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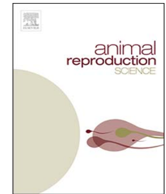

# Mating induces production of MMP2 in the llama oviduct: Analysis of MMP2 effect on semen

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ABSTRACT

Ovulation of South American Camelids is induced by mating. After copulation, sperm are stored into the oviduct to be released near ovulation time. To study whether copulation induces matrix metalloproteinase-2 (MMP2) secretion in the oviduct, the occurrence of MMP2 in oviductal tissue and oviductal fluid (OF) from 24 h post-mated was compared with non-mated llama females. There was an incremental increase of MMP2 in the oviductal epithelial cells, and MMP2 activity in OF after copulation. Additionally, MMP2 activator (MMP14), inducer (EMMPRIN) and inhibitor (TIMP2) were present in the oviductal epithelial cells of both non-mated and post-mated females. A post-mating segment-specific regulation occurred because relative abundance of TIMP2 mRNA was greater in the utero tubal-junction which was accompanied with a reduced amount of MMP14 in the ampulla in comparison with the non-mated females. To examine the effect of MMP2 on semen liquefaction and sperm physiology, the effects of addition of recombinant human MMP2 was evaluated. The MMP2 had no effect on semen thread formation and seminal plasma protein profile. Sperm viability and plasma membrane function were not influenced by the enzyme treatment either. In summary, in llamas the oviductal microenvironment changes in response to stimuli induced by copulation, increasing the production and secretion of MMP2.

## 1. Introduction

South American camelids (SACs) are induced-ovulating species. During mating, the male deposits semen deep inside the uterine horns of the female with ovulation occurring 26–42 h later (San-Martin et al., 1968; Ratto et al., 2006). A protein in the seminal plasma of llamas and alpacas, identified as β-Nerve Growth Factor (β-NGF), induces ovulation by stimulating pituitary LH secretion (Adams and Ratto, 2013; Berland et al., 2016). After insemination, sperm is stored in the utero-tubal-junction (UTJ) segment of the

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llama oviduct, and it is released 28 h post-mating and transport to the site of fertilization continues (Apichela et al., 2009). The oviduct environment is favourable for fertilization to occur (Hunter, 2005; Rodriguez-Martinez, 2007). There, however, is a lack of knowledge about the varying oviduct functions and timing of these functions, particularly in induced-ovulating species because the oviductal microenvironment likely changes in response to stimuli induced by copulation.

In previous studies, the two metalloproteinases (MMP2 and MMP9) were present in the llama oviduct and oviductal fluid (OF) of non-pregnant and non-mated females, and there was a greater amount of MMP2 activity than MMP9 in the OF (Zampini et al., 2014, 2017). The MMP2 is an important contributor to oocyte release from preovulatory follicles of rats, mice, sheep, and humans (Curry and Osteen, 2003). While the role of MMP2 in the ovary is well defined, its role in the oviduct remains unknown. In the present study, the hypothesis was that mating would induce MMP2 secretion in the oviduct. To assess this hypothesis, in the current study, the presence of MMP2 in oviductal epithelial cells and OF in 24 h post-mated was compared with non-mated llamas. In addition, to enhancing the understanding of the MMP2 function in the llama oviduct, the effect of addition of MMP2 on sperm and seminal plasma was evaluated, as well as presence of modulators of MMP2 activity in the oviduct after mating.

## 2. Materials and methods

### 2.1. Animals

Fertile, non-lactating, 5–8 year-old female llamas (*Lama glama*, $n = 11$) used in this study were provided by the Instituto Nacional de Tecnología Agropecuaria (INTA) Abra Pampa, located on the high Andean plateau in the Argentine northwest.

Fertile male llamas ($n = 3$), ranging between 5 and 8 years of age, used in the study belonged to the Faculty of Veterinary Sciences of the University of Buenos Aires, in Buenos Aires, Argentina.

### 2.2. Oviducts, oviductal epithelial cells (OECs) and oviductal fluid (OF)

Llama oviducts from 24 h post-mated females ($n = 3$) were obtained immediately after slaughtering, in accordance with protocols approved by the local institutional animal care committee. To minimize hormonal influences only the oviducts ipsilateral to dominant follicles and having dominant follicles smaller than 7 mm were used. Oviducts were dissected and OF was obtained by perfusion with 100 μl of 10 mM Tris-HCl, 150 mM NaCl (pH 7.4) at 4 °C. The perfused solution was subsequently centrifuged to remove cellular debris. Oviducts were subsequently separated into ampulla, isthmus and UTJ segments, and fixed with 4% formaldehyde in PBS (pH 7.4) (Zampini et al., 2017). Ipsilateral oviducts and OF from non-mated females were used as the control ($n = 3$). For RT-PCR assays, OECs from oviductal segments from five additional llamas, three non-mated and two post-mated, were used. Epithelial cells from ampulla, isthmus and UTJ segments were separately collected by gently scraping the mucosal epithelial layer with the blunt side of a sterile scalpel (Apichela et al., 2009). The OECs were lysed in RNA Lysis Buffer solution (SV total RNA isolation system, Promega, Madison, WI, USA), and stored in liquid nitrogen until RNA isolation.

### 2.3. Semen collection

Semen collections were conducted between the months of August and October by electroejaculation with animals being under general anaesthesia using a P-T Electronics 304 electroejaculator (Oregon, USA) with a #4 probe and three ventral electrodes. Electrical stimulation was performed as previously described by Director et al. (2007). All procedures were approved by the Committee for the Care and Use of Laboratory Animals (CICUAL) of the Faculty of Veterinary Sciences of the University of Buenos Aires (protocol 2010/24).

### 2.4. Experiment I – RNA relative abundance of MMP2 and related genes in OECs of mated females

#### 2.4.1. RNA isolation and cDNA synthesis

Total RNA from OECs (ampulla, isthmus and UTJ) was isolated using the SV total RNA isolation system (Promega, Madison, WI, USA) according to the manufacturer's instructions. The RNA was quantified spectrophotometrically at 260 nm, and RNA integrity was examined by electrophoresis on 1.5% agarose gels, stained with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA).

Reverse transcription was performed using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA) and oligo $(dT)_{15}$ primer. The reaction mixture (25 μl) consisted of 1 μg of RNA, 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM of each dNTP, 25 pmol of oligo $(dT)_{15}$, 200 units of reverse transcriptase, and RNase-free water. Reactions were performed in a thermal cycler at 42 °C for 90 min, followed by enzyme inactivation at 94 °C for 5 min.

#### 2.4.2. Semi-quantitative PCR

Relative abundance of MMP2, MMP14, TIMP2 and EMMPRIN mRNA was analyzed by semi-quantitative PCR in 24 h post-mated llama OECs (ampulla, isthmus and UTJ).

The MMP2 and TIMP2 primers were designed based on llama MMP2 and TIMP2 sequences previously identified (Zampini et al., 2014). The MMP14, EMMPRIN and ACTB (β-actin) primers were designed using predicted *Vicugna pacos* nucleotide sequences. Primer sequences are shown in Table 1.

Amplifications were conducted in a final volume of 10 μl containing 1 μl of cDNA, 2 μl of 5X Green GoTaq Reaction Buffer (pH

**Table 1**
Primers used in RT-PCR.

| | Primer sequences (5´-3´) | GenBank accession number | Amplicon size (bp) |
|---|---|---|---|
| MMP2 forward | CATGATGGAGAGGCTGACAT | GQ244429.1 | 148 |
| MMP2 reverse | GCTCATCGTCATCAAAGTGG | | |
| MMP14 forward | GAGGTTCTACGGTCTGCGAG | XM_006217304.2 | 193 |
| MMP14 reverse | GGGGGTGTAGTTCTGGATGC | | |
| EMMPRIN forward | AGGTGCTTGTGCTGGTCACT | XM_015240959.1 | 137 |
| EMMPRIN reverse | GCCTTTCTTGCCTTTGTCGT | | |
| TIMP2 forward | GCACCACCCAGAAGAAGAGC | KC425455.1 | 117 |
| TIMP2 reverse | CCATCCAGAGGCACTCATCC | | |
| ACTB forward | GCGGGACCACCATGTACC | XM_006210388.1 | 183 |
| ACTB reverse | ACTCCTGCTTGCTGATCCAC | | |

8.5), 0.2 mM of each dNTP, 2.5 units of GoTaq DNA polymerase (Promega, Madison, WI, USA), and 1 µM of each primer pair. Reactions were conducted in a Techne TC-512 Thermal Cycler. Amplification conditions were as follow: 94 °C for 5 min; 10 cycles of 94 °C for 10 s, 65 °C for 5 s, and 72 °C for 10 s; 20 cycles of 94 °C for 10 s, 65 °C for 5 s, and 72 C for 10 s, followed by a final step at 72 °C for 5 min. ACTB cDNA was amplified as °internal control. The PCR products were analyzed on 1.5% agarose gels, and visualized with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA).

For semi-quantitative expression analysis, gel images were captured with a Pentax Optio M90 digital camera, and the optical density of PCR products was quantified using ImageJ 1.42q software (NIH, Bethesda, Maryland, USA). The relative abundance of each transcript was normalized against that of ACTB (reference gene), and the transcript/ACTB ratio was calculated for each oviduct segment analyzed and state.

### *2.5. Experiment II – detection of MMP2 in oviducts from mated females*

Ampulla, isthmus and UTJ oviductal segments were examined immunohistochemically. Oviduct sections (7 µm) were mounted on positively-charged slides (HDA microscope slides, Cat. No. HDAS001A, Yancheng Huida Medical Instruments Co., Ltd., China). After deparaffinisation and rehydration, sections were rinsed with PBS. Prior to immunolabelling, antigen retrieval was performed as follows: slides were submerged in 10 mM sodium citrate (pH 6.1) and kept in a 90 °C water bath for 20 min. Slides were subsequently taken out of the water bath and were allowed to cool to room temperature.

The sections were subsequently subjected to the following immunohistochemical staining schedule: (a) rinse with PBS, (b) blockage with 5 mg/ml skim milk-PBS solution for 30 min at room temperature, (c) incubation with anti MMP2 polyclonal antibody (dilution 1:100, AB19167, Millipore, Bedford, MA, USA) for 1 h at room temperature and afterwards overnight in a humidified chamber at 4 °C, (d) rinse two times with 0.02% PBS-Tween, (e) incubation at room temperature for 30 min with biotin-conjugated anti-rabbit immunoglobulin G (IgG) antibody (dilution 1:200, B8895, Sigma, St. Louis, MO, USA), (f) incubation at room temperature for 30 min with streptavidin-alkaline phosphatase conjugate (dilution 1:500, E2636, Sigma, St. Louis, MO, USA), (g) incubation with SigmaFast substrate (BCIP/NBT, B5655, Sigma, St. Louis, MO, USA) until colour development (15 min for UTJ sections and 30 min for ampulla and isthmus sections). Sections were left unstained or counterstained with nuclear fast red (N3020, Sigma, St. Louis, MO, USA), dehydrated and mounted. The controls sections were processed by replacing primary antibody with blocking buffer. Samples were observed using a Leica DM 500 light microscope and images were captured with a Leica ICC50 HD camera. Staining and image acquisition were performed in parallel for the entire set. Identical image acquisition settings and exposure times were applied.

The ImageJ 1.42q software (NIH, MD, USA) was used to measure the labeled area of the oviductal epithelium according to Jensen (2013). For the analysis, three different photographs of the no-counterstained oviductal sections were quantified. Three areas of each image were selected for evaluation. Data were expressed as pixels/µm$^2$.

### *2.6. Experiment III – MMP2 activity in OF from mated females*

Gelatine zymography was used to compare gelatinase activity of matrix metalloproteinases in OF from 24 h post-mated and non-mated llamas. Total protein was determined using a Micro BCA kit (Thermo Fisher Scientific, Rockford, USA). For zymography, 30 µg of total proteins were separated in non-reducing conditions on 8% polyacrylamide gels containing 1 mg/ml gelatine. Five µl of a PageRuler Unstained Broad Range Protein Ladder (Thermo Fisher Scientific, Rockford, USA) were loaded onto each gel in a separate well as described by Zampini et al. (2014). Briefly, gels were run at 150 V at room temperature. After electrophoresis, gels were washed six times for 15 min with enzyme renaturing buffer (200 mM NaCl, 5 mM $CaCl_2$, 5 µM $ZnCl_2$, 2.5% Triton X-100, 0.02% $NaN_3$, 50 mM Tris-HCl, pH 7.5) to remove SDS, and incubated in developing buffer (200 mM NaCl, 5 mM $CaCl_2$, 5 µM $ZnCl_2$, 0.02% $NaN_3$, 50 mM Tris-HCl, pH 7.5) for 42 h at 37 °C. Gels were then stained for 40 min with 0.125% Coomassie Brilliant Blue R-250 (Sigma, St. Louis, MO, USA) in 50% methanol and 20% acetic acid under gentle agitation, and de-stained in 30% methanol and 10% acetic acid until clear bands were visible against a dark blue background.

The molecular weight of the bands was calculated using GelAnalyzer freeware software (http://www.gelanalyzer.com, version 2010 by Istvan Lazar and Dr. Istvan Lazar, Hungary).

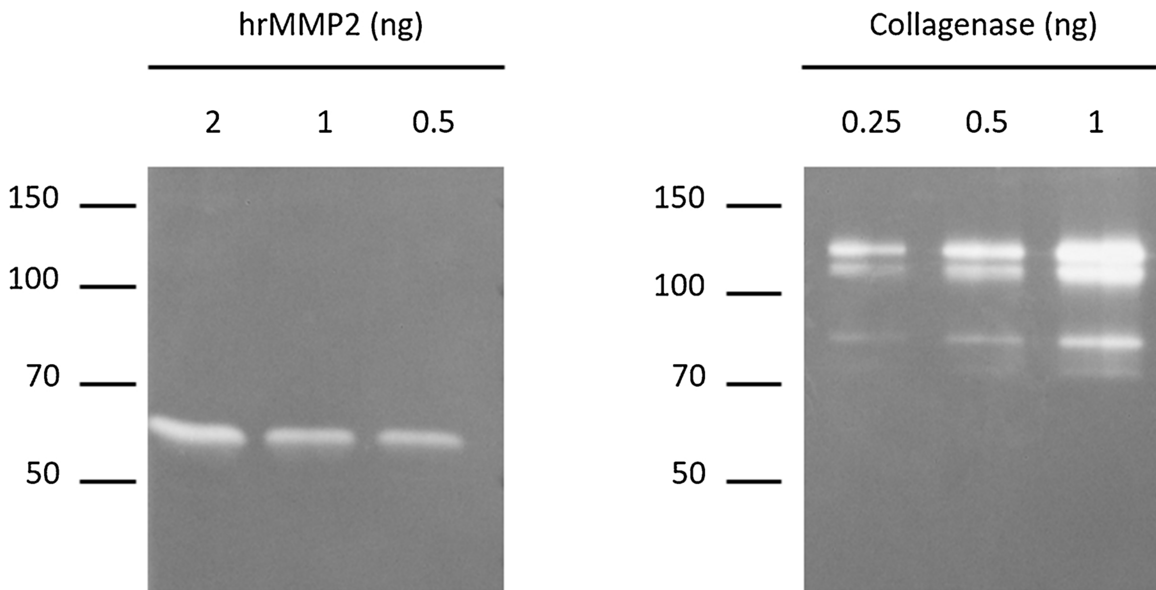


**Fig. 1.** Gelatine zymography of hrMMP2 and collagenase. Different amounts of total protein were loaded into the wells of polyacrylamide gels. hrMMP2 appears as a single band of about 62 kDa, whereas Type I collagenase has four-band patterns (122, 112, 84 and 74 kDa).

Densitometric analysis of the gelatinase bands was performed using ImageJ 1.42q software (NIH, Bethesda, Maryland, USA).

### *2.7. Experiment IV – effect of MMP2 on semen thread formation*

To determine whether MMP2 affects thread formation, ejaculates ($n = 3$) were divided into four aliquots; (1) MMP2: 50 µl of MMP2 diluted in 15 mM HEPES-buffered Tyrode's albumin lactate pyruvate medium plus 3 mg/ml bovine serum albumin (H-TALP-BSA) was added to 50 µl of semen, to achieve a final MMP2 concentration of 0.035 µg/µl, (2) Control without MMP2: 50 µl of semen plus 50 µl of H-TALP-BSA, (3) Positive control: 50 µl of semen plus 50 µl of collagenase diluted in H-TALP-BSA, to achieve a final enzyme concentration of 0.01 µg/µl, (4) Control without dilution: 100 µl of raw semen, that was utilized to calculate relative units of thread formation as subsequently described.

Human recombinant MMP2 (hrMMP2, SRP3118, Sigma, St. Louis, MO, USA), and Type I collagenase (Clostridium peptidase A from Clostridium histolyticum, Sigma, St. Louis, MO, USA) were used for the experiment. hrMMP2 and collagenase activity was confirmed by gelatine zymography as previously described (Fig. 1).

Samples were incubated for 4 h at 37 °C. The viscoelastic properties of the semen were assessed at 0–4 h, using the thread formation test as previously described by Bravo et al. (2000). Briefly, 20 µl of semen were pipetted onto a glass slide, and the pipette was moved vertically from the glass slide forming a thread of semen. The length at which the thread snapped was measured in millimeters using a ruler. Two measurements were taken for each sample at each evaluation time. Thread formation values were normalized against those of control without dilution (raw semen) at each sampling point.

### *2.8. Experiment V – effect of MMP2 on sperm function*

To determine whether MMP2 affects sperm viability and membrane function, ejaculates ($n = 3$) were divided into two aliquots; (1) MMP2: 50 µl of MMP2 diluted in H-TALP-BSA was added to 50 µl of semen, to achieve a final MMP2 concentration of 0.035 µg/µl, (2) Control without MMP2: 50 µl of semen plus 50 µl of H-TALP-BSA.

Samples were incubated for 4 h at 37 °C. Live spermatozoa and the membrane function were assessed at the beginning and end of the incubation period (0 and 4 h).

The hypoosmotic swelling (HOS) test was used for assessing membrane function, and the fluorochromes 6-Carboxyfluorescein Diacetate (CFDA) and Propidium Iodide (PI) were used for assessing membrane integrity (viability). These techniques were conducted according to Giuliano et al. (2008).

For the HOS test, semen (12.5 µl) was incubated at 37 °C for 20 min in 50 µl of hypoosmotic solution: fructose (2.45 mg/ml) – sodium citrate (4.5 mg/ml) in Mili Q deionized water, adjusted to 50 mOsm. After incubation, a minimum of 200 spermatozoa were evaluated using a phase contrast microscope (400×). Sperm showing the characteristic swelling of the tail were classified as HOS positive, having a functional plasma membrane. Osmolarity of the solutions was measured using an automatic cryoscopic osmometer (Osmomat® 030, Gonotec, Berlin, Germany).

For evaluating membrane integrity, 12.5 µl of semen were incubated at 37 °C for 10 min in 127.5 µl of staining medium. This medium contained 2.5 µl of a solution of CFDA (0.5 mg/ml in dimethylsulphoxide) and 125 µl of saline medium (described by

Harrison and Vickers, 1990). After the first 10 min of incubation, 2.5 µl of a solution of PI (0.5 mg/ml in isotonic saline) were added and incubated for another 10 min at 37 °C. A minimum of 200 spermatozoa were evaluated using an epifluorescence microscope with a rhodamine and standard fluorescein filter set (400 ×). Spermatozoa that fluoresced green throughout their length were classified as being viable (intact membrane) while sperm nuclei that fluoresced red were classified as non-viable (damaged membrane).

### 2.9. *Experiment VI – effect of MMP2 on llama seminal plasma protein profile*

To obtain seminal plasma (SP), a total of three ejaculates were collected from three male llamas. To separate SP, ejaculates were subjected to successive centrifugations at 12,000 × *g* for 30 min until the absence of sperm in the supernatant was confirmed using a phase contrast microscope (400 ×). SP from the different males was pooled and kept at −20 °C until further assays.

To determine the effect of MMP2 on llama seminal plasma proteins, a pool of SP containing 40 µg of total proteins was treated with MMP2, previously diluted in 0.1% BSA-PBS (final MMP2 concentration was 0.2 µg/µl), and incubated for 4 h at 37 °C. SP without enzyme, diluted in 0.1% BSA-PBS, was also incubated for 4 h at 37 °C and used as control.

Samples were loaded onto a 4% stacking polyacrylamide gel, which was overlaid on top of an 8% or 15% resolving gel to resolve proteins larger and smaller than 40 kDa, respectively. Five µl of a PageRuler Unstained Broad Range Protein Ladder (Thermo Fisher Scientific, Rockford, USA) was loaded in a separate well. Gels were processed at room temperature at 150 V until completion, and stained with colloidal Coomassie Blue. Gels were fixed in a 30% methanol and 2% phosphoric acid solution for 3 h, and equilibrated for 1 h with a solution containing 17% ammonium sulphate, 18% methanol and 2% phosphoric acid. Coomassie Brilliant Blue G250 powder (0.5 g/l) was subsequently added and the gel was stained for 2 days.

### 2.10. *Statistical analysis*

One-way ANOVA was used to calculate statistical differences. Fisher's LSD test was used to determine the level of significance. Results were considered statistically significant at $P < 0.05$.

## 3. Results

### 3.1. *Relative abundance of MMP2, MMP14, TIMP2 and EMMPRIN in llama post-mated OECs*

Transcripts of MMP2, MMP14, TIMP2 and EMMPRIN were detected in the epithelial cells from ampulla, isthmus and UTJ of both

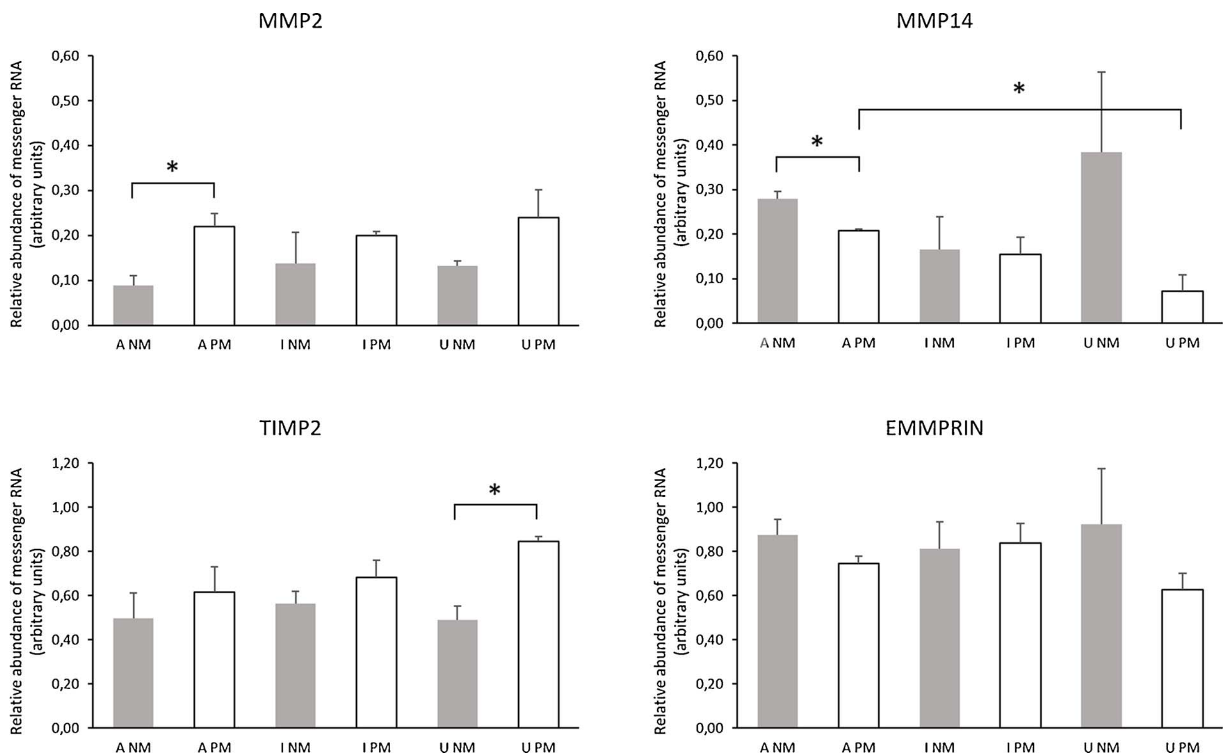


**Fig. 2.** Relative abundance of messenger RNA (mRNA) for MMP2, MMP14, TIMP2 and EMMPRIN in post-mated (PM) and non-mated (NM) llama OECs from ampulla (A), isthmus (I) and UTJ (U). Data are shown as mean relative abundances of mRNA (normalized to ACTB reference gen) ± standard error of the mean (SEM). The asterisks denote differences ($P < 0.05$).

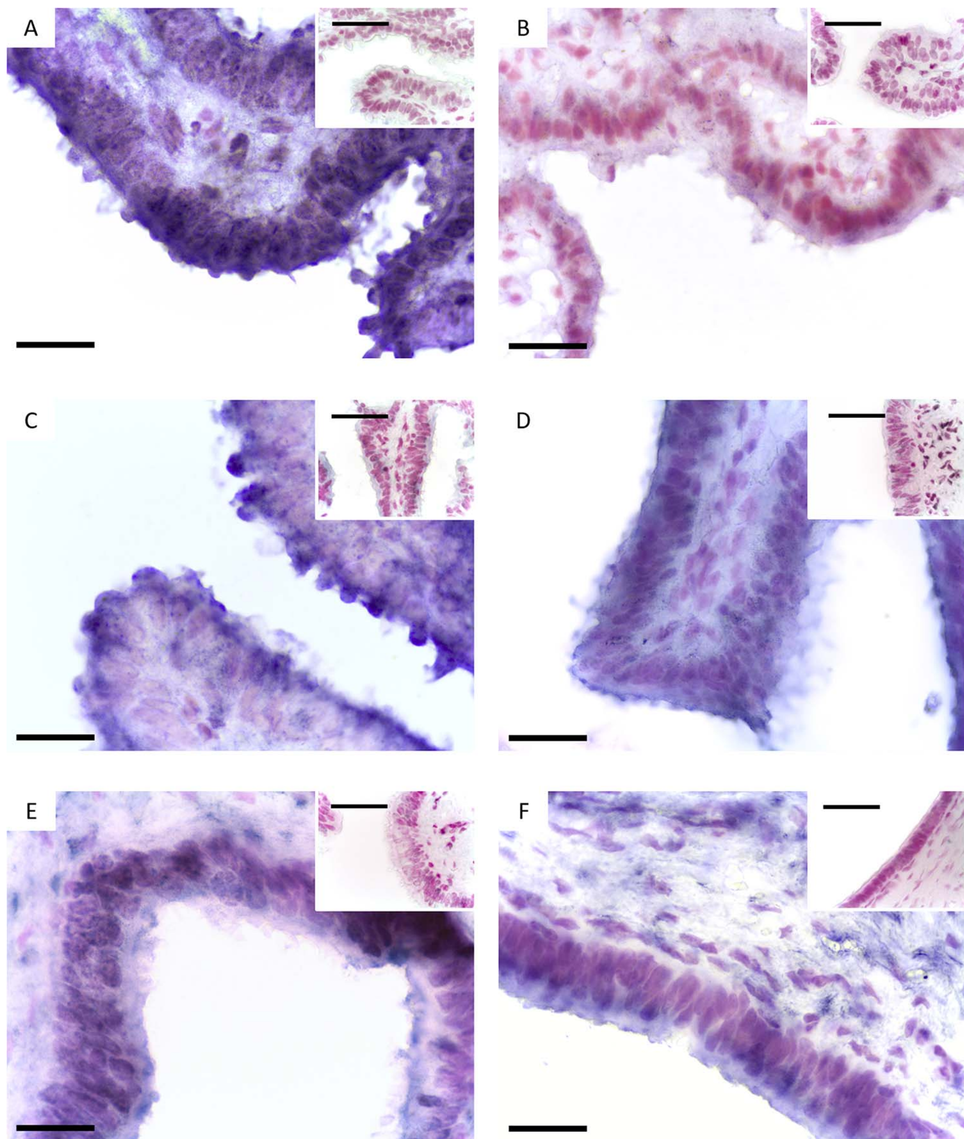


**Fig. 3.** Immunohistochemical localisation of MMP2 in post-mated llama oviductal segments: ampulla (A), isthmus (C), and UTJ (E). Controls of non-mated oviductal segments: ampulla (B), isthmus (D) and UTJ (F). Insets correspond to negative controls without primary antibody. Tissue sections were counterstained with nuclear fast red. Scale bar, 50 μm.

non-mated and post-mated llamas (Fig. 2). Of these genes, MMP2 mRNA was of a greater relative abundance in post-mated females, with statistical significance in ampulla; while, in isthmus and UTJ there was a tendency for a greater relative abundance. In UTJ of post-mated llamas, the relative abundance of TIMP2 mRNA was greater than in non-mated females. While, relative abundance of MMP14 mRNA was different among the anatomic regions assayed in post-mated (ampulla compared with UTJ, −0.42), and between the physiological states in the ampulla segment (non-mated compared with post-mated, +1.87). The relative abundance of EMMPRIN mRNA was at steady-state amounts between physiological states and among the oviductal segments.

### *3.2. Immunolocalization of MMP2 in the oviduct of mated llamas*

There was a marked immuno-staining of MMP2 in the oviductal mucosa of mated females. The ampulla had an irregularly distributed immuno-staining for MMP2 in the apical cytoplasm of epithelial cells, and a moderate and irregular signal in sub-epithelial connective tissue (Fig. 3A). In isthmus sections, there was a marked evenly distributed immuno-staining in the apical cytoplasm of epithelium, with distinct bulbous projections of epithelial cell immuno-staining. Sub-epithelial connective tissue had a moderate uneven distribution of immuno-staining (Fig. 3C). In the UTJ, there was a marked homogenous immuno-staining in the cytoplasm of epithelial cells. In contrast to the other segments, the UTJ also had distinct perinuclear immuno-staining. In sub-

**Table 2**
MMP2 signal intensity in pixels/μm$^2$ (mean ± SE) of ampulla, isthmus and UTJ of non-mated and post-mated females. Within a column, significant differences are indicated with different letters (P < 0.05).

| | Ampulla | Isthmus | UTJ |
|---|---|---|---|
| Non-mated | 1.41 ± 0.22a | 10.19 ± 7.75a | 2.73 ± 1.02a |
| Post-mated | 26.41 ± 8.95b | 51.50 ± 0.5b | 29.06 ± 5.34b |

epithelial connective tissue the immuno-staining signal was less than in the other two segments (Fig. 3E).

There were notable differences in immuno-staining intensity when comparing sections of ampulla, isthmus and UTJ of mated and non-mated females with very marked staining in mated females (Table 2).

### 3.3. Gelatinase activity in oviductal fluid of mated llamas

Oviductal fluid of post-mated llamas had eight proteolytic bands (350, 280, 130, 120, 94, 75, 62 and 53 kDa), whereas OF of non-mated females had only six gelatinolytic bands that were also present in the mated llamas (350, 120, 94, 75, 62 and 53 kDa; Fig. 4A). Particularly, gelatinolytic activity of the 62 kDa MMP2 was 1.17 greater in OF post-mating than in OF of non-mated females ($P < 0.05$; Fig. 4B).

### 3.4. Effect of MMP2 on thread formation and sperm function

Semen thread formation was not affected by hrMMP2 as there was no differences observed between hrMMP2 of treated and control samples (no hrMMP2 supplement) for the incubation periods. As expected, the seminal thread was completely eliminated by collagenase treatment (positive control; Fig. 5).

The percentage of live spermatozoa and the sperm plasma membrane function were not altered after addition of hrMMP2 during the incubation periods (Table 3).

### 3.5. Effect of MMP2 on protein profile of llama seminal plasma

The protein profile of seminal plasma treated with hrMMP2 and the control (without enzyme) were similar and hrMMP2 treatment did not affect the seminal plasma protein profile (Fig. 6).

## 4. Discussion

The present study focused on expression of the MMP2 gene in the oviduct in response to the mating stimulus. The MMP2 gene expression in the oviduct 24 h post-mating and in non-mated llamas was compared by assessing relative abundance of mRNA and in

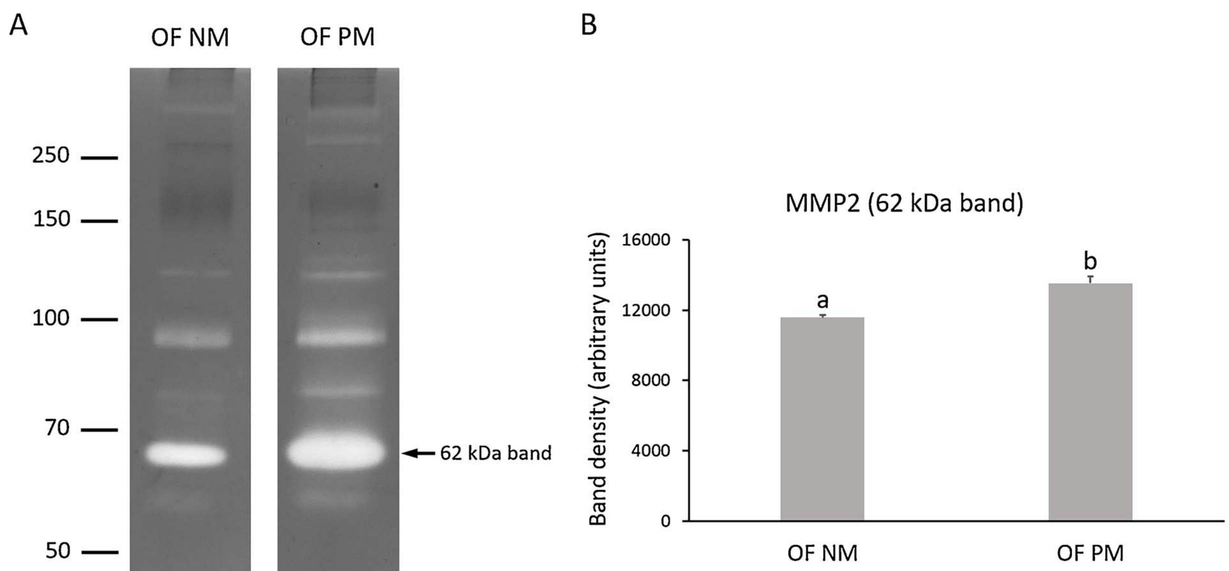


**Fig. 4.** Gelatine zymography of llama OF from non-mated (OF NM), and post-mated (OF PM) animals (A). Densitometric analysis of 62 kDa (B) protein bands (mean ± SEM). Different letters above bars indicate differences ($P < 0.05$) in values for samples.

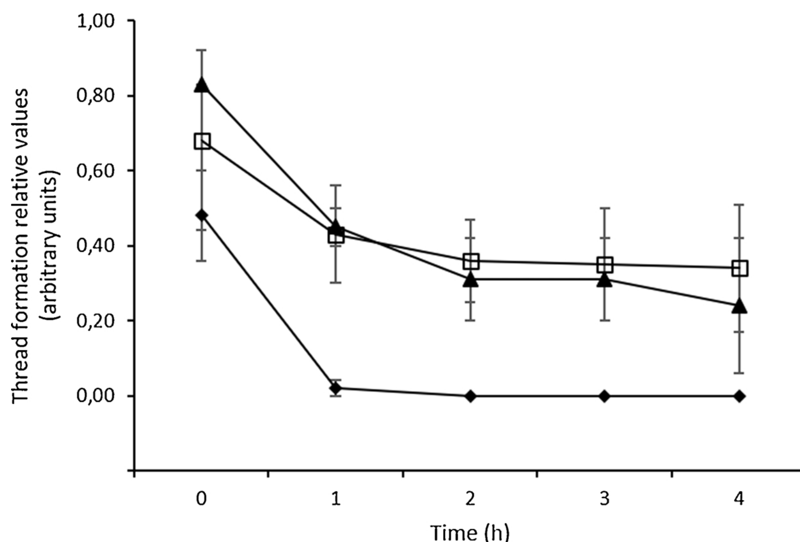


**Fig. 5.** Thread formation relative values (mean ± SEM) of llama semen treated with MMP2 (□), positive control (with collagenase) (◆), and control (without MMP2) (▲) after 0, 1, 2, 3 and 4 h of treatment. Data were normalized against those of control without dilution (raw semen) at each sampling point.

**Table 3**
Percentage of live spermatozoa and sperm plasma membrane functionality in semen incubated at 37 °C during 4 h (samples with hrMMP2 and diluted samples without hrMMP2). The values are expressed as mean ± SEM.

| | with hrMMP2 | | without hrMMP2 | |
|---|---|---|---|---|
| | 0 h | 4 h | 0 h | 4 h |
| Live spermatozoa (%) | 54.5 ± 5.0 | 27.5 ± 16.0 | 48.8 ± 10.9 | 49.7 ± 4.2 |
| Sperm swelling (%) | 34.2 ± 8.8 | 23.3 ± 6.3 | 33.7 ± 2.6 | 31.8 ± 12.6 |

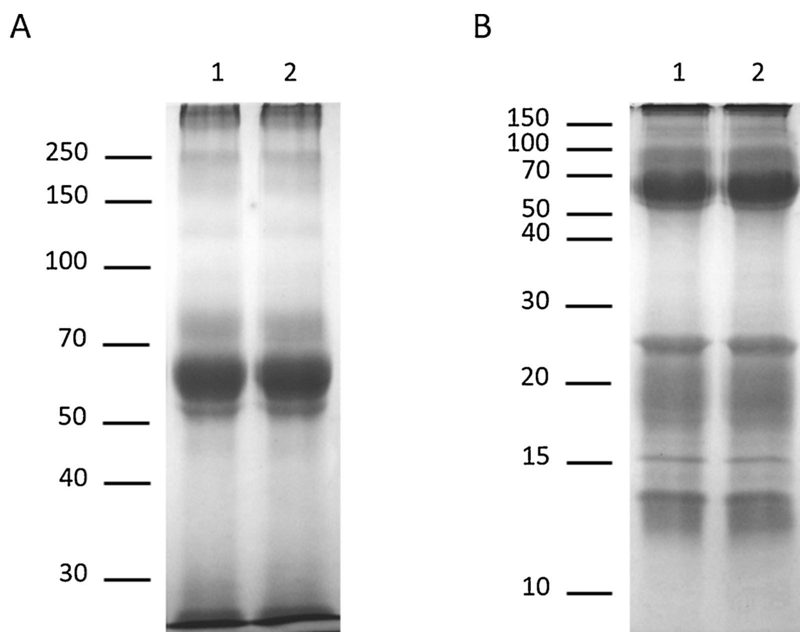


**Fig. 6.** Electrophoretic profiles of llama semen incubated with MMP2 (2) and without MMP2 (1) at 37 °C for 4 h. Samples were loaded onto a 8% (A) or 15% (B) resolving gel.

evaluation of amount of MMP2 protein by immunohistochemistry assays. There were distinct differences observed in samples from mated and non-mated animals. Results of the present study are consistent with those by Gabler et al. (2001) with the greatest relative abundance of MMP2 mRNA and amount of protein being present during the pre-ovulatory phase in cattle around the time when the LH surge occurred.

In addition, there was a qualitative (8 compared with 6 bands) and a quantitative (regarding MMP2) increase in gelatinolytic activity in the OF from post-mated llamas in the present study. In previous studies, it was proposed that OECs had an active role in the secretion of MMP2 into the oviductal fluid (Zampini et al., 2014, 2017). Results from the present study provide evidence that OECs have a role in regulation of MMP2 secretion and that an increase in MMP2 in the oviductal cells was consistent with the MMP2 content in OF.

Moreover, it was important to ascertain whether the MMP2 system is present in the oviduct and whether the relative amount of gene expression varies in response to mating. Interesting, the MMP2 inhibitor, TIMP2; MMP2 activator, MMP14; and MMP2 inductor, EMMPRIM, were present in all the oviductal segments from non-mated and mated llamas in the present study. There appears to be a segment-specific regulation during mating because the amount of TIMP2 was greater in the UTJ; while, MMP14 gene expression was less in the ampulla in comparison to that of the non-mated females. These findings indicate a well-regulated control of MMP2 proteolytic activity in the llama oviduct microenvironment 24 h after mating when the oviduct is undergoing changes in the oviductal fluids to enhance oocyte viability and be conducive for fertilization.

Hormones released in response to mating may affect MMP2 production in the llama oviduct. In camelids mating induces a pre-ovulatory surge release of LH in blood plasma that is necessary for ovulation (Ratto et al., 2006; Berland et al., 2016). The effect of LH on MMP2 gene expression has been studied in the ovary, and an increase in MMP2 in response to LH has been shown to occur in several mammals. In rodents, for example, expression of MMP2 gene was increased after the endogenous LH surge or administration of hCG to immature PMSG-primed animals (Reich et al., 1991; Cooke et al., 1999). The relative abundance MMP2 mRNA was also increased in preovulatory granulosa cells in macaques in response to hCG administration *in vivo* (Chaffin and Stouffer, 1999). Similarly, the pre-ovulatory LH surge induced an increase MMP2 activity in sheep follicular extracts (Gottsch et al., 2000). There are LH receptors (LHR) in the oviduct, suggesting that LH may also contribute to the regulation of oviduct development and secretions in pigs, cattle and humans (Gawronska et al., 1999; Ziecik et al., 2007; Li et al., 2014). Interestingly, the llama ovulation-inducing factor, β-NGF, enhances MMP2 gene expression and activity in the human pancreas and endothelial cells (Okada et al., 2004; Park et al., 2007). Delivery of β-NGF through male seminal plasma could, therefore, also induce an increase in MMP2 in the llama oviduct.

The greatest amount of MMP2 was present 24 h post-mating in the present study and this could be associated with reproductive events taking place in the oviductal lumen near ovulation. Camelid males ejaculate a highly mucous semen in which sperm are trapped and in which these cells have oscillatory movements without progressive motility (Lichtenwalner et al., 1996; Bravo et al., 1997; Giuliano et al., 2010; Casaretto et al., 2012). During copulation, male llamas deposit semen deep inside the uterine horns, and the UTJ functions as a sperm reservoir in these animals (Apichela et al., 2009, 2010). Spermatozoa remain adhered to the llama UTJ for at least 28 h after mating (Apichela et al., 2009), and seminal plasma, particularly secretions from the bulbourethral glands is present during sperm storage coating the mucosal layer of the UTJ (Apichela et al., 2014).

Semen proteolysis and spermatozoa release from oviductal reservoirs is thought to be under oviduct control, and the timing of the release is associated with the time of ovulation so as to enhance the probability of fertilization. The highly glycosylated protein mucin 5B has been proposed to be responsible for mucous consistency in alpaca semen (Kershaw-Young and Maxwell, 2012) and this seems to agree with findings that several proteases such as collagenase, trypsin, and papain are effective viscosity reducers of camelid seminal plasma (Bravo et al., 2000; Giuliano et al., 2010; Kershaw-Young et al., 2013). The protease responsible for elimination of camelid seminal plasma viscoelastic properties *in vivo*, however, is still unknown.

To examine whether MMP2 is involved in *in vivo* semen liquefaction, the effect of hrMMP2 on semen thread formation and sperm function was analyzed in the present study. Addition of hrMMP2 at the tested concentration had no effect on semen thread formation or the seminal plasma protein profile. The percentage of live spermatozoa and sperm plasma membrane function were not affected by the enzyme treatment. These results may indicate that MMP2 as a sole enzyme does not have an adequate amount of activity to produce semen liquefaction to release sperm from oviductal reservoirs.

## 5. Conclusions

The current study indicates mating produces an increase in the production of MMP2 and its secretion into the llama oviduct. Additionally, expression of genes involved in induction, inhibition and activation of MMP2 may indicate that MMP2 activity in each oviductal llama segment is highly regulated.

This is the first study that has the role of MMP2 in the oviduct. Under the current assay conditions, MMP2 alone did not affect seminal plasma or sperm variables.

## Conflicts of interest

None.

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