# Leptin and leptin receptor are detectable in equine spermatozoa but are not involved in *in vitro* fertilization

Running head: Leptin in equine *in vitro* fertilization

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## Abstract

In ~~human~~ people and swine, ~~leptin~~ leptin (OB) has been ~~demonstrated~~ identified~~to be present~~ in seminal plasma and leptin receptors (OB-R) on ~~sperm~~ the cell surface of spermatozoa, indicating that~~the~~ spermatozo~~a~~a~~on~~ a~~re~~s a target for OB. This hormone ~~is~~ has also ~~present~~ been detected in follicular fluid (FF), ~~as detected~~ in wom~~e~~an and mare~~s~~, although its role ~~should be~~requires further stud~~i~~y~~ed~~. The aims of this study were to investigate the immunolocalization and the expression of OB and OB-R in equine spermatozoa and to evaluate the involvement of OB in equine *in vitro* fertilization (IVF). Since progesterone (P) and OB are both found in FF, the individual and combined effects of these two hormones were studied in equine IVF comparing the results ~~obtained~~ to those ~~found using~~from the use of FF for *in vitro* sperm preparation. For the first time, ~~in equine spermatozoa,~~ we ~~describe the presence of~~were able to identify *OB* and *OB-R* mRNA and their corresponding proteins in equine spermatozoa. When spermatozoa were treated with OB, there was a decrease in the three motility parameters VSL, STR, and LIN, commonly associated ~~by CASA analysis to~~with hyperactivation, ~~decreased~~ whil~~s~~te the acrosome reaction rate increased ($P<0.05$). The fertilization rate was 51% with FF, 46.15% with P, 43.64% with P combined with OB, and 0% with OB alone. The ~~rate~~ percentage of 8 cell stage embryos was 18.7% with FF, 17.1% with P, and 16.7% with OB combined with P. OB alone did not permit ~~fertilize~~ oocyte~~s~~ fertilization indicating that, in the horse, OB is involved in capacitation and hyperactivation but not in spermatozoa penetration.


**Additional Keywords**: leptin, progesterone, fertilization, spermatozoa, horse

## Introduction

In ~~the~~ equine species, *in vitro* assisted reproduction techniques are challenging ~~and complicated~~.

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Only two papers report the birth of foals from *in vitro* fertilization (IVF). In both cases, *in vivo* matured oocytes collected by ovum pick-up were used (Palmer *et al.* 1991; Bezard, 1992). T~~992).~~ ~~It is considered that t~~he main limiting factors appear to be the ~~rel~~reliance on~~y in~~ the zona pellucida for hardening of oocytes and ~~in the~~ reduced *in vitro* capacitation of ~~the~~ stallion spermatozoa. Heparin, equine proteins present in zona pellucida (also called zona proteins), caffeine, and lysophospholipids have been shown to increase the percentage~~s~~ of capacitated and acrosome-reacted spermatozoa, although they do not facilitate ~~the~~ spermatozoa penetration into *in vitro* matured oocytes (Graham, 1996). Following incubation of spermatozoa-oocyte complexes for 1 hour ~~Only a~~there is a low incidence (20%) of the acrosomal reaction (AR) ~~is observed~~ in stallion spermatozoa bound to the zona pellucida~~, following 1 hour incubation of spermatozoa-oocyte complexes~~ (Ellington *et al*. 1993; Cheng *et al.* 1996; Meyers *et al.* 1996). The reported low incidence of AR in zona-bound spermatozoa suggests that several other localized biological agents ~~localized nearby the ovulated oocyte~~ contribute to induction of the AR ~~induction~~ required for ~~the~~ successful fertilization *in vivo*. Follicular fluid (FF) represents one of these agents~~m~~ as, at ovulation, it is transported, together with the cumulus-oocyte complex, from the follicle to the oviductal ampulla, where fertilization occurs. ~~Within FF, P~~progesterone (P) has been suggested to be the main active component in FF responsible for the induction of the AR. When charcoal treatment is used to remove steroid hormones ~~are removed~~ from ~~the~~ FF ~~by charcoal treatment~~, ~~its~~ the FF becomes inactive~~effect disappears~~, ~~whereas it~~efficacy is restored when the charcoal-treated FF is supplemented with P (Cheng *et al.* 1998). ~~Moreover~~Furthermore, progesterone receptors have been detected on the surface of spermatozoa in different species including ~~hu~~man (Kirkman-Brown *et al.* 2002) and equines (Cheng *et al*, 1998).

Another hormone found in the FF is leptin (OB). This hormone is the 167-amino acid product of *Ob* gene expression (Zhang *et al.* 1994) and is involved in the regulation of energy metabolism.

OB is ~~predominantly s~~synthesized predominantly by adipocytes and has been shown to be involved in the regulation of ~~the~~ various reproductive functions (Chehab et al., 1996). OB has been quantitatively assayed in human (De Placido *et al.* 2006), pig (Lackey *et al.* 2002) and mare FF (Lange-Consiglio *et al.* 2012). Both, P and OB have been reported to be involved in ~~the~~ capacitation and acrosome reactions of spermatozoa. Many studies confirm~~ed~~ that the effects of P on human spermatozoa are mediated ~~essentially~~ by the increase of intracellular calcium concentrations (Blackmore *et al.* 1990), the stimulation of activity of phospholipases (Murase and Roldan, 1996), the phosphorylation of proteins (Tesarik *et al.* 1993; Luconi *et al.* 1995; Emiliozzi *et al.* 1996) and the efflux of chloride (Meizel, 1997). It is known that OB is also involved in protein phosphorylation~~, too~~. Tyrosine phosphorylation of spermatozoan proteins during capacitation has been reported in ~~mouse~~mice, ~~human~~people, bulls, hamsters (Visconti *et al.* 1995; Leclerc *et al.* 1996; Galantino-Homer *et al.* 1997), pigs (Kalab *et al.* 1998; Flesch *et al.* 1999; Tardif *et al.* 2003) and in horses (Gonzalez-Fernandez et al., 2013). Furthermore, the Janus kinases and signal transducers and activators of transcription pathways (JAK/STAT) represent the main signaling cascades activated by OB (Thomas, 2004). The binding between OB and OB-R activates JAK2 kinase ~~that~~ causing phosphorylation of~~es~~ several tyrosine residues including those on the functional OB-R. These phosphorylative mechanisms provide binding sites for STAT3 protein that, following activation, is translocated to the nucleus where transcription of target genes is stimulated.

By this mechanism it is conceivable that OB increases protein tyrosine phosphorylation and affects both the capacitation and the acrosome reactions (Lampaio e du Plessis, 2008). In pigs and horses, as in ~~humans~~man, OB has been found in seminiferous tubules and in seminal plasma (Lackey *et al.* 2002; Aquila *et al.* 2005). OB receptors (OB-R) were~~as~~ detected on ~~human spermatozoa~~ the cell surface of human spermatozoa indicating that ~~the spermatozoon~~these may

be~~as~~ a possible target for OB in the male genital tract (Jope *et al.* 2003). In the horse, there are as yet no ~~information have been reported~~reports ~~so far about~~of the presence of OB-R on the surface of spermatozoa, ~~and~~ nor any ~~about~~ on the effects of OB on capacitation and fertilization. In this context, ~~in the present work~~ we investigated whether *OB* and *OB-R* are detectable at molecular level and by immunocytochemistry in equine spermatozoa. Since OB and P are found in FF, we also aimed to assess the individual and combined effect of OB and P in comparison to FF to better understand the role of OB in equine IVF~~ we also aimed to assess the individual and combined effect of OB and P in comparison to FF~~.

**Materials and methods**

*Materials and animals*

All reagents were purchased from Sigma Aldrich Chemical (Milano, Italy, http://www.sigmaaldrich.com/italy.html) unless otherwise specified.

Fresh semen was collected by means of an artificial vagina from three adult stallions of proven fertility. All collections were performed according to approved animal care and following protocols of the Bioethics Committee of Milan University.

Equine epididymides (N = 6) and ovaries (N = 375) were collected at a local abattoir from horses slaughtered for reasons other than the present ~~experiments~~study.

Experimental design

There were 3 parts to this study~~is study is organized in three experiments~~: in the first, ~~we assessed~~ OB and OB-R on equine spermatozoa were assessed by immunocytochemistry, Western Blot analysis and molecular biology; in the second, ~~we studied~~ the effect of OB on capacitation and hyperactivation of equine spermatozoa was studied by motility assessment, fluorescent staining,

and detection of apoptosis ~~detection~~. In this step, the individual effect of OB wa~~i~~s compared to that induced by P or by the combination of OB and P, or by FF. In the third ~~experiment~~part, ~~the~~ IVF was performed using media supplemented with OB or P or OB combined with P or FF.

Experiment 1: detection of OB and OB-R on equine spermatozoa by immunocytochemistry, Western blot analysis and molecular biology

*Immunocytochemistry*

Immunocytochemical detection of OB and OB-R was performed on fresh ejaculated spermatozoa selected by a swim-up procedure and on spermatozoa collected from the caput epididymid~~i~~s of three different stallions, pooled, and washed three times with Tris-Buffered Saline (TBS: 0.05 M Tris/HCl, 0.15 M NaCl; pH 7.5). Ten µl of concentrated cell suspension were smeared onto clean glass slides. The smears were dried and fixed in cold absolute methanol for 7 min at -20°C. After methanol removal, spermatozoa were washed in TBS and placed in a blocking solution overnight. The blocking solution consisted of 0.1 M glycine, 1% goat serum, 0.01% Triton X-100, 1% powdered non-fat dry milk, 0.5% BSA, and 0.02% sodium azide in PBS (Euroclone, Milan, Italy). After blocking, OB and OB-R staining were carried out overnight using anti-OB (A-20) and anti-OB-R (M-18) affinity purified rabbit polyclonal antibodies (code sc-842 and sc-1834-R, respectively; Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) diluted 1:100 in PBS containing 1% Triton X-100 (PBS-T).

The specificity of the immunostaining was proven using non-immune rabbit serum (DakoCytomation, Glostrup, Denmark) instead of specific antiserum or omission of the primary antibody.

Spermatozoa were then washed 4x15 min in PBS-T and incubated for 4 h with goat anti-rabbit fluorescein isothiocyanate (FITC)-conjugate secondary antibody, diluted 1:100 in PBS-T (Santa

Cruz Biotech Inc.). Slides were examined by conventional fluorescence analysis using a BX 51 microscope (Olympus) equipped with DMU filter set. Three hundred spermatozoa per slide were analyzed by a combination of 488/650 nm emission wavelength (100x objective). Similar staining on histological slides of equine adipose tissue was used as positive control.

*Western Blot analysis*

Swim-up purified spermatozoa were washed and centrifuged for 5 min at 5000 × *g* and the pellet was shaken in lysis buffer [60mM Tris-HCl (pH 6.8), 50 mM DTT, 2% SDS, 10% glycerol, 1 mM PMSF] for 15 min. Protein concentration was evaluated using Bradford's assay and an equal quantity of proteins (20µg) were diluted and boiled for 10 min in 2× Laemmli SDS buffer. Samples were electrophoresed on 10% SDS-polyacrylamide gels and electroblotted onto Bio-Rad Nitrocellulose membranes 0,2um using a Bio-Rad trans-blot electrophoretic cell and reagents. The primary antibodies [anti-OB (A-20) and anti-OB-R (M-18) affinity purified rabbit polyclonal antibodies; Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA] were diluted 1:1000 in a solution containing 2% BSA, 0.01% NaN3 in PBS and were incubated for 2 h at about 20°C and rinsed 3 times with PBS plus 0.05% Tween 20. Anti-β-actin antibody (ab8226 from ABCAM) was used to normalize the sample loading. The secondary antibody reactions were performed using Immun-Star™ Goat Anti-Rabbit or Goat Anti-Mouse AP Detection Kit reagents for Western blot and ChemiDoc™ MP imager (Bio-Rad). Adipose tissue lysate was used as a positive sample and, the negative control was swim-up spermatozoa lysate immunodepleted with anti-OB or anti-OB-R after immune-precipitation with protein A-G Agarose.

*Ob and Ob-R mRNA detection*

Total RNA from equine adipose tissue, ejaculated spermatozoa after swim-up and immature spermatozoa from epididymides was isolated using TRIZOL® reagent (Invitrogen Life Technologies Italia, Monza, Italy) following the manufacturer's protocol and according to Das *et al.* ( 2010). After treating the samples with DNase, RNA concentration and purity were measured using a NanoDrop Spectrophotometer (NanoDrop® ND1000, Wilmington, DE, USA). Complementary DNA was synthesized from 500 ng of total RNA using TaqMan® Reverse Transcription reagents (Applied Biosystems Italia, MB, Italy). Conditions used were 25°C for 5 min, 42°C for 30 min and 85°C for 5 min. Qualitative PCR was performed using 1 µl of the obtained cDNA in 25 µl final volume with Jumpstart™ Taq ReadyMix™ under the following conditions: initial denaturation at 94°C for 2 min, 35 cycles at 94°C for 30 sec (denaturation), 60°C for 30 sec (annealing), 72°C for 2 min (elongation) and final elongation at 72°C for 5 min. PCR products were analyzed by gel electrophoresis and visualized using an UV Gel Doc trans-illuminator (Bio-Rad Life Science, Segrate, Mi, Italy). Equine specific oligonucleotides primers were designed based on NCBI *Equus caballus* available sequences or on Mammal multi-aligned sequences. Primers were used at 300 nM final concentration and their sequences are as~~the~~ follow~~sing~~: Glycerhaldehyde-3-phosphate dehydrogenase *(GAPDH):* forward 5'-AGATCAAGAAGGTGGTGAAG-3' and reverse 5'-TTGTCATACCAGGAAATGAGC-3' (Product size: 170bp), leptin-Receptor (*Ob-R*): forward 5'-TCCAAGTCACATCTGGTGGA-3' and reverse 5'-GGTAAAAGTGTTGGGCTGGA-3' (Product size: 154bp), leptin (*Ob*): forward 5'-GCACTGTGGACCCCTGTGC-3' and reverse 5'-TGGAGGAGACTGACTGCGTG-3' (Product size: 180bp). Equine adipose tissue was employed as a positive control.

Experiment 2: capacitation and hyperactivation of equine spermatozoa assessed by motility, fluorescent staining and apoptosis

*Spermatozoa Culture Media*

In this study, the modified Whittens non-capacitating medium (MW: 100 mM NaCl, 4.7 mM KCl, 1.2 mM $MgCl_2$, 5.5 mM glucose anhydrous, 22 mM HEPES, 4.8mM lactic acid hemicalcium salt and 1.0 mM pyruvic acid; Travis *et al.* 2004) was used. To obtain the capacitating conditions, non-capacitating MW base medium was supplemented with 25 mM $NaHCO_3$ and 7 mg/mL BSA. This medium was called capacitating medium (CM). For both media, the final pH was 7.25.

Another medium used in this study was the FF. FF was drawn from preovulatory follicles of mares' ovaries during~~at~~ estrous collected at the local abattoir. The assessment of the diameter of the follicle (about 4.5 cm) along with the visual analysis of the presence of uterine endometrial folds and by the value of circulating progesterone (<1 ng/ml) confirmed that mares were in estrus. Pooled FF was centrifuged (1300 g, 10 min, room temperature) to remove cells, and then frozen at -80°C until P assays were performed. P concentration was determined on pooled FF using a quantitative test based on the ELFA (Enzyme Linked Fluorescent Assay; Mini-Vidas, bioMérieux Italia S.p.A., Florence, Italy) technique (Anckaert *et al.* 2002).

Other media employed in this study were: CM supplemented with 10ng/mL of recombinant human OB (Sigma L4146), CM supplemented with 200 ng/mL of P (Sigma P8783), and CM supplemented with 10 ng/mL of OB combined to 200 ng/mL of P.

R~~In this study, r~~ecombinant human OB was used because, after multiple sequence alignments studies, this OB ~~showed the higher similarity~~was most similar ~~in respect~~ to ~~the~~ horse OB ~~species~~.

*Semen Preparation*

Preparation of fresh semen was performed as previously reported (McPartlin *et al.* 2009) with some modifications. Spermatozoa motility and concentration of each sample diluted in pre-warmed non-capacitating MW and kept at 37°C were analyzed by computer assisted semen analysis (CASA). A customized CASA system was assembled with an Olympus BX 51 microscope fitted with a warming stage, negative phase contrast optics (20x objective and 10x ocular) and a Basler (model A6021-2) video camera interfaced with a computer to digitize and analyze the image. The software used for image acquisition and analysis was Image-Pro Plus 5.1-Media Cybernetics (Immagini & Computer, Bareggio, Milano, Italy).

The instrument setting for computerized semen analysis is reported in Ttable 1.

An aliquot of 5 µl of diluted semen was pipetted into a pre-warmed 20µm-depth counting chamber (Cell-Vu Chambers, Fertility Technologies Inc., IMV, Piacenza, Italy). Spermatozoa motility was assessed within 20 s. For each sample, ten microscope fields were analyzed. Some parameters were measured directly on the digital images (velocity parameters and movements of the head) whilst others were calculated from the measurements, *e.g.* the straightness of movement and the percentage of motile or progressively motile spermatozoa. The cell track was reconstructed on sequential digital images by the accompanying software.

*Apoptotic rate*

The percentage of apoptotic spermatozoa was assessed using an Annexin-V-FITC Apoptosis Detection KIT following the manufacturers' instructions. Samples were analyzed every two hours from 0 h to 6 h of incubation under capacitating conditions: 1) CM, 2) FF, 3) CM supplemented with OB (10ng/mL), 4) CM supplemented with P (200 ng/mL); 5) CM supplemented with OB (10 ng/mL) combined with P (200 ng/mL). Reactions wereas performed on 500 µl of semen. Three hundred spermatozoa were analyzed usingby a combination of

488/560 nm emission. Spermatozoa at the early stage of apoptosis~~apoptotic process~~ stained positively only for the annexin V-FITC, necrotic spermatozoa for PI and Annexin V-FITC, and live spermatozoa did not stain positively for either PI or Annexin V-FITC.

*Capacitation of spermatozoa*

Pooled samples of ejaculated spermatozoa from three stallions, in triplicate, were centrifuged in 15 mL conical tubes at 100xg for 1 min (at 37°C) to remove particulate matter and dead spermatozoa. The supernatant was transferred to a 14 mL round-bottom centrifuge tube and centrifuged at 600xg for 5 min (at 37°C). The pellet of pooled spermatozoa was resuspended in CM to a final concentration of $10x10^6$ spermatozoa/mL and 500 µL aliquots were incubated for 6 h in polyvinyl alcohol coated 5-mL round-bottom tubes (Holmquist, 1982) at 37°C in a humidified air atmosphere.

*Hyperactivation of spermatozoa*

Hyperactivation of spermatozoa resuspended in CM was induced by incubation for an additional 6 h in different media: 1) CM (control: CTR), 2) FF, 3) CM supplemented with OB (10ng/mL), 4) CM supplemented with P (200 ng/mL), 5) CM supplemented with OB (10 ng/mL) combined with P (200 ng/mL). For each condition the final cell concentration was standardized to $1x10^6$ spermatozoa/mL and spermatozoa motility was analyzed by CASA every two hours from 0 h to 6 h of incubation ~~by CASA~~. In addition to the percentage of motile spermatozoa, five motility parameters were evaluated: the average path velocity (VAP, µm/sec; the average velocity of the smoothed cell path), the straight-line velocity (VSL, µm/sec; the average velocity measured in a straight line from beginning to end of the track), the amplitude of lateral head displacement (ALH, µm; the mean width of head oscillations), the straightness (STR, %; the average value of

the ratio VSL/VAP x 100) and the linearity index (LIN, %; the average of the ratio VSL/VCL x 100).

~~At a preliminary level~~First, a dose-response study on the effects of recombinant human OB on spermatozoa was performed in order to identify the best concentration of this hormone to be used for the following experiments. Spermatozoa were incubated in CM supplemented with OB at different concentrations, from 0 to 50 ng/mL at 5ng/mL interval. Spermatozoa motility assessment, fluorescent staining (FITC-PNA/PI) for viability and acrosome reaction, and apoptotic rate evaluation were performed every two hours from 0 h to 6 h after treatments.

The following experiments were performed using ~~the~~ OB at ~~the concentration of~~ 10 ng/mL based on the optimal results obtained for motility, viability, acrosome reaction and the lower response of apoptotic rate (data not shown).

*Fluorescent staining (FITC-PNA/PI) for viability and acrosome reaction*

Fluorescein isothiocyanate-conjugated peanut agglutin (FITC-PNA) was used to determine the acrosome status of viable spermatozoa in all the conditions of hyperactivation tested: 1) CM, 2) FF, 3) CM supplemented with OB (10 ng/mL), 4) CM supplemented with P (200 ng/mL), 5) CM supplemented with OB (10 ng/mL) combined with P (200 ng/mL). Propidium iodide (PI) staining was used to detect apoptotic spermatozoa. FITC-PNA intensely labeled the acrosome region of acrosome-reacted spermatozoa (green), whereas PI stained the head of dead spermatozoa (red). Evaluation was performed every two hours from 0 h to 6 h of incubation. Briefly: 500µl of semen were diluted in HEPES/BSA solution (130 mM NaCl, 4 mM potassium chloride, 14 mM fructose, 10 m*M* HEPES, 1 mM calcium chloride, 0.5 mM magnesium chloride, 0.1% BSA) to reach the concentration of $15x10^6$ spermatozoa/mL. Samples were then incubated with 2.5µl of propidium iodide (PI, 1 µg/mL) and 2.5 µl FITC-PNA (1 µg/mL) at 37°C for 5 min under light-

proof conditions. Spermatozoa were fixed in 10% formalin solution. Three hundred spermatozoa per slide were immediately examined using a BX 51 microscope equipped with DMU filter set using a simultaneous combination of excitation and emission filters at 488/650 nm at 100x magnification.

Experiment 3: *in vitro* fertilization

*Recovery and maturation of oocytes*

Ovaries were obtained during the natural reproductive season (from March to August) immediately after slaughter, maintained at 30°C in PBS supplemented with antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin sulphate) and processed within 6 h of slaughtering. Cumulus-oocyte complexes (COCs) were harvested by scraping the surface of obvious follicles. Only compact cumulus oocytes (CCOs) with at least three layers of cumulus cells were assigned for IVM. CCOs were washed three times in basic TCM199 medium with Earle's salts, buffered with 4.43 mM HEPES, supplemented with 10% fetal bovine serum and 25 µg/µmL gentamicin (M199). IVM was performed following the ~~procedure~~ method previously described (Dell'Aquila *et al.* 2003). Medium TCM-199 with Earle's salts, buffered with 4.43 mM HEPES and 33.9 mM sodium bicarbonate and supplemented with 0.1 g/l L-glutamine, 2 mM sodium pyruvate, 2.92 mM calcium-L-lactate pentahydrate (Fluka 21175 Serva Feinbiochem GmbH & Co) and 50 µg/mL gentamicin was used. pH was adjusted to 7.18 and the medium was filtered through 0.22-µm filters (No.5003-6, Lida Manufacturing Corp) and stored at 4°C until use (for a maximum of one week). On the day of IVM, the medium was further supplemented with 20% (v/v) fetal calf serum. Then, gonadotrophins (10 µg/mL ovine follicle-stimulating hormone, FSH, and 20 µg/mL ovine luteinizing hormone, LH) and 1 µg/mL17β-estradiol were added. The m~~M~~edium was re-filtered and equilibrated for 1 h under 5% $CO_2$ in air before use.

Compact COCs were washed three times in the culture medium and groups of up to 10 COCs were placed in 400 µl of medium/well of a four-well dish (Nunc Intermed), covered with pre-equilibrated lightweight paraffin oil and cultured for 29 h at 38.5°C under 5% $CO_2$.

The protocol for the oocytes maturation was standardized in our laboratory and, as reported by Lange-Consiglio et al. (2009), the maturation rate of compact cumulus oocytes reached the 43-45%.

*In vitro fertilization (IVF)*

$10x10^6$ spermatozoa/mL were pre-incubated for 6 h in CM, then diluted to $1x10^6$ spermatozoa/mL with: 1) CM, 2) FF, 3) CM supplemented with OB (10ng/mL), 4) CM supplemented with P (200 ng/mL), 5) CM supplemented with OB (10 ng/mL) combined with P (200 ng/mL). A sample of semen was pre-incubated in non-capacitating medium for 6h and then diluted in each of the five mediaum mentioned above, and used for IVF.

Five mature mare oocytes were transferred into droplets of 100µl of each spermatozoa suspensions and incubated for 18 h at 38.5°C in 5% $CO_2$. Oocytes were then transferred into DMEM/F-12 for three days to evaluate the rate of embryo developments. For each experimental condition, a spermatozoa-free control was performed to assess parthenogenesis. The presence of two pronuclei and a spermatozoa tail before cleavage were evaluated, on a sample of oocytes, at the end of IVF by aceto-orcein stain (1% orcein in 45% acetic acid, followed by aceto-glycerol (glycerol 20%, acid acetic 20%, distilled water 60%). Moreover, cleavage was evaluated by Hoechst staining on a sample of oocytes after 24 hours of co-incubation with spermatozoa.

Oocytes were considered fertilized if one or more decondensing spermatozoa heads or pronuclei were observed, or if they cleaved to the two-cell stage. Degenerating oocytes containing no

chromatin or fragmented chromatin, and oocytes that failed to mature to metaphase II were not counted in the assessment of fertilization rates.

Statistical Analysis

The experiments on semen were repeated three times on pooled ejaculates from three stallions. The values reported represent the mean values. The IVF study was repeated four times. Data were analyzed by one-way ANOVA with post-test using standard parametric methods through a system of linear model analysis of variance. When significant differences ($P<0.05$) were detected, the Student-Newman-Keuls method was applied to assess all pair-wise multiple comparisons.

The statistical analyses were carried out using GraphPad Instat 3.00 for Windows (GraphPad Software, La Jolla, CA, USA).

**Results**

Experiment 1: detection of OB and OB-R on equine spermatozoa by immunocytochemistry, Western blot analysis and molecular biology

*Leptin OB and OB-R localization and expression in equine spermatozoa*

~~The presence of~~ OB and OB-R were~~as~~ weakly detected in ejaculated spermatozoa in the post-acrosomal region and in the middle piece of equine spermatozoa (Fig. 1 A). The adipose tissue, used as positive control, stained positively for both OB and OB-R (Figure 1B). The negative controls ~~resulted in the absence of~~ demonstrated no immunoreactivity.

At mRNA level, *Ob* was detected in ejaculated spermatozoa but RT-PCR failed to detect signal for the *Ob-R* transcripts in the same samples (Fig. 1 C). *Ob* and *Ob-R* expression was confirmed in immature spermatozoa collected from the ~~caput~~ head of the epididym~~id~~is (Fig. 1 D).

*Western Blot analysis*

The presence of OB protein in equine spermatozoa was also investigated by Western blotting. ~~One~~ A single lane corresponding to the molecular weight of 16 kDa was observed in the lysate obtained from equine sperm samples ~~corrisponding~~corresponding to that observed in the adipocyte extract used as positive control (Fig. 2A). Some weak immunoreactive lanes associated ~~to~~ with different isoforms were obtained for OB-R. For this protein, one main band corresponding to the molecular weight of 90 kDa was also found (Fig. 2B).

Experiment 2: capacitation and hyperactivation of equine spermatozoa assessed by motility, fluorescent staining and apoptosis.

To evaluate the effects of P in our experimental settings, we measured the levels of P in FF. The mean concentration of P evaluated by quantitative test was 200 ng/mL.

*Apoptotic rate*

~~Six hours of incubation in FF, OB or P did not~~ There was no e~~a~~ffect on the apoptotic rate of spermatozoa after six hours of incubation in FF, OB or P when compared to those ~~–~~ in CM, demonstrating no cytotoxic effect (Table 2).

*Hyperactivation of spermatozoa*

The addition of different media to stallion spermatozoa at 0 h induced an immediate change in motility. This change was characterized by the decrease ($P<0.05$) of VSL, STR, and LIN, increase of AHL ($P<0.05$) and ~~acquisition of~~onset of circling motione ~~motility~~. After 6 hours

incubation in the tested media, spermatozoa were slower compared to those incubated in CM medium, ~~as detected by~~ showing a further decrease ($P<0.05$) ~~of~~ in VSL, STR, and LIN. (Table 3).

*Fluorescent staining (FITC-PNA/PI) for viability and acrosome reaction, and apoptosis*

Immunofluorescent patterns of vitality and acrosome reaction staining of equine spermatozoa are shown~~ed~~ in Table 4. As determined by FITC-PNA/PI staining, at the beginning of incubation on ~~the~~ average 66.1%, 61.6%, 61.11%, 60.3% and 60.8% of viable spermatozoa were detected respectively ~~detected~~ in CM, FF, CM with OB, CM with P, and CM with a combination of OB and P, ~~without~~ with no significant difference ~~among~~ between media. Following 6 hours incubation, spermatozoa viability progressively decreased to 48.8%, 26.3%, 14.3%, 29.7%, and 25.5% respectively. Effects in each ~~condition differed~~sample differing significantly from the control.

The initial percentage of AR in live equine spermatozoa at time 0 of incubation was 10.7%, 11.4%, 9.2%, 10.5% and 12.7%~~–~~ in CM, FF, CM with OB, CM with P, or CM with a combination of OB and P, respectively. No significant difference was found ~~among~~ between the different culture conditions. After 6 hours of incubation, the number of capacitated spermatozoa, expressed as live acrosome-reacted (AR), increased significantly ($P<0.05$) reaching the ~~rates~~ percentages of 44.6%, 35.3%, 46.1% and 44.8% respectively in FF, CM with OB, CM with P, or CM with a combination of OB and P. In CM, the ~~rate~~ percentage of AR spermatozoa remained constant until the last time point. In FF the ~~rate~~ percentage of live AR spermatozoa was achieved after 4 hours of incubation, when the percentage of dead spermatozoa was lower. The incidence of AR spermatozoa in the OB treated sample was lower and significantly different from FF, P and OB combined with P.

Experiment 3: in vitro fertilization

~~As~~ Fertilization was confirmed~~evidenced~~ by either pronuclear formation (Fig. 3) or cleavage to the two-cell stage, the fertilization rates obtained were 51% ± 4.83% (26/51 oocytes) for IVF with FF, 46.15% ± 3.18% (24/52) with P and 43.64% ± 3.63% (24/55) with P combined with OB. No fertilization was achieved using OB (0/55) or CM (0/42). Our IVF setting only allowed for the attainment of 8 cell stage embryos which were obtained when combining the capacitating conditions with the induction of hyperactivation. The 8 cell stage embryos were produced in four independent replicates at the following rates: 18.7±1.90% using FF, 17.1% ± 1.15 in presence of P, and 16.7% ± 0.51 when OB was combined to P. Spermatozoa incubated in OB alone and in the other conditions (in CM alone, or in non capacitating medium supplemented with FF or OB or P or OB-P combination), did not fertilize oocytes (Table 5, Fig. 4) as demonstrated by the Hoechst staining. These data are net of the rate of parthenogenesis that was about 6% in each experimental condition as calculated on test sample oocytes.

**Discussion**

In this study, we investigated whether *OB* and *OB-R* were detectable in equine spermatozoa. ~~The~~ Immunocytochemical analysis detected the presence of OB and OB-R in~~at~~ the post-acrosomal region, as well as in the middle piece of equine spermatozoa. Antibod~~y~~ies~~s~~ specificity was supported by the positive controls on equine adipose tissues. The Western Blot analysis ~~sustained~~ supporteed this result. At the molecular level, however, the expression of *Ob-R* was only confirmed in ejaculated spermatozoa letting us speculate that, during spermatozoa maturation, ~~the~~ *Ob-R* transcription stops. ~~In this perspective,~~This suggests that the presence of protein is mainly due to the translation process occurring during the earlier stages of spermatogenesis. The

expression of *Ob-R* found in immature spermatozoa collected from the ~~caput~~ head of the epididymis confirmed our hypothesis.

The expression pattern of one isoform at 90 kDa, observed in this study, is different from t~~w~~hat reported for pig spermatozoa by Aquila *et al.* (2008) ~~that~~ who observed different isoforms and by De Ambrogi *et al*. (2007) ~~that~~ who demonstrated one isoform at 382bp, although Aquila et al. (2008) claim that the pattern of OB-R expression is species-specific.

To our knowledge, this is the first study reporting the presence of *Ob* and *Ob-R* mRNA in equine ejaculated spermatozoa. Aquila et al. (2005b) were the first to demonstrate ~~a~~ direct production of OB by human ejaculated spermatozoa indicating that spermatozoa are effective in secreting OB, despite their supposed inability to translate transcripts. In contrast to Grunewald *et al.* (2005), Aquila *et al.* (2005a) proposed that some of these transcripts could encode for proteins that are essential in early embryo development. More recently, the presence of *Ob* mRNA was also detected ~~also~~ in bull spermatozoa (Abavisani *et al.* 2011) supporting the idea that spermatozoa are able to transcribe *Ob* and translate it.

As demonstrated in pig spermatozoa, OB affects both capacitation and acrosome reaction through its receptor (Aquila *et al.* 2008). After detecting OB and OB-R in ejaculated spermatozoa by immunocytochemistry and gene expression, ~~for a deeper understanding of the involvement of OB in equine IVF,~~ we investigated its effect alone or in combination with P in the challenging settings of equine IVF to gain a deeper understanding of the involvement of OB in equine IVF ~~settings~~. Results were then compared to those obtained when ~~inducing~~ *in vitro* capacitation was induced using the FF conditions.

Based on the analysis of the motion parameters currently used to define hyperactivated motility in other species (Mortimer and Mortimer, 1990; Suarez *et al.* 1992; Ho and Suarez, 2001; Marquez and Suarez, 2004; Baumber and Meyers, 2006; Marquez and Suarez, 2007; Marquez *et*

*al.* 2007), ~~the~~ incubation with FF, or OB, or P or their combination ~~-~~significantly affected stallion spermatozoa motility. Stallion spermatozoa can be defined as hyperactivated when a VSL< 46.5 µm/sec, a STR< 46.6% and a LIN< 20.2% are detected (McPartlin *et al.* 2009). As shown in this study, spermatozoa tracks shortened, became more curved and the decrease in VSL, LIN and STR was consistent with the acquisition of hyperactivation.

Although the ability of FF to induce capacitation and motility changes has already been established, as well as the potential of P to increase the permeability of the plasma membrane to calcium (Therien and Manjunath, 2003), there ~~are~~ is no evidence~~s about~~ of the involvement of OB in equine spermatozoa hyperactivation and fertilization. Despite a claim by Li *et al.* (2009) ~~claimed no significant effect of~~that ~~-~~OB had no significant effect on ~~human~~ ejaculated human spermatozoa motility~~;~~, our study suggests a regulatory role of OB signaling in spermatozoa motility. This hypothesis comes from the expression of OB-R observed in the middle piece of equine spermatozoa~~-~~, which is the region that mainly contributes to spermatozoa motility.

Capacitation is not only associated with hyperactivation, but also with the acquired capability of spermatozoa to undergo the acrosome reaction after binding to the zona pellucida. Following incubation in FF~~F~~, or in CM supplemented with OB, or P or the combination of OB and P, the number of capacitated spermatozoa (expressed as live acrosome-reacted) increased significantly ($P < 0.05$). After 6 hours of incubation, these ~~rates~~ percentages reached 44.6% for FF, 35.3% for OB, 46.1% for P and 44.8% for OB combined with P. Conversely, in CM alone, the rate of AR spermatozoa remained constant overtime. According to Cheng et al (1998), P~~,~~ alone, or combined with OB, increased ~~-~~the proportion of physiological AR spermatozoa to the same level as~~of~~ FF ~~compared~~ in contrast to the effects of OB alone. Moreover, our results showed that spermatozoa pre-incubated in CM for 6h and then exposed to fertilizing medium supplemented with FF, P or the combination of OB and P for 18h, fertilized oocytes leading to 8 cell stage embryo

development at the rate of 18.7%, 17.1% and 16.7%, respectively. On the other hand, spermatozoa in medium supplemented with OB did not fertilize any oocytes.

It can be argued that P facilitated IVF by promoting both hyperactivation and acrosome reaction. Many studies report that this hormone, secreted by cumulus cells and contained in the FF, induces AR in human (Baldi *et al.* 1998; Meizel and Turner, 1991), mouse (Roldan *et al.* 1994), boar (Melendrez *et al.* 1994), stallion (Cheng *et al.* 1998; Meyers *et al.* 1995), golden hamster (Llanos and Anabalon, 1996), dog (Brewis *et al.* 2001), and caprine (Somanath *et al.* 2000) spermatozoa. P also affects human spermatozoa capacitation (Foresta *et al.* 1992; Uhler *et al.* 1992; Luconi *et al.* 1995) and increases the ability of mouse spermatozoa ~~ability~~to respond to the zona pellucida (Roldan *et al.* 1994). Steroid effects are mediated by proteins acting as receptors on the cell surface (Meizel and Turner, 1991). The acrosome reactions produced by the exposure of capacitated spermatozoa to P could explain the rate of penetration in our IVF setting. Thus, because neither incubation of spermatozoa in capacitation conditions alone nor the treatment of non capacitated spermatozoa with P supported IVF in our experiments, these results additionally support the hypothesis that the pre-incubation step in capacitation medium is required for the activation of functional receptors evoked by ~~the~~ P binding. As reported by~~ccording to~~ McPartlin *et al.* (2009), ~~the evidences we provide in our study~~these results support the hypothesis that capacitation and hyperactivation are ~~separable~~ separate and independent events. Regarding OB, our results are in agreement with those reported for capacitation and acrosome reaction in pig spermatozoa (Aquila *et al.* 2008), although the rate of AR spermatozoa was lower compared to that obtained with P. The AR rate was ~~figured~~ calculated on live spermatozoa after OB treatment, highlighting the effect of OB compared to the control. In any case, after IVF no spermatozoa penetration ~~was detected~~occurred and, therefore, no embryo was obtained.

It has been demonstrated that pig seminal plasma contains a significant amount of OB and that this amount is lower in the FF (Lackey *et al.* 2002). As spermatozoa leave seminal plasma during ~~their~~ transit in the female reproductive tract, they are exposed to decreased OB concentrations. Aquila *et al.* (2008) speculated that the high OB concentrations in seminal plasma might contribute to maint~~ain~~enance of spermatozoa in a quiescent metabolic condition. ~~On the contrary~~Conversely, the low OB concentrations in the ~~pig~~ secretions of the female pig reproductive tract ~~secretions~~ (Gregoraszczuk *et al.* 2004) could ~~contribute to~~ induce spermatozoa activation, by facilitating their capacitation and acquisition of fertilizing ability. Previous results from our laboratory indicated that supplementation with OB in the range between 10 and 1000 ng/mL increases the maturation rate of equine oocytes and enhances the fertilization rate after ICSI, thus confirming the stimulatory effect of OB on oocyte quality and fertilization rate after ICSI (Lange-Consiglio *et al.* 2009). Based on this~~e~~ information, OB supplementation ~~it would be anticipated a~~ would be expected to have a positive influence ~~of OB supplementation~~ in standard equine IVF. Since no embryos were obtained, this hypothesis remains open to question~~s~~. It is possible to speculate that although ~~from~~ previous experiments ~~10 ng/mL was set as the~~suggested ~~the~~ an optimal concentration of 10 ng/mL (data not shown), this dosage was not able to support oocyte fertilization in the co-culture system. Recently we evaluated ~~the~~ OB levels in mare preovulatory FF (Lange-Consiglio *et al.* 2012) and, in that case, ~~the~~ concentrations ranged ~~obtained fluctuated~~ between 3.36 to 5.72 ng/mL in adult Standardbred and draft mares, respectively but, by the dose response in this study, the same concentrations did not activate equine spermatozoa *in vitro*. It remains to be ~~established~~ seen whether ~~any~~ interaction between the OB-R on spermatozoa and the OB produced by the female genital tract ~~is occurring in driving~~drives fertilization *in vivo*.

In conclusion, in this study we induced hyperactivation and acrosome reaction to equine spermatozoa by FF, OB, P, and OB combined with P. We obtained 8 cell stage embryos by using FF, P or a combination of OB and P. Interestingly, the supplementation of CM with OB in our IVF conditions did not help ~~in~~ oocyte~~s~~ fertilization. Based on these results, we can speculate that P, but not OB, is the key factor in FF to achieve IVF ~~in this study~~.

This finding may indicate that, equine OB is specifically involved in the improvement of ooplasmic maturity (Lange-Consiglio *et al.* 2009) even though during co-culture with spermatozoa no effects were seen ~~were revealed~~ on th~~os~~e external ~~investments~~ layers of the oocyte (corona radiata, intercellular matrix, zona pellucida and oolemma)~~,~~ which are involved in spermatozoa penetration, ~~neither~~ nor were effects on spermatozoa ~~were evident~~. This observation is a significant finding as OB produced no toxic effects ~~of OB occurred~~ as demonstrated ~~by~~ in our previous study ~~about~~ of apoptosis. Nevertheless, it is important not to rule out the potential action of OB, nor its possible involvement in ~~other~~ spermatozoan functions other than thos~~e ones~~e studied, such as sperm-zona pellucida binding and/or zona pellucida-induced acrosome reaction.

This ~~paper~~ study confirms the difficulties in achieving ~~overcoming the inability to~~ appropriate~~ly~~ capacitation~~e~~ and/or hyperactivation of~~e~~ stallion spermatozoa *in vitro*. It supports the hypothesis that capacitation and hyperactivation are required for a successful IVF in ~~the equine species~~ horses and, for the first time, it demonstrates the presence of *Ob* and *Ob-R* mRNA and their proteins in equine spermatozoa. Further studies are required to determine the role of these ~~although their role needs to be further assessed~~.

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**References**

Abavisani, A., Baghbanzadeh. A., Shayan. P., Dehghani. H. (2011). Leptin mRNA in bovine spermatozoa. *Res Vet Sci* **90**, 439–442.

Anckaert, E., Mees, M., Schiettecatte, J., Smitz, J. (2002). Clinical validation of a fully automated 17beta-estradiol and progesterone assay (VIDAS) for use in monitoring assisted reproduction treatment. *Clin Chem Lab Med* **40**, 824–831.

Aquila, S., Gentile, M., Middea, E., Catalano, S., Andò, S. (2005a). Autocrine regulation of insulin secretion in human ejaculated spermatozoa. *Endocrinology* **146**, 552–557.

Aquila, S., Gentile, M., Middea, E,, Catalano, S., Morelli, C., Pezzi, V., Andò, S. (2005b). Leptin secretion by human ejaculated spermatozoa. *J Clin Endocrinol Metab* **90**, 4753–4761.

Aquila, S., Rago, V., Guido, C., Casaburi, I., Zupo, S., Carpino, A. (2008). Leptin and leptin receptor in pig spermatozoa: evidence of their involvement in sperm capacitation and survival. *Reproduction* **136**, 23–32.

Baldi, E., Luconi, M., Bonaccorsi, L., Forti, G. (1998). Nongenomic effects of progesterone on spermatozoa: mechanisms of signal transduction and clinical implications. *Front Biosci* **3**, D1051–1059.

Baumber, J., Meyers, S.A. (2006). Hyperactivated motility in rhesus macaque (Macaca mulatta) spermatozoa. *J Androl* **27**, 459–468.

Bezard, J., (1992). In vitro fertilization in the mare. In: Proceedings of the International Scientific *Conference on Biotechnics in Horse Reproduction. Agricultural University of Cracow, Poland*, Abstract 12.

570 Blackmore, P.F., Beebe, S.J., Danforth, D.R., Alexander, N. (1990). Progesterone and 17 alpha-
571 hydroxyprogesterone. Novel stimulators of calcium influx in human sperm. *J Biol Chem* **265**,
572 1376–1380.
573 Brewis, I.A., Morton, I.E., Moore, H.D., England, G.C. (2001). Solubilized zona pellucida
574 proteins and progesterone induce calcium influx and the acrosome reaction in capacitated dog
575 spermatozoa. *Mol Reprod Dev* **60**, 491–497.
576 Chehab, F.F., Lim, M.E., Lu, R: (1996). Correction of the sterility defect in homozygous obese
577 female mice by treatment with the human recombinant leptin. *Nat Genet* **12,** :318-320.
578 Cheng, F.P., Fazeli, A.R., Voorhout, W.F., Marks, A., Bevers, M.M., Colenbrander, B. (1996).
579 Use of peanut agglutinin to assess the acrosomal status and the zona pellucida-induced
580 acrosome reaction in stallion spermatozoa. *J Androl* **17**, 674–682.
581 Cheng, F.P., Fazeli, A.R., Voorhout, W.F., Tremoleda, J.L., Bevers, M.M., Colenbrander, B.
582 (1998). Progesterone in mare follicular fluid induces the acrosome reaction in stallion
583 spermatozoa and enhances in vitro binding to the zona pellucida. *Int J Androl* **21**, 57–66.
584 Das, P.J., Paria, N., Gustafson-Seabury, A., Vishnoi, M., Chaki, S.P., Love, C.C., Varner, D.D.,
585 Chowdhary, B.P., Raudsepp, T. (2010). Total RNA isolation from stallion sperm and testis
586 biopsies. *Theriogenology* **74**, 1099–1106.
587 Dell'Aquila, M.E., Albrizio, M., Maritato, F., Minoia, P., Hinrichs, K. (2003). Meiotic
588 competence of equine oocytes and pronucleus formation after intracytoplasmic sperm
589 injection (ICSI) as related to granulosa cell apoptosis. *Biol Reprod* **68**, 2065–2072.
590 De Placido, G., Alviggi, C., Clarizia, R., Mollo, A., Alviggi, E., Strina, I., Fiore, E., Wilding, M.,
591 Pagano, T., Matarese, G. (2006). Intra-follicular leptin concentration as a predictive factor for
592 in vitro oocyte fertilization in assisted reproductive techniques. *J Endocrinol Invest* **29**,719–
593 726.

De Ambrogi, M., Spinaci, M., Galeati, G., Tamanini C. (2007) Leptin receptor in boar spermatozoa. *In J Androl* **30**, 458–461.

Ellington, J.E., Ball, B.A., Yang,X. (1993). Binding of stallion spermatozoa to the equine zona pellucida after coculture with oviductal epithelial cells. *J Reprod Fertil* **98**, 203–208.

Emiliozzi, C., Cordonier, H., Guérin, J.F., Ciapa, B., Benchaïb, M., Fénichel, P. (1996). Effects of progesterone on human spermatozoa prepared for in-vitro fertilization. *Int J Androl* **19**, 39–47.

Flesch, F,M,, Colenbrander, B,, van Golde, L.M., Gadella, B.M. (1999). Capacitation induces tyrosine phosphorylation of proteins in the boar sperm plasma membrane. *Biochem Biophys Res Commun* **262**, 787–792.

Foresta, C., Rossato, M., Mioni, R., Zorzi, M. (1992). Progesterone induces capacitation in human spermatozoa. *Andrologia* **24**, 33–35.

Galantino-Homer, H.L., Visconti, P.E., Kopf, G.S. (1997). Regulation of protein tyrosine phosphorylation during bovine sperm capacitation by a cyclic adenosine 3'5'-monophosphate-dependent pathway. *Biol Reprod* **56**, 707–719.

Gonzalez-Fernandez, L., Macıas-Garcıa, B. Loux, S.C., Varner, D.D., Hinrichs, K (2013). Focal adhesion kinases and calcium/calmodulin-dependent protein kinases regulate protein tyrosine phosphorylation in stallion sperm. *Biol Reprod* **88**, 1-12.

Graham, J.K. (1996). Methods for induction of capacitation and the acrosome reaction of stallion spermatozoa. *Vet Clin North Am Equine Pract* **12**, 111–117.

Gregoraszczuk, E.L., Ptak, A., Wojtowicz, A.K., Gorska, T., Nowak, K.W. (2004). Estrus cycle-dependent action of leptin on basal and GH or IGF-I stimulated steroid secretion by whole porcine follicles. *Endocr Regul* **38**, 15–21.

617 Grunewald, S., Paasch, U., Glander, H.J., Anderegg, U. (2005). Mature human spermatozoa do
618 not transcribe novel RNA. *Andrologia* **37**, 69–71.
619 Ho, H.C., Suarez, S.S. (2001). An inositol 1,4,5-trisphosphate receptor-gated intracellular Ca(2+)
620 store is involved in regulating sperm hyperactivated motility. *Biol Reprod* **65**, 1606–1615.
621 Holmquist, L. (1982). Surface modification of Beckman Ultra-Clear centrifuge tubes for density
622 gradient centrifugation of lipoproteins. *J Lipid Res* **23**, 1249–1250.
623 Jope, T., Lammert, A., Kratzsch, J., Paasch, U., Glander, H.J. (2003). Leptin and leptin receptor
624 in human seminal plasma and in human spermatozoa. *Int J Androl* **26**, 335–341.
625 Kalab, P., Peknicová, J., Geussová, G., Moos, J. (1998). Regulation of protein tyrosine
626 phosphorylation in boar sperm through a cAMP-dependent pathway. *Mol Reprod Dev* **51**,
627 304–314.
628 Kirkman-Brown, J.C., Punt, E.L., Barratt, C.L. Publicover, S.J. (2002). Zona pellucida and
629 progesterone-induced Ca21 signaling and acrosome reaction in human spermatozoa. *J Androl*
630 **23**, 306–315.
631 Lackey, B.R., Gray, S.L., Henricks, D.M. (2002). Measurement of leptin and insulin-like growth
632 factor-I in seminal plasma from different species. *Physiol Res* **51**, 309–311.
633 Lange-Consiglio, A., Arrighi, S., Fiandanese, N., Pocar, P., Aralla, M., Bosi, G., Borromeo, V.,
634 Berrini, A., Meucci, A., Dell'aquila, M.E., Cremonesi, F. (2012). Follicular fluid leptin
635 concentrations and expression of leptin and leptin receptor in the equine ovary and in vitro-
636 matured oocyte with reference to pubertal development and breeds. *Reprod Fertil Dev*
637 http://dx.doi.org/10.1071/RD12188.
638 Lange-Consiglio, A., Dell'Aquila, M.E., Fiandanese, N., Ambruosi, B., Cho, Y.S., Bosi, G.,
639 Arrighi, S., Lacalandra, G.M., Cremonesi, F. (2009). Effects of leptin on in vitro maturation,

fertilization and embryonic cleavage after ICSI and early developmental expression of leptin (Ob) and leptin receptor (ObR) proteins in the horse. *Reprod Biol Endocrinol* **7**:113.

Lampaio, F., du Plessis, S.S. (2008). Insulin and leptin enhance human sperm motility, acrosome reaction and nitric oxide production. *Asian J Androl* **10**: 799-807.

Leclerc, P., de Lamirande, E., Gagnon, C. (1996). Cyclic adenosine 3',5'monophosphate-dependent regulation of protein tyrosine phosphorylation in relation to human sperm capacitation and motility. *Biol Reprod* **55**, 684–692.

Li, H.W., Chiu, P.C., Cheung, M.P., Yeung, W.S., O, W.S. (2009). Effect of leptin on motility, capacitation and acrosome reaction of human spermatozoa. *Int J Androl* **32**, 687–694.

Llanos, M,N,, Anabalón, M,C. (1996). Studies related to progesterone-induced hamster sperm acrosome reaction. *Mol Reprod Dev* **45**, 313–319.

Luconi, M., Bonaccorsi, L., Krausz, C., Gervasi, G., Forti, G., Baldi, E. (1995). Stimulation of protein tyrosine phosphorylation by platelet-activating factor and progesterone in human spermatozoa. *Mol Cell Endocrinol* **108**, 35–42.

Marquez, B., Ignotz, G., Suarez, S.S. (2007). Contributions of extracellular and intracellular Ca2+ to regulation of sperm motility: Release of intracellular stores can hyperactivate CatSper1 and CatSper2 null sperm. *Dev Biol* **303**, 214–221.

Marquez, B., Suarez, S.S. (2007). Bovine sperm hyperactivation is promoted by alkaline-stimulated Ca2+ influx. *Biol Reprod* **76**, 660–665.

Marquez, B., Suarez, S.S. (2004). Different signaling pathways in bovine sperm regulate capacitation and hyperactivation. *Biol Reprod* **70**, 1626–1633.

McPartlin, L.A., Suarez, S.S., Czaya, C.A. Hinrichs, K., Bedford-Guaus, S.J. (2009). Hyperactivation of stallion sperm is required for successful in vitro fertilization of equine oocytes. *Biol Reprod* **81**, 199–206.

664 Meizel, S. (1997). Amino acid neurotransmitter receptor/chloride channels of mammalian sperm
665 and the acrosome reaction. *Biol Reprod* **56**, 569–574.
666 Meizel, S., Turner, K.O. (1991). Progesterone acts at the plasma membrane of human sperm. *Mol*
667 *Cell Endocrinol* **77**, R1-5.
668 Melendrez, C.S., Meizel, S., Berger, T. (1994). Comparison of the ability of progesterone and
669 heat solubilized porcine zona pellucida to initiate the porcine sperm acrosome reaction in
670 vitro. *Mol Reprod Dev* **39**, 433–438.
671 Meyers, S.A., Liu, I.K., Overstreet, J.W., Vadas, S., Drobnis, E.Z. (1996). Zona pellucida binding
672 and zona-induced acrosome reactions in horse spermatozoa: comparisons between fertile and
673 subfertile stallions. *Theriogenology* **46**, 1277–1288.
674 Meyers, S.A., Overstreet, J.W., Liu, I.K., Drobnis, E.Z. (1995). Capacitation in vitro of stallion
675 spermatozoa: comparison of progesterone-induced acrosome reactions in fertile and subfertile
676 males. *J Androl* **16**, 47–54.
677 Mortimer, S.T., Mortimer, D. (1990). Kinematics of human spermatozoa incubated under
678 capacitating conditions. *J Androl* **11**, 195–203.
679 Murase, T., Roldan, E.R. (1996). Progesterone and the zona pellucida activate different
680 transducing pathways in the sequence of events leading to diacylglycerol generation during
681 mouse sperm acrosomal exocytosis. *Biochem J* **320** (Pt 3), 1017–1023.
682 Palmer, E., Bézard, J., Magistrini, M., Duchamp, G. (1991). In vitro fertilization in the horse. A
683 retrospective study. *J Reprod Fertil* Suppl **44**, 375–384.
684 Roldan, E.R., Murase, T., Shi, Q.X. (1994). Exocytosis in spermatozoa in response to
685 progesterone and zona pellucida. *Science* **266**, 1578–1581.
686 Somanath, P.R., Suraj, K., Gandhi, K.K. (2000). Caprine sperm acrosome reaction: promotion by
687 progesterone and homologous zona pellucida. *Small Rumin Res* **37**, 279–286.

688 Suarez, S,S., Dai, X.B., DeMott, R.P., Redfern, K., Mirando, M.A. (1992). Movement
689 characteristics of boar sperm obtained from the oviduct or hyperactivated in vitro. *J Androl* **13**,
690 75–80.
691 Tardif, S., Dubé, C., Bailey, J.L. (2003). Porcine sperm capacitation and tyrosine kinase activity
692 are dependent on bicarbonate and calcium but protein tyrosine phosphorylation is only
693 associated with calcium. *Biol Reprod* **68**, 207–213.
694 Tesarik, J., Moos, J., Mendoza, C. (1993). Stimulation of protein tyrosine phosphorylation by a
695 progesterone receptor on the cell surface of human sperm. *Endocrinology* **133**, 328–335.
696 Thérien, I., Manjunath, P. (2003). Effect of progesterone on bovine sperm capacitation and
697 acrosome reaction. *Biol Reprod* **69**, 1408–1415.
698 Thomas, T. (2004). The complex effects of leptin on bone metabolism through multiple
699 pathways. *Curr Opin Pharmacol* **4**, 295–300.
700 Travis, A.J., Tutuncu, L., Jorgez, C.J., Ord, T.S., Jones, B.H., Kopf, G.S., Williams, C.J. (2004).
701 Requirements for glucose beyond sperm capacitation during in vitro fertilization in the mouse.
702 *Biol Reprod* **71**, 139–145.
703 Uhler, M.L., Leung, A., Chan, S.Y., Wang, C. (1992). Direct effects of progesterone and
704 antiprogesterone on human sperm hyperactivated motility and acrosome reaction. *Fertil Steril*
705 **58**, 1191–1198.
706 Visconti, P.E., Bailey, J.L., Moore, G.D., Pan, D., Olds-Clarke, P., Kopf, G.S. (1995).
707 Capacitation of mouse spermatozoa. I. Correlation between the capacitation state and protein
708 tyrosine phosphorylation. *Development* **121**, 1129–1137.
709 Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L., and Friedman, J.M. (1994)
710 Positional cloning of the mouse obese gene and its human homologue. *Nature* **372**, 425-432.

711

712 **Table 1.** Instrument setting for computerized semen analysis

| Parameter | Value |
|---|---|
| Number of frames to analyze | 20 frames |
| Sampling frequency | 30 frames/s |
| Minimum sampling points for motility | 1 point |
| Minimum sampling points for velocity | 3 points |
| Maximum velocity | 150 μ/s |
| Threshold velocity | 8 μ/s |
| Minimum sampling points for calculatinge AHL | 7 points |
| Minimum velocity for calculatinge AHL | 20 μ/s |
| Minimum linearity for calculatinge AHL | 3.5 |
| Pixel scale | 0.688 μ/pixel |
| Cell size range | 4-15 pixel |

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**Table 2. Percentage of apoptotic spermatozoa after exposure to different capacitation media.**

Data are expressed as mean ± standard deviation. Different small letters superscripts (a,b,c) indicate statistically different comparisons ($p<0.05$) between lines. Different capital letter superscripts (A,B,C,D) indicate statistically different comparisons ($P<0.05$) between columns.

| Time of exposure (h) | CM | CM+FF | CM+P | CM+OB | CM + OB and P |
|---|---|---|---|---|---|
| **0** | $16\pm0.55^{acA}$ | $18\pm0.42^{bA}$ | $16\pm0.55^{acA}$ | $15\pm0.33^{aA}$ | $17\pm0.64^{cA}$ |
| **2** | $17\pm0.53^{aAB}$ | $16\pm1.11^{aA}$ | $16\pm0.27^{aA}$ | $18\pm1.65^{aA}$ | $17\pm0.72^{aA}$ |
| **4** | $18\pm0.77^{aB}$ | $17\pm1.31^{aA}$ | $18.\pm1.73^{aA}$ | $23\pm2.31^{bB}$ | $19\pm0.61^{aB}$ |
| **6** | $21\pm0.72^{aC}$ | $18\pm0.44^{aA}$ | $17\pm0.79^{bcA}$ | $25\pm2.91^{dB}$ | $20\pm0.48^{abB}$ |

CM:capacitating medium; FF: follicular fluid; P: progesterone; OB: leptin

737 **Table 3. Stallion sperm hyperactivation in different capacitation media at two set times**

738 Data are expressed as mean ± standard deviation. Different small letters superscripts (a,b) indicate statistically different comparisons
739 ($p<0.05$) between 0h and 6h in each employed medium. Different capital letter superscripts (A,B) indicate statistically different
740 comparisons ($P<0.05$) at 6h between CM and other media
741

| Media employed | VAP (µm/s) | | VSL (µm/s) | | AHL (µm) | | STR (%) | | LIN (%) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **0h** | **6h** | **0h** | **6h** | **0h** | **6h** | **0h** | **6h** | **0h** | **6h** |
| **CM** | $141.5\pm4.8^{a}$ | $124.2\pm9.22^{bA}$ | $87.4\pm9.44^{a}$ | $67.6\pm5.34^{bA}$ | $6.12\pm0.22^{a}$ | $6.33\pm0.76^{bA}$ | $61.77\pm0.32^{a}$ | $54.00\pm0.46^{bA}$ | $34.33\pm0.27^{a}$ | $29.11\pm0.11^{bA}$ |
| **FF** | $167.3\pm13.8^{a}$ | $110.1\pm9.52^{bB}$ | $66.9\pm6.9^{a}$ | $40.5\pm4.10^{bB}$ | $7.57\pm0.43^{b}$ | $10.42\pm0.94^{bB}$ | $40.00\pm0.7^{a}$ | $36.72\pm0.37^{bB}$ | $19.71\pm0.12^{a}$ | $17.23\pm0.21b^{B}$ |
| **CM+ P** | $161.1\pm5.6^{a}$ | $113.4\pm4.33^{bB}$ | $63.2\pm5.3^{a}$ | $42.3\pm3.98^{bB}$ | $7.42\pm0.61^{b}$ | $9.44\pm0.82^{bB}$ | $39.23\pm1.3^{a}$ | $38.22\pm1.51^{aB}$ | $21.33\pm0.65^{a}$ | $19.35\pm0.23b^{B}$ |
| **CM + OB** | $135.0\pm6.22^{a}$ | $115.6\pm10.11^{bB}$ | $85\pm5.77^{a}$ | $47.7\pm3.83^{bB}$ | $8.04\pm0.51^{b}$ | $9.91\pm0.49^{bB}$ | $63.00\pm0.16^{a}$ | $41.22\pm0.24^{bB}$ | $31.01\pm0.18^{a}$ | $23.00\pm0.14b^{B}$ |
| **CM+ OB and P** | $164.2\pm4.22^{a}$ | $112.2\pm5.92^{bB}$ | $64.3\pm3.22^{a}$ | $43.4\pm4.22^{bB}$ | $7.22\pm0.58^{b}$ | $10.37\pm0.99^{bB}$ | $38.62\pm0.33^{a}$ | $37.71\pm0.77^{aB}$ | $22.46\pm0.48^{a}$ | $18.87\pm0.52b^{B}$ |

742 CM:capacitating medium; FF: follicular fluid; P: progesterone; OB: leptin
743 VAP: average path velocity; VSL: straight-line velocity; AHL: average laterale head;STR: straightness; LIN: linearity index.

**Table 4. Fluorescent staining (FITC-PNA) for acrosome reaction (AR) in different capacitation media.**

Data are expressed as mean ± standard deviation. Different small letters superscripts (a,b,c) indicate statistically different comparisons (P<0.05) between lines. Different capital letter superscripts (A,B,C,D) indicate statistically different comparisons (P<0.05) between columns.

| Type of spermatozoa | Time of exposure (h) | (CM) | CM+FF | CM+ P | CM+OB | CM+OB and P |
|---|---|---|---|---|---|---|
| **Viable** | 0 | 66.1±3.54$^{aA}$ | 61.6±4.33$^{aA}$ | 60.3±3.43$^{aA}$ | 61.1±4.24$^{aA}$ | 60.8±2.88$^{aA}$ |
| | 2 | 62.1±4.72$^{aAC}$ | 52.7±3.63$^{bA}$ | 54.6±2.94$^{bA}$ | 49.4±3.55$^{bB}$ | 51.7±3.72$^{bA}$ |
| | 4 | 54.2±4.55$^{aBC}$ | 26.9±3.22$^{bB}$ | 28.3±3.12$^{bB}$ | 34.3±2.76$^{bC}$ | 27.4±4.11$^{bB}$ |
| | 6 | 48.8±2.65$^{aB}$ | 26.3±5.62$^{bB}$ | 29.7±3.66$^{bB}$ | 14.3±1.65$^{bD}$ | 25.5±3.52$^{bB}$ |
| **Viable with AR** | 0 | 10.7±2.02$^{aA}$ | 11.4±1.22$^{aA}$ | 10.7±1.55$^{aA}$ | 9.2±2.11$^{aA}$ | 12.7±1.76$^{aA}$ |
| | 2 | 14.6±2.55$^{aA}$ | 15.4±2.63$^{aA}$ | 18.5±3.63$^{aB}$ | 15.9±1.67$^{aB}$ | 16.7±1.43$^{aB}$ |
| | 4 | 16.0±4.12$^{aA}$ | 48.4±1.69$^{bB}$ | 47.4±1.76$^{bcC}$ | 25.3±2.87$^{dC}$ | 35.6±1.52$^{eC}$ |
| | 6 | 15.8±5.02$^{aA}$ | 44.6±2.59$^{bB}$ | 46.±1.99$^{bC}$ | 35.0±2.98 $^{cD}$ | 44.8±1.88$^{bD}$ |
| **Dead** | 0 | 10.7±1.74$^{aA}$ | 13.6±3.04$^{aA}$ | 12.7±1.59$^{aA}$ | 12.5±1.87$^{aA}$ | 13.0±1.04$^{aA}$ |
| | 2 | 9.2±0.76$^{aA}$ | 17.3±1.65$^{bcA}$ | 13.5±1.63$^{bA}$ | 16.3±2.08$^{bcA}$ | 17.9±1.92$^{cB}$ |
| | 4 | 12.2±0.99$^{aAC}$ | 12.9±1.43$^{aA}$ | 13.7±1.72$^{abA}$ | 16.0±1.66$^{abA}$ | 17.0±1.74$^{bB}$ |
| | 6 | 15.3±2.86$^{aBC}$ | 14.3±3.74$^{aA}$ | 11.6±1.02$^{aA}$ | 27.0±2.85$^{bB}$ | 16.1±1.92$^{aAB}$ |
| **Dead with AR** | 0 | 12.4±2.10$^{aA}$ | 13.4±1.33$^{abA}$ | 16.4±1.87$^{abA}$ | 17.1±1.55$^{bA}$ | 13.5±1.22$^{abA}$ |
| | 2 | 14.0±1.98$^{aAC}$ | 14.7±2.75$^{aA}$ | 13.4±1.04$^{aAC}$ | 18.4±2.84$^{aA}$ | 13.6±0.68$^{aA}$ |
| | 4 | 17.6±2.33$^{aBC}$ | 11.8±1.53$^{bA}$ | 10.7±1.99$^{bBC}$ | 24.3±2.88$^{cB}$ | 20.0±1.79$^{aB}$ |
| | 6 | 20.1±1.35$^{aB}$ | 14.8±2.98$^{bA}$ | 10.6±1.65$^{bBC}$ | 23.7±1.99$^{cB}$ | 13.5±1.11$^{bA}$ |

CM:capacitating medium; FF: follicular fluid; P: progesterone; OB: leptin.

**Table 5. Development to 8 cell-stage embryos.**

Data are expressed as mean ± standard deviation. Values labeled with different letters are statistically different (P<0.05).

| Replicate | CM (%) | CM+FF (%) | CM+P (%) | CM+OB (%) | CM+OB and P (%) | Non CM+ FF (%) | Non CM+ P (%) | Non CM+ OB (%) | Non CM+ OB and P (%) |
|---|---|---|---|---|---|---|---|---|---|
| **1** | 0/10 (0.0) | 4/19 (21.1) | 3/18 (16.7) | 0/22 (0.0) | 5/29 (17.2) | 0/11 (0.0) | 0/12 (0.0) | 0/13 (0.0) | 0/11 (0.0) |
| **2** | 0/12 (0.0) | 4/24 (16.7) | 5/27 (18.5) | 0/25 (0.0) | 4/25 (16.0) | 0/10 (0.0) | 0/14 (0.0) | 0/10 (0.0) | 0/12 (0.0) |
| **3** | 0/13 (0.0) | 4/21 (19.1) | 3/19 (15.8) | 0/18 (0.0) | 4/24 (16.7) | 0/10 (0.0) | 0/12 (0.0) | 0/11 (0.0) | 0/13 (0.0) |
| **4** | 0/13 (0.0) | 3/17 (17.7) | 4/23 (17.4) | 0/20 (0.0) | 3/18 (16.7) | 0/12 (0.0) | 0/9 (0.0) | 0/10 (0.0) | 0/11 (0.0) |
| **average** | 0.0±0.0 | 18.65±1.90[a] | 17.1±1.15[a] | 0.0±0.0 | 16.7±0.51[a] | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0± (0.0) |

CM:capacitating medium; FF: follicular fluid; P: progesterone; OB: leptin; Non CM: non capacitating medium.

**Figure legends**

**Fig. 1.** Immunocytochemistry and molecular expression of leptin (OB) and leptin receptor (OB-R). A) Immunolocalization in the post-acrosomal region and in the tail in equine ejaculated spermatozoa. B) Immunolocalization of OB and OB-R in equine adipose tissue used as control. Magnification 20x. C) *Ob* and *Ob-R* expression at mRNA level in equine spermatozoa. Signal for *GAPDH* (employed as reference gene) and leptin transcripts observed in adipose tissue (a: positive control), total ejaculate (b), sperm after swim-up (c) and sperm after swim-up and following 6 hours of incubation with exogenous leptin (d). e: blank. D) *GAPDH*, *OB* and *OB-R* transcripts observed in immature spermatozoa from head of epididymis ~~head~~. Marker: 100bp.

**Fig. 2.** Western Blot to assess the presence of A) OB and B) OB-R on two horse spermatozoa samples (S1,S2). Adipose tissue (AT) lysate was used as positive sample and sperm lysate immunodepleted of OB or OB-R was employed as negative control (ctrl). β-actin represents the reference marker.

**Fig. 3.** Acteo-orcein stain showing presence of two pronuclei. The arrow at the left indicates the female pronucleus and the arrow at the right the male pronucleus.

**Fig. 4.** Representative *in vitro* fertilized mare oocytes. A) Light microscopy of two-cell stage embryos (Day 1). B) Hoechst 33258 staining showing two pronuclei. C) Light microscopy of eight-cell stage embryos (Day 3). D) Hoechst 33258 staining showing eight pronuclei. Original magnification x20. Scale bar represent 20µm.40 mins