Universidade Federal do Pará
Núcleo de Ciências Agrárias e Desenvolvimento Rural
Empresa Brasileira de Pesquisa Agropecuária - Amazônia Oriental
Universidade Federal Rural da Amazônia
Programa de Pós-Graduação em Ciência Animal

Karol Guimarães Oliveira

Biometria testicular, caracterização e congelamento de sêmen de macacos-de-cheiro de vida livre (*Saimiri vanzolinii, S. cassiquiarensis* e *S. macrodon*) e cativeiro (*S. collinsi*) em água de coco em pó (ACP-118®)

Belém
2014

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Tese apresentada para obtenção do grau de Doutor em Ciência Animal. Programa de Pós-Graduação em Ciência Animal. Núcleo de Ciências Agrárias e Desenvolvimento Rural. Universidade Federal do Pará. Empresa Brasileira de Pesquisa Agropecuária - Amazônia Oriental. Universidade Federal Rural da Amazônia.
Área de concentração: Produção Animal.
Orientador: Profa. Dra. Sheyla F. S. Domingues

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MS - SECRETARIA DE VIGILÂNCIA EM SAÚDE

INSTITUTO EVANDRO CHAGAS

Parecer de Aprovação Nº 0010/2011/CEPAN/IEC/SVS/MS

Protocolo CEPAN - nº 0005/2011

Ananindeua/PA, 10 de maio de 2011.

Projeto: **"Congelação de sêmen de macaco-de-cheiro (*Saimiri sciureus*) em diluidores à base de água de coco em pó (ACP® -118) e TES-TRIS em três concentrações de glicerol".**

Pesquisador Responsável: **SHEYLA FARHAYLDES SOUZA DOMINGUES**

Conforme decisão do Comitê de Ética em Pesquisa com Animais-CEPAN do Instituto Evandro Chagas, cientificamos que o projeto acima **foi aprovado.**

Recomendamos ao coordenador responsável que mantenha atualizados todos os documentos pertinentes ao projeto.

Os relatórios parciais deverão ser encaminhados a este Comitê, anualmente, a partir do início do projeto.

Atenciosamente,

Nelson Antonio Bailão Ribeiro

NELSON ANTONIO BAILÃO RIBEIRO
Coordenador do CEPAN/IEC

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Ministério do Meio Ambiente - MMA
Instituto Chico Mendes de Conservação da Biodiversidade - ICMBio
Sistema de Autorização e Informação em Biodiversidade - SISBIO

Autorização para atividades com finalidade científica

Número: 29906-1	Data da Emissão: 22/07/2011 10:24

Dados do titular

Nome: Fernanda Pozzan Paim	CPF: 953.933.740-20
Título do Projeto: UMA ESTRATÉGIA DE CONSERVAÇÃO EX-SITU PARA ESPÉCIES AMEAÇADAS DA VÁRZEA AMAZÔNICA ? BIOTECNOLOGIA DA REPRODUÇÃO COMO MITIGADORA DO IMPACTO DAS MUDANÇAS CLIMÁTICAS	
Nome da Instituição : Instituto de Desenvolvimento Sustentavel Mamiraua	CNPJ: 03.119.820/0001-95

SISBIO

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Captura e coleta de Saimiri sciureus cassiquiarensis	11/2011	11/2011
2	Captura e coleta de Saimiri sciureus macrodon	10/2012	10/2012
3	Captura de Saimiri vanzolinii	10/2013	10/2013

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Equipe

#	Nome	Função	CPF	Doc. Identidade	Nacionalidade
1	Stefânia Araújo Miranda	Veterinária	752.895.472-15	3076625 SEGUP-PA	Brasileira
2	Sheyla Farhayldes Souza Domingues	Veterinária	480.204.273-68	89120020241062 SSP-CE	Brasileira
3	Michele Araujo	Bióloga	062.387.986-70	MG11356667 SSP-MG	Brasileira
4	Fernanda Lopes Roos	Veterinária	007.297.410-95	2080648815 sjs-RS	Brasileira
5	Louise Maranhão de Melo	Veterinária	035.694.314-36	5068643 SSP-PE	Brasileira
6	Helder Lima de Queiroz	Biólogo	245.207.131-53	638709 SSP-DF	Brasileira
7	João Valsecchi do Amaral	Biólogo	268.127.908-93	27320958-9 SSP - SP-SP	Brasileira
8	KAROL GUIMARÃES OLIVEIRA	Bióloga	795.566.042-00	4315710 SEGUP-PA	Brasileira
9	Julianne Silva de Lima	Bióloga	820.341.332-34	4410652 ssp-PA	Brasileira

Locais onde as atividades de campo serão executadas

#	Município	UF	Descrição do local	Tipo
1	UARINI	AM	Reserva de Desenvolvimento SustentÃ¡vel MamirauÃ¡	Fora de UC

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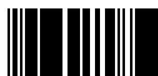

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Autorização para atividades com finalidade científica

Número: 31542-1	Data da Emissão: 02/05/2012 17:41

Dados do titular

Nome: KAROL GUIMARÃES OLIVEIRA	CPF: 795.566.042-00
Título do Projeto: CONGELAÇÃO DO SÊMEN DE MACACO-DE-CHEIRO (Saimiri sciureus) EM DILUIDORES À BASE DE ÁGUA DE COCO EM PÓ (ACP®-118) E TES-TRIS EM TRÊS CONCENTRAÇÕES DE GLICEROL	
Nome da Instituição : UNIVERSIDADE FEDERAL DO PARÁ	CNPJ: 34.621.748/0001-23

SISBIO

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Treinamento laboratorial	04/2012	04/2012
2	Seleção do grupo experimental no Centro Nacional de Primatas	04/2012	05/2012
3	Coleta e congelação do sêmen. Avaliação do protocolo de criopreservação mais eficiente.	05/2012	11/2012
4	Publicação dos resultados preliminares	07/2012	01/2013
5	Coleta e congelação de sêmen no Centro Nacional de Primatas e Bosque Rodrigues Alves	01/2013	07/2013
6	Análise de dados e Publicação dos resultados finais	10/2013	03/2014

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Equipe

#	Nome	Função	CPF	Doc. Identidade	Nacionalidade
1	Regiane Rodrigues dos Santos	Cooperação	730.277.743-87	93002694006 ssp-CE	Brasileira
2	Julianne Silva de Lima	Cooperação	820.341.332-34	4410652 ssp-PA	Brasileira
3	DÉBORA DA VERA CRUZ ALMEIDA	Cooperação	875.101.382-72	5251633 SSP-PA	Brasileira
4	Sheyla Farhayldes Souza Domingues	Orientador	480.204.273-68	89120020241062 SSP-CE	Brasileira
5	DANUZA LEITE LEÃO	Cooperação	948.066.912-91	4841018 SEGUP-PA	Brasileira
6	Stefânia Araújo Miranda	Cooperação	752.895.472-15	3076625 SEGUP-PA	Brasileira
7	Yago Pinto da Silva	Cooperação	009.154.502-16	6159916 P. Civil-PA	Brasileira
8	Adriel Behn de Brito	Cooperação	664.287.422-68	2975003 SEGUP-PA	Brasileira
9	Sarah Scalercio	Cooperação	850.450.632-00	4077026 SEGUP-PA	Brasileira

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Karol Guimarães Oliveira

Biometria testicular, caracterização e congelamento de sêmen de macacos-de-cheiro de vida livre (*Saimiri vanzolinii, S. cassiquiarensis* e *S. macrodon*) e cativeiro (*S. collinsi*) em água de coco em pó (ACP-118®)

Tese apresentada para obtenção do grau de Doutor em Ciência Animal. Programa de Pós-Graduação em Ciência Animal. Núcleo de Ciências Agrárias e Desenvolvimento Rural. Universidade Federal do Pará. Empresa Brasileira de Pesquisa Agropecuária-Amazônia Oriental. Universidade Federal Rural da Amazônia.
Área de concentração: Produção Animal.

Data da aprovação. Belém - PA: 24/06/2014

Banca Examinadora

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Profa. Dra. Sheyla F. S. Domingues (Presidente)
Instituição: UFPA

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Profa. Dra. Anita Stone (Membro Titular)
Instituição UFPA/UFRA/MPEG

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Prof. Dr. Helder Queiroz (Membro Titular)
Instituição: UFPA/IDSM/MPEG

____
Prof. Dr. Moysés Miranda (Membro Titular)
Instituição: UFPA

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Profa. Dra. Simone Damasceno (Membro Titular)
Instituição: UFPA

Aos meus pais e irmão,
com todo amor e carinho.

AGRADECIMENTOS

A DEUS, por permitir que eu chegasse até aqui, por nunca me abandonar e sempre me dar alívio nos momentos difíceis.

À minha mãe Eleonora (Léo), por toda a dedicação, por me apoiar incondicionalmente e sempre estar disposta a me ouvir. Pelas inúmeras injeções de ânimo sempre que eu desanimava, e pelo colo reconfortante (via telefone) quando me desesperava. Por tanto orar por mim e acima de tudo, pelo seu imensurável amor. Tenho plena certeza de que não teria conseguido sem ela. Meu exemplo de ser humano!

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Aos membros da banca examinadora: Profa. Dra. Anita Stone, Prof. Dr. Helder Queiroz, Prof. Dr. Moysés Miranda e Profa. Dra. Simone Damasceno pela honra da presença e pelas valiosas contribuições.

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A Sarah Scalercio pelo incentivo e companheirismo, muito importantes nos momentos decisivos do desenvolvimento da tese.

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Ao Rodrigo Rodrigues, amigo e funcionário do PPGCA, pela disponibilidade e profissionalismo.

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Ao Centro Nacional de Primatas (IEC-SVS) e ao Instituto de Desenvolvimento Sustentável Mamirauá, pelo apoio financeiro e logístico, fundamentais para a realização desta pesquisa.

À UFPA e ao Programa de Pós-Graduação em Ciência Animal.

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RESUMO

Recentemente, o táxon *Saimiri sciureus* (macaco-de-cheiro) foi revisado e algumas subespécies são agora classificadas, por alguns autores, como espécies, por exemplo: o *Saimiri collinsi*, o *S. cassiquiarensis* e o *S. macrodon*. Características fenotípicas nestas espécies são bem determinadas. No entanto, informações sobre aspectos reprodutivos são ainda bastante escassas. O objetivo da primeira fase deste trabalho foi descrever e correlacionar a biometria testicular com a qualidade do sêmen de macacos-de-cheiro de cativeiro (*S. collinsi*) e de vida-livre (*S. vanzolinii, S. cassiquiarensis* e *S. macrodon*). Foram mensurados comprimento, largura, altura, circunferência e volume testiculares, bem como motilidade, vigor, integridade de membrana plasmática e morfologia espermática para a correlação de dados. Não foi observada diferença entre os testículos direito e esquerdo dentro de cada espécie. Nem diferença entre as espécies com relação às medidas testiculares. O sêmen, que foi coletado por meio de eletroejaculação com probe retal, foi constituído por uma fração coagulada e uma fração líquida ou ambas. A qualidade do ejaculado foi semelhante entre as espécies. Não sendo observada correlação entre o volume testicular e o volume seminal de *S. collinsi* e *S. vanzolinii*, tanto com relação à fração líquida quanto à coagulada. A segunda fase do trabalho teve como objetivo propor um protocolo de congelamento de sêmen de *S. collinsi* em diluidor a base de ACP-118® (água de coco em pó) testando duas concentrações de glicerol (1,5 e 3%), em laboratório. E a terceira fase objetivou testar um protocolo de resfriamento de sêmen sem adição de gema de ovo, seguido do congelamento com a melhor concentração de glicerol observada no experimento anterior, e aplicar esses protocolos testados em *S. collinsi* mantidos em cativeiro, nas espécies *S. vanzolinii, S. cassiquiarensis* e *S. macrodon* em condições de campo. O sêmen de *S. collinsi* pôde ser resfriado em diluidor a base de ACP-118® sem ovo gema. O protocolo de congelamento desenvolvido demonstrou ser útil para a criopreservação de sêmen de *S. collinsi*, embora requeira melhoria para permitir melhor manutenção da qualidade espermática após o descongelamento. O procedimento demonstrou ser aplicável também em condições de campo em animais de vida livre, fornecendo resultados bastante animadores para a formação de bancos de germoplasma de espécies vulneráveis à extinção, como o *S. vanzolinii.*

Palavras-chave: Macaco-de-cheiro. Sêmen. Eletroejaculação. Criopreservação. ACP.

ABSTRACT

Recently, *Saimiri sciureus* taxon (squirrel monkey) was revised and some subspecies are now classified by some authors as species, for example: *Saimiri collinsi, S. cassiquiarensis* and *S. macrodon*. Phenotypically these species are well determined. However, information on reproductive aspects is still quite scarce. The objective of the first phase of this study was to describe and correlate the testis with the seminal quality from captive (*S. collinsi*) and free-living (*S. vanzolinii, S. cassiquiarensis* and *S. macrodon*) squirrel monkeys. Length, width, height, circumference and testicular volume were measured as well as motility, vigour, plasma membrane integrity and morphology, for correlation data. No difference was observed between the right and left testicles within species, neither difference between species concerning to testicular measures. Semen, which was collected by electroejaculation with rectal probe, was constituted by a coagulated fraction, a liquid fraction or both. Seminal quality was similar among species. There is no correlation between testicular volume and seminal volume (of liquid or coagulated fraction) in *S. collinsi* and *S. vanzolinii*. The second phase of this work aimed to propose a semen freezing protocol in an extender based in ACP-118® (powdered coconut water), testing two glycerol concentrations (1.5 and 3%) for captive *S. collinsi.* The third phase aimed to test a semen cooling protocol without addition of egg yolk (extracellular cryoprotector) followed by freezing with the best glycerol concentration observed in the preceding experiment and apply these protocols, previously tested in captive *S. collinsi,* in free-living males from *S. vanzolinii, S. cassiquiarensis* and *S. macrodon* in field conditions. Semen from *S. collinsi* could be cooled in ACP-118® without egg yolk. The freezing protocol developed proved to be useful for semen cryopreservation of *S. collinsi*, although requiring improved to allow better maintenance of sperm quality after thawing. The procedure proved to be also applicable under field conditions on free-living animals, providing very encouraging results for the formation of genebanks for species vulnerable to extinction as the *S. vanzolinii*.

Key Words: Squirrel monkey. Semen. Electroejaculation. Cryopreservation. ACP.

SUMÁRIO

1 INTRODUÇÃO 13
2 OBJETIVOS 15
2.1 GERAL 15
2.2 ESPECÍFICOS 15
3 REVISÃO DE LITERATURA 16
3.1 CLASSIFICAÇÃO E MORFOLOGIA DE PRIMATAS DO NOVO MUNDO 16
3.2 O GÊNERO *Saimiri* (VOIGT, 1831) 16
3.2.1 A espécie *S. cassiquiarensis* 18
3.2.2 A espécie *S. macrodon* 19
3.2.3 A espécie *S. vanzolinii* 19
3.2.4 A espécie *S. collinsi* 20
3.3 ASPECTOS REPRODUTIVOS DE *Saimiri* 21
3.3.1 Sazonalidade reprodutiva 21
3.3.2 Características do aparelho reprodutor masculino 22
3.4 O COÁGULO SEMINAL DE PRIMATAS 23
3.4.1 **O sêmen de *Saimiri*** 25
3.5 MÉTODOS DE COLETA DE SÊMEN DE PRIMATAS 25
3.6 A CÉLULA ESPERMÁTICA 27
3.6.1 **O espermatozoide de *Saimiri*** 28
3.7 CRIOPRESERVAÇÃO DO SÊMEN 28
3.7.1 **O meio diluidor seminal e seus componentes** 30
3.7.1.1 Crioprotetores 30
3.7.1.2 Diluidor a base de água de coco em pó (ACP®) 32
4 CAPÍTULO 1- TESTICULAR BIOMETRY AND SEMEN CHARACTERISTICS IN CAPTIVE AND WILD SQUIRREL MONKEY SPECIES (*Saimiri* sp.) ……….. 35
5 CAPÍTULO 2- SEMINAL CHARACTERISTICS AND CRYOPRESERVATION OF SPERM FROM THE SQUIRREL MONKEY, *Saimiri collinsi* 65
6 CAPÍTULO 3- COOLING AND FREEZING OF SPERM FROM CAPTIVE, FREE-LIVING AND ENDANGERED SQUIRREL MONKEY SPECIES 89
7 CONCLUSÕES ……….. 111
REFERÊNCIAS 112

1 INTRODUÇÃO

Os primatas Neotropicais compõem um grupo bastante diversificado e ecologicamente importante, mas que vem enfrentando sérios problemas de conservação, como o aquecimento global, a destruição ou fragmentação de *habitats*, a introdução de espécies exóticas nos ecossistemas, a caça ou outras pressões antrópicas, que provocam uma drástica diminuição ou até mesmo a extinção de espécies antes mesmo de serem conhecidas (FICKEL et al., 2007; TORRE, 2012). Esse cenário pode ser atenuado através da manutenção da variabilidade genética desses animais, em seus *habitats* naturais (*in situ*), ou pela manutenção das condições adequadas em cativeiro (*ex situ*), e ainda, pela formação de bancos de germoplasma, no qual gametas e células somáticas podem ser utilizados em programas de biotecnologia reprodutiva (DOMINGUES; CALDAS-BUSSIERE, 2006).

O macaco-de-cheiro (*Saimiri* sp.) é um dos primatas Neotropicais mais comumente utilizados em pesquisas nas mais diversas áreas do conhecimento devido às suas características físicas, como o reduzido tamanho corporal e a facilidade de manutenção em cativeiro (ABEE, 2000). A espécie *S. sciureus*, cujo *status* de conservação é menos preocupante do que de outras espécies do gênero, tem sido há anos utilizada como modelo experimental para o estudo da fisiologia e desenvolvimento de biotécnicas da reprodução (DOMINGUES; CALDAS-BUSSIERE, 2006), como coleta de sêmen por electroejaculação (BENNETT, 1967a), por vibroestimulação peniana (KUGELMEIER, 2011), inseminação artificial (BENNETT, 1967b) e estimulação hormonal ovariana (BENNETT, 1967c), fecundação *in vitro* (CLINE, 1972; GOULD et al.,1973; KUEHL; DUKELOW, 1975a) e maturação *in vitro* de oócitos (KUEHL; DUKELOW, 1975b). Embora haja um grande número de pesquisas com a espécie, pouco se tem avançado com relação às técnicas de conservação de gametas, sendo que somente um trabalho descreve a criopreservação de gameta masculino desse gênero (DENIS et al.,1976).

Uma recente reclassificação taxonômica sofrida por essa espécie elevou três subespécies ao nível de espécie: *S. collinsi, S. cassiquiarensis, S. macrodon* (LAVERGNE et al., 2010; MERCÊS, 2013; MITTERMEIER et al., 2013). Isso estimulou ainda mais a necessidade de estudos relacionados a aspectos reprodutivos específicos para cada espécie do gênero *Saimiri*, uma vez que a maioria desses estudos é relacionada às espécies *S. sciureus* e *S. boliviensis.*

Nesse contexto, as seguintes hipóteses científicas foram propostas e testadas: o volume testicular e o volume do ejaculado das espécies de *Saimiri* estudadas são medidas positivamente correlacionadas; o sêmen de *Saimiri collinsi* não coagula totalmente após a

ejaculação; é possível congelar o sêmen de *Saimiri* em diluidor a base de água de coco em pó (ACP®) utilizando as concentrações de glicerol 1,5 ou 3% e obter bons resultados pós-congelamento; o sêmen de *Saimiri* suporta o resfriamento sem a utilização da gema de ovo; é possível executar os protocolos de criopreservação de sêmen desenvolvidos em laboratório nas condições de campo, nas espécies de vida livre.

Desta forma, a proposta desse trabalho é contribuir para o conhecimento da fisiologia reprodutiva de machos das espécies *S. collinsi* de cativeiro e *S. vanzolinii, S. cassiquiarensis* e *S. macrodon* de vida-livre, visando o aprimoramento do método de seleção de animais para pesquisas em reprodução, por meio da avaliação de características físicas, como massa corporal, volume testicular e da qualidade do sêmen, bem como o desenvolvimento de um método de criopreservação do sêmen dessas espécies.

2 OBJETIVOS

2.1 GERAL

Ampliar os conhecimentos acerca da fisiologia reprodutiva de machos das espécies *Saimiri collinsi* (em cativeiro), *S. vanzolinii, S. cassiquiarensis* e *S. macrodon* (de vida-livre) visando o desenvolvimento de biotécnicas de reprodução.

2.2 ESPECÍFICOS

- Descrever parâmetros testiculares (comprimento, largura, altura, circunferência e volume) e aspectos seminais (características macro e microscópicas) nos machos das espécies *S. vanzolinii, S. cassiquiarensis*, *S. macrodon,* de vida livre e *S. collinsi*, criados em cativeiro;

- Investigar correlações entre as espécies no que diz respeito aos aspectos seminais e testiculares;

- Propor um protocolo de congelamento de sêmen de *S. collinsi* em diluidor a base de ACP-118® (água de coco em pó) testando duas concentrações de glicerol (1,5 e 3%);

- Propor um protocolo de resfriamento de sêmen diluído ACP-118® sem adição de gema de ovo;

- Testar em *S. vanzolinii, S. cassiquiarensis* e *S. macrodon* de vida livre os protocolos de criopreservação de sêmen desenvolvidos nos animais de cativeiro (*S. collinsi*).

3 REVISÃO DE LITERATURA

3.1 CLASSIFICAÇÃO E MORFOLOGIA DE PRIMATAS DO NOVO MUNDO

Os primatas do Novo Mundo (infraordem Platyrrhini) atualmente estão distribuídos entre 152 espécies (204 espécies e subespécies), 20 gêneros e cinco famílias: Cebidae, Aotidae, Pitheciidae, Atelidae e Callitrichidae (RYLANDS et al., 2012) . Nos últimos 30 anos, o número de espécies têm aumentado consideravelmente, principalmente, devido à adoção do conceito filogenético de espécie, estudos citogenéticos e biomoleculares, que contribuiram para a descoberta de 31 novas espécies e subespécies (RYLANDS et al., 2012).

De um modo geral, esses animais caracterizam-se morfologicamente pela presença de três pré-molares, nariz achatado e narinas abertas para os lados. A cauda, em alguns gêneros, apresenta-se preênsil e é geralmente longa, com exceção do gênero *Cacajao* (Uacari) que possui cauda mais curta (GROVES, 2005).

3.2 O GÊNERO *Saimiri* (VOIGT, 1831)

O gênero *Saimiri* tem taxonomia ainda não claramente determinada. Estudos biomoleculares e morfológicos apresentam resultados ainda conflitantes em relação à sua classificação taxonômica e distribuição geográfica (LAVERGNE et al., 2010; PAIM et al., 2013). Portanto, a nomenclatura utilizada neste estudo será a proposta por Lavergne et al. (2010) e Mittermeier et al. (2013), que consideram as seguintes espécies de *Saimiri: S. cassiquiarensis, S. vanzolinii, S. macrodon, S. boliviensis, S. sciureus, S. ustus, S. oerstedii* e *S. collinsi,* recentemente validado como táxon

Os macacos-de-cheiro ou macacos-mão-de-ouro, como são popularmente conhecidos os primatas do gênero *Saimiri*, têm ampla distribuição geográfica, ocorrendo em toda a Amazônia, incluindo as áreas de transição com o Cerrado e parte da América Central (HERSHKOVITZ, 1984; SILVA JÚNIOR, 1992). As maiores densidades populacionais concentram-se em florestas alagáveis e margens de corpos d'água (SILVA JÚNIOR, 1992). São primatas arborícolas, ocupando o estrato médio da floresta e ocasionalmente descendo até o solo ou ocupando estratos mais elevados da copa (BOINSKI, 1989).

Quanto à pelagem, são caracterizados pela coroa e dorso amarelo-acinzentados, braços, pernas, mãos e pés amarelo-alaranjados, e no terço distal da cauda possuem um pincel negro. A boca apresenta-se com contorno na cor preta (Fig. 1), e na região orbital apresentam arcos superciliares que, segundo Hershkovitz (1984), classificariam as espécies em dois grupos: **Romano**, com pêlos brancos ao redor dos olhos e pêlos escuros na cabeça formando

um padrão arredondado entre os olhos (Fig 2 A), e **Gótico**, formando um “V” profundo entre os olhos (Fig 2 B).

Figura 1- Exemplares do gênero *Saimiri* em ambiente natural: *Saimiri* sp. (A) e *S. vanzolinii* (B) e em cativeiro: *S. collinsi* (C e D).
Fonte: photographersdirect.com (A); Fernanda Paim (B) e Tatyana Pinheiro (C e D).

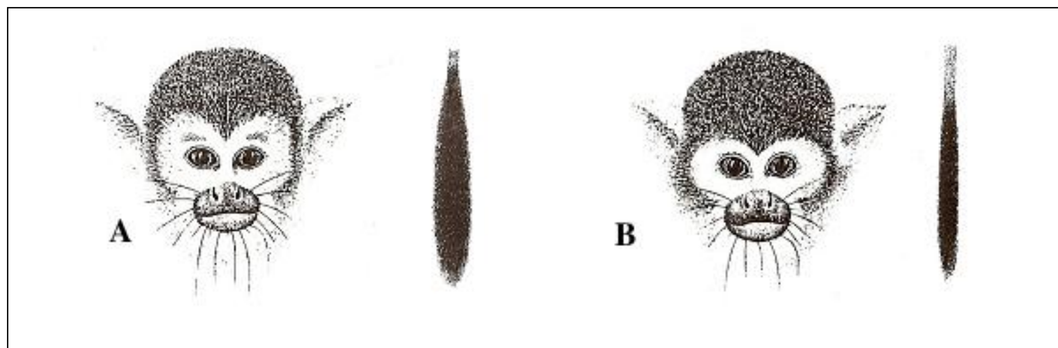

Figura 2- Arcos superciliares e pincel caudal do grupo Gótico (A) e Romano (B).
Fonte: Hershkovitz (1984) *apud* Paim (2008).

São animais onívoros, alimentando-se basicamente de frutos, pequenos vertebrados ou flores (MITTERMEIER; VAN ROOSMALEN, 1981; STONE, 2007), embora já tenham sido

avistados consumindo folhas em ambiente de semi-cativeiro (RAMALHO et al., 2005). Durante a estação seca, quando a disponibilidade de frutos é menor, os insetos passam a constituir boa parte da dieta (STONE, 2007).

Esses animais são gregários e em ambiente natural formam grandes grupos multimacho e multifêmea de 25 a 75 indivíduos (BOINSKI, 1999) com predominância de fêmeas (MITCHELL, 1990). Em cativeiro geralmente os grupos sociais são mantidos com apenas um ou dois machos por grupo, com o objetivo de evitar agressões entre eles. O tamanho do grupo pode variar de um único par até no máximo 35 a 50 animais, com base nas dimensões físicas do recinto e a que o cativeiro se destina (TARDIF et al., 2006).

A maturidade sexual das fêmeas ocorre por volta de dois e meio a três anos de idade, enquanto os machos atingem a maturidade sexual somente por volta dos cinco anos (TARDIF et al., 2006). As fêmeas têm ciclos estrais muito curtos, com duração média de sete a oito dias, que ocorrem apenas durante a época de acasalamento, e gestação com duração média de 150 (137-175) dias (ROSENBLUM, 1968). A cria nasce com aproximadamente 15% do peso corporal da mãe (DUKELOW), justificando a gestação de um único filhote por ano (BALDWIN, 1985). Apesar disso, o seu desenvolvimento é bastante rápido, de forma que cinco a seis meses após o nascimento, os filhotes já estão completamente desmamados (ROSENBLUM, 1968).

3.2.1 A espécie *S. cassiquiarensis*

Espécie cuja distribuição abrange a Amazônia colombiana, o sul da Venezuela e Amazônia brasileira ao norte do Rio Solimões (Figura 3) (MITTERMEIER et al., 2013).

No que diz respeito à reprodução, a espécie *S. cassiquiarensis* segue o padrão sazonal do gênero. Durante a gravidez e a amamentação, as fêmeas são menos ativas, se alimentam mais lentamente e descansam mais que os outros membros do grupo, e tendem a manter associações com grupos de outra espécie de primata, *Sapajus macrocephalus* (MITTERMEIER et al., 2013).

De acordo com a Red List (2010) da International Union for Conservation of Nature (IUCN) seu *status* de conservação é pouco preocupante.

Figura 3- Distribuição geográfica da espécie *Saimiri cassiquiarensis.*
Fonte: Mittermeier et al. (2013).

3.2.2 A espécie *S. macrodon*

Distribui-se do oeste da Amazônia brasileira, estendendo-se à parte da Colômbia, leste do Equador, ao norte e a leste do Peru (Figura 4). São bastante semelhantes ao *Saimiri sciureus*, porém apresentam o dorso mais escuro (MITTERMEIER et al., 2013). O *status* de conservação dessa espécie é considerado pouco preocupante (IUCN, 2010).

Figura 4- Distribuição geográfica da espécie *Saimiri macrodon*.
Fonte: Mittermeier et al. (2013).

3.2.3 A espécie *S. vanzolinii*

Os animais desse gênero têm uma das menores distribuições geográficas de todas as espécies de primatas Neotropicais, em torno de 870 km², restrita à porção sudeste da Reserva de Desenvolvimento Sustentável Mamirauá na Amazônia brasileira, ao norte do Rio Solimões (Figura 5). Esse grau de endemismo confirma a extrema vulnerabilidade dessa espécie à

extinção (IUCN, 2013; PAIM et al., 2013). Estimativas recentes indicam uma população mínima de 5.500-10.900 indivíduos. A possibilidade de híbridos e uma progressiva invasão de sua distribuição natural têm causado preocupação (MITTERMEIER et al., 2013).

Figura 5- Distribuição geográfica da espécie *Saimiri vanzolinii.*
Fonte: Mittermeier et al. (2013).

Sem dimorfismo sexual, ambos os sexos apresentam porte ligeiramente reduzido. (MITTERMEIER et al., 2013).

3.2.4 A espécie *S. collinsi*

A distribuição geográfica de *S. collinsi* abrange o arquipélago do Marajó e a região situada ao sul do rio Amazonas, entendendo-se até o Maranhão, nas matas de transição entre a Amazônia e o Cerrado. Ainda não se tem bem definida qual seria sua limitação ao sul. A porção oeste da distribuição de *S. collinsi* se estende até a margem esquerda do rio Tapajós, sendo o município de Juruti-PA a localidade mais ocidental (MERCÊS, 2013) (Figura 6).

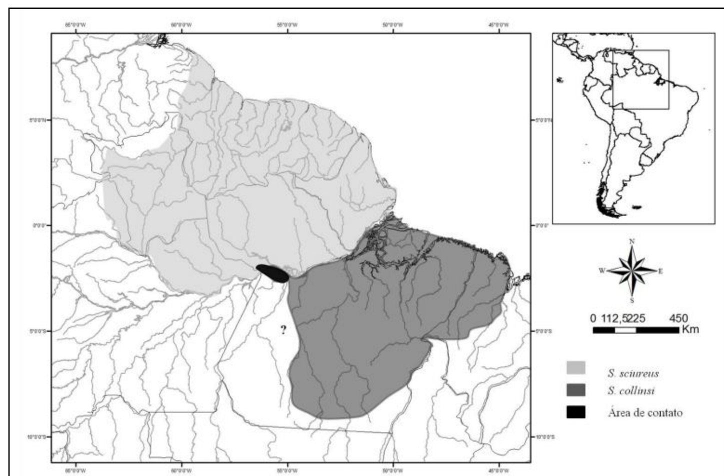

Figura 6- Distribuição geográfica da espécie *Saimiri collinsi* (cinza escuro).
Fonte: Mercês (2013).

3.3 ASPECTOS REPRODUTIVOS DE *Saimiri*

3.3.1 Sazonalidade reprodutiva

Os macacos-de-cheiro possuem a reprodução mais sazonal dentre os primatas Neotropicais (DI BITETTI; JANSON, 2000). De maneira geral, as temporadas de nascimentos são curtas e ocorrem geralmente durante a estação chuvosa, pouco antes ou durante o pico de disponibilidade de frutas e insetos (TREVINO, 2007). Na região amazônica, os acasalamentos ocorrem na estação seca e o final da gestação, parto e lactação coincidem com a estação mais chuvosa, de forma que o nascimento da progênie culmine com o período mais favorável à sua sobrevivência, devido à disponibilidade de alimentos (DUKELOW, 1983; STONE, 2006).

Boinski (1987) define uma hierarquia de dois fatores seletivos que afetam a estação de nascimentos de *S. oerstedi*. A disponibilidade de alimentos como fator principal, que estabelece o período durante o qual a maioria dos nascimentos ocorrerá, e a pressão de predação, como um segundo fator, que promove alta sincronia de nascimento dentro dos grupos durante o período de abundância de alimentos. Macacos-de-cheiro de vida livre exibem também um aumento na sazonalidade reprodutiva (decréscimo da duração da reprodução) com a latitude, devido à disponibilidade de recursos ser imprevisível (DI BITETTI; JANSON, 2000; TREVINO, 2007).

Estudando *S. boliviensis*, Dumond (1968) encontrou uma correlação positiva entre a quantidade de chuva e a estação reprodutiva, tanto em animais de vida livre (Amazônia) quanto de cativeiro (sul da Flórida). Entretanto, segundo Trevino (2007), em cativeiro os padrões de sazonalidade reprodutiva dos macacos-de-cheiro variam de acordo com o tipo de recinto. Em recintos onde os animais sejam expostos a elementos ambientais (condições de semi-cativeiro) eles exibem estações bem definidas de acasalamentos e nascimentos, restritas a uma faixa de meses. Porém, quando mantidos sob condições controladas, tendem a continuar acasalando e reproduzindo durante todo o ano.

Além dos fatores ambientais, diversos processos fisiológicos e comportamentais regulam o padrão anual do ciclo reprodutivo desses primatas. Acredita-se que uma comunicação social complexa esteja envolvida na sincronização da atividade sexual de ambos os sexos (COE; ROSENBLUM, 1978). Segundo Baldwin (1970) as fêmeas seriam mais sensíveis aos fatores ambientais e estimulariam sexualmente os machos através de feromônios e/ou outros canais de comunicação. Antes da temporada de acasalamento os machos adultos, individualmente ou em grupo, são observados aproximando-se das fêmeas adultas para cheirar seus órgãos genitais e outras partes do corpo, vocalizando e exibindo ereção peniana.

Uma forma de comunicação utilizada por *S. boliviensis*, tanto por machos quanto por fêmeas, porém mais comum em machos, é o "display" genital, que consiste na abertura de uma ou ambas as coxas e exibição da genitália. Esse é um comportamento observado com maior frequência durante a estação de acasalamento (NEWMAN, 1985).

A estação de acasalamentos é precedida e acompanhada por alterações morfofisiológicas e comportamentais tanto nos machos quanto nas fêmeas (CHEN et al., 1981). Nos machos de todas as espécies de *Saimiri* estudadas até o momento, tanto em vida livre quanto em cativeiro, ocorre um fenômeno conhecido como *fatting*, supostamente relacionado à seleção sexual (STONE, 2014). Esse fenômeno consiste no aumento do peso corporal (85 a 222 g) (DUMOND; HUTCHISON, 1967) através do acúmulo de gordura e retenção água, principalmente na região superior do dorso, membros superiores e ombros durante período de acasalamento (MITCHELL, 1990; STONE, 2004), que varia de 2 a 8 semanas (IZAR et al., 2008). Essa condição de *fatting* é restrita à estação reprodutiva, fora desse período o macho demonstra menor interesse pela fêmea e assume uma posição menos central dentro do grupo (BALDWIN, 1985).

O estado de *fatting* não está relacionado ao consumo de alimentos (STONE, 2004). Ele é controlado por um aumento sazonal de testosterona e estrogênio, além da elevação dos níveis de hormônios tireoidianos (COE et al., 1985), cortisol (SCHIML et al., 1996) e androstenediona (CHEN et al., 1981). Sendo que o pico da concentração de testosterona coincide com a fase em que as fêmeas retomam a atividade ovariana e a posterior redução da concentração desse hormônio provoca um declínio da frequência de cópulas e de ejaculações (WIEBE et al., 1988). Essas mudanças endócrinas estariam relacionadas também ao aumento sazonal da espermatogênese, do volume do ejaculado e do tamanho testicular (DUMOND; HUTCHISON, 1967; BALDWIN, 1970; CHEN et al., 1981).

3.3.2 Características do aparelho reprodutor masculino

O pênis de *Saimiri* tem comprimento médio de 21 mm (LOPES, dados não publicados), apresenta osso peniano (báculo) que em *Saimiri boliviensis* mede cerca de 9,0 mm (DIXSON, 1987) e algumas espículas queratinizadas vestigiais laterais ao seu corpo, sendo encoberto por um prepúcio retrátil. A bolsa escrotal é semi pendulosa e assimétrica, proporcionalmente grande considerando-se o porte desses animais (STEINBERG et al., 2005). Testículo e epidídimo juntos formam uma massa relativamente pequena e globular. Na borda ventral do testículo podem ser observadas veias helicoidais e proeminentes. As vesículas seminais são tubulares e não ramificadas. A próstata localiza-se na parte proximal

da uretra, imediatamente distal à bexiga (HILL, 1960) (Figura 7). Poucos trabalhos se concentraram na biometria testicular desse gênero.

Figura 7- Aparelho reprodutor masculino de *S. cassiquiarensis.*
Fonte: Cedido por Gerson Lopes (dados não publicados).

3.4 O COÁGULO SEMINAL DE PRIMATAS

O sêmen de diversas espécies de primatas apresenta-se sob a forma de um coágulo que supostamente seja formado a partir de reações das enzimas prostáticas com as proteínas, frutose e outros numerosos compostos bioquímicos constituintes do líquido seminal (DIXSON; ANDERSON, 2001, 2002).

O coágulo seminal pode assumir diferentes consistências conforme o regime de acasalamento da espécie. Sendo assim, em regimes monogâmicos ou poligínios, ambos em que a fêmea tem somente um parceiro sexual, o coágulo tende a ser menos consistente ou até mesmo ausente, em contraste a regimes poligâmicos (quando não há o estabelecimento de casais) em que atinge consistência quase sólida. Essa pressão de seleção sexual teria levado também ao desenvolvimento de testículos maiores em relação ao peso corporal, permitindo uma maior capacidade de produção espermática devido à maior quantidade de túbulos seminíferos (DIXSON; ANDERSON, 2001).

O coágulo também pode servir como um veículo dos espermatozóides para o interior da cérvix da fêmea, constituindo uma proteção física e química, pois apresenta pH alcalino,

aumentando o índice de sobrevivência dos espermatozóides no meio ácido vaginal (DIXSON; ANDERSON, 2001).

Hernández-López et al. (2008), analisando o ejaculado de *Atelles geoffroyi* durante duas épocas do ano, verificaram aumento do volume de coágulo seminal durante as estações secas em relação às chuvosas. Há evidências de que a produção desse coágulo seja um processo que exija algum gasto energético pelo macho, pois é consideravelmente maior quando as fêmeas encontram-se mais receptivas ao coito do que nas outras épocas do ano (HERNÁNDEZ-LÓPEZ et al., 2008). Entretanto, ainda inexistem estudos suficientes para elucidar o processo de produção do coágulo seminal entre as espécies de primatas (DIXSON; ANDERSON, 2002).

Entretanto, o coágulo seminal se tornou um dos principais problemas da avaliação e processamento do sêmen da maioria dos primatas (VALLE et al., 2004; ARAÚJO et al., 2009; OLIVEIRA et al., 2011). Diversas pesquisas são realizadas com o intuito de promover sua dissolução e maximizar seu aproveitamento.

Alguns protocolos sugerem a liquefação desta fração por meio da imersão do tubo coletor em banho-maria a 37 °C por 30 minutos para o sêmen de *Macaca fascicularis* (macaco-cauda-longa) (LI et al., 2003; LI et al., 2005b). Valle e colaboradores (2004) alcançaram a dissolução do coágulo de *Alouatta caraya* (bugio preto e dourado) após incubação a 37º C por 30 minutos em ringer lactato. Outros recomendam a liquefação enzimática do coágulo seminal em pronase e quimotripsina, como descrito para *C. jacchus* (MORRELL; HODGES, 1998). Para a espécie *A. geoffroyi* é descrita a utilização das enzimas tripsina ou a associação de tripsina e hialuronidase, embora ambas tenham afetado a motilidade e o vigor espermático, além de não desfazerem totalmente o coágulo (HERNÁNDEZ-LÓPEZ et al., 2002).

Algumas técnicas para dissolução do coágulo seminal de *Sapajus apella* (macaco-prego) também foram descritas. Paz et al. (2006) utilizaram meio TCM 199 acrescido das enzimas tripsina e hialuronidase e observaram que em ambos os casos houve uma redução significativa da motilidade e do vigor espermático em comparação à fração líquida de acordo com o tempo de exposição, além de não promoverem a completa dissolução do coágulo. Alguns protocolos sugerem sua dissolução sem o uso de enzimas proteolíticas, utilizando apenas solução salina a 0,9% (NAGLE; DENARI, 1983), diluidor a base de água de coco *in natura* (ARAÚJO et al., 2009), ou em pó (OLIVEIRA et al., 2010), todos associados à fragmentação mecânica do coágulo com o uso de pipeta e incubação em banho-maria a 37 °C por 2 horas, quando se atingiu a completa dissolução do coágulo seminal.

3.4.1 O sêmen de *Saimiri*

Nos primeiros estudos acerca do sêmen dos animais desse gênero ele é descrito como sendo constituído por duas porções distintas: uma fração líquida e rica em espermatozoides; e outra coagulada, transparente, incolor e azoospérmica (BENNETT, 1967). Porém, atualmente sabe-se que o sêmen de *Saimiri*, assim como o de diversas outras espécies de primatas, tem consistência líquida, sofrendo completa coagulação imediatamente após a ejaculação (DUKELOW, 1983; SCHNEIDERS et al., 2004; VALLE et al., 2004; WOLF, 2009).

Algumas tentativas de dissolução desse coágulo seminal foram descritas para *S. sciureus* e *S. boliviensis*. Por meio da incubação a 37 ºC ou em temperatura ambiente, por 30 a 60 minutos, utilizando ou não enzimas proteolíticas (BENNETT, 1967; ACKERMAN; ROUSSEL, 1968; DENIS et al.,1976; YEOMAN et al., 1998) e mais recentemente, Kugelmeier (2011) testou três concentrações de tripsina (0,5; 1 e 5%) associadas à incubação, porém essa enzima não foi eficaz na dissolução do coágulo, embora não tenha causado danos significativos aos espermatozoides.

3.5 MÉTODOS DE COLETA DE SÊMEN DE PRIMATAS

A primeira tentativa bem sucedida de coletar sêmen de primatas não-humanos foi relatada por Mastroianni e Manson (1963) com *Macaca mulatta*. Foi utilizada uma corrente alternada monofásica aplicada através de dois eletrodos, um na base do pênis e outro contra o aspecto ventral da glande próximo ao frênulo. Weisbroth e Young (1965) posteriormente descreveram o uso de uma probe retal para estimular a ejaculação de sêmen em várias espécies primatas do Velho Mundo.

Atualmente existem diversos outros métodos de coleta de sêmen de primatas não-humanos, como vagina artificial, lavagem vaginal após a cópula, punção do epidídimo, masturbação e estimulação peniana. A micropunção de cabeça e cauda de epidídimo é um método usualmente empregado em situações especiais, como em animais recém-falecidos, e foi descrito em algumas espécies do Velho e Novo Mundo: *M. fascicularis* (MAHONY et al., 1993), *Pan troglodytes* (YOUNG et al.,1994), *M. fuscata* (SANKAI et al., 1997) e *C. jacchus* (MORRELL et al., 1997). A técnica de vagina artificial e masturbação são métodos que exigem o condicionamento do animal, uma vez que é necessário estimulá-lo (vagina artificial) ou orientá-lo a fazê-lo (masturbação). Dessa forma, ambas as técnicas dispensam a contenção química (VALLE, 2002), assim como a técnica de lavagem vaginal após a cópula, que teve eficácia confirmada em *C. jacchus* (KUEDERLING et al., 1996; VALLE, 2002). A vibroestimulação peniana (VEP), desenvolvida inicialmente para humanos, foi testada com

sucesso em primatas Neotropicais e consiste na estimulação direta do pênis, que é introduzido em um tubo de silicone conectado a uma unidade vibratória com frequência e amplitude reguláveis. Durante a execução da técnica o animal é contido em uma cadeira adaptada sem que haja necessidade do uso de sedativo, sendo mais compatível a espécies de pequeno porte e fácil contenção física como *C. jacchus* (YEOMAN et al., 1998) e *S. sciureus* (KUEDERLING et al., 2000; SCHNEIDERS et al., 2004). Embora a necessidade de condicionamento adequado dos animais à cadeira de contenção dificulte a aplicação dessa técnica. Os bons resultados alcançados com a VEP podem estar relacionados à melhor estimulação do pênis por inteiro e à diminuição da contaminação seminal por urina (VANDEVOORT, 2004).

Porém, dentre todos esses métodos de coleta seminal, a eletroejaculação com probe retal (EEJ) ainda é o mais difundido, com aplicação em várias espécies de primatas não-humanos. O protocolo de EEJ (voltagem, amperagem, quantidade e duração dos estímulos) pode variar consideravelmente entre espécies. Em *A.geoffroyi* foram aplicados estímulos de 1 a 7 volts e 10 a 100 mA, aumentado 1 volt a cada 2 ou 3 tentativas sucessivas, além de estimulação manual do pênis (HERNÁNDEZ-LÓPEZ et al., 2002). Para o mesmo gênero, Valle et al. (2004) utilizaram estímulos de 0 a 8 volts com aumentos subsequentes de 0,5 volt a cada série de 30 estímulos com duração média de 2 a 3 segundos e intervalos de 1 a 2 segundos. Já na espécie *S. apella*, Barnabe et al. (2002) descreveram um protocolo no qual são realizadas 5 séries de 20 estímulos cada, em um nível progressivo de intensidade, de 50 a 300 mA. Contudo, Araújo et al. (2009) demonstraram que é possível induzir a ejaculação nessa espécie com estímulos que variam de 12,5 a 100 mA, com intervalos de 30 segundos entre cada série de estímulos. Esses intervalos otimizaram o processo de ejaculação, sendo possível obter frações de sêmen líquidas e coaguladas nos intervalos entre cada série.

No gênero *Saimiri* o primeiro relato de coleta de sêmen foi feito por Bennett (1967) por meio de EEJ com probe retal bipolar de 12,5 cm de comprimento e 0,6 cm de diâmetro e voltagem que variava de 0 a 10 V, resultando em um volume satisfatório de sêmen contendo alta concentração de espermatozoides móveis. A EEJ é utilizada como método de coleta na maioria das pesquisas com sêmen *Saimiri* (YEOMAN; 1998), embora a VEP esteja se tornando cada vez mais difundida (YEOMAN et al., 1998; KUEDERLING et al., 2000; SCHNEIDERS et al., 2004; VALLE et al., 2007; KUGELMEIER, 2011; VIANA, 2013). Quando comparada ao método de EEJ, a VEP mostrou-se mais eficiente por proporcionar ejaculados com menor quantidade de coágulo seminal e com melhor qualidade, atestando a

influência direta do método de coleta sobre as características do sêmen (YEOMAN et al., 1998).

3.6 A CÉLULA ESPERMÁTICA

O espermatozoide é revestido mais externamente pela membrana plasmática, que é constituída por uma dupla camada lipídica (fosfolipídios, glicolipídios e colesterol) entremeada por moléculas de proteína, formando o chamado “mosaico fluido” (SINGER; NICHOLSON, 1972), onde os fosfolipídios apresentam uma camada hidrofílica externa e uma cadeia de ácidos graxos que se estende para o interior da membrana. Esta organização faz com que as cadeias de ácidos graxos promovam uma barreira hidrofóbica dificultando a passagem de água e outras moléculas. O transporte molecular é feito através de canais ou poros formados pelas proteínas, existindo pouco ou nenhum transporte de moléculas hidrofílicas em regiões da membrana sem poros ou canais. Alterações de temperatura podem provocar mudanças na conformação da membrana, que resultam em arranjos anormais dos fosfolipídios e proteínas, e permitem rápida passagem de moléculas que normalmente passariam vagarosamente através da membrana. A regulação das concentrações adequadas de íons (sódio, potássio, cálcio e magnésio) no meio intra e extracelular é mantida por bombas iônicas proteicas presentes nas membranas (AMANN; PICKETT, 1987).

A integridade estrutural da membrana plasmática é determinada pela temperatura e pela solução em que se encontra; na temperatura corporal a membrana está fluida. Essa característica é decorrente da ampla mobilidade lateral dos fosfolipídios que, entretanto, não possuem a mesma facilidade de se movimentar entre as faces externa e interna (COOPER, 1996). A fluidez da membrana plasmática é determinada por sua composição lipídica (STRYER, 1988). O colesterol age na regulação dessa fluidez, posicionando-se com sua cadeia longa entre as cadeias lineares dos fosfolipídios e interferindo na interação entre elas. Porções de membrana que possuem relação elevada entre colesterol e fosfolipídios são mais resistentes às mudanças de temperatura (AMANN; PICKET,1987; STRYER, 1988). Dessa forma, a membrana plasmática tem papel fundamental nos procedimentos de resfriamento e congelamento de sêmen, uma vez que durante a redução ou o aumento da temperatura do sêmen, ocorrem alterações nas associações dos lipídios com as proteínas e entre si, que são necessárias para um funcionamento normal da membrana (PARKS; GRAHAM, 1992).

3.6.1 O espermatozoide de *Saimiri*

A espermatogênese do macaco-de-cheiro dura cerca de 39 dias (30,5 dias da condição de célula primária até o estágio de espermátide e 8,5 dias para a formação da célula madura) (BARR, 1973). A ativação do processo de espermatogênese ocorre na estação de acasalamentos, evidenciada pelo aumento do diâmetro dos tubos seminíferos, e a inativação ocorre fora dessa fase (DUMOND; HUTCHINSON, 1967; PASQUALINI et al., 1986).

O espermatozoide de *Saimiri sciureus* (Figura 8) tem em média 69,24 ± 0,15 µm de comprimento total. A cabeça tem aproximadamente 5,11 ± 0,2 µm de comprimento e é achatada lateralmente com um leve intumescimento apical, sua área superficial é pequena e a margem posterior do acrossomo tem aparência serrilhada ou de microvilosidades; a peça intermediária insere-se de forma excêntrica na porção posterior da cabeça e mede 9,03 ± 0,12 µm de comprimento. As mitocôndrias são finas no corte transversal e fazem múltiplas voltas ao redor da peça intermediária; a cauda ou flagelo do espermatozoide tem comprimento médio de 55,34 ± 0,13 µm (DUKELOW, 1983).

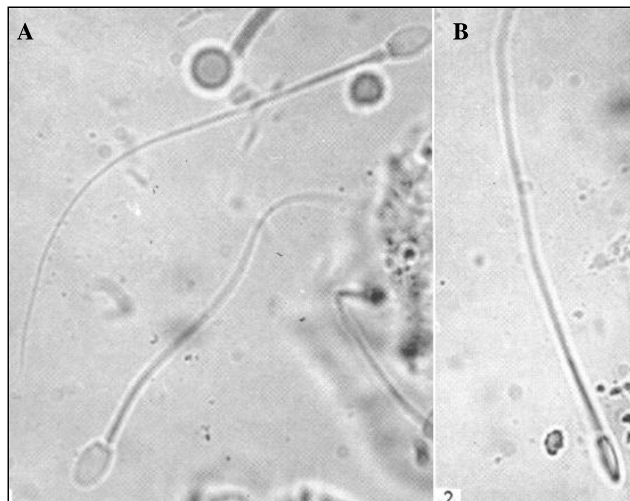

Figura 8: Espermatozoide de *S. sciureus*. Vista frontal (A) e vista lateral (B) da cabeça (x665).
Fonte: Bennett (1967).

3.7 CRIOPRESERVAÇÃO DO SÊMEN

O metabolismo celular gera certos produtos, como o ácido láctico e o gás carbônico, que podem aumentar a acidez do sêmen, desencadeando danos celulares irreversíveis (MCKINNON, 1996). A peroxidação dos lipídios da membrana leva à perda da sua integridade, o que prejudica a função celular e, consequentemente, diminui a motilidade do espermatozoide (AURICH et al., 1996).

Durante o resfriamento há redução da temperatura e a célula chega a um estado de

quiescência, onde há redução do metabolismo e diminuição dos gastos energéticos e da produção de catabólitos tóxicos, contribuindo para a preservação da célula espermática. Entretanto essa diminuição da temperatura pode também acelerar determinadas reações bioquímicas celulares, que variam entre as espécies animais, entre indivíduos da mesma espécie e ainda entre os compartimentos do espermatozoide, podendo até levar à morte celular (HOLT, 2000).

Se o resfriamento do espermatozoide, da temperatura corpórea até temperaturas próximas a 5 °C, é conduzido de maneira abrupta, ocorre um evento denominado "choque térmico ou *stress* térmico", que resulta em danos espermáticos irreversíveis caracterizados por alterações nos padrões normais de motilidade (movimento circular ou retrógrado), perda rápida de motilidade, danos do metabolismo, da membrana plasmática e do acrossoma (GRAHAM, 1996; HOLT, 2000).

Após essa faixa de temperatura e alcançada a temperatura de congelação (abaixo de 0 ºC), a célula espermática será exposta à alterações de osmolaridade do meio extracelular, uma vez que parte da água presente no meio, encontrada na forma de solução, congela. Consequentemente, ocorre o aumento da saturação de solutos e o meio extracelular se torna hipertônico. Então ocorre um fluxo de água da célula para o meio externo buscando atingir o equilíbrio osmótico. Este é um dos eventos mais críticos do processo de congelação, pois na tentativa de promover o equilíbrio osmótico, a célula pode sofrer efeitos deletérios devido à exposição a um meio extremamente saturado e à desidratação severa, que promove a desnaturação das macromoléculas e encolhimento excessivo da célula até ocorrer um colapso da membrana, é o chamado "Efeito solução" (WATSON, 1995; HOLT, 2000). Por isso a curva de congelação deve ser lenta para evitar o congelamento da água intracelular e rápida o suficiente para evitar o contato da célula desidratada com o meio hiperosmótico, embora a perda de água seja um evento benéfico, por reduzir a probabilidade de formação de grandes cristais de gelo dentro da célula, que causariam danos às estruturas internas e/ou a membrana plasmática (SQUIRES, 1999).

Portanto, para o sucesso da criopreservação de espermatozoides, é necessário seguir uma série de passos que visam à redução dos danos celulares e que assegurem a longevidade *in vitro* e *in vivo* da célula espermática. Um desses passos é a utilização de meios diluidores e crioprotetores com o intuito de proteger os espermatozoides dos choques térmicos e osmóticos que ocorrem durante esse processo (CONCANNON; BATTISTA, 1989).

3.7.1 O meio diluidor seminal e seus componentes

Os diluidores seminais são meios que devem conferir proteção aos diferentes compartimentos espermáticos durante as etapas da criopreservação: resfriamento, congelação e descongelação. Assim, sua constituição básica deve garantir nutrição adequada, proteção contra o choque térmico, balanço eletrolítico (pH e osmolaridade) e inibição do crescimento bacteriano. A existência de diferenças na composição lipídica da membrana plasmática do espermatozoide entre as espécies, raças e ainda entre indivíduos da mesma espécie, pode explicar o maior ou menor efeito protetor de um diluente (HOLT, 2000). Assim, a composição do diluente é de extrema importância e deve ser espécie-específica.

Para ser completo e eficiente, o diluidor seminal deve ser constituído por determinadas substâncias, tais como: agentes crioprotetores (lactose, gema de ovo, glicerol, DMSO, etilenoglicol, etc), substâncias tampão (TRIS, TES, ringer lactato, água de coco, citrato, fosfato de sódio, etc), fonte energética (frutose ou inositol) e antibióticos (estreptomicina, gentamicina, penicilina, etc) (HOLT, 2000).

3.7.1.1 Crioprotetores

De acordo com a capacidade de atravessar ou não a membrana plasmática, os crioprotetores podem ser classificados como penetrantes (intracelulares) e não penetrantes (extracelulares). Os crioprotetores penetrantes são moléculas pequenas como o glicerol, etilenoglicol, propilenoglicol, propanodiol, dimetilsulfoxido e dimetilformamida que atravessam a membrana plasmática e atuam no meio intracelular, sendo essenciais para minimizar ou prevenir a formação de cristais de gelo no interior da célula. Estas substâncias agem como solvente e soluto, contribuindo com a pressão osmótica. Entre estes crioprotetores, destaca-se o glicerol por apresentar melhores resultados na criopreservação do sêmen de muitas espécies (GRAHAM, 1996).

Em relação aos crioprotetores não penetrantes ou extracelulares, estes não atravessam a membrana plasmática, mas auxiliam na sua estabilização durante o processo de congelação/descongelação. São formados por grandes moléculas como proteínas (gema de ovo e leite desnatado), polímeros sintéticos (polivinilpirrolidona e metilcelulose) e açúcares (GRAHAM, 1996).

Na criopreservação de sêmen de primatas não-humanos os crioprotetores não-penetrantes e penetrantes mais comumente utilizados são a gema de ovo e o glicerol, respectivamente, em proporções variadas (MORREL; HODGES, 1998).

A gema de ovo é um dos crioprotetores mais utilizados como base dos diluentes de sêmen, sendo incorporada de maneira habitual na maioria dos protocolos de conservação. Age protegendo a célula espermática do choque térmico, preservando a motilidade, prevenindo a liberação da enzima hialuronidase do acrossoma e na manutenção da integridade de membranas mitocondriais (SALAMON; MAXWELL, 2005), além de atuar como tampão osmótico conferindo maior resistência às células tanto para meios hipotônicos quanto para hipertônicos (JONES; MARTIN, 1973).

A proteção contra o choque pelo frio, conferida pela gema de ovo, deve-se à interação dos fosfolipídeos presentes na superfície celular com os componentes lipoprotéicos de baixa densidade da gema, que possibilita a aderência das lipoproteínas à membrana plasmática do espermatozoide, conferindo-lhe proteção durante a criopreservação (DROBNIS et al., 1993; FASTAD, 1996).

Apesar das vantagens, por ser um produto de origem animal, a gema traz riscos de contaminação microbiana para o diluidor de sêmen, que pode afetar negativamente a fertilidade, tanto pela própria presença das bactérias, como pela produção de toxinas, pela degradação dos componentes do meio, ou ainda, pela utilização de substratos metabólicos. Essa situação determina a necessidade de incorporar substâncias de efeito antimicrobiano aos diluentes (WATSON, 1990). Outro inconveniente da utilização da gema de ovo em meios diluidores é a dificuldade de padronização das amostras, uma vez que a composição dos ovos pode variar dependendo da alimentação e da genética animal (AMIRAT et al., 2005).

O glicerol ($CH_3H_8O_3$) é o crioprotetor mais empregado na congelação do sêmen de diferentes espécies de primatas e possui a capacidade de penetrar através das membranas celulares, permanecendo tanto na membrana quanto no citoplasma, impedindo, dessa forma, a formação de grandes cristais de gelo intracelulares (WATSON, 2000). Contudo, há relatos de que o glicerol exerça efeitos tóxicos sobre os espermatozoides, alterando os aspectos físico-químicos da célula, o que pode levar à ruptura da membrana plasmática, à remoção de importantes proteínas de membrana ou provocar danos acrossomais (LOVELOCK; POLGE, 1954). Diante disso, a concentração final de glicerol no diluente deverá ser aquela em que há uma predominância de seus efeitos protetores sobre os efeitos tóxicos. Essa concentração poderá ser influenciada por diversos fatores como, por exemplo: outros componentes do diluidor, padrão de resfriamento e métodos de congelação e descongelação. No entanto, as características seminais de cada espécie podem ser consideradas como os fatores decisivos para definir a quantidade do glicerol a ser adicionada ao diluente (WATSON, 2000).

Alguns trabalhos com primatas demonstraram que concentrações excessivas desse

crioprotetor podem causar diminuição da capacitação espermática (LI et al., 2005a), como demonstrado em estudo com espermatozoides de *C. jacchus*, onde o glicerol tornou-se tóxico quando utilizado fora dos limites da concentração adequada para essa espécie (2,5-7%) (MORREL et al., 1997; MORREL; HODGES, 1998), intervalo que compreende a concentração adequada também para *Macaca mulatta* (5%) (SI et al., 2004 *apud* SILVA, 2005). A glicerolização (adição do glicerol ao meio) tem sido realizada em diferentes momentos durante o processo de criopreservação, tanto em primatas do Novo quanto do Velho Mundo. Conradie et al. (1994) diluíram sêmen de *Cercopithecus aethiops* em solução com glicerol a 32 °C antes de resfriar a 5 °C, similarmente ao protocolo utilizado com sêmen de *Gorilla gorila* (LANZENDORF et al., 1992). Contudo, em ejaculados de *Macaca fascicularis*, a adição do glicerol ocorreu a 4 °C somente após 2 horas de resfriamento (LI et al., 2005). Com a espécie *C. jacchus* obteve-se bons resultados adicionando o glicerol antes do envase e resfriamento do sêmen (VALLE, 2007). Entretanto, com *S. apella*, a adição do glicerol antes do resfriamento pode ter contribuído para a total perda da motilidade e vigor espermáticos verificados após a criopreservação, possivelmente devido ao longo tempo de exposição dos espermatozoides aos efeitos tóxicos do glicerol (OLIVEIRA et al., 2010).

Na tabela 2 são listados os principais crioprotetores, diluidores (tampões) e resultados obtidos após o congelamento do sêmen de diferentes espécies de primatas.

3.7.1.2 Diluidor a base de água de coco em pó (ACP®)

Estudos realizados a partir da década de 80 constataram que a água de coco (*Cocos nucifera,* família Palmae) pode ser uma alternativa como diluidor de sêmen de várias espécies. Ela é uma solução natural, ligeiramente ácida, estéril, composta de sais, proteínas, açúcares, vitaminas, gorduras neutras, indutores da divisão celular e eletrólitos diversos (BLUME; MARQUES JR, 1994), fornecendo os nutrientes necessários para a conservação de células espermáticas. Sua fração ativa foi identificada como um fitormônio promotor de crescimento celular denominado ácido 3-indol-acético (NUNES; COBARNOUS, 1995).

Os excelentes resultados obtidos nos primeiros estudos com a água de coco *in natura* na preservação do sêmen de caprinos (SALLES, 1989), ovinos (ARAÚJO, 1990), suínos (TONIOLLI, 1989), cães (MONTEZUMA JR et al., 1994; CARDOSO et al., 2003; CARDOSO et al., 2006), e diversas outras espécies, levaram à elaboração de um meio de conservação de sêmen à base de água de coco em pó (ACP®), como modo alternativo à utilização da água de coco *in natura*.

A ACP® é produzida a partir da extração do líquido endospérmico do coco (água de

coco) sob forma asséptica. O líquido é filtrado e bombeado para o sistema de secagem. A amostra seca é transformada em pó fino e uniforme, amorfo, destituído de água livre, com alta solubilidade. A uniformidade do produto, obtida mediante rigoroso controle de processamento leva à manutenção dos valores agregados do endosperma líquido do coco (CARDOSO, 2005). Esse diluente seminal foi testado com sucesso na criopreservação do sêmen de diferentes espécies animais (SAMPAIO NETO et al., 2002, SALGUEIRO et al., 2002; CARDOSO et al., 2005, RONDON et al., 2008). Em primatas, a ACP® foi utilizada na espécie *S. apella* (macaco-prego) (OLIVEIRA et al., 2010) somente na dissolução do coágulo seminal em diferentes temperaturas. Nesta mesma espécie, a água de coco foi testada para a criopreservação seminal, mas na sua forma *in natura* (OLIVEIRA et al., 2011).

Tabela 2- Composição dos diluidores utilizados no congelamento do sêmen de diferentes espécies de primatas.

Espécie	Tampão	Crioprotetor	Viabilidade (%)	Motilidade (%)	Referências
Callithrix jacchus	TES-TRIS	3% glicerol	34	44	**O'Brien et al., 2003**
Cercopithecus aethiops	Glutamato de sódio	14% glicerol	53	28	**Roussel e Austin, 1967**
Erythrocebus patas	Glutamato de sódio	14% glicerol	51	23	**Roussel e Austin, 1967**
Macaca assamensis	TES-TRIS	5% glicerol	39	69	**Li et al., 2005**
Macaca fascicularis	TES-TRIS	3% glicerol	NI	65	**Tollner et al., 1990****
	TRIS	5% glicerol	62	61	**Li et al., 2005**
	TRIS	6% glicerol	45	29	**Feradis et al., 2001**
Macaca fuscata	TES-TRIS	5% glicerol	60	65	**Sankai et al., 1997**
Macaca mulatta	Glutamato de sódio	14% glicerol	50	27	**Roussel e Austin,1967**
	TES-TRIS	12% glicerol	NI	27	**Dong et al., 2009**
	TES-TRIS	3% glicerol	NI	54	**Dong et al., 2009**
	TRIS	5% glicerol	74	85	**Sanchez-Partida et al., 2000****
Macaca speciosa	Glutamato de sódio	14% glicerol	50	25	**Roussel e Austin, 1967**
Papio anubis	Glutamato de sódio	Gema de ovo	65	44	**Kraemer e Vera Cruz, 1969**
	TES-TRIS	3% glicerol	47	83	**O'Brien et al., 2003**
Pan troglodytes	Glutamato de sódio	14% glicerol	54	27	**Roussel e Austin, 1967**
	TES-TRIS	5% glicerol	21*	30*	**Kusunoki et al., 2001**
	TES-TRIS	2,5% glicerol	46*	28*	**Kusunoki et al., 2001****
	TES-TRIS	3% glicerol	28	43	**O'Brien et al., 2003**
Sapajus apella	TES-TRIS	3,5% glicerol e gema de ovo	26	0	**Oliveira et al., 2011**
	Água de coco	2,5% glicerol e gema de ovo	13	0	
	ACP-118®	3% glicerol e gema de ovo	18	19	**Leão et al., 2013**

NI: Não informado
* Valores médios calculados do epidídimo direito e esquerdo
** Nascimentos após inseminação artificial com sêmen descongelado.

4 CAPÍTULO 1- TESTICULAR BIOMETRY AND SEMEN CHARACTERISTICS IN CAPTIVE AND WILD SQUIRREL MONKEY SPECIES (*Saimiri* sp.)

Artigo I

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THERIOGENOLOGY

Similarities in testicular and seminal aspects in four squirrel monkeys' species

Karol G. Oliveira[a,b] E-mail: karolgoliveira83@gmail.com

Regiane R. Santos[a,c*] E-mail: r.rodriguesdossantos@pq.cnpq.br

Danuza L. Leão[a] E-mail: danleao.88@gmail.com

Helder L. Queiroz[d] E-mail: helder@mamiraua.org.br

Fernanda P. Paim[d] E-mail: fernanda@mamiraua.org.br

João L.S.G. Vianez-Júnior[e] E-mail: vianez.iec@gmail.com

Sheyla F. S. Domingues[a] E-mail: shfarha@ufpa.br

[a]Laboratory of Amazon Animal Biotechnology and Medicine, Federal University of Pará, Castanhal, PA, Brazil, CEP 68746-360

[b]National Primate Center, Ananindeua, PA, Brazil, CEP 66645-000

[c]Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

[d]Mamirauá Institute for Sustainable Development, Tefé, AM, Brazil, CEP 69470-000

[e]Center for Technological Innovation of Evandro Chagas Institute, Ananindeua, PA, Brasil, CEP 66645-000

*Correspondence to

Regiane R Santos

Faculty of Veterinary Medicine

Yalelaan 104, 104, 3584 CM, Utrecht, The Netherlands.

Tel.: +31 30 2531078; fax: +31 30 2534125

Abstract

Differential phenotypic characteristics for taxonomic diagnosis purposes are well determined in the genus *Saimiri* (squirrel monkey). However, data on its reproductive characteristics are lacking. Our aim was to determine testicular biometry and correlate with seminal analysis in captive (*S. collinsi*) and free living (*S. vanzolinii, S. cassiquiarensis* and *S. macrodon*) squirrel monkeys. Testicular length, width, height, circumference and volume were measured. Testicular biometry showed no differences between right and left testicles within the same species, as well as among species. Semen collected by electroejaculation was constituted of a liquid and coagulated fraction, or only one of them. No significant difference was observed between volumes of these fractions within species or when each fraction was compared among the studied species. No correlation between testicular volume and seminal volume was observed. Seminal quality was similar among species. The method of electroejaculation yielded satisfactory results on these species, under field conditions.

Key-words: *Saimiri*, testicular biometry, sperm, electroejaculation.

1. Introduction

Squirrel monkeys (*Saimiri* sp.) are Neotropical primates whose taxonomy was recently revised. Although molecular and morphological studies have presented conflicting results regarding the taxonomic organization and geographical distribution [1,2], the most updated classification proposed by Mittermeier et al. considers the following species of *Saimiri: S. cassiquiarensis, S. vanzolinii, S. macrodon, S. boliviensis, S. sciureus, S. ustus* and *S. oerstedii* [3]. Additionally, the taxon *S. collinsi* has been recently recognized through molecular studies [1,4].

We are performing the first study on testicular biometry and seminal characterization based on this new taxonomic organization with the following species: *S. collinsi, S. vanzolinii*, *S. cassiquiarensis* and *S. macrodon*. Phenotypic differences among these species are more related to colour and pelage patterns as depicted in Figure 1. The conservation status of these species on IUCN red list is considered as of least concern, except for *S. vanzolinii* (Black-headed squirrel monkey), which is listed as vulnerable and its entire population is confined to the Mamirauá Reserve [5].

Squirrel monkeys are widely used as animal models for a variety of research areas [6]. Such application can be explained by their small size and ease of handling in captivity [6-8]. Males of *S. sciureus* and *S. boliviensis* are the most studied intensely [6,9], and from which semen has been obtained by electroejaculation (EEJ) [10-15] and penile vibratory stimulation (PVS) [15] and characterized (Table 1).

Paim et al. investigated morphological and ecological differences among *S. vanzolinii, S. cassiquiarensis* and *S. macrodon* living in Mamirauá Sustainable Development Reserve, but specific reproductive aspects of these recent recognized species are still unknown [2]. Studies in reproduction focused on the species can be more relevant than those focused on the genus, since some parameters can be different among species of a single genus, such as body

weight, hormonal response, behavior, growth and development. These differences have to be taken under consideration in research projects, for they might introduce new biases in the results [6].

Table 1. Mean (± SD) or range values of seminal volume and sperm concentration, plasma membrane integrity (PMI), sperm motility and normal sperm morphology (NSM) in semen collected from *S. sciureus* and *S. boliviensis.*

Species	**Volume (µL)**	**Concentration (x 10^6sperm/mL)**	**PMI (%)**	**Motility (%)**	**NSM (%)**	**Refs**
S. sciureus*	50.79-81.29	106	NI	NI	NI	[10]
	100	0.295 ± 13	79 ± 2	NI	NI	[11]
	NI	153 ± 96.73	36.75 ± 18.99	35 ± 11.8	55.75	[12]
	159.5 ± 57	427.3± 160.6	NI	65.85 ± 15.35	NI	[13]
	80-300	NI	NI	40-80	< 51	[14]
S. boliviensis*	205 ± 25	2.8 ± 1.7	NI	44.1 ± 11.4	NI	[15]
S. boliviensis**	436 ± 90	77.1 ± 20.4	NI	80.6 ± 4.3	NI	[15]

* Semen collected by rectal electroejaculation
** Semen collected by penile vibrostimulation
NI: Not informed

In the present work we aim to describe seminal characteristics, testicular parameters and their correlations for *S. vanzolinii, S. cassiquiarensis* and *S. macrodon*, captured in wild, and for *S. collinsi*, maintained in captivity. Therefore, our main goal is to provide biologic information on these species, to support in the future, development of successful protocols for conservation of squirrel monkeys' sperm.

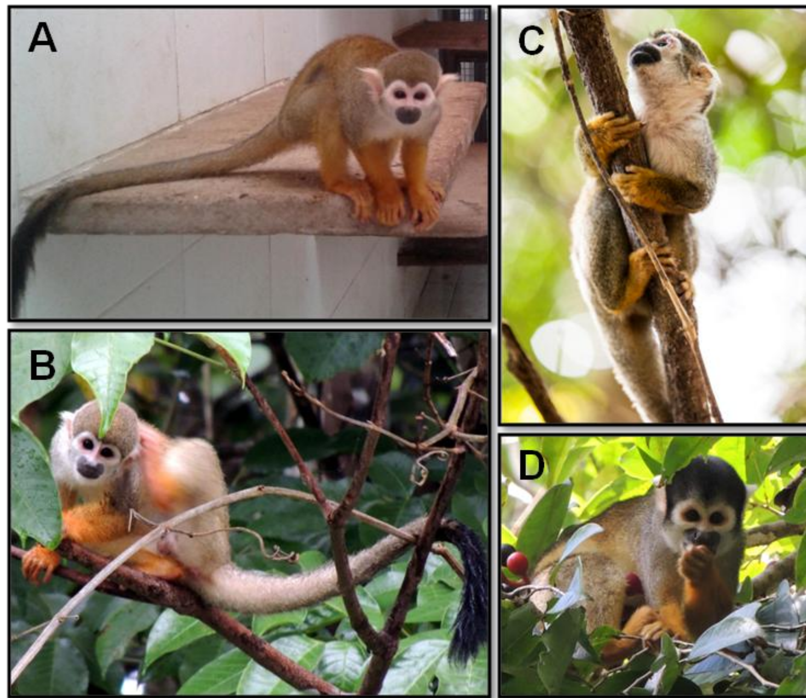

Figure 1. Pictures from *S. collinsi* (A) by Tatyana Pinheiro; *S. cassiquiarensis* (B) by Fernanda Paim; *S. macrodon* (C) by Sônia Vill and *S. vanzolinii* (D) by Fernanda Paim.

2. Materials and Methods

2.1. Study Site

We conducted our study at two different locations. The captive males (*S. collinsi*) were examined and maintained at National Primate Center (CENP), Ananindeua, Brazil (1°22’58’’S and 48°22’51’’W), where the climate is humid tropical, with an average annual temperature of 28 °C. The free-living males (*S. vanzolinii, S. cassiquiarensis* and *S. macrodon*) were captured at Mamirauá Sustainable Development Reserve (Figure 2). The reserve is a protected area located at the confluence of the Solimões and Japurá rivers (03°02’22”S and 64°51’41”W), covering a total of 1,124,000 ha of floodplain ecosystems [2]. Monthly average precipitation is 131.1 mm, and average temperature is 27.5 °C (min. average 23.02 °C and max. average 31.86 °C) [16].

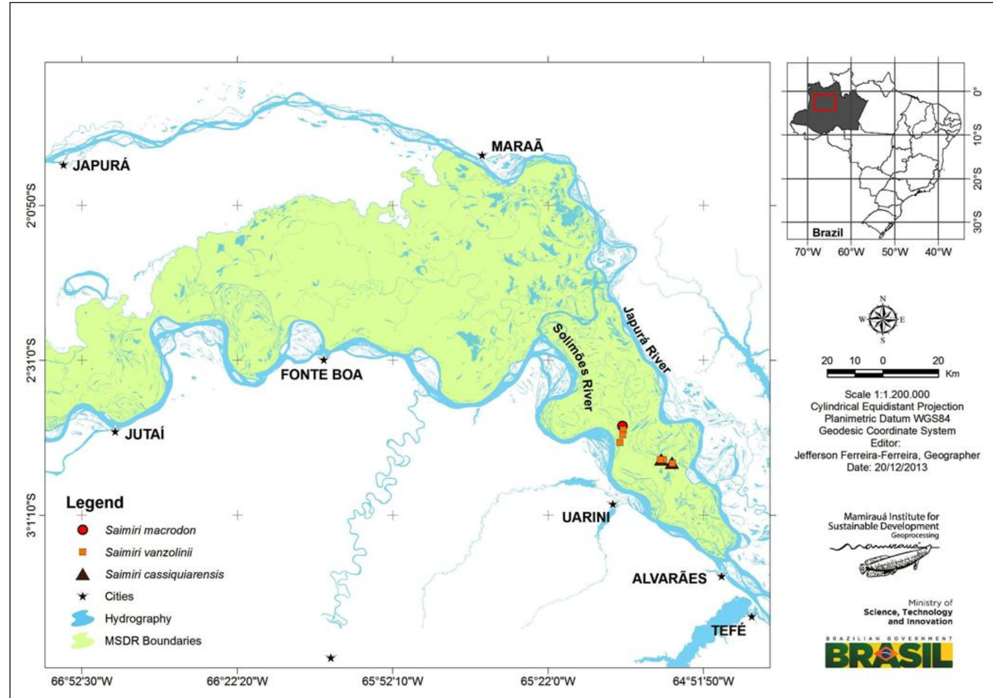

Figure 2. Distribution of capture sites for *S. vanzolinii, S. cassiquiarensis* and *S. macrodon*. Source: Mamirauá Institute for Sustainable Development, Remote sensing and geoprocessing group, 2013.

2.2. Study Animals

All experimental protocols were approved by environmental authorities (Ministério do Meio Ambiente - Sistema de Autorização e Informação em Biodiversidade - SISBIO/ICMBio/MMA nº 31542-2 for captive animals/nº 29906-3 for wild animals), by the Ethical Committee in Animal Research (nº 0010/2011/CEPAN/IEC/SVS/MS) (for captive animals) and Ethics and Research Committee and the Animal Use Ethical Committee of the Mamirauá Institute for Sustainable Development (nº 002/2012) (for wild animals). Adult males (> 5 years old) [17] from four species of the genus *Saimiri* were selected for our study: *S. collinsi* (n= 13), *S. vanzolinii* (n= 10), *S. cassiquiarensis* (n= 5) and *S. macrodon* (n= 9). The age of all animals was estimated based on dentition considering tooth eruption, intra-osseous tooth formation and tooth wear [18]. Only adult animals were selected for the present study.

S. collinsi males were captive animals from CENP, selected by their physical characteristics, and clinical parameters such as complete hemogram, hepatic and renal function (supplementary Tables 1 and 2). External genitalia were evaluated and andrologic examination (i.e. inspection and palpation of the testes to verify size, consistency, symmetry and mobility) was performed. 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 diet consisted of fresh fruit, milk and commercial pellet chow (MEGAZOO™ P18, Protein 18%, Fiber Maxi. 6.5%, Betim, MG, Brazil) and cricket larvae (*Zophobas morio*). Vitamins, minerals and eggs were supplied once a week and water was available *ad libitum*. The physical restraint was done with netting and leather glove, by a trained animal caretaker. Semen was collected at the same period of the day, i.e. in the morning before feeding, and throughout two months (October and November) of 2011 and 2012.

S. vanzolinii, *S. cassiquiarensis* and *S. macrodon* males were captured using a Tomahawk Live Trap (0.7 m x 0.4 m x 0.4 m; length, width and height, respectively) in two field expeditions in November 2012 and October 2013. Traps were set up in the early morning and checked after four hours and at mid-afternoon. The animals caught were handled by a trained animal caretaker wearing leather gloves.

2.3. Chemical Restraint and Semen Collection

After physical restraint, all studied animals were anesthetized with ketamine hydrochloride (Vetanarcol 15 mg/kg im; König S.A., Avellaneda, Argentina) and xylazine hydrochloride (Kensol 1 mg/kg im; König S.A.) by a veterinarian [19]. Achieved total anaesthetic effect, the animals were weighed. Average body weights (grams) were 868 (705 – 1125), 818 (580 – 1055), 614 (555 – 675), and 777 (578 – 1005) for *S. collinsi*, *S. vanzolinii*, *S. cassiquiarensis*, and *S. macrodon*, respectively (Supplementaty Table 3). Subsequently,

the males were placed in dorsal recumbence; both testes were evaluated and measured: length (cranial-caudal), width (medial-lateral) and height (dorsal-ventral) with a calliper rule, and circumference with a tape-measure. The testicular volume was calculated by the formula: length × width × height × 0.524. 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 by EEJ (Autojac-Neovet, Uberaba, Brazil) with a rectal probe as indicated by Bennett: 0.6 cm diameter and 12.5 cm length with a rounded end, bearing two metal plates (2 cm in length and 0.8 cm wide) on opposite sides [10]. The 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 to 8 minutes), composed of 35 electrical stimuli (12.5 to 100 mA) within an interval of 30 s between series [19].

Ejaculate (liquid and coagulated fractions) was collected into micro-centrifuge tubes (1.5 mL). If a male did not ejaculate after the session, no further attempts were made to collect semen in the case of wild animals. In the case of captive animals, another EEJ was attempt after intervals of at least 30 days.

In captivity, semen sampling was performed in a collection room. In the wild, it was performed near capture points, to avoid the removal of the caught animals from their place of origin. Rectal temperature was measured prior to the EEJ procedure. A veterinarian monitored the animals during EEJ as well as after recovering from anaesthesia.

2.4. Seminal Evaluation

Immediately after ejaculation in a graduated microtube, semen was placed in a water bath at 37 °C. Appearance and consistency were evaluated subjectively by a same measurer,

i.e. colour (colourless, yellowish or whitish), opacity (opaque or transparent), appearance (amorphous or filamentary seminal coagulum) and degree of coagulation (four-point scale) according Dixson and Anderson [20]. The seminal liquid fraction was measured with the aid of a pipette. Coagulum volume was calculated as the total volume minus the liquid volume. Subsequently, the extender ACP-118™ (powdered coconut water; ACP Biotecnologia, Fortaleza, Ceará, Brazil) was added to a microtube, which was maintained in the water bath (37 °C) for a period of 1 to 1.5 hour to allow coagulum liquefaction [19]. During incubation, periodically (every 15 minutes) gentle mixing of the samples with the help of a pipette tip was performed to improve sample homogeneity.

Sperm motility was expressed as the percentage of sperm actively moving in a forward direction. Sperm vibrating in a place were not considered to be motile. To measure sperm motility, 10 µL of semen was placed in a pre-warmed (37 °C) glass slide with cover slip and evaluated. Sperm vigour was subjectively scored on a scale from 0 to 5 [19]. Sperm morphology and plasma membrane integrity were evaluated by a smear prepared adding 5 µL of eosin-nigrosine stain (Vetec, Rio de Janeiro, Brazil) to 5 µL of semen on a pre-warmed (37 °C) glass slide. Morphologic defects detected in sperm were classified as major or minor [21]. Sperm concentration was determined in a Neubauer chamber after dilution of 1 µL semen in 99 µL formalin solution 10%. Seminal pH was measured with a pH strip (Merck Pharmaceuticals, Darmstadt, Germany). All evaluations were performed under a light microscope (Nikon, Tokyo, Japan), at a magnification X 100, by a same measurer.

2.5. Data Analysis

All data are expressed as mean ± standard deviation (SD). Testicular biometry and seminal data were evaluated using one-way ANOVA followed by Tukey multiple comparisons *post hoc* test. The Spearman rank-order correlation coefficient was used to measure correlation between parameters. Statistical significance was obtained whenever P <

0.05. For all statistical analyses, software R, version 3.1.2, was used.

3. Results

Total time for anaesthetic effect (approximately 4 to 5 minutes) and rectal temperature (38.6 ± 1.1 °C) were not species-specific. Animals selected for this study presented testes with normal consistency and mobility. Testicular biometry showed no differences between right and left testicles within the same species, neither among species, which presented all similar testicular biometry (Figure 3). The testicular circumference was not measured in *S. cassiquiarensis*. Hence, there is no data available.

Penile erection began more frequently between the 13rd and 20th electrical stimuli of each series, and ejaculation occurred on average between 15th and 18th stimuli of each series, in all species. The collected semen was constituted by a liquid and coagulated fraction or only one (Table 2). Ejaculation was almost always initiated by the liquid fraction that often was partially or totally coagulated after about 10 seconds. Liquid and coagulated fractions were transparent or opaque and colourless, whitish or yellowish in all studied species. This wide variation in appearance and constitution of ejaculated among collections was regardless of species, and no pattern was observed within species. Coagulated fraction presented filamentary or amorphous appearance and the most frequent coagulation degree observed was 3, where semen coagulates so that the ejaculate forms a whitish, non-fluid, non-gelatinous mass, but no a compact plug, and was not spontaneously dissolved, according to Dixson and Anderson's scale [20] (Figure 4). The number of animals of each species in which EEJ was performed, as well as number of ejaculations are listed in Table 2. Concerning volume of liquid and coagulated fractions, no significant difference was observed among species within each compared fraction or between fractions within the same species (Table 2).

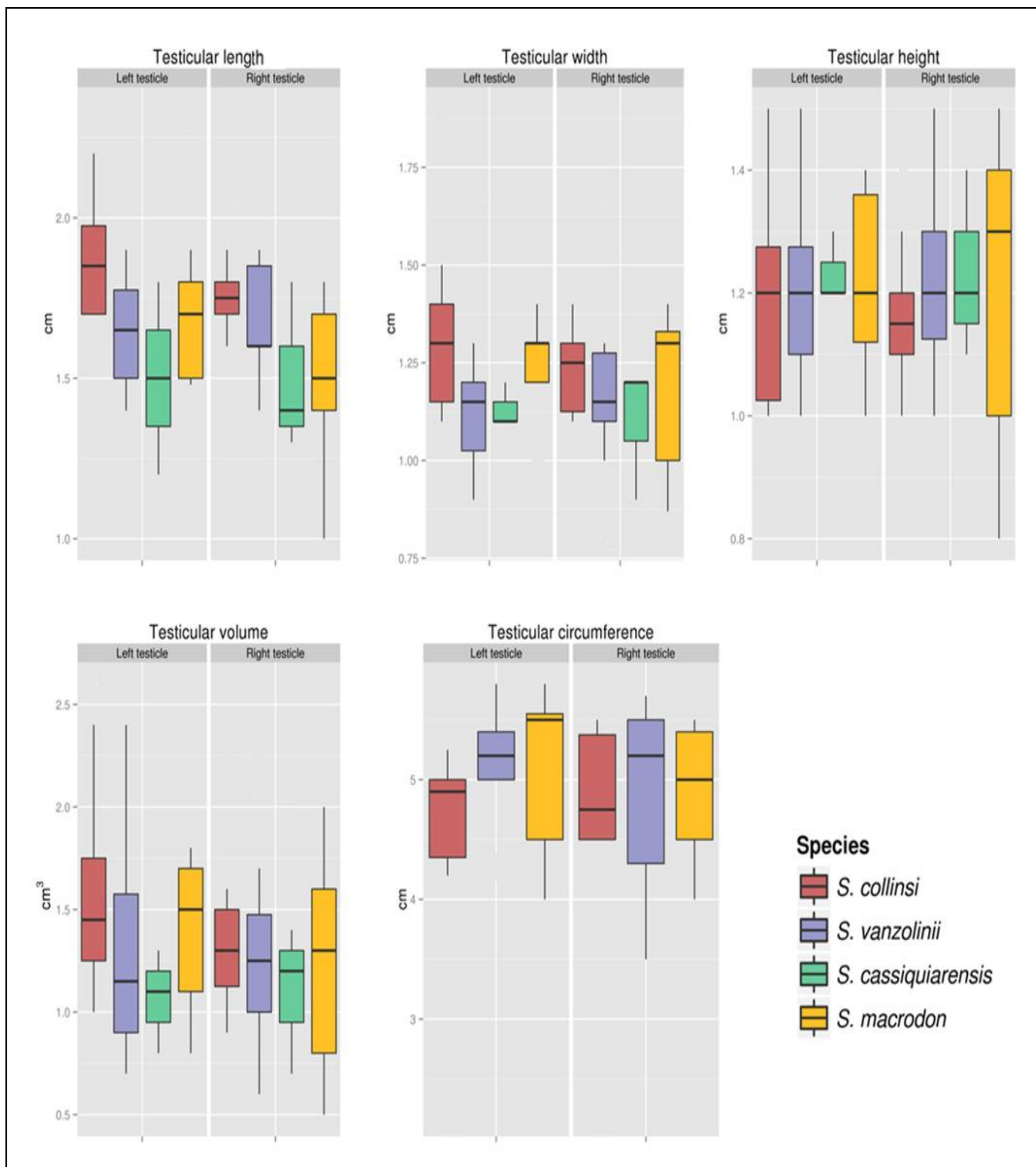

Figure 3. Box-plot of parameters of testicular biometry of *S. collinsi, S. vanzolinii, S. cassiquiarensis* and *S. macrodon* males. No significant differences were observed within and among species regarding seminal liquid and coagulated fractions.

Table 2. Number of animals submitted to electroejaculation (N), total number of trials (EEJ), trials with ejaculates (total, containing both liquid and coagulated fractions, only liquid, or only coagulated fraction), ejaculates presenting sperm, and mean (± SD) seminal volume of liquid and coagulated fractions.

Species	N	EEJ	trials with ejaculates (fractions)				ejaculates with sperm (n)	liquid fractions (µL)	coagulated fractions (µL)
			total	liquid + coagulated	liquid	coagulated			
S. collinsi	13	27	13	4	7	2	9	49.2 ± 68.9	65.4 ± 142.1
S. vanzolinii	8	8	6	3	0	3	3	28.3 ± 59.8	125.8 ± 142.5
S. cassiquiarensis	5	5	2	1	0	1	1	5 ± 7.1	175 ± 176.8
S. macrodon	1	1	1	0	0	1	1	0*	500*

* Only one sample obtained.
No significant differences were observed within and among species regarding seminal liquid and coagulated fractions.

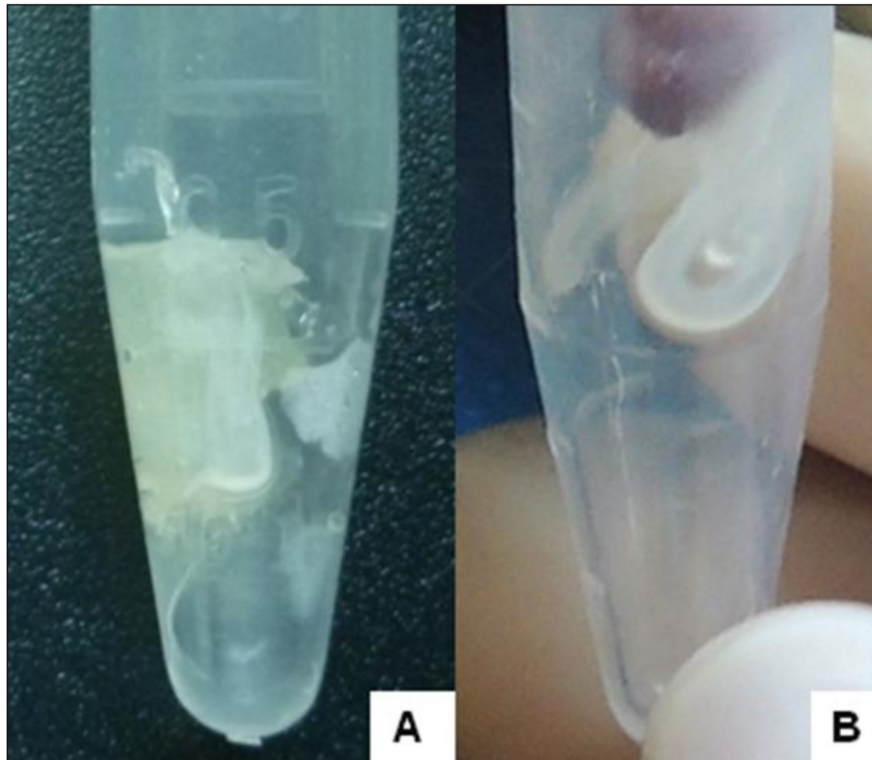

Figure 4. Amorphous (A) and filamentous (B) coagulated fraction from *S. macrodon* and *S. vanzolinii*, respectively, collected by electroejaculation.

There was no correlation between the testicular volume and seminal volume, when liquid (R= 0.31, *p*= 0.25) and coagulated (R= 0.32, *p*= 0.25) fractions from *S. collinsi* were evaluated. Likewise, liquid and coagulated fractions from *S. vanzolinii* were not correlated with testicular volume (R= -0.69, *p*= 0.12 and R= -0.37, *p*= 0.49, respectively). Correlations in *S. cassiquiarensis* and *S. macrodon* were not performed due to the insufficient number of seminal samples of both species (Figure 5).

Sperm motility, vigour and plasma membrane integrity were similar among species (Figure 6). Due to the limited number of seminal samples (n= 1) obtained from *S. macrodon*, sperm morphology was evaluated only on *S. collinsi, S. vanzolinii* and *S. cassiquiarensis* and no differences among these species was observed (Table 3).

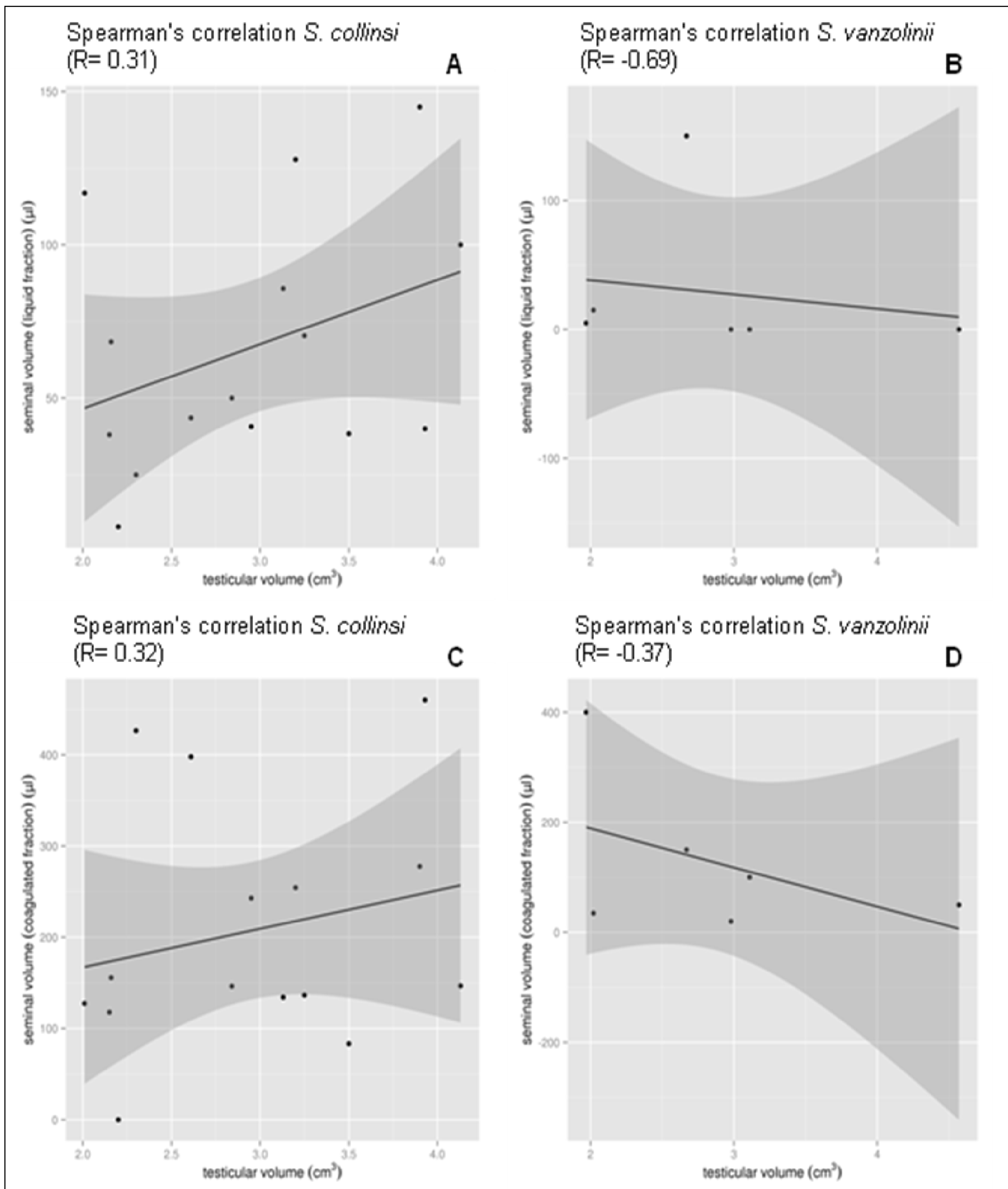

Figure 5. Correlation found between testicular volume and seminal volume (liquid or coagulated fractions) in *S. collinsi* (A and C) and *S. vanzolinii* (B and D).

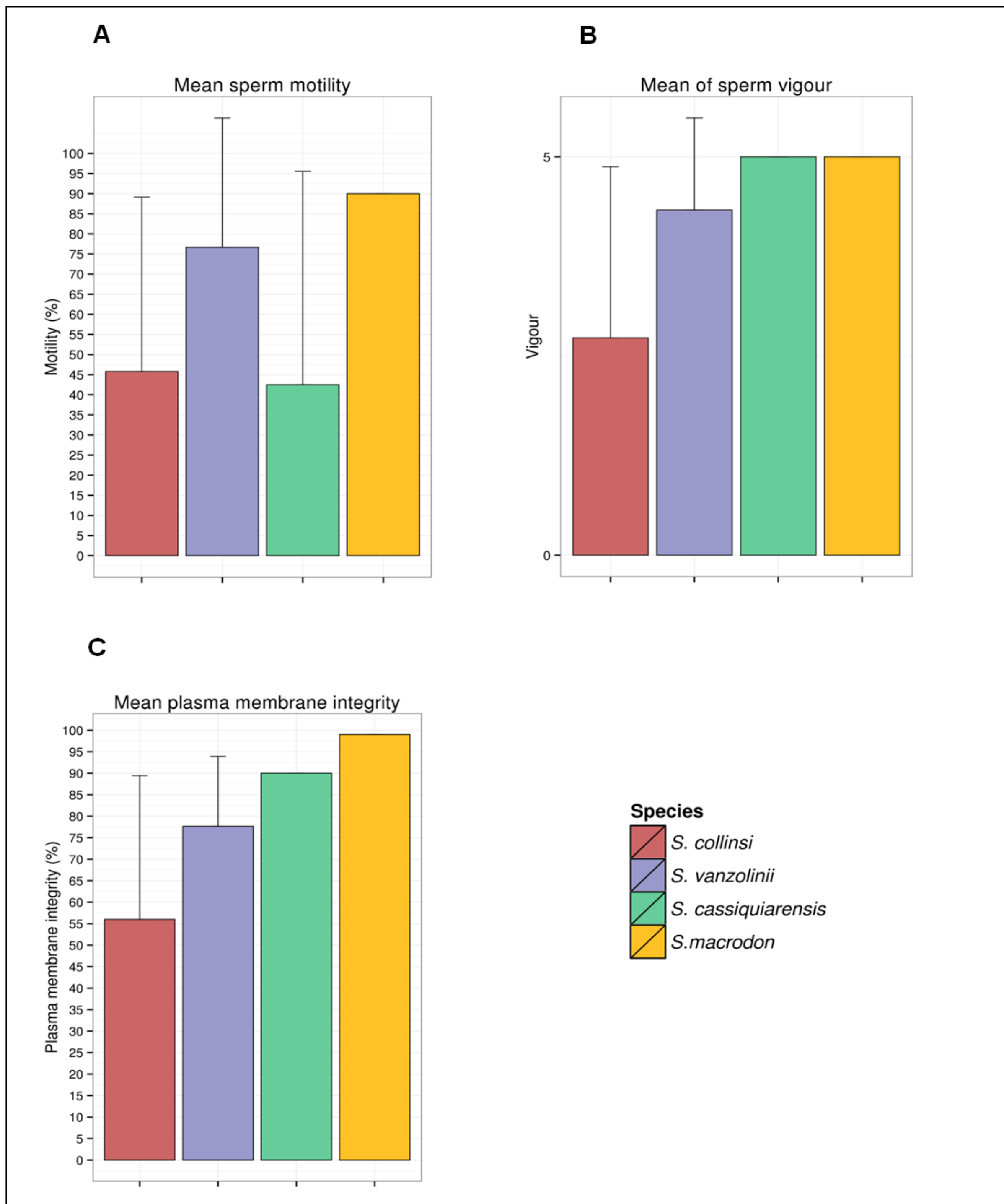

Figure 6. Mean sperm motility (A), vigour (B) and plasma membrane integrity (C) of *S. collinsi, S. vanzolinii, S. cassiquiarensis* and *S. macrodon.*

The most common defects were observed in the tail with a predominance of secondary defects, i.e. coiled tail (see Figure 7). Sperm concentration was measured in semen from captive animals, and the mean (± SD) value for liquid fraction of *S. collinsi* was 88.31 ± 36.64

x 10^6 sperm/mL. Mean (± SD) seminal pH of total ejaculate (liquid and coagulated fraction) was 7.43 ± 0.63. For none of these parameters, differences among species were observed.

Table 3: Mean percentages (± SD) of normal sperm and sperm with major and minor pathologic defects in fresh semen (liquid fraction) of *S. collinsi, S. vanzolinii* and *S. cassiquiarensis.*

Morphology	**Species**		
	S. collinsi	***S. vanzolinii***	***S. cassiquiarensis****
Normal	69.06 ± 21.18	90 ± 9.19	81
Major pathologic defects			
Strongly coiled tail	8.14 ± 12.07	3.50 ± 3.54	0
Proximal droplet	0.24 ± 0.93	0.5 ± 0.70	0
Pseudo droplet	0.17 ± 0.48	0	0
Small abnormal head	0.02 ± 0.15	0	0
Narrow at base	0.02 ± 0.13	0	0
Pear-shaped defect	0.02 ± 0.15	0	0
Minor pathologic defects			
Terminally coiled tail	11.26 ± 8.72	4.50 ± 6.36	10
Simple bent tail	10.88 ± 8.87	1 ± 0	9
Distal droplet	0.19 ± 0.47	0	0
Forked tail	0	0.5 ± 0.70	0

* Only one sample evaluated.
It was not possible to evaluate sperm morphology of *S. macrodon* due to inexistence of liquid fraction.

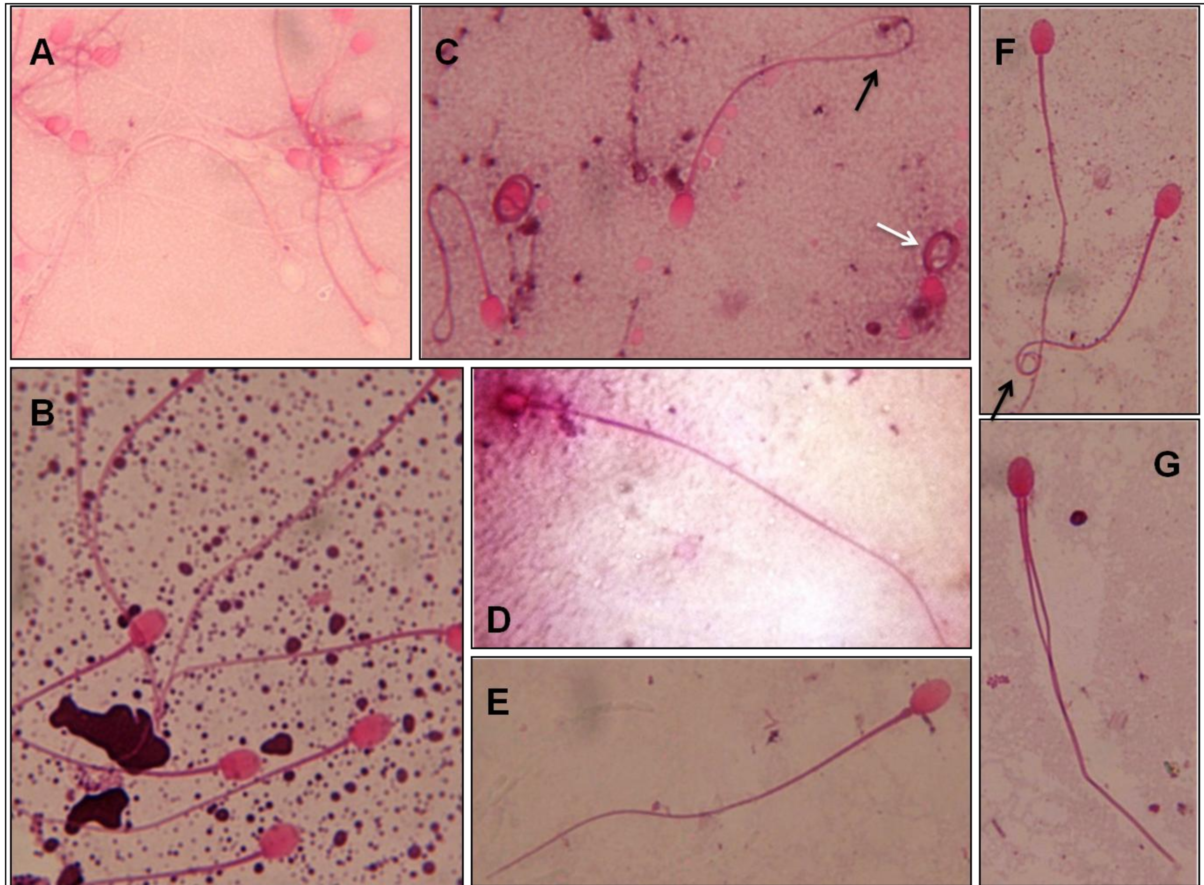

Figure 7. Sperm morphology. A: *S. collinsi* sperm with intact (not stained) and injured (stained) plasma membrane; B: abaxial insertion of *S. vanzolinii*; C: Strongly coiled (white arrow) and simple bent tail (black arrow) of *S. collinsi*; D: Small abnormal head of *S. collinsi*; E: Proximal droplet of *S. vanzolinii*; F: normal and terminally coiled tail (black arrow) of *S. vanzolinii*; G: Forked tail of *S. vanzolinii*.

Supplementary table 1. Hemogram results from *S. collinsi* males (n= 10). Values are expressed as mean ± SD.

Parameter	**Results**	**Reference values***
Hematocrit (%)	43.2 ± 3.6	44.00 ± 0.6
Red blood cells (x10^6/mL)	07.2 ± 0.4	07.1 ± 0.1
Hemoglobin (g/dL)	13.9 ± 1.2	13.8 ± 0.2
Mean corpuscular volume (fL)	59.9 ± 2.8	61.9 ± 0.6
Mean corpuscular hemoglobin (pg)	19.1 ± 1.1	19.4 ± 0.2
Mean corpuscular hemoglobin concentration (%)	31.8 ± 1.0	31.5 ± 0.2
White blood cells (x10^3/mL)	11.0 ± 4.1	10.5 ± 0.6
Platelets (million/mm^3)	268 ± 93	-
Basophils (%)	0.44 ± 0.7	0.0 ± 0.2
Eosinophils (%)	01.9 ± 0.9	01.0 ± 0.2
Neutrophils (%)	51.5 ± 11.7	35.0 ± 3.2
Lymphocytes (%)	44.1 ± 11.2	61.0 ± 3.1
Monocytes (%)	02.1 ± 1.6	02.0 ± 0.3

*Source of reference values: Kakoma I, James MA, Jackson W, Bennett G, Ristic M. 1985. Hematologic values of normal bolivian squirrel monkeys (*S. sciureus*): a comparison between wild-caught and laboratory-bred male animals. Folia Primatologica 44:102-107.

Supplementary table 2. Biochemical analysis of plasma from *S. collinsi* males (n= 10). Values are expressed as mean ± SD.

Parameters	**Results**	**Reference values**
Glucose (mg/dL)	108 ± 42.4	103 ± 30.3
Blood urea nitrogen (BUN) (mg/dL)	48.8 ± 37.7	38.7 ± 10
Cholesterol (mg/dL)	188.3 ± 43.0	151 ± 64.7
Triglycerides (mg/dL)	72.5 ± 40.4	74.9 ± 32.7
Creatinine (mg/dL)	0.6 ± 0.1	0.9 ± 0.2
Total Bilirrubin (mg/dL)	< 0.10	0.8 ± 0.6
Phosphatase (U/L)	249 ± 308	358 ± 175
Glutamic Oxaloacetic Transaminase (U/L)	190 ± 56.6	185 ± 95.3
Glutamic Pyruvic Transaminase (U/L)	201 ± 75.4	184 ± 110
Total Protein (g/dL)	6.5 ± 0.7	6.9 ± 1.0
Calcium (mg/dL)	9.7 ± 0.9	9.6 ± 0.9
Albumina (g/dL)	3.7 ± 0.3	4.2 ± 0.6
Carbon Dioxide	21	11.1 ± 3.9
Potassium	3.5	5.7 ± 1.0
Thyroxine-Binding Globulin	2.8 ± 0.5	-
Very Low Density Lipoprotein	17.3 ± 7.6	-
Ammonia	46.5 ± 55.9	-
Phosphorus	5.3 ± 0.2	-
Magnesium	2.7 ± 0.3	-
Iron	154 ± 94.1	-

*Source reference values: KCCMR. Michale E. Keeling Center for Comparative Medicine and Research. [Internet]. Texas: The University of Texas MD. Anderson Cancer Center. [cited 2014 March 2]. Available from: http://www.mdanderson.org/education-and-research/departments-programs-and-labs/programs-centers-institutes/michale-e-keeling-center-for-comparative-medicine-and-research/animal-resources/squirrel-monkey-diagnostic-reference-values.html.

Supplementary table 3. Body weight per animal, as well as mean (± SD) and range values of studied males from *S. collinsi* (n= 13), *S. vanzolinii* (n= 10), *S. cassiquiarensis* (n= 5) and *S. macrodon* (n= 9) species.

Individual	Weight (grams)	Range
S. collinsi	868 ± 104	705 – 1125
1	1055	
2	740	
3	717	
4	975	
5	915	
6	850	
7	878	
8	782	
9	748	
10	935	
11	845	
12	990	
13	852	
S. vanzolinii	818 ± 136	580 – 1055
1	715	
2	835	
3	1055	
4	875	
5	860	
6	775	
7	675	
8	905	
9	906	
10	580	
S. cassiquiarensis	614 ± 44	555 – 675
1	675	
2	605	
3	600	
4	555	
5	635	
S. macrodon	777 ± 170	578 – 1005
1	720	
2	860	
3	1000	
4	905	
5	590	
6	615	
7	1005	
8	578	
9	723	

4. Discussion

This is the first report describing seminal and testicular parameters in free-living *S. vanzolinii, S. cassiquiarensis* and *S. macrodon*. Previous studies on these themes were focused on *S. collinsi* [19], *S. sciureus* [22] and *S. boliviensis* [15]. Besides its substantial importance for the evaluation of male fertility, characterization of semen from neotropical non-human primates is still scarce [23].

Observed symmetry between right and left testicles within a same individual of all studied species was expected because the testicles are paired organs and there are no major differences between them if organogenesis occurs without anomalies as monorchidism, cryptorchidism or hypoplasia. Total testicular volume of *S. collinsi* (2934 ± 831 mm³; mean ± SD), *S. vanzolinii* (2552 ± 900 mm³), *S. cassiquiarensis* (2192 ± 619 mm³) and *S. macrodon* (2608 ± 748 mm³) presented greater measurements than those described in *S. sciureus* by Pasqualini et al. (951-1792 mm³; min-max) [24] and Viana (1515 ± 87.18 mm³; mean ± SD) [25], and were similar to the recent measurement reported for *S. collinsi* (2967 ± 635 mm³; mean ± SD) [19]. Even though wild monkeys may present different patterns of reproductive season, and captive monkeys are usually able to mate and reproduce all year [22, 25], no effect on testicular biometry was observed.

Positive correlation between ejaculate and testicular volumes observed in *S. collinsi* but not in *S. cassiquiarensis* would be not very significant, due to the wide variation in sperm quality between individuals. Besides the fact that many ejaculates despite the large volume did not contain viable sperm or even were oligospermic. This would have relation with: the position of the stimulating probe within the rectum relative to adjacent prostate, loss of part of ejaculate into the bladder (retrograde ejaculation) due anaesthetic effect or electrical surge [15, 26], or simply a possible masturbation or copula previously to EEJ.

Many attempts of semen collection were made during the present study. However, the

number of ejaculates presenting good spermatic parameters was relatively small. This was previously observed in *S. boliviensis*, when males were rectally stimulated during EEJ, and presented modest penile erection (frequently faded) and ejaculates were predominantly composed by a coagulated fraction [15]. Although these authors found that more than half of the ejaculates (60%) failed to contain motile sperm, and besides the risk of both equipment and stimulation procedure affecting seminal quality [27], semen collection with EEJ is still conventionally applied especially for neotropical primates. Furthermore, EEJ was applied because other methods were not available, and conditioning free-living animals was not an option, since it would affect their daily habits. Other successful semen collection methods are already described for essentially terrestrial species, as *Macaca fuscata* [23], differently of *Saimiri sp.*, which is basically arboreal.

With some exceptions such as the *Alouatta caraya,* species, which does not present semen coagulum following ejaculation [28], semen from most primates coagulates during or after ejaculation, being difficult to evaluate seminal quality [10, 13, 15, 26, 27, 29, 30]. In the present study, one part of the ejaculate coagulated but another part remained liquid, without significant difference between the volumes of these two fractions. The seminal coagulum apparently has as main function to act as a reservoir of sperm and protects them against adverse vaginal conditions, e.g. acid pH acid [31]. The necessary time after ejaculation for spontaneous coagulum liquefaction is variable and species-specific [32]. In the present study, no spontaneous liquefaction occurred. Addition of proteolytic enzymes, e.g. trypsin, has been reported to be effective for liquefaction of seminal coagulum from several primate species [32]. However, some studies have shown that proteolytic enzymes can cause lesions in sperm membrane, compromising fertility capacity [33]. Therefore, we did not use proteolytic enzymes to accelerate liquefaction of seminal coagulum, but we diluted the coagulated in ACP-118™ (powdered coconut water). This extender was previously tested for *S. collinsi* [19]

and *S. apella* [29], whose semen also does not liquefy spontaneously. ACP-118^{TM} was used in association with mechanical fragmentation and incubation in water bath at 37 °C. ACP-118^{TM} contains ascorbic acid and polyphenol oxidases, which are antioxidants that supports sperm quality during and after incubation [29, 30]. ACP-118^{TM} is also composed by different bioactive enzymes, e.g phosphatase, catalase, dehydrogenase, which may support coagulum liquefaction.

Collected semen was in general similar to that described in *S. sciureus* and *S. boliviensis* studies (employing EEJ or PVS) [10-15] (Table 1). Mean seminal volume collected in this study was higher than that observed in the first seminal description of the genus *Saimiri* (in *S. sciureus*) [10]. In this former study, semen was also collected by EEJ, and Bennett described two seminal fractions: a colourless sperm-free coagulum and a sperm-rich fluid [10]. However, in our study, we observed sperm in both fractions. Ackerman and Roussel [12] described liquefaction of coagulum seminal by trypsin resulting in lower sperm motility and plasma membrane integrity than those obtained in the present and recent study with *S. collinsi* [19], maybe due a detrimental effect of trypsin on sperm quality. Others scientific reports on seminal characteristics of *Saimiri* species do not cite addition of proteolic enzymes for coagulum liquefaction [13, 15, 26].

The observed sperm motillity was similar to that reported in other primate species when EEJ was applied. For instance, it was similar to the findings in fresh semen from *Papio anubis* (48-92%) [34] or from *Ateles geoffroyi* (~ 56%) [32] both collected by EEJ. However, when the PVS method was applied, sperm motility was improved in semen collected from in *Callithrix jacchus* [35] and *Macaca mulatta* [36], both with 71% of motility. Semen pH was similar to the reported for *S. collinsi* [19], *Alouatta caraya* [27] and for *Ateles geoffroyi* [37] and *Callithrix jacchus* (7.6) [35]. Mean (± SD), sperm concentration of liquid fraction was similar to observed in ejaculates collected both by EEJ as by PVS, of *S. sciureus* [10,12] and

S. boliviensis [15], despite the large variation between the ejaculates analyzed in this study (0-965 x 10^6 sperm/mL).

The percentage of normal sperm (69% *S. collinsi*, 90% *S. vanzolinii* and 81% *S. cassiquiarensis*) was superior to that previously reported in *S. sciureus* (49-65%) trypsin-digested semen [12] and similar to described in some Neotropical primates in semen collected by EEJ: *S. collinsi* (74.7%) [19] and *Sapajus apella* (81.7%) [29] after semen liquefaction in ACP-118™, *Callithrix jacchus* (87.6%) [38], *Ateles geoffroyi* at dry season (73%) [39], *Alouatta caraya* (78.7%) [27] and *Sapajus apella* after semen liquefaction in coconut water solution (78.2%) or TES-TRIS (83.7%) [30].

Spermatic pathologies observed in that study were classified according Blom [21], whose classification system is based on the relative importance of the sperm abnormality to fertility. Thus, while the major abnormalities have been correlated to impaired fertility, minor defects do not necessarily indicate a disturbance of spermatogenesis, but nevertheless, could cause a reduction in fertility if they are present in large proportions within the ejaculate [40]. The highest percentage of major pathological defect was observed in *S. collinsi* (about 8.61%), being a relatively low value, which do not compromise the use of that semen in biotechnologies of reproduction. We observed the eccentric insertion (abaxial) of the middle piece in the posterior portion of the head in all species, as previously described for other species of this genus [14]. Number of tail defects was greater than head defects in this study, as described in *S. collinsi* (strongly coiled tail 8.3%, coiled tail 9.3% and bent tail 7% vs. 0.33% pear-shaped defect) [19], *Sapajus apella* (strongly coiled tail 9%, bent tail 11% and coiled tail 7% vs. 0 head defects) [29] and *Callithrix jacchus* (50% tail defects vs. 4.5% head defects) [41].

5. Conclusions

Despite the variation between ejaculates quality and number of ejaculates, EEJ yielded satisfactory results, and these data expand the knowledge of reproductive biology in male squirrel monkeys. Furthermore, studies in free-living species during other periods of the year in order to meet the seasonal variations of the reproductive physiology are also necessary. Knowledge on semen characteristics will support the development of procedures for semen preservation and further fundamental studies on seminal characteristics within free-living non-human primates, which is extremely important, especially for the conservation of endangered species like *S. vanzolinii.*

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5 CAPÍTULO 2- SEMINAL CHARACTERISTICS AND CRYOPRESERVATION OF SPERM FROM THE SQUIRREL MONKEY, *Saimiri collinsi*

Artigo II

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Seminal characteristics and cryopreservation of sperm from the squirrel monkey, *Saimiri collinsi*

K.G. Oliveira[1], D.L. Leão[1], D.V.C. Almeida[1], R.R. Santos[1, 2*], S.F.S. Domingues[1,3]

[1]Laboratory of Wild Animal Biology and Medicine, Federal University of Pará, Belém, Pará, Brazil;

[2]Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands;

[3]Faculty of Veterinary Medicine, Federal University of Pará, Castanhal, Pará, Brazil.

*Corresponding author: Regiane R Santos, DMV, PhD, Faculty of Veterinary Medicine, Yalelaan 114. 3584CM, Utrecht, The Netherlands, Tel +31 30 2531078, Email: R.Rodriguesdossantos@pq.cnpq.br

Abstract

The Neotropical non-human primate squirrel monkey (*Saimiri* sp) is one of most commonly used species in research in several areas of knowledge. However, little progress has been reported in respect to techniques for preservation of their gametes. Thus, the main objectives of this study were (i) to describe testicular and seminal aspects of a new species *Saimiri collinsi*, (ii) to preserve semen of this species by cooling or freezing using ACP-118™ (powdered coconut water), and (iii) to test two glycerol (GLY) concentrations (1.5 or 3%) for semen freezing in presence of ACP-118™. Experimental group started with 14 captive males, but only 11 were suitable to collect ejaculates containing sperm. After anaesthesia both testes were evaluated: length, width, height and testicular circumference. Semen was collected by electroejaculation (EEJ) and evaluated, followed by dilution, cooling and freezing. Seminal parameters and sperm motility, vigour, plasma membrane integrity and normal morphology were evaluated after each step; functionality was also checked in fresh and frozen-thawed sperm. Sperm motility, plasma membrane integrity and normal sperm in cooled semen (n= 11) were respectively 44.1 ± 34.0, 63.1 ± 15.6 and 73.8 ± 19.8, with vigour ranging of 2-3. Sperm motility, plasma membrane integrity, normal and functional sperm in frozen semen (n= 05) were respectively 0.6 ± 1.3 (1.5 and 3% GLY), 4.4 ± 4.9 (1.5% GLY) and 6.6 ± 7.2 (3% GLY); 86.8 ± 3.0 (1.5% GLY) and 88.8 ± 5.1 (3% GLY); 13.3 ± 11.9 (1.5% GLY) and 14.3 ± 13.5 (3% GLY), and vigour 0 for both 1.5% and 3% GLY. No significant difference between glycerol concentrations was observed. We concluded that EEJ was efficient for semen collection of *S. collinsi* and tested cooling protocol allowed to recover a satisfactory percentage (63%) of membrane intact sperm. However, freezing protocol was not appropriate to sperm preservation.

Key-words: Squirrel monkey, semen, freezing, coconut water, glycerol.

1. Introduction

Neotropical non-human primates (NHP) have been facing serious conservation problems, due to a drastic decrease in their population or even extinction of species, sometimes before being characterized [1, 2]. To overcome or control this genetic loss, it is crucial to preserve and to restore preservation areas. In the meanwhile, preserving germplasm of NHP appears as an alternative to safeguard genetic material for future support in maintaining biodiversity [3].

Cryopreservation of male gametes has been described for different NHP species [4-16], with glycerol (GLY) being the most used permeable cryoprotectant at variable concentrations (2.5 – 14%) and TES-TRIS as the extender (Table 1). Besides TES-TRIS, a coconut water solution (CWS) for semen coagulum liquefaction appears as a promising extender [16]. A standardized powdered coconut water (ACP-118™, ACP Biotecnologia, Fortaleza, Ceará, Brazil), which contains vitamins, minerals, amino acids, carbohydrates, growth factors, phytohormones, and saturated fatty acids [17] has been successfully used as semen extender in other animal species such as dog [18, 19], stallion [20], fish [17, 21], agouti [22] and collared peccary [23]. Therefore, we considered it as a candidate to be tested in the NHP *Saimiri collinsi*.

Among the NHP species, the squirrel monkey (*Saimiri* sp) has been used in laboratory studies for more than 40 yr, resulting in vast information on their physiology and reproduction [24], but little is known in respect to gametes preservation. After a recent taxonomic review through molecular studies [25, 26], *S. collinsi* has been recognized as a new species. Up to now, *S. sciureus* and *S. boliviensis* are the most studied species [27, 28], from which semen obtained by electroejaculation (EEJ) and penile vibratory stimulation (PVS) was characterized

[5, 15, 29-33] (Table 2). Only two studies have described semen cryopreservation for these two species [14, 15]. No seminal description or semen preservation techniques are available for *S. collinsi*.

Table 1. Commonly used extenders and glycerol concentrations for the cryopreservation of non-human primate semen.

Species	**Extender**	**Cryoprotectant**
Callithrix jacchus	TES-TRIS [4]	3% glycerol
Cercopithecus aethiops	Sodium glutamate [5]	14% glycerol
Erythrocebus patas	Sodium glutamate [5]	14% glycerol
Macaca fascicularis	TRIS [6]	5% glycerol
	TES-TRIS [7]	3% glycerol
	TRIS [8]	6% glycerol
Macaca fuscata	TES-TRIS [9]	5% glycerol
Macaca mulatta	Sodium glutamate [5]	14% glycerol
	TES-TRIS [10]	12% glycerol
	TES-TRIS [10]	3% glycerol
	TRIS [11]	5% glycerol
Macaca speciosa	Sodium glutamate [5]	14% glycerol
Papio anubis	Sodium glutamate [12]	Egg yolk
	TES-TRIS [4]	3% glycerol
Pan troglodytes	Sodium glutamate [5]	14% glycerol
	TES-TRIS [13]	5% glycerol
	TES-TRIS [13]	2.5% glycerol
	TES-TRIS [4]	3% glycerol
Saimiri boliviensis	TES-TRIS [14]	8% glycerol and egg yolk
Saimiri sciureus	Lactose [15]	4% glycerol and egg yolk
Sapajus apella	TES-TRIS [16]	3.5% glycerol and egg yolk
	Coconut water – CWS [16]	2.5% glycerol and egg yolk

Table 2. Mean (± SD) or range values of seminal volume (µL), sperm concentration (x10^6 sperm/mL), plasma membrane integrity (PMI) (%), sperm motility (%) and normal sperm morphology (NSM) (%) in fresh semen collected from *S. sciureus* and *S. boliviensis.*

Species	Seminal volume	Sperm concentration	PMI	Sperm motility	NSM
S. sciureus*	50.8 - 81.3 [29]	106	NI	NI	NI
	100 [30]	0.295 ± 13	79 ± 2	NI	NI
	400 [5]	205.9	NI	52	NI
	NI [31]	153 ± 96	36.8 ± 19	35 ± 11.8	55.8
	159.5 ± 57 [15]	427.3± 160.6	NI	65.9 ± 15.4	NI
	80-300 [32]	NI	NI	40 - 80	< 51
S. boliviensis*	205 ± 25 [33]	2.8 ± 1.7	NI	44.1 ± 11.4	NI
S. boliviensis**	436 ± 90[33]	77.1 ± 20.4	NI	80.6 ± 4.3	NI

* Semen was collected by penile electroejaculation
** Semen was collected by penile vibrostimulation
NI: Not informed

We aimed to describe seminal characteristics of the new species *S. collinsi*, to test cooling and freezing protocols using ACP-118™ as extender, and to test two GLY concentrations (1.5 or 3.0%) for the cryopreservation of *S. collinsi* semen.

2. Materials and Methods

2.1. Animals and Semen Collection

This study was conducted with institutional approval from Ministério do Meio Ambiente - Sistema de Autorização e Informação em Biodiversidade - SISBIO/ICMBio/MMA nº 31542-2 and by Ethical Committee in Animal Research of Instituto Evandro Chagas (nº 0010/2011/CEPAN/IEC/SVS/MS).

The males were originated from Marajó archipelago (0°58'S and 49°34'W) and were maintained in captivity at National Primate Center (CENP), Ananindeua, Brazil (1°22'58''S

and 48°22’51’’ W) [34]. The climate is humid tropical, with an average annual temperature of 28 °C. The experimental group (n=14 males; ~ 15 yr) was selected by physical characteristics, clinical parameters such as complete hemogram and blood biochemical analysis. Animal body weight, external genitalia and andrologic examination, i.e. inspection and palpation of the testes to verify size, consistency, symmetry and mobility, were also used to select animals.

Animals were collectively housed in cages of 4.74 m x 1.45 m x 2.26 m (length, width and height, respectively), under natural photoperiod (12 h of light and 12 h of dark). The diet consisted of fresh fruits, vegetables, milk, commercial pellet chow for primates and larvae cricket (*Zophobas morio*); water was available *ad libitum*.

Physical restraint was performed by a trained animal caretaker wearing leather gloves. Semen was collected at the same period of the day, i.e. in the morning before feeding. After physical restraint, animals were anesthetized with ketamine hydrochloride (15 mg/kg; IM; Vetanarcol, Köning S.A., Avellaneda, Argentina) and xylazine hydrochloride (1 mg/kg; IM; Köning S.A.) by a veterinarian. Achieved total anaesthetic effect, the animals were placed in dorsal recumbence; both testes were evaluated and length (cranial-caudal), width (medial-lateral), height (dorsal-ventral) and total testicular circumference were measured. Genital region was then sanitized with a mild soap and distilled water (1:10) and gauze. Prepuce was retracted with the thumb and index fingers for an efficient cleaning of the penis.

Males were stimulated by EEJ (Autojac-Neovet, Uberaba, Brazil) with a rectal probe: 0.6 cm diameter and 12.5 cm length, with a rounded end, bearing two metal plates (2 cm in length and 0.8 cm width) on opposite sides [29]. The 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 increasing electrical stimuli (12.5-100 mA) with an interval of 30 s between series [16]. If a male was unable to ejaculate after the

session, no further attempts were made to collect semen, and intervals between semen collections were at least 30 d. A veterinarian monitored continually the animals during as well as after recovering from anaesthesia.

2.2. Extenders

Two extenders were prepared containing A and B-fractions. A-fraction consisted of 5.84 g ACP-118™ (ACP Biotecnologia) diluted in 50 mL ultra-pure water. B-fraction was constituted by 60% A-fraction plus 40% egg yolk. Final concentration of egg yolk was 20%. Osmolarity of A and B-fractions was 300 and 353 mOsm/L, respectively. Egg yolk was obtained at University Farm School, from chicken (*Gallus gallus domesticus*) eggs laid not more than 12 hours.

2.3. Seminal Evaluation

Eppendorfs containing semen were placed in a water bath at 37 °C immediately after ejaculation. Volumes of liquid and coagulated fractions were evaluated in a graduated tube, with the aid of a pipette. Appearance and consistency were assessed subjectively, i.e. colour (colourless, yellowish or whitish), opacity (opaque or transparent) and appearance (amorphous or filamentary seminal coagulum). Vigour was subjectively evaluated on a scale of 0 to 5 [15]. In brief, no motility was considered 0, slight movement with >75% of sperm showing vibration only was represented by 1, moderate forward movement in about >50% of sperm was represented by 2, forward movement in about 70% of sperm was represented by 3, and when ~90% or >95% of sperm presented very active forward movement, scales 4 and 5 were used, respectively. Sperm motility was expressed as the percentage of cells actively moving in a forward direction. Sperm vibrating in place were not considered to be motile [3]. To measure percentage of progressive forward motility, 10 μL of semen was placed in a pre-warmed (37 °C) glass slide with cover slip and 200 sperm were counted, as described by Oliveira et al. [16]. Sperm concentration was determined in a Neubauer chamber after dilution

of 1 μL semen in 99 μL formalin solution 10%. Sperm morphology and integrity of plasmatic membrane were evaluated by a smear prepared 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. Morphologic defects detected in sperm were classified as primary or secondary [16]. Sperm membrane functionality was assessed by hypo-osmotic swelling test (HOST) after dilution of 5 μL of semen in 45 μL of hypo-osmotic solution (108 mOsm/L). Seminal pH was measured with a pH strip (Merk Pharmaceuticals, Darmstadt, Germany). All evaluations were performed under a light microscope (Nikon, Tokyo, Japan), at a magnification X 100. Semen was assessed directly after collection (fresh), after dilution and before cooling (pre-cooled), after cooling, and after freezing in the presence of 1.5 or 3% GLY.

2.4. Semen cooling and freezing

After evaluation, fresh semen was diluted in A-fraction (1:1), placed in water bath at 37 °C until a sufficient volume of liquid fraction for cooling (above 50 μL) was obtained; this procedure took approximately 1 hr. This limiting time of dissolution was chosen to preserve sperm motility. Seminal samples were diluted in B-fraction (1:1) and microscopically evaluated. Only samples (n=11) presenting at least motility 20%, vigour 3, 50% of sperm membrane integrity and 30% of normal sperm morphology were cooled. Samples were cooled in covered microtubes, following a curve of 37 to 4 °C within 1.5 hr. After cooling, sperm was evaluated as described above. Cooled semen samples (n = 5) that displayed the least 5% motile sperm, sperm vigour of 2 or more, plasma membrane integrity of 40% and with a minimum of 30% sperm with normal morphology were then cryopreserved. For this, cooled semen was divided into two equal aliquots with a range in sperm concentration of 13 – 29 x 10^6 sperm/ml. To one aliquot was added GLY to a final concentration of 1.5%, and to the other aliquot to a final concentration of 3% GLY. This cryoprotectant was added directly to the semen in three steps, with 30 s interval each. Thereafter, aliquots were drawn into 0.12

mL plastic straws (IMV, L'Aigle, France), sealed with a metal bead and stored horizontally in vapour of liquid nitrogen (-60 °C) for 20 min, then plunged into liquid nitrogen (-196 °C). After one month storage, straws were kept in a water bath (37 °C) for 30 s [10] and the thawed semen was microscopically evaluated.

2.5. Statistical Analysis

All data are expressed as mean ± standard deviation (SD) and analysed by the Statview 5.0 program (SAS Institute Inc. Cary, NC, USA), except vigour, which was expressed as moda. The effect of storage temperature on sperm motility, plasma membrane integrity, morphology and functionality was evaluated by ANOVA. Comparisons between cryopreservation steps were performed using Fisher's test. The same effects on vigour were evaluated using Kruskal-Wallis test. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Testicular biometry and semen analysis

Selected males for this study (n=14) were healthy, as confirmed by their complete hemogram and blood biochemical analyses (Supplementary tables 1 and 2). Mean (± SD) body weight was 886 ± 113 grams (620 - 1115 grams; min – max). All animals presented testes with normal consistency, symmetry and mobility. However, one male was diagnosed with unilateral cryptorchidism, and hence it was removed from the experiment, despite the fact that measurements of his testis were equivalent to the average of the sample group. Testicular biometry of the 13 remaining males is presented in Table 3.

Table 3. Mean (± SD) of length, width, height, volume of right and left testicles and total testicular circumference of *Saimiri collinsi*; (n = 13 males)

	Length (cm)	**Width (cm)**	**Height (cm)**	**Volume (cm^3)**	**Total vol. (cm^3)**	**Total circumf. (cm)**
Right	1.86 ± 0.21	1.23 ± 0.11	1.16 ± 0.05	1.40 ± 0.26	2.96 ± 0.63	7.38 ± 0.42
Left	1.83 ± 0.24	1.34 ± 0.14	1.18 ± 0.09	1.55 ± 0.42		

From the 13 males, one did not ejaculate. A total of 32 semen collection trials (at least two attempts in each of the 12 males) were performed resulting in 20 ejaculates (62.5%). From the 20 ejaculates, three did not contain sperm and one animal presenting azoospermia was excluded. Therefore, 17 ejaculates were used in the present study (see details in Table 4). Ejaculation was almost always initiated by a liquid fraction often partially or totally coagulated after 10 s. Mean (± SD) collected volume of liquid and coagulated seminal fractions were 51.8 ± 49.5 μL (5 – 200 μL) and 304 ± 283.6 μL (10 – 1100 μL), respectively. Liquid and coagulated fractions were transparent or opaque and colourless, whitish or yellowish. It was observed a wide variation in appearance and constitution of ejaculates between collections regardless the male. Coagulated fraction presented filamentary or amorphous appearance (Fig. 1). Microscopic parameters of collected fresh semen are depicted in Table 4.

1 **Table 4.** Mean (± SD) values of ejaculates (number), sperm motility (%) and vigour (grade), sperm plasma membrane integrity (PMI; %), normal
2 sperm morphology (NSM; %), sperm concentration (x 10^6sperm/mL) and seminal pH of fresh semen collected. Data from each male; (n = 11).
3

Animal	Ejaculates	Motility	Vigour	PMI	NSM	Concentration	pH
AAA	1	100	5	75	81	98	7.0
AHR	1	45	3	49	68	-	6.5
AIM	2	70 ± 28.3	5	86.5 ± 13.4	67 ± 8.5	50 ± 7.1	7.5
BAA	2	50 ± 56.6	4	67.5 ± 17.7	80.5 ± 0.7	-	7.5
BAD	1	85.0	5	90	75	68	7.0
BAH	2	80 ± 14.1	4	83.5 ± 16.3	69 ± 0.9	53 ± 15	8.0
BAJ	2	80 ± 14.1	5	72.5 ± 10.6	66 ± 0.6	69 ± 8.6	7.5
BAO	1	50	3	54	77	49	7.8
BBC	1	80	5	88	61	102	7.0
BEX	2	95 ± 27.1	5	83.2 ± 16.7	61 ± 6.0	87	7.5
BEZ	2	90 ± 14.1	5	90 ± 14.1	78	72 ± 9.6	7.5

4
5
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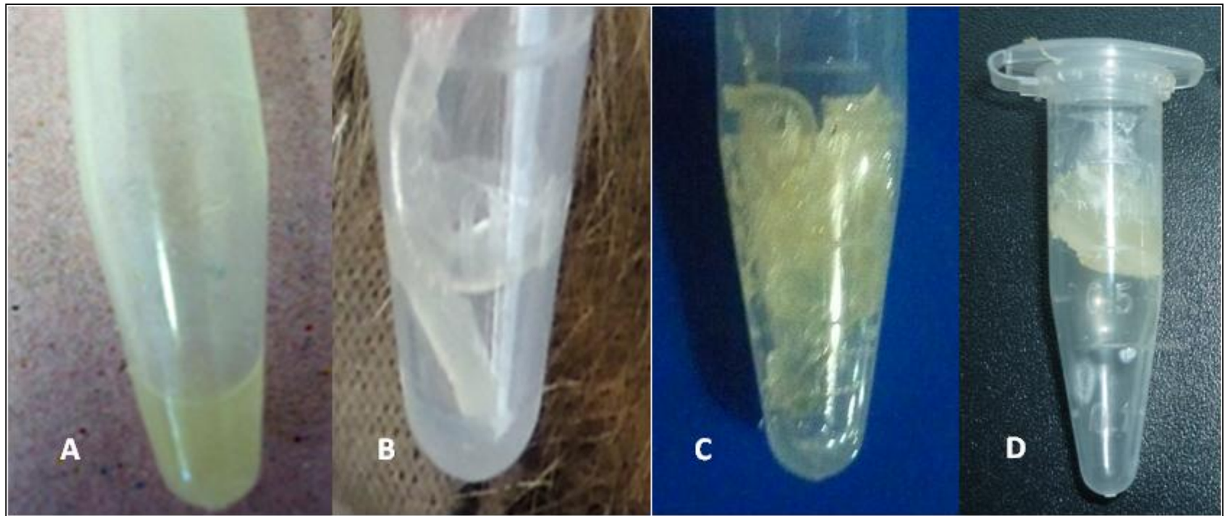

Figure 1. Representative images of opaque liquid fraction (A), filamentary (B) and amorphous (C) fractions of semen collected from *S. collinsi* by electroejaculation.

3.2. Semen cooling and freezing

From the 20 ejaculates, 11 (55%) provided semen presenting sufficient quality to be cooled, i.e. at least sperm motility of 20%, vigour 3, 50% of sperm membrane integrity and 30% of normal sperm morphology. From the 11 cooled ejaculates, 5 (46%) provided semen presenting quality to be frozen, i.e. at least sperm motility of 5%, vigour 2, 40% of sperm membrane integrity and 30% of normal sperm morphology. Sperm motility and plasma membrane integrity were not affected during semen dilution (pre-cooled semen) when compared to fresh semen, but this procedure decreased ($p < 0.01$) vigour. Cooling, on the other hand, resulted in decreased ($p < 0.01$) sperm motility, vigour and plasma membrane integrity when compared to fresh ($p < 0.001$) semen. Independently on the GLY concentration (1.5 or 3%), all the evaluated parameters (sperm motility, vigour, plasma membrane integrity and functionality) were negatively affected ($p < 0.001$) by freezing, except the percentages of morphologically normal sperm that remained the same in all steps (Figure 2). The most common observed sperm morphological changes were coiled and strongly coiled tail (Table 5).

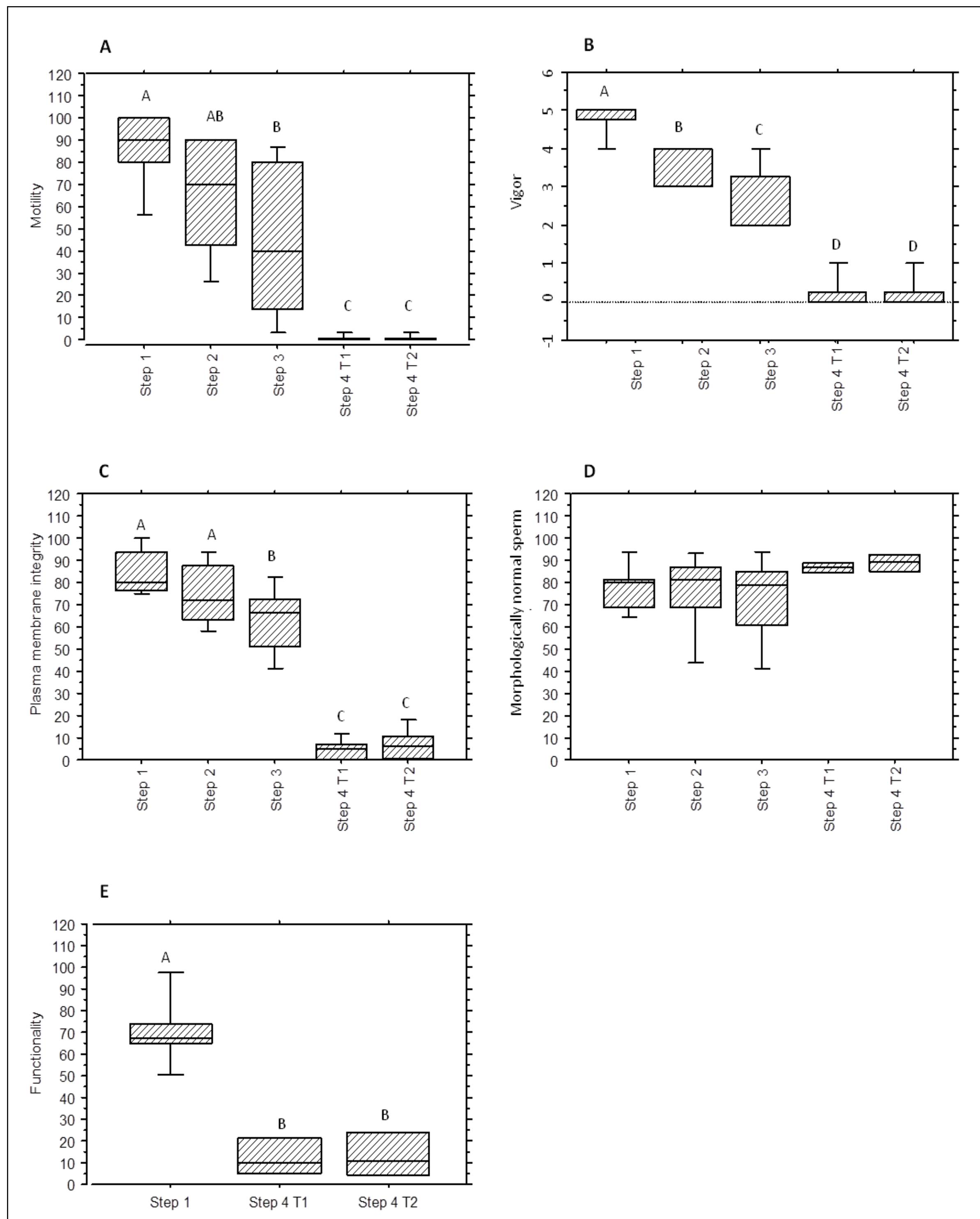

Figure 2. Sperm parameters of fresh (n= 11), pre-cooled (n= 11), cooled (n= 11) and frozen semen using 1.5 (n= 05) or 3% (n= 05) glycerol. A: Sperm motility, B: Sperm vigour, C: Plasma membrane integrity, D: Normal sperm morphology and E: Plasma membrane functionality*.

*evaluated only in fresh and frozen-thawed sperm.

A-D: different upper-case letters indicates significant differences among groups within each parameter. ($p < 0.05$).

Table 5. Mean (± SD) percentages of normal sperm and sperm morphology (major and minor pathologic defects) in fresh (n = 11 males) and frozen (n = 5 males) semen of *S. collinsi* in 1.5 or 3% glycerol.

Morphology	**Fresh**	**Frozen**	
		1.5% GLY	**3.0% GLY**
Normal	74.7 ± 7.8	87 ± 1.4	89.5 ± 2.1
Major pathologic defects			
Strongly coiled tail	8.3 ± 13.6	5 ± 7.1	5.5 ± 7.8
Pear-shaped defect	0.33 ± 0.6	0	0
Minor pathologic defects			
Coiled tail	9.3 ± 5.5	5 ± 7.1	2.5 ± 3.5
Bent tail	7 ± 1.7	3 ± 1.4	2.5 ± 2.1
Axial tail	0.3 ± 0.6	0	0

Supplementary table 1. Hemogram results from *S. collinsi* males (n = 13).

Parameter	**Results**	**Reference values**
Hematocrit (%)	43.2 ± 3.6	44.00 ± 0.6
Red blood cells (x10^6/mL)	07.2 ± 0.4	07.1 ± 0.1
Hemoglobin (g/dL)	13.9 ± 1.2	13.8 ± 0.2
Mean corpuscular volume (fL)	59.9 ± 2.8	61.9 ± 0.6
Mean corpuscular hemoglobin (pg)	19.1 ± 1.1	19.4 ± 0.2
Mean corpuscular hemoglobin concentration (%)	31.8 ± 1.0	31.5 ± 0.2
White blood cells (x10^3/mL)	11.0 ± 4.1	10.5 ± 0.6
Platelets (m/mm^3)	268 ± 93	-
Basophils (%)	0.44 ± 0.7	0.0 ± 0.2
Eosinophils (%)	01.9 ± 0.9	01.0 ± 0.2
Neutrophils (%)	51.5 ± 11.7	35.0 ± 3.2
Lymphocytes (%)	44.1 ± 11.2	61.0 ± 3.1
Monocytes (%)	02.1 ± 1.6	02.0 ± 0.3

Values are expressed as mean ± SD.
Source: Kakoma I, James MA, Jackson W, Bennett G, Ristic M. 1985. Hematologic values of normal bolivian squirrel monkeys (*S. sciureus*): a comparison between wild-caught and laboratory-bred male animals. Folia Primatologica 44:102-107.

Supplementary table 2. Biochemical analysis of plasma from *S. collinsi* males (n= 13). Values are expressed as mean ± SD.

Parameters	Results	Reference values
Glucose (mg/dL)	108 ± 42.4	103 ± 30.3
Blood urea nitrogen (BUN) (mg/dL)	48.8 ± 37.7	38.7 ± 10
Cholesterol (mg/dL)	188.3 ± 43.0	151 ± 64.7
Triglycerides (mg/dL)	72.5 ± 40.4	74.9 ± 32.7
Creatinine (mg/dL)	0.6 ± 0.1	0.9 ± 0.2
Total Bilirrubin (mg/dL)	< 0.10	0.8 ± 0.6
Phosphatase (U/L)	249 ± 308	358 ± 175
Glutamic Oxaloacetic Transaminase (U/L)	190 ± 56.6	185 ± 95.3
Glutamic Pyruvic Transaminase (U/L)	201 ± 75.4	184 ± 110
Total Protein (g/dL)	6.5 ± 0.7	6.9 ± 1.0
Calcium (mg/dL)	9.7 ± 0.9	9.6 ± 0.9
Albumina (g/dL)	3.7 ± 0.3	4.2 ± 0.6
Carbon Dioxide	21	11.1 ± 3.9
Potassium	3.5	5.7 ± 1.0
Thyroxine-Binding Globulin	2.8 ± 0.5	-
Very Low Density Lipoprotein	17.3 ± 7.6	-
Ammonia	46.5 ± 55.9	-
Phosphorus	5.3 ± 0.2	-
Magnesium	2.7 ± 0.3	-
Iron	154 ± 94.1	-

Source: KCCMR. Michale E. Keeling Center for Comparative Medicine and Research. [Internet]. Texas: The University of Texas MD. Anderson Cancer Center. [cited 2014 March 2]. Available from: http://www.mdanderson.org/education-and-research/departments-programs-and-labs/programs-centers-institutes/michale-e-keeling-center-for-comparative-medicine-and-research/animal-resources/squirrel-monkey-diagnostic-reference-values.html.

4. Discussion

Despite the studies on *S. sciureus* and *S. boliviensis* [14, 15, 29-33], this is the first report describing seminal and andrologic parameters of *S. collinsi*. Mean body weight was similar to that of other *Saimiri* species [35], but total testicular volume of *S. collinsi* (2.96 ± 0.63 cm³; mean ± SD) was greater than in *S. sciureus* (0.95 - 1.79 cm³; min-max) [36].

Anaesthetic and EEJ protocol applied during this experiment allowed semen collection, although only 55% of the ejaculates were of sufficient quality that they were then cooled. In a study by Yeoman et al. [33], PVS was found to improve sperm recovery compared to EEJ in *S. boliviensis*. However, the use of PVS requires animal conditioning, which means its application in captive males. Although the present study was performed using captive *S. collinsi*, our main goal was to develop a protocol to be applied under field conditions for free living animals. In this case, semen collection must be performed without conditioning and, hence, by EEJ under anaesthesia.

Microscopic characteristics of fresh semen was similar to those previously described for semen of *S. sciureus* collected by EEJ [31] and of *S. boliviensis* collected by EEJ and PVS [33], regarding plasma membrane integrity, sperm motility, morphology and concentration. Seminal volume collected in this work was similar to that previously reported in *S. sciureus* [5, 15, 30, 32] and *S. boliviensis* [33]. Seminal pH was similar as reported for *Ateles geoffroyi* [37]. There is no available report on the seminal pH in other *Saimiri* species.

Differently from *S. sciureus* and *S. boliviensis*, from whose collected semen (EEJ or PVS) coagulates immediately after ejaculation [29, 33], we observed coagulation only 10 s after *S. collinsi* semen collection, with part of the ejaculate remaining liquid. No relationship between ejaculate constitution and individual was observed. The time spent for incubation (max 60 min) with extender did not affect sperm motility, but appeared to contribute with an impairment of sperm vigour as previously observed in *Sapajus apella* [16]. Although a

decrease in seminal quality post-cooling was observed, parameters values are still acceptable and this procedure appears as a tool for the short-term storage of semen from *S. collinsi*.

Unfortunately, the present freezing procedure was unable to preserve sperm quality. It is difficult to point out the exact cause of this failure once we were testing ACP-118™ for the first time, but some explanations can be proposed. Sperm concentration in the frozen samples in the present study was ranging of 13 - 29 x 10^6 sperm/mL, which is low when compared to other NHP species when submitted to a similar procedure. For instance, cryopreserved semen from common squirrel [15] and rhesus [38] monkeys often contains a sperm concentration of 50 - 100 x 10^6 sperm/mL, from which motility appears preserved after thawing. In the present study, centrifuging the semen to obtain a greater sperm concentration was leading to semen coagulation. Due the low sperm concentration obtained, it was not possible to include extra treatments or experiments to compare ACP-118™ with the routine extender TES-TRIS. This challenge should be solved in future studies. In some investigations with primate sperm, GLY was diluted in a part of the used medium before being added to the semen prior to cooling [4, 6, 10, 16, 39-41]. In the present study GLY was added after cooling, based on a study with *Sapajus apella* sperm, where prolonged exposure to GLY was detrimental to sperm quality [16]. Maybe this also had affected the success of the present procedure.

5. Conclusions

In conclusion, EEJ was efficient for semen collection of squirrel monkey and tested cooling allowed the recovery of a satisfactory percentage of sperm with intact plasma membrane. The possibility to preserve semen from *S. collinsi* at low temperatures, even for a short period, facilitates transport and handling of sperm from this Neotropical NHP. Although the present freezing protocol using ACP-118™ was not appropriate for the preservation of *S. collinsi* sperm, future studies including the improvement of the sperm concentration and the

adequate moment of cryoprotectant addition to the medium might help to develop an efficient freezing protocol for semen from these species.

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6 CAPÍTULO 3- COOLING AND FREEZING OF SPERM FROM CAPTIVE, FREE-LIVING AND ENDANGERED SQUIRREL MONKEY SPECIES

Artigo III

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COOLING AND FREEZING OF SPERM FROM CAPTIVE, FREE-LIVING AND ENDANGERED SQUIRREL MONKEY SPECIES

Karol G. Oliveira[a,b]. E-mail: karolgoliveira83@gmail.com

Regiane R. Santos[a,c*]. E-mail: regianerdossantos@gmail.com

Danuza L. Leão[a]. E-mail: danleao.88@gmail.com

Adriel B. Brito[a]. E-mail: adrielbehn@yahoo.com.br

Julianne S. de Lima[a]. E-mail: juliannesilvabio@gmail.com

Wlaísa V. Sampaio[a]. E-mail: wlaisa.sampaio@gmail.com

Sheyla F. S. Domingues[a]. E-mail: shfarha@gmail.com

[a]Laboratory of Wild Animal Biology and Medicine, Federal University of Pará, Castanhal, PA, Brazil, CEP 68740-970

[b]National Primate Center, Ananindeua, PA, Brazil, CEP 66645-000

[c]Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

*Correspondence to Regiane R Santos

Federal University of Pará, Laboratory of Wild Animal Biology and Medicine

BR 316 Km 61, CEP 68740-970, Castanhal, Pará, Brazil.

Tel.: +55 91 33114707

ABSTRACT

Germoplasm banking is an important tool for the preservation of genetic material from endangered species, such as *Saimiri vanzolinii* (Black-headed squirrel monkey). That species is the Neotropical primate with a lower incidence area, just 870 km of floodplains in southern part of the Mamirauá Sustainable Development Reserve. Therefore, in the present study we aimed to develop a sperm cryopreservation protocol comparing sperm cooling in presence (T1) and absence (T2) of egg yolk, and to test freezing protocols to preserve semen from captive (*Saimiri collinsi*), and free-living (*S. vanzolinii*, *S. cassiquiarensis* and *S. macrodon*) New World non-human primates. Cooling preserved sperm of *S. collinsi* in all evaluated microscopic parameters, except for sperm motility. No differences were observed among the treatments, indicating that semen of this species can be cooled without egg yolk. Freezing did not affect sperm quality of *S. collinsi*, except plasma membrane integrity that was negatively affected. Generally a good maintenance rate was observed between cooling and thawing of semen for the four species, showing the positive translational application of protocols from *S. collinsi* to the free-living species. Developed freezing protocol proved to be useful for sperm cryopreservation of *S. collinsi* and in field conditions.

Keywords: *Saimiri*, Neotropical primate, semen, sperm cryopreservation, coconut water, ACP, glycerol

INTRODUCTION

Currently, several New World Primate species are listed in the Red List of Threatened Species as vulnerable to extinction [10]. *Saimiri vanzolinii* (Black-headed squirrel monkey) is one of those vulnerable species with their entire group geographically limited to the Mamirauá Sustainable Development Reserve and surroundings [19]. Therefore, the formation of germplasm banks of gametes and somatic cells appear as an alternative to preserve genetic diversity to be used in breeding biotechnology programs [4].

Unfortunately, the application of reproductive biotechnologies focusing on the conservation of male gametes of the genus *Saimiri*, popularly named “squirrel monkeys”, have not yet been settled on a laboratory routine basis. Sperm freezing has been reported only in *S. sciureus* [3], *S. boliviensis* [24] and *S. collinsi* [17] (Table 1), and it is not known if data on those species can be translated to all *Saimiri* species. Although using different protocols (extenders and glycerol concentrations), freezing of *S. sciureus* and *S. boliviensis* semen resulted in a sperm viability of approximately 50%, while this viability rate decreased to less than 10% in *S. collinsi*. Semen cooling also consists in an additional method to preserve sperm for a short period, once it facilitates the semen transport and handling in the field [12], as well as it is known that some males present semen with poor freezability [8].

Recently, we reported the use of the purified and lyophilized coconut water extender (ACP-118™, ACP Biotecnologia, Fortaleza, Ceará, Brazil) for *S. collinsi* sperm cooling and freezing. Combined with this extender, egg yolk (20%) and glycerol (1.5 or 3%) were used as extra- and intracellular cryoprotectants, respectively [17]. Probably because of the prolonged semen exposure to glycerol, i.e. direct exposure in the fraction A before freezing, frozen-thawed sperm presented a very poor quality. In the present study, GLY was added only diluted in the fraction B.

Table 1: Mean (± SD) of plasma membrane integrity (PMI), sperm motility and sperm motility recovery rate (MRR) in frozen-thawed semen of different species of squirrel monkeys, extenders and cryoprotectants extracellular (EC) and intracellular (IC).

Species	PMI	Motility	MRR	Extender	EC	IC
Saimiri sciureus[3]	NI	53.8 ± 17.1 51.8 ± 22.9	83.4 ± 33.7 87.9 ± 44.8	-	20% egg yolk 11% lactose	4% glycerol
Saimiri boliviensis[24]	NI	41	56	TES-Tris	egg yolk	8% glycerol
Saimiri collinsi[17]	4.4 ± 4.9 6.6 ± 7.2	0.6 ± 1.3	NI	ACP-118™	20% egg yolk	1.5% glycerol 3% glycerol

NI: No informed data.

Besides the vulnerable species *S. vanzolinii*, the other *Saimiri* species such as *S. cassiquiarensis* and *S. macrodon* represent other monkeys from the *Saimiri* genus, from which no semen is banked or evaluated. The *S. collinsi* comprises an optimal model to develop semen biotechnologies. For a field application of the procedure one must bear in mind the difficulties and risks to use non-fresh egg yolk for semen cooling. Therefore, in this study we aimed (i) to compare two sperm cooling procedures using ACP-118™ added or not by egg yolk on the quality of sperm from captive *S. collinsi*; (ii) to test a sperm freezing protocol in *S. collinsi* and iii) to evaluate the success of the application of the *S. collinsi* semen preservation protocols under field conditions in three free-living *Saimiri* species (*S. vanzolinii*, *S. cassiquiarensis* and *S. macrodon*).

MATERIAL AND METHODS

Animals and semen collection

This study was conducted at two different locations. Captive males (*S. collinsi*) were maintained at National Primate Center (CENP), Ananindeua, Brazil (1°22'58"S and 48°22'51"W), where the climate is humid tropical, with an average annual temperature of 28 °C. Free-living males (*S. vanzolinii, S. cassiquiarensis* and *S. macrodon*) were captured at

Mamirauá Sustainable Development Reserve (Figure 1). The reserve is a protected area located at the confluence of the Solimões and Japurá rivers (03°02'22"S and 64°51'41"W), covering a total of 1,124,000 ha of floodplain ecosystems [19]. Monthly average precipitation is 131.1 mm, and average temperature is 27.5 °C (min. average 23.02 °C and max. average 31.86 °C) [9].

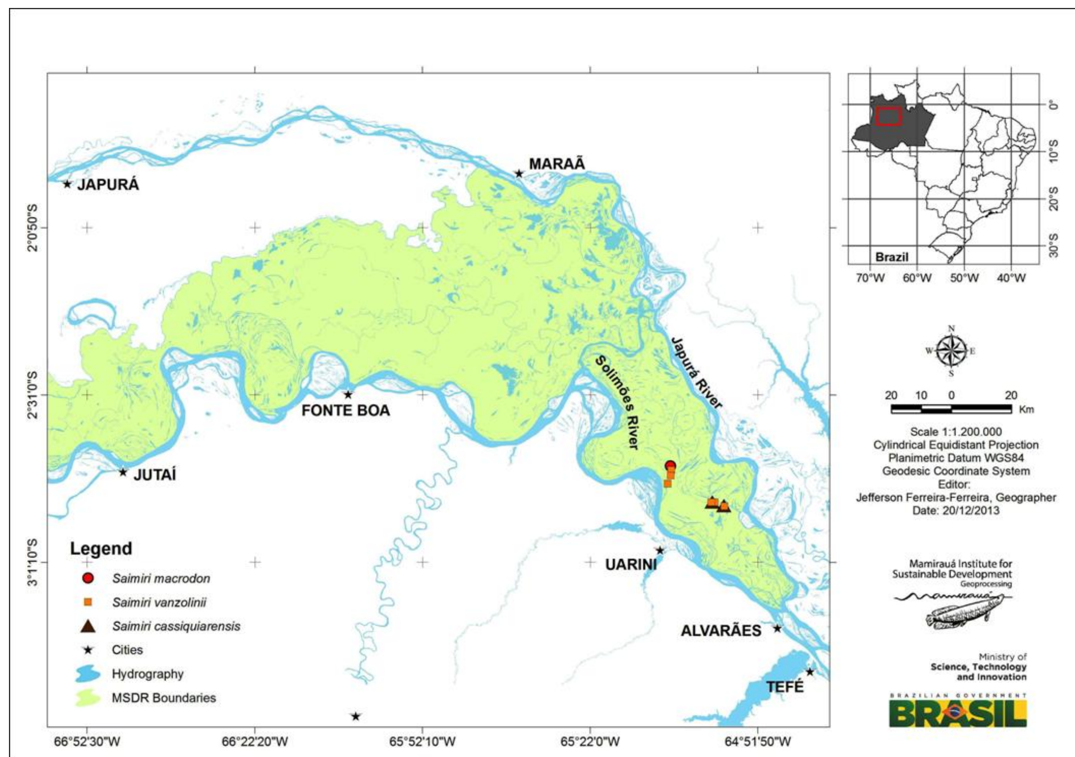

Figure 1: Capture points of *S. vanzolinii*, *S. cassiquiarensis* and *S. macrodon.* Source: Mamirauá Institute for Sustainable Development Geoprocessing, 2013.

All experimental protocols were approved by environmental authorities (Ministry of the Environment - System of Authorization and Information on Biodiversity - SISBIO/ICMBio/MMA nº 31542-2, for captive animals/nº 29906-3, for wild animals), by the Ethical Committee in Animal Research (nº 0010/2011/CEPAN/IEC/SVS/MS, for captive animals) and Ethics and Research Committee and the Animal Use Ethical Committee of the Mamirauá Institute for Sustainable Development (nº 002/2012, for wild animals).

S. collinsi males (n= 12) were selected by their physical characteristics, and clinical

parameters such as complete hemogram, hepatic and renal function. External genitalia were evaluated and andrologic examination (i.e. inspection and palpation of the testes to verify size, consistency, symmetry and mobility) was performed. 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 diet consisted of fresh fruit, milk and commercial pellet chow (MEGAZOO P18™, Protein 18%, Fiber Maxi. 6.5%, Betim, MG, Brazil) and cricket larvae (*Zophobas morio*). Vitamins, minerals and eggs were supplied once a week and water was available *ad libitum*. The physical restraint was done with netting and leather glove, by a trained animal caretaker. Semen was collected at the same period of the day (in the morning before feeding) and throughout four months (August to November) of 2012.

S. vanzolinii (n= 6), *S. cassiquiarensis* (n= 5) and *S. macrodon* (n= 1) males were captured using a Tomahawk Live Trap (0.7 m x 0.4 m x 0.4 m) (length, width and height, respectively) in two field expeditions, in November 2012 and October 2013. Traps were set up in the early morning (5 a.m.) and checked after 4 h and at mid-afternoon. The animals caught were handled by a trained animal caretaker wearing leather gloves.

After physical restraint, all study animals were anesthetized with ketamine hydrochloride (15 mg/kg; IM; Vetanarcol, König S.A., Avellaneda, Argentina) and xylazine hydrochloride (1 mg/kg; IM; Kensol, König S.A.) by a veterinarian [17]. Achieved total anaesthetic effect, animals were placed in dorsal 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 by electroejaculation (EEJ) (Autojac-Neovet, Uberaba, Brazil) with a rectal probe produced according Bennett [2]. The 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 30 s between series [17].

Ejaculates (liquid and coagulated fractions) were collected into microtubes (1.5 mL). If a wild male did not ejaculate after the sessions, no further attempts were made to collect semen. In the case of captive animals, another EEJ was executed after at least 30 days. In captivity, semen sampling was performed in a collection room. In the wild it was performed near the capture points to avoid the removal of the caught animals from their place of origin. Rectal temperature was measured prior to the EEJ procedure. A veterinarian monitored the animals during EEJ as well as after recovering from anaesthesia.

Extenders

Two extenders were prepared: A and B-fractions. A-fraction was utilized for the first dilution, for seminal liquefaction, and consisted of 5.84 g ACP-118™ diluted in 50 mL ultrapure water. B-fraction was utilized for the second dilution and was constituted by 0.598 mL A-fraction plus 0.4 mL egg yolk and 0.002 mL gentamicin. Osmolarities of A and B-fractions were 300 and 353 mOsm/L, respectively.

Seminal evaluation

Microtubes containing semen were placed in a water bath at 37 °C immediately after ejaculation. Volumes of liquid and coagulated fractions were evaluated in a graduated tube, with the aid of a pipette. Appearance and consistency were assessed subjectively, i.e. colour (colourless, yellowish or whitish), opacity (opaque or transparent) and appearance (amorphous or filamentary seminal coagulum).

Vigour was subjectively evaluated on a scale of 0 to 5 as previously described [17]. In brief, no sperm motility was considered 0, slight movement with >75% of sperm showing

vibration only was represented by 1, moderate forward movement in about >50% of sperm was represented by 2, forward movement in about 70% of sperm was represented by 3, and when ~90% or >95% of sperm presented very active forward movement, scales 4 and 5 were used, respectively. Sperm motility was expressed as the percentage of cells actively moving in a forward direction. Sperm vibrating in place were not considered to be motile [4]. To measure percentage of progressive forward sperm motility, 10 μL of semen was placed in a pre-warmed (37 °C) glass slide with cover slip and 200 sperm were counted. Normal sperm morphology and plasma membrane integrity were evaluated by a smear prepared 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. Morphologic defects detected in sperm were classified as primary or secondary [18]. Plasma membrane functionality was assessed by hypo-osmotic swelling test (HOST) after dilution of 5 μL of semen in 45 μL of hypo-osmotic 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 10 μL of this solution in a pre-warmed (37 °C) glass slide with cover slip, and at least 100 spermatozoa were counted. Spermatozoa with functional plasma membrane were those presenting coiled tail.

Sperm concentration was determined in a Neubauer chamber after dilution of 1 μL semen in 99 μL formalin solution 10%. Seminal pH was measured with a pH strip (Merk Pharmaceuticals, Darmstadt, Germany). All evaluations were performed under a light microscope (Nikon, Tokyo, Japan), at a magnification X 100. Semen was assessed directly after collection (fresh), after dilution and before cooling (pre-cooled), after cooling and after freezing in the treatments 1 and 2.

Seminal liquefaction and sperm cooling

After evaluation, fresh semen was diluted in A-fraction (1:1), placed in water bath at 37 °C until a sufficient volume of liquid fraction for cooling (above 50 μL) was obtained; this procedure took approximately 1.5 h. This limiting time of dissolution was chosen to preserve sperm motility. Only samples presenting at least sperm motility 50%, vigour 3, 60% of plasma membrane integrity and 80% of normal sperm morphology were selected for cooling (*S. collinsi*, n= 4; *S. vanzolinii*, n= 2; *S. cassiquiarensis*, n= 1; *S. macrodon*, n= 1).

Samples were equally divided in two aliquots. Two portions of B-fraction were prepared, each one containing the same volume of the seminal aliquots, for use in two treatments. In treatment 1 (T1) the seminal sample was diluted in B-fraction at 1 part sample to 0.75 part extender (1:0.75). The remainder 0.25 part of B-fraction was reserved for a later dilution. In treatment 2 (T2) the seminal sample was not diluted in B-fraction. Samples were cooled in covered microtubes, following a curve of 37 to 4 °C within 1.5 h. After cooling, sperm was evaluated as described above and submitted to cryopreservation.

Sperm freezing and thawing

For freezing, cooled semen with a range in sperm concentration of 15 to 40 x 10^6 sperm/mL was used. For T1, the remainder part of B-fraction (0.25) was added with glycerol to a final concentration of 3%. Dilution was performed within 30 s. For T2, the sample was diluted in B-fraction (1:1) containing glycerol. Likewise, this dilution was performed three times at 30 s interval and final glycerol concentration was 3%.

Thereafter, samples from both treatments were separately drawn into 0.12 mL plastic straws (IMV, L'Aigle, France), sealed with a metal bead and stored horizontally in vapour of liquid nitrogen (-60 °C) for 20 min, then plunged into liquid nitrogen (-196 °C). After two months storage, straws were kept in a water bath (37 °C) for 30 s and the thawed semen was microscopically evaluated.

Statistical analysis

All data are expressed as mean ± standard deviation (SD) and analysed by the Statview 5.0 program (SAS Institute Inc. Cary, NC, USA), except vigour, which was expressed as moda. The effect of storage temperature on sperm motility, plasma membrane integrity, normal sperm morphology and plasma membrane functionality was evaluated by Students'test. Vigour data were evaluated using Kruskal-Wallis test. Effect of extenders was evaluated by ANOVA, and differences were located with a Fisher's protected least significant difference (LSD) post hoc test. $p < 0.05$ was considered as statistically significant.

RESULTS

Seminal analysis

Selected males for this study (*S. collinsi* n= 12, *S. vanzolinii* n= 6, *S. cassiquiarensis* n= 5 and *S. macrodon* n= 1) were healthy, as confirmed by their clinical and haematological analyses. Means (± SD) of body weights per species were 913.75 ± 116.29 grams (725 – 1125 grams; min – max, *S. collinsi*), 852.5 ± 115.57 (715 – 1055, *S. vanzolinii*), 598.75 ± 33.01 (555 – 635, *S. cassiquiarensis*) and 720 (*S. macrodon*). All animals presented testes with normal consistency, symmetry and mobility.

A total of 39 semen collection attempts in 24 males resulted in 21 ejaculates (seven males did not ejaculate). From the 21 ejaculates, 4 contained scarce sperm and 3 were azoospermic , whence were excluded from this study (see Table 2). Therefore, 8 ejaculates were used in the present study (see details in Table 2). Ejaculation was almost always initiated by a liquid fraction often partially or totally coagulated after 10 s. Mean (± SD) collected volume of liquid and coagulated seminal fractions were 40.71 ± 66.83 µL (0 – 250 µL) and 170.24 ± 165.59 µL (0 – 500 µL), respectively. Liquid and coagulated fractions were transparent or opaque and colourless, whitish or yellowish. It was observed a wide variation

in appearance and constitution of ejaculates between collections regardless the male or species.

Table 2: Number of males submitted to electroejaculation (N), total number of trials (EEJ), number of ejaculations, number of selected seminal samples and mean (± SD) volumes of liquid and coagulated seminal fractions of the selected samples in studied species.

Species	N	EEJ	Ejaculations	Samples	LF volume (µL)	CF volume (µL)
S. collinsi	12	27	12	4	100 ± 106.14	287.5 ± 184.28
S. vanzolinii	6	6	6	2	85 ± 91.92	90 ± 84.85
S. cassiquiarensis	5	5	2	1	10	50
S. macrodon	1	1	1	1	0	500
Total	**24**	**39**	**21**	**8**	**-**	**-**

Sperm cooling and freezing

S. collinsi

Results of seminal parameters assessed after each step of cryopreservation (initial evaluation of fresh semen, dilution/incubation, cooling and freezing) are depicted in Figure 2. Sperm motility was not affected by dilution and liquefaction, being similar to the fresh semen (p= 0.08). No difference was observed between treatments (T1 and T2), for both cooling (p= 0.06) and freezing (p= 0.2) procedures. Sperm motility was not impaired by freezing in T2, but significantly decreased when submitted to T1 (Figure 2A). Regarding sperm vigour, no difference was observed between treatments or cryopreservation steps (p= 0.06) (Figure 2B).

Plasma membrane integrity was unaffected by dilution (p= 0.22), as well as before and after cooling in T1 (p= 0.44) and T2 (p= 0.46). However, a significant decline on plasma membrane integrity was observed after freezing at T1 (p= 0.002) and T2 (p= 0.03), with no difference between treatments (p= 0.33) (Figure 2C). Normal sperm morphology percentage remained constant before and after cooling in both T1 (p= 0.79) and T2 (p= 0.83), as well as

before and after freezing at T1 (p= 0.79) and T2 (p= 0.89) (Figure 2D).

The percentage of sperm with functional plasma membrane was unchanged before and after dilution (p= 0.15), before and after cooling in both T1 (p= 0.89) and T2 (p= 0.77), with no difference between treatments (p= 0.46). Significant difference was observed only after freezing at T1 (p= 0.02), although it remained similar to control at T2 (p= 0.34). There was no significant difference between treatments after freezing (p= 0.5) (Figure 2E).

S. vanzolinii, S. cassiquiarensis **and** ***S. macrodon***

Due to the small number of samples of free living species, statistical comparisons among species were not performed. Only two ejaculates of *S. vanzolinii*, one of *S. cassiquiarensis* and one of *S. macrodon* (Table 3) had sperm quality to be cooled and frozen.

Sperm morphology in fresh, diluted, cooled and frozen semen in T1 and T2, per male of each species is detailed in Table 4.

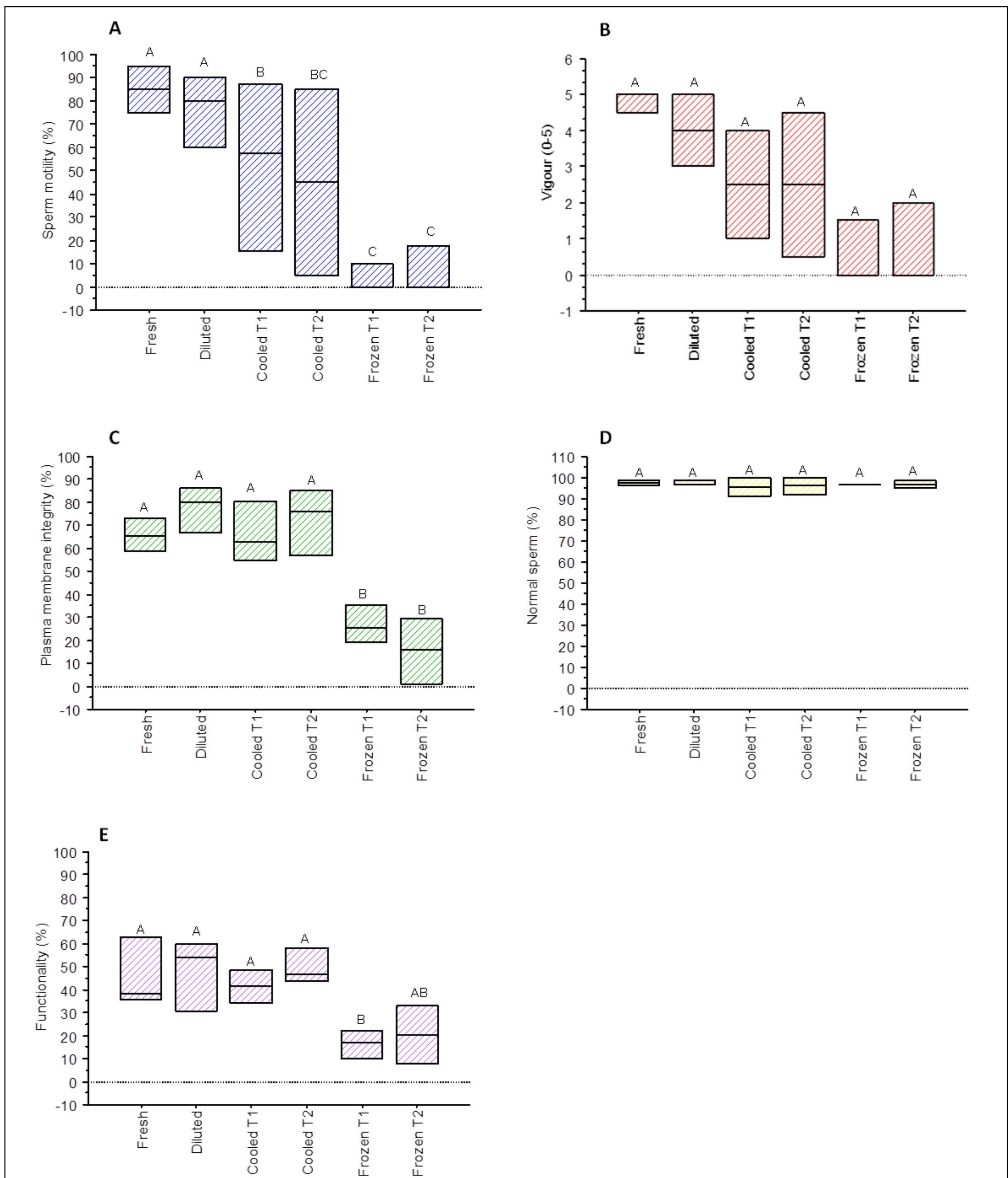

Figure 2: Mean percentage of sperm motility (A), sperm vigour (B), plasma membrane integrity (C), normal sperm (D) and plasma membrane functionality (E) in fresh semen, diluted (pre cooling), cooled in T1, cooled in T2, frozen in T1 and frozen in T2 of *S. collinsi* (n= 4).

Table 3: Sperm motility, vigour, plasma membrane integrity (PMI) and plasma membrane functionality in fresh, diluted, cooled and frozen semen in T1 and T2, per male of *S. vanzolinii* (Svan; n= 2), *S. cassiquiarensis* (Scas; n= 1) and *S. macrodon* (Smac; n= 1).

	Svan.1	Svan.2	Scas	Smac
Fresh				
Motility	90	100	80	90
Vigour (0-5)	5	5	5	5
PMI	81	69	62	98
Diluted				
Motility	90	NA	20	70
Vigour (0-5)	5	NA	2	5
PMI	79	NA	43	85
Cooled T1				
Motility	30	30	10	60
Vigour (0-5)	3	2	3	5
PMI	67	52	21	90
Cooled T2				
Motility	30	30	0	40
Vigour (0-5)	3	2	0	4
PMI	75	37	25	71
Frozen T1				
Motility	30	30	6	10
Vigour (0-5)	2	3	2	3
PMI	32	48	3	34
Functionality*	15	31	0	44
Frozen T2				
Motility	60	30	6	10
Vigour (0-5)	4	2	2	5
PMI	55	2	7	38
Functionality*	NA	24	20	49

*Functionality was evaluated only in frozen semen.

NA: Not assessed.

Table 4: Sperm morphology in fresh, diluted, cooled and frozen semen in T1 and T2, per male of *S. collinsi* (Scol; n= 4), *S. vanzolinii* (Svan; n= 2), *S. cassiquiarensis* (Scas; n= 1) and *S. macrodon* (Smac; n= 1).

	Scol	Svan	Scas	Smac
Fresh				
Normal	99	95 ± 4.24	NA	NA
Strongly coiled tail	0.5 ± 0.71	0.5 ± 0.71	NA	NA
Coiled tail	0.5 ± 0.71	4 ± 5.65	NA	NA
Bent tail	0	0.5 ± 0.71	NA	NA
Diluted				
Normal	98 ± 1.41	97	91	NA
Strongly coiled tail	0.25 ± 0.5	0	3	NA
Coiled tail	0.75 ± 0.95	2	5	NA
Bent tail	1 ± 1.41	1	1	NA
Cooled T1				
Normal	95.5 ± 4.95	98 ± 2.83	94	NA
Strongly coiled tail	0	0.5 ± 0.71	3	NA
Coiled tail	1.5 ± 2.12	1.5 ± 2.12	0	NA
Bent tail	3 ± 4.24	0	3	NA
Cooled T2				
Normal	96.5 ± 5.65	95 ± 1.41	94	NA
Strongly coiled tail	1 ± 1.41	0.5 ± 0.71	0	NA
Coiled tail	1 ± 1.41	1.5 ± 2.12	0	NA
Bent tail	1.5 ± 2.12	3 ± 4.24	6	NA
Frozen T1				
Normal	97.5 ± 0.71	99	89	93
Strongly coiled tail	0	1	0	0
Coiled tail	0.5 ± 0.71	0	5	4
Bent tail	2 ± 1.41	0	6	3
Frozen T2				
Normal	97.5 ± 2.12	95 ± 1.41	83	87
Strongly coiled tail	0	1.5 ± 2.12	0	0
Coiled tail	0	2.5 ± 0.70	10	3
Bent tail	2.5 ± 2.12	1	7	10

NA: Not assessed.

DISCUSSION

In this work our main goal was to establish and to test a sperm cryopreservation protocol for *S. collinsi* in captivity and the possibility to translate this method to other free living (*S. cassiquiarensis* and *S. macrodon*) and vulnerable (*S. vanzolinii*) *Saimiri* species.

Percentage of successful semen collection of *S. collinsi* in this work (about 14.81%) has been previously reported by our team for this species at same captivity conditions and EEJ protocol [17]. Semen collection was more successful for free-living species. This difference may be due to fact that field collections were performed during dry season (October-November), which probably is the reproductive season of those free-living species (Mamirauá Institute records - non published data). Differently from free-living species, captive squirrel monkeys do not presented a specific period for reproductive activities and births, being spread throughout the whole year.

It was observed an elevated number of oligospermic ejaculates in *S. collinsi*, probably due to the collection method [24, 25]. Seminal volume, sperm motility, plasma membrane integrity, percentage of normal sperm morphology in fresh semen had been similar to previously described for semen of *Saimiri sciureus* [1-3, 6, 7, 11, 20], *S. boliviensis* [25] and *S. collinsi* [17] collected by EEJ, and of *S. boliviensis* [25] collected by penile vibrostimulation. Such evaluations providing information about seminal characteristics and sperm quality for each species are crucial for the development of effective strategies for assisted reproduction. Hence, it must be performed both for captive as for free-living populations [23].

None of the evaluated sperm parameters of *S. collinsi* were impaired by dilution in ACP-118™ as well as by period on water bath, which were statistically similar to those exhibited in fresh semen. ACP-118™ is rich in membrane stabilizers [22], vitamin C, and whose capacity of support sperm quality has recently been confirmed in *Sapajus apella*

(capuchin monkey) by Leão et al. [12].

Sperm of many mammal species can be successfully frozen, however the recovery of motile sperm at the end of cryopreservation process is lower than 50% in most mammals [15]. Information on the cryoprotective mechanisms of certain externders'compounds is lacking [15], and there are species-specific low freezability semen characteristics. Therefore, cooling semen becomes an important alternative for facilitating semen transport and handling at field conditions [12], which can be followed by IVF or artificial insemination [16].

In all descriptions of sperm cryopreservation of squirrel monkey, cooling step had been performed with egg yolk [3, 17, 25]. On sperm cryopreservation of *Macaca fascicularis* (cynomolgus monkey) using TEST (a standard extender) egg yolk-free and 3% glycerol, post-thaw sperm motility and plasma membrane integrity were 34.23 ± 3.43 and 41.37 ± 3.42, respectively [15], superior to our findings. Dong et al. [5], in a detailed study about egg yolk effects on sperm cryopreservation of *Macaca mulatta* (rhesus monkey), reported that time delay (1- 5 h) after ejaculation before adding egg yolk to semen and dilution method had no significant effect on post-thaw sperm motility. However, they also demonstrated that egg yolk, even at low concentrations, played an important role in sperm freezing, since samples frozen with egg yolk presented significantly higher sperm motility after thawing than those samples without egg yolk. In our study we tested egg yolk addition before or after cooling, however in both treatments it was added for freezing without difference between treatments.

Prior to freezing glycerol was diluted in a part of extender before being added to semen, differently from a previous work by our team, when it was added directly in the semen [17]. This procedure probably affected quality of frozen-thawed semen due to cytotoxic action of glycerol, such as alteration of sperm membrane structure, disturbance of bioenergetic balance and decreasing of sperm motility and fertility [21]. In the present study it was observed a superior maintenance of the evaluated spermatic parameters, compared with

Oliveira et al. [17]. However, sperm motility and plasma membrane integrity in frozen-thawed semen of *S. collinsi* were inferior when compared with results previously reported of other *Saimiri* species (Table 1) and *Sapajus apella* (sperm motility: 34 ± 10.2 and plasma membrane integrity: 37.7 ± 13.8) in ACP-118™ [12].

Despite the poor sperm quality observed after freezing-thawing process, the rate of maintenance of this quality was high between cooling and thawing, especially in free-living species. Moreover, it was also observed that results of sperm cryopreservation greatly vary because of variation among semen donor individuals. Previous studies had already shown that there are significant differences in the response to sperm cryopreservation among individual males [8, 13, 14]. However, individual ejaculates from the same male do not differ in their tolerance to freezing [8].

CONCLUSION

Semen of *S. collinsi* can be cooled in an extender based in ACP-118™ egg yolk-free. Developed freezing protocol has been shown to be useful for sperm cryopreservation of *S. collinsi*, although requires improvement to allow a better recovery of sperm quality after thawing. The procedure appears to be properly translated to the free-living species with satisfactory sperm quality after freezing-thawing in both treatments. This is of special importance in field conditions for wild animals, as *Saimiri vanzolinii*, which is vulnerable to extinction.

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7 CONCLUSÕES

Neste estudo foi observada considerável variação entre as características dos ejaculados da espécie *S. collinsi*. Essa variação ocorreu tanto entre ejaculados do mesmo indivíduo quanto entre indivíduos. Com relação às espécies de vida livre estudadas, um maior número de observações durante outros períodos do ano são necessárias, a fim de compreender com mais clareza as variações sazonais da fisiologia reprodutiva.

A EEJ demonstrou ser eficiente para a coleta de sêmen dessas espécies. O protocolo de congelamento testado no Capítulo 2 (Artigo II) não foi adequado provavelmente devido à maneira como foi realizada a adição do glicerol ao meio diluidor. Porém, o resfriamento garantiu boa qualidade espermática do sêmen resfriado. Entretanto, os melhores resultados foram obtidos quando a adição do glicerol à amostra seminal foi realizada após a sua prévia diluição em uma fração do meio diluidor utilizado, em ambos os protocolos testados (resfriamento com e sem a gema de ovo) Capítulo 3 (Artigo III).

Embora necessite de modificações para oferecer melhor qualidade espermática pós-congelamento, o protocolo de congelamento desenvolvido demonstrou ser útil e possível de ser utilizando em condições de campo.

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