billion stallion sperm cells and all of the stud dog sperm cells were diluted to a total volume of 40 mL with Easy Mixin CST (Animal Reproduction Systems, Chino, CA, USA). Samples (1 mL) of the extended semen were cooled to a temperature of 4 °C and stored for 48 h. After incubation, the semen was evaluated for motility, and CFU counts were performed. The remainder of the extended semen was centrifuged for 15 min at 5000 rpm and the supernatant removed. The sperm pellet was resuspended in 3.5 mL of Easy Freezin LE (Animal Reproduction Systems) for the stallion and 2.0 mL for the stud dog, drawn into straws and cryopreserved. After cryopreservation, the semen was thawed in a 32–37 °C water bath for 10–15 s. The semen was evaluated for motility, and CFU counts were performed. All semen samples had motility that was within normal ranges for the procedures performed. The results are shown in the chart below. Expected CFU/mL numbers were calculated from the inoculums and were based on the expectation that centrifugation would concentrate fungal organisms. In this study, both the hyphae and conidia of *A. fumigatus* and the yeast of *C. albicans* survived cool-extension and cryopreservation. Centrifugation during the cryopreservation process concentrated the fungal organisms as well as the sperm cells.

Sample	*Candida yeast*		*Aspergillus conidia*		*Aspergillus hyphae*	
	Expected CFU (mL)	Actual CFU (mL)	Expected CFU (mL)	Actual CFU (mL)	Expected CFU (mL)	Actual CFU (mL)
Inoculum	1×10^7	0.6×10^7	0.7×10^7	1.5×10^7	Unknown	1.0×10^3
Horse RI	1×10^6	0.9×10^6	1×10^6	2.5×10^6	1×10^2	1.8×10^2
Dog RI	1×10^6	0.8×10^6	1×10^6	2.8×10^6	1×10^2	3.0×10^2
Horse CE	1.8×10^5	2.4×10^5	1×10^5	3.2×10^4	1.8×10^2	0.5×10^2
Dog CE	1.4×10^5	1.1×10^5	1×10^5	2.8×10^4	0.1×10^2	0.2×10^2
Horse CP	4.0×10^6	3.0×10^6	1.1×10^6	2.7×10^5	2.0×10^2	1.8×10^2
Dog CP	3.0×10^6	2.0×10^6	2.0×10^6	3.6×10^5	2.0×10^2	2.5×10^2

Colony forming unit count results. RI = raw inoculated, CE = cool-extended, CP = cryopreserved.

Keywords: Semen cryopreservation; Cool-extension; Equine; Canine; Fungus

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EFFECTS OF AGE AND FSH ON COLLECTION OF EQUINE OOCYTES AND DEVELOPMENTAL COMPETENCY AFTER INTRACYTOPLASMIC SPERM INJECTION

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Reduced fertility in aged mares is associated with retarded early embryonic development, potentially associated with delayed fertilization or reduced developmental competence. Intracytoplasmic sperm injections (ICSI) could reduce fertilization delays, and exogenous FSH could maximize oocyte and embryo production. Objectives were to compare oocytes collected per cycle and embryonic development after ICSI for young (4–9 years) and old (>20 years) mares, with or without FSH. Treatment with FSH (equine FSH, 6.25 mg, im, bid; Bioniche Animal Health Inc., Athens, GA, USA) began when at least one follicle >20 mm was detected during diestrus. Treated and non-treated cycles were alternated. Oocytes were collected from follicles >30 mm 20–24 h after LH (EquiPure LH™, 0.75 mg, iv; AspenBio Pharma Inc., Castle Rock, CO, USA) using transvaginal aspirations. Oocytes were matured further in TCM199 at 38.5 °C in 6% CO_2 in air. Sperm were injected 40 ± 1 h after LH, using frozen sperm from a single ejaculate. Resulting zygotes were incubated in DMEM/F12 at 38.5 °C in 5% CO_2, 5% O_2 and 90% N_2. Blastocysts considered viable (formation before 9 days and adequate quality) were transferred non-surgically into recipients 3–7 days after ovulation. Follicle and oocyte data were analyzed using two-way ANOVA for main effects of age and FSH (and their interaction); Tukey's hsd was used to separate means. Developmental data were compared

Table 1
Effects of mare age and FSH treatment on numbers of oocytes collected per cycle, cleavage and blastocysts per oocyte, and pregnancies with a heartbeat per embryo transfer

	Young mares ($n = 22$)		Old mares ($n = 15$)	
	eFSH	No eFSH	eFSH	No eFSH
Oocytes per cycle*	1.08 ± 0.1^{a}	0.74 ± 0.1^{b}	1.08 ± 0.1^{a}	0.86 ± 0.1^{ab}
Cleavage rate at 48 h after ICSI (%)	74 (14/19)	52 (14/27)	62 (13/21)	67 (18/27)
Blastocysts per oocyte injected (%)	42 (8/19)	30 (8/27)	24 (5/21)	22 (6/27)
Pregnancies per embryo transfer (%)	50 (3/6)	33 (2/6)	40 (2/5)	60 (3/5)

Values with different superscripts (a and b) within rows differ ($P < 0.05$).
*LS means ± S.E.M.

using Fisher's Exact Test (SAS Institute Inc., Cary, NC, USA). More ($P = 0.01$) oocytes were collected per cycle from mares treated with FSH than not treated (LS means ± S.E.M. of 1.1 ± 0.1 and 0.8 ± 0.1, respectively). Cleavage, blastocyst and pregnancy rates were not affected ($P > 0.05$) by age or FSH treatment (Table 1). Numbers of delayed embryos (<4 cells at 48 h) were similar ($P = 0.8$) for young and old mares (8/28, 29% and 10/31, 32%). In conclusion, FSH increased the number of oocytes collected per cycle. Embryo development was not different after ICSI of oocytes from young and old mares. We inferred that ICSI could be used to alleviate fertilization delays, and potentially improve early embryo development in old mares.

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Keywords: Oocyte; FSH; Aging; Embryo; Intracytoplasmic sperm injection

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DOSE RESPONSE TO OVULATION-INDUCING FACTOR (OIF) IN LLAMAS

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The existence of an ovulation-inducing factor (OIF) has been documented recently in the seminal plasma of llamas and alpacas—a discovery that challenges the concept that physical stimulation is responsible for eliciting ovulation in these species [1]. The objectives of the study were to determine if the dose of OIF needed to elicit ovulation is physiologically relevant (i.e., what proportion of an ejaculatory dose), and to test the hypothesis that CL form and function is affected by OIF in a dose-dependant manner. Female llamas were assigned randomly to five groups ($n = 10$/group) and given a single im dose of 500, 250, 125 or 60 μg of purified OIF (representative of 1/2 to 1/16 that is present in a single ejaculate), or 1 mL phosphate-buffered saline (controls). Ovulation and CL development were monitored by transrectal ultrasonography. Blood samples were taken every-other-day from Days 0 (the day of treatment) to 16 for measurement of plasma progesterone concentrations. More frequent blood sampling (16 samples in 12 h) was done on four animals per group to determine changes in plasma concentrations of LH. Ovulation rates were compared among groups by Chi-square analysis. Single-point measurements were compared among groups by analysis of variance, and serial data were compared by analysis of variance for repeated measures using Tukey's multiple comparisons as a post hoc test (GraphPad Prism Software, Version 3.0, San Diego, CA, USA). The diameter of the largest follicle at the time of treatment did not differ among groups ($P = 0.30$). Ovulation rates were 9/10, 9/10, 7/10, 3/10, 0/10 in groups given decreasing doses of OIF and the control group, respectively ($P < 0.05$). The maximum diameter of the CL was greatest ($P < 0.05$) in the high-dose group (mean ± S.E.M.; 13.5 ± 0.5, 10.9 ± 0.7, 11.6 ± 0.7, 10.9 ± 1.0 mm). The day-to-day profiles of CL diameter and plasma progesterone concentration were highest in the high-dose group, and decreased in a dose-dependent manner (day effect, $P < 0.01$; group effect, $P < 0.01$; day-by-group interaction, $P < 0.01$). Similarly, plasma LH concentrations during the 12-h period after treatment were highest in the high-dose group ($P < 0.05$), and decreased in a dose-dependent manner (time effect, $P < 0.01$; group effect, $P = 0.18$; day-by-group interaction, $P < 0.01$). A rise in plasma LH concentration was apparent in all llamas that ovulated, and absent in all but one llama (treated with 60 μg of OIF) that failed to ovulate. Among only those llamas that ovulated, the CL was detected earliest (2.1 ± 0.1, 2.5 ± 0.2, 2.3 ± 0.2, 3.3 ± 0.3 days after treatment, respectively; $P < 0.05$),