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PII: S0093-691X(23)00517-4

DOI: https://doi.org/10.1016/j.theriogenology.2023.12.033

Reference: THE 16927

To appear in: *Theriogenology*

Received Date: 20 July 2023

Revised Date: 28 December 2023

Accepted Date: 28 December 2023

Please cite this article as: Carrasco RA, Bogle OA, Ratto MH, Ulloa-Leal C, Mamani-Cato RH, Adams GP, Predictive characteristics of male fertility in alpacas with special reference to seminal NGF, *Theriogenology* (2024), doi: https://doi.org/10.1016/j.theriogenology.2023.12.033.

Predictive characteristics of male fertility in alpacas with special reference to seminal NGF

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Declaration of conflict of interest: none

Abstract

Recent studies document the LH-releasing pathway of nerve growth factor (NGF) in male camelids and that the LH response to seminal NGF is associated with elevated plasma testosterone concentration. Results provide rationale for the hypothesis that NGF in semen is associated with male fertility. In Experiment 1, the association between the amount of NGF in the ejaculate and characteristics of the male reproductive system was examined in alpacas. The concentration of NGF was measured by radioimmunoassay in semen samples collected from male alpacas (n=47) and correlated with sperm morphology and motility, and measurements of the male reproductive anatomy. Most ejaculates had NGF concentrations that, based on previous studies, triggered ovulation in female camelids, however, we only found a positive correlation between NGF concentration with sperm concentration, thread formation and total NGF, and a negative correlation with pH. In Experiment 2, a retrospective analysis was carried out to determine if breeding performance during the previous season was related to recent concentrations of seminal NGF in male alpacas (n=22). Birth rates tended to be correlated with sperm concentration and total amount of NGF in the ejaculate (P=0.09). Experiment 3 was a prospective study to determine the relationship between seminal NGF (n=8 male alpacas) and ovulation and pregnancy rates in a breeding trial. No association was detected between seminal NGF concentration and ovulation rate, pregnancy rate, or LH response in the female. We conclude that among the breeding males used in our study, the abundance of seminal NGF was correlated with sperm concentration and thread formation, however, it was not predictive of male fertility in alpacas. Examination of males not previously selected as breeding stock may be

expected to include a broader range of seminal NGF and provide a more comprehensive understanding of the relationship between seminal NGF and male fertility.

Keywords: alpaca, semen, NGF, fertility.

1. Introduction

South American camelids are classified as induced ovulators [1]. As such, the delivery of competent sperm for fertilization and the release of the oocyte from the ovarian follicle are initiated through the process of mating. Coitus was associated with a subsequent increase in the circulating concentration of luteinizing hormone (LH) which drives ovulation [2–4]. Interestingly, a similar LH secretion profile was observed in females after either copulation (with an intact or vasectomized male) [2,4,5] or after intramuscular administration of seminal plasma [6–9]. Hence, it is unlikely that penile stimulation of somatosensory nerves in the female is a primary stimulus for ovulation in these animals. In this regard, alpacas ovulated after endometrial abrasion and intrauterine deposition of seminal plasma but not after abrasion and deposition of saline solution [10]. Consistent with this observation, results of a more recent study showed that llamas mated with a urethrostomized male failed to ovulate while llamas mated with an intact male did ovulate [11]. Initially the seminal factor responsible was referred to as ovulation-inducing factor (OIF) [reviewed in 9], but has since been identified as the neurotrophin, nerve growth factor (NGF) [12]. The potency of this factor has been demonstrated in several studies [12–14] where more than 90% of female llamas or alpacas, isolated from male contact, ovulated after a single dose of

seminal plasma or purified NGF. A dose representing 1/200th (60 µg) of NGF found in a normal ejaculate (as measured from protein chromatography) elicited ovulation in 30% of female llamas and increasing the dose to 1/50th (250 µg) resulted in ovulation in ≥ 90% of females [13]. In addition, a luteotrophic effect first described in relation to prolonged LH release in llamas treated with seminal plasma [6], was confirmed in a follow-up study in which there was a dose-related increase in corpus luteum (CL) diameter and plasma progesterone concentration in response to NGF treatment [13]. The luteotrophic effect of NGF in llamas has been confirmed in three subsequent studies [15–17]. The pre-ovulatory LH surge induced by NGF is more sustained than that observed after GnRH administration [6,16,18], suggesting that the luteotrophic effect of NGF could be due to a prolonged LH secretion profile.

The presence of the high affinity receptor (trkA) and low affinity receptor (p75) for NGF in male reproductive organs and sperm of different species including llama, suggest a role for NGF in sperm function [19–24]. The addition of exogenous NGF to golden hamster semen increased sperm motility in a dose- and time-dependent manner, and increased the percentage of sperm that underwent the acrosome reaction [25]. The addition of NGF to llama sperm during cooling increased the percentage of total motility and vigor [26]. Few reports are available on the concentration of NGF in mammalian semen or its relationship with sperm morphology and function. In one study, NGF concentration in human seminal plasma was quantified using an enzyme-linked immunosorbent assay with values that ranged from 0.13 to 1.4 ng/mL [27]. In another report in humans, mean NGF concentrations in the seminal plasma were not different among fertile, oligoasthenozoospermic or asthenozoospermic men (0.82 ± 0.1, 0.68 ± 0.2 and

84 0.79 ± 0.1 ng/mL, respectively) [28]. In bovine seminal plasma, an estimated 1 mg of purified NGF
85 was isolated from 1 g of lyophilized seminal plasma (10 mL of semen), while in rabbits, an induced
86 ovulator species NGF sperm concentration ranged from 1895 pg/ml to 152 µg/ml, representing
87 1.5% of total protein content in seminal plasma [29,30]. Studies in rabbits have shown the
88 presence of NGF in the prostate and that blood plasma and seminal plasma content of NGF does
89 not change during sexual maturation [31]. Interestingly, NGF receptors were found in the
90 reproductive tract of male rabbits, and administration of NGF intravaginally triggered ovulation
91 in rabbits [23,32]. However, no reports were found regarding factors related to NGF
92 concentration in seminal plasma, nor on its correlation with sperm characteristics in camelids.

93

94 In an early study involving four estrous periods in each of 25 dairy heifers, ovulation was hastened
95 by 2 hours when heifers were bred with a vasectomized bull than when nonbred [33], and
96 administration of seminal plasma in beef and dairy cows tended to improve pregnancy rates in
97 herds with compromised fertility (<50% fertility, [34]). In llamas, NGF and its high-affinity
98 receptor were found in the female reproductive tract, and breeding induced expression of NGF
99 in the oviduct [35]. Additionally, intrauterine administration of NGF increased endometrial
100 vascularization bilaterally 12 hours after administration, suggesting a role on uterine function
101 [36]. In a recent report, administration of NGF to male llamas and alpacas increased circulating
102 LH concentrations in a surge-like manner, and maintained elevated circulating testosterone
103 concentrations [37]. The latter suggests a functional role of NGF in the male camelid reproductive
104 physiology via an endocrine or an exocrine route.

105

106 In the present study, we tested the hypothesis that NGF in semen is associated with male fertility
107 in South American camelids. The objectives were to determine the relationship between seminal
108 NGF and the morpho-functional characteristics of the male reproductive system (anatomy,
109 testosterone production, and semen and sperm parameters; Experiment 1), and examine the
110 association between the abundance of seminal NGF and breeding performance during the
111 previous breeding season (retrospective analysis; Experiment 2) and current breeding season
112 (prospective analysis; Experiment 3).

113

114 **2. Material and Methods**

115 *2.1. Animals*

116 The study was conducted from January to April at the Quimsachata Research Station in the
117 Department of Puno, Peru (15°S, 71°W, and 4500 m above sea level). Animal procedures were
118 performed in accordance with the guidelines of the Canadian Council on Animal Care and were
119 approved by the University of Saskatchewan Protocol Review Committee.

120

121 *2.2. Experiment 1 - Relationship between seminal NGF concentration, reproductive anatomy, and*
122 *sperm characteristics in male alpacas*

123 *2.2.1. Semen collection*

Male alpacas (n=47) 4-14 years of age and 45-74 kg body weight were maintained on natural pasture throughout the study. For a three-week period before sample collections, males were trained to use a phantom mount equipped with an artificial vagina [38]. Acceptance of the phantom mount was encouraged by applying freshly collected vaginal swabs or urine from receptive females to the phantom mount and orifice of the artificial vagina. In some instances, a live receptive female was placed beside the phantom mount to train the male to associate it with copulation. To facilitate the workload, males were divided into one of three collection periods (first group: n=16 males; second group: n=16; third group: n=15). For each group, ejaculates were collected on the same day using four separate phantom mounts. Mount time and semen characteristics were recorded for 6 or 7 ejaculates per male, with at least one full day of rest between collection days.

After each semen collection, a blood sample was collected by jugular venipuncture in a heparinized tube (BD Biosciences, Mississauga, Canada) and centrifuged at 1500 x g for 15 minutes. The blood plasma was harvested and stored at -20°C until testosterone assay.

2.2.2. Analysis of ejaculates

Ejaculates were evaluated for semen volume, froth volume, thread formation, pH, and sperm concentration, motility, viability and morphology in field conditions. Semen volume and froth volume were determined directly from the graduated collection tube (BD Biosciences, San Jose, California). Semen volume was defined as the liquid portion of the ejaculate at the bottom of the

collection tube; froth volume was the foamy portion that was not liquefied. Thread formation was determined by aspirating semen through an 18 G needle, placing a drop onto a glass slide and pulling the semen mass upwards (adapted from [39]). The distance from the initial drop of semen on the slide to the maximum distance before the semen retracted back to the slide was taken as the thread formation measurement (in mm). Samples in which thread formation resembled that of water were given a measurement of 0 mm. Semen pH was measured using a pH-meter (Hanna Instruments, Premier Farnell UK Limited, Leeds, United Kingdom) and confirmed with litmus paper (Whatman/GE Healthcare, Mississauga, Canada). Sperm concentration was estimated using a Neubauer hemocytometer. A 10 µL aliquot of the fresh ejaculate was placed on each side of the hemocytometer chamber (duplicate counts), and the mean sperm count of the two chambers was taken as the concentration [40]. Total sperm motility was determined by observing the oscillatory and progressive movement of the sperm at a minimum of 10 random microscopic fields using a light microscope (Zeiss Axioskop 40; Thornwood, New York, USA) at a magnification of 400 x as previously described [41]. Sperm viability and morphology was evaluated using eosin-nigrosin stain. A minimum of 20 fields and 200 sperm per sample were assessed for both live-dead and sperm morphology counts under 1000x with immersion oil. The sperm were examined for defects of the head, mid piece, principal piece and acrosome. Samples with a low sperm concentration (where 200 sperm were not counted) were excluded from morphology analysis. Analysis of all ejaculates was done by the same operator.

166 An aliquot of each ejaculate was centrifuged at 500 x g for 15 minutes to remove spermatozoa
167 without disrupting the sperm cell membrane. The supernatant was collected and centrifuged
168 again at 1500 x g for 15 minutes to ensure only seminal plasma remained. Samples were stored
169 in liquid nitrogen until NGF assay. The protein concentration of seminal plasma was quantified
170 using Bradford's method according to the manufacturer's directions (Bio-Rad Laboratories,
171 Mississauga, Ontario, Canada).

172

173 *2.2.3. Morphometry of testis and accessory sex glands*

174 Testis area (cm^2) was calculated by measurements taken at the greatest width using vernier
175 calipers and testicular area was calculated using the area of a circumference: Area = π $radius^2$.
176 The maximum height and width of the right and left lobes of the compact portion of the prostate
177 gland and the right and left bulbourethral glands were measured by transrectal ultrasonography
178 using integrated electronic calipers (7.5 MHz linear array transducer, MyLab Five, Esaote, Italy;
179 Figure 1). The area for an ellipse was used to estimate the area of each accessory gland: Area
180 =π(radius of long axis)(radius of short axis). The areas (cm^2) of the left and right lobes of the
181 prostate gland and left and right bulbourethral glands, respectively, were summed to provide a
182 total area measurement for each gland.

183

184 *2.2.4. Hormone assays*

Seminal plasma NGF concentration was measured by validated double-antibody radioimmunoassay, as previously described [38]. A subset of 3 to 4 samples were measured from each male. Frozen seminal plasma samples were thawed and diluted with PBS to a concentration that was within the range of assay detection. Samples were analyzed in duplicate in a single assay with a standard curve ranging from 0 to 200 µg/mL. The lowest detectable limit of the assay was 10 µg/mL. The intra-assay coefficients of variation for the low (25 µg/mL) and high (100 µg/mL) reference values were 10% and 6%, respectively. Ejaculates were diluted with PBS to fit within the standard curve. Values were then corrected by the dilution factor to calculate the concentration found within seminal plasma. Total NGF abundance within an ejaculate was the product of the concentration measured and the total volume of semen collected.

Circulating testosterone concentrations were measured using a commercial radioimmunoassay kit (Siemens Medical Solutions USA, Inc., Malvern, PA, USA). All samples were measured in duplicate in a single assay. The intra-assay coefficients of variation for the low, medium and high references were 14%, 6% and 5%, respectively. The minimum detectable limit of the assay was 0.1 ng/mL.

2.3. Experiment 2 - Seminal NGF concentration and previous live birth rates (retrospective)

Semen was collected and analyzed, as described in Experiment 1 (n=22 alpacas; 3-6 ejaculates per male) and correlated with respective birthing rates from the previous breeding season. Mean seminal plasma NGF concentration, calculated from the concentration measured in individual

206 ejaculates, represented individual values for each male; these concentrations were used to
207 correlate the number of offspring produced with respect to the number of females bred (n=171).
208 Nonpregnancy was diagnosed (or assumed) if the female was re-bred during the same breeding
209 season or failed to give birth, and pregnancy was confirmed by live birth during the following
210 birthing season. Breeding was controlled by staff from the Quimsachata Research Station and
211 identification of male / female pairings was recorded. Live birth rates were calculated as the
212 number of live offspring observed from the number of females that were bred.

213

214 *2.4. Experiment 3 – Prospective study using a breeding trial*

215 A randomized blind trial was done in which the breeding history of males and females, as well as
216 the seminal NGF concentration, were unknown to the researchers. Healthy adult male alpacas
217 were selected during the current breeding season (n=8). Semen was collected (n≥3
218 ejaculates/male) and processed, as described in Experiment 1, and seminal NGF concentrations
219 were analyzed (as in Experiment 1) after the completion of the experiment. For data analysis,
220 males were grouped based on seminal NGF concentration into low (n=2 males; 0.8 ± 0.4 mg/mL),
221 medium (n=4 males; 3.7 ± 0.7 mg/mL) and high (16.2 ± 5.7 mg/mL; n=2 males) groups.

222

223 Mature non-pregnant, non-lactating, female alpacas (n=160) ranging in age from 3 to 12 years
224 were used. The alpacas were selected from a group of 200 based on an initial examination to
225 confirm that they were in adequate body condition and were non-pregnant with no apparent
226 pathological conditions of the reproductive tract, as detected by transrectal ultrasonography (7.5

MHz linear array transducer, MyLab Five, Esaote, Italy). Ovarian follicle development was examined once daily [42] for at least three consecutive days by transrectal ultrasonography before breeding. Alpacas with a growing follicle ≥ 7mm in diameter in either ovary were assigned randomly to one of the eight males for breeding. Males were allowed to breed the female once, for a maximum of 30 minutes, with continuous examination during copulation to ensure proper vaginal intromission. Two females were bred to one male per day: one female in the morning and one in the afternoon. At least one full day of rest was given to males before the next set of breedings. A total of 160 copulations were monitored (20 females/male x 8 males).

Females were examined by transrectal ultrasonography on Day 3 (Day 0= day of copulation) to detect ovulation. Ovulation was defined as the disappearance of a growing follicle which had been present at the time of breeding and the subsequent formation of a corpus luteum detected on Day 3 [6]. The ovaries were examined again on Day 7 and Day 20 to determine CL diameter. Pregnancy was determined by transrectal ultrasonography on Day 20 (defined as the detection of an embryo proper) and confirmed on Day 25 (detection of the embryonic heartbeat).

A subset of female alpacas (n=2 females per male) were used to evaluate the interval from copulation to ovulation, luteal dynamics and systemic LH and progesterone concentrations. The subset of animals was examined by transrectal ultrasonography every 4 h after copulation (Day 0 = breeding) until ovulation was detected, and every-other-day until Day 20 to monitor CL development. A blood sample was taken immediately before and at 1, 2, 3, 4 and 8 hours after

248 copulation to determine changes in plasma LH concentration, and every-other-day until Day 20
249 to determine plasma progesterone concentration. Blood samples were collected into heparinized
250 tubes by jugular venipuncture (BD Biosciences, Mississauga, Canada). Samples were centrifuged
251 at 1500 x g for 15 minutes and the cellular portion was decanted, and the supernatant was stored
252 frozen at -20°C.

253

254 *2.4.1. Hormone analysis*

255 Plasma LH was measured using a double-antibody radioimmunoassay [43]. Samples were
256 analyzed in duplicate in a single assay. The minimum detectable limit was 0.1 ng NIAMDDK-oLH-
257 24. The range of the standard curve was 0.06 ng (80% ligand-labeled LH) to 8.0 ng (20% ligand-
258 labeled LH). The intra-assay coefficients of variation for the low (0.45 ng/mL) and high (2.2 ng/mL)
259 reference standards were 6% and 9%, respectively.

260

261 Plasma progesterone concentrations were measured in duplicate using a commercially available
262 double-antibody radioimmunoassay kit ([6]; Coat-A-Count Total Progesterone; Diagnostic
263 Products Corporation, Los Angeles, USA). Samples were analyzed in three consecutive assays with
264 an inter-assay coefficient of variation of 4%, 2% and 6% for reference concentrations of 1.9, 3.6
265 and 16.6 ng/mL, respectively.

266

267 *2.5. Statistical Analyses*

Statistical analyses were done using the Statistical Analysis Software package (SAS Learning Edition, 9.1; SAS Institute Inc., Cary, NC, USA). Data are reported as mean ± SEM. Differences with a P-value of ≤ 0.05 were considered significant, whereas P >0.05 but ≤ 0.10 were considered trends approaching significance.

Experiment 1: The concentration of NGF and total NGF among males were compared by Kruskal-Wallis nonparametric test. The degree of heterogeneity of NGF concentration and total abundance within males were assessed by Kruskal Wallis nonparametric test of the absolute values of residuals from the mean. Stepwise regression was used to build a prediction model for total NGF per ejaculate. Spearman's rank correlation was used to determine the relationship between variables.

Experiment 2: Associations between NGF concentration or total NGF abundance in semen with live birth rates were assessed using nonparametric Spearman rank correlation tests, given the non-normal distribution of these variables.

Experiment 3: T-test was used to compare breeding duration (i.e., morning vs. afternoon) of males. Data from males with similar abundance of NGF in their ejaculates were combined and are presented as low (n=2 males), medium (n=4) and high (n=2) NGF groups. NGF concentration and total abundance were compared among groups by Kruskal-Wallis nonparametric test.

Ovulation and pregnancy rates were compared among groups by Chi-square analysis (proc genmod procedure, SAS). Spearman rank-correlation tests were used to evaluate correlations among all endpoints. Non-serial data (i.e., breeding duration, follicle diameter on the day of breeding (Day 0), CL diameter on Day 7 and maximum CL diameter) were compared among NGF groups by one–way ANOVA. For the subset of females in Experiment 3, the interval to ovulation was compared among respective treatment groups by one-way ANOVA. Plasma LH and progesterone concentrations, luteal dynamics were compared among groups by ANOVA for repeated measures.

3. Results

3.1. Experiment 1 – Relationship between seminal NGF concentration, reproductive anatomy, and sperm characteristics in male alpacas

Of 316 collection attempts, 243 ejaculates were collected and analyzed. Failed collections (n=73) were due to 1) inclement weather (38/73), lack of libido (26/73), failed ejaculation (6/73) and urine contamination (3/73). Only 9.1% (22 of 243) of ejaculates were azoospermic. Nearly all ejaculates had froth (241/243; 99%).

The mean concentration of NGF per ejaculate was 6.1 ± 0.7 mg/mL and the total NGF per ejaculate was 7.0 ± 1.1 mg (n=153). Protein concentration and total protein content per ejaculate were 11.6 ± 1.4 mg/mL and 15.5 ± 1.9 mg, respectively. The amount of NGF per ejaculate and per

mL of seminal plasma were different among males (P=0.03), and differed among ejaculates of the same males as reflected in the analysis of absolute residuals for each male (P<0.0001). The majority of ejaculates (>90%) contained more than 250 µg of NGF (Table 1).

Correlations among male reproductive parameters are shown in Table 2. NGF concentration was positively correlated with sperm concentration, thread formation and total NGF, but negatively correlated with pH. In addition, NGF concentration tended to be correlated with seminal volume, total prostate area and total bulbourethral area (Table 2). The application of stepwise multiple regression analysis revealed 3 independent variables predictive of total NGF per ejaculate, namely NGF concentration, semen volume and prostate area (R2=0.56; Table 3). An overview of male reproductive endpoints and sperm morphology are shown in Supplementary Tables 1-2 and Supplementary Figures 1, 2.

3.2. Experiment 2 – Seminal NGF concentration and previous live birth rates (retrospective)

According to the reproductive records provided by the research station, the number of females bred per male during the previous breeding season ranged from 3 to 13. The live birth rates obtained from males (n=22) for the previous breeding season ranged from 14% to 100%. The number of live births per male was negatively correlated with semen thread formation (Spearman ρ= -0.51; P=0.02) and tended to be positively correlated with NGF concentration (Spearman ρ=0.38; P=0.09) and total NGF per ejaculate (Spearman ρ=0.36; P=0.09).

328

329 *3.3. Experiment 3 – Prospective study using a breeding trial*

330 The age of the females, copulation time, and follicle diameter at breeding was similar among low,
331 medium and high seminal NGF groups (Table 4). Ovulation and pregnancy rates were not
332 different among males with low, medium or high amounts of NGF in the ejaculate (Table 4).
333 Ovulation rates induced by individual males ranged from 40% to 100% and were not correlated
334 with seminal NGF concentration (ρ=0.33; P=0.42) or pregnancy rate (ρ=0.05; P=0.45). Pregnancy
335 rates were strongly correlated with sperm concentration (ρ=0.65; P=0.04) and viability (ρ=0.85;
336 P=0.004), and tended to be correlated with sperm motility (ρ=0.55; P=0.08) and morphology
337 (ρ=0.49; P=0.10; Table 5). The morphologic abnormalities most closely associated with pregnancy
338 rate in the present study were head size (Spearman ρ = -0.44; P= 0.01), multiple tails (Spearman
339 ρ = -0.36; P=0.04) and coiled tails (Spearman ρ =0.41; P= 0.02). Also, pregnancy rates were
340 negatively correlated with age (ρ=-0.78; P=0.01).

341

342 The interval from breeding to ovulation was longest to shortest in the low, medium, and high
343 NGF groups, respectively (29.8 ± 1.4, 28.2 ± 0.9 and 26.5 ± 1.5 hours), but differences were not
344 significant (P=0.28). Plasma LH concentrations peaked between 1 and 3 hours after breeding and
345 did not differ among groups. In all groups, plasma LH concentrations had not yet returned to
346 basal levels by 8 hours after treatment. (Fig. 2).

347

Although NGF did not influence Day 7 CL diameter in pregnant animals, low NGF-mated females had a greater CL diameter on Days 18 and 20 (treatment effect P=.79; Day effect P≤0.0001; Interaction P≤0.0001; Fig. 2). Plasma progesterone concentrations in pregnant and nonpregnant alpacas were not different among the high-, medium- and low-NGF male groups (Fig. 2). There was a tendency (P=0.08) for a lower mean progesterone concentration in pregnant animals that were mated with males in the high NGF group, but there was no difference in pregnancy rates.

Discussion

The present series of experiments were done to determine seminal predictors of reproductive performance in alpacas, in particular the abundance of NGF in the ejaculate. In Experiment 1, we examined the relationship between seminal NGF and male reproductive characteristics. Although NGF constituted 45 to 54% of total protein content of seminal plasma, high individual variability existed not only in seminal plasma NGF concentration but also in protein concentration. In subsequent experiments, we examined the relationship between the abundance of seminal NGF and the live birth rates in the preceding breeding season (Experiment 2 – retrospective) and the pregnancy rate in the current season (Experiment 3 – prospective). Results of the retrospective and prospective studies did not support the hypothesis that the abundance of NGF in seminal plasma is reflective of fertility within the context of typical field conditions.

Results of previous studies demonstrated that administration of seminal plasma or purified NGF from seminal plasma triggered LH release, ovulation and CL development in a dose-dependent

manner [13,44]. Based on a dose-response relationship, we hypothesized that alpacas with ejaculates containing low amounts of NGF may contribute to ovulation and pregnancy failure in bred females. A dose of 60 μg administered intramuscularly corresponded with a 30% ovulation rate; increasing the dose to 250 μg induced ovulation in 90% of treated llamas [13]. However, an important limitation of the present study is that only 2.6% (4/153) of ejaculates contained less than 60 μg of NGF (Experiment 1), and the mean amount of seminal NGF measured for the low, medium and high NGF groups (Experiment 3) was well above the minimum 250 μg threshold reported to generate a 90% ovulation rate in llamas given seminal NGF intramuscularly [13]. The extent to which this was a result of selective inclusion of males of known fertility (annual selection of herd sires) in the present study is unknown, but worthy of future investigation.

Another factor that may have contributed to under-estimating seminal NGF content in the present study was that most if not all ejaculates contained a volume of froth, typical of ejaculates collected with an artificial vagina. In a previous study, approximately 11% of ejaculates contained ≥ 40% of foam when artificial vagina collection method was used [45]. Possibly the foam/froth volume is liquefied in vivo and could potentially increase total semen volume and total NGF abundance. Thus, it is a plausible explanation as to why correlations between total NGF in the ejaculate and sperm parameters such as motility and concentration were not observed. Based on this, the amount of NGF deposited into the uterus at copulation was likely greater than that estimated by measurement in the fluid fraction of the ejaculate. In previous work, intrauterine deposition of 1 mL diluted seminal plasma (1:1) did not induce ovulation in alpacas [6] but ovulations (41%) occurred when the volume increased to 2 mL diluted seminal plasma (1:1) [10].

391 It is worth noting that in such previous studies [6,10], the quantity of seminal NGF used was not
392 measured, however, the volume of diluted seminal plasma was below the mean volume of
393 undiluted semen used in the present study (1.7 mL, data not shown). In a more recent study in
394 which a high dose of NGF (20 mg) was deposited into the uterus of llamas [46], 100% ovulated,
395 confirming a dose-response through the intrauterine route. Factors influencing absorption of
396 NGF by the endometrium are worthy of further investigation.

397

398 An important finding of the present study was that ovulation rate was not directly correlated
399 with pregnancy rate. Induction of ovulation ranged from 40% to 100% among males, and while
400 the male with the most NGF in his ejaculate also had the highest ovulation rate, his was among
401 the lowest pregnancy rates. Analysis of his semen revealed low sperm concentration and
402 numerous sperm defects. In contrast, the ovulation induction rate of the male with the least NGF
403 per ejaculate was not statistically lower than that of the male with the most, but had a greater
404 percentage of normal sperm and a higher pregnancy rate (35% vs. 60% pregnant for the high NGF
405 and low NGF males, respectively). The mean percentage of morphologically normal sperm per
406 ejaculate in the present study (37%) was similar to that reported by Lichtenwalner et al., [47],
407 but considerably lower than that reported by Bravo et al., [41]. Although the impact of each
408 abnormality on male fertility was not investigated in the present study, the most frequent defects
409 observed were related to the shape of the sperm head, detached heads and midpiece reflexes.
410 In cattle, sperm with pyriform heads were associated with a lower pregnancy rate, as a result of
411 reduced ability to bind and penetrate the zona pellucida, and a higher pregnancy loss rate to Day
412 60 [48]. Narrow sperm heads in cattle had a negative effect on fertility only when extreme

413 narrowness is observed in the post acrosome region [49]. Head shape was not significantly
414 correlated with lower pregnancy rates in the present study; however, it is worth noting that all
415 head shape defects (e.g., tapered, pyriform) were included in a single category, and the degree
416 and site of narrowing was not taken into consideration. We speculate that sperm defects are
417 derived from insults which occurred during spermatogenesis or during epididymal
418 transport/storage. Thus, our results warrant further investigation on the effects of NGF signalling
419 at the sperm level in South American camelids, particularly on its effects on fertilization or
420 motility.

421

422 A relationship between seminal NGF and sperm characteristics was observed in Experiment 1. In
423 azoospermic samples, NGF comprised only 10% of total protein content of seminal plasma, and
424 azoospermic samples were found in 9% of ejaculates collected. The percentage of azoospermic
425 samples in Experiment 1 is consistent with previous reports in which azoospermic ejaculates in
426 camelids ranged from 5% to 22% [40,45,47,50]. In one study, 14% of ejaculates collected using
427 an artificial vagina had very few or ¨scarce¨ sperm [50]. Reasons for variation among studies may
428 include collection method and frequency, and season of collection (dry vs. rainy season).

429

430 The primary source of NGF in camelids is the prostate gland [38]. Correspondingly, a significant
431 relationship between total NGF per ejaculate and male prostate size was observed in the present
432 study. In camelids, the body of the prostate is composed of two large lobes (right and left)
433 connected by a smaller isthmus. Perhaps a refined method of ultrasound morphometry would

reveal an even stronger correlation; i.e., individual lobe measurement, volume instead of area measurement, and variation in echotexture. Further, since NGF was detected in a greater proportion of arterial and venous vessels in the disseminate part than in the body of the prostate [38], assessment of the degree of vascularization (as indicated by power flow Doppler imaging) may be predictive.

We consider that it is unlikely that breeding frequency impacted the results of the present study. Male alpacas permitted to breed twice a day for nine consecutive days induced an 81% ovulation rate and 78% pregnancy rate [51]. Although ovulation rates were not different when breeding frequency was increased to four and six times a day, a decline in the number of pregnant animals was observed with six breedings per day [51]. In the present study, males were: 1) allowed to breed only two females per day, 2) breedings were separated by a minimum of four hours, and 3) breeding days were not consecutive but were separated by one full day. Consequently, the duration of breeding, pregnancy and ovulation rates were not influenced by the schedule or day of breeding. Of the 160 pairings, there was only one (0.6%) isolated event when a male showed no libido and did not mate the female.

In summary, despite that seminal NGF represents half of the total protein content within the ejaculate, the degree of variability in amount of NGF in semen within and among alpacas precluded its use as a tool to distinguish fertility among males in a field setting. A paucity of seminal NGF was associated with azoospermia, but seminal NGF content was not otherwise

associated with sperm motility, morphology or fertilizability. Seminal characteristics correlated positively to fertility included sperm concentration and viability, and those negatively correlated included morphologic abnormalities of the sperm head and midpiece. A positive correlation between prostate morphometry and seminal NGF content may form the basis of future methods of screening for the latter.

Acknowledgements

We thank Dr. Wilfredo Huanca, Guillermo Bonilla, Nathalie Zirena Arana, Jesus Enrique Turin Vilca, Gerardo Mamani, Renato Sanchez, Judith Karina Vasquez Laqui, Alejandra Ugarelli and Keli McIvor for their help with data collection. We thank Dr. Peter Flood for graciously providing the photograph of the alpaca male reproductive tract.

Funding

This research was supported by a grant from the Natural Sciences and Engineering Research Council of Canada and by the Chilean National Science and Technology Research Council (Fondecyt N° 1120518).

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628 **Tables**

629 Table 1. Distribution of NGF abundance in alpaca seminal plasma collected by artificial vagina
630 (Experiment 1).

631

Quantity of NGF per ejaculate	Proportion of ejaculates (%)
≤ 60 µg	4/153 (2.6%)
> 60 ≤125 µg	6/153 (3.9%)
>125 ≤250 µg	5/153 (3.3%)
> 250 µg	138/153 (90.2%)

632

633 Table 2. Pearson's correlation coefficients among male reproductive parameters in alpacas
634 (P<0.05; n=47 male alpacas, n=153 ejaculates; Experiment 1): A = age of male; B = weight of male;
635 C = semen collection length; D = NGF concentration; E = total NGF per ejaculate; F = sperm
636 concentration; G = pH; H = volume of semen; I = sperm motility; J = seminal thread formation; K
637 = plasma testosterone concentration; L = total testis area; M = total prostate area; N = total
638 bulbourethral area; O = % live sperm per ejaculate; P = % normal sperm per ejaculate.

	A	B	C	D	E	F	G	H	I	L	M	N	O	P
A						-0.19*								
B						0.33								
C					0.36				-	0.25		0.28		
D						0.27	-0.24							
E				0.70										
F	-0.19*	0.33		0.27			-0.41							
G				-0.24		-0.41		0.32	-0.33					
H				-0.19*	0.49		0.32							
I		0.28				0.37	-0.33							
J		-0.35		0.26		-0.19*		0.23						
K														
L														
M	0.37	0.24		0.21*	0.22									0.19*
N	0.38	0.02*		-0.19*										
O	-0.20*					0.47	-0.33		0.76	0.29				0.24
P		0.22				0.34	-0.26*							

639 * Tendency for a correlation (P<0.10)

640

641 Table 3. Association between total NGF per ejaculate (dependent variable) and NGF
642 concentration, semen volume and prostate area (independent variables), as assessed by
643 stepwise multiple regression analysis (n=153; Experiment 1).

Independent variables	β- weight	t value	P-value
NGF Concentration	0.63	8.11	<0.0001
Semen volume	0.424	5.54	<0.0001
Prostate area	0.22	2.84	0.006
Constant	-	-3.25	0.002

644 **model R= 0.75; R2=0.56; Adjusted R2=0.54; F= 33.22; P<0.0001

645

646

647 Table 4. The ovarian response (mean±SEM) and pregnancy rate in female alpacas (n=160) bred
648 once to a male with a low, medium, or high concentration of NGF in the ejaculate (n=2, 4, and 2
649 males, respectively; based on n=3 ejaculates per male; Experiment 3). No statistical differences
650 among groups for any endpoint.

	Male group [seminal NGF]		
	Low	Medium	High
Endpoint	[0.8 ± 0.4 mg/mL]	[3.7 ± 0.7 mg/mL]	[16.2 ± 5.7 mg/mL]
Number of females	40	80	40
Age of females (years)	6.6 ±0.39	6.5±0.41	6.9±0.42
Duration of breeding (min)	22.1±1.08	21.9±0.98	23.1±1.20
Follicle diameter at breeding (mm)	9.2±0.23	9.1±0.26	9.4±0.23
Ovulation rate	32/40 (80%)	62/80 (78%)	36/40 (90%)
Pregnancy rate	18/40 (45%)	41/80 (51%)	16/40 (40%)
CL diameter on Day 7* (mm)	13.0±0.30	12.9±0.25	12.9±0.28

651 *Day 0 = day of breeding

652

653

654 Table 5. Correlations between seminal characteristics, testicular area, age of females and
655 pregnancy rate in alpacas (Experiment 3).

Factor	Spearman's-rank correlation coefficient ρ	P-value
Total NGF in ejaculate	0.05	0.45
Sperm concentration	0.65	0.04*
Sperm motility	0.55	0.08
Sperm viability	0.85	0.004*
Semen pH	-0.76	0.01*
Total testis area	-0.68	0.03*
Normal sperm morphology	0.49	0.1
Midpiece defect	-0.72	0.02*
Proximal droplet	-0.6	0.06
Acrosome defect	-0.88	0.002*
Age of female	-0.78	0.01*

656 *Correlation is significant at the 0.05 level (2-tailed)

657

658

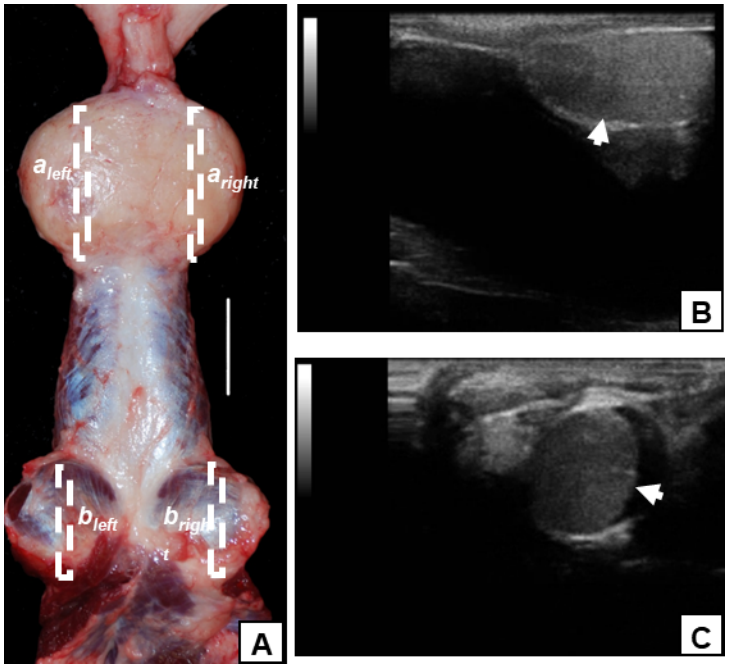
659 **Figures**

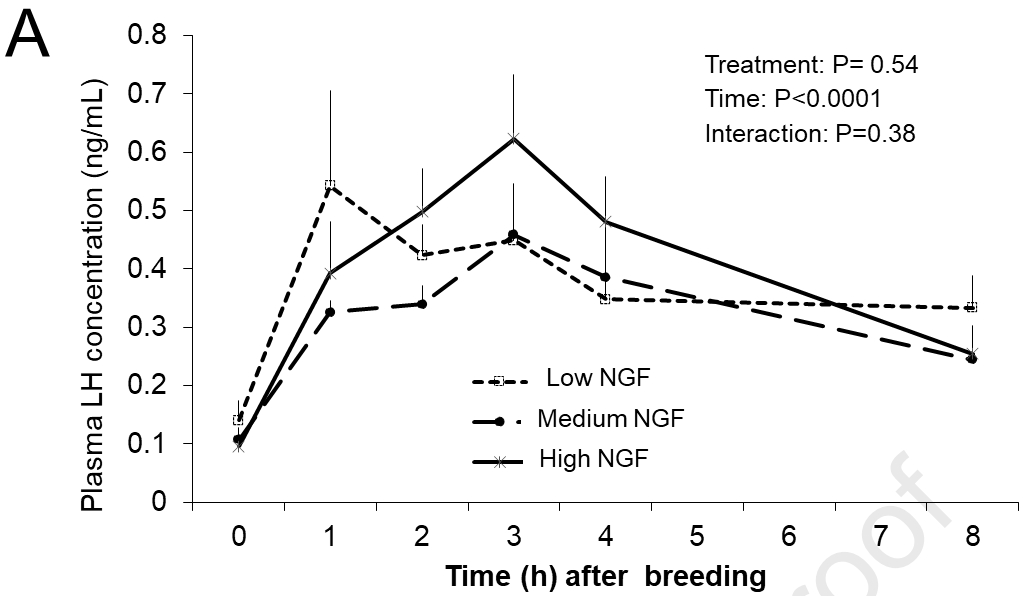
660 Figure 1. Gross and ultrasonographic morphology of the pelvic urethra and accessory glands of
661 a male alpaca. A) Dissection of pelvic urethra (dorsal view) showing the left and right lobes of
662 the compact portion of the prostate (a) and the left and right lobes of the bulbourethral gland
663 (b). B) Ultrasound image of a lobe of the prostate gland. C) Ultrasound image of a lobe of the
664 bulbourethral gland. Ultrasound images were taken in a sagittal plane, indicated by the dashed
665 lines in A. Caliper marks outline the glandular tissue. Note the crescent-shaped anechoic region
666 along the caudal aspect of the bulbourethral gland (arrow heads, C). Distance between major
667 scale lines is 1 cm. Ultrasound images taken using a transrectal linear-array 7.5 MHz transducer.

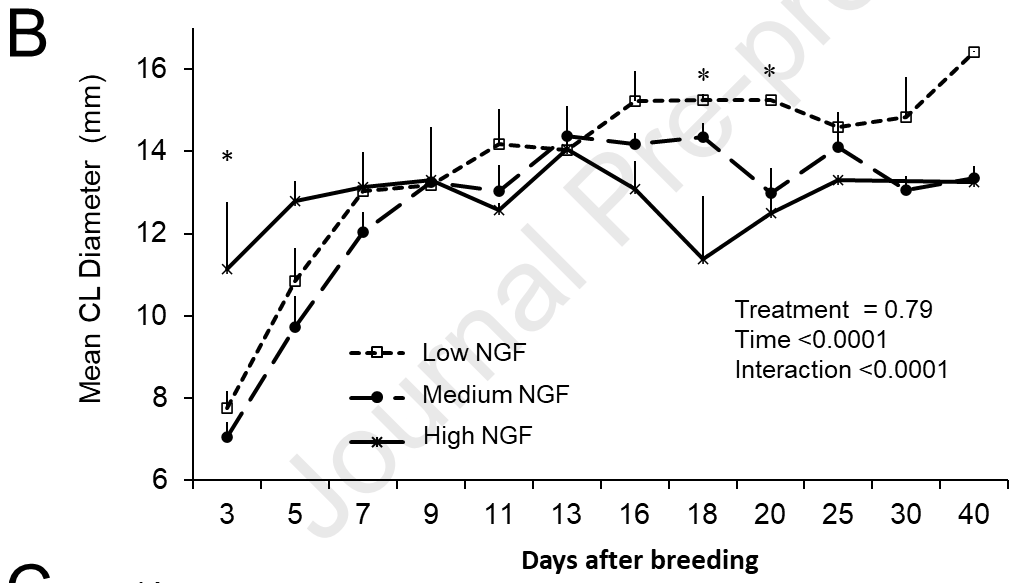
668

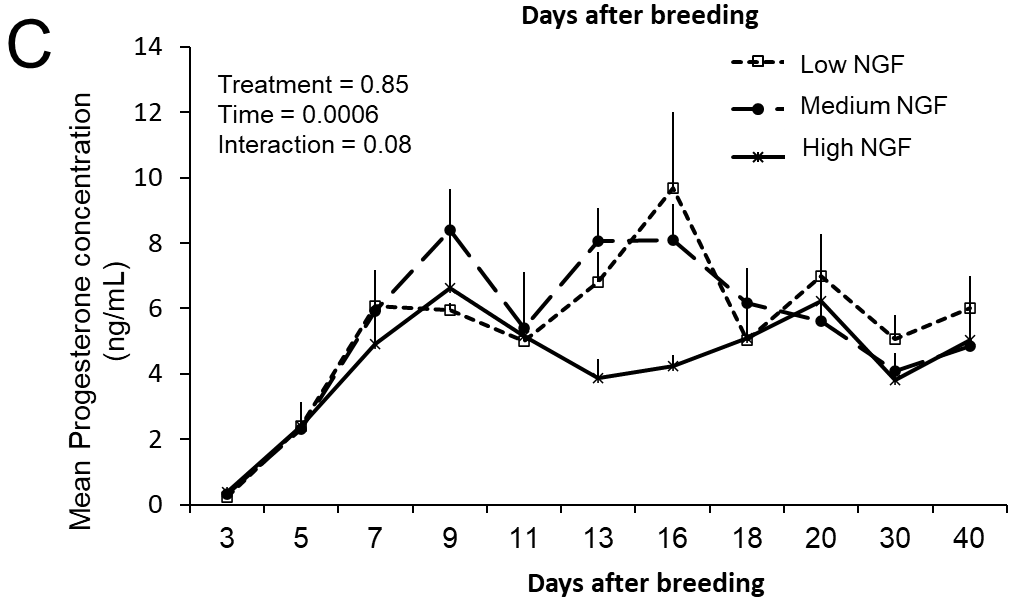
669 Figure 2. Endocrine and ovarian response (mean ± SEM) in female alpacas bred to males
670 containing low, medium and high concentrations of NGF in the ejaculate (subset from Experiment
671 3). A) Plasma LH concentrations (n=4 females per group). B) CL diameter and C) plasma
672 progesterone concentrations in pregnant female alpacas that ovulated following breeding (n = 3
673 for low, n=8 for medium, n=3 for high NGF groups).

674









Highlights:

- NGF quantity in semen is not predictive of male alpaca fertility.
- Most of male alpaca had enough NGF in semen to induce ovulation in female alpaca.
- NGF did not correlate with sperm motility, viability and morphology.