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

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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.

43 Introduction

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

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

57 Although studies on the squirrel monkey have already shown correlations between
 58 reproductive seasonality and spermatogenesis (*Saimiri sciureus*) [12] and the gonadal hormones (*S.*
 59 *sciureus*) [13], only one study has reported the seasonal influence on seminal quality (*S. sciureus*)
 60 [14]. However, nothing is known about the protein composition of spermatozoa in these Neotropical
 61 primates, or of the differences in the sperm protein profile between the breeding and non-breeding
 62 seasons. In domestic animals, proteomic studies have shown the upregulation and downregulation of
 63 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 μ L of 1% eosin (Vetec, Rio de Janeiro, Brazil) and 5 μ L of 1% nigrosine (Vetec, Rio de Janeiro, Brazil) to 5 μ L of semen on a prewarmed (37°C) glass slide. The sperm concentration was determined in a Neubauer chamber after the dilution of 1 μ L of semen in 99 μ L of 10% formalin solution. The plasma membrane functionality was assessed with the hypoosmotic swelling test after the dilution of 5 μ L of semen in 45 μ L 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 μ L 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 $\times$. The semen was assessed directly both after collection (fresh) and after dilution in ACP-118.

Sperm separation and freeze-drying

Owing to the occurrence 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 $\pm$ 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 cm³, respectively) than in the dry season (816.10 ± 6.85 g and 1.91 ± 0.13 cm³) (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 ($\pm$ SEM) values of the body weight, testicular volume (cm³), 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 ($\mu\text{g/mL}$) 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 web-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

298 line color represents the types of evidence for the association. Green text; black
 299 coexpression; blue databases; and pink experiments. AKAP3 A-kinase anchor protein 3;
 300 SPA17 Sperm surface protein Sp17; CABYR Calcium-binding tyrosine phosphorylation-
 301 regulated protein; RHPN1 Rhophilin-1; RSPH3 Radial spoke head protein 3 homolog;
 302 CCDC63 Coiled-coil domain-containing protein 63; TRPV6 Transient receptor potential
 303 cation channel subfamily V member 6; DNALI1 Axonemal dynein light intermediate
 304 polypeptide 1; TEKT3 Tektin-3; CFAP36 Cilia- and flagella-associated protein 36; GSR
 305 Glutathione reductase, mitochondrial; GRSF1 G-rich sequence factor 1; GSS Glutathione
 306 synthetase; SOD2 Superoxide dismutase [Mn], mitochondrial; GSTO2 Glutathione S-
 307 transferase omega-2; GGT7 Glutathione hydrolase 7; GGT1 Glutathione hydrolase 1
 308 proenzyme; SOD1 Superoxide dismutase [Cu-Zn]; GGT5 Glutathione hydrolase 5
 309 proenzyme; GGT6 Glutathione hydrolase 6; DNAJB6 DnaJ (Hsp40) homolog, subfamily B,
 310 member 6; DNAJB1 DnaJ (Hsp40) homolog, subfamily B, member 1; HSPH1 Heat shock
 311 105kDa/110kDa protein 1; DNAJC7 DnaJ (Hsp40) homolog, subfamily C, member 7;
 312 GRPEL1 GrpE-like 1, mitochondrial (E. coli); HSPA9 Heat shock 70kDa protein 9
 313 (mortalin); HSPA1A Heat shock 70kDa protein 1A; HSPA8 Heat shock 70kDa protein 8;
 314 HSP90AA1 Heat shock protein 90kDa alpha (cytosolic), class A member 1; DNAJB12 DnaJ
 315 (Hsp40) homolog, subfamily B, member 12 (409 aa); ROPN1 Rhophilin associated tail protein
 316 1 (212 aa); AKAP3 A kinase (PRKA) anchor protein 3; ROPN1L Rhophilin associated tail
 317 protein 1-like (230 aa); AKAP14 A kinase (PRKA) anchor protein 14; ZPBP Zona pellucida
 318 binding protein; EFCAB7 EF-hand calcium binding domain 7 (629 aa); GAS2L2 Growth

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

321

322 **Discussion**

323 **Proteins associated with spermatogenesis and sperm motility**

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

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

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

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

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

360 In this context, Imai et al. [60] showed that the failure to express phospholipid
361 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

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

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

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

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

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

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

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.

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Supporting information

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

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

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

Biological process

Cellular component

Molecular function

Figure 1

A **B** **C** **D**

Figure 2

Table 1: Mean ($\pm$ 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 $\pm$ 6.85	883.15 $\pm$ 14.50	0.0002
Testicular volume (cm³)			
Right testicular volume	0.95 $\pm$ 0.06	1.19 $\pm$ 0.07	0.02
Left testicular volume	0.96 $\pm$ 0.07	1.22 $\pm$ 0.08	0.03
Total testicular volume	1.91 $\pm$ 0.13	2.42 $\pm$ 0.11	0.01
Fresh sample			
Seminal parameters			
pH	7.96 $\pm$ 0.10	7.30 $\pm$ 0.11	0.0002
Seminal volume (μ L)	114.70 $\pm$ 16.93	145.29 $\pm$ 28.14	0.35
Total sperm count ($\times 10^6$ /ml)	14,196 $\pm$ 3,08	19,036 $\pm$ 8,89	0.67
Motility	63 $\pm$ 7.56	80 $\pm$ 5.39	0.76
Vigour	3.4 $\pm$ 0.28	3.85 $\pm$ 0.28	0.23
PMF ¹	74 $\pm$ 2.17	73.25 $\pm$ 4.67	0.87
PMI ²	64 $\pm$ 5.45	59.28 $\pm$ 6.94	0.59
Normal sperm	71.40 $\pm$ 2.85	71.64 $\pm$ 5.34	0.83
After dilution in ACP-118			
Seminal parameters			
Motility	44.18 $\pm$ 7.82	63.46 $\pm$ 7.99	0.10
Vigour	3 $\pm$ 0.24	3.15 $\pm$ 0.29	0.89
*PMF	67 $\pm$ 4.14	73.60 $\pm$ 8.41	0.46
**PMI	77.66 $\pm$ 3.74	67.76 $\pm$ 7.62	0.26
Normal sperm	72.58 $\pm$ 2.71	70.75 $\pm$ 5.14	0.76

*Plasma membrane functionality (PMF; %)

**Plasma membrane integrity (PMI; %)

Table 2. Upregulation or downregulation of sperm protein expression ($\mu\text{g/mL}$) in *Saimiri collinsi* during the rainy (non-breeding) and dry (breeding) seasons.

Protein function	Accession Number	Rain season	Dry season	<i>P</i> -value	
<i>Cell adhesion</i>					
T-complex protein 11 homolog isoform X1	XP_010332353.1	-0.0418158	-0.0646175	-0.0228018	Downregulation
<i>Transport</i>					
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
<i>Enzymatic action</i>					
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 dehydrogenase 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	
<i>Metabolic process</i>					
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
<i>Signal Transduction</i>					
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
<i>Binding</i>					
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
<i>Structural</i>					
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