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Protein profile of the ovarian follicular fluid of brown brocket deer (*Mazama gouazoubira*; Fisher, 1814)

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Short Communication

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Summary

The aim of this study was to characterize the protein profile of ovarian follicular fluid (FF) of brown brocket deer (*Mazama gouazoubira*). Five adult females received an ovarian stimulation treatment and the FF was collected by laparoscopy from small/medium (≤3.5 mm) and large (>3.5 mm) follicles. Concentrations of soluble proteins in FF samples were measured and proteins were analyzed by 1-D SDS-PAGE followed by tryptic digestion and tandem mass spectrometry. Data from protein list defined after a Mascot database search were analyzed using the STRAP software tool. For the protein concentration, no significant difference ($P > 0.05$) was observed between small/medium and large follicles: 49.2 ± 22.8 and 56.7 ± 27.4 µg/µl, respectively. Mass spectrometry analysis identified 13 major proteins, but with no significant difference ($P > 0.05$) between follicle size class. This study provides insight into elucidating folliculogenesis in brown brocket deer.

Introduction

The brown brocket deer (*Mazama gouazoubira*) is a species that adapts itself to a wide range of environments (Black-Décima *et al.*, 2010). According to the IUCN *Red List of Threatened Species* (IUCN, 2016), it is classified as 'least concern'. However, in some regions of Brazil, it is considered as 'endangered' (SEMA, 1998). *In situ* and *ex situ* conservation programmes can benefit from reproductive biotechnologies, including artificial insemination and *in vivo* embryo production. Therefore, some recent studies have focused on its reproduction and physiology (Pereira *et al.*, 2006) as well as assisted reproductive techniques (ART; Zanetti and Duarte, 2011).

The determination of follicular fluid (FF) composition may be important to ascertain the microenvironment involved in follicle development and as an indication of the nutritional needs of the oocyte. In addition, selection of competent oocytes requires the identification of molecular markers present in the FF (Fahiminiya and Gérard, 2010), Several studies on the proteome of FF have been performed in bovine (Mortarino *et al.*, 1999), canine (Fahiminiya *et al.*, 2010), equine (Fahiminiya *et al.*, 2011), swine (Sun *et al.*, 2011), bubaline (Fu *et al.*, 2016) and ovine (Wu *et al.*, 2018).

This work aimed to perform a preliminary study on the protein profile of FF of brown brocket deer and to verify possible differences between small/medium and large follicles.

Materials and methods

Experimental animals and FF collection

Five adult females received hormonal treatment by insertion of intravaginal devices impregnated with 330 mg progesterone (CIDR-G, Zoetis, Kalamazoo, MI, USA) for 8 days. On the day of CIDR insertion, females also received 0.25 mg of estradiol benzoate (Estrogin, Biofarm, Jaboticabal, Brazil) and on the fourth day of treatment 700 IU eCG (Folligon, Intervet, Boxmeer, The Netherlands). Females were fasted for 24 h, followed by anaesthesia with 5.0 mg/kg of ketamine hydrochloride (Vetbrans Saúde Animal, Jacareí, Brazil), 0.3 mg/kg of xylazine hydrochloride (Mallinckrodt Vet, Cotia, Brazil) and 0.5 mg/kg of medazolan (Cristália, Itapira, Brazil), by intravenous (i.v.) injection. Next, females were intubated and maintained on a superficial plane using isoflurane (Abbott, São Paulo, Brazil) according to Zanetti *et al.* (2010). Laparoscopy was performed using a 5-mm Hopkins laparoscope

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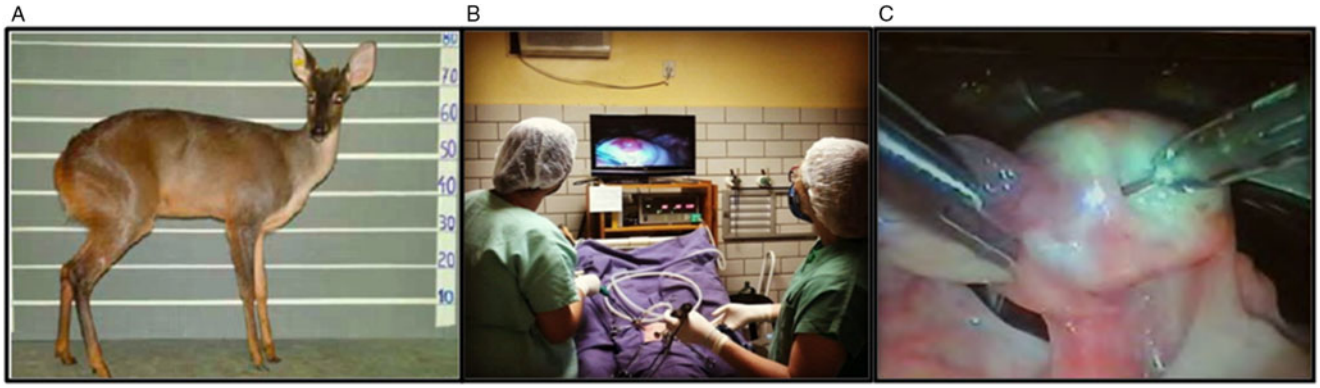

Figure 1. Adult *Mazama gouazoubira* female (*A*), videolaparoscopy (*B*) and follicular puncture for fluid collection (*C*).

(Karl Storz, Tuttlingen, Germany). Follicles were visualized, punctured and fluid was collected using a 22-G needle connected to a vacuum pump (WTA, Cravinhos, Brazil) adjusted to 35 mmHg. For fluid collection, follicles were measured and classified as small/medium (≤ 3.5 mm) or large (>3.5 mm) (Figure 1). A skilled and experienced operator scrupulously avoided contamination with blood. Collection of FF without visible signs of blood (pink or red colour) was considered a success. Samples of FF were individualized (per animal and follicle size) and mixed with a protease inhibitor cocktail (10 µl/ml) (Sigma-Aldrich, St. Louis, MO, USA), according to Aquino-Cortez *et al.* (2017). The samples was centrifuged at 3000 *g* at 4°C for 30 min and the supernatant was stored at −80°C until analysis.

One-dimensional gel electrophoresis SDS-PAGE

The samples from one female were discarded due contamination with blood. Soluble protein content in FF samples was determined according to the Bradford method (Bradford, 1976). A volume of sample containing 15 µg of protein was mixed with sample buffer (0.125 M Tris–HCl, pH 6.8, 4% SDS, 20% (v/v) glycerol, 0.2 M DTT, 0.02% bromophenol blue), boiled for 90 s, and loaded into the wells of a stacking gel (4% acrylamide), laid on top of a 10% polyacrylamide resolving gel (GE Life Sciences, Piscataway, NJ, USA). In one well of the stacking gel, 10 µl of a 180–19 kDa molecular weight standard mix (BenchMark Prestained Protein Ladder, Invitrogen, CA, USA) was loaded to allow molecular weight estimation of the protein bands. An initial current at 150 V, 50 mA and 90 W was applied for approximately 1 h 45 min to enable proteins to migrate through the stacking gel. Gels were stained with colloidal Coomassie Blue G-250, rinsed with deionized water, and scanned using an Image Scanner II (GE Life Sciences). The gel images were analyzed using Quantity One 4.5 software (Bio-Rad, Hercules, CA, USA). After analysis of the digitalized gel images and identification of the bands of interest, bands were cut from the gels, sliced and subjected to in-gel digestion with trypsin (Promega, Madison, WI, USA). Briefly, bands were excised from the gels and transferred to clean tubes, and washed three times (15 min each wash) with 400 µl of a solution containing 50% acetonitrile and 50% ammonium bicarbonate (25 mM at pH 8.0). Then, the gel pieces were washed twice with 200 µl pure acetonitrile for 5 min and air dried at room temperature (20–25 °C). Then, the gel pieces were incubated with trypsin (166 ng per band; Promega) for 20 h at 37 °C. After incubation, peptides were extracted by three washes with 5% trifluoroacetic acid, and 50% acetonitrile in 50 mM ammonium bicarbonate. Supernatants containing the peptides were concentrated in the microtubes using a speed vacuum concentrator (Eppendorf, Hauppauge, NY, USA).

Mass spectrometry analysis and protein identification

Digested peptides were analyzed using an electrospray ionization-quadrupole-time of flight (ESI-Q-TOF) mass spectrometer (Thermo Scientific, Bremen, Germany). The peptides were injected the nano Acquity ultrapure liquid chromatography (UPLC) sample manager and chromatographic separation was performed using an EasySpray column (C18, 2 mm beads, 75 mm inner diameter) (Thermo Scientific) with a capillary of 25 cm bed length and flow rate of 0.3 ml/min. Mobile phases A and B consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. All solvents were mass spectrometry grade quality (Sigma-Aldrich). The flow rate was 0.3 ml/min, and the solvent gradient was 2% B to 30% B in 60 min, followed by 90% B for 20 min. Data were processed using a Protein Lynx Global Server (Waters Co., Milford, MA, USA) and were converted to peak list text files for database searching. Peptides were identified using the Mascot database (Matrix Science, Boston, MA, USA) by searching against the NCBI database. Searches were made according to the following criteria: maximum of one missed trypsin cleavage, mono-isotopic peptides with charge +1, +2 and +3, with variable modification of protein *N*-acetylation and oxidized methionine (M) residues and fixed variation of carbamidomethyl (C) residues using a tandem mass spectrometry (MS/MS) ion searching mode. Peptide mass tolerance and fragment mass tolerance were ±1.2 Da and ± 0.6 Da, respectively.

Statistical analysis

All data were compared by unpaired *t*-test. Data were expressed as mean ± standard deviation (SD) and a P-value < 0.05 was considered to be significant.

Results

From five females, 55 follicles were visualized and 44 (21 small/medium and 23 large) were punctured. The mean (± SD) number of small/medium and large follicles was 6.0 ± 4.4 and 5.0 ± 2.4, respectively ($P > 0.05$). Although care was taken to avoid blood contamination, for further analysis, samples from one female were discarded due to this condition.

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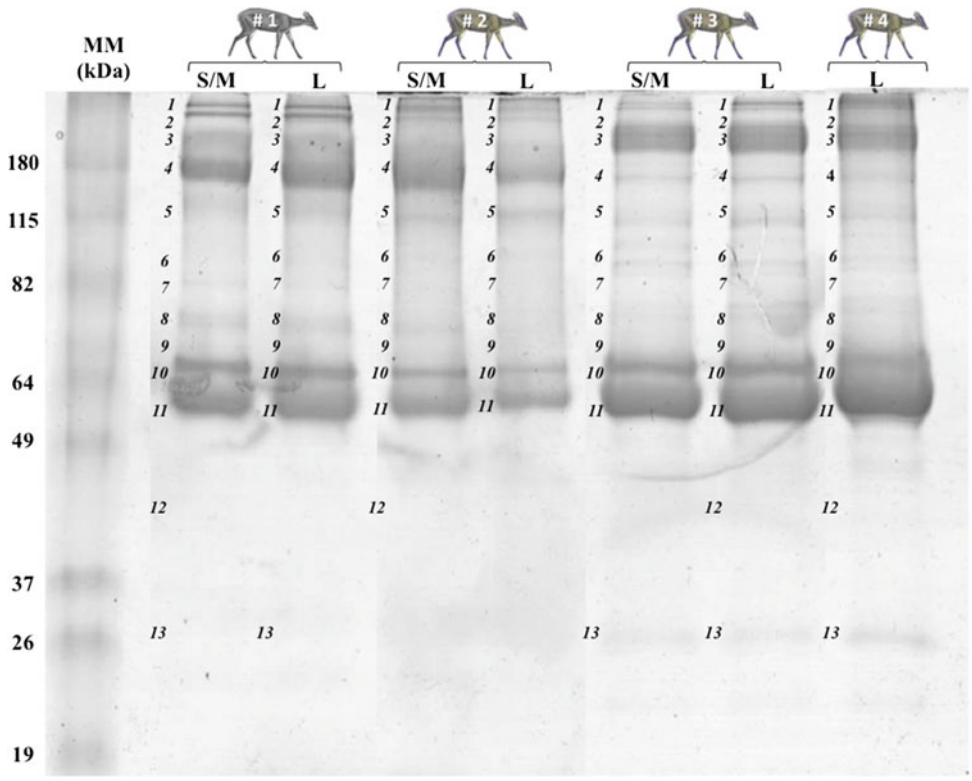

Figure 2. SDS-PAGE analysis of follicular fluid of *Mazama gouazoubira* females (♯1–4). S/M (small/medium) and L (large) follicles. Numbers in italic shows the different bands identified on the gel. MM: molecular mass (180–186 kDa).

On average, 71.5 µl of FF was obtained, distributed in small/medium (25.0 ± 15.0 µl) and large follicles (113.8 ± 98.1 µl). Protein concentration was similar ($P > 0.05$) in the two follicle categories: 49.2 ± 22.8 µg/µl (small/medium) and 56.7 ± 27.4 µg/µl (large).

Female #4, there did not have sufficient volume for small/medium follicles analysis. Therefore, for this female, visualization of the bands is not present in the gel (Figure 2). There were no significant differences in band intensities between the two follicular categories. The analysis of 1-D gels allowed the detection of 17 bands common to small/medium and large follicles. The best-defined bands ($n = 13$) were identified by MS/MS (Figure 2). Table S1 shows a detailed list of proteins with their accession numbers, scores, sequence coverage and molecular mass.

Discussion

The study of FF components may contribute to an understanding of reproductive physiology of deer and for further use in ART. To our knowledge, there has been no previous information on the protein profile of FF in any species of deer. Therefore, this is the first study using SDS-PAGE combined with LC-MS/MS.

Follicular puncture by laparoscopy was efficient for FF collection. According to Ginther *et al.* (1997), an unsuccessful aspiration was the result of blood contamination of the FF. However, visual inspection provided a fast and relatively reliable method for determination of blood-contaminated FF (Levay *et al.*, 1997).

Proteins derived from blood serum (albumin, serotransferrin, inter-alpha-trypsin, haptoglobin, apolipoprotein and ceruloplasmin) were found in *M. gouazoubira* and had previously been described in FF of other species, such as bubaline (Fu *et al.*, 2016), bovine (Ferrazza *et al.*, 2017), caprine (Paula Junior *et al.*, 2018) and ovine (Wu *et al.*, 2018). During the final stage of folliculogenesis, the composition of FF is influenced by the blood–follicle barrier, which allows passage of low-molecular-weight proteins (Fahiminiya and Gérard, 2010).

Other detected proteins were plasma proteins from the acute phase of inflammation, such as complement factor (complement C3). In goats, this protein significantly increased as follicles developed from medium to large size (Paula Junior *et al.*, 2018), explaining the inflammation process promoting follicle wall breakdown during ovulation (Fu *et al.*, 2016).

Alpha-2-macroglobulin and haptoglobin, which are expressed during the physiological response to inflammation (Handrieder *et al.*, 2008) were also observed in the FF of bubaline (Fu *et al.*, 2016) and bovine (Ferrazza *et al.*, 2017). Proteins with enzyme inhibitory function (serpin A3 and inter-alpha trypsin) were detected in this study as observed in buffalo females (Fu *et al.*, 2016).

Interestingly, the FF of *M. gouazoubira* contained thyroxine-binding globulin and lactotransferrin, respectively, the principal carrier of thyroid hormone in serum (Murata *et al.*, 1986) and a multifunctional protein occurring in many biological secretions (Sánchez *et al.*, 1992). However, these two proteins have not been described in the FF of farm animals.

This study was not able to detect any significant difference in protein content in the FF as follicles developed from small/medium to large size. We hypothesized that this result may be due to: (i) joining of small and medium follicles into one group; or (ii) if such differences occurred, it would probably be at a time very close to ovulation.

Finally, the current study may contribute to a further understanding of the physiological mechanisms underlying folliculogenesis. It would be interesting to use quantitative techniques such as shotgun proteomics to obtain more detailed information on the FF of brown brocket deer.

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Conflicts of interest. The authors hereby declare that there are no conflicting interests.

Ethics statement. This study was approved by the Ethical Committee for the Animal Use of the State University of Ceará (2437412/2016) and the Biodiversity Information and Authorization System (45727-1).

References

Aquino-Cortez A, Pinheiro BQ, Lima DBC, Silva HVR, Mota-Filho AC, Martins JAM, Rodriguez-Villamil P, Moura AA and Silva LDM (2017) Proteomic characterization of canine seminal plasma. *Theriogenology* **95**, 178–86.

Black-Décima P, Rossi RV, Vogliotti A, Cartes JL, Maffei L, Duarte JMB and Juliá JP (2010) Brown brocket deer *Mazama gouazoubira* (Fischer 1814). In Duarte JMD and González S (eds) *Neotropical Cervidology*. Jaboticabal: FUNEP, pp 119–32.

Bradford MM (1976) A rapid and sensitive method for the quantization of microgram quantities of protein utilizing the principle of dye binding. *Anal Biochem* **72**, 248–54.

Fahiminiya S and Gérard N (2010) Follicular fluid in mammals. *Gynecol Obstet Fertil* **38**, 402–4.

Fahiminiya S, Reynaud K, Labas V, Batard S, Chastant-Maillard S and Gérard N (2010) Steroid hormones content and proteomic analysis of canine follicular fluid during the preovulatory period. *Reprod Biol Endocrinol* **8**, 132–46.

Fahiminiya S, Labas V, Roche S, Dacheux JL and Gérard N (2011) Proteomic analysis of mare follicular fluid during late follicle development. *Proteome Sci* **9**, 54–73.

Ferrazza RA, Garcia HDM, Schmidt EMDS, Mihm Carmichael M, Souza FF, Burchmore R, Sartori R, Eckersall PD and Ferreira JCP (2017) Quantitative proteomic profiling of bovine follicular fluid during follicle development. *Biol Reprod* **97**, 835–49.

Fu Q, Huang Y, Wang Z, Chen F, Huang D, Lu Y, Liang X and Zhang M (2016) Proteome profile and quantitative proteomic analysis of buffalo (*Bubalus bubalis*) follicular fluid during follicle development. *Int J Mol Sci* **17**, 618–38.

Ginther OJ, Kot K, Kulick LJ and Wiltbank MC (1997) Sampling follicular fluid without altering follicular status in cattle: oestradiol concentrations early in a follicular wave. *J Reprod Fert* **109**, 181–6.

Handrieder J, Nyakas A, Naessén T and Bergquist J (2008) Proteomic analysis of human follicular fluid using an alternative bottom-up approach. *J Proteome Res* **7**, 443–9.

IUCN *Red List of Threatened Species* (2016) Available at http://wwwiucnredlistorg. Accessed 4 January 2019.

Levay PF, Huyser C, Fourie FL and Rossouw DJ (1997) The detection of blood contamination in human follicular fluid. *J Assist Reprod Genet* **14**, 212–7.

Mortarino M, Vigo D, Maffeo G and Ronchi S (1999) Two-dimensional polyacrylamide gel electrophoresis map of bovine ovarian fluid proteins. *Electrophoresis* **20**, 866–9.

Murata Y, Magner JA and Refetoff S (1986) The role of glycosylation in the molecular conformation and secretion of thyroxine-binding globulin. *Endocrinology* **118**, 1614–21.

Paula Junior AR, Van Tilburg MF, Lobo MDP, Monteiro-Moreira AC, Moreira RA, Melo CH, Souza-Fabjan JM, Araújo AA, Melo LM, Teixeira DIA, Moura AA and Freitas VJF (2018) Proteomic analysis of follicular fluid from tropically-adapted goats. *Anim Reprod Sci* **188**, 35–44.

Pereira RJ, Polegato BF, Souza S, Negrão JA and Duarte JM (2006) Monitoring ovarian cycles and pregnancy in brown brocket deer (*Mazama gouazoubira*) by measurement of fecal progesterone metabolites. *Theriogenology* **65**, 387–99.

Sánchez L, Calvo M and Brock JH (1992) Biological role of lactoferrin. *Arch Dis Child* **67**, 657–61.

SEMA Portaria SEMA nº 001 de 9 de junho de 1998; Rio de Janeiro: 1998 Lista das Espécies Ameaçadas de Extinção no Estado do Rio de Janeiro.

Sun YL, Ping ZG, Li CJ, Sun YF, Yi KL, Chen L, Li XY, Wang XL and Zhou X (2011) Comparative proteomic analysis of follicular fluids from normal and cystic follicles in sows. *Reprod Domest Anim* **46**, 889–95.

Wu Y, Lin J, Han B, Wang L, Chen Y, Liu M and Huanget J (2018) Proteomic profiling of follicle fluids after superstimulation in one-month-old lambs. *Reprod Domest Anim* **53**, 186–94.

Zanetti ES, Polegato BF and Duarte JM (2010) Comparison of two methods of synchronization of estrus in brown brocket deer (*Mazama gouazoubira*). *Anim Reprod Sci* **117**, 266–74.

Zanetti ES and Duarte JM (2011) Comparison of three protocols for superovulation of brown brocket deer (*Mazama gouazoubira*). *Zoo Biol* **31**, 642–55.