Protein profile of the seminal plasma of collared peccaries (*Pecari tajacu* Linnaeus, 1758)

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Abstract

This study was conducted to characterize the major proteins of the peccary seminal plasma, based on the semen samples collected from nine adult and reproductively sound animals. Our approach included the use of two-dimensional electrophoresis followed by Coomassie blue staining and analysis of polypeptide maps with PDQuest Software (Bio-Rad). Proteins were identified by tandem mass spectrometry (LC-MS/MS). We detected 179 protein spots per gel and 98 spots were identified by mass spectrometry, corresponding to 23 different proteins. The combined intensity of those spots accounted for $56.2 \pm 6\%$ of the intensities of all spots and 60.9% of the intensities of spots presented in every protein map. Protein spots identified as clusterin represented $19.7 \pm 8.3\%$ of the integrated optical densities of all spots detected in the seminal plasma maps. There was a negative association ($r = -0.87$; $P < 0.05$) between the intensity of a clusterin spot and the percentage of sperm with functional membrane. Spermadhesin porcine seminal plasma protein 1 and bodhesin 2 comprised 5.4 ± 1.9 and $8.8 \pm 3.9\%$ of the total intensity of all spots respectively. Many proteins appeared in a polymorphic pattern, such as clusterin (27 spots), epididymal secretory glutathione peroxidase (ten spots), inter- α -trypsin inhibitor (12 spots), and IgG-binding protein (ten spots), among others. In conclusion, we presently describe the major seminal plasma proteome of the peccary, which exhibits a distinct high expression of clusterin isoforms. Knowledge of wild species reproductive biology is crucial for an understanding of their survival strategies and adaptation in a changing environment.

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Introduction

For decades, native American communities have used wildlife as a source of animal protein. Despite prohibitive laws, poaching is still occurring worldwide and this has been a major problem in populated areas. To overcome this situation, at least partially, adaptation of wild species to captivity is proving to be a promising solution. The collared peccary (*Pecari tajacu* Linnaeus, 1758) is a clear example as it constitutes a potential resource for commercial exploitation, production of meat, and leather. Moreover, the International Union for Conservation of Nature (IUCN) classifies the peccaries as a least concerned species but emphasizes that the population is disappearing in some regions of their natural habitats (IUCN, http://www.iucn.org/about/work/programmes/species/our_work/the_iucn_red_list/, access in: 18th September 2013).

Knowledge on the reproductive aspects of collared peccaries is of crucial importance for the development of appropriate management practices (Mayor *et al.* 2007).

Several researches have been demonstrating important aspects of their reproduction. For instance, the peccary spermatogenesis presents a meiotic index of 1:2.7, which is very similar to that of swine, a closely related domestic species (Costa *et al.* 2004). As in the swine, the peccary ejaculate is composed of three fractions: i) clear and colorless, ii) rich in spermatozoa, and iii) the jelly fraction (Hellgren *et al.* 1989). Fractions that constitute the seminal plasma are mainly originated from the prostate, seminal vesicles, and bulbourethral glands (Garcia *et al.* 2000).

In the domestic swine, recent studies have been focused on origin and function of seminal plasma proteins (Huang *et al.* 2000). Two spermadhesins presenting 26 kDa (pI 6.2) and 55 kDa (pI 4.5) were identified as potential markers for fertility in this species, as its presence is associated with high prolificity rates (Flowers & Turner, 2001). In addition, the 26 kDa protein was related to better sperm membrane integrity after freezing–thawing processes (Bianchi *et al.* 2008). The characterization of proteins present in the seminal plasma may

provide essential information for identification of molecular determinants of fertility. It would contribute for the knowledge on reproductive aspects of the peccaries, in which there is still a lack of such information. This knowledge would be useful for the selection of the more appropriate stud males for use in natural reproduction and for the formation of germplasm banks. Therefore, this study was conducted to characterize the major proteins of the seminal plasma and their associations with semen characteristics in collared peccaries.

Materials and methods

Animals

All experimental protocols and animal care procedures were approved by the Ethics Committee of the Federal University of the Semi-arid (UFERSA) for handling animals (process no. 23 091.0254/11-88). Collared peccaries (*P. tajacu*) used in the experiment were provided by the Center for Multiplication of Wild Animals (UFERSA), located in the northeast of Brazil (Mossoró, RN, Brazil; 5°10'S and 37°10'W). The climate of this region is typically semi-arid, with an average annual temperature of 27 °C. Nine healthy and sexually mature males aged 21 ± 1 months and each weighing 20.1 ± 0.1 kg were used. They were isolated from females 6 months before the beginning of the study and maintained under a 12-h natural photoperiod throughout the research. They were maintained in paddocks (20 × 3 m), with a covered area (3 × 3 m) and fed sow food and fruits, with free access to water.

Semen collection

For semen collection, males were physically restrained by a capture net and then anesthetized by i.v. administration of propofol (Propovan, Cristalia, Fortaleza, Brazil) on a dose of 5 mg/kg of body weight, as previously described (Souza *et al.* 2009). Semen was collected with the aid of an electroejaculator (Autojac, Neovet, Campinas, SP, Brazil) connected to a 12 V source (Castelo *et al.* 2010). The stimulatory cycle comprised ten stimuli in each voltage, starting from 5 V, followed by a voltage increase in steps of 1 V, up to 12 V. Each electrical stimulus lasted 3 s, with intermittent breaks of 2 s. The stimuli cycles were maintained for 10 min from the beginning of the procedure. The dimension of the electroejaculator probe was 15 × 1.3 cm, and 12 cm of the probe was inserted into the animal rectum. Semen was collected in plastic tubes and mixed with a cocktail of protease inhibitors (Sigma–Aldrich, catalog no. P8340). Aliquots of semen samples were taken for semen analysis and the remaining samples, centrifuged at 700 *g* for 10 min, were transferred to clean tubes and stored at –20 °C until further analysis.

Semen evaluation

The volume of semen was measured using micropipettes and we recorded the aspect of the ejaculates. The percentage of motile sperm and sperm vigor was immediately assessed using light microscopy at 100× and 400× magnification.

For determination of sperm vigor, we used a 0–5 scale, where 0 was given to sperm with no movement and 5 characterized the maximum sperm progressive forward movement. Bromophenol blue-stained smears were prepared using 5 µl semen for evaluation of sperm morphology under light microscopy (1000×) and viability (400×), counting 200 cells/slide. Sperm concentration was determined using a Neubauer counting chamber. Sperm functional integrity was analyzed by the hypo-osmotic swelling test, using distilled water as the hypo-osmotic solution (Castelo *et al.* 2010), counting 200 cells.

Determination of seminal plasma protein profile

Two-dimensional gel electrophoresis

For two-dimensional (2D) electrophoresis, seminal plasma samples from nine individuals were individually thawed at room temperature and centrifuged at 10 000 *g* for 1 h at 4 °C (Moura *et al.* 2006). Samples containing 400 µg protein (Bradford 1976) were mixed with re-hydration buffer (7 M urea, 2 M thiourea, 0.5% CHAPS, 0.5% immobilized pH gradient (IPG) buffer, pH 4–7, 65 mM dithiothreitol (DTT), and traces of bromophenol blue) in an amount enough to make 250 µl (Souza *et al.* 2012). Then, the mixture was incubated with 13 cm IPG strips in the pH range of 4–7 (GE Life Sciences, Piscataway, NJ, USA) and allowed to re-hydrate for 20 h. Isoelectric focusing was performed in an Ettan IPGphor 3 apparatus (GE Life Sciences), at 100 V for 2 h, 500 V for 1 h, 1000 V for 30 min, 5000 V for 2 h, and 8000 V for 1 h, in a total of 24 000 Vh. After focusing, seminal plasma proteins were equilibrated in equilibration buffer I (6 M urea, 50 mM Tris–HCl, pH 8.8, 29.3% glycerol, 2% SDS, and 1% DTT) for 15 min and re-equilibrated thereafter for an additional 15 min in equilibration buffer II (similar to equilibration buffer I, but containing 2.5% iodoacetamide instead of DTT). After equilibration, IPG strips were placed on the top of SDS–PAGE gels, containing a homogeneous concentration of acrylamide (12.5%). Standards from 97 to 14 kDa (Sigma Chemical Co.) were used and gels were run in an SE 600 Ruby apparatus (12.5% T/2.6% C, 250 V, and 25 mA/gel) for ~5 h. Reagents for electrophoresis were purchased from GE Life Sciences or Sigma–Aldrich.

Gels were stained by colloidal Coomassie blue (Souza *et al.* 2012), scanned at 300 dpi (ImageScanner II; GE Life Sciences), and analyzed using PDQuest Software, version 7.3.0 (Bio-Rad Laboratories). For the set of nine seminal plasma protein maps, a master gel was generated by the software, based on a reference gel, which represented the best pattern of spots in all samples of the study. Additional spots consistently present in other gels were also added to the master and proteins in key regions of the gels were used as landmarks. Control of spot matches was done by checking each spot, in each gel, with the respective pattern in the master. Protein quantities in the gels were given as parts per million (ppm) of the total integrated optical density of the spots, according to PDQuest Software (Moura *et al.* 2007, 2010, Souza *et al.* 2012).

Protein identification

Proteins separated by 2D-PAGE were subjected to in-gel trypsin digestion as described elsewhere (Souza *et al.* 2012).

Briefly, excised gel pieces were washed three times with 400 μ l of a solution containing ammonium bicarbonate (25 mM) and acetonitrile (50%), pH 8.0, to remove SDS and dye, dehydrated after two washes with 200 μ l of absolute acetonitrile, and completely dried under vacuum. Gel pieces were then incubated ($\sim$ 20 h) at 37 °C with trypsin (166 ng/spot, Promega, catalog no. V5111). Peptides were then extracted from gel pieces by washing three times with 25 μ l of trifluoroacetic acid (5%) in ammonium bicarbonate (50 mM) and acetonitrile (50%) for 30 min. The extracts were dried under vacuum again and re-suspended in 10 μ l of injection buffer (95% water, 5% acetonitrile, and 0.1% formic acid). A piece of blank gel and a piece of albumin from the molecular weight marker were also digested and used as negative and positive controls respectively.

For tandem mass spectrometry (LC–MS/MS), the digested samples were injected using the nanoAcquity UPLC sample manager and the chromatographic separation was performed using a UPLC C18 column (75 μ m $\times$ 10 cm) with a flow of 0.6 μ l/min (Souza *et al.* 2012). The mass spectra were acquired in a Synapt G2 HDMS instrument (Waters Co., Milford, MA, USA) using a data-dependent acquisition, where the three top peaks were subjected to MS/MS. Mobile phases A and B consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile respectively. The gradient conditions used were as follows: 0 min with 3% of B, increasing linearly to 30% B in 20 min, then it increased up to 70% B in 40 min where it remained until 50 min and in the next minute it was decreased to 3% of B. The data were processed using Mascot Distiller (Matrix Science Ltd; www.matrixscience.com) and subjected to database search using Mascot Server 2.3, using mammalia as the taxon. The searches were made with the assumption that there was a maximum of one missed trypsin cleavage and that peptides were mono-isotopic and using partially oxidized methionine residues and completely carbamidomethylated cysteine residues. Peptide mass tolerance and fragment mass tolerance were initially set to $\pm$ 0.1 Da respectively for MS/MS ion searching. However, candidate peptide IDs were only accepted if the m/z values were observed within 0.1 Da (typically <0.05 Da) of the theoretical mass of the candidate ID, as determined when manually reviewing MASCOT search results.

Gene ontology analysis

Data from the seminal plasma protein list obtained after MASCOT search were analyzed using the Software for Researching Annotations of Proteins (STRAP), an open-source application, as previously reported (Souza *et al.* 2012). Gene ontology terms for biological process and molecular function were obtained from UniProtKB and EBI GOA databases. Gene ontology associated with peccary seminal plasma proteins was also compared with that of other species such as rams (Souza *et al.* 2012) and domestic boar (*Sus scrofa*; V R Cadavid, J M Martins & A A Moura 2013, unpublished results). Comparisons were only made using gene ontology analyses that were previously conducted with the same software used in this study.

Protein interaction analysis

Analysis of known and predicted protein–protein interactions were run for clusterin using the open-access database-based

online tool STRING (<http://string-db.org>), version 9.05. In this case, analyses were conducted assuming *Bos taurus* as the organism. In the evidence view, interactions were based in co-expression (black lines), databases (light blue), text-mining (yellow), and experimental (purple) data. In the action mode, interactions represent reaction (black lines), binding (blue), or catalysis (purple). Only proteins with a confidence score above 0.900 were selected for analysis.

Statistical analyses

The experiment followed a completely randomized design, where each animal was considered as a random factor. Semen parameters and spot intensities from protein maps were tested for normality, using the Shapiro–Wilk's test, and for

Figure 1 Seminal plasma proteins of adult *Pecari tajacu*. (A) Represents the map used as a reference for the match set. (B) Master gel generated by the PDQuest Software using gels from nine animals. Gels were stained with Coomassie blue and proteins identified by mass spectrometry. Spot numbers refer to those in Table 1 and Supplementary Table 1.

Table 1 Seminal plasma proteins of collared peccaries (*Pecari tajacu*) identified by two-dimensional electrophoresis and tandem mass spectrometry. Table includes spots detected in 13 cm gels, within the 4–7 pH range. Spot numbers refer to those in Fig. 1.

Protein and spot numbers	NCBI accession number	MS/MS protein score	Sequence covered (%)	Relative quantity (mean $\pm$ S.D. %)
Bodhesin 2				8.8 $\pm$ 3.9
Spot 01	121484235	92	27	
Epididymal secretory protein E1				1.2 $\pm$ 0.7
Spot 02	47523496	121	28	
Spot 03	47523496	148	28	
Spot 04	47523496	125	28	
Spermadhesin PSP-1				5.4 $\pm$ 1.9
Spot 05	108346	47	9	
Spot 06	108346	82	9	
Spot 07	108346	44	9	
Casein α S1				2.7 $\pm$ 1.5
Spot 08	30794348	43	5	
Epididymal secretory glutathione peroxidase				6.1 $\pm$ 1
Spot 09	47523090	75	19	
Spot 10	47523090	186	22	
Spot 11	47523090	95	24	
Spot 12	47523090	94	24	
Spot 13	47523090	116	19	
Spot 14	47523090	78	24	
Spot 15	47523090	81	19	
Spot 16	47523090	159	22	
Spot 17	47523090	128	17	
Spot 18	47523090	47	5	
Clusterin				19.74 $\pm$ 8.32
Spot 19	47522770	346	12	
Spot 20	47522770	220	8	
Spot 21	47522770	200	8	
Spot 22	47522770	160	8	
Spot 23	47522770	135	15	
Spot 24	47522770	152	8	
Spot 25	47522770	136	12	
Spot 26	47522770	94	10	
Spot 27	47522770	203	11	
Spot 28	47522770	189	11	
Spot 29	47522770	409	10	
Spot 30	47522770	465	14	
Spot 31	47522770	279	12	
Spot 32	47522770	81	4	
Spot 33	47522770	32	4	
Spot 34	47522770	60	2	
Spot 35	47522770	39	2	
Spot 36	47522770	99	2	
Spot 37	47522770	46	2	
Spot 38	47522770	93	4	
Spot 39	47522770	331	10	
Spot 40	47522770	106	4	
Spot 41	47522770	268	8	
Spot 42	50979240	79	6	
Spot 43	47522770	231	7	
Spot 44	47522770	239	4	
Spot 95	47522770	88	4	
Albumin				2.5 $\pm$ 2
Spot 45	301786252	298	9	
Spot 46	30962111	256	7	
Spot 96	3319897	135	3	
Spot 47	301786252	89	4	
Spot 48	126723746	884	31	
Spot 49	126723746	435	15	
Annexin 5				2.1 $\pm$ 1.5
Spot 50	291401824	1231	55	
Spot 36	291401824	600	43	
Spot 37	291401824	801	47	
Acetylcholine receptor subunit α -7				0.4 $\pm$ 0.2
Spot 51	27806239	35	1	
Inter- α -trypsin inhibitor heavy chain H4 precursor				1.8 $\pm$ 0.8
Spot 52	48374067	381	8	
Spot 53	48374067	420	10	

Table 1 Continued.

Protein and spot numbers	NCBI accession number	MS/MS protein score	Sequence covered (%)	Relative quantity (mean $\pm$ s.d. %)
Spot 54	48374067	373	8	
Spot 55	48374067	263	6	
Spot 56	48374067	376	9	
Spot 57	48374067	284	7	
Spot 58	48374067	288	9	
Spot 59	48374067	284	8	
Spot 49	48374067	67	3	
Spot 42	48374067	328	6	
Spot 60	48374067	332	6	
Spot 61	48374067	303	5	
Transferrin				0.3 $\pm$ 0.3
Spot 62	6175087	349	12	
Spot 63	6175087	97	4	
Spot 64	6175087	191	7	
Spot 65	6175087	338	9	
ζ -globin-like				0.7 $\pm$ 1
Spot 66	291415430	75	20	
Ceruloplasmin precursor				0.6 $\pm$ 0.4
Spot 67	390517044	254	5	
Spot 68	390517044	381	6	
Spot 69	390517044	328	7	
T-complex protein 1 subunit gamma				0.1 $\pm$ 0.06
Spot 70	164448698	37	1	
Spot 71	164448698	37	1	
Spot 72	164448698	37	1	
Fc fragment of IgG-binding protein				1.0 $\pm$ 0.9
Spot 73	291414125	64	0.5	
Spot 74	297485627	138	1	
Spot 75	297485627	86	0.5	
Spot 76	297485627	78	0.5	
Spot 77	297485627	60	0.5	
Spot 78	297485627	73	0.5	
Spot 79	297485627	78	0.5	
Spot 80	297485627	78	0.5	
Spot 81	297485627	62	0.5	
Spot 97	297485627	77	0.5	
Carboxypeptidase E precursor				0.2 $\pm$ 0.2
Spot 82	148228720	65	5	
Spot 83	148228720	88	5	
(Predicted) hypothetical protein				0.6 $\pm$ 0.3
Spot 84	291391030	302	9	
Actin				0.4 $\pm$ 0.2
Spot 85	268607671	73	8	
Spot 86	45269029	255	18	
Spot 94	45269029	526	33	
Lipocalin-15				0.4 $\pm$ 0.7
Spot 87	76631203	96	12	
Cathepsin L1 precursor				0.2 $\pm$ 0.07
Spot 88	47522698	160	11	
Hexosaminidase B				0.8 $\pm$ 0.3
Spot 89	262072808	111	6	
Spot 90	262072808	141	7	
Spot 91	262072808	155	8	
Spot 92	262072808	74	4	
Laminin α 5-like				0.1 $\pm$ 0.08
Spot 93	296484172	47	1	
Tetraspanin CD63				0.04 $\pm$ 0.01
Spot 98	126723685	88	10	

asymmetry and kurtosis, using the UNIVARIATE procedure with NORMAL and PLOT options of the Statistical Analysis System (SAS 2003). Variables without a normal distribution (Shapiro-Wilk's, $P < 0.05$) were log (log ($x+1$)) or angularly (arcsin $\sqrt{x/100}$) transformed. Associations between seminal plasma parameters and the intensities of the spots detected

in the 2D maps of the animals were estimated by Pearson's (when two parametric variables were associated) or Spearman's (when at least one non-parametric variable was used in the association) method (using the CORR procedures, SAS (2003)). Only spots expressed in all gels were included in the analysis of correlation.

Results

All semen collection attempts conducted in the nine animals resulted in ejaculation. All ejaculates were white, watery, and contained sperm. Total volume of ejaculates was 2 ± 1.1 ml, with a sperm concentration of $207 \pm 160.7 \times 10^6$ spermatozoa/ml. Sperm motility was $83 \pm 20.9\%$, with vigor of 4.2 ± 1.1 . Percentages of viable sperm (sperm membrane integrity), morphologically normal sperm, and sperm presenting functionally intact membrane were 72.6 ± 10.4 , 80 ± 21 , and $80.4 \pm 14.1\%$ respectively.

The average protein concentration in seminal plasma samples from collared peccaries was 34.1 ± 14 mg/ml. Based on the analysis of a PDQuest Software match set with nine gels constructed within the 4–7 pH range, we detected 179 protein spots per gel (Fig. 1A and B). The master gel consistently showed 82 spots (46% of all spots) present in every protein map and intensities of these spots corresponded to 92.3% of the total intensity of all spots depicted in the seminal plasma maps. Ninety-eight spots were identified by MS/MS, corresponding to 23 different proteins (Fig. 1, Table 1, and Supplementary Table 1, see section on supplementary data given at the end of this article). The combined intensity of those spots accounted for 56.2% of the intensities of the all spots and 60.9% of the intensities of spots presented in every protein map.

Spots identified as clusterin represented $19.7 \pm 8.3\%$ of the integrated optical densities of all spots detected in the seminal plasma maps. There was a negative association ($r = -0.87$; $P < 0.05$) between the intensity of a clusterin spot (spot no. 27; Fig. 1) and the percentage of sperm with functional membrane. In fact, that was the unique significant association found between a seminal plasma protein and a semen characteristic in the peccary.

Spermadhesins, identified as spermadhesin porcine seminal plasma protein 1 (PSP-1) and bodhesin 2 (Bdh-2), comprised 5.4 ± 1.9 and $8.8 \pm 3.9\%$ of the total intensity of all valid spots in the gels respectively. Other major proteins found in the peccary seminal plasma include epididymal secretory glutathione peroxidase (GPX, $6.1 \pm 1.3\%$), albumin ($2.5 \pm 2\%$), and annexin 5 (ANX5, $2.1 \pm 1.5\%$). Many proteins appeared in a polymorphic pattern in the seminal plasma map, as trains of isoforms, such as clusterin (27 spots), epididymal secretory GPX (ten spots), inter- α -trypsin inhibitor (12 spots), IgG-binding protein (IgGBP, ten spots), PSP-1 (three spots), albumin (six spots), transferrin (four spots), and hexosaminidase B (four spots), among others. Other proteins identified in the seminal plasma of collared peccaries include ζ -globin, ceruloplasmin, carboxypeptidase E, and T complex-containing chaperonins (Fig. 1, Table 1, and Supplementary Table 1).

Biological processes of peccary seminal plasma proteins are linked to cellular process (22%), regulation

Figure 2 Gene ontology annotations of the *Pecari tajacu* seminal plasma proteome based on their biological process and molecular function. Protein data were analysed using the Software for Researching Annotations of Proteins. Gene ontology terms were obtained from UniProtKB and EBI GOA databases. Gene ontology associated with peccary seminal plasma proteins was also compared with that of rams (Souza *et al.* 2012) and domestic boar (*S. scrofa*; V R Cadavid, J M Martins & A A Moura 2013, unpublished results). Comparisons were only made using gene ontology analysis previously conducted with the same software used in the present study.

(17%), response to stimulus (14%), localization, and reproduction (11%). As a reference, biological processes of seminal proteins were mainly associated with cellular process, response to stimulus, regulation, and reproduction as found in the ram and domestic swine (Fig. 2). As for molecular functions, peccary seminal proteins mostly participate in binding (52%), followed by catalytic (16%) and antioxidant activities (8%), and enzyme regulatory and molecular transducer activity (4%). Gene ontology annotations defined as antioxidant, catalytic, and molecular transducer activity appear more distinct when compared to those of ram and swine proteins (Fig. 2).

Network analysis (Fig. 3) showed that clusterin interacts with a wide range of protein groups, such as α -2-plasmin inhibitor (SERPIN2), tissue inhibitor of metalloproteinase 1 (TIMP-1), fibronectin precursor (FN1), plasminogen precursor (PLG), α -2-macroglobulin (A2M), and secreted protein acidic and rich in cysteine (SPARC), among others.

Figure 3 Schematic view of known and predicted protein interactions for clusterin, according to the STRING database (v. 9.05). Given the absence of *Pecari tajacu* clusterin sequence, we selected the information from *Bos taurus*. Only interactions with the highest confidence score (0.900) are shown. Interactions include physical and functional associations, showing the evidence (A) and action (B) views. CLU, clusterin; SERPINF2, α -2-plasmin inhibitor; TIMP-1, tissue inhibitor of metalloproteinase 1; FN1, fibronectin precursor; PLG, plasminogen precursor; A2M, α -2-macroglobulin; SPARC, secreted protein acidic and rich in cysteine; APP, amyloid β 4 protein; VWF, Von Willebrand factor; SERPINE1, plasminogen activator inhibitor 1; TGFB3, transforming growth factor β 3.

Discussion

A combination of 2D electrophoresis, computerized analysis of gel images, and MS/MS enabled us to compile a comprehensive profile of the major seminal plasma proteins of *P. tajacu*. To our knowledge, this is the first survey of the seminal plasma proteome of adult and reproductively sound peccaries. Peccary seminal proteins are diverse and potentially affect reproduction by several mechanisms. Below, we discuss the main attributes of such proteins according to their functional groups.

Proteins associated with protection of sperm integrity and function

In the present work, specific proteins were associated with sperm protection, acting as chaperones and antioxidants. As chaperones, the peccary seminal plasma expresses clusterin and T-complex protein 1, subunit gamma, while proteins with antioxidant properties include selenium-independent GPX5, ceruloplasmin, albumin, and transferrin. In several domestic mammals, the epididymis is a major source of scavengers for reactive oxygen species in the reproductive tract and contains appreciable amounts of superoxide dismutase, catalase, and GPX (Dacheux *et al.* 2006, Moura *et al.* 2010). As shown for other species, it is likely that GPX5 protects peccary sperm cells against hydrogen peroxide and lipoperoxides, maintaining membrane functionality and preventing loss of motility (Alvarez & Storey 1989) and premature acrosome reaction (Vernet *et al.* 1999).

As it is well known, albumin is secreted in nearly all body fluids and, in semen, it protects sperm by binding to free fatty acid hydroperoxides, preventing oxidative damages (Storey 1997). Accessory sex glands and epididymides secrete albumin in several domestic mammals (Dacheux *et al.* 2006, Moura *et al.* 2007, 2010, Souza *et al.* 2012). This protein interacts with the

sperm membrane during epididymal transit and sperm-bound albumin participates in the fertilization process as well (Gonçalves *et al.* 2008). Transferrin, in turn, is an iron-binding protein found in the peccary seminal plasma and probably acts by chelating excess of free ionic iron, protecting sperm against lipid peroxidation (Wakabayashi *et al.* 1999), and preventing bacterial growth (Farnaud & Evans 2003). However, transferrin only reacts with ferric iron and ferrous iron must then be converted to the ferric state in order to bind transferrin. This conversion is catalyzed by ceruloplasmin, a copper-containing enzyme with ferroxidase activity, identified in the peccary seminal plasma (Hellman & Gitlin 2002). In addition, ceruloplasmin has antioxidant properties, acting as an effective scavenger for superoxide anions (Goldstein *et al.* 1979). Seminal plasma transferrin correlates with sperm counts and fertility in men (Foresta *et al.* 1986) and lactotransferrin is associated with sperm motility in Asian elephants (Kiso *et al.* 2013). TCP1-containing chaperonins (CCTs) are present on the sperm surface of mice and boars (Belleannée *et al.* 2011) and mediate sperm–oocyte interaction (Dun *et al.* 2011). Interestingly, the presence of CCTs 5 and 8 in bull sperm membrane was negatively correlated with fertility. A possible explanation for such relationship would be related to the failure in removal of CCTs in the final stages of spermatogenesis (D'amours *et al.* 2010). The precise role played by CCT3 chaperonin identified in the peccary seminal plasma remains unclear, but one possibility is that CCT3 interacts with other chaperones, such as clusterin, preventing protein degradation during epididymal maturation.

Clusterin was the most polymorphic (27 spots) and abundant protein group in the peccary seminal plasma gels. Even when compared with clusterin expression in the reproductive fluids of other species, we have studied using the same 2D SDS–PAGE approach, such as the bovine (Moura *et al.* 2007, 2010), ovine (Souza *et al.* 2012), and domestic swine (*S. scrofa*; V R Cadavid,

J M Martins & A A Moura 2013, unpublished results), clusterin abundance and isoform diversity in the peccary seminal plasma is much greater and, thus, unique. However, the precise functional meaning of such event is unclear. Clusterin is multifunctional, acting as a chaperone (Humphreys *et al.* 1999), protecting reproductive tract cells against apoptosis (Bailey *et al.* 2002) or complement attack (Meri & Jarva 2001), and assisting with removal of damaged sperm in the epididymis of men (Zalata *et al.* 2012). The chaperone activity of clusterin is consistent with its propensity to interact with different proteins *in vivo* (Carver *et al.* 2003) and in accordance with the network analysis we presently show. Based on such analysis, clusterin can interact with regulators of cell growth (such as SPARC), as well as with proteases and protease inhibitors, such as PLG, A2M, TIMP-1, α -2-antiplasmin precursor, and plasminogen activator inhibitor 1. Clusterin also has potential links to fibronectins, which participate in cell adhesion, healing of wounds, and maintenance of cell structure, GTP protein-coupled receptors (amyloid β A4 protein), and modulators of cell growth, such as TGFB3. Thus, pronounced expression and isoform diversity of clusterin in the peccary seminal plasma may be related to its broad functional attributes. Additionally, we report that the intensity of a clusterin spot (no. 27, Fig. 1) was negatively associated with the percentage of sperm with intact membrane. In bulls and rams, sperm with morphologic defects, in contrast with normal cells, exhibit extensive clusterin binding (Ibrahim *et al.* 2001a,b). This association probably occurs as a result of clusterin's ability to bind damaged portions of sperm membrane hydrophobic regions (Bailey & Griswold 1999). Although significant, the correlation presently shown between clusterin and a sperm parameter was based on a group of only eight animals and it needs, therefore, to be confirmed in experiments with larger samples.

H4 heavy chain (ITI4) is a member of the inter- α -trypsin inhibitor family acting rather as an acute-phase protein (Piñeiro *et al.* 2004). Some of these proteins, such as α -1-antitrypsin, have been described a component of the reproductive tract fluids of bulls (Moura *et al.* 2010), where they modulate host-defensive actions to inflammatory reactions and infections. Similar to clusterin, ITI4 inhibits complement activation and complement-associated phagocytosis and damage (Garantziotis *et al.* 2008). Thus, it is possible that these proteins work together to protect sperm against inflammatory reactions.

In humans and animals, antisperm antibodies can react with sperm surface proteins and impairs fertilization. To mitigate such effects, at least in humans, seminal plasma contains IgGBP, which bind IgGs via the Fc fragment and prevent them from reaching the sperm (Chiu & Chamley 2003). To the best of our knowledge, this is the first report of IgGBP in the seminal plasma of either domestic or wild animals. Molecular weights of

IgGBP identified in the present work are similar to those of human IgGBPs (Witkin *et al.* 1983) and likely play a similar role.

Lipophilic proteins

Lipocalin-15 protein is the boar homolog of the lipocalin-type prostaglandin-D-synthase (PGDS). These proteins belong to the lipocalin family, which consists of small secretory proteins sharing a common feature of binding and transporting lipophilic molecules (Pervaiz & Brew 1987). In bulls, PGDS binds to the acrosomal cap of cauda epididymal sperm and PGDS spot intensity in seminal plasma 2D maps is positively associated with bull fertility (Gerena *et al.* 2000). Moreover, treatment of sperm or oocytes with antibodies against bovine PGDS decreases fertilization rates and early embryo development (Gonçalves *et al.* 2008). It is therefore possible that PGDS, a typical epididymal protein, influences events related to the fertilization process. Epididymal secretory protein – E1 (HE1), another lipophilic component detected in the peccary seminal fluid, has been found in the bull (Moura *et al.* 2010), boar (Okamura *et al.* 1999), and human (Dacheux *et al.* 2006) cauda epididymal fluid, and in the seminal plasma of rams (Souza *et al.* 2012). HE1 is also involved in sperm membrane modification during epididymal maturation (Okamura *et al.* 1999).

Annexins are calcium-dependent, phospholipid-binding components and ANX5 is a component of human seminal plasma, protecting both DNA and sperm membrane integrity against oxidative damage (Lu *et al.* 2011). ANX5 is also the major protein of rabbit seminal plasma (Arruda-Alencar *et al.* 2012), where it is mainly expressed as 66–70 kDa isoforms, making nearly 38% of all spot intensities in 2D gels. In the peccary, ANX5 represents only 2% of all spot intensities, but the three ANX5 spots have kDa and pH values similar to those found in the rabbit seminal plasma gels. ANX5 function in the peccary semen is still unknown, but it is possible that it has a protective role, as shown for humans (Lu *et al.* 2011).

Proteins mediating formation of the oviduct reservoir and sperm–oocyte interaction

The PSP-1 and Bdh-2 are members of the spermadhesin family detected in the collared peccaries seminal plasma. Spermadhesins are secreted by both the porcine epididymis and accessory sex glands (Ekhlas-Hundrieser *et al.* 2002) and, in the seminal fluid, PSP-1 forms a heterodimer with PSP-2 (Calvete *et al.* 1995), which binds to the sperm acrosome. PSP-1/PSP-2 complex is the most abundant protein group of the boar seminal plasma, corresponding to at least 50% of all proteins (Calvete *et al.* 1995, V R Cadavid,

J M Martins & A A Moura 2013, unpublished results). This contrasts with the analysis of peccary seminal fluid, where clusterin is the predominant protein and spermadhesins are in much lower concentration. In the swine, ejaculates with higher percentage of PSP-1/PSP-2-positive cells have higher proportion of motile and metabolically active sperm (Caballero *et al.* 2006) and these properties are mostly due to PSP-1/PSP-2 decapacitating ability (García *et al.* 2006). PSP-1/PSP-2 also stimulates leukocyte migration to the uterus shortly after ejaculation (Assreuy *et al.* 2003), thus providing a uterine environment free of microorganisms. In the domestic swine, purified PSP-1/PSP-2 have beneficial effects on sperm viability when added to semen extenders (Centurion *et al.* 2003). Particularly in the boar, spermadhesins are involved in the formation of the oviduct reservoir and sperm–egg binding, interacting with oocyte receptors through its CUB domain (Topfer-Petersen *et al.* 2008). Therefore, it is possible that spermadhesins of the peccary seminal plasma bind to sperm and affect such events before and during fertilization as well.

Acetylcholine receptors (AChR) are present in the midpiece of mouse sperm, where they regulate motility probably due to its ability to increase calcium uptake (Bray *et al.* 2005). Additionally, AChR functions in zona pellucida-induced acrosome reaction, involving the increase in intra-sperm calcium content and phospholipase C activation (Jaldety *et al.* 2012). Such roles in sperm function are critical, since $\alpha 7$ -null mouse sperm have impaired motility (Bray *et al.* 2005). Given that CHRNA7 was found in the peccary seminal plasma as a single spot and representing only 0.2% of all spot intensities, it is possible that this protein probably derives from a sperm cell and may not represent a component truly secreted in the seminal plasma.

Glycosidases, such as galactosidase, fucosidase, and mannosidase, among others, are common components of the seminal plasma of several species, such as rams (Souza *et al.* 2012), humans (Corrales *et al.* 2002), and bulls (Kelly *et al.* 2006). Glycosidases mostly come from the epididymis, where they modify sperm surface glycoproteins during maturation (Dacheux *et al.* 2006). Additionally, some glycosidases bind to sperm membrane glycosidic residues and act as sites to either recognize the oviductal epithelium (Talevi & Gualtieri 2010) or to participate in human sperm–egg interaction through zona-pellucida glycoproteins (Miranda *et al.* 2000). Hexosaminidases bind to human sperm membrane during epididymal transit (Perez Martinez *et al.* 2008) and participate in sperm–zona pellucida interaction (Miranda *et al.* 2000). In the peccary seminal fluid, hexosaminidase is expressed as four isoforms with 102 kDa, very similar to the boar seminal hexosaminidase (Daron & Aull 1985). It is likely therefore that this protein participates in sperm–egg recognition, as shown for other species.

Tetraspanins have transmembrane domains and are involved in protein trafficking. Some tetraspanins, such as CD9 and CD81, play a role in sperm–egg fusion, probably through links with integrins (Ziyyat *et al.* 2006). Such proteins are present in human prostasomes and epididymosomes, structures that mediate the transfer of components to sperm, either during epididymal maturation or after ejaculation. This study is the first to describe tetraspanin CD63 in the seminal plasma of any species but its precise function in reproductive events is still unclear.

α -S1-casein was found in the peccary seminal plasma. As well established, caseins are major proteins of milk, but α -S1-casein has been detected in human prostate secretions (Xu *et al.* 2012) and in the membrane of ejaculated sperm, participating in bovine (Pate *et al.* 2008) and swine (Mori *et al.* 1996) sperm–egg fusion. Given that S1-casein stabilizes calcium ions in solution and enhances calcium incorporation in numerous cell types, such protein likely plays a role in the acrosome reaction (Mori *et al.* 1996).

Proteases

Carboxypeptidase E and cathepsin L1 are proteases identified in the peccary seminal plasma. The former is synthesized in the boar epididymis (Guyonnet *et al.* 2009) and possibly modifies sperm membrane proteins during epididymal maturation (Métayer *et al.* 2002). Cathepsins, in turn, are cysteine proteases and cathepsin L1 precursor (procathepsin L) identified in the present work likely comes from the epididymis, as also found in the rat (Tomomasa *et al.* 1994), bull (Moura *et al.* 2010), and boar (Okamura *et al.* 1995). Pro-enzymes are activated to mature cathepsins in epididymal fluid (Tomomasa *et al.* 1994) and believed to affect sperm maturation and its fertilizing capacity. Indeed, cauda epididymal fluid cathepsin D is positively associated with fertility of dairy bulls (Moura *et al.* 2006).

Other proteins from the seminal plasma

Actin is a putative intracellular protein, associated with the cytoskeleton, and act as sperm undergo capacitation and acrosome reaction (Brener *et al.* 2002). Nevertheless, this protein is consistently found as a component of ram seminal plasma (Souza *et al.* 2012) and cauda epididymal fluid of bulls (Moura *et al.* 2010). Actin has no confirmed function in the reproductive fluids and its presence in such secretions is more likely the result of leakage from damaged or dead sperm. A ζ -globin-like protein has been recently reported as one of the major seminal plasma proteins in rabbits (Arruda-Alencar *et al.* 2012), but there are no reports on such protein in the seminal plasma of other species.

In conclusion, this study describes the major proteome of the seminal plasma of *P. tajacu*. Gene ontology analysis confirms similarities and differences with the ram, a ruminant, and the domestic swine. Also in comparison with the *S. scrofa*, the closely related species, the peccary seminal fluid shows a distinct high expression of clusterin isoforms and less amount of spermadhesins. As for other species, seminal plasma proteins of the peccary are potentially involved in several reproductive processes, such as sperm protection, modification of sperm membrane, formation of the oviduct reservoir and sperm–egg binding. Knowledge of wild species reproductive biology is crucial for a complete understanding of their survival strategies and adaptation in a changing environment. Also, it sets the foundation for assisted reproduction techniques such as gamete cryopreservation, artificial insemination, and IVF.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-13-0220>.

Declaration of interest

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

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