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ORIGINAL ARTICLE

Llama oviductal sperm reservoirs: involvement of bulbourethral glands

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Summary

The aim of this study was to elucidate the role of llama seminal plasma in the formation of oviductal sperm reservoirs. Female llamas with follicles in the mature phase were mated with a bulbourethral glands-removed male. Females mated with nonbulbourethral glands-removed males were used as control. Oviducts were obtained by surgery 24 h after mating. The uterotubal junction and isthmus were examined by scanning electron microscopy, and mucopolysaccharides were identified by Alcian blue staining. To know the proteins probably involved in sperm reservoir formation, SDS-PAGE of seminal plasma (8% and 18% resolving gel) was made. Spermatozoa only adhered to the oviductal mucosa surface of uterotubal junction of females mated with nonbulbourethral glands-removed males confirming that seminal plasma and, in particular, bulbourethral secretions are related with the oviductal sperm reservoir formation. Histological sections showed sperm in the lumen, immersed in substance, positive for acid mucopolysaccharides. Alcian blue staining of seminal plasma proteins SDS-PAGE showed a band of high molecular weight containing mucopolysaccharides, only present in nonbulbourethral glands-removed males. Bulbourethral glands would secrete at least eight different proteins that most likely participate in the process of sperm storage in the oviduct.

Introduction

The behavioural characteristics and reproductive ~~physiology~~ of South American Camelids (SACs) differ from other domestic livestock (Vaughan, 2011). Females exhibit waves of ovarian follicular growth and are induced ovulators, and therefore, they do not exhibit oestrous cycles in the manner of spontaneously ovulating species such as sheep and cattle. The interval between mating and ovulation is approximately 26 h in the alpaca (San-Martin *et al.*, 1968) and 30 h in the llama (Ratto *et al.*, 2006). Such a long time between copulation and ovulation implies that the preservation of viability of sperm, waiting for the ovum in the reproductive tract, is a critical reproductive factor in this species. In previous studies, it was demonstrated that the uterotubal junction (UTJ) acts as a sperm reservoir in llamas (Apichela *et al.*, 2010). We also confirmed that the UTJ acts as an anatomical barrier facilitating sperm storage and that UTJ epithelial cells show greater ability to bind sperm than isthmus cells (Apichela *et al.*, 2010). On the other hand, observations of the sperm reservoir using scanning electron microscopy showed sperm adhered to the UTJ mucosa and sperm covered by a mucus-like secretion, whose source has not been elucidated yet. This could be an additional mechanism of entrapment of sperm in the reservoir, which is consistent with speculations about the role of semen as a sperm reservoir in the genital tract of female camelids (Bravo *et al.*, 1997; Brown, 2000). SAC semen is highly viscous (Casaretto *et al.*, 2012), and although the physiological function of this viscosity has not been ~~studied~~ thoroughly (Tibary & Vaughan, 2006), this particular characteristic has been attributed to the bulbourethral glands (Gonzales *et al.*, 2003). The aim of this study was to elucidate the role of llama seminal plasma in the formation of a sperm reservoir. Whether sperm from males

with surgically removed bulbourethral glands is able to form a sperm reservoir in the caudal oviduct was ~~examined~~ using scanning electron microscopy. In addition, this study enhances the knowledge of the seminal proteins involved in this process.

Materials and methods

Animals

Fertile male and female llamas between 5 and 10 years old were used in this study. The animals were kept at the School of Veterinary Sciences, University of Buenos Aires (Buenos Aires, Argentina). Llamas were kept in pens, which provided shade, and animals fed on pasture, supplemented with bales of alfalfa. They had free access to fresh water.

Surgery

Animals were deprived of solid food and water, 24 h and 18 h before surgery respectively. General anaesthesia was induced by IV administration of 0.2 mg kg^{-1} xylazine (Rompun®; Bayer, Buenos Aires, Argentina), 1.5 mg kg^{-1} ketamine hydrochloride (Ketamina®; Holliday, Buenos Aires, Argentina) and 0.1 mg kg^{-1} butorphanol (Torbutrol plus®; Fort Dodge, La Plata, Argentina). Local anaesthesia of the surgical area was carried out using 2% lidocaine (Equi Systems®, Buenos Aires, Argentina). ~~General~~ anaesthesia was administered IV injecting doses of ketamine hydrochloride and xylazine, according to individual response. The bulbourethrectomy procedure involved a 5–7 cm incision under the anal sphincter muscle, until the dorsal side of the pelvic urethra and the bulbourethral glands became visible. Bulbourethral glands were removed by separating the bulbourethral muscle. Dissection was made by an incision at the base of the gland at the excretion conduct level.

Collection of oviducts by surgery

Ovaries and oviducts were carefully exposed with manual transrectal aid through an 8 to 10 cm long surgical incision in the left flank. Oviducts were removed by slicing up the caudal portion of the uterine horns.

Experiment 1: Protocol to study involvement of seminal plasma in the formation of oviductal sperm reservoirs

Breeding

Ovarian follicular dynamics were monitored by ultrasonography using a Berger LC 2010 plus with a 5 MHz linear array electronic transducer (Buenos Aires, Argentina). Once the follicles reached a diameter ≥ 7 mm (pre-ovulatory sise), mating was allowed. Two fertile females were mated once with a bulbourethral glands-removed (BR) male. As control group, two females were mated with two different non-BR males. Males were allowed to copulate for 20–30 m. In all the cases, an operator controlled the effectiveness of the copula. Oviducts were collected 24 h after mating. Isthmus and UTJ were separated and fixed in 10% buffered formaldehyde, or longitudinally opened and fixed in Karnovsky solution for scanning electron microscopy studies.

Scanning Electron Microscopy of the oviductal mucosa

Isthmus and UTJ samples of both experimental groups were postfixed overnight in 1% osmium tetroxide and subsequently treated with an aqueous solution of 2% uranyl acetate for 40 min. Then, samples were serially dehydrated in ethanol, passed through acetone, critical point dried, mounted on aluminium stubs, coated with gold and finally examined under a Jeol CF 35 scanning electron microscope (Apichela *et al.*, 2009).

Histochemical evaluation

Fixed isthmuses and UTJs of both experimental groups were embedded in paraplast. Seven μm sections were stained with 1% Alcian blue 8Gx, pH 2.5 (Biopack, Buenos Aires, Argentina) (Apichela *et al.*, 2006). Counterstaining was carried out with Nuclear Fast Red Solution (Sigma-Aldrich, Sigma, Saint Louis, MO, USA). Slides were examined under an Olympus BX40 microscope (Japan).

Experiment 2: Preliminary studies of seminal proteins involved in sperm reservoir formation. Seminal plasma protein profile by 1D electrophoresis

Sperm characteristics were evaluated as follows: Sperm motility was examined under a phase contrast microscope (100×) with a warmed stage (37 °C); the percentage of live spermatozoa was analysed using a supravital stain with the following fluorochromes: 6-carboxyfluorescein diacetate and propidium iodide (Giuliano *et al.*, 2008). Sperm concentration was measured using a hemocytometer. Semen thread formation was evaluated with a Pasteur pipette.

Seminal plasma

Ejaculates of the BR male and non-BR males were obtained by electroejaculation (Director *et al.*, 2007). Semen was centrifuged at 8000 ***g*** for 10 min.

Supernatants were separated and kept at −20 °C until assayed, and absence of sperm was controlled microscopically. Total proteins were determined using a Micro BCA protein assay kit (Thermo Fisher Scientific, USA).

1D electrophoresis

Three BR seminal plasma samples, each containing 100 μg of protein, were pooled; the same procedure was carried out with three non-BR semen samples. Electrophoresis was carried out according to Gevaert & Vandekerckhove (2000) as follows: Thirty μg protein of each pool was prepared by dilution (v/v) with a sample buffer (0.1 M Tris–HCl, pH 6.8, 2% sodium dodecyl sulphate (SDS), 1% 2-β–Mercaptoethanol, 30% glycerol and 0.05% bromophenol blue) and loaded onto a 4% stacking polyacrylamide gel, which was overlaid on top of an 8% or 18% resolving gel to resolve proteins larger and smaller than 50 kDa respectively. Another 18% resolving gel with 60 μg of protein of each pool was run for glycoproteins. Five μl of a PageRuler Unstained Broad Range Protein Ladder (Thermo Fisher Scientific) with 250, 150, 100, 70, 50, 40, 30, 20, 15, 10 and 5 kDa sise markers was loaded in a separate well. Gels were run at room temperature at 150V until completion, and then fixed in a 30% methanol −10% acetic acid solution. Gels were stained with a Promega s Silver Sequence kit according to the manufacturer's indications (Promega, Madison, WI, USA). For Alcian blue staining, gels were fixed overnight with 12.5% trichloroacetic acid, rapidly washed twice with water, treated with 1% periodic acid in 3% acetic acid for 2 h, again thoroughly washed and finally incubated with sodium metabisulphite in 3% acetic acid for 1 h. Then gels were stained overnight with 0.5% Alcian blue (pH 2.5) in 3% acetic acid and washed with 3% acetic acid until no background was detectable. Gels were photographed with a digital Olympus C-5060 camera (Japan), and the molecular weight of the bands was calculated using GelAnalyzer 2010a freeware software.

Results

Bulbourethrectomy

After surgery, the BR male responded favourably to semen collection. Ejaculates did not form a thread when they were pipetted, unlike seminal plasma of non-BR males. The average BR and non-BR semen characteristics such as motility and percentage of morphologically normal sperm are within the ranges reported by different authors (Lichtenwalner *et al.*, 1996; Aller *et al.*, 2003; Director *et al.*, 2007) (Table 1).

Participation of seminal plasma in oviductal sperm reservoir formation

Sperm – oviduct interaction was observed by scanning electron microscopy in oviducts collected 24 h after mating. None of the cases showed any signs of ovulation in the ovaries. Oviducts of females mated with non-BR males clearly showed the presence of clusters of sperm attached to the UTJ mucosa and sperm ~~covered~~ by a sheet-like substance, distributed in patches (Fig. 1). However, in the isthmus, no spermatozoa or presence of the adherent sheet-like substance was observed. In contrast, neither sperm nor the adherent substance was observed in both the UTJ mucosa (Fig. 1) and isthmus of females mated with the BR male. In addition, cross-sections of histochemical assays showed sperm in the oviducts of females mated with non-BR males, which was located in the UTJ lumen and entrapped by an Alcian blue stained substance, indicating the presence of acid mucopolysaccharides (Fig. 2). Similar staining was observed in the glycocalix of UTJ epithelial cells.

Seminal plasma proteins

Gel electrophoresis of seminal plasma from the BR male resulted in a protein band pattern that was markedly different from that of non-BR males (Fig. 3). Attention was paid to bands that were present in the seminal plasma protein profile of non-BR males, and not or with markedly less intensity in the BR male. A pattern of 18 protein bands was observed in seminal plasma from both the BR male and non-BR males (>300, 85, 80, 70, 60, 54, 53, 46, 38, 34, 26, 23, 17, 16, 15, 14, 12 and 11 kDa), whereas eight additional bands were only present in non-BR males (254, 231, 203, 169, 148, 130, 118 and 49 kDa). Only the clearly visible high molecular weight band (>300 kDa) from non-BR males was positive for mucopolysaccharide acid staining (Fig. 2).

Table 1 ~~Xxxxxxxxxxxx~~

Males	Volume (ml)	% Motility	Concentration 10^6 sp	% Live sperm	*n*
BR male	1.73 ± 0.78	26.4 ± 21.33	107.97 ± 171.82	35.05 ± 12.45	13
Non-BR male 1	5.35 ± 2.34	25.31 ± 23.34	52.42 ± 46.80	63.25 ± 10.01	11
Non-BR male 2	2.32 ± 1.18	22.06 ± 25.76	67.68 ± 71.75	52.25 ± 19.60	15

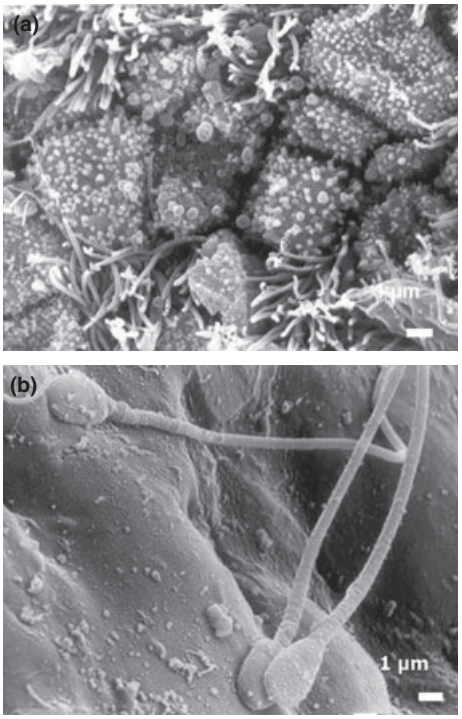

Fig. 1 Scanning electron micrograph of the uterotubal junction mucosal surface from mated females. (a) 24 h after mating with the bulbourethral gland removed male. (b) 24 h after mating with a non bulbourethral gland removed male (normal male). Bar: 1 µm.

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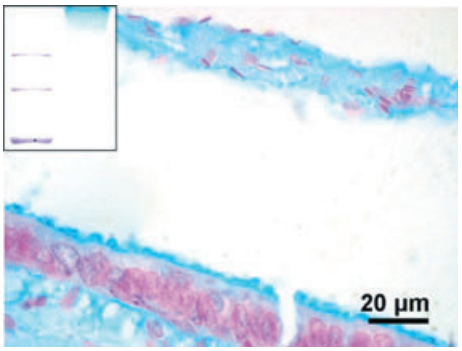

Fig. 2 Alcian blue staining of uterotubal junction epithelium cross-section 24 h postmating. The female was mated with a non bulbourethral glands removed male male. Insert: Alcian blue staining of an 18% SDS-PAGE gel. A band of high molecular weight that stains for mucopolysaccharides can be observed. Bar: 20 µm.

Discussion

This is the first study that evidences the role of seminal plasma in the formation of an oviductal sperm reservoir. In previous studies, we already mentioned the oviductal UTJ as the site of sperm storage in llamas (Apichela *et al.*, 2009, 2010). We also observed a substance that

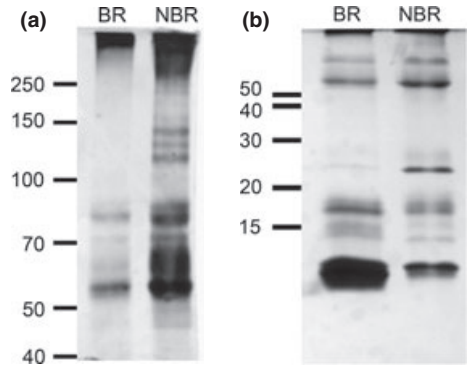

Fig. 3 SDS-PAGE of llama seminal plasma. (a) 8% SDS-PAGE. (b) 18% SDS-PAGE. NBR, normal or nonbulbourethral glands removed males; BR, bulbourethral glands removed male.

participates in the sperm adhesion to oviductal epithelial cells, but only in mated female UTJs (not in unpaired female controls) (Apichela *et al.*, 2009). Taking into account that llama seminal plasma is particularly mucus, we proposed a seminal origin for the adherent material observed. It is well known that camelid males ejaculate highly mucus semen in which sperm is trapped (Lichtenwalner *et al.*, 1996; Bravo *et al.*, 1997; Casaretto *et al.*, 2012). This characteristic is generally attributed to the presence of glycosaminoglycans produced by secretion of the bulbourethral gland (Kershaw-Young *et al.*, 2012), which can be surgically removed without affecting semen quality (Gonzales *et al.*, 2003). Our results coincide with findings by these authors, because only thread formation diminished after bulbourethrectomy, without affecting other seminal parameters. When females were mated with the BR male, no sperm or adherent substance was found in the oviductal mucosa 24 h postmating. Under normal conditions, sperm maintains adhered to the llama oviduct UTJ at least 28 h after mating (Apichela *et al.*, 2009). Our observations could indicate a diminished capacity of sperm to stay in the sperm reservoirs due to the lack of the sticky substance provided by the bulbourethral glands, and consequently, llama seminal plasma would participate in the sperm reservoir formation. Our study is in agreement with previous suggestions made in camels. Deen *et al.* (2005) proposed that semen could act as a sperm reservoir in the genital tract of female camels because of its particular consistency and that it would have certain protective action on sperm viability. This shows the importance of the seminal factor, which can be modified when semen is diluted with extenders, resulting in a diminished adhesion of sperm to the oviductal mucosa and thus a shorter life-span in the female tract. Perhaps, this explains why artificial insemination with diluted semen is more efficient when insemination is performed

near ovulation (Giuliano *et al.*, 2012). These findings would reinforce the importance to study the role of seminal plasma in the regulation of sperm functioning and its use in assisted reproductive technology.

Many proteins from seminal plasma have been related to either fertility, like in bull (Killian *et al.*, 1993), boar (Flowers, 1998), stallion (Brandon *et al.*, 1999) and buffalo (Harshan *et al.*, 2009), freezability, as in bulls (Asadpour *et al.*, 2007) or viability, like in bulls and rams (Barrios *et al.*, 2000). Addition of plasma proteins to semen probably improves the function of semen extenders and consequently fertility. There is very little information available about the seminal plasma protein profile or its functions in camelids. The most notable feature in the anatomy of the internal genitalia of camelids is the absence of vesicular glands (Tibary & Anouassi, 1997); they only possess a prostate and two bulbourethral glands. In most mammalians, secretion by the vesicular glands accounts for all the major seminal plasma proteins (Bergeron *et al.*, 2005). Moreover, bovine seminal plasma proteins (BSP), secreted by vesicular glands, mediate sperm recognition and binding to the oviductal epithelium to form a reservoir (Gwathmey *et al.*, 2006). During ejaculation, spermatozoa mix with BSPs. These proteins remove some cholesterol from the plasmatic membrane and subsequently bind to choline phospholipids. This replacement process does not allow free movement of phospholipids, and consequently stabilises the plasmatic membrane (Villemure *et al.*, 2003). The current study has demonstrated that the bulbourethral glands secrete molecules that facilitate sperm adhesion to the oviduct in camelids and that several proteins are involved. At least eight protein bands have been found that would be secreted by these glands. Some of them seem related to the ability of sperm to form an oviductal reservoir, and hence, they would prolong spermatozoa life in the female genital tract. This differs llamas from other species, such as boar, in which bulbourethral secretions do not contain proteins (they were not detected electrophoretically or by gel filtration) (Schellpfeffer & Hunter, 1970). Our findings also suggest that the high molecular weight band (>300 kDa) in seminal plasma from non-BR males could be a glycosaminoglycan (GAG), because of its staining with Alcian blue at pH 2.5. The predominant GAG in alpaca seminal plasma is keratan sulphate (KS) (Kershaw-Young *et al.*, 2012), and its concentration positively correlates with semen thread formation. KS exists as a proteoglycan attached to a core protein and interacts with other molecules forming cross-links within the extracellular matrix. It is probable that this molecule binds to the oviductal extracellular matrix, thus facilitating semen adhesion to the UTJ. Recently, by means of mass spectrometry and ITRAQ analysis, four seminal plasma proteins have been identified in alpaca (Kershaw-Young & Maxwell, 2012). One of them, Mucin 5B, is a gel-forming protein produced by the bulbourethral glands and has been related to semen viscosity. Mucin 5B could be responsible for the laminar substance that entraps spermatozoa in the sperm reservoir following mating. This protein probably degrades by some mechanism of sperm release trigged by the oviduct, although allowing spermatozoa ascend to the site of fertilisation. These results indicate that the camelid bulbourethral glands seem to produce molecules with particular functions and, even more important, that processes assumed in other mammalian cannot be extrapolated to camelids.

In conclusion, our results indicate that seminal plasma, and in particular secretions by the bulbourethral glands, participates in the process of sperm storage in the oviduct and that it is an important factor to be considered in the development of protocols for biotechnology.

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References

Aller JF, Rebuffi GE, Cancino AK, Alberio RH (2003) Influencia de la criopreservación sobre la motilidad, viabilidad y fertilidad de espermatozoides de llama (Lama glama). *Arch Zootec* 52:15–23.

Apichela S, Jimenez-Diaz M, Shuster S, Sinowatz F, Miceli DC (2006) Vicuna oviduct mucosa: ultrastructure and lectin affinities. *Small Rumin Res* 66:164–168.

Apichela S, Jiménez-Díaz MA, Roldan-Olarte E, Valz-Gianinet J, Miceli DC (2009) *In vivo* and *In vitro* sperm interaction with oviductal epithelial cells of llama. *Reprod Domest Anim* 44:943–951.

Apichela S, Valz-Gianinet JN, Schuster S, Jiménez-Díaz MA, Roldán-Olarte M, Miceli DC (2010) Lectin binding patterns and carbohydrate mediation of sperm binding to llama oviductal cells *in vitro*. *Anim Reprod Sci* 118: 344–353.

Asadpour R, Alavi-Shoushtari S, Rezaii S, Ansari M (2007) SDS-polyacrylamide gel electrophoresis of buffalo bulls seminal plasma proteins and their relation with semen freezability. *Anim Reprod Sci* 102:308–313.

Barrios B, Pérez-Pé R, Gallego M, Tato A, Osada J, Muiño-Blanco T (2000) Seminal plasma proteins revert the cold-shock damage on ram sperm membrane. *Biol Reprod* 63:1531–1537.

Bergeron A, Villemure M, Lazure C, Manjunath P (2005) Isolation and characterization of the major proteins of ram seminal plasma. *Mol Reprod Dev* 71:461–470.

Brandon CI, Heusner GL, Caudle AB, Fayrer-Hosken RA (1999) Two-dimensional polyacrylamide gel electrophoresis of equine seminal plasma proteins and their correlation with fertility. *Theriogenology* 52:863–873.

Bravo PW, Flores U, Garnica J, Ordoñez C (1997) Collection of semen and artificial insemination of alpacas. *Theriogenology* 47:619–626.

Brown BW (2000) A review on reproduction in south american camelids. *Anim Reprod Sci* 58:169–195.

Casaretto C, Martínez Sarrasague M, Giuliano S, Rubin de Celis E, Gambarotta M, Carretero I, Miragaya M (2012) Evaluation of Lama glama semen viscosity with a cone-plate rotational viscometer. *Andrologia* 44:334–341.

Deen A, Vyas S, Sahani MS (2005) Problems of artificial insemination in dromedarius camel - failure of ovulation and entrapment of spermatozoa in gelatinous camel semen. *Veterinarski Arhiv* 75:293–301.

Director A, Giuliano S, Trasorras V, Carretero MI, Pinto M, Miragaya M (2007) Electroejaculation in llama (Lama glama). *J Camel Pract Res* 14:203–206.

Flowers WL (1998). Boar fertility and artificial insemination. In: Abstracts of the 15th International Pig Veterinary Society Congress, 1998, Birmingham, England. Birmingham: IPVS, pp 45–52.

Gevaert K, Vandekerckhove J (2000) Protein identification methods in proteomics. *Electrophoresis* 21:1145–1154.

Giuliano S, Director A, Gambarrota M, Trasorras V, Miragaya M (2008) Collection method, season and individual variation on seminal characteristics in the llama (Lama glama). *Anim Reprod Sci* 104:359–369.

Giuliano SM, Chaves MG, Trasorras VL, Gambarotta M, Neild D, Director A, Pinto M, Miragaya MH (2012) Development of an artificial insemination protocol in llamas using cooled semen. *Anim Reprod Sci* 131:204–210.

Gonzales V, Copa S, Ochoa R (2003). Efecto de la bulbouretrectomía y periodicidad de colección den las características macro y microscópicas del eyaculado en llamas de tres edades. Proceedings of III Congreso Mundial de Camélidos TII, pp 743–746.

Harshan HM, Sankar S, Singh LP, Singh MK, Sudharani S, Ansari MR, Singh SK, Majumdar AC, Joshi P (2009) Identification of PDC-109-like protein(s) in buffalo seminal plasma. *Anim Reprod Sci* 115:306–311.

Kershaw-Young CM, Maxwell WM (2012) Seminal plasma components in camelids and comparisons with other species. *Reprod Domest Anim* 47:369–375.

Kershaw-Young CM, Evans G, Maxwell WM (2012) Glycosaminoglycans in the accessory sex glands, testes and seminal plasma of alpaca and ram. *Reprod Fertil Dev* 24: 362–369.

Killian GJ, Chapman DA, Rogowski LA (1993) Fertility-associated proteins in Holstein bull seminalplasma. *Biol Reprod* 49:1202–1207.

Lichtenwalner AB, Woods GL, Weber JA (1996) Seminal collection, seminal characteristics and pattern of ejaculation in llamas. *Theriogenology* 46:293–305.

Ratto M, Huanca W, Singh J, Adams GP (2006) Comparison of the effect of natural mating, LH and GnRH on interval to ovulation and luteal function in llamas. *Anim Reprod Sci* 91:299–306.

San-Martin M, Copaira M, Zuniga J, Rodreguez R, Bustinza G, Acosta L (1968) Aspects of reproduction in the alpaca. *J Reprod Fertil* 16:395–399.

Schellpfeffer DA, Hunter AG (1970) Electrophoretic and gel filtration behaviour of boar seminal plasma proteins before and after removal of accessory sex glands. *J Reprod Fertil* 23:291–298.

Tibary A, Anouassi A (1997) Theriogenology in Camelidae. Actes Editions, Rabat, Morocco, p. 33.

Tibary A, Vaughan J (2006) Reproductive physiology and infertility in male South American camelids: a review and clinical observations. *Small Rumin Res* 61:283–298.

Vaughan J (2011) Ovarian function in South American camelids (alpacas, llamas, vicunas, guanacos). *Anim Reprod Sci* 124:237–243.

Villemure M, Lazure C, Manjunath P (2003) Isolation and characterization of gelatin-binding proteins from goat seminal plasma. *Reprod Biol Endocrinol* 1:39–48.

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