Seminal quality and global proteomic analysis of spermatozoa from captive Amazon squirrel monkeys (*Saimiri collinsi* Osgood, 1916) during the dry and rainy seasons

Danuza Leite Leão[1,2*], Sheyla Farhayldes Souza Domingues[1,2,3], Patrícia da Cunha Sousa[1], Wlaisa Vasconcelos Sampaio[1,2], Fábio Roger Vasconcelos[4], Arlindo Alencar Moura[4], Regiane Rodrigues dos Santos[1], Morten Skaugen[5], Irma Caroline Oskam[6]

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

[2] Federal Rural University of the Amazon, Belém, Pará, Brazil

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

[4] Laboratory of Animal Physiology, Department of Animal Science, Federal University of Ceará, Fortaleza, Ceará, Brazil

[5] Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway

[6] The Animal Production Experimental Center, Norwegian University of Life Sciences, Ås, Norway

*Corresponding author:

E-mail: danleao.88@gmail.com

Short title: Differential expression of *Saimiri collinsi* sperm proteins during the dry and rainy seasons

Abstract

The squirrel monkey (*Saimiri collinsi*), a Neotropical primate endemic to the Amazon in Brazil, is used as a biological model for reproductive research on the genus *Saimiri*. Although this animal is known to exhibit reproductive seasonality, nothing is known about the differences in its seminal quality, sperm protein composition, or sperm protein profile between the breeding (dry) and non-breeding (rainy) seasons. Thus, the aims of this study were to evaluate the quality of *S. collinsi* semen during the dry and rainy seasons and to describe the global sperm proteomics and expression variations in the sperm proteins during the two seasons. Aside from the pH, there was no difference in the seminal quality between the dry and rainy seasons. The study approach based on bottom-up proteomics allowed the identification of 2343 proteins present in the sperm samples throughout these two seasons. Of the 79 proteins that were differentially expressed between the two seasons, 39 proteins that were related to spermatogenesis, sperm motility, capacitation, fecundation, and defense systems against oxidative stress were upregulated in the dry season. Knowledge on the sperm proteins provides crucial information for elucidating the underlying mechanisms associated with sperm functionality. Thus, our results help to advance our understanding of the reproductive physiology of *S. collinsi*, providing valuable information for the improvement of protocols used in assisted reproduction techniques for the conservation of endangered *Saimiri* species.

Introduction

The squirrel monkey (*Saimiri collinsi*), a Neotropical primate endemic to the Amazon in Brazil [1], is commonly used as an experimental model for reproductive research on the genus *Saimiri* [2-4]. According to the International Union for Conservation of Nature's Red List of Threatened Species, two *Saimiri* species are ranked as vulnerable (*Saimiri oerstedii* and *Saimiri vanzolini*) and one species as almost threatened (*Saimiri ustus*) to extinction [5].

Primates of the genus *Saimiri* exhibit reproductive seasonality. In the free-living animals, the breeding season (mating) and births occur during the dry season and rainy season, respectively. Supposedly, the rainy season is when there is more food available for the newborn [6-8]. However, *Saimiri* monkeys that are held in captivity without variations in their environment and food supply express less of a seasonality pattern by continuing to mate and reproduce throughout the year [9]. Because of the conflicting observations between free-range and captive individuals, it is obvious that the effects of environmental factors (e.g., rainfall, temperature, photoperiod, and food supply) on reproductive seasonality need to be more fully understood [9-11].

Although studies on the squirrel monkey have already shown correlations between reproductive seasonality and spermatogenesis (*Saimiri sciureus*) [12] and the gonadal hormones (*S. sciureus*) [13], only one study has reported the seasonal influence on seminal quality (*S. sciureus*) [14]. However, nothing is known about the protein composition of spermatozoa in these Neotropical primates, or of the differences in the sperm protein profile between the breeding and non-breeding seasons. In domestic animals, proteomic studies have shown the upregulation and downregulation of expression of some sperm proteins when the breeding and non-breeding seasons are compared [15].

Mammalian male fertility depends on physiological events that begin with spermatogenesis and culminate with successful adhesion/signaling between the sperm membrane and the extracellular coat of the oocyte, followed by adhesion/fusion between the oocyte and sperm membranes during fertilization in the female reproductive tract [16, 17]. Proteins expressed by spermatozoa and those from the seminal plasma that bind to the sperm plasma membrane render the spermatozoa capable of fertilizing a mature oocyte [18, 19]. Studies in animals and humans have described sperm proteins that have significant associations with sperm motility (i.e., L-lactate dehydrogenase and dynein heavy chain 1 (DNAH1)) [20, 21], sperm capacitation (i.e., clusterin, spermadhesin, and mitochondrial peroxiredoxin-5) [22, 23], and fertility (i.e., enolase 1, ropporin-1-like protein (ROPN1), and Izumo sperm–egg fusion 1 (IZUMO1)) [24, 25].

In non-human primates, sperm proteomics has been carried out only in Old World primates for characterization of the sperm protein profile [18, 26-29]. Although these studies have been carried out in the genus *Macaca*, which also exhibits reproductive seasonality [30], nothing is known about the changes that may occur in the sperm protein profile during the non-breeding and breeding seasons, and the influence of these changes on the seminal quality of these animals. Knowledge about the absence, presence, underexpression, or overexpression of these sperm proteins could help to further our understanding of the mechanisms behind the reduction in the fertilization ability of sperm [19, 31].

Defining the sperm protein profiles of *Saimiri collinsi* in the breeding (dry season) and non-breeding (rain season) seasons may provide us with a better understanding about the reproductive physiology of these animals, as well as whether the sperm cells could be used in assisted reproduction techniques throughout the year rather than being restricted only to the breeding period.

Therefore, the aims of this study were to (i) evaluate the quality of *S. collinsi* semen during the dry and rainy seasons, (ii) describe the global sperm proteomics in *S. collinsi*, (iii) describe the variations of the proteins in sperm collected during the dry and rain seasons, and (iv) evaluate the potential correlation between the expression of the sperm proteins and the seminal quality in *S. collinsi.*

Methods

Study design

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

Animal ethics statement

The animal study was approved by the Ethical Committee in Animal Research (Approval No. 02/2015/CEPAN/IEC/SVS/MS) and by the System of Authorization and Information in

Biodiversity (SISBIO/ICMBio/MMA No. 47051-2), and carried the license of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES/IBAMA/Permit No. 17BR025045-DF). All procedures were performed under the supervision of a veterinarian.

Animals

S. collinsi males (N = 4) that originated from the Marajó Archipelago (0°58′S and 49°34′W) and were maintained in captivity at the Centro Nacional de Primatas, Brazil (1°22′58″S and 48°22′51″W) were used for the semen collection. The average age of the animals was 15 years. The external genitalia of each animal were evaluated and an andrology examination (i.e., inspection and palpation of the testes to verify the size, consistency, and symmetry) was performed.

Housing conditions

The animals were housed collectively in cages (4.74 m × 1.45 m × 2.26 m), with 12 h of natural light each day. The mixed animal groups typically consisted of three males and three females and their juvenile offspring. The region is defined by the Köppen-Geiger climate classification system as having a tropical rainforest climate (AF), with an average annual temperature of 28°C (maximum of 32°C and minimum of 24°C) [32]. The animals were fed fresh fruits, vegetables, commercial pellet chow specific for Neotropical non-human primates (18% protein, 6.5% fiber; Megazoo, Minas Gerais, Brazil), and cricket larvae (*Zophobas morio*). Vitamins, minerals, and eggs were supplied once a week, and water was available *ad libitum*.

Body weight, testicular biometry, and semen collection

Semen was collected monthly from June 2015 to May 2016, every morning before feeding, making up a total of 48 semen collections (12 per animal). For the semen collection, physical restraint of each animal was performed by a trained animal caretaker wearing leather gloves, and all animals were anesthetized with ketamine hydrochloride (20 mg/kg; intramuscularly (IM); Vetanarcol, König S.A., Avellaneda, Argentina) and xylazine hydrochloride (1 mg/kg; IM; Kensol, König S.A.) and monitored by a veterinarian. After anesthesia, the animals were weighed using a weight balance, and the testicular length, width, height, and circumference were measured using a universal caliper. The testicular volume was calculated according to the method described by Oliveira et al. [4]. After the animal had been placed in dorsal recumbency, the genital region was sanitized with a mild soap and distilled water (1:10) and the prepuce was retracted for a more efficient cleaning of the penis with saline solution. The animal was then stimulated according to the rectal electroejaculation procedure described by Oliveira et al. [2-4]. In brief, an electroejaculator (Autojac-Neovet, Uberaba, Brazil) rectal probe was smeared with a sterile lubricant gel (KY Jelly, Johnson & Johnson Co., Arlington, TX, USA) and introduced into the rectum (~2.5 cm deep) and electrical stimuli were then delivered. The stimulation session consisted of three series (7 and 8 min), composed of 35 electrical stimuli (12.5 and 100 mA), with an interval of 30 s between the series. The ejaculates (liquid and coagulated fractions) were collected into microtubes (1.5 mL).

Semen evaluation

The 1.5-mL conical microtubes containing the semen were placed in a water bath at 37°C immediately after collection for evaluation of the seminal volume, color, and viscosity. The volumes of the liquid and coagulated fractions were evaluated in a graduated tube, with the aid of a pipette. The appearance was assessed subjectively for color (colorless, yellowish, or whitish) and opacity (opaque or transparent) [2-4]. The seminal pH was measured with a pH strip (Merck Pharmaceuticals, Darmstadt, Germany).

The sperm motility, vigor, and morphology were evaluated according to the methods described by Oliveira et al. [2-4]. For evaluation of the normal sperm morphology and plasma membrane integrity, a smear sample was prepared by adding 5 µL of 1% eosin (Vetec, Rio de Janeiro, Brazil) and 5 µL of 1% nigrosine (Vetec, Rio de Janeiro, Brazil) to 5 µL of semen on a prewarmed (37°C) glass slide. The sperm concentration was determined in a Neubauer chamber after the dilution of 1 µL of semen in 99 µL of 10% formalin solution. The plasma membrane functionality was assessed with the hypoosmotic swelling test after the dilution of 5 µL of semen in 45 µL of hypoosmotic solution (0.73 g of sodium citrate, 1.35 g of fructose, and 100 mL of ultrapure water; pH 7.2 and 108 mOsm/L). After a 45-min incubation in a water bath (37°C), 10 µL of this solution was placed on a prewarmed (37°C) glass slide and covered with a coverslip, and at least 200 spermatozoa were counted to determine the number with coiled tails (indicative of spermatozoa with a functional plasma membrane). All evaluations were performed under a light microscope (E400; Nikon, Tokyo, Japan) at a magnification of 100×. The semen was assessed directly both after collection (fresh) and after dilution in ACP-118.

Sperm separation and freeze-drying

Owing to the occurance of seminal coagulation in *S. collinsi*, the semen sample was diluted 1:1 in ACP-118 (300 mOsm/kg and pH 6.42), incubated in a water bath (Biomatic, Porto Alegre, Rio Grande do Sul, Brazil) at 37°C for 1 h, and then separated on 45%/90% Percoll gradient media (centrifugation at 10,000 *g*, 15 min, 12°C). Thereafter, the samples were washed in Tris-NaCl medium (centrifugation at 8000 *g*, 5 min, 12°C), and the separated sperm fraction (pellet) was stored in microtubes, together with Tris-NaCl and a protease inhibitor (1:1000; P8340 catalog, Sigma-Aldrich, St. Louis, MO, USA), in liquid nitrogen or a –80°C freezer. For lyophilization, the frozen sperm samples were placed in a freeze dryer (FreeZone 2.5 Liter Benchtop Freeze Dry System; Labconco, Kansas City, MO, USA) for 10 h at a temperature of –55°C and vacuum pressure of 0.025 mbar.

Liquid chromatography-mass spectrometry

Each individual dried sperm sample was resuspended in 50 µL of lysis buffer (0.1 M Tris-Cl (pH 8.0), 4% sodium dodecyl sulfate, and 10 mM dithiothreitol) and centrifuged at 5000 *g* for 1 h at 4°C. The supernatant was reserved for the preparation of suspension samples for bottom-up proteomic analysis with tryptic digestion, using the method established by Zougman et al. [33]. The extracted peptides were analyzed on an UltiMate 3000 RSLCnano/Q-Exactive system (Thermo Fisher Scientific, Bremen, Germany) that was set up with a Nanospray Flex ion source. The tryptic peptides (~1 µg loaded) were separated on a 50 cm × 75 µm (i.d.) column (Thermo Fisher Scientific) using a 120 min gradient of 12–45% acetonitrile. The mass spectrometry (MS) and

tandem mass spectrometry (MS/MS) data were recorded using a standard data-dependent acquisition method, with the following conditions: *m/z* range of 300–1600; Automatic Gain Control targets of 3×10^6 (MS) and 5×10^4 (MS/MS); resolutions of 70 K (MS) and 35 K (MS/MS); dynamic exclusion set to 20 s, and normalized collision energy set to 28. Xcalibur software (v. 3.1; Thermo Fisher Scientific) was used to evaluate the raw data, which were converted to *mgf* format (for Mascot database searching) using the MS convert module of ProteoWizard (v. 3.0.9016). The Mascot (v. 2.6) searches were performed on an in-house server against an online *Saimiri boliviensis boliviensis* (Bolivian squirrel monkey) database (National Center for Biotechnology Information, Bethesda, MD, USA). MaxQuant software (v. 1.6.1.0) [34] was used for the label-free quantification.

Protein categorization

The protein information obtained by Mascot was analyzed using the STRuctural Analysis Programs (STRAP) for searching annotations of proteins. STRAP automatically obtains Gene Ontology (GO) terms associated with proteins in an identification list of results based on homology search analysis using various freely accessible databases [335].

In silico protein network analysis

Protein–protein networks were retrieved from the STRING database (v. 10.0), which consists of known and predicted protein interactions collected from direct (physical) and indirect (functional) associations. The database quantitatively integrates interaction data from four sources: a

genomic context, high-throughput experiments, co-expression data, and previous knowledge from research publications [36]. The STRING program was set to show no more than 10 interactions and medium confidence. Pathways not described for *S. boliviensis boliviensis* were analyzed using those for other non-human primate species and *Homo sapiens*.

Statistical analysis

All seminal quality data are expressed as the mean ± standard error of the mean and were analyzed using the StatView 5.0 program (SAS Institute Inc., Cary, NC, USA). Data were checked for normality using the Kolmogorov-Smirnov test. The effects of the dry and rainy seasons on the seminal quality were evaluated by analysis of variance, and differences were determined with Fisher's protected least significant difference *post hoc* test. A p value of <0.05 was considered as being statistically significant. With regard to the differences in protein expression between the dry and rainy seasons, the protein concentration data were logarithmically transformed and two-sample tests were performed using Perseus software (v. 1.6.1.1; Max Planck Institute of Biochemistry, Planegg, Germany).

Results

Characteristics of the Amazon monkeys and semen

The body weights of the male monkeys and their total testicular volumes were significantly higher in the rainy season (883.15 ± 14.50 g and 2.42 ± 0.11 cm^3, respectively) than in the dry season (816.10 ± 6.85 g and 1.91 ± 0.13 cm^3) (Table 1). Semen collection was successful in 42 of the 48 attempts (88%) because four ejaculates did not contain sperm; of these, 39 samples were used for the experiments. The highest percentage of ejaculates in both the liquid and coagulated fractions was 59%. With regard to the semen color and opacity, 10% of the samples were colorless, 33% were whitish, 57% were yellowish, 46% were transparent, and 54% were opaque. There was a statistical difference ($p = 0.0002$) in the seminal pH between the dry (7.96 ± 0.10) and rainy (7.30 ± 0.11) seasons in the liquid fraction (fresh sample). With regard to the other seminal parameters, there were no changes in the seminal volume, total sperm count, and sperm motility, vigor, plasma membrane functionality, and integrity as well as in the normal sperm regardless of the period of the year (dry or rainy season) (Table 1).

Table 1. Mean (±SEM) values of the body weight, testicular volume (cm^3), and seminal parameters of *Saimiri collinsi* during the dry (breeding) and rainy (non-breeding) seasons.

*Plasma membrane functionality (PMF; %)

**Plasma membrane integrity (PMI; %)

Sperm proteomics

The study approach based on bottom-up proteomics allowed the identification of 2343 proteins in the sperm samples (Supporting Information S1 Table). Of the total proteins identified, 223 were determined to participate in important reproductive events, such as spermatogenesis (67 proteins), sperm motility (42 proteins), capacitation/acrosome reaction (20 proteins), and fertilization (32 proteins) (Supporting Information S2 Table).

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

Fig 1. **Gene Ontology annotation of the cellular component (A), biological process (B), and molecular function (C) classes of identified *Saimiri collinsi* sperm proteins analyzed by STRAP.** The Gene Ontology terms were obtained from the UniProtKB database.

We also identified 79 sperm proteins that were differentially expressed between the dry (breeding season) and rainy seasons (non-breeding season). Of these, 39 were upregulated in the dry season, with the main protein functions being for enzymatic activity (i.e., deoxyguanosine kinase

and matrix metalloproteinase-7), cellular regulation (i.e., amine oxidase and serine protease 30-like), and immune system processes (i.e., heat shock 70 kDa protein 1A/1B and clusterin) (Table 2 and Supporting Information S3 Table). With regard to proteins that participate in important events in reproduction, 10 that were increased during the dry season were related to spermatogenesis (i.e., cat eye syndrome critical region protein 5, heat shock-related 70 kDa protein 2 (Hsp70.2/HSPA2), and peroxidase (GPX4), sperm motility (i.e., ADP/ATP translocase 4, ROPN1L, and tektin-5), capacitation (i.e., ROPN1L), and fecundation (i.e., sperm surface protein Sp17 (SPA17)), or were important defense systems against oxidative stress (i.e., nucleoside diphosphate kinase homolog 5 and catalase).

Table 2. **Upregulation or downregulation of sperm protein expression (µg/mL) in *Saimiri collinsi* during the rainy (non-breeding) and dry (breeding) seasons.**

In silico protein network analysis indicated that the proteins that were upregulated during the dry (breeding) season, such as ROPN1L, phospholipid hydroperoxide glutathione, HSPA2, and SPA17, interacted with 10 other proteins. Among these interactions, only ROPN1L and phospholipid hydroperoxide glutathione interacted with each other (Fig 2).

Fig 2. Protein interaction analysis. Proteins were analyzed with the wed-based STRING software. Analyzed proteins were: a- Ropporin-1-like; b- Phospholipid hydroperoxide glutathione; c- Heat shock proteins 70kDa protein 2; d- Sperm surface protein Sp17. Different

line color represents the types of evidence for the association. Green textming; black coexpression; blue databases; and pink experiments. AKAP3 A-kinase anchor protein 3; SPA17 Sperm surface protein Sp17; CABYR Calcium-binding tyrosine phosphorylation-regulated protein; RHPN1 Rhophilin-1; RSPH3 Radial spoke head protein 3 homolog; CCDC63 Coiled-coil domain-containing protein 63; TRPV6 Transient receptor potential cation channel subfamily V member 6; DNALI1 Axonemal dynein light intermediate polypeptide 1; TEKT3 Tektin-3; CFAP36 Cilia- and flagella-associated protein 36; GSR Glutathione reductase, mitochondrial; GRSF1 G-rich sequence factor 1; GSS Glutathione synthetase; SOD2 Superoxide dismutase [Mn], mitochondrial; GSTO2 Glutathione S-transferase omega-2; GGT7 Glutathione hydrolase 7; GGT1 Glutathione hydrolase 1 proenzyme; SOD1 Superoxide dismutase [Cu-Zn]; GGT5 Glutathione hydrolase 5 proenzyme; GGT6 Glutathione hydrolase 6; DNAJB6 DnaJ (Hsp40) homolog, subfamily B, member 6; DNAJB1 DnaJ (Hsp40) homolog, subfamily B, member 1; HSPH1 Heat shock 105kDa/110kDa protein 1; DNAJC7 DnaJ (Hsp40) homolog, subfamily C, member 7; GRPEL1 GrpE-like 1, mitochondrial (E. coli); HSPA9 Heat shock 70kDa protein 9 (mortalin); HSPA1A Heat shock 70kDa protein 1A; HSPA8 Heat shock 70kDa protein 8; HSP90AA1 Heat shock protein 90kDa alpha (cytosolic), class A member 1; DNAJB12 DnaJ (Hsp40) homolog, subfamily B, member 12 (409 aa); ROPN1 Rhophilin associated tail protein 1 (212 aa); AKAP3 A kinase (PRKA) anchor protein 3; ROPN1L Rhophilin associated tail protein 1-like (230 aa); AKAP14 A kinase (PRKA) anchor protein 14; ZPBP Zona pellucida binding protein; EFCAB7 EF-hand calcium binding domain 7 (629 aa); GAS2L2 Growth

arrest-specic 2 like 2; RASL10A RAS-like, family 10, member A; GAS2L1 Growth arrest-specic 2 like 1; PRSS50 Protease, serine, 50.

Discussion

Proteins associated with spermatogenesis and sperm motility

In *S. collinsi*, it was possible to verify the upregulation of important proteins that participated in spermatogenesis and sperm motility in the dry season (breeding season), such as ROPN1L, HSPA2, cat eye syndrome critical region protein 5, and phospholipid hydroperoxide glutathione. In mice, the loss of ROPN1L impairs sperm motility, cAMP-dependent protein kinase phosphorylation, and fibrous sheath integrity [37]. ROPN1L is a sperm flagellar protein that binds A-kinase anchoring protein (AKAP) 3 and 4, which are primary components of the sperm fibrous sheath. The fibrous sheath is a flagellar cytoskeletal structure unique to sperm that surrounds the outer dense fibers and axoneme [37, 38]. The degradation of AKAP3 and subsequent dephosphorylation of tyrosine result in sperm capacitation [39].

Heat shock proteins (HSPs) are chaperone proteins that are expressed in response to cell stress [40, 41]. Several HSP family members are expressed in the sperm, such as HSP 70 kDa (HSP70), which appears in the acrosome membranes. HSP 60 kDa (HSP60) is located primarily in the sperm midpiece, in association with the mitochondria, whereas HSP 90-alpha (HSP90AA1) is located in the sperm flagellum [42]. HSP60, HSP70, and HSP90AA1 are known components of sperm in different species, such as humans [43], rams [44], bulls, stallions, cats, and dogs [45]. The acrosomal HSP70 has a role in gamete interaction and fertilization [46], whereas HSP90AA1

expression has been correlated with the resistance of sperm to freezing [47, 48] since this protein is characterized as a ubiquitous molecular chaperone that provides protection and protein folding during thermal stress and resistance against cell oxidative stress [49].

HSPA2, which is a molecular chaperone that assists in the folding, transport, and assembly of proteins in the cytoplasm, mitochondria, and endoplasmic reticulum and is a testis-specific member of the 70-kDa family [50], has been suggested to be crucially involved in spermatogenesis and meiosis [51]. In humans, the downregulation of HSPA2 mRNA was observed in testes with abnormal spermatogenesis, and the protein expression was high in normal spermatogenesis and low in spermatogenesis arrest [52]. Human HSPA2 was shown to regulate the expression of the sperm surface receptors involved in sperm-oocyte recognition [53], and its depression in the testes was also associated with spermatogenic impairment and the fertilization rate in men with azoospermia who were treated with intracytoplasmic sperm injections [54].

Sperm motility is driven mainly by the energy produced by the mitochondria present in the intermediate piece of the male gamete [55]. However, the axoneme is another important cellular component that is directly associated with sperm motility. The dynein heavy chains have been annotated as subunits of the axonemal dynein complexes, which are multisubunit axonemal ATPase complexes that generate the force for cilia motility and govern the beat frequency [56]. DNAH1 is related to spermatogenesis and cell proliferation [57]. In humans, mutations in DNAH1 cause multiple morphologic abnormalities of the sperm flagella, leading to male infertility [21]. The radial spoke proteins play a key role in regulating dynein activity and flagellar motility [58, 59].

In this context, Imai et al. [60] showed that the failure to express phospholipid hydroperoxide glutathione peroxidase (GPX4) caused human male infertility, with 30% of men

diagnosed with oligoasthenozoospermia showing a significant decrease in the level of the enzyme. Those authors also found a significantly lower number of spermatozoa in the semen and significantly lower motility of the spermatozoa than those seen in fertile men. GPX4 is an intracellular antioxidant that directly reduces peroxidized phospholipids and is strongly expressed in the mitochondria of the testis and spermatozoa. In bulls, GPX4 is considered a unique marker for seminal quality analysis owing to the direct correlation between the selenoperoxidase and the progressive motility of the sperm [61].

Capacitation and the acrosome reaction

The acrosome, which is a membrane-bound exocytotic vesicle that is located over the anterior portion of the nucleus, contains the hydrolytic enzymes that are required for the acrosome reaction, binding of the zona pellucida (ZP), penetration through the ZP, and sperm–egg membrane fusion, all of which are indispensable events during the fertilization process [62]. In the acrosome membrane (internal and external membranes), the sperm acrosome membrane-associated family (i.e., SPACA3, SPACA1, and SPACA4) [63, 64] are sperm surface membrane proteins that may be involved in the adhesion and fusion of the sperm to the egg prior to fertilization [65]. SPACA1 and SPACA3 are localized in the acrosomal matrix, including the principal segment and equatorial segment, and are proteins characterized as membrane antigens [63, 65, 66]. SPACA1 may be involved in sperm fusion with the oölemma, since treatment of human sperm with the anti-SPACA1 antibody prevented sperm penetration into zona-free hamster eggs [63]. Fujihara et al. [67] demonstrated that the SPACA1 protein was indispensable for the normal shaping of the sperm heads

during spermiogenesis in mice. In humans, this protein was identified as a sperm membrane antigen, with a molecular mass ranging from 32 to 34 kDa [63].

Sperm–egg fusion

Membrane fusion is a key event in the fertilization process that culminates in the merger of the male–female gamete membranes and cytoplasm and fusion of the genomes, thereby initiating embryonic development [68]. In humans, a change in the expression of the sperm proteins may be a major cause of subfertility in men with normozoospermia [69]. In this context, research has been focusing on the identification of the key molecular players and their functions, and several proteins in the egg or the spermatozoa have been found to be essential for fertilization.

Until now, IZUMO1 has been found to be the essential protein on the sperm side for the fusion process. As a testis-specific protein, IZUMO was discovered on the equatorial segment of the acrosome-reacted mouse spermatozoa through proteomic analysis of the antigen recognized by the monoclonal anti-mouse sperm antibody [70]. IZUMO is present in both mouse (~56 kDa protein) and human (~38 kDa protein) sperm [71]. In mice, immunization with the IZUMO protein caused a contraceptive effect in females, which was due to the significantly inhibited fusion of sperm to the zona-free mouse eggs with the anti-PrimeB antibody. However, no effect on sperm motility was observed [72]. IZUMO2, IZUMO3, and IZUMO4 have significant homology with the N-terminal domain of IZUMO1 [73]. Inoue et al. [24] showed the interaction between angiotensin-converting enzyme-3 located on the sperm acrosomal cap and IZUMO1 in the fertilization process. However, it was reported that angiotensin-converting enzyme-3 disappears from the membrane after the acrosome reaction. Nevertheless, the *in silico* protein interaction analysis of IZUMO1 revealed its

405 association with the CD9 molecule, folate receptor 4 (delta) homolog (mouse), folate receptor 1
406 (adult), folate receptor 2 (fetal), SPACA1, SPACA4, IZUMO family member 4, zona pellucida
407 binding protein 2, and metallopeptidase domain 2.

408 After the acrosome reaction, the C-terminal calmodulin domain (20 kDa) of SPA17 (located
409 on the external side of the sperm plasma membrane) is proteolytically cleaved to 17 kDa and then
410 binds to the extracellular matrix of the oocyte. This C-terminus of SPA17 plays a role in cell–cell
411 adhesion [74, 75]. In our study, SPA17 was shown to be upregulated during the dry season,
412 implying that this protein could also be involved in the fertilization processes in the breeding season
413 of *S. collinsi*.

414 Our results on the seminal quality also showed that proteomics is an important
415 complementary tool for use toward understanding and elucidating the influence of seasonality on
416 the sperm cells in *S. collinsi*, since it was not possible to verify this influence by classic seminal
417 analysis for this species. Additionally, it is important to mention that the results of the seminal
418 parameters analyzed (viz., appearance, semen volume, pH, and sperm concentration, motility, vigor,
419 and morphology) were similar to those previously reported for fresh Amazon squirrel monkey
420 sperm (liquid fraction) and sperm from the coagulated fraction after dilution in ACP-118 [2-4].
421 However, this was the first time that a comparison of these parameters during the dry and rainy
422 seasons was performed for this species.

423 Although the seminal pH was higher in the dry season, it was slightly alkaline during both
424 seasons and similar to the range reported elsewhere for *S. collinsi* (pH 6.5–8.0) [2-4] as well as for
425 the Neotropical primates *Alouatta caraya* (pH 8.1) [76], *Ateles geoffroyi* (pH 8.0) [77], *Callithrix*
426 *jacchus* (pH 7.4–7.6) [78, 79], *Callithrix penicillata* [80], and *Callimico goeldii* (pH 6.1) [81]. In

427 women, the acidic vaginal environment is toxic to sperm because the optimal pH for sperm viability
428 ranges from 7.0 to 8.5, and a reduction in sperm motility is seen at a pH of less than 6.0. However,
429 during human sexual intercourse, the vaginal epithelium produces a transudate that lubricates the
430 vagina and elevates the vaginal pH to 7.0 [82]. This physiological modification to accommodate the
431 alkaline pH of semen temporarily protects the spermatozoa and creates an optimal environment in
432 the cervix for sperm motility [83].

433 It is worth mentioning that measurement of the seminal pH in our study was only possible
434 with the liquid fraction, as it was necessary to dilute the coagulated fraction in order to establish its
435 pH value. The ACP-118 extender used for non-human primates, including species of the genus
436 *Saimiri* [2-4], has a pH (6.5) that is compatible to the liquid fraction of *S. collinsi* semen. ACP-118
437 is composed of different bioactive enzymes (e.g., phosphatase, catalase, and dehydrogenase), which
438 may support coagulum liquefaction. This extender also contains ascorbic acid and polyphenol
439 oxidases, which are antioxidants that maintain the sperm quality during and after incubation [84,
440 85]. In this way, the ACP-118 composition may have affected the quality of the *S. collinsi* sperm,
441 since there was no difference in the sperm parameters analyzed between the dry and rainy seasons.
442 Thus, our results showed that the ACP-118 extender used for coagulum liquefaction was able to
443 maintain similar sperm qualities in both seasonal periods.

444

445

446

447

448

Conclusions

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

Acknowledgments

The authors thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001, and Conselho Nacional de Desenvolvimento Científico e Tecnológico (Projeto Universal 01-2016/ Processo No. 421649/2016-0) for their financial support. We would also like to thank the National Primate Center (Conselho Executivo das Normas-Padrão, Brazil) and Norwegian University of Life Sciences (Norway) for the technical support provided during this research.

References

1. Mercês MP, Paula WS, Júnior JSS. New records of *Saimiri collinsi* Osgood, 1916 (Cebidae, Primates), with comments on habitat use and conservation. Mammalia. 2017; 85: 1-5.

2. Oliveira KG, Leão DL, Almeida DV, Santos RR, Domingues SF. Seminal characteristics and cryopreservation of sperm from the squirrel monkey, *Saimiri collinsi*. Theriogenology. 2015; 84: 743-749.

3. Oliveira KG, Santos RR, Leão DL, Brito AB, Lima JS, Sampaio WV, et al. Cooling and freezing of sperm from captive, free-living and endangered squirrel monkey species. Cryobiology. 2016a; 72: 283-289.

4. Oliveira KG, Santos RR, Leão DL, Queiroz HL, Paim FP, Vianez-Júnior JLSG, et al. Similarities in testicular and seminal aspects in four squirrel monkeys' species. Theriogenology. 2016b; 86: 879-887.

5. Boubli, J.P., Rylands, A.B., 2008. Samiri vanzolinii. In IUCN 2011. IUCN Red List of Threatened Species, Version 2011.2. Diponible in www.iucnredlist.org Acessed in 20/11/2015

6. Wolf RH, Harrison RM, Martin TW. A review of reproductive patterns of New World monkeys. Lab Anim Sci. 1975; 25: 814-821.

491 7. Lindburg DG. Seasonality of reproduction in primates. In: Mitchell G, Erwin J, editors.
492 Comparative primate biology: behavior, cognition, and motivation. New York: Alan R. Liss.
493 1987; pp. 167-218.
494
495 8. Logdberg B. Methods for timing of pregnancy and monitoring of fetal body and brain growth in
496 squirrel monkeys. J Med Primatol. 1993; 22: 374-379.
497
498 9. Trevino HS. Seasonality of reproduction in captive squirrel monkeys (*Saimiri Sciureus*). Am J
499 Primatol. 2007; 69: 1001-1012.
500
501 10. Du Mond FV. The squirrel moneky in a seminatural environment. In: Rosenblum LA, Cooper
502 RW. The Squirrel Monkey. New York: London: Academic Press; 1968. pp 87-145.
503
504 11. Harrison RM, Dukelow WR. Seasonal adaptation of laboratory maintained squirrel monkeys
505 (*Saimiri sciureus*). J Med Primatol. 1973; 2: 277-283.
506
507 12. Du Mond FV, Hutchinson TC. Squirrel monkey reproduction: the "fatted" male phenomenon
508 and seasonal spermatogenesis. Science. 1967; 158: 1067-1070.
509
510 13. Mendoza SP, Lowe EL, Resko JR, Levine S. Seasonal variations in gonadal hormones and
511 social behavior in squirrel monkeys. Physiol Behav. 1978; 20: 515-522.
512

513 14. Chen JJ, Smith ER, Gray GD, Davidson JM. Seasonal changes in plasma testosterone and
514 ejaculatory capacity in squirrel monkeys (*Saimiri sciureus*). Primates. 1981; 22: 253-260.
515
516 15. van Tilburg MF, Salles MGF, Silva MM, Moreira RA, Moreno FB, Monteiro-Moreira ACO, et
517 al. Semen variables and sperm membrane protein profile of Saanen bucks (*Capra hircus*) in dry
518 and rainy seasons of the northeastern Brazil (3°S). Int J Biometeorol 2015; 59: 561-573.
519
520 16. Sutovsky P. Sperm-egg adhesion and fusion in mammals. Expert Rev Mol Med. 2009; 11:e11.
521
522 17. Ashrafzadeh A, Karsani SA, Nathan S. Mammalian sperm fertility related proteins. Int J Med
523 Sci. 2013; 10: 1649-1657.
524
525 18. Zhou T, Wang G, Chen M, Zhang M, Guo Y, Yu C, et al. Comparative analysis of macaque and
526 human sperm proteomes: Insights into sperm competition. Proteomics. 2015; 15: 1564-1573.
527
528 19. Agarwal A, Bertolla RP, Samanta L. Sperm proteomics: potential impact on male infertility
529 treatment. Expert Rev Proteomics. 2016; 13: 285-296.
530
531 20. Légaré C, Droit A, Fournier F, Bourassa S, Force A, Cloutier F, et al. Investigation of male
532 infertility using quantitative comparative proteomics. J Proteome Res. 2014; 13: 5403-5414.
533

534 21. Khelifa MB, Coutton C, Zouari R, Karaouzene T, Rendu J, Bidart M, et al. Mutations in
535 DNAH1, which encodes an inner arm heavy chain dynein, lead to male infertility from multiple
536 morphological abnormalities of the sperm flagella. Am J Hum Genet. 2014; 94: 95-104.
537
538 22. Rahman MS, Kwon WS, Pang MG. Prediction of male fertility using capacitation-associated
539 proteins in spermatozoa. Mol Reprod Dev. 2017; 84: 749-759.
540
541 23. Hernández-Silva G, López-Araiza JFE, López-Torres AS, Larrea F, Torres-Flores V, Chirinos
542 M. Proteomic characterization of human sperm plasma membrane associated proteins and their
543 role in capacitation. Andrology. 2019; 1-10.
544
545 24. Inoue N, Kasahara T, Ikawa M, Okabe M. Identification and disruption of sperm-specific
546 angiotensin converting enzyme-3 (ACE3) in mouse. PLoS One. 2010; 5: e10301.
547
548 25. Park YJ, Kwon WS, Oh SA, Pang MG. Fertility-related proteomic profiling bull spermatozoa
549 separated by Percoll. J Proteome Res. 2012; 11: 4162-4168.
550
551 26. Srivastav A. Maturation-dependent glycoproteins containing both N- and O-linked
552 oligosaccharides in epididymal sperm plasma membrane of rhesus monkeys (*Macaca mulatta*). J
553 Reprod Fertil. 2000; 119: 241-252.
554

555 27. Skerget S, Rosenow M, Polpitiya A, Petritis K, Dorus S, Karr T. The Rhesus macaque (*Macaca*
556 *mulatta*) sperm proteome. Mol Cell Proteomics. 2013; 12: 3052-3067.
557
558 28. Kawase O, Cao S, Xuan X. Sperm membrane proteome in wild Japanese macaque (*Macaca*
559 *fuscata*) and Sika deer (*Cervus nippon*). Theriogenology. 2015; 83: 95-102.
560
561 29. Liu X, Jin SH, Liu XX, Wang WJ, Liu FJ. Proteome profiling of the sperm maturation milieu in
562 the rhesus monkey (*Macaca mulatta*) epididymis. Reprod Fertil Dev. 2016; 28: 732-741.
563
564 30. Zamboni L, Conaway CH, Van Pelt L. Seasonal changes in production of semen in free-ranging
565 rhesus monkey. Biol Reprod. 1974; 11: 251-267.
566
567 31. Jodar M, Soler-Ventura A, Oliva R. Semen proteomics and male infertility. J Proteom. 2017;
568 162: 125-134.
569
570 32. Kottek M, Grieser J, Beck C, Rudolf B, Rubel F. World Map of the Köppen-Geiger climate
571 classification updated. Meteorol Z. 2006; 15: 259-263.
572
573 33. Zougman A, Selby PJ, Banks RE. Suspension trapping (STrap) sample preparation method for
574 bottom-up proteomics analysis. Proteomics. 2014; 14: 1006-1010.
575

576 34. Tyanova S, Temu T, Carlson A, Sinitcyn P, Mann M, Cox, J. Visualization of LC-MS/MS
577 proteomics data in MaxQuant. Proteomics. 2015; 15: 1453-1456.
578
579 35. Bhatia VN, Perlman DH, Costello CE, McComb ME. Software Tool for Researching
580 Annotations of Proteins (STRAP): Open-Source Protein Annotation Software with Data
581 Visualization. Anal Chem. 2009; 81: 9819-9823.
582
583 36. Snel B, Lehmann G, Bork P, Huynen MA. STRING: a web-server to retrieve and display the
584 repeatedly occurring neighbourhood of a gene. Nucleic Acids Res. 2000; 28: 3442-3444.
585
586 37. Fiedler SE, Dudiki T, Vijayaraghavan S, Carr DW. Loss of R2D2 proteins ROPN1 and
587 ROPN1L causes defects in murine sperm motility, phosphorylation, and fibrous sheath integrity.
588 Biol Reprod. 2013; 88: 1-10.
589
590 38. Eddy EM, Toshimori K, O'Brien DA. Fibrous sheath of mammalian spermatozoa. Microsc Res
591 Tech. 2003; 61: 103-115.
592
593 39. Vizel R, Hillman P, Ickowicz D, Breitbart H. AKAP3 degradation in sperm capacitation is
594 regulated by its tyrosine phosphorylation. Biochim Biophys Acta. 2015; 1850: 1912-1920.
595
596 40. Mayer MP, Bukau B. Hsp70 chaperones: Cellular functions and molecular mechanism. Cell Mol
597 Life Sci. 2005; 62: 670-684.

41. Mortensen CJ, Choi YH, Ing NH, Kraemer DC, Vogelsang MM, Hinrichs K. Heat shock protein 70 gene expression in equine blastocysts after exposure of oocytes to high temperatures in vitro or in vivo after exercise of donor mares. Theriogenology. 2010; 74: 374-383.

42. Naaby-Hansen S, Herr JC. Heat shock proteins on the human sperm surface. J Reprod Immunol. 2010; 84: 32-40.

43. Miller D, Brough S, Al-Harbi O. (1992). Characterization and cellular distribution of human spermatozoal heat shock proteins. Hum Reprod. 1992; 7: 637-645.

44. van Tilburg MF, Rodrigues MAM, Moreira RA, Moreno FB, Monteiro-Moreira ACO, Cândido, MJ, et al. Membrane-associated proteins of ejaculated sperm from Morada Nova rams. Theriogenology. 2013; 79: 1247-1261.

45. Volpe S, Galeati G, Bernardini C, Tamanini C, Mari G, Zambelli D, et al. Comparative immunolocalization of heat shock proteins (HSP)-60, -70, -90 in boar, stallion, dog and cat spermatozoa. Reprod Dom Anim. 2008; 43: 385-392.

46. Spinaci M, Volpe S, Bernardini C, Ambrogi M, Tamanini C, Seren E, et al. Immunolocalization of heat shock protein 70 (HSP 70) in boar spermatozoa and its role during fertilization. Mol Reprod Dev. 2005; 72: 534-541.

620

621 47. Casas I, Sancho S, Ballester J, Briz M, Pinart E, Bussalleu E, et al. The HSP90AA1 sperm
622 content and the prediction of the boar ejaculate freezability. Theriogenology. 2010; 74: 940-950.

623

624 48. Wang S, Wang W, Xu Y, Tang M, Fang J, Sun H, et al. Proteomic characteristics of human
625 sperm cryopreservation. Proteomics. 2010; 14: 298-310.

626

627 49. Fukuda A, Osawa T, Oda H, Tanaka T, Toyokuni S, Uchida K. Oxidative stress response in
628 iron-induced acute nephrotoxicity: enhanced expression of heat shock protein 90. Biochem
629 Biophys Res Commun. 1996; 219: 76-81.

630

631 50. Huszar G, Stone K, Dix D, Vigue L. Putative creatine kinase Misoform in human sperm is
632 identifiedas the 70-kilodalton heat shock protein HspA2. Biol Reprod. 2000; 63: 925-932.

633

634 51. Zhu D, Dix DJ, Eddy EM. HSP70-2 is required for CDC2 kinase activity in meiosis I of mouse
635 spermatocytes. Development. 1997; 124: 3007-3014.

636

637 52. Son WY, Han CT, Hwang SH, Lee JH, Kim S, Kim, YC. Repression of hspA2 messenger RNA
638 in human testes with abnormal spermatogenesis. Fertil Steril. 2000; 73: 1138-1144.

639

640 53. Redgrove KA, Anderson AL, McLaughlin EA, O'Bryan MK, Aitken RJ, Nixon B. Investigation
641 of the mechanisms by which the molecular chaperone HSPA2 regulates the expression of sperm

surface receptors involved in human sperm-oocyte recognition. Mol Hum Reprod. 2013; 19: 120-135.

54. Tian Y, Zhang F, Zhang X, Li L, Wang L, Shi B, et al. Depression of HspA2 in human testis is associated with spermatogenic impairment and fertilization rate in ICSI treatment for azoospermic individuals. J Assist Reprod Genet. 2014; 31: 1687-1693.

55. Piomboni P, Focarelli R, Stendardi A, Ferramosca A, Zara V. The role of mitochondria in energy production for human sperm motility. Int J Androl. 2012; 35: 109-124.

56. Castleman VH, Romio L, Chodhari R, Hirst RA, Castro SC, Parker KA, et al. Mutations in radial spoke head protein genes RSPH9 and RSPH4A cause primary ciliary dyskinesia with central-microtubular-pair abnormalities. Am J Hum Genet. 2009; 84: 197-209.

57. Meng Y, Zhang W, Zhou J, Liu M, Chen J, Tian S, et al. Genome-wide analysis of positively selected genes in seasonal and non-seasonal breeding species. PLoS One. 2015; 10: e0126736.

58. Smith EF, Yang P. The radial spokes and central apparatus: mechano-chemical transducers that regulate flagellar motility. Cell Motil Cytoskeleton. 2004; 57: 8-17.

59. Wemmer KA, Marshall WF. Flagellar motility: all pull together. Curr Biol. 2004; 14: R992–R993.

60. Imai H, Suzuki K, Ishizaka K, Ichinose S, Oshima H, Okayasu I, et al. Failure of the expression of phospholipid hydroperoxide glutathione peroxidase in the spermatozoa of human infertile males. Biol Reprod. 2001; 64:674-683.

61. Stradaioli G, Sylla L, Monaci M, Maiorino M. Phospholipid hydroperoxide glutathione peroxidase in bull spermatozoa provides a unique marker in the quest for semen quality analysis. Theriogenology. 2009; 72: 91-98.

62. Hardy DM, Oda MN, Friend DS, Huang TT Jr. A mechanism for differential release of acrosomal enzymes during the acrosome reaction. Biochem J. 1991; 275: 759-766.

63. Hao Z, Wolkowicz MJ, Shetty J, Klotz K, Bolling L, Sen B, et al. SAMP32, a testis-specific, isoantigenic sperm acrosomal membrane-associated protein. Biol Reprod. 2002; 66: 735-744.

64. Ogura Y, Takagishi Y, Harayama H. Changes in the distribution and molecular mass of boar sperm acrosome-associated 1 proteins during the acrosome reaction; their validity as indicators for occurrence of the true acrosome reaction. Anim Reprod Sci. 2016; 172: 94-104.

65. Mandal A, Klotz KL, Shetty J, Jayes FL, Wolkowicz MJ, Bolling LC, et al. SLLP1, a unique, intra-acrosomal, non-bacteriolytic, c lysozyme-like protein of human spermatozoa. Biol Reprod. 2003; 68: 1525-1537.

686

687 66. Kishida K, Harayama H, Kimura F, Murakami T. Individual differences in the distribution of
688 sperm acrosome associated 1 proteins among male patients of infertile couples; possible their
689 impacts on outcomes of conventional *in vitro* fertilization. Zygote. 2016; 24: 654-661.

690

691 67. Fujihara Y, Satouh Y, Inoue N, Isotani A, Ikawa M, Okabe M. SPACA1-deficient male mice are
692 infertile with abnormally shaped sperm heads reminiscent of globozoospermia. Development.
693 2012; 139: 3583-3589.

694

695 68. Yanagimachi R. Sperm-egg fusion. Curr Top Membr Trans. 1998; 32: 3-43.

696

697 69. Xu W, Hu H, Wang Z, Chen X, Yang F, Zhu Z, et al. Proteomic characteristics of spermatozoa
698 in normozoospermic patients with infertility. J Proteome Res. 2012; 75: 5426-5436.

699

700 70. Inoue N, Yamaguchi R, Ikawa M, Okabe M. Sperm-egg interaction and gene manipulated
701 animals. Soc Reprod Fertil Suppl. 2007; 65: 363-371.

702

703 71. Inoue N, Ikawa M, Isotani A, Okabe M. The immunoglobulin superfamily protein Izumo is
704 required for sperm to fuse with eggs. Nature. 2005; 434: 234-238.

705

706 72. Wang M, Lv Z, Shi J, Hu Y, Xu C. Immunocontraceptive potential of the Ig-like domain of
707 Izumo. Mol Reprod Dev. 2009; 76: 794-801.
708
709 73. Ellerman D, Pei J, Gupta S, Snell W, Myles D, Primakoff P. Izumo is a part of a multiprotein
710 family whose members form large complexes on mammalian sperm. Mol Reprod Dev. 2009;
711 76: 1188-1199.
712
713 74. Wen Y, Richardson RT, Widgren, EE, O'Rand MG. Characterization of Sp17: A ubiquitous
714 three domain protein that binds heparin. Biochem J. 2001; 357: 25-31.
715
716 75. Frayne J, Hall L. A re-evaluation of sperm protein 17 (Sp17) indicates a regulatory role in an A-
717 kinase anchoring protein complex, rather than a unique role in sperm-zona pellucida binding.
718 Reproduction. 2002; 124: 767-774.
719
720 76. Valle RR, Guimarães MABV, Muniz JAPC, Barnabe RC, Vale WG. Collection and evaluation
721 of semen from captive howler monkeys (*Alouatta caraya*). Theriogenology. 2004; 62: 131-138.
722
723 77. Hernández-López L, Cerda-Molina AL, Páez-Ponce D, Mondragón- Ceballos R. The seminal
724 coagulum favours passage of fast-moving sperm into the uterus in the black-handed spider
725 monkey. Reproduction. 2008;136: 411-421.
726

78. Ceballos R. The seminal coagulum favours passage of fast-moving sperm into the uterus in the black-handed spider monkey. Reproduction. 2008; 136: 411-421.

79. Valle RR, Valle CM, Nichi M, Muniz JAPC, Nayudu PL, Guimarães MABV. Semen characteristics of captive common marmoset (*Callithrix jacchus*): a comparison of a German with a Brazilian colony. J Med Primatol. 2014; 43: 225-30.

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

81. Arakaki PR, Carvalho FM, Castro PHG, Muniz JAPC, Valle RDRD. Collection, evaluation, and coagulum dissolution of semen from Goeldi's Monkey, *Callimico goeldii*. Folia Primatol. 2017; 88: 334-343.

82. Nakano FY, Leão RBF, Esteves SC. Insights into the role of cervical mucus and vaginal pH in unexplained infertility. MedicalExpress 2015; 2: 1-8.

83. Mishra AK, Kumar A, Swain DK, Yadav S, Nigam R. Insights into pH regulatory mechanisms in mediating spermatozoa functions. Vet World. 2018; 11: 852-858.

749 84. Oliveira KG, Miranda SA, Leão DL, Brito AB, Santos RR, Domingues SFS. Semen coagulum
750 liquefaction, sperm activation and cryopreservation of capuchin monkey (*Cebus apella*) semen
751 in coconut water solution (CWS) and TES-TRIS. Anim Reprod Sci. 2011; 123: 75-80.

752

753 85. Leão DL, Miranda AS, Brito AB, Lima JS, Santos RR, Domingues, SFS. Efficacious long-term
754 cooling and freezing of *Sapajus apella* semen in ACP-118®. Anim Reprod Sci. 2015; 159: 118-
755 123.

756

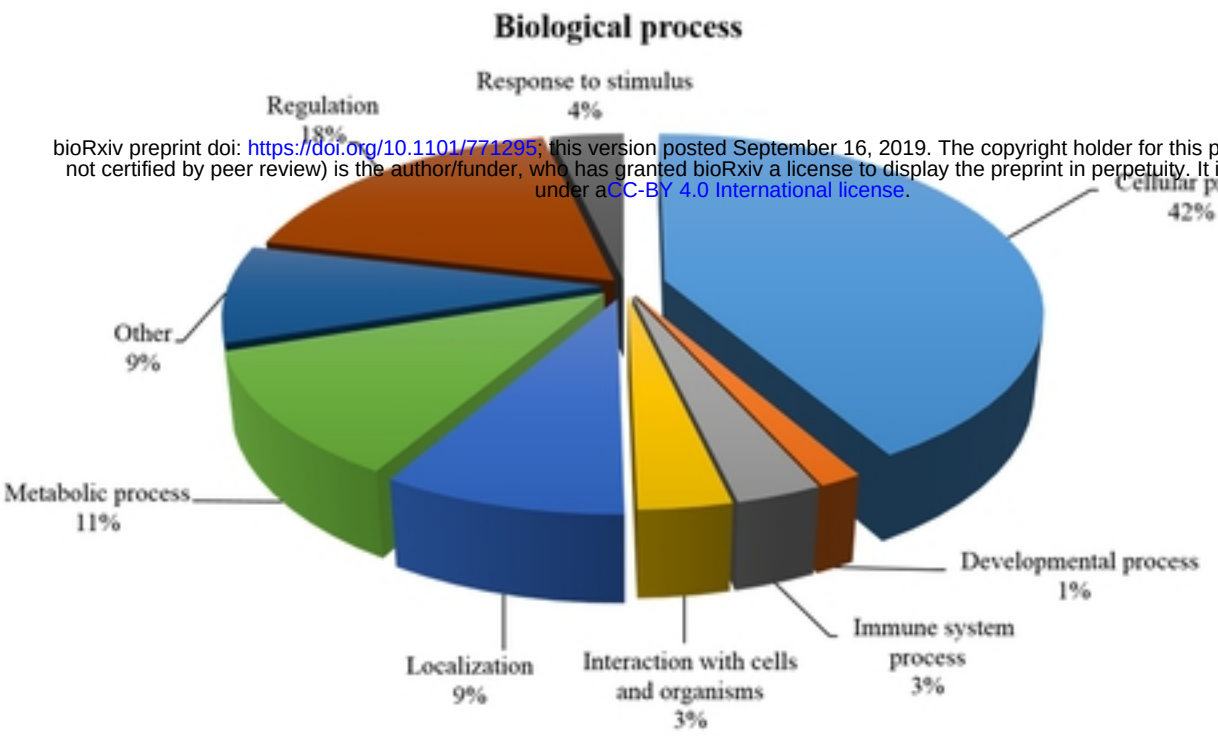
757 Supporting information

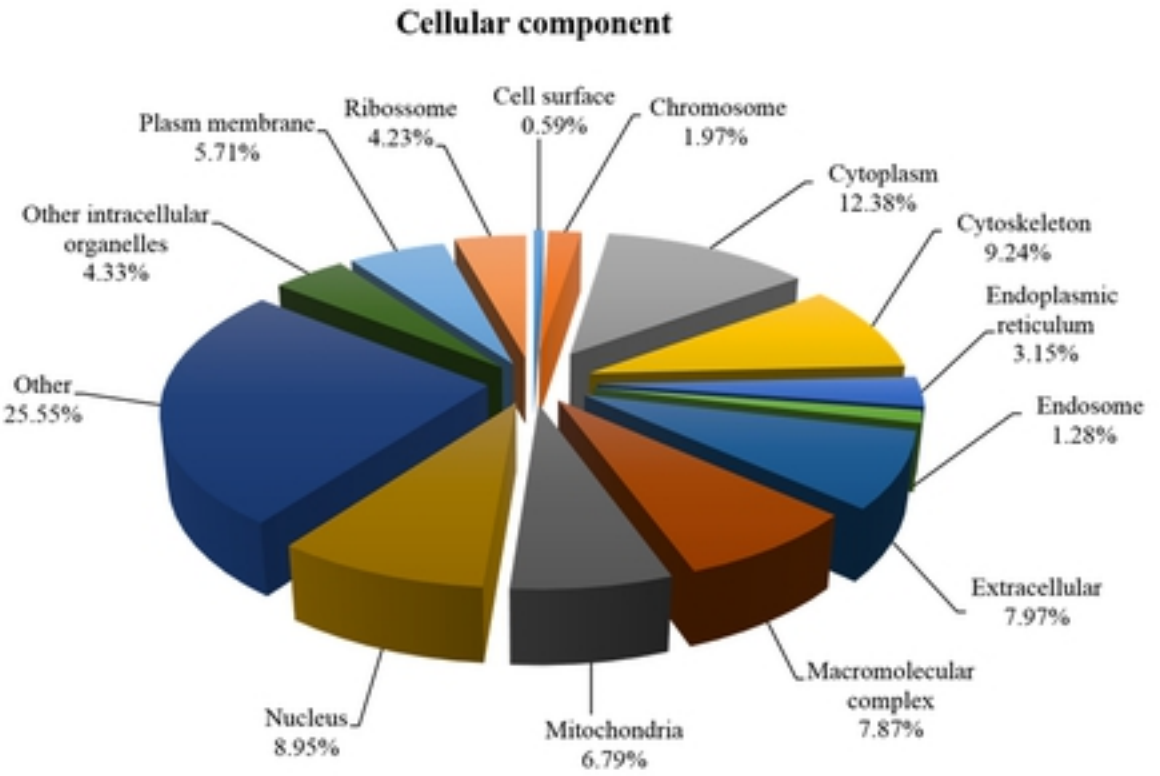
758 **S1 Table. Spectral count of *Saimiri collinsi* sperm protein throughout an entire year (.XLS).**

759 **S2 Table. Sperm proteins of *Saimri collinsi* that participate in important reproductive events**
760 **(.XLS).**

761 **S3 Table. Two-sample tests of the sperm protein concentrations in *Saimiri collinsi* during the**
762 **dry and rainy seasons.**

763





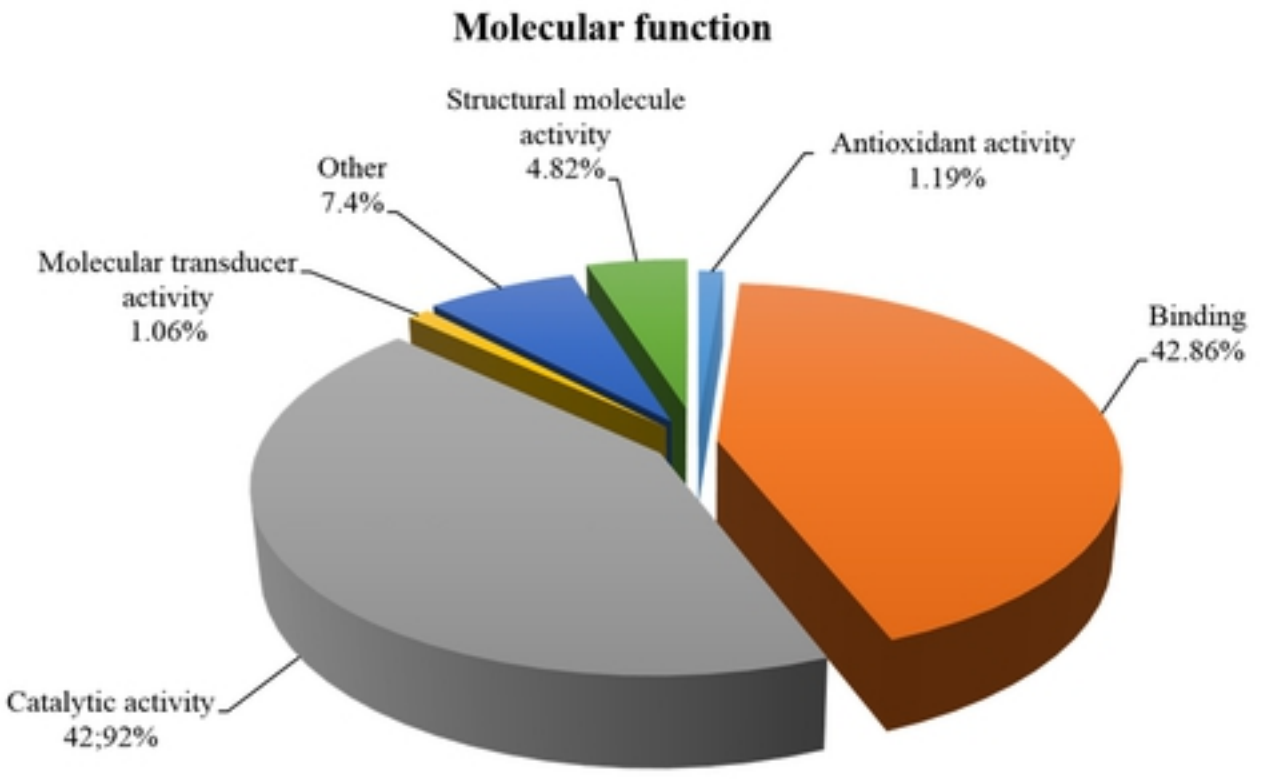
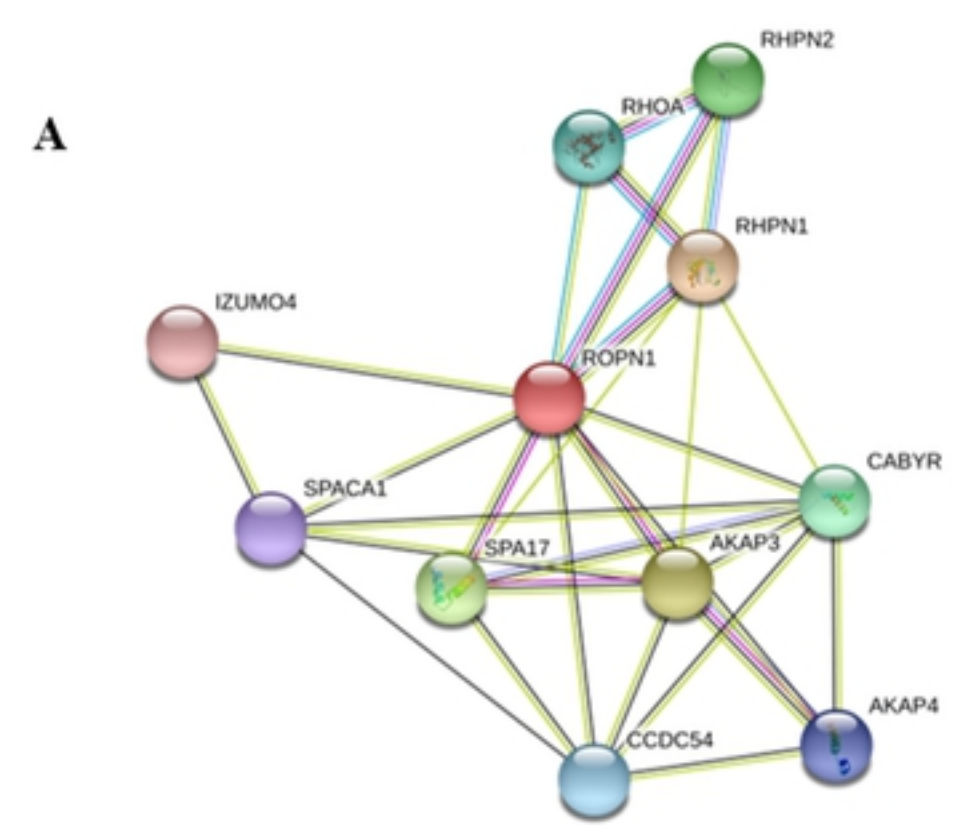
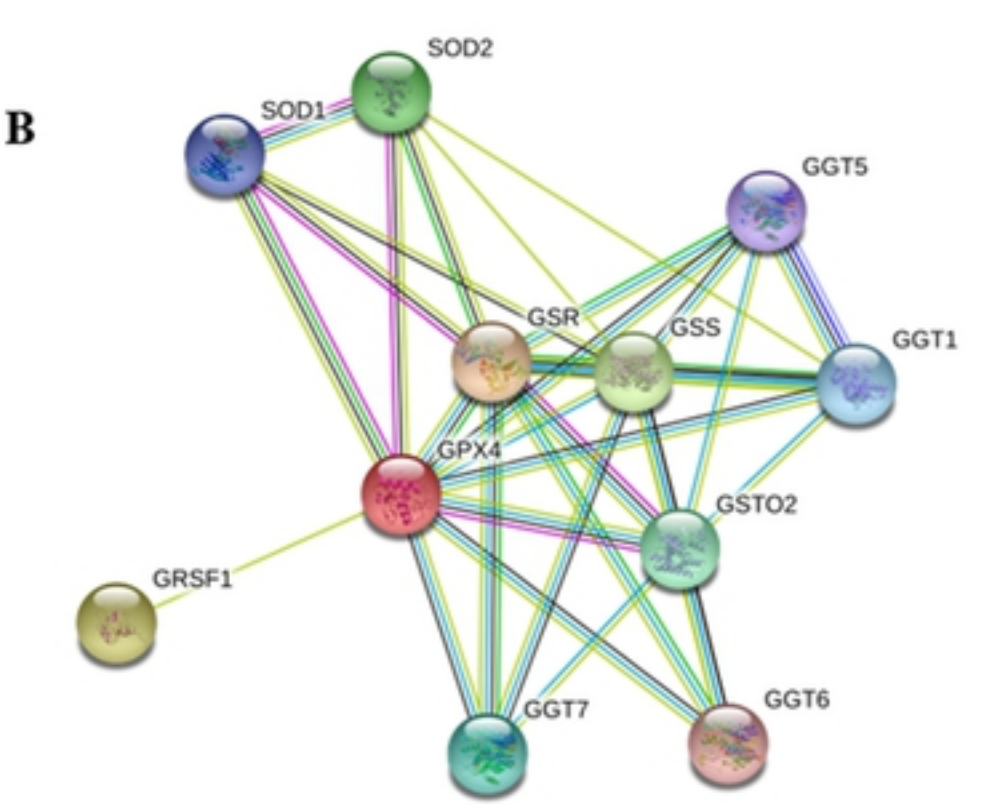
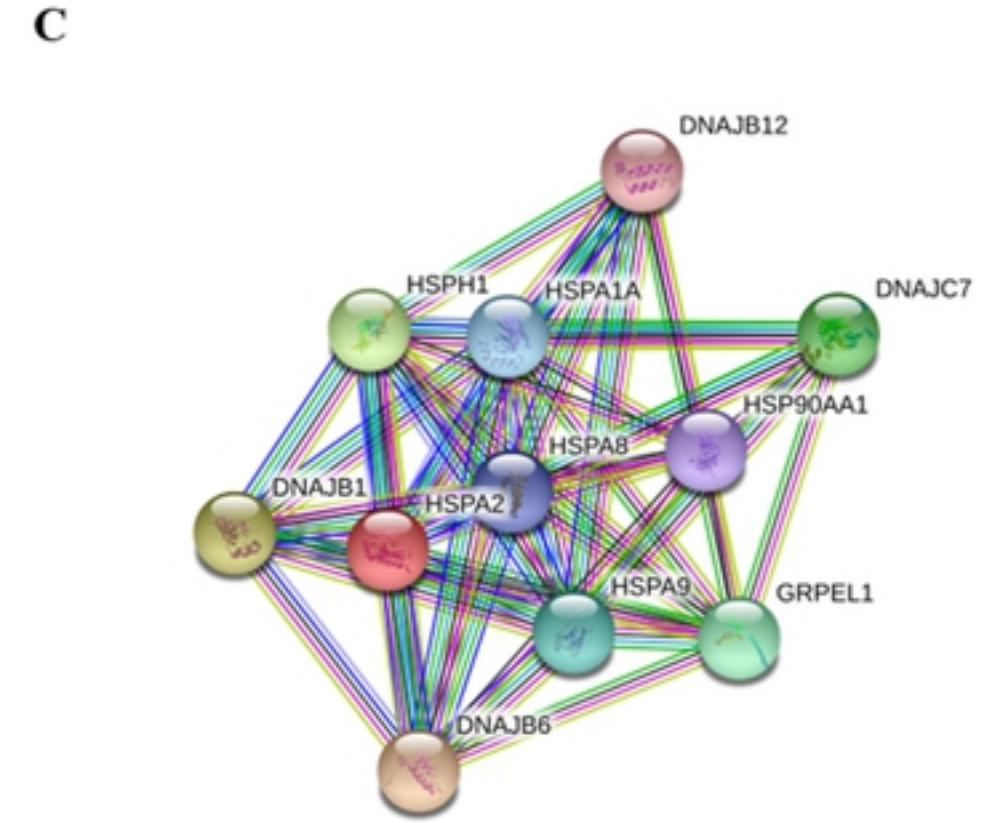


Figure 1


A
RHPN2
RHOA
RHPN1
IZUMO4
ROPN1
CABYR
SPACA1
SPA17
AKAP3
AKAP4
CCDC54


B
SOD2
SOD1
GGT5
GSR
GSS
GGT1
GPX4
GSTO2
GRSF1
GGT7
GGT6


C
DNAJB12
HSPH1
HSPA1A
DNAJC7
HSP90AA1
HSPA8
DNAJB1
HSPA2
HSPA9
GRPEL1
DNAJB6

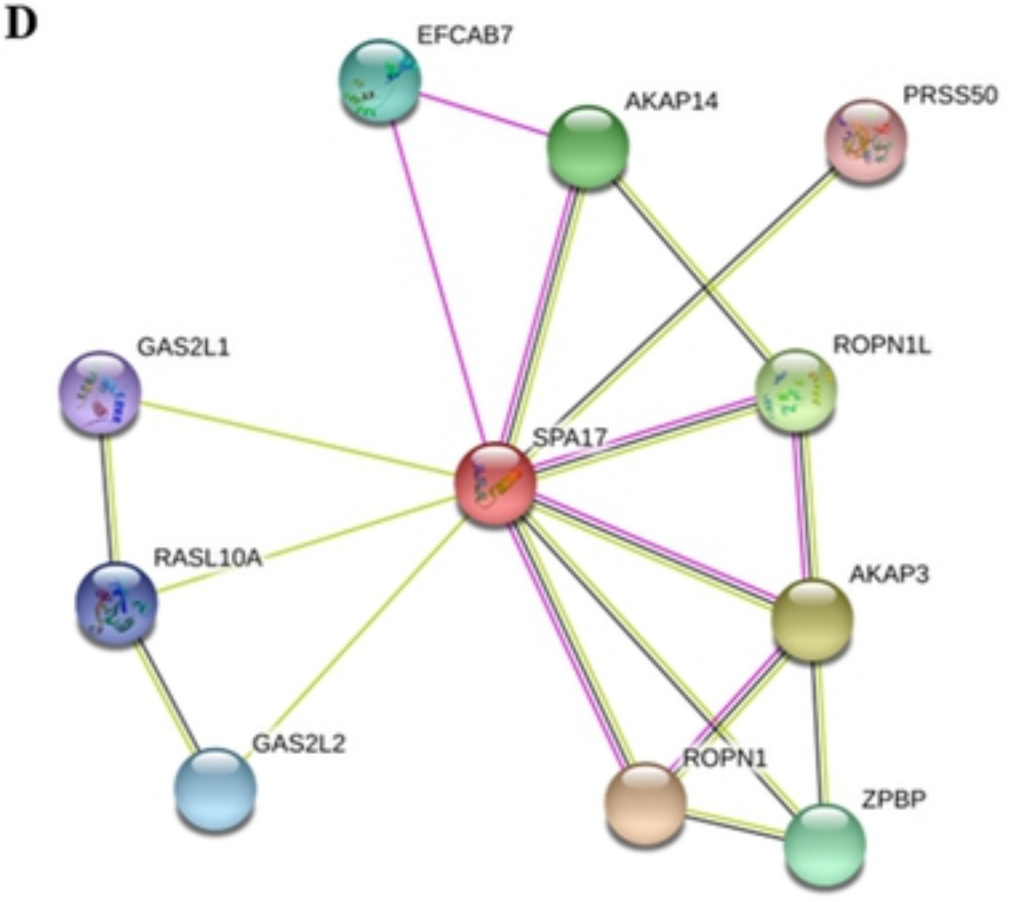

D
EFCAB7
AKAP14
PRSS50
GAS2L1
ROPN1L
SPA17
RASL10A
AKAP3
GAS2L2
ROPN1
ZPBP

Figure 2

Table 1: Mean (±SEM) values of the body weight, testicular volume (cm3), and seminal parameters of *Saimiri collinsi* during the dry (breeding) and rainy (non-breeding) seasons.

	Dry season	**Rain season**	***P*-value**
Body weight (g)	816.10±6.85	883.15±14.50	0.0002
Testicular volume (cm^3)			
Right testicular volume	0.95±0.06	1.19±0.07	0.02
Left testicular volume	0.96±0.07	1.22±0.08	0.03
Total testicular volume	1.91±0.13	2.42±0.11	0.01
Fresh sample			
Seminal parameters			
pH	7.96±0.10	7.30±0.11	0.0002
Seminal volume (µL)	114.70±16.93	145.29±28.14	0.35
Total sperm count (x 10^6/ml)	14,196±3,08	19,036±8,89	0.67
Motility	63±7.56	80±5.39	0.76
Vigour	3.4±0.28	3.85±0.28	0.23
PMF[1]	74±7.17	73.33±4.67	0.87
PMI[2]	64±5.45	59.28±6.94	0.59
Normal sperm	71.40±2.85	71.64±5.34	0.83
After dilution in ACP-118			
Seminal parameters			
Motility	44.18±7.82	63.46±7.99	0.10
Vigour	3±0.24	3.15±0.29	0.89
*PMF	67±4.14	73.60±8.41	0.46
**PMI	77.66±3.74	67.76±7.62	0.26
Normal sperm	72.58±2.71	70.75±5.14	0.76

*Plasma membrane functionality (PMF; %)
**Plasma membrane integrity (PMI; %)

Table 1

Table 2. Upregulation or downregulation of sperm protein expression (μg/mL) in *Saimiri collinsi* during the rainy (non-breeding) and dry (breeding) seasons.

Protein function	Accession Number	Rain season	Dry season	*P-value*	
Cell adhesion					
T-complex protein 11 homolog isoform X1	XP_010332353.1	-0.0418158	-0.0646175	-0.0228018	Downregulation
Transport					
Metaxin-2	XP_003921884.1	-0.110764	-0.1303	-0.0195369	Downregulation
AP-1 complex subunit gamma-1 isoform X2	XP_003939997.1	0.00478612	-0.00957221	-0.0143583	Downregulation
Desmoglein-1	XP_003924808.1	-0.233383	-0.259419	-0.0260363	Downregulation
Cytochrome c oxidase subunit 4 isoform 1	XP_010331988.1	0.154676	0.178389	0.0237126	Upregulation
ADP/ATP translocase 4	XP_003927767.1	0.188749	0.233035	0.0442863	Upregulation
Solute carrier family 2, facilitated glucose transporter member 3 isoform X2	XP_010338156.1	0.204773	0.164742	-0.0400316	Downregulation
Enzymatic action					
Inactive hydroxysteroid dehydrogenase-like protein 1	XP_003922852.1	0.0530542	0.00802451	-0.0450296	Downregulation
Nucleoside diphosphate kinase homolog 5	XP_003933998.1	0.0517683	0.0970006	0.0452323	Upregulation
Deoxyguanosine kinase	XP_003922648.1	-0.0198231	0.00259574	0.0224188	Upregulation
Beta-hexosaminidase subunit alpha	XP_003929709.1	-0.0187426	0.0282641	0.0470066	Upregulation
Neutrophil elastase	XP_010330763.1	-0.0881865	-0.0872099	0.000976637	Downregulation
Cathepsin G	XP_003924684.1	0.000703451	-0.0432964	-0.0439998	Downregulation
NADH dehydrogenase subunit 4	YP_006493379.1	-0.018066	-0.00610582	0.0119601	Downregulation
ATPase inhibitor, mitochondrial	XP_003944631.1	-0.08326	-0.133093	-0.049833	Upregulation
Matrilysin	XP_003923823.1	-0.0658871	-0.0392313	-0.0658871	Upregulation
Catalase	XP_003920025.1	-0.0442755	-0.021024	0.0266558	Upregulation

Alcohol dehydrogenase class-3	XP_003929577.2	-0.110761	-0.120986	-0.0102248	Upregulation
Pyruvate dehyrogenase phosphatase catalytic subunit 1 isoform X1	XP_010347702.1	-0.111713	-0.0830047	0.0287079	Downregulation
Phospholipase A1 member A isoform X1	XP_003935462.1	-0.0228687	-0.0505345	-0.0276657	Downregulation
L-lactate dehydrogenase A-like 6A	XP_010348467.1	-0.0667417	-0.0488062	0.0179355	Upregulation
6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 2 isoform X2	XP_003930467.1	0.0514503	0.0460828	-0.00536754	Downregulation
Glutaredoxin-3	XP_010331632.1	-0.00368504	0.0240983	0.0277833	Upregulation
Fructose-bisphosphate aldolase A	XP_010339012.1	0.244215	0.275165	0.0309499	Upregulation
L-lactate dehydrogenase C chain isoform X1	XP_003919941.1	0.331268	0.293222	-0.0380465	Downregulation
ATP synthase subunit alpha, mitochondrial	XP_003924745.1	0.289526	0.253334	-0.0361922	Downregulation
Nicotinamide mononucleotide adenylyltransferase 3 isoform X1	XP_010328728.1	-0.0188498	-0.0614076	-0.0425578	Downregulation
Carboxypeptidase A5	XP_010341383.1	0.175417	0.155398	-0.0200193	Downregulation
Epididymis-specific alpha-mannosidase	XP_003934701.1	-0.0246382	-0.00485184	0.0197863	
Metabolic process					
Prosaposin isoform X3	XP_003921884.1	-0.0257112	-0.0655105	-0.0397993	Upregulation
Apolipoprotein E	XP_010349445.1	0.00325406	-0.0301955	-0.0334496	Downregulation
Prostaglandin E synthase 3	XP_003945090	-0.00495669	0.00443556	0.00939225	Upregulation
Glutathione S-transferase Mu 3	XP_010342304.1	0.301758	0.262642	-0.0391165	Downregulation
Signal Transduction					
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	XP_010348645.1	0.0070466	-0.00720209	-0.0142487	Downregulation
Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	XP_010344866.1	-0.331463	-0.290751	0.0407123	Upregulation
Succinate-semialdehyde dehydrogenase,	XP_003927461.1	-0.0770785	-0.0849022	-0.00782367	Upregulation

mitochondrial					
Regulation					
Histone deacetylase 11 isoform X1	XP_010335234.1	0.0196068	0.0254748	0.005868	Upregulation
Putative alpha-1-antitrypsin-related protein	XP_010337932.1	-0.0468411	-0.0630148	-0.0161737	Downregulation
Adenylyl cyclase-associated protein 1 isoform X1	XP_010345853.1	0.0995165	0.124495	0.024978	Upregulation
Gasdermin-A	XP_003942931.1	-0.306126	-0.342276	-0.0361497	Upregulation
Serine/threonine-protein phosphatase PP1-beta catalytic subunit	XP_010350608.1	0.0431037	0.023024	-0.0200797	Downregulation
Serine/threonine-protein phosphatase PP1-gamma catalytic subunit	XP_003932298.1	0.127303	0.162706	0.0354028	Upregulation
Serine protease 30-like	XP_010337384.1	0.107069	0.130249	0.0231806	Upregulation
Amine oxidase	XP_003939638.1	-0.0154437	0.0185325	0.0339763	Upregulation
Ropporin-1-like	XP_010350743.1	0.200637	0.218699	0.0180613	Upregulation
Probable C-mannosyltransferase DPY19L2 isoform X1	XP_003930783.1	0.28643	0.242708	-0.0437227	Downregulation
Cat eye syndrome critical region protein 5	XP_010331313.1	-0.0809455	-0.0461215	0.034824	Upregulation
Actin-related protein T2	XP_003939685.1	0.0629516	0.0138987	-0.0490529	Downregulation
Immune system process					
Clusterin	XP_003937256.1	-0.0631874	-0.051801	0.0113864	Upregulation
Saoe class I histocompatibility antigen, A alpha chain-like	XP_010331157.1	0.0015255	-0.00101701	-0.00254251	Downregulation
Heat shock cognate 71 kDa protein	XP_003923627.1	-0.0294348	-0.0662507	-0.036815	Downregulation
Heat shock 70 kDa protein 13	XP_003934369.1	-0.214329	-0.231181	-0.0168523	Downregulation
Heat shock 70 kDa protein 1A/1B	XP_010330550.1	0.110054	0.0649447	-0.0451095	Upregulation
Heat shock-related 70 kDa protein 2	XP_003924476.1	-0.0426444	0.0048713	0.0475157	Upregulation

Heat shock protein HSP 90-alpha-like	XP_010331234.1	0.200363	0.215513	0.0151495	Upregulation
26S proteasome non-ATPase regulatory subunit 6	XP_003940262.1	-0.126224	-0.173192	-0.0469682	Downregulation
Mucin-7	XP_003931974.1	0.00693327	-0.0291519	-0.0360851	Downregulation
T-complex protein 1 subunit gamma	XP_010346914.1	0.0982317	0.1061	0.00786838	Upregulation
26S proteasome non-ATPase regulatory subunit 11	XP_003933069.1	-0.00172181	0.00344362	0.00516543	Upregulation
Phospholipid hydroperoxide glutathione peroxidase, mitochondrial	XP_003944526.1	0.247381	0.295112	0.0477303	Upregulation
Binding					
Fatty acid-binding protein, epidermal	XP_003940229.1	-0.178573	-0.1364	0.0421733	Upregulation
BPI fold-containing family A member 1	XP_003932174.1	0.0510516	0.0562223	0.00517074	Upregulation
Pancreatic secretory granule membrane major glycoprotein GP2 isoform X2	XP_010339045.1	0.0359252	0.0129086	-0.0230166	Upregulation
ATP-dependent RNA helicase DDX3X isoform X2	XP_010333111.1	0.059067	0.0213557	-0.0377113	Downregulation
EF-hand calcium-binding domain-containing protein 5	XP_003931501.1	0.0106187	0.0144026	0.0037839	Upregulation
Barrier-to-autointegration factor-like protein	XP_003933224.1	-0.107591	-0.141747	-0.0341563	Downregulation
Fascin-3	XP_003921070.1	0.031443	-0.00888356	-0.0403266	Downregulation
Sperm surface protein Sp17	XP_010332707.1	-0.00367783	-0.00266121	0.00101662	Upregulation
IQ domain-containing protein F5-like	XP_003936750.1	-0.0514445	-0.00810819	0.0433363	Downregulation
Plasma membrane calcium-transporting ATPase 4 isoform X1	XP_010347205.1	0.0938473	0.0803806	-0.0134667	Downregulation
Coiled-coil domain-containing protein 136	XP_010341530.1	0.0791321	0.0806699	0.00153776	Downregulation
Structural					
40S ribosomal protein S13	XP_003919926.1	0.349783	0.3246	-0.0251828	Downregulation
Actin-like protein 10	XP_003932104.1	-0.112513	-0.0969337	0.015579	Downregulation

40S ribosomal protein S11	XP_003940405.1	0.00987448	-0.0294448	-0.0393193	Downregulation
Outer dense fiber protein 2 isoform X5	XP_010349590.1	0.258377	0.297718	0.0393412	Upregulation
Tektin-5	XP_003938786.1	0.192894	0.2234	0.0305061	Upregulation
Tektin-3	XP_003929310.1	0.156527	0.146821	-0.00970651	Downregulation
Dynein heavy chain 8, axonemal	XP_010332512.1	0.0487448	0.0329032	0.028147	Downregulation
Dynein heavy chain 12, axonemal	XP_010345476.1	0.0791321	0.0806699	-0.0158416	Upregulation