein genes RSPH9 and RSPH4A cause primary ciliary dyskinesia with
617 central-microtubular-pair abnormalities. Am J Hum Genet 2009;84:197-209.
618 https://doi.org/10.1016/j.ajhg.2009.01.011.
619
620 [46] Meng Y, Zhang W, Zhou J, Liu M, Chen J, Tian S, et al. Genome-wide analysis of
621 positively selected genes in seasonal and non-seasonal breeding species. PLoS One
622 2015;10:e0126736. https://doi.org/10.1371/journal.pone.0126736.
623
624 [47] Smith EF, Yang P. The radial spokes and central apparatus: mechano-chemical transducers
625 that regulate flagellar motility. Cell Motil Cytoskeleton 2004;57:8-17.
626 https://doi.org/10.1002/cm.10155.
627

1 Este artigo está submetido na Theriogenology, seguindo suas normas de apresentação.

628 [48] Wemmer KA, Marshall WF. Flagellar motility: all pull together. Curr Biol 2004;14:R992–3.
629 https://doi.org/10.1016/j.cub.2004.11.019.
630
631 [49] Imai H, Suzuki K, Ishizaka K, Ichinose S, Oshima H, Okayasu I, et al. Failure of the
632 expression of phospholipid hydroperoxide glutathione peroxidase in the spermatozoa of human
633 infertile males. Biol Reprod 2001;64:674-83. https://doi.org/10.1095/biolreprod64.2.674.
634
635 [50] Stradaioli G, Sylla L, Monaci M, Maiorino M. Phospholipid hydroperoxide glutathione
636 peroxidase in bull spermatozoa provides a unique marker in the quest for semen quality analysis.
637 Theriogenology 2009;72:91-8. https://doi.org/10.1016/j.theriogenology.2009.02.006.
638
639 [51] Hardy DM, Oda MN, Friend DS, Huang TT Jr. A mechanism for differential release of
640 acrosomal enzymes during the acrosome reaction. Biochem J 1991;275:759-66.
641 https://doi.org/10.1042/bj2750759.
642 [52] Hao Z, Wolkowicz MJ, Shetty J, Klotz K, Bolling L, Sen B, et al. SAMP32, a testis-specific,
643 isoantigenic sperm acrosomal membrane-associated protein. Biol Reprod 2002;66:735-44.
644 https://doi.org/10.1095/biolreprod66.3.735.
645
646 [53] Ogura Y, Takagishi Y, Harayama H. Changes in the distribution and molecular mass of boar
647 sperm acrosome-associated 1 proteins during the acrosome reaction; their validity as indicators
648 for occurrence of the true acrosome reaction. Anim Reprod Sci 2016;172:94-104.
649 https://doi.org/10.1016/j.anireprosci.2016.07.006.
650
651 [54] Mandal A, Klotz KL, Shetty J, Jayes FL, Wolkowicz MJ, Bolling LC, et al. SLLP1, a
652 unique, intra-acrosomal, non-bacteriolytic, c lysozyme-like protein of human spermatozoa. Biol
653 Reprod. 2003;68:1525-37. https://doi.org/10.1095/biolreprod.102.010108.
654
655 [55] Kishida K, Harayama H, Kimura F, Murakami T. Individual differences in the distribution
656 of sperm acrosome associated 1 proteins among male patients of infertile couples; possible their
657 impacts on outcomes of conventional *in vitro* fertilization. Zygote 2016;24:654-61.
658 https://doi.org/10.1017/S0967199415000623.

1 Este artigo está submetido na Theriogenology, seguindo suas normas de apresentação.

659

660 [56] Fujihara Y, Satouh Y, Inoue N, Isotani A, Ikawa M, Okabe M. SPACA1-deficient male mice
661 are infertile with abnormally shaped sperm heads reminiscent of globozoospermia. Development
662 2012;139:3583-89. https://doi.org/10.1242/dev.081778.
663
664 [57] Yanagimachi R. Sperm-egg fusion. Curr Top Membr Trans 1998;32:3-43.
665 https://doi.org/10.1016/S0070-2161(08)60129-X.
666
667 [58] Xu W, Hu H, Wang Z, Chen X, Yang F, Zhu Z, et al. Proteomic characteristics of
668 spermatozoa in normozoospermic patients with infertility. J Proteome Res 2012;75:5426-36.
669 https://doi.org/10.1016/j.jprot.2012.06.021.
670
671 [59] Inoue N, Yamaguchi R, Ikawa M, Okabe M. Sperm-egg interaction and gene manipulated
672 animals. Soc Reprod Fertil Suppl 2007;65:363-71.
673
674 [60] Inoue N, Ikawa M, Isotani A, Okabe M. The immunoglobulin superfamily protein Izumo is
675 required for sperm to fuse with eggs. Nature 2005;434:234-38.
676 https://doi.org/0.1038/nature03362.
677
678 [61] Wang M, Lv Z, Shi J, Hu Y, Xu C. Immunocontraceptive potential of the Ig-like domain of
679 Izumo. Mol Reprod Dev 2009;76:794-801. https://doi.org/10.1002/mrd.21027.
680
681 [62] Ellerman D, Pei J, Gupta S, Snell W, Myles D, Primakoff P. Izumo is a part of a
682 multiprotein family whose members form large complexes on mammalian sperm. Mol Reprod
683 Dev 2009;76:1188-99. https://doi.org/10.1002/mrd.21092.
684
685 [63] Wen Y, Richardson RT, Widgren, EE, O'Rand MG. Characterization of Sp17: A ubiquitous
686 three domain protein that binds heparin. Biochem J 2001;357:25-31.
687 https://doi.org/10.1042/0264-6021:3570025.
688

1 Este artigo está submetido na Theriogenology, seguindo suas normas de apresentação.

689 [64] Frayne J, Hall L. A re-evaluation of sperm protein 17 (Sp17) indicates a regulatory role in an
690 A-kinase anchoring protein complex, rather than a unique role in sperm-zona pellucida binding.
691 Reproduction 2002;124:767-74. https://doi.org/10.1530/rep.0.1240767.
692
693
694 **Supporting information**
695 **S1 Table. Spectral count of *Saimiri collinsi* sperm protein throughout an entire year (.XLS).**
696 **S2 Table. Sperm proteins of *Saimri collinsi* that participate in important reproductive**
697 **events (.XLS).**

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CONCLUSÕES GERAIS

De um total de 2.342 proteínas identificadas por meio da cromatografia líquida acoplada à espectrometria de massas em tandem dos espermatozoides de *Saimiri collinsi*. Essas proteínas foram relacionadas a importantes eventos reprodutivos como a espermatogênese, motilidade espermática, capacitação e fecundação.

No tocante as eletromicrografias obtidas por microscopia eletrônica de varredura e transmissão, essas imagens nos permitiram caracterizar e avaliar de forma mais ampla os espermatozoides de *S. collinsi*, fator este que nos auxilia na detecção de possíveis anormalidades espermáticas que podem afetar e diminuir a capacidade fecundante dos espermatozoides.

O conhecimento adquirido sobre as proteínas espermáticas juntamente com a análise micromorfológica e ultraestrutural da célula espermática, é um avanço significativo no sentido de ajudar a compreender a biologia reprodutiva do gênero *Saimiri*, pois fornece informações cruciais para a elucidação dos mecanismos subjacentes associados à função espermática, proporcionando ampliar os avanços da pesquisa biotecnológica em reprodução animal para a conservação de espécies ameaçadas de extinção e fornece uma referência para estudos semelhantes sobre outros primatas neotropicais.

Nesse sentido, complementarmente, a busca por parâmetro não invasivo, como a distância anogenital, que auxiliam no exame andrológico para a seleção de primatas não-humanos é de suma importância, visto que podem predizer a baixa qualidade seminal e inviabilizaria o uso destes animais em programas de biotecnologia para a conservação de espécies ameaçadas de extinção.