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DETERMINING THE ROLE OF NERVE GROWTH FACTOR-BETA IN SEMINAL
PLASMA ON BOVINE REPRODUCTION

BY

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DISSERTATION

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ABSTRACT

Nerve growth factor- β (NGF) is a protein found in the seminal plasma of a variety of mammalian species. While initially known for regulating neuronal survival and differentiation, it was later discovered to interact within the endocrine and reproductive systems as well. Most notable is its function in camelids as an ovulation inducing agent with a dose-dependent luteotrophic effect. While also present in the seminal plasma of spontaneously ovulating species, such as the bull, the role of NGF was initially thought to be limited to regulating sperm physiology. However, upon discovering its effect on ovulation induction in camelids, the potential functions of NGF within the female reproductive system of other species came to light.

The luteotrophic effect of NGF is of much interest to the cattle industry where sustainability relies on reproductive success. While many researchers have investigated factors such as nutrition and estrous cycle manipulation, there appears to still be a bottleneck in improving reproductive efficiency in cows due to early embryonic losses. The majority of conception failure in cattle is thought to occur prior to maternal recognition of pregnancy as a result of luteal insufficiency. When corpus luteum formation and progesterone production are inadequate following fertilization, the conceptus will fail to produce the threshold level of interferon tau needed to signal for maternal recognition of pregnancy. As a result, luteolysis and embryonic loss will occur. There is, therefore, a need to uncover novel techniques that will minimize these potential losses in cattle.

Increasing evidence is available to suggest that seminal plasma proteins play an important role in regulating female fertility, such as the luteotrophic effect of NGF. However, with the rapid expansion of artificial insemination use in the cattle industry, these physiological mechanisms may

become lost. Frozen semen used in artificial insemination is extended to achieve the lowest effective breeding dose and maximize the number of straws acquired per collection. However, this practice also dilutes out the seminal plasma proteins to sub-physiological concentrations. Given that many of these proteins, such as NGF, have been associated with bull fertility, more work is needed to understand their specific roles in both male and female reproduction. Not only could these factors improve sperm function, but they may also regulate events throughout ovulation and fertilization within the female reproductive tract that contribute to successful pregnancies. Therefore, it is necessary to evaluate the effects of NGF within the female reproductive system and as an additive to semen extender to determine its potential usefulness within the cattle industry.

It was hypothesized that intramuscular administration of purified bovine NGF to cows at artificial insemination would improve corpus luteum function and conceptus development. To test this hypothesis, Angus crossbred cows underwent estrus synchronization and were randomly allocated to one of two treatment groups: (1) CONT (n = 30) - 12 mL phosphate-buffered saline; or (2) NGF (n = 30) - 296 µg purified NGF in 12 mL phosphate-buffered saline administered intramuscularly at artificial insemination (day 0). Blood samples were collected to measure plasma progesterone concentrations (days 0, 3, 7, 10, 14, 19), plasma pregnancy-specific protein B concentrations (day 24), and expression of interferon-stimulated genes in peripheral blood leukocytes (day 19). Corpus luteum size (days 0, 3, 7, 10, 14, 19), pregnancy status (days 28, 45, 66), and embryonic/fetal crown-rump length (days 28, 45, 66) were assessed using transrectal ultrasonography. It was found that despite no change in corpus luteum size between treatments, NGF cows had increased plasma progesterone concentrations over CONT cows from days 10 to 19. Pregnancy rates were 75% in NGF cows and 59% in CONT cows. Fold-change expression of interferon-stimulated genes *Isg15* and *Mx2* at day 19 were greater in pregnant NGF cows than in

pregnant CONT cows. Consistently, plasma pregnancy-specific protein B concentrations at day 24 were greater in pregnant NGF than in pregnant CONT cows, and fetal crown rump length tended to be increased in pregnant NGF cows at day 66. Herein, it was demonstrated that systemic administration of NGF to cows at artificial insemination improves corpus luteum function and enhances conceptus development as determined by upregulation of interferon-stimulated genes and increased pregnancy-specific protein B production.

Upon confirming the luteotrophic effect of NGF in cows, my next interest was uncovering its mechanism of action. Therefore, it was hypothesized that NGF interacts with the pre-ovulatory follicle to increase vascularity and enhance steroidogenesis, which persists throughout ovulation to create a highly functional and vascular corpus luteum. To test this hypothesis, two Holstein heifers underwent estrus synchronization and were ovariectomized upon formation of a pre-ovulatory follicle (>12 mm). Pieces of follicle wall tissue (theca + granulosa cells) were excised and incubated in Eagle's MEM culture media supplemented with 1% L-glutamine, 1% nonessential amino acids, 1% penicillin-streptomycin, 1% insulin-transferrin-selenium, 10% fetal bovine serum, 40 ng/mL cortisol, 4 ng/mL luteinizing hormone, and 4 ng/mL follicle stimulating hormone. Culture wells were either supplemented with 100 ng/mL NGF (n = 12) or left as an untreated control (n = 12). Culture media was withdrawn and replaced with fresh media at 3, 6, 12, 24, 48, and 72 hours and frozen for subsequent steroid hormone measurement. Follicle tissue pieces were flash-frozen at completion of culture (72 hours) to assess expression of steroidogenic and angiogenic genes. Treatment with NGF increased testosterone production and upregulated steroidogenic enzyme 17 beta-hydroxysteroid dehydrogenase (*17 β -hsd*) gene expression at 72 h in the follicle wall extracts. There was no effect of NGF treatment on production of progesterone and estradiol-17 β or gene expression of other steroidogenic enzymes in the follicle tissue, suggesting

that the increased androgen production may be secondary to theca cell proliferation. Additionally, treatment with NGF downregulated gene expression of the angiogenic fibroblast growth factor 2 (*Fgf2*), but did not alter expression of other angiogenic factors. The reduced expression of fibroblast growth factor 2 in NGF treated cells suggests a possible hastened onset of cell remodeling that occurs during early corpus luteum development. Overall, this study demonstrated that NGF interacts directly with the bovine pre-ovulatory follicle to increase thecal cell androgen production and alter expression of enzymes involved with follicular to luteal reconstruction.

Next, heifers underwent estrus synchronization and were randomly allocated to one of two treatment groups: (1) Control (n=12)- 12 mL phosphate-buffered saline; or (2) NGF (n=12)- 250 µg purified NGF in 12 mL phosphate-buffered saline administered intramuscularly at day 0 (presence of pre-ovulatory follicle). Transrectal ultrasonography was performed every 4 hours to measure follicle size and vascularity and determine the time to ovulation. Blood was collected every 4 hours up to 32 hours to measure serum estradiol-17β concentrations. After ovulation, transrectal ultrasonography was performed daily to evaluate corpus luteum size and vascularity, and blood was collected every 2 days to measure serum progesterone concentrations. Corpus luteum biopsies were performed on days 9 and 14 to assess gene expression of steroidogenic and angiogenic enzymes, oxytocin, and luteinizing hormone receptor, and to measure the percentage of small luteal cells over large luteal cells. Follicle diameter at treatment was ~13 mm in both groups. There was a main effect of NGF treatment on the pre-ovulatory follicle diameter; however, follicle vascular area and serum estradiol-17β concentrations were unaffected. The average time to ovulation was 13.8 hours in NGF heifers versus 17.5 hours in CONT heifers. There tended to be an effect of NGF treatment on corpus luteum diameter, but no differences in its vascular area. Serum progesterone concentrations were higher in NGF heifers from days 10 to 12 compared to

control heifers. Gene expression of steroidogenic enzymes, steroidogenic acute regulatory protein (*Star*) and 3-beta-hydroxysteroid dehydrogenase (*3 β -hsd*), were both upregulated in the corpus luteum of NGF heifers, whereas angiogenic enzymes, prostaglandin E₂ synthase (*Pges*) and its receptor (*Pger*), were downregulated. There was a tendency for upregulated luteinizing hormone receptor (*Lhcgr*) gene expression in the corpus luteum of NGF heifers, consistent with an increased percentage of small luteal cells. In conclusion, NGF treatment affected follicle size, which may be attributed to increased follicular edema. Contrary to our hypothesis, NGF did not affect vascularity of the follicle or corpus luteum. Rather, the luteotrophic effect of NGF appears to be mediated through increased small luteal cell number and enhanced steroidogenic function in the corpus luteum. This change is likely secondary to the interactions between NGF and the pre-ovulatory follicle that alter the mechanisms of luteal construction following ovulation.

Furthermore, it was hypothesized that NGF is produced predominantly in the bull vesicular gland, is positively associated with sire conception rate scores, and improves sperm cryotolerance. To test this hypothesis, accessory sex glands (ampulla, bulbourethral, prostate, vesicular glands) were harvested post-mortem from three mature bulls and either frozen or formalin-fixed for *Ngf* gene expression and NGF protein localization. Seminal plasma NGF concentrations were measured in the pre-ejaculate and sperm-rich fraction from bulls collected by electroejaculation. Additionally, seminal plasma NGF concentrations were measured in semen collected by artificial vagina at a commercial bull-stud. These bulls were allocated to two categories based on calculated sire conception rate scores: (1) negative/0 deviations; or (2) positive deviations. Lastly, sperm acquired by both electroejaculation and epididymal harvest from 10 bulls were incubated with 0 ng/mL (CONT), 0.5 ng/mL (LOW), 5 ng/mL (MED), or 50 ng/mL (HIGH) purified NGF and manually frozen in 0.5-mL straws to evaluate effects on sperm cryotolerance. Pre-freeze sperm

motility was assessed in each sample prior to treatment using computer-aided sperm analysis. Frozen straws were thawed and incubated at 37°C for post-thaw motility assessment in 30 min intervals for 4 h. Samples were stained with fluorescent probes for evaluation of post-thaw sperm viability (SYBR-14/PI), acrosomal integrity (FITC-PNA/PI), and chromatin stability (acridine orange) using flow cytometry. Gene expression of *Ngf* was highest in the vesicular gland, intermediate in the ampulla, and lowest in the prostate and bulbourethral glands. Consistently, highest NGF staining intensity was detected in the ampulla and vesicular glands. Seminal plasma NGF concentrations were higher in the sperm-rich fraction than in the pre-ejaculate and positively associated with sire conception rate scores in bulls. Supplementation of extender with HIGH concentrations of NGF decreased post-thaw curvilinear velocity and amplitude of lateral head displacement and increased linearity in ejaculated, but not epididymal, sperm. These findings suggest a potential role in preventing premature sperm hyperactivation and capacitation that relies on the presence of other seminal plasma constituents. Post-thaw viability, acrosome integrity, and DNA fragmentation index were not affected by NGF treatment in either ejaculated or epididymal sperm. In conclusion, treatment with NGF did not significantly improve cryotolerance of sperm collected by electroejaculation or epididymal harvest in bulls. Collectively, these results demonstrate that NGF is positively associated with bull fertility, likely attributable to its beneficial effects within the female reproductive tract rather than on spermatozoal functions alone.

To my Mother, Father, Brother, Sister, and Husband

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Chapter 1:

Overview

1.1 Overview

Nerve growth factor- β (NGF) is a protein found in the seminal plasma of a variety of species, including the bull [1]. Though the roles of NGF were initially thought to be limited to regulating neuronal survival and differentiation, studies have since demonstrated its broader physiological implications, including those within the reproductive and endocrine systems [2]. One of the most profound functions of NGF discovered yet is its ability to elicit luteinizing hormone (LH) secretion from the anterior pituitary and induce ovulation in camelids [3–5]. When seminal plasma is introduced into the camelid uterus at copulation, NGF is absorbed into the bloodstream [6]. Interestingly, NGF travels across the blood-brain barrier and stimulates gonadotropin-releasing hormone (GnRH) secretion from the hypothalamus, triggering the pre-ovulatory LH surge [7]. Systemic administration of NGF results in prolonged LH secretion and a dose-dependent luteotrophic effect in camelids [3]. In spontaneously ovulating species, NGF signaling in the ovary is critical for the first ovulation to occur [14], yet we have only recently begun to truly understand the interaction between the ovary and seminal plasma derived NGF.

One study demonstrated that intramuscular administration of purified llama NGF to cyclic heifers resulted in larger corpus luteum (CL) diameter and greater concentrations of plasma progesterone following ovulation [15]. In cows, CL size is correlated with plasma progesterone levels until the beginning of the luteal stasis phase at day 7, after which luteal blood flow becomes a more reliable predictor of progesterone production [8]. This finding suggests that the degree of luteal vascularity may better predict luteal function in the early diestrous phase of cattle. In camelids, luteal vascularity and plasma progesterone concentrations were higher in NGF versus

GnRH-treated animals 6 days after ovulation [9]. The effects of NGF on luteal vascularity in cows remains undetermined, though *in vitro* studies have demonstrated that NGF directly stimulates prostaglandin E₂ production in bovine theca cells [10]. Prostaglandin E₂ acts as a pro-angiogenic molecule by inducing endothelial cell migration and pseudocapillary formation [11]. Therefore, it seems likely that NGF may exert some of its reported luteotrophic properties in cattle through improved luteal vascularity and enhanced LH signaling [3,9,12,13].

Enhanced CL formation with subsequent increased progesterone production may help reduce early embryonic death in cattle [14]. Reports estimate that up to 80% of bovine embryo losses occur by day 16 of pregnancy [15–17]. Many of these losses are attributed to luteal phase dysfunction and the downstream effects on histotroph secretion into the uterine lumen [18,19]. Growth and survival of the preimplantation blastocyst rely on the production of these embryotrophic factors from the uterus, which ultimately affects interferon tau secretion [20,21]. A threshold concentration of interferon tau is required to effectively block luteolysis and achieve maternal recognition of pregnancy in ruminants [22]. Therefore, if fertilization occurs, but progesterone and interferon tau production are inadequate, luteolysis and early embryonic death will ensue. A recent study found that initiating progesterone supplementation on day 4 post-estrus resulted in significantly greater embryo length compared to control heifers with experimentally induced sub-luteal function [23]. Although some studies have demonstrated the beneficial effects of using supplemental progesterone early in the luteal phase, others reported negative effects associated with improper timing of administration [24–26]. Treatment with NGF would mimic the uptake of seminal plasma proteins near ovulation, providing a novel technique to improve endogenous progesterone production without the concerns of inappropriate timing.

The role of seminal plasma proteins in regulating fertility is of special relevance to the cattle industry where artificial insemination has advanced genetic selection of desirable traits such as milk production and meat quality [27]. Historically, bull studies have focused on improving semen cryopreservation techniques and determining the minimum effective breeding dose. Meanwhile, the effects of semen processing on the function of seminal plasma proteins have not been fully investigated [28]. When processing for cryopreservation, semen is extended to the lowest effective sperm dose, which may dilute proteins to sub-physiologic levels [29] and contribute to lower fertility [30–32]. Several studies have demonstrated positive associations between seminal plasma proteins and bull fertility [33–38], with reported beneficial effects on spermatozoal transport, survival, and fertilization capability within the female reproductive tract [33,35]. Consistently, spermatozoal *Ngf* gene abundance has been associated with positive sire conception rates in bulls [35], and NGF supplementation to frozen-thawed bovine sperm altered leptin secretion and viability [39]. Further studies are necessary to improve semen cryopreservation in bulls, bringing forth a need to understand the role that individual seminal plasma proteins, such as NGF, play in sperm cryotolerance and fertility.

Thus, the overall goal of my doctoral dissertation work was to determine the role of NGF on ovulation, CL function, and conceptus development in cows/heifers and understand its production dynamics and effect on sperm cryotolerance in the bull. The NGF used in this study was purified from bull seminal plasma using a multi-step protocol that yielded a purity of 59.35%. The contaminants identified would have minimal interaction with the effects we are studying, and we felt it was important to determine its role specifically within the bovine species.

To complete my dissertation work, three main hypotheses were tested: 1) NGF improves CL function and conceptus development in cows, 2) NGF increases vascularity and

steroidogenesis within both the pre-ovulatory follicle (POF) and subsequent CL of heifers, and 3) NGF is positively associated with sire conception rates and can be used to improve sperm cryotolerance in bulls. To test these hypotheses, the following specific aims were completed:

Specific Aim 1: Determine the effects of NGF on corpus luteum function and conceptus development in cows.

To complete this aim, cows underwent estrus synchronization and were randomly allocated to 1 of 2 treatment groups: (1) CONT- 12 mL phosphate-buffered saline; or (2) NGF- 296 µg purified NGF in 12 mL phosphate-buffered saline administered intramuscularly at time of artificial insemination (day 0). Blood samples were collected to measure plasma concentrations of progesterone (days 0, 3, 7, 10, 14, 19) and pregnancy-specific protein B (day 24) and expression of interferon-stimulated genes in peripheral blood leukocytes (day 19). Transrectal ultrasonography was used to determine CL size (days 0, 3, 7, 10, 14, 19), diagnose pregnancy (days 28, 45, 66), and measure embryonic/fetal crown-rump length (days 28, 45, 66). Plasma progesterone levels were higher in NGF cows from days 10 to 19 compared to CONT, despite no difference in CL size. Pregnancy rates were 75% in NGF cows and 59% in CONT cows, which did not differ significantly. Further, conceptus development was enhanced in pregnant NGF cows as determined by upregulated expression of interferon-stimulated genes at day 19, increased plasma pregnancy-specific protein B levels at day 24, and a tendency for increased fetal crown-rump length at day 66. Improved CL function around maternal recognition of pregnancy (~day 16) with NGF treatment likely contributed to enhanced conceptus development. These results are presented in Chapter 3.

Specific Aim 2: Characterize the changes in pre-, peri-, and post-ovulatory dynamics following NGF treatment in heifers.

To complete this aim, heifers underwent estrus synchronization and were ovariectomized upon development of a POF. Pieces of follicle wall tissue (theca + granulosa cells) were cultured in media supplemented with LH, follicle stimulating hormone, +/- NGF. Culture media was replaced and frozen at 3, 6, 12, 24, 48, and 72 hours for steroid hormone measurement. Follicle wall tissue pieces were collected and flash-frozen at completion of culture (72 hours) to assess steroidogenic and angiogenic gene expression. Next, heifers underwent estrus synchronization and were randomly allocated to 1 of 2 treatment groups: (1) Control- 12 mL phosphate-buffered saline; or (2) NGF- 250 μ g purified NGF in 12 mL phosphate-buffered saline administered intramuscularly at day 0 (presence of a POF). Transrectal ultrasonography was performed every 4 hours to assess POF size and vascularity and determine the time to ovulation. Blood was collected every 4 hours up to 32 hours to measure serum estradiol-17 β concentrations. After ovulation, transrectal ultrasonography was performed daily to look at CL size and vascularity, and blood was collected every 2 days until day 14 to measure serum progesterone concentrations. Corpus luteum biopsies were performed on days 9 and 14 to assess gene expression of steroidogenic and angiogenic enzymes, oxytocin, and LH receptor, and to measure the ratio of small to large luteal cells. Direct treatment of follicular tissue *in vitro* resulted in upregulated steroidogenic enzyme 17 beta-hydroxysteroid dehydrogenase (*17 β -hsd*) gene expression and downregulated angiogenic enzyme fibroblast growth factor 2 (*Fgf2*) gene expression. Consistently, NGF treatment increased testosterone production from the follicle tissue over untreated controls. Follicle diameter at the time of systemic NGF administration to heifers did not differ between treatments (~13 mm). There was an effect of NGF treatment on the POF diameter; however, follicle vascular area and serum

estradiol-17 β concentrations were unaffected. The average time to ovulation was 13.8 hours in NGF heifers versus 17.5 hours in control heifers, which did not differ significantly. There was a tendency for increased CL diameter in NGF heifers, but no difference in its vascular area. Serum progesterone concentrations were increased in NGF heifers from days 10 to 12. Gene expression of steroidogenic enzymes, steroidogenic acute regulatory protein (*Star*) and 3-beta-hydroxysteroid dehydrogenase (*3 β -hsd*), were upregulated in the CL of NGF heifers, whereas angiogenic enzymes, prostaglandin E₂ synthase (*Pges*) and its receptor (*Pger*), were downregulated. There was a tendency for increased LH receptor (*Lhcgr*) gene expression in the CL of NGF heifers, consistent with an increased percentage of small luteal cells observed. In conclusion, these studies demonstrated that NGF interacts directly with the bovine POF to influence CL formation. The luteotrophic effect of NGF is a result of increased small luteal cell number and improved steroidogenic enzyme activity in the CL. These results are presented in Chapters 4 and 5.

Specific Aim 3: Determine the biology of NGF production in the bull and its effects on fertility traits.

To complete this aim, accessory sex glands (ampulla, bulbourethral, prostate, vesicular glands) were harvested post-mortem from mature bulls and either frozen or formalin-fixed for *Ngf* gene expression and NGF protein localization. Seminal plasma NGF concentrations were measured in the pre-ejaculate and sperm-rich fraction from bulls collected by electroejaculation. Additionally, seminal plasma NGF concentrations were measured from semen collected by artificial vagina at a commercial bull-stud. These bulls were allocated to two categories based on calculated sire conception rate scores: (1) negative/0 deviations; or (2) positive deviations. Lastly, sperm collected by both electroejaculation and epididymal harvest were incubated with 0 ng/mL (CONT), 0.5 ng/mL (LOW), 5 ng/mL (MED), or 50 ng/mL (HIGH) purified NGF prior

cryopreservation to assess its effect on sperm cryotolerance. Relative *Ngf* gene expression was highest in the vesicular gland, intermediate in the ampulla, and lowest in the prostate and bulbourethral glands. Consistently, the highest NGF staining intensity was detected in the ampulla and vesicular glands. Seminal plasma NGF concentrations were significantly higher in the sperm-rich fraction than in the pre-ejaculate and positively associated with sire conception rates. Despite these findings, supplementing freezing extender with NGF did not significantly improve post-thaw sperm motility, viability, acrosome integrity, or chromatin stability in ejaculated or epididymal derived sperm. Collectively, these results demonstrated that NGF is secreted into the sperm-rich fraction of the ejaculate and is associated with bull fertility. Though NGF alone did not improve sperm cryotolerance, future studies should evaluate different combinations of proteins and effects on survivability within the female reproductive tract to better understand its positive association with sire fertility. These results are summarized in Chapters 6 and 7.

To summarize, Chapter 1 provides an outline of my dissertation. Chapter 2 provides a background on NGF and its effects on reproduction across species. Chapter 3 describes the results from testing the hypothesis that NGF improves CL function and conceptus development in cows. Chapter 4 describes the results from testing the hypothesis that NGF alters steroidogenesis and angiogenic markers in the pre-ovulatory follicle. Chapter 5 describes the results from testing the hypothesis that NGF alters the peri-ovulatory dynamics that act downstream to influence CL function. Chapter 6 describes the results from testing the hypothesis that NGF is produced in the bull vesicular gland and positively influences sire conception rates, whereas Chapter 7 describes the results from testing the hypothesis that supplementing freezing extender with NGF alters sperm cryotolerance. Lastly, Chapter 8 summarizes the results of my dissertation work and provides insight on future projects based on the findings presented herein.

1.2 References

- [1] Harper GP, Glanville RW, Thoenen H. The purification of nerve growth factor from bovine seminal plasma. Biochemical characterization and partial amino acid sequence. *J Biol Chem* 1982;257:8541–8.
- [2] Tessarollo L. Pleiotropic functions of neurotrophins in Development. *Cytokine Growth Factor Rev* 1998;9:125–37. doi:10.1016/S1359-6101(98)00003-3.
- [3] Adams GP, Ratto MH, Huanca W, Singh J. Ovulation-inducing factor in the seminal plasma of alpacas and llamas. *Biol Reprod* 2005;73:452–7. doi:10.1095/biolreprod.105.040097.
- [4] Ratto MH, Leduc YA, Valderrama XP, van Straaten KE, Delbaere LTJ, Pierson RA, et al. The nerve of ovulation-inducing factor in semen. *Proc Natl Acad Sci U S A* 2012;109:15042–7. doi:10.1073/pnas.1206273109.
- [5] Kershaw-Young CM, Druart X, Vaughan J, Maxwell WMC. β -Nerve growth factor is a major component of alpaca seminal plasma and induces ovulation in female alpacas. *Reprod Fertil Dev* 2012;24:1093–7. doi:10.1071/RD12039.
- [6] Berland MA, Ulloa-Leal C, Barria M, Wright H, Dissen GA, Silva ME, et al. Seminal plasma induces ovulation in llamas in the absence of a copulatory stimulus: role of nerve growth factor as an ovulation-inducing factor. *Endocrinology* 2016;157:3224–32. doi:10.1210/en.2016-1310.
- [7] Silva ME, Smulders JP, Guerra M, Valderrama XP, Letelier C, Adams GP, et al. Cetorelix suppresses the preovulatory LH surge and ovulation induced by ovulation-inducing factor (OIF) present in llama seminal plasma. *Reprod Biol Endocrinol* 2011;9:74. doi:10.1186/1477-7827-9-74.
- [8] Herzog K, Brockhan-Lüdemann M, Kaske M, Beindorff N, Paul V, Niemann H, et al. Luteal blood flow is a more appropriate indicator for luteal function during the bovine estrous cycle than luteal size. *Theriogenology* 2010;73:691–7. doi:10.1016/j.theriogenology.2009.11.016.
- [9] Ulloa-Leal C, Bogle OA, Adams GP, Ratto MH. Luteotrophic effect of ovulation-inducing factor/nerve growth factor present in the seminal plasma of llamas. *Theriogenology* 2014;81:1101–1107.e1. doi:10.1016/j.theriogenology.2014.01.038.
- [10] Dissen GA, Parrott JA, Skinner MK, Hill DF, Costa ME, Ojeda SR. Direct effects of nerve growth factor on thecal cells from antral ovarian follicles. *Endocrinology* 2000;141:4736–50. doi:10.1210/endo.141.12.7850.
- [11] Finetti F, Solito R, Morbidelli L, Giachetti A, Ziche M, Donnini S. Prostaglandin E2 regulates angiogenesis via activation of fibroblast growth factor receptor-1. *J Biol Chem* 2008;283:2139–46. doi:10.1074/jbc.M703090200.
- [12] Tanco VM, Van Steelandt MD, Ratto MH, Adams GP. Effect of purified llama ovulation-inducing factor (OIF) on ovarian function in cattle. *Theriogenology* 2012;78:1030–9. doi:10.1016/j.theriogenology.2012.03.036.
- [13] Tanco VM, Ratto MH, Lazzarotto M, Adams GP. Dose-response of female llamas to ovulation-inducing factor from seminal plasma. *Biol Reprod* 2011;85:452–6.

doi:10.1095/biolreprod.111.091876.

- [14] Lonergan P. Influence of progesterone on oocyte quality and embryo development in cows. *Theriogenology* 2011;76:1594–601. doi:10.1016/j.theriogenology.2011.06.012.
- [15] Sreenan J., Diskin MG. The extent and timing of embryonic mortality in cattle. Dordrecht, The Netherlands: Martinus Nijhoff Publishers; 1986.
- [16] Diskin MG, Murphy JJ, Sreenan JM. Embryo survival in dairy cows managed under pastoral conditions. *Anim Reprod Sci* 2006;96:297–311. doi:10.1016/j.anireprosci.2006.08.008.
- [17] Diskin M, Morris D. Embryonic and early foetal losses in cattle and other ruminants. *Reprod Domest Anim* 2008;43:260–7. doi:10.1111/j.1439-0531.2008.01171.x.
- [18] Kimura M, Nakao T, Moriyoshi M, Kawata K. Luteal phase deficiency as a possible cause of repeat breeding in dairy cows. *Br Vet J* 1987;143:560–6. doi:10.1016/0007-1935(87)90047-9.
- [19] Gray CA, Taylor KM, Ramsey WS, Hill JR, Bazer FW, Bartol FF, et al. Endometrial glands are required for preimplantation conceptus elongation and survival. *Biol Reprod* 2001;64:1608–13. doi:10.1095/biolreprod64.6.1608.
- [20] Spencer TE, Sandra O, Wolf E. Genes involved in conceptus-endometrial interactions in ruminants: Insights from reductionism and thoughts on holistic approaches. *Reproduction* 2008;135:165–79. doi:10.1530/REP-07-0327.
- [21] Spencer TE, Forde N, Lonergan P. The role of progesterone and conceptus-derived factors in uterine biology during early pregnancy in ruminants. *J Dairy Sci* 2016;99:5941–50. doi:10.3168/jds.2015-10070.
- [22] Mann GE, Lamming GE. Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. *Reproduction* 2001;121:175–80.
- [23] Parr MH, Scully S, Lonergan P, Evans ACO, Crowe MA, Diskin MG. Establishment of critical timing of progesterone supplementation on corpus luteum and embryo development in beef heifers. *Anim Reprod Sci* 2017. doi:10.1016/j.anireprosci.2017.02.005.
- [24] Mann GE, Fray MD, Lamming GE. Effects of time of progesterone supplementation on embryo development and interferon-tau production in the cow. *Vet J* 2006;171:500–3. doi:10.1016/j.tvjl.2004.12.005.
- [25] O'Hara L, Forde N, Carter F, Rizos D, Maillo V, Ealy AD, et al. Paradoxical effect of supplementary progesterone between day 3 and day 7 on corpus luteum function and conceptus development in cattle. *Reprod Fertil Dev* 2014;26:328–36. doi:10.1071/RD12370.
- [26] Monteiro Jr PLJ, Ribeiro ES, Maciel RP, Dias ALG, Solé Jr E, Lima FS, et al. Effects of supplemental progesterone after artificial insemination on expression of interferon-stimulated genes and fertility in dairy cows. *J Dairy Sci* 2014;97:4907–21. doi:http://dx.doi.org/10.3168/jds.2013-7802.
- [27] Hayes BJ, Chamberlain AJ, Maceachern S, Savin K, McPartlan H, MacLeod I, et al. A

- genome map of divergent artificial selection between *Bos taurus* dairy cattle and *Bos taurus* beef cattle. *Anim Genet* 2009;40:176–84. doi:10.1111/j.1365-2052.2008.01815.x.
- [28] Schjenken JE, Robertson SA. Seminal fluid and immune adaptation for pregnancy - comparative biology in mammalian species. *Reprod Domest Anim* 2014;49:27–36. doi:10.1111/rda.12383.
 - [29] Westfalewicz B, Dietrich MA, Ciereszko A. Impact of cryopreservation on bull (*Bos taurus*) semen proteome. *J Anim Sci* 2015;93:5240–53. doi:10.2527/jas.2015-9237.
 - [30] Lima FS, Risco CA, Thatcher MJ, Benzaquen ME, Archbald LF, Santos JEP, et al. Comparison of reproductive performance in lactating dairy cows bred by natural service or timed artificial insemination. *J Dairy Sci* 2009;92:5456–66. doi:10.3168/jds.2009-2197.
 - [31] Dalton JC, Nadir S, Bame JH, Noftsinger M, Nebel RL, Saacke RG. Effect of time of insemination on number of accessory sperm, fertilization rate, and embryo quality in nonlactating dairy cattle. *J Dairy Sci* 2001;84:2413–8. doi:10.3168/jds.S0022-0302(01)74690-5.
 - [32] Pelissier CL. Dairy cattle breeding problems and their consequences. *Theriogenology* 1976;6:575–83. doi:10.1016/0093-691X(76)90125-4.
 - [33] Gwathmey TM, Ignatz GG, Mueller JL, Manjunath P, Suarez SS. Bovine seminal plasma proteins PDC-109, BSP-A3, and BSP-30-kDa share functional roles in storing sperm in the oviduct. *Biol Reprod* 2006;75:501–7. doi:10.1095/biolreprod.106.053306.
 - [34] Killian GJ, Chapman DA, Rogowski LA. Fertility-associated proteins in Holstein bull seminal plasma. *Biol Reprod* 1993;49:1202–7. doi:10.1095/biolreprod49.6.1202.
 - [35] Kasimanickam V, Kasimanickam R, Arangasamy A, Saberivand A, Stevenson JS, Kastelic JP. Association between mRNA abundance of functional sperm function proteins and fertility of Holstein bulls. *Theriogenology* 2012;78:2007–19. doi:10.1016/j.theriogenology.2012.07.016.
 - [36] Erikson DW, Way AL, Chapman DA, Killian GJ. Detection of osteopontin on Holstein bull spermatozoa, in cauda epididymal fluid and testis homogenates, and its potential role in bovine fertilization. *Reproduction* 2007;133:909–17. doi:10.1530/REP-06-0228.
 - [37] Gerena RL, Irikura D, Urade Y, Eguchi N, Chapman DA, Killian GJ. Identification of a fertility-associated protein in bull seminal plasma as lipocalin-type prostaglandin D synthase. *Biol Reprod* 1998;58:826–33. doi:10.1095/biolreprod58.3.826.
 - [38] Patel M, Gandotra VK, Cheema RS, Bansal AK, Kumar A. Seminal plasma heparin binding proteins improve semen quality by reducing oxidative stress during cryopreservation of cattle bull semen. *Asian-Australasian J Anim Sci* 2016;29:1247–55. doi:10.5713/ajas.15.0586.
 - [39] Li C, Sun Y, Yi K, Ma Y, Sun Y, Zhang W, et al. Detection of nerve growth factor (NGF) and its specific receptor (TrkA) in ejaculated bovine sperm, and the effects of NGF on sperm function. *Theriogenology* 2010;74:1615–22. doi:10.1016/j.theriogenology.2010.06.033.

Chapter 2:

Nerve Growth Factor- β , Ovulation, and Potential Roles for Improved Male and Female Fertility

2.1 Background on Nerve Growth Factor- β

Nerve growth factor- β (NGF) is a member of the neurotrophin gene family also consisting of brain-derived neurotrophic factor, neurotrophin-3, and neurotrophin-4/5 that exist as basic, homodimeric, secretory proteins with a molecular mass of ~26 to 27 kDa [1,2]. Nerve growth factor was initially identified in the early 1950s when researchers demonstrated that a specific growth factor from a mouse sarcoma directly stimulated neurite outgrowth in the chick embryo [3–5]. It was subsequently discovered that NGF plays a critical role in vertebrate sympathetic and sensory neuron development [6–10]. The most abundant sources of NGF protein reported initially were snake venom [11] and male mouse submaxillary gland [12]. However, after observing responses to mouse NGF treatment in other mammals [9,13], it was suggested that this protein may play a significant role across species. A major setback was the lack of detectable NGF in the salivary glands of these other species (rat, guinea pig, cow, pig, rabbit, and man) [14], which led to the discovery of the guinea pig prostate as an alternative NGF-rich source [15,16]. Using both a biological assay and two-site radioimmunoassay, moderate levels of NGF were also detected in rabbit and bull prostate glands [17]. Interestingly, using these techniques, no detectable NGF activity was found in the sex organs of the mouse, rat, hamster, or human [17]. The next crucial discovery was that the bull seminal plasma was another rich source of NGF [18], with the vesicular gland being the principal site of production as determined by biological assay and gel electrophoresis [19].

Nerve growth factor was suggested to exist in crude bovine seminal plasma as a high molecular weight complex [18,19]. In mice, the high molecular weight isoform of NGF is known as 7S-NGF, has a molecular mass of 140 kDa, and is composed of three protein structures, α , β , and γ [20]. After translation, this isoform is cleaved upon translocation into the endoplasmic reticulum to produce an unprocessed NGF precursor (proNGF) of ~50 kDa [21]. The highly basic (pI 9.3) and biologically active β -subunit ($M_r = 26$ kDa) dissociates from this complex at a pH 4.0 under experimental conditions in the mouse [22]. Likewise, dissociation of a high molecular weight complex in bull seminal plasma reportedly occurs at pH 3.0 and also produces a basic (pI 9.5 to 10), low molecular weight, homodimeric β -subunit, consisting of two identical and non-covalently linked polypeptide chains (monomeric $M_r = 15$ kDa) [18]. Little information is available regarding the high molecular weight complex in bulls. One study suggested that a 7S complex is not formed in bulls due to the lack of an arginine-arginine protease cleavage site at carboxy-terminus of bovine NGF [23] where processing by an associated γ -subunit occurs in the mouse [24]. Another study used tandem mass spectrometry and reported the presence of an NGF precursor in the seminal plasma of stallions, rams, bulls, and camelids, but not in boars or bucks [25]. Using western immunoblot analysis with a polyclonal mouse antibody against NGF, a prominent band was detected in both llama and bull seminal plasma at ~13 kDa (consistent with a reduced NGF monomer), with less distinct bands visible at ~60 kDa [2], potentially a proNGF isoform [26]. While the relevance of proNGF in the seminal plasma has yet to be determined, its roles within the central nervous system include inducing apoptosis [27] and influencing the mature NGF moiety [21].

Despite indistinguishable biological activity, mouse anti-bovine monoclonal antibodies exhibited poor binding to mouse NGF compared to native bovine NGF [28]. Though the biological

activity of bovine NGF was not affected by antibody binding, the data still suggested the existence of some structural differences between bovine and mouse NGF [28]. In comparison to NGF isolated from mouse submaxillary glands, amino acid composition in bovine NGF has higher proportions of aspartate, glutamate, proline, glycine, leucine, and tyrosine residues and lower threonine residues [18]. Additionally, bovine NGF contains two intra-chain disulfide bridges per peptide chain instead of the three bridges per chain observed in mouse [18] and llamas [2]. The NH₂-terminal amino acid sequence seems to be highly conserved across mammals, with bovine NGF differing from mouse NGF only by the presence of serine (position 3), arginine (position 9), and isoleucine (position 18), rather than threonine, methionine and valine, respectively [18].

The first successful purification of NGF from bovine seminal plasma was described in length by Harper *et al.* in 1982 [18]. Prior to dissociation, initial runs through diethylaminoethyl-cellulose (DEAE-C) and carboxymethyl-cellulose (CM-C) columns allowed for removal of other acidic and basic seminal plasma proteins, respectively, since the high molecular weight NGF complex does not bind to DEAE-C or CM-C [18]. Dissociation of the high molecular weight NGF complex within the obtained fraction was performed using a sodium citrate buffer (pH 3.0) [18]. This fraction was then run through a sulfopropyl-Sephadex (SP-S) column (a strong cation exchanger), which allowed for binding and isolation of the purified low molecular weight bovine NGF [18]. A similar NGF purification protocol using strong anion and cation exchangers with gradient elution was developed and described in Chapter 3 for use throughout my dissertation. Initially, attempts to purify this protein based on a previous protocol derived from camel studies using high performance liquid chromatography were unsuccessful [29]. Using this protocol, we found major contamination with a similar sized protein, Caltrin (Fig. 1), as distinguished by liquid chromatography-mass spectrometry. The difficulty in purifying NGF from bovine seminal plasma

as compared to camelids likely relates to differences in both protein abundance and biochemical characteristics. In camelids, NGF represents ~27% of total protein, whereas it represents less than 1% of total protein in bull ejaculates [30]. Regardless, the differences observed highlight the need to evaluate this seminal plasma protein separately amongst species.

2.2 Nerve Growth Factor- β in Induced Ovulators

Induced ovulation is defined as the phenomenon where coitus is required to produce the signals that initiate ovulation; whereas spontaneous ovulation occurs at a regular frequency regardless of copulation [31]. The rationale for why some species evolved these mechanisms for ovulation are unknown. A multi-species study observed an increased ability for males of species with induced ovulation to monopolize paternity over those with spontaneous ovulation, suggesting a mechanism of paternal control [83]. In cats, ovulation is initiated by the coitus-induced neuroendocrine reflex that synapses within the hypothalamus to stimulate a surge of gonadotropin releasing hormone (GnRH) that is sufficient to signal for a pre-ovulatory luteinizing hormone (LH) surge [32]. This ovulation induction mechanism was suggested to provide queens with a defense against “forced copulation” from undesirable males [33]. Since multiple copulations are required to produce sufficient GnRH and LH surges to induce ovulation in cats [32], the queen can breed with multiple toms while in estrus to allow for selection of dominant genetics.

This ovulation inducing mechanism is in contrast to camelids, which undergo prolonged copulation duration and require the presence of an “ovulation-inducing factor” (OIF) within the seminal plasma [34]. Early evidence of such a factor came from the discovery in 1985 that ovulation could be induced in Bactrian camels by intravaginal, intrauterine, or intramuscular administration of seminal plasma rather than by mechanical stimulation [35–37]. Approximately

20 years after its initial discovery in camels, the presence of OIF in seminal plasma was reported in llamas and alpacas in 2005 [34], reigniting interest in its identity and mechanism of action. A wide range of seminal plasma signaling molecules have been identified across mammalian species, with localized effects ranging from sperm protection to increasing oviductal embryotrophic cytokines [38]. These mechanisms seemed to have formed in parallel with invertebrates, such as *Drosophila*, where seminal fluid influences ovulation and female tract remodeling [39].

Early studies reported that >90% of alpacas and llamas ovulated in response to a single intramuscular dose of llama seminal plasma versus 0% that were administered phosphate-buffered saline (PBS) [34]. It is important to note that in all of these studies, treatment was administered only when a growing follicle capable of ovulation was detected. Interestingly, no alpacas responded to intrauterine administration of either seminal plasma or PBS, which was likely due to lack of systemic absorption of OIF from the uterus [34]. In camelids, prolonged copulation causes uterine hyperemia and inflammation due to the mechanical stimulus of the penis and semen deposition into the uterine horns, which was hypothesized to facilitate uptake of seminal plasma constituents from the uterus [40,41]. To test this hypothesis, a follow-up study performed endometrial curettage prior to intrauterine infusion of either seminal plasma or PBS in alpacas to mimic the copulation stimulus [42]. The authors reported that while intramuscular administration of seminal plasma produced the highest ovulation rate (93%), endometrial curettage increased ovulation rate from 41% to 67% following intrauterine infusion of seminal plasma [42].

Another interesting finding from the initial study by Adams et al. (2005) was a tendency for the corpus luteum (CL) to have a greater diameter and prolonged growth in llamas with OIF-induced versus GnRH-induced ovulations [34]. The increase in CL size was attributed to an

observed prolonged elevation of plasma LH concentrations and resulted in increased plasma progesterone concentrations in the OIF-treated llamas [34]. It was subsequently reported that exposure of rat pituitary cells to alpaca seminal plasma stimulated dose-dependent LH secretion, independent of GnRH signaling [43]. In 2010, researchers were able to identify OIF from llama and alpaca seminal plasma as a protein molecule with a molecular mass of ≥ 30 kDa [44]. The subsequent year, these researchers purified OIF from llama seminal plasma and confirmed its specific ability to stimulate LH secretion, induce ovulation, and alter CL function in camelids [45]. Using purified OIF, it was discovered that this protein exerted a dose-dependent luteotrophic effect when administered systemically to llamas [46]. Consistent with the previous studies, Bogle et al. (2012) reported that treatment of both llama and bovine pituitary cell cultures with purified llama OIF directly stimulated LH release, suggesting a direct role of OIF on stimulating LH production from the anterior pituitary [47].

An elaborate follow-up study was performed in llamas to determine if there is involvement from the hypothalamus in the stimulation of LH secretion from the anterior pituitary by purified llama OIF [48]. Consistent with other studies [34,46], llamas treated with 1 mg OIF systemically exhibited higher plasma progesterone concentrations when compared to the llamas treated with 50 μ g GnRH [48]. Pre-treatment with a GnRH antagonist (cetrorelix) blocked the ability of both GnRH and OIF to induce an LH surge and ovulation, providing evidence of hypothalamic involvement in OIF-signaling pathway [48]. One limitation of this study is that it could not determine whether OIF was still acting directly at the level of the hypothalamus. Rather, the results suggested that the presence of GnRH is necessary for OIF to exert its effects. It is possible that OIF directly stimulates GnRH production from the hypothalamus, which then signals for the pre-ovulatory LH surge. Alternatively, GnRH could alter the expression of OIF receptors in the

anterior pituitary and work synergistically with OIF to induce LH production. In another study, there was a tendency for ovariectomy to affect LH concentrations in llamas treated with purified OIF that was partially restored when animals were pre-treated with estrogens [49]. The authors concluded that peripheral estradiol concentrations modulated the effects of OIF on pituitary LH secretion [49]. Since estradiol directly stimulates the hypothalamic GnRH release necessary for the LH surge to occur, this data supported the idea that OIF was acting at the level of the hypothalamus. However, removing estradiol likely alters the pulsatile secretory activity of GnRH, which would affect any synergistic effect it may have with OIF in the anterior pituitary for stimulating LH production. Still, since the stimulatory action of estradiol on the hypothalamus functions mainly through kisspeptin neurons [50], there could be a role of OIF via this route.

The breakthrough discovery came when OIF from camelid seminal plasma was identified as nerve growth factor- β (NGF) in 2012 [2,51], broadening our understanding of this molecule amongst mammals. The NGF receptor, tyrosine kinase A (TrkA), has been detected in the hypothalamus of the rat [52] and llama [53]. However, a low proportion of GnRH neurons (2.5%) were found to be immunoreactive to TrkA, suggesting that OIF/NGF may not directly interact with GnRH neurons [54]. Although the llamas used were reported as non-pregnant and non-lactating, there was no mention of their ovarian or follicular status. Since many hormones and receptors within the hypothalamic-pituitary-gonadal axis exhibit temporal expression throughout the estrous cycle (e.g., kisspeptin in the hypothalamus is lower during diestrus in cattle [55]; TrkA expression greater in developing bovine CL versus mature or regressing CL [56]), it would be worthwhile to evaluate the presence of receptors based on follicular status. It has been suggested that the camelid OIF/NGF may interact with GnRH neurons through an intermediate cell type, such as kisspeptin [54,57], which plays a role in inducing ovulation in the musk shrew [58]. Though increases in

circulating NGF have been reported within 15 minutes of intrauterine infusion of seminal plasma in llamas [59], it is unclear through which route NGF reaches the hypothalamus. Adams et al. [53] suggested that NGF may cross the blood-brain barrier into cerebral spinal fluid, which has been observed in other species [60–62].

In addition to the effects on the hypothalamus and/or pituitary gland, studies have indicated that the luteotrophic effect of NGF may be related to changes in ovarian blood flow of the pre-ovulatory follicle. Ulloa-Leal et al. (2014) reported that administration of purified OIF/NGF to llamas resulted in greater vascularity of the pre-ovulatory follicle at 4 hours after treatment compared to GnRH-treated llamas [63]. Consistently, there was greater vascular area in the luteal tissue and higher progesterone concentrations in OIF/NGF-treated llamas [63]. Interestingly, administration of a second dose of OIF/NGF at the time of ovulation increased CL diameter, CL vascularity, and progesterone concentrations compared to one pre-ovulatory dose [64]. In a follow-up study, females were subjected to ovariectomies, where it was determined that pre-ovulatory NGF/OIF treatment upregulated gene expression of cytochrome p450, family 11, subfamily A, polypeptide 1 (*Cyp11a1*) and steroidogenic acute regulatory protein (*Star*) in the CL [65]. These findings provide a reasonable explanation for increased progesterone production since StAR transports cholesterol into the mitochondria and CYP11A1 converts cholesterol into pregnenolone, the precursor to progesterone (Fig. 2) [66,67]. These studies attributed the increased follicular and CL vascularity to the NGF/OIF induced changes in the secretion pattern of LH. However, since neurotrophins and their receptors have been identified in mammalian follicles [68], a direct effect of NGF/OIF on the llama ovary cannot be ruled out.

Studies in rabbits, another induced ovulator, have implicated NGF as a potential ovulation-inducing factor, similar to llamas, due its abundance in seminal plasma [69]. In contrast to llamas, it appears that the OIF in rabbits exerts its effect through both an endocrine- and nervous-mediated pathway, since epidural anesthesia drastically reduced ovulatory responses in response to intravaginal insemination with raw semen [70]. In an attempt to identify NGF as the OIF in rabbits, 24 µg recombinant mouse NGF was administered intramuscularly to rabbits [71]. Treatment with NGF did not increase ovulation rate (30%) when compared to saline controls (25%) and was much less effective than those administered gonadorelin (100% ovulation rate) [71]. The same research group reported that ovulation occurred in 100% of rabbits treated with gonadorelin, but only in 33% treated with recombinant mouse NGF, 25% treated with saline, and 0% treated with centrifuged raw camel seminal plasma [72]. Further studies using different doses and sources of NGF are needed to determine its potential role in ovulation induction of rabbits.

2.3 Nerve Growth Factor-β in Spontaneous Ovulators

As previously mentioned, NGF was first identified in the seminal plasma of bulls in the 1980s [18]. Subsequent studies in spontaneously ovulating species focused on its role within the male reproductive tract. It wasn't for another decade that the mammalian ovary was discovered to contain both NGF and its receptor, TrkA [73–76]. Another neurotrophin, NT-4, is abundantly expressed in the ovary and oocytes of *Xenopus*, suggesting a conserved evolutionary non-neuronal mechanism amongst these proteins [77]. Interestingly, neurotrophins (including NGF) and their receptors have been implicated in the mechanism surrounding seasonality in sheep [78]. Increased gene expression of *Ngf* and *Trka* in the arcuate nucleus and dopaminergic A15 of the hypothalamus were observed prior to transitional estrus in ewes, which affected responsiveness to estradiol

negative feedback typical of seasonal species [78]. Similarly, localization patterns of NGF and its receptors in the ovary and uterus of ground squirrel were correlated with increases in plasma gonadotropins, estradiol, and progesterone within the breeding season, suggesting potential involvement in the regulation of seasonal reproductive changes [79–81].

NGF has been localized to ovarian granulosa cells prior to formation of the first primordial follicles in neonatal mice [82] and rats [74] and is involved initiating folliculogenesis [83]. There was a reduction in the number of primary follicles despite normal presence of primordial follicles in NGF-null mice, suggesting a critical role of NGF in controlling differentiation of granulosa cell shape and proliferation [82,84]. Additionally, *in vitro* treatment with NGF improved survival of goat pre-antral follicles in a dose-dependent manner [85]. The role of NGF in promoting differentiation to primary and secondary follicles may be of importance for litter-bearing species, as multiple follicles need to be selected for dominance. The *Ngf* gene has since been implicated as a potential marker for litter size after finding an association between *Ngf* gene expression and phenotypic litter size in goats [86,87]. On the contrary, excessive NGF production impairs the bidirectional communication between oocyte and cumulus cells, inhibiting oocyte meiotic maturation [88]. This disruption in oocyte competence facilitates the development of ovarian cysts in conjunction with elevated plasma LH and may contribute to the development or progression of polycystic ovarian syndrome in humans [89,90].

Nerve growth factor- β may contribute to the cascade of events leading to follicular rupture at ovulation [91]. Inhibiting NGF/TrkA signaling reduced the rate of ovulation in rats, indicating a critical role in mammalian ovulation [92]. Though present during late fetal development, expression of NGF and TrkA in the ovary decreases postnatally between 24 and 48 hours after

birth and remains low until puberty [74]. At the time of the first pre-ovulatory LH surge, a transient activation of NGF/TrkA occurs and is integral to the process of follicular cytodifferentiation proceeding the first ovulation [92]. In bovine theca cells, NGF induced prostaglandin E₂ (PGE) synthesis [93], which promotes follicular rupture at ovulation [94]. Additionally, NGF/TrkA signaling in theca cells contributes to the loss of gap junctions within the follicular wall that precedes ovulation [95]. The presence of both LH and follicle stimulating hormone (FSH) *in vitro* were necessary to stimulate NGF secretion from medium to large follicles in ewes [96]. Consistently, follicular NGF concentrations increased following the gonadotropin surge in ewes, and *in vitro* treatment with NGF induced marked cumulus expansion and progressive cumulus-oocyte uncoupling that is typical before ovulation [97].

While there is clear evidence that the mammalian ovary can produce NGF (at least in spontaneously ovulating species), there may be a synergistic role for the seminal plasma NGF introduced during copulation to enhance steroidogenesis within the follicle, advance time to ovulation, and alter subsequent CL function (Fig. 3). Given the presence of hundreds of seminal plasma molecules, including NGF, it seems naïve to assume they exert no effects on the female reproductive tract when introduced during copulation. This notion is especially relevant given the systemic role of NGF in stimulating the pre-ovulatory LH surge in camelids [2,51]. The bovine uterus maintains a local countercurrent exchange between the uterine venous drainage and the ovarian artery that allows transport of prostaglandin F_{2α} from the uterus to the ovary during luteolysis [98,99]. This exchange could also provide a local route by which seminal plasma factors can reach the ovary [100]. In ruminants, semen deposition occurs in the vagina rather than the uterus (as is the case with camelids), so further studies are needed to determine if seminal plasma proteins are able to be “carried” to the uterus with spermatozoa or if systemic uptake of molecules

from the vagina is possible [101]. In camelids, circulating NGF can be detected within 15 minutes of copulation [59], and local inflammation produced from coitus seems to be crucial for ensuring systemic NGF absorption from the uterus [42]. However, whether a similar systemic absorption of NGF occurs in spontaneously ovulating species remains to be determined.

More recently, studies have looked at the systemic effects of NGF on bovine reproduction. One study showed an increase in FSH production in heifers treated systemically with llama-derived NGF on days 6 or 9 of the follicular wave, corresponding with dominant follicle sizes of 11.6 and 12.1 mm, respectively [102]. Subsequently, CL diameter was larger in heifers treated on day 6, with increased plasma progesterone levels in both groups compared to untreated controls [102], consistent with luteotrophic effects reported in camelids [46]. Similarly, heifers treated systemically with bovine seminal plasma containing 250 µg NGF within 4 hours of ovulation tended to have higher plasma progesterone concentrations, despite no differences in CL size [103]. In this same study, heifers treated before ovulation (follicle size ~10.9 mm), all ovulated within a 4 hour period (30 to 34 h after LH administration) versus a 22 hour period (26 to 44 hours after LH administration) [103]. Since whole seminal plasma was administered, it is difficult to ascertain whether this synchrony was due to NGF or another seminal plasma constituent (such as estradiol). Treatment of bovine theca cells with recombinant NGF increased androstenedione production [93], which is used as a precursor for estradiol production in the granulosa cell via the two-cell, two-gonadotropin theory (Fig. 2). Ovarian estradiol released into circulation is crucial for regulating the hypothalamic events that lead to ovulation [50]. In the human pre-ovulatory follicle, NGF stimulated estradiol secretion directly and indirectly through upregulation of FSH receptors [104]. Similar to results seen in camelids, NGF is able to directly stimulate LH production from bovine pituitary cells *in vitro*, suggesting another potential sequela to systemic absorption [47].

Interestingly, gene expression of the NGF receptor, *Trka*, is upregulated in response to LH treatment in bovine theca cells *in vitro* [93], and TrkA protein is increased in the dominant follicle and early CL *in vivo* [56], suggesting a local effect of NGF on early luteal formation. This data, coupled with the ability of NGF to directly stimulate LH production in bovine pituitary cells, suggests that there may be a multimodal effect of NGF systemically and locally that upregulates its signaling pathway to advance ovulation and improve luteal function in ruminants (Fig. 3).

Improved luteal function may be attributed to the direct effects of NGF on stimulating PGE production and theca cell proliferation from the bovine antral follicle [93]. After ovulation, theca cells differentiate into small luteal cells that, in response to LH binding, produce an early rise in progesterone that is essential for supporting initial embryonic growth [105]. Increased PGE production in the pre-ovulatory follicle also provides another possible rationale for improved steroidogenesis within the CL. This pro-angiogenic molecule increases vascularity [106], which would improve the ability for signaling molecules (such as LH) to reach ovarian tissues. Indeed, enhanced PGE production may explain why llamas treated with NGF experienced increased CL vascularity and progesterone concentrations when compared to GnRH-treated controls [63]. While this particular mechanism has yet to be studied in cattle, it is known that CL blood flow is significantly correlated with progesterone production in the early luteal phase [107]. Interestingly, NGF directly stimulated progesterone production from the bovine CL, with high doses (100 ng/mL) able to promote oxytocin release [108]. Further studies are necessary to better understand the underlying luteotrophic mechanism of NGF within the complex hypothalamic-pituitary-gonadal axis in cattle.

2.4 Nerve Growth Factor- β and Early Embryonic Development

Enhancement of CL development with subsequent increased progesterone production may help reduce early embryonic death in cattle [109]. Reports have estimated that up to 80% of bovine embryo losses occur by day 16 of pregnancy and may be attributed to the downstream effects of inadequate circulating progesterone concentrations on histotroph secretion into the uterine lumen [109,110]. Holstein heifers with low circulating progesterone concentrations had reduced histidine, threonine, and asparagine in histotrophic secretions on Day 13 post-ovulation when compared to those with normal or high progesterone concentrations [111]. This alteration in histotroph composition could affect its role as a nutritional source for conceptus development. Growth and survival of the preimplantation blastocyst rely on the production of these embryotrophic factors from the uterus, which ultimately affects embryonic interferon tau secretion [112,113]. Threshold levels of interferon tau are required to effectively block luteolysis and achieve maternal recognition of pregnancy in ruminants [114]. Therefore, if fertilization is achieved, but progesterone and interferon tau concentrations are inadequate, luteolysis and early embryonic death will occur.

Early embryonic death due to luteal insufficiency seems to be especially important in high-producing dairy cows that yield increased proportions of poor quality embryos [115,116]. Consistently, negative associations have been found between the concentration of milk progesterone at days 4 to 6 post-ovulation and the probability of embryo survival [117]. Of interest, lactating cows have decreased maximal progesterone concentrations when compared to non-lactating heifers, despite having larger volumes of luteal tissue [118]. This finding implies that there is either a dysfunction in steroidogenesis of the CL or an increased rate of steroid metabolism

by the liver [119–121]. Corpus luteum size is correlated with plasma progesterone up until the beginning of the luteal stasis phase at day 7 in cattle, after which luteal blood flow becomes a more reliable predictor of plasma progesterone concentrations [107]. This finding suggests that the degree of luteal vascularity may affect progesterone production in the early diestrus phase and may be better predictive of CL function. It seems that NGF may exert some of its reported luteotrophic properties through improved luteal vascularity, which could enhance LH signaling [34,46,63,102]. Regardless of the cause of luteal insufficiency, there is increasing evidence to suggest that progesterone supplementation can overcome this effect by increasing embryo developmental capacity and interferon tau production [122].

Although studies have shown the benefits of using supplemental progesterone early in the luteal phase to improve pregnancy per artificial insemination, timing of administration appears to be crucial for enhancing embryonic development [122–124]. Supplementing progesterone from days 4 to 7 and 4 to 10 post-estrus in heifers with experimentally induced subluteal function resulted in significantly greater embryo length compared to untreated subluteal control heifers [125]. On the contrary, supplementing progesterone from days 7 to 10 in this study did not increase embryo length [125]. While short-term progesterone supplementation of heifers from days 3 to 7 after estrus increased conceptus size and interferon tau production, it also led to a paradoxical increased number of short cycles [123]. Likewise, heifers supplemented with progesterone from days 4 to 7, 4 to 10, and 7 to 10 all had decreased progesterone concentrations at day 15 in comparison to untreated subluteal control heifers [125]. Therefore, progesterone supplementation does not help to overcome compromised luteal function and may require long-term administration. Strategies aimed at improving development of the endogenous CL or administration of luteotrophic agents have been proposed as an alternative. A commonly described mechanism for

increasing peripheral progesterone concentrations is administration of human chorionic gonadotropin (hCG) [126]. This LH-like hormone induces ovulation of the first wave dominant follicle to form accessory CL that will produce supplemental progesterone [109]. While administration of hCG from 5 to 7 days after artificial insemination increases progesterone concentrations in dairy cows, the effects on pregnancy rates have been inconsistent [127,128]. Similar to exogenous progesterone supplementation, timing of hCG administration seems to be a contributing factor to its variable success rates [128].

Administration of NGF would mimic the endogenous uptake of seminal plasma proteins that occur at natural mating and could provide a novel technology to improve CL function and reduce pregnancy losses in cattle. Circulating NGF concentrations in female llamas increased within 15 minutes of insemination and were sustained for at least 3 hours [59]. Though NGF directly stimulates LH production from bovine pituitary cells *in vitro* [47], this mechanism has yet to be determined *in vivo*. The ability of NGF to stimulate LH release, coupled with research showing that LH can increase abundance of NGF receptors (TrkA) in bovine theca cells [93], suggest a potential upregulation mechanism within the NGF signaling cascade that could lead to downstream effects on CL function. Systemic administration of NGF would ensure a rational use of the molecule to act at both the ovary and the anterior pituitary. Additionally, this strategy would eliminate the concern about timing of administration that is present with other methods [122,125,126] and reduce stress associated with excessive handling after insemination [129].

While improved luteal function may play a big role in improving conceptus development, there is some indication that NGF may have the ability to act directly on the embryo. Rabbit zygotes incubated with NGF had improved numbers of hatching blastocysts and increased total

cell number in the blastocysts [130]. Similarly, incubation of sheep oocytes with NGF during *in vitro* fertilization induced early cleavage and improved embryo development [131]. When combined with other paracrine factors, NGF treatment of sheep oocytes during *in vitro* maturation promoted cumulus cell expansion and inhibited oocyte/cumulus apoptosis, which prevented embryo apoptosis and allowed more embryos to develop into blastocysts [132]. While *in vitro* studies help to understand direct effects of NGF signaling, further studies looking at *in vivo* effects of NGF treatment on embryonic development are needed.

2.5 Nerve Growth Factor- β in the Male

Before we can truly understand the role of NGF within the female reproductive tract, we must first have an in depth understanding of its production and roles within the male reproductive tract. Nerve growth factor was localized to the Sertoli cells in rats as early as embryonic day 14 and identified as one of the regulators of paracrine cell to cell interactions that result in morphological sex determination and perinatal testis growth [133]. Gene expression of NGF was demonstrated in the mouse testis along with the ability for both germ and non-germ cells to synthesize NGF and signal in an autocrine/paracrine manner [134,135]. Additional studies identified a NGF-responsive lamina-propria cell that influenced tubular wall and seminiferous epithelium in human testis [136]. In addition to testis and epididymis, NGF and its receptors have been localized to the seminal vesicles, prostate, and coagulating gland of rats [137,138] and increased expression of androgen binding protein mRNA in the Sertoli cells of rat testis [139]. Similar to the effects reported on theca cells [93], NGF appears to have proliferation and differentiation-promoting effects on adult stem Leydig cells [140,141]. The observation of NGF in different compartments of the male reproductive tract may reflect different functions in

regulating germ cell differentiation, influencing motility of mature spermatozoa, or signaling within the female reproductive tract [142].

Early studies identified vesicular glands of the bulls as the primary source of NGF production using biological assays [19]. More recently, this finding was confirmed using immunohistochemistry, with strongest immunoreactivity localized to the glandular epithelium and lumen of both bull ampulla and vesicular glands [30]. Species-dependent variations were identified in regard to NGF localization in the male. The main source of NGF was identified as the prostate in llamas, which lack a vesicular gland, and both prostate and ampulla in cervids (elk and white-tailed deer) [30]. In rabbits, another induced ovulator, the primary source of NGF was also identified as the prostate gland using both immunohistochemistry and gene expression [69,71]. Interestingly, NGF was not detected in either stallion or boar seminal plasma by either PC₁₂ differentiation bioassay or by radioimmunoassay [30]. Consistently, the use of western blot identified NGF in the seminal plasma of alpaca, camels, and bulls, but not in boars, bucks, rams, or stallions [25]. These distinct species-specific variations in NGF presence and localization are likely a manifestation of differences in copulation mechanisms. Bulls secrete fractions of seminal plasma with the prostate contributing mostly to pre-ejaculate and the vesicular gland contributing mostly to the sperm-rich fraction [143]. While bulls copulate within a matter of seconds, copulation lasts around 30 minutes in llamas, with sperm ejection not occurring for approximately 5 to 10 minutes [144]. Since llamas undergo multiple emissions and ejaculations during this prolonged copulation period, NGF can be distributed throughout the entire ejaculate rather than needing to be concentrated into a specific fraction, as in bulls.

It is worth noting that the previous study did not mention the season for sample collection of the seasonally-dependent elk and white-tailed deer evaluated [30]. Seasonal changes in *Ngf* mRNA and NGF protein expression have been reported in the reproductive tract of both male and female ground squirrels [79,145]. Seasonal changes in seminal plasma composition have been implicated for variations observed in semen quality throughout the breeding season of white-tailed deer based on changes observed in accessory sex gland size and ejaculate appearance [146,147]. In rams, motility was found to be improved after incubating frozen-thawed sperm with seminal plasma harvested in autumn and winter, but not spring or summer, suggesting seasonal variability in its composition [148]. Though bulls are not considered seasonal species, studies have reported seasonal fluctuations in testosterone production [149–151], which could correspond with changes in seminal plasma composition. Interestingly, in three separate proteomic studies using Holstein, *Bos taurus*, and *Bos indicus* bulls, only 22 out of hundreds of proteins identified in bull seminal plasma overlapped [152–154]. Additionally, NGF immunolocalization in rabbit accessory sex glands changed with age, potentially in relation to changes in serum testosterone [155]. Collectively, these findings all suggest that there could be variations in seminal plasma composition based on species, breed, age, season, and/or collection technique. Therefore, there is a need to evaluate each of these factors separately to better understand the biology of NGF production within the male and its potential use in reproductive technologies.

Several studies have demonstrated an association between seminal plasma proteins and bull fertility [5–9]. The effects of seminal plasma constituents on sperm transport, capacitation, and fertilization have been well described previously [162–164]. The role of seminal plasma proteins in regulating fertility is of special relevance to the cattle industry where artificial insemination has advanced genetic selection of desirable traits such as milk production and meat

quality [165]. Historically, bull semen studies have focused on improving semen freezing techniques and determining the minimum effective breeding dose, while overlooking the effects that semen processing may have on the function of seminal plasma proteins [166]. When processing semen for cryopreservation, samples are extended, which may dilute seminal plasma proteins to sub-physiologic levels [167] and contribute to lower fertility [168–170].

Compared to other seminal plasma proteins, relatively little work has looked at the effects of NGF on spermatozoa. Ejaculated bovine sperm have been found to have NGF-immunoreactivity localized to the sperm head and tail, while its receptor (TrkA) is localized to the acrosomal cap, nucleus, and tail regions [171]. In addition to several other proteins, sperm *Ngf* mRNA abundance in bulls was strongly associated with positive sire conception rates [158], which was attributed to beneficial effects of NGF on sperm membrane integrity [171]. However, as mentioned previously, NGF may play a pivotal role within the female reproductive tract as well. In golden hamsters, NGF and its receptor are broadly distributed throughout the female reproductive tract (ovary, uterus, oviducts) [172]. It was also determined that NGF increased sperm motility and acrosome reaction in golden hamster sperm in a time- and dose-dependent manner [173]. Consistently, NGF and its receptor have both been localized to the bovine oviduct and may regulate its function in conjunction with gonadotropins [174]. Collectively, these findings suggest that there may be a temporal and/or spatial mechanism of NGF signaling between the male and female reproductive systems that has yet to be fully elucidated amongst mammalian species.

More recently, seminal plasma *Ngf* mRNA expression was found to be positively associated with the maintenance of post-thaw functional membrane integrity in bull sperm, suggesting that it could be used to assess cryotolerance [175]. Another study reported higher *Ngf*

mRNA expression in spermatozoa of good semen-producing (<25% discarded ejaculates) versus poor semen-producing (>40% discarded ejaculates) bulls, though they found no correlation with field conception rates [176]. Relative *Ngf* expression levels in spermatozoa were also positively associated with pre-freeze mitochondrial membrane potential and post-thaw sperm velocity parameters in *Bos taurus* bulls [176]. Based on these findings, it seems that NGF is an optimal candidate protein to evaluate for improving sperm cryopreservation techniques in bulls. In men with normozoospermia and asthenozoospermia, supplementation of freezing extender with NGF at 0.5 ng/mL improved sperm viability and motility and decreased DNA fragmentation [177,178]. Consistently, treatment of bovine sperm cells with 40 to 80 ng/mL NGF increased leptin secretion and sperm membrane integrity [171]. Additional information regarding the production of NGF in the bull and its role in sperm physiology are necessary to determine how it can most effectively be used in assisted reproductive techniques.

2.6 Figures

Figure 1. (A) Absorbance profile for one-step HPLC purification protocol. (B) Peaks corresponding with D1, D2, D4, D5, D7, D12, E4, and E6 fractions were run on SDS-Page gel and stained with Coomassie Blue. D5 fraction showed a prominent band (~15 kDa) with less prominent bands staining in fractions D4 & D6. These fractions were subjected to trypsin digestion and liquid chromatography-mass spectrometry (LC/MS). Majority of NGF in fraction D4, with lower concentrations in D5 & D6. Major contaminant, Caltrin, highly concentrated in D5 (prominent band observed).

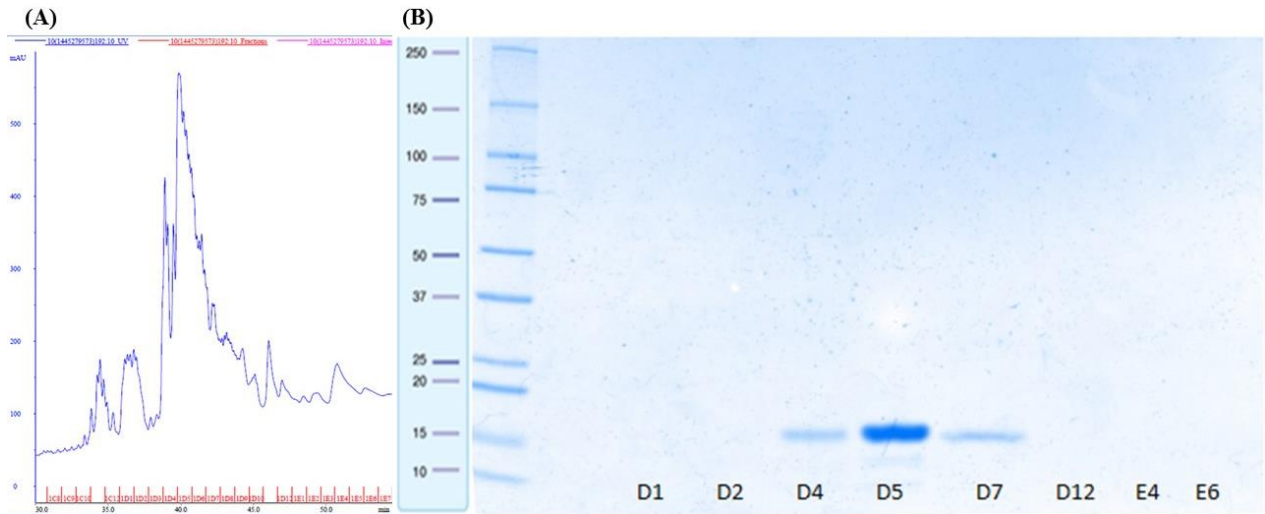


Figure 2. Depiction of the steroidogenic enzymes involved in the two-cell, two-gonadotropin theory for ovarian steroid production.

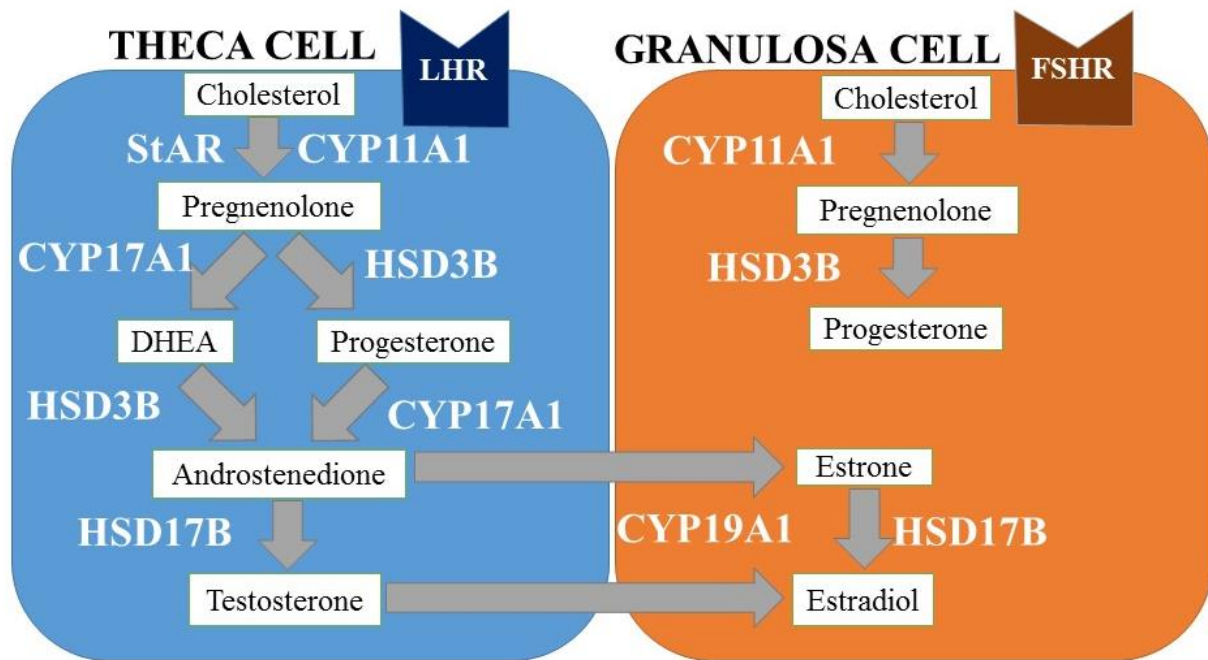
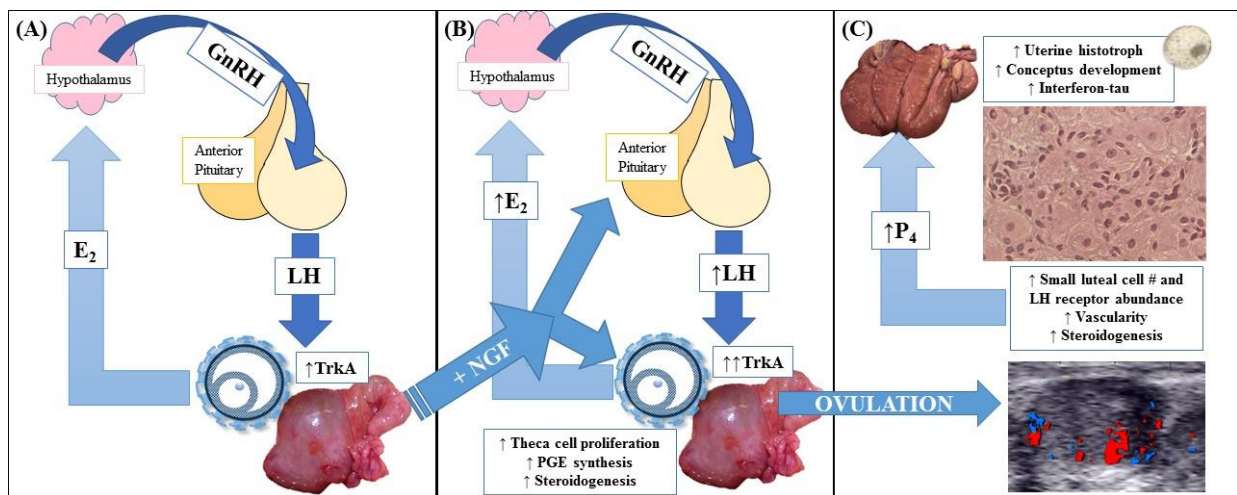


Figure 3. Schematic of the potential mechanism of NGF in improving luteal function. **(A)** Pre-ovulatory hormone signaling within the hypothalamic-pituitary-ovarian axis begins as estradiol (E_2) is produced from the growing follicle and stimulates an increase in gonadotropin releasing hormone (GnRH) pulse frequency from the hypothalamus. GnRH acts on the anterior pituitary to stimulate follicle stimulating and luteinizing hormone (LH) production. LH signaling upregulates TrkA expression in the theca and granulosa cells of the bovine pre-ovulatory follicle [56]. **(B)** Upon copulation, NGF is potentially absorbed into systemic circulation, where it could act on the anterior pituitary to increase LH production [47], further upregulating TrkA expression in the ovary. NGF likely also travels to the ovary, where it can bind to its now upregulated TrkA receptor and stimulate increased theca cell proliferation, prostaglandin E_2 (PGE) synthesis, and steroidogenesis [93]. **(C)** Following ovulation, increased vascularity could be present due to angiogenesis stimulated from PGE production [106]. Additionally, the proliferated theca cells will luteinize and become small luteal cells, which produce large amounts of progesterone (P_4) in response to LH signaling during early luteal formation [179]. This combination of effects leads to increased P_4 production, enhancing conceptus growth and interferon-tau production, which signals for maternal recognition of pregnancy [109].



2.7 References

- [1] Kolbeck R, Jungbluth S, Barde Y-A. Characterisation of neurotrophin dimers and monomers. *Eur J Biochem* 1994;225:995–1003. doi:10.1111/j.1432-1033.1994.0995b.x.
- [2] Ratto MH, Leduc YA, Valderrama XP, van Straaten KE, Delbaere LTJ, Pierson RA, et al. The nerve of ovulation-inducing factor in semen. *Proc Natl Acad Sci U S A* 2012;109:15042–7. doi:10.1073/pnas.1206273109.
- [3] Levi-Montalcini R, Hamburger V. Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. *J Exp Zool* 1951;116:321–61. doi:10.1002/jez.1401160206.
- [4] Levi-Montalcini R, Hamburger V. A diffusible agent of mouse sarcoma, producing hyperplasia of sympathetic ganglia and hyperneurotization of viscera in the chick embryo. *J Exp Zool* 1953;123:233–87. doi:10.1002/jez.1401230203.
- [5] Cohen S, Levi-Montalcini R, Hamburger V. A nerve growth-stimulating factor isolated from sarcomas 37 and 180. *Proc Natl Acad Sci* 1954;40:1014–8. doi:10.1073/pnas.40.10.1014.
- [6] Levi-Montalcini R. The nerve growth factor: its mode of action on sensory and sympathetic nerve cells. *Harvey Lect* 1966;60:217–59.
- [7] Levi-Montalcini R. The nerve growth factor: thirty-five years later. *Biosci Rep* 1987;7:681–99. doi:10.1007/BF01116861.
- [8] Levi-Montalcini R, Booker B. Destruction of the sympathetic ganglia in mammals by an antiserum to a nerve-growth protein. *Proc Natl Acad Sci U S A* 1960;46:384–91. doi:10.1073/pnas.46.3.384.
- [9] Levi-Montalcini R, Booker B. Excessive growth of the sympathetic ganglia evoked by a protein isolated from mouse salivary glands. *Proc Natl Acad Sci U S A* 1960;46:373–84.
- [10] Levi-Montalcini R. Growth control of nerve cells by a protein factor and its antiserum. *Science* (80-) 1964;143:105–10.
- [11] Cohen S, Levi-Montalcini R. A nerve growth-stimulating factor isolated from snake venom. *Proc Natl Acad Sci* 1956;42:572–4. doi:10.1073/pnas.42.9.571.
- [12] Cohen S. Purification of a nerve-growth promoting protein from the mouse salivary gland and its neuro-cytotoxic antiserum. *Proc Natl Acad Sci U S A* 1960;46:302–11. doi:10.1073/pnas.46.3.302.
- [13] Angeletti PU, Levi-Montalcini R, Kettler R, Thoenen H. Comparative studies on the effect of the nerve growth factor on sympathetic ganglia and adrenal medulla in newborn rats. *Brain Res* 1972;44:197–206. doi:10.1016/0006-8993(72)90375-7.
- [14] Harper GP, Pearce FL, Vernon CA. The production and storage of Nerve Growth Factor *in vivo* by tissues of the mouse, rat, guinea pig, hamster, and gerbil. *Dev Biol* 1980;77:391–402. doi:10.1016/0012-1606(80)90483-2.
- [15] Harper GP, Barde YA, Burnstock G, Carstairs JR, Dennison ME, Suda K, et al. Guinea pig prostate is a rich source of nerve growth factor. *Nature* 1979;279:160–2. doi:10.1038/279160a0.

- [16] Banks BEC, Banthorpe D V., Charlwood KA, Pearce FL, Vernon CA, Edwards DC. Distribution and immunology of mammalian nerve growth factor. *Nature* 1973;246:503–4. doi:10.1038/246503a0.
- [17] Harper GP, Thoenen H. The distribution of nerve growth factor in the male sex organs of mammals. *J Neurochem* 1980;34:893–903. doi:10.1111/j.1471-4159.1980.tb09663.x.
- [18] Harper GP, Glanville RW, Thoenen H. The purification of nerve growth factor from bovine seminal plasma. Biochemical characterization and partial amino acid sequence. *J Biol Chem* 1982;257:8541–8.
- [19] Hofmann H-D, Unsicker K. The seminal vesicle of the bull: a new and very rich source of nerve growth factor. *Eur J Biochem* 1982;128:421–6. doi:10.1111/j.1432-1033.1982.tb06981.x.
- [20] Varon S, Nomura J, Shooter EM. Reversible dissociation of the mouse nerve growth factor protein into different subunits. *Biochemistry* 1968;7:1296–303. doi:10.1021/bi00844a008.
- [21] Paoletti F, Malerba F, Kelly G, Noinville S, Lamba D, Cattaneo A, et al. Conformational plasticity of proNGF. *PLoS One* 2011;6:e22615. doi:10.1371/journal.pone.0022615.
- [22] Server AC, Shooter EM. Nerve growth factor. *Adv Protein Chem* 1977;31:339–409. doi:10.1016/S0065-3233(08)60221-1.
- [23] Meier R, Becker-André M, Götz R, Heumann R, Shaw A, Thoenen H. Molecular cloning of bovine and chick nerve growth factor (NGF): delineation of conserved and unconserved domains and their relationship to the biological activity and antigenicity of NGF. *EMBO J* 1986;5:1489–93. doi:10.1002/j.1460-2075.1986.tb04387.x.
- [24] Edwards RH, Selby MJ, Garcia PD, Rutter WJ. Processing of the native nerve growth factor precursor to form biologically active nerve growth factor. *J Biol Chem* 1988;263:6810–5. doi:10.1016/j.juro.2010.12.067.
- [25] Druart X, Rickard JP, Mactier S, Kohnke PL, Kershaw-Young CM, Bathgate R, et al. Proteomic characterization and cross species comparison of mammalian seminal plasma. *J Proteomics* 2013;91:13–22. doi:10.1016/j.jprot.2013.05.029.
- [26] Reinshagen M, Geerling I, Eysselein VE, Adler G, Huff KR, Moore GP, et al. Commercial recombinant human β -nerve growth factor and adult rat dorsal root ganglia contain an identical molecular species of nerve growth factor prohormone. *J Neurochem* 2000;74:2127–33. doi:10.1046/j.1471-4159.2000.0742127.x.
- [27] Pedraza CE, Podlesniy P, Vidal N, Arévalo JC, Lee R, Hempstead B, et al. Pro-NGF isolated from the human brain affected by Alzheimer’s disease induces neuronal apoptosis mediated by p75NTR. *Am J Pathol* 2005;166:533–43. doi:10.1016/S0002-9440(10)62275-4.
- [28] Steele JG, Dalton BA, Hoffman H, Underwood PA, Rathjen D. Monoclonal antibodies to nerve growth factor from bovine seminal plasma. *Mol Immunol* 1985;22:1061–72. doi:10.1016/0161-5890(85)90109-9.
- [29] Pan G, Chen Z, Liu X, Li D, Xie Q, Ling F, et al. Isolation and purification of the ovulation-inducing factor from seminal plasma in the bactrian camel (*Camelus bactrianus*). *Theriogenology* 2001;55:1863–79. doi:10.1016/S0093-691X(01)00528-3.

- [30] Bogle OA, Carrasco RA, Ratto MH, Singh J, Adams GP. Source and localization of ovulation-inducing factor/nerve growth factor in male reproductive tissues among mammalian species. *Biol Reprod* 2018. doi:10.1093/biolre/iy149.
- [31] Conaway CH. Ecological adaptation and mammalian reproduction. *Biol Reprod* 1971;4:239–47. doi:10.1093/biolreprod/4.3.239.
- [32] Wildt DE, Seager SWJ, Chakraborty PK. Effect of copulatory stimuli on incidence of ovulation and on serum luteinizing hormone in the cat. *Endocrinology* 1980;107:1212–7. doi:10.1210/endo-107-4-1212.
- [33] Larivière S, Ferguson SH. Evolution of induced ovulation in North American carnivores. *J Mammal* 2003;84:937–47. doi:10.1644/BME-003.
- [34] Adams GP, Ratto MH, Huanca W, Singh J. Ovulation-inducing factor in the seminal plasma of alpacas and llamas. *Biol Reprod* 2005;73:452–7. doi:10.1095/biolreprod.105.040097.
- [35] Chen BX, Yuen ZW, Pan GW. Semen-induced ovulation in the bactrian camel (*Camelus bactrianus*). *J Reprod Fertil* 1985;73:335–9. doi:10.1530/jrf.0.0740335.
- [36] Xu YS, Wang HY, Zeng GQ, Jiang GT, Gao YH. Hormone concentrations before and after semen-induced ovulation in the bactrian camel (*Camelus bactrianus*). *J Reprod Fertility* 1985;74:341–6. doi:10.1530/jrf.0.0740335.
- [37] Pan G, Zhao X, Chen S, Jiang S, Huang Y, Zu Y, et al. The ovulation inducing effect of seminal plasma in the Bactrian camel. In: Allen WR, editor. *Proc. first Int. Camel Conf.*, Newmarket, England: R&W Publications; 1992, p. 159–61.
- [38] Bromfield JJ, Schjenken JE, Chin PY, Care AS, Jasper MJ, Robertson SA. Maternal tract factors contribute to paternal seminal fluid impact on metabolic phenotype in offspring. *Proc Natl Acad Sci USA* 2014;111:2200–5. doi:10.1073/pnas.1305609111.
- [39] Wolfner MF. The gifts that keep on giving: physiological functions and evolutionary dynamics of male seminal proteins in *Drosophila*. *Heredity (Edinb)* 2002;88:85–93. doi:10.1038/sj/hdy/6800017.
- [40] Bravo PW, Moscoso J, Ordoñez C, Alarcon V. Transport of spermatozoa and ova in female alpaca. *Anim Reprod Sci* 1996;43:173–9. doi:10.1016/0378-4320(95)01465-9.
- [41] Bravo PW, Fowler ME, Stabenfeldt GH, Lasley B. Endocrine responses in the llama to copulation. *Theriogenology* 1990;33:891–9. doi:10.1016/0093-691X(90)90824-D.
- [42] Ratto MH, Huanca W, Singh J, Adams GP. Local versus systemic effect of ovulation-inducing factor in the seminal plasma of alpacas. *Reprod Biol Endocrinol* 2005;3:29. doi:10.1186/1477-7827-3-29.
- [43] Paolicchi F, Urquieta B, Del Valle L, Bustos-Obregón E. Biological activity of the seminal plasma of alpacas: stimulus for the production of LH by pituitary cells. *Anim Reprod Sci* 1999;54:203–10. doi:10.1016/S0378-4320(98)00150-X.
- [44] Ratto MH, Huanca W, Adams GP. Ovulation-inducing factor: a protein component of llama seminal plasma. *Reprod Biol Endocrinol* 2010;8:44. doi:10.1186/1477-7827-8-44.
- [45] Ratto MH, Delbaere LT, Leduc YA, Pierson RA, Adams GP. Biochemical isolation and purification of ovulation-inducing factor (OIF) in seminal plasma of llamas. *Reprod Biol*

- Endocrinol 2011;9:24. doi:10.1186/1477-7827-9-24.
- [46] Tanco VM, Ratto MH, Lazzarotto M, Adams GP. Dose-response of female llamas to ovulation-inducing factor from seminal plasma. *Biol Reprod* 2011;85:452–6. doi:10.1095/biolreprod.111.091876.
 - [47] Bogle OA, Ratto MH, Adams GP. Ovulation-inducing factor (OIF) induces LH secretion from pituitary cells. *Anim Reprod Sci* 2012;133:117–22. doi:10.1016/j.anireprosci.2012.06.006.
 - [48] Silva ME, Smulders JP, Guerra M, Valderrama XP, Letelier C, Adams GP, et al. Cetorelix suppresses the preovulatory LH surge and ovulation induced by ovulation-inducing factor (OIF) present in llama seminal plasma. *Reprod Biol Endocrinol* 2011;9:74. doi:10.1186/1477-7827-9-74.
 - [49] Silva ME, Recabarren MP, Recabarren SE, Adams GP, Ratto MH. Ovarian estradiol modulates the stimulatory effect of ovulation-inducing factor (OIF) on pituitary LH secretion in llamas. *Theriogenology* 2012;77:1873–82. doi:10.1016/j.theriogenology.2012.01.004.
 - [50] Pinilla L, Aguilar E, Dieguez C, Millar RP, Tena-Sempere M. Kisspeptins and reproduction: physiological roles and regulatory mechanisms. *Physiol Rev* 2012;92:1235–316. doi:10.1152/physrev.00037.2010.
 - [51] Kershaw-Young CM, Druart X, Vaughan J, Maxwell WMC. β -Nerve growth factor is a major component of alpaca seminal plasma and induces ovulation in female alpacas. *Reprod Fertil Dev* 2012;24:1093–7. doi:10.1071/RD12039.
 - [52] Gibbs RB, Pfaff DW. In-situ hybridization detection of *trkA* messenger-RNA in brain-distribution, colocalization with p75NGFR and up-regulation by nerve growth factor. *J Comp Neurol* 1994;341:324–39. doi:10.1002/cne.903410304.
 - [53] Adams GP, Ratto MH, Silva ME, Carrasco RA. Ovulation-inducing factor (OIF/NGF) in seminal plasma: a review and update. *Reprod Domest Anim* 2016;51:4–17. doi:10.1111/rda.12795.
 - [54] Carrasco RA, Singh J, Adams GP. The relationship between gonadotropin releasing hormone and ovulation inducing factor/nerve growth factor receptors in the hypothalamus of the llama. *Reprod Biol Endocrinol* 2018;16:83. doi:10.1186/s12958-018-0402-6.
 - [55] Tanco VM, Whitlock BK, Jones MA, Wilborn RR, Brandebourg TD, Foradori CD. Distribution and regulation of gonadotropin-releasing hormone, kisspeptin, RF-amide related peptide-3, and dynorphin in the bovine hypothalamus. *PeerJ* 2016;4:e1833. doi:10.7717/peerj.1833.
 - [56] Carrasco R, Singh J, Adams GP. The dynamics of *trkA* expression in the bovine ovary are associated with a luteotrophic effect of ovulation-inducing factor/nerve growth factor (OIF/NGF). *Reprod Biol Endocrinol* 2016;14:47. doi:10.1186/s12958-016-0182-9.
 - [57] El Allali K, El Bousmaki N, Ainani H, Simonneaux V. Effect of the camelid's seminal plasma ovulation-inducing factor/ β -NGF: a kisspeptin target hypothesis. *Front Vet Sci* 2017;4. doi:10.3389/fvets.2017.00099.
 - [58] Inoue N, Sasagawa K, Ikai K, Sasaki Y, Tomikawa J, Oishi S, et al. Kisspeptin neurons mediate reflex ovulation in the musk shrew (*Suncus murinus*). *Proc Natl Acad Sci U S A*

- 2011;108:17527–32. doi:10.1073/pnas.1113035108.
- [59] Berland MA, Ulloa-Leal C, Barria M, Wright H, Dissen GA, Silva ME, et al. Seminal plasma induces ovulation in llamas in the absence of a copulatory stimulus: role of nerve growth factor as an ovulation-inducing factor. *Endocrinology* 2016;157:3224–32. doi:10.1210/en.2016-1310.
 - [60] Loy R, Taglialatela G, Angelucci L, Heyer D, Perez-Polo R. Regional CNS uptake of blood-borne nerve growth factor. *J Neurosci Res* 1994;39:339–46. doi:10.1002/jnr.490390311.
 - [61] Pan W, Banks WA, Kastin AJ. Permeability of the blood-brain barrier to neurotrophins. *Brain Res* 1998;788:87–94. doi:10.1016/S0006-8993(97)01525-4.
 - [62] Berland MA, Guerra M, Bogle OA, Vio K, Adams GP, Ratto MH. 107 Detection of biotinylated-ovulation-inducing factor (OIF) in cerebrospinal fluid and its ability to induce ovulation. *Reprod Fertil Dev* 2012;25:201. doi:10.1071/RDv25n1Ab107.
 - [63] Ulloa-Leal C, Bogle OA, Adams GP, Ratto MH. Luteotrophic effect of ovulation-inducing factor/nerve growth factor present in the seminal plasma of llamas. *Theriogenology* 2014;81:1101–1107.e1. doi:10.1016/j.theriogenology.2014.01.038.
 - [64] Fernández A, Ulloa-Leal C, Silva M, Norambuena C, Adams GP, Guerra M, et al. The effect of repeated administrations of llama ovulation-inducing factor (OIF/NGF) during the peri-ovulatory period on corpus luteum development and function in llamas. *Anim Reprod Sci* 2014;149:345–52. doi:10.1016/j.anireprosci.2014.08.001.
 - [65] Silva M, Ulloa-Leal C, Valderrama XP, Bogle OA, Adams GP, Ratto MH. Nerve growth factor from seminal plasma origin (sp β -NGF) increases CL vascularization and level of mRNA expression of steroidogenic enzymes during the early stage of corpus luteum development in llamas. *Theriogenology* 2017;103:69–75. doi:10.1016/j.theriogenology.2017.07.041.
 - [66] Stocco DM. StAR protein and the regulation of steroid hormone biosynthesis. *Annu Rev Physiol* 2001;63:193–213. doi:10.1146/annurev.physiol.63.1.193.
 - [67] Niswender GD. Molecular control of luteal secretion of progesterone. *Reproduction* 2002;123:333–9. doi:10.1530/rep.0.1230333.
 - [68] Streiter S, Fisch B, Sabbah B, Ao A, Abir R. The importance of neuronal growth factors in the ovary. *Mol Hum Reprod* 2016;22:3–17. doi:10.1093/molehr/gav057.
 - [69] Maranesi M, Zerani M, Leonardi L, Pistilli A, Arruda-Alencar J, Stabile AM, et al. Gene expression and localization of NGF and its cognate receptors NTRK1 and NGFR in the sex organs of male rabbits. *Reprod Domest Anim* 2015;50:918–25. doi:10.1111/rda.12609.
 - [70] Maranesi M, Petrucci L, Leonardi L, Piro F, Rebollar PG, Millán P, et al. New insights on a NGF-mediated pathway to induce ovulation in rabbits (*Oryctolagus cuniculus*). *Biol Reprod* 2018;98:634–43. doi:10.1093/biolre/iyoy041.
 - [71] Garcia-Garcia RM, Masdeu M del M, Sanchez Rodriguez A, Millan P, Arias-Alvarez M, Sakr OG, et al. β -nerve growth factor identification in male rabbit genital tract and seminal plasma and its role in ovulation induction in rabbit does. *Ital J Anim Sci* 2018;17:442–53. doi:10.1080/1828051X.2017.1382315.

- [72] Masdeu M, Garcia-Garcia RM, Cardinali R, Millan P, Arias Alvarez M, Castellini C, et al. 271 Induction of ovulation in rabbit does using purified nerve growth factor and camel seminal plasma. *Reprod Fertil Dev* 2015;27:224. doi:10.1071/RDv27n1Ab271.
- [73] Ernfors P, Wetmore C, Olson L, Persson H. Identification of cells in rat brain and peripheral tissues expressing mRNA for members of the nerve growth factor family. *Neuron* 1990;5:511–26. doi:10.1016/0896-6273(90)90090-3.
- [74] Dissen GA, Hirshfield AN, Malamed S, Ojeda SR. Expression of neurotrophins and their receptors in the mammalian ovary is developmentally regulated: changes at the time of folliculogenesis. *Endocrinology* 1995;136:4681–92. doi:10.1210/en.136.10.4681.
- [75] Anderson RA, Robinson LLL, Brooks J, Spears N. Neurotrophins and their receptors are expressed in the human fetal ovary. *J Clin Endocrinol Metab* 2002;87:890–7. doi:10.1210/jcem.87.2.8221.
- [76] Ren L, Medan MS, Weng Q, Jin W, Li C, Watanabe G, et al. Immunolocalization of nerve growth factor (NGF) and its receptors (TrkA and p75LNGFR) in the reproductive organs of shiba goats. *J Reprod Dev* 2005;51:399–404. doi:10.1262/jrd.16082.
- [77] Hallböök F, Ibáñez CF, Persson H. Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in xenopus ovary. *Neuron* 1991;6:845–58. doi:10.1016/0896-6273(91)90180-8.
- [78] Lehman MN, Ladha Z, Coolen LM, Hileman SM, Connors JM, Goodman RL. Neuronal plasticity and seasonal reproduction in sheep. *Eur J Neurosci* 2010;32:2152–64. doi:10.1111/j.1460-9568.2010.07530.x.
- [79] Li B, Sheng X, Bao L, Huang S, Li Q, Liu Y, et al. Seasonal changes in expression of nerve growth factor and its receptors TrkA and p75 in the ovary of wild ground squirrel (*Citellus dauricus* Brandt). *J Ovarian Res* 2014;7:1–9. doi:10.1186/1757-2215-7-3.
- [80] Song M-S, Ma X-T, Weng J, Li B, Weng Q, Taya K. Expression of nerve growth factor and its receptors NTRK1 and TNFRSF1B in the uteri of wild female ground squirrels (*Citellus dauricus* Brandt). *Biol Reprod* 2010;83:434–434. doi:10.1093/biolreprod/83.s1.434.
- [81] Li B, Sheng X, Song M, Zhang H, Weng J, Zhang M, et al. Expression of nerve growth factor and its receptors TrkA and p75 in the uterus of wild female ground squirrel (*Citellus dauricus* Brandt). *Gen Comp Endocrinol* 2012;176:62–9. doi:10.1016/j.ygcen.2011.12.024.
- [82] Dissen GA, Romero C, Hirshfield AN, Ojeda SR. Nerve growth factor is required for early follicular development in the mammalian ovary. *Endocrinology* 2001;142:2078–86. doi:10.1210/endo.142.5.8126.
- [83] Ojeda SR, Romero C, Tapia V, Dissen GA. Neurotrophic and cell–cell dependent control of early follicular development. *Mol Cell Endocrinol* 2000;163:67–71. doi:10.1016/S0303-7207(99)00242-7.
- [84] Hirshfield AN. Development of follicles in the mammalian ovary. *Int Rev Cytol* 1991;124:43–101. doi:10.1016/S0074-7696(08)61524-7.
- [85] Chaves RN, Alves AMC V, Duarte ABG, Araújo VR, Celestino JJH, Matos MHT, et al. Nerve growth factor promotes the survival of goat preantral follicles cultured *in vitro*.

- Cells Tissues Organs 2010;192:272–82. doi:10.1159/000317133.
- [86] An X, Bai L, Hou J, Zhao H, Peng J, Song Y, et al. Molecular cloning, tissue expression and SNP analysis in the goat nerve growth factor gene. *Mol Biol Rep* 2013;40:857–63. doi:10.1007/s11033-012-2126-6.
 - [87] Naicy T, Venkatachalapathy RT, Aravindakshan TV, Radhika G, Raghavan KC, Mini M, et al. Nerve Growth Factor gene ovarian expression, polymorphism identification, and association with litter size in goats. *Theriogenology* 2016;86:2172–8. doi:10.1016/j.theriogenology.2016.07.011.
 - [88] Zhai Y, Yao G, Rao F, Wang Y, Song X, Sun F. Excessive nerve growth factor impairs bidirectional communication between the oocyte and cumulus cells resulting in reduced oocyte competence. *Reprod Biol Endocrinol* 2018;16:28. doi:10.1186/s12958-018-0349-7.
 - [89] Dissen GA, Garcia-Rudaz C, Paredes A, Mayer C, Mayerhofer A, Ojeda SR. Excessive ovarian production of nerve growth factor facilitates development of cystic ovarian morphology in mice and is a feature of polycystic ovarian syndrome in humans. *Endocrinology* 2009;150:2906–14. doi:10.1210/en.2008-1575.
 - [90] Wilson JL, Chen W, Dissen GA, Ojeda SR, Cowley MA, Garcia-Rudaz C, et al. Excess of nerve growth factor in the ovary causes a polycystic ovary-like syndrome in mice, which closely resembles both reproductive and metabolic aspects of the human syndrome. *Endocrinology* 2014;155:4494–506. doi:10.1210/en.2014-1368.
 - [91] Dissen GA, Mayerhofer A, Ojeda SR. Participation of nerve growth factor in the regulation of ovarian function. *Zygote* 1996;4:309–12. doi:10.1017/S0967199400003300.
 - [92] Dissen GA, Hill DF, Costa ME, Dees W Les, Lara HE, Ojeda SR. A role for TrkA Nerve Growth Factor receptors in mammalian ovulation. *Endocrinology* 1996;137:198–209. doi:10.1210/en.137.1.198.
 - [93] Dissen GA, Parrott JA, Skinner MK, Hill DF, Costa ME, Ojeda SR. Direct effects of nerve growth factor on thecal cells from antral ovarian follicles. *Endocrinology* 2000;141:4736–50. doi:10.1210/endo.141.12.7850.
 - [94] Tsafiriri A, Lindner HR, Zor U, Lamprecht SA. Physiological role of prostaglandins in the induction of ovulation. *Prostaglandins* 1972;2:1–10. doi:10.1016/0090-6980(72)90024-X.
 - [95] Mayerhofer A, Dissen GA, Parrott JA, Hill DF, Mayerhofer D, Garfield RE, et al. Involvement of nerve growth factor in the ovulatory cascade: *trkA* receptor activation inhibits gap junctional communication between thecal cells. *Endocrinology* 1996;137:5662–70. doi:10.1210/endo.137.12.8940397.
 - [96] Mattioli M, Barboni B, Gioia L, Lucidi P. Nerve growth factor production in sheep antral follicles. *Domest Anim Endocrinol* 1999;17:361–71. doi:10.1016/S0739-7240(99)00056-9.
 - [97] Barboni B, Mattioli M, Gioia L, Turriani M, Capacchietti G, Berardinelli P, et al. Preovulatory rise of NGF in ovine follicular fluid: possible involvement in the control of oocyte maturation. *Microsc Res Tech* 2002;59:516–21. doi:10.1002/jemt.10230.
 - [98] Knickerbocker JJ, Thatcher WW, Foster DB, Wolfenson D, Bartol FF, Caton D. Uterine prostaglandin and blood flow responses to estradiol-17 β in cyclic cattle. *Prostaglandins* 1986;31:757–76. doi:10.1016/0090-6980(86)90179-6.

- [99] Krzymowski T, Stefańczyk-Krzymowska S, Koziorowski M. Counter current transfer of PGF2 alpha in the mesometrial vessels as a mechanism for prevention of luteal regression in early pregnancy. *Acta Physiol Pol* 1989;40:23–34.
- [100] Schjenken JE, Robertson SA. Seminal fluid signalling in the female reproductive tract: Implications for reproductive success and offspring health. In: Bronson R., editor. *Male Role Pregnancy Loss Embryo Implant. Fail. Adv. Exp. Med. Biol.* vol 868, Springer, Cham; 2015, p. 127–58. doi:10.1007/978-3-319-18881-2_6.
- [101] Robertson SA. Seminal plasma and male factor signalling in the female reproductive tract. *Cell Tissue Res* 2005;322:43–52. doi:10.1007/s00441-005-1127-3.
- [102] Tanco VM, Van Steelandt MD, Ratto MH, Adams GP. Effect of purified llama ovulation-inducing factor (OIF) on ovarian function in cattle. *Theriogenology* 2012;78:1030–9. doi:10.1016/j.theriogenology.2012.03.036.
- [103] Tribulo P, Bogle OA, Mapletoft RJ, Adams GP. Bioactivity of ovulation inducing factor (or nerve growth factor) in bovine seminal plasma and its effects on ovarian function in cattle. *Theriogenology* 2015;83:1394–401. doi:10.1016/j.theriogenology.2014.12.014.
- [104] Salas C, Julio-Pieper M, Valladares M, Pommer R, Vega M, Mastronardi C, et al. Nerve growth factor-dependent activation of trkA receptors in the human ovary results in synthesis of follicle-stimulating hormone receptors and estrogen secretion. *J Clin Endocrinol Metab* 2006;91:2396–403. doi:10.1210/jc.2005-1925.
- [105] Harrison LM, Kenny N, Niswender GD. Progesterone production, LH receptors, and oxytocin secretion by ovine luteal cell types on days 6, 10 and 15 of the oestrus cycle and day 25 of pregnancy. *J Reprod Fert* 1987;79:539–48. doi:10.1530/jrf.0.0790539.
- [106] Finetti F, Solito R, Morbidelli L, Giachetti A, Ziche M, Donnini S. Prostaglandin E2 regulates angiogenesis via activation of fibroblast growth factor receptor-1. *J Biol Chem* 2008;283:2139–46. doi:10.1074/jbc.M703090200.
- [107] Herzog K, Brockhan-Lüdemann M, Kaske M, Beindorff N, Paul V, Niemann H, et al. Luteal blood flow is a more appropriate indicator for luteal function during the bovine estrous cycle than luteal size. *Theriogenology* 2010;73:691–7. doi:10.1016/j.theriogenology.2009.11.016.
- [108] Miyamoto A, Okuda K, Schweigert FJ, Schams D. Effects of basic fibroblast growth factor, transforming growth factor- β and nerve growth factor on the secretory function of the bovine corpus luteum *in vitro*. *J Endocrinol* 1992;135:103–14. doi:10.1677/joe.0.1350103.
- [109] Lonergan P. Influence of progesterone on oocyte quality and embryo development in cows. *Theriogenology* 2011;76:1594–601. doi:10.1016/j.theriogenology.2011.06.012.
- [110] Sreenan J., Diskin MG. The extent and timing of embryonic mortality in cattle. Dordrecht, The Netherlands: Martinus Nijhoff Publishers; 1986.
- [111] Mullen MP, Bazer FW, Wu G, Parr MH, Evans ACO, Crowe MA, et al. Effects of systemic progesterone during the early luteal phase on the availabilities of amino acids and glucose in the bovine uterine lumen. *Reprod Fertil Dev* 2014;26:282–92. doi:10.1071/RD12319.
- [112] Spencer TE, Sandra O, Wolf E. Genes involved in conceptus-endometrial interactions in

- ruminants: Insights from reductionism and thoughts on holistic approaches. *Reproduction* 2008;135:165–79. doi:10.1530/REP-07-0327.
- [113] Spencer TE, Forde N, Lonergan P. The role of progesterone and conceptus-derived factors in uterine biology during early pregnancy in ruminants. *J Dairy Sci* 2016;99:5941–50. doi:10.3168/jds.2015-10070.
 - [114] Mann GE, Lamming GE. Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. *Reproduction* 2001;121:175–80.
 - [115] Sartori R, Sartor-Bergfelt R, Mertens SA, Guenther JN, Parrish JJ, Wiltbank MC. Fertilization and early embryonic development in heifers and lactating cows in summer and lactating and dry cows in winter. *J Dairy Sci* 2002;85:2803–12. doi:10.3168/jds.S0022-0302(02)74367-1.
 - [116] Wiebold JL. Embryonic mortality and the uterine environment in first-service lactating dairy cows. *J Reprod Fertil* 1988;84:393–9. doi:10.1530/jrf.0.0840393.
 - [117] McNeill RE, Diskin MG, Sreenan JM, Morris DG. Associations between milk progesterone concentration on different days and with embryo survival during the early luteal phase in dairy cows. *Theriogenology* 2006;65:1435–41. doi:10.1016/j.theriogenology.2005.08.015.
 - [118] Sartori R, Haughian JM, Shaver RD, Rosa GJM, Wiltbank MC. Comparison of ovarian function and circulating steroids in estrous cycles of Holstein heifers and lactating cows. *J Dairy Sci* 2004;87:905–20. doi:10.3168/jds.S0022-0302(04)73235-X.
 - [119] Sangsritavong S, Combs DK, Sartori R, Armentano LE, Wiltbank MC. High feed intake increases liver blood flow and metabolism of progesterone and estradiol-17 β in dairy cattle. *J Dairy Sci* 2002;85:2831–42. doi:10.3168/jds.S0022-0302(02)74370-1.
 - [120] Vasconcelos JLM, Sangsritavong S, Tsai SJ, Wiltbank MC. Acute reduction in serum progesterone concentrations after feed intake in dairy cows. *Theriogenology* 2003;60:795–807. doi:10.1016/S0093-691X(03)00102-X.
 - [121] Wiltbank M, Lopez H, Sartori R, Sangsritavong S, Gümen A. Changes in reproductive physiology of lactating dairy cows due to elevated steroid metabolism. *Theriogenology* 2006;65:17–29. doi:10.1016/j.theriogenology.2005.10.003.
 - [122] Mann GE, Fray MD, Lamming GE. Effects of time of progesterone supplementation on embryo development and interferon-tau production in the cow. *Vet J* 2006;171:500–3. doi:10.1016/j.tvjl.2004.12.005.
 - [123] O’Hara L, Forde N, Carter F, Rizos D, Maillou V, Ealy AD, et al. Paradoxical effect of supplementary progesterone between day 3 and day 7 on corpus luteum function and conceptus development in cattle. *Reprod Fertil Dev* 2014;26:328–36. doi:10.1071/RD12370.
 - [124] Monteiro Jr PLJ, Ribeiro ES, Maciel RP, Dias ALG, Solé Jr E, Lima FS, et al. Effects of supplemental progesterone after artificial insemination on expression of interferon-stimulated genes and fertility in dairy cows. *J Dairy Sci* 2014;97:4907–21. doi:http://dx.doi.org/10.3168/jds.2013-7802.
 - [125] Parr MH, Scully S, Lonergan P, Evans ACO, Crowe MA, Diskin MG. Establishment of

- critical timing of progesterone supplementation on corpus luteum and embryo development in beef heifers. *Anim Reprod Sci* 2017. doi:10.1016/j.anireprosci.2017.02.005.
- [126] Rizos D, Scully S, Kelly AK, Ealy AD, Moros R, Duffy P, et al. Effects of human chorionic gonadotrophin administration on Day 5 after oestrus on corpus luteum characteristics, circulating progesterone and conceptus elongation in cattle. *Reprod Fertil Dev* 2012;24:472–81. doi:10.1071/RD11139.
 - [127] Santos JEP, Thatcher WW, Pool L, Overton MW. Effect of human chorionic gonadotropin on luteal function and reproductive performance of high-producing lactating Holstein dairy cows. *J Anim Sci* 2001;79:2881–94. doi:10.2527/2001.79112881x.
 - [128] Stevenson JS, Portaluppi MA, Tenhouse DE, Lloyd A, Eborn DR, Kacuba S, et al. Interventions after artificial insemination: conception rates, pregnancy survival, and ovarian responses to gonadotropin-releasing hormone, human chorionic gonadotropin, and progesterone. *J Dairy Sci* 2007;90:331–40. doi:10.3168/jds.S0022-0302(07)72634-6.
 - [129] Dobson H, Tebble JE, Smith RF, Ward WR. Is stress really all that important? *Theriogenology* 2001;55:65–73. doi:10.1016/S0093-691X(00)00446-5.
 - [130] Pei Y. Effect of nerve growth factor (NGF) on the development of preimplantation rabbit embryos *in vitro*. *Vet Res Commun* 2010;34:11–8. doi:10.1007/s11259-009-9325-1.
 - [131] Crispo M, dos Santos-Neto PC, Vilariño M, Mulet AP, de León A, Barbeito L, et al. RAPID COMMUNICATION: Nerve growth factor influences cleavage rate and embryo development in sheep. *J Anim Sci* 2016;94:4447–51. doi:10.2527/jas.2016-0736.
 - [132] Wang DH, Ren J, Zhou CJ, Han Z, Wang L, Liang CG. Supplementation with CTGF, SDF1, NGF, and HGF promotes ovine *in vitro* oocyte maturation and early embryo development. *Domest Anim Endocrinol* 2018;65:38–48. doi:10.1016/j.domaniend.2018.05.003.
 - [133] Cupp AS, Kim GH, Skinner MK. Expression and action of neurotrophin-3 and nerve growth factor in embryonic and early postnatal rat testis development. *Biol Reprod* 2000;63:1617–28. doi:10.1095/biolreprod63.6.1617.
 - [134] Ayer-LeLievre C, Olson L, Ebendal T, Hallböök F, Persson H. Nerve growth factor mRNA and protein in the testis and epididymis of mouse and rat. *Proc Natl Acad Sci U S A* 1988;85:2628–32. doi:10.1073/PNAS.85.8.2628.
 - [135] Seidl K, Buchberger A, Erck C. Expression of nerve growth factor and neurotrophin receptors in testicular cells suggest novel roles for neurotrophins outside the nervous system. *Reprod Fertil Dev* 1996;8:1075. doi:10.1071/RD9961075.
 - [136] Seidl K, Holstein A-F. Evidence for the presence of nerve growth factor (NGF) and NGF receptors in human testis. *Cell Tissue Res* 1990;261:549–54. doi:10.1007/BF00313534.
 - [137] Li C, Watanabe G, Weng Q, Jin W, Furuta C, Suzuki AK, et al. Expression of nerve growth factor (NGF), and its receptors TrkA and p75 in the reproductive organs of the adult male rats. *Zoolog Sci* 2005;22:933–7. doi:10.2108/zsj.22.933.
 - [138] Djakiew D, Pflug B, Dionne C, Onoda M. Postnatal expression of nerve growth factor receptors in the rat testis. *Biol Reprod* 1994;51:214–21. doi:10.1095/biolreprod51.2.214.

- [139] Lönnerberg P, Söder O, Parvinen M, Martin Ritzén E, Persson H. β -nerve growth factor influences the expression of androgen-binding protein messenger ribonucleic acid in the rat testis. *Biol Reprod* 1992;47:381–8. doi:10.1095/biolreprod47.3.381.
- [140] Zhang L, Wang H, Yang Y, Lui H, Zhang Q, Xiang Q, et al. NGF induces adult stem Leydig cells to proliferate and differentiate during Leydig cell regeneration. *Biochem Biophys Res Commun* 2013;436:300–5. doi:10.1016/J.BBRC.2013.05.098.
- [141] Müller D, Davidoff MS, Bargheer O, Paust H-J, Pusch W, Koeva Y, et al. The expression of neurotrophins and their receptors in the prenatal and adult human testis: evidence for functions in Leydig cells. *Histochem Cell Biol* 2006;126:199–211. doi:10.1007/s00418-006-0155-8.
- [142] Li CL, Zhou X. The potential roles of neurotrophins in male reproduction. *Reproduction* 2013;145:R89–95. doi:10.1530/REP-12-0466.
- [143] Seidel GEJ, Foote RH. Compartmental analysis of sources of the bovine ejaculate. *Biol Reprod* 1970;2:189–96. doi:10.1095/biolreprod2.2.189.
- [144] Lichtenwalner ABB, Woods GLL, Weber JAA. Seminal collection, seminal characteristics and pattern of ejaculation in llamas. *Theriogenology* 1996;46:293–305. doi:10.1016/0093-691X(96)00186-0.
- [145] Zhang H, Wang Y, Zhan J, Wang L, Li Q, Sheng X, et al. Testicular expression of NGF, TrkA and p75 during seasonal spermatogenesis of the wild ground squirrel (*Citellus dauricus* Brandt). *Eur J Histochem* 2015;59:16–22. doi:10.4081/ejh.2015.2522.
- [146] Stewart JL, Shipley CF, Ellerbrock RE, Schmidt L, Lima FS, Canisso IF. Physiological variations in reproductive and metabolic features of white-tailed deer (*Odocoileus virginianus*) bucks throughout the rutting season. *Theriogenology* 2018;114:308–16. doi:10.1016/j.theriogenology.2018.04.015.
- [147] Stewart JL, Shipley CF, Ellerbrock RE, Lima FS, Canisso IF. Variation in post-thaw sperm quality of white-tailed deer bucks (*Odocoileus virginianus*) during rut. *Anim Reprod Sci* 2018;195:121–30. doi:10.1016/j.anireprosci.2018.05.014.
- [148] Domínguez MP, Falcinelli A, Hozbor F, Sánchez E, Cesari A, Alberio RH. Seasonal variations in the composition of ram seminal plasma and its effect on frozen-thawed ram sperm. *Theriogenology* 2008;69:564–73. doi:10.1016/j.theriogenology.2007.11.010.
- [149] Chacur MGM, Mizusaki KT, Filho LRA, Oba E, Ramos AA. Seasonal effects on semen and testosterone in zebu and taurine bulls. *Acta Sci Vet* 2013;41:1–5.
- [150] Jiménez-Severiano H, Quintal-Franco J, Vega-Murillo V, Zanella E, Wehrman ME, Lindsey BR, et al. Season of the year influences testosterone secretion in bulls administered luteinizing hormone. *J Anim Sci* 2003;81:1023–9. doi:10.2527/2003.8141023x.
- [151] Stewart JL, Shipley CF, Ireland FA, Jarrell VL, Timlin CL, Shike DW, et al. Long-term effects of pyrethrin and cyfluthrin, a type II synthetic pyrethroid, insecticide applications on bull reproductive parameters. *Reprod Domest Anim* 2016;51. doi:10.1111/rda.12729.
- [152] Westfalewicz B, Dietrich MA, Mostek A, Partyka A, Bielas W, Nizański W, et al. Analysis of bull (*Bos taurus*) seminal vesicle fluid proteome in relation to seminal plasma proteome. *J Dairy Sci* 2016;100:1–17. doi:10.3168/jds.2016-11866.

- [153] Kelly VC, Kuy S, Palmer DJ, Xu Z, Davis SR, Cooper GJ. Characterization of bovine seminal plasma by proteomics. *Proteomics* 2006;6:5826–33. doi:10.1002/pmic.200500830.
- [154] Rego JPA, Crisp JM, Moura AA, Nouwens AS, Li Y, Venus B, et al. Seminal plasma proteome of electroejaculated *Bos indicus* bulls. *Anim Reprod Sci* 2014;148:1–17. doi:10.1016/j.anireprosci.2014.04.016.
- [155] Sanchez-Rodriguez A, Arias-Alvarez M, Rebollar PG, Lorenzo PL, Garcia-Garcia RM. 148 Immunolocalization of β -nerve growth factor (NGF) in male reproductive tract and NGF levels in serum and seminal plasma at puberty and adulthood in rabbit. *Reprod Fertil Dev* 2018;30:214–214. doi:10.1071/RDv30n1Ab148.
- [156] Gwathmey TM, Ignatz GG, Mueller JL, Manjunath P, Suarez SS. Bovine seminal plasma proteins PDC-109, BSP-A3, and BSP-30-kDa share functional roles in storing sperm in the oviduct. *Biol Reprod* 2006;75:501–7. doi:10.1095/biolreprod.106.053306.
- [157] Killian GJ, Chapman DA, Rogowski LA. Fertility-associated proteins in Holstein bull seminal plasma. *Biol Reprod* 1993;49:1202–7. doi:10.1095/biolreprod49.6.1202.
- [158] Kasimanickam V, Kasimanickam R, Arangasamy A, Saberivand A, Stevenson JS, Kastelic JP. Association between mRNA abundance of functional sperm function proteins and fertility of Holstein bulls. *Theriogenology* 2012;78:2007–19. doi:10.1016/j.theriogenology.2012.07.016.
- [159] Erikson DW, Way AL, Chapman DA, Killian GJ. Detection of osteopontin on Holstein bull spermatozoa, in cauda epididymal fluid and testis homogenates, and its potential role in bovine fertilization. *Reproduction* 2007;133:909–17. doi:10.1530/REP-06-0228.
- [160] Gerena RL, Irikura D, Urade Y, Eguchi N, Chapman DA, Killian GJ. Identification of a fertility-associated protein in bull seminal plasma as lipocalin-type prostaglandin D synthase. *Biol Reprod* 1998;58:826–33. doi:10.1095/biolreprod58.3.826.
- [161] Patel M, Gandotra VK, Cheema RS, Bansal AK, Kumar A. Seminal plasma heparin binding proteins improve semen quality by reducing oxidative stress during cryopreservation of cattle bull semen. *Asian-Australasian J Anim Sci* 2016;29:1247–55. doi:10.5713/ajas.15.0586.
- [162] Poiani A. Complexity of seminal fluid: a review. *Behav Ecol Sociobiol* 2006;60:289–310. doi:10.1007/s00265-006-0178-0.
- [163] Bedford JM. The Functions—or Not—of Seminal Plasma? *Biol Reprod* 2015;92. doi:10.1095/biolreprod.114.126045.
- [164] Bromfield JJ. A role for seminal plasma in modulating pregnancy outcomes in domestic species. *Reproduction* 2016;152:R223–32. doi:10.1530/REP-16-0313.
- [165] Hayes BJ, Chamberlain AJ, Maceachern S, Savin K, McPartlan H, MacLeod I, et al. A genome map of divergent artificial selection between *Bos taurus* dairy cattle and *Bos taurus* beef cattle. *Anim Genet* 2009;40:176–84. doi:10.1111/j.1365-2052.2008.01815.x.
- [166] Schjenken JE, Robertson SA. Seminal fluid and immune adaptation for pregnancy - comparative biology in mammalian species. *Reprod Domest Anim* 2014;49:27–36. doi:10.1111/rda.12383.

- [167] Westfalewicz B, Dietrich MA, Ciereszko A. Impact of cryopreservation on bull (*Bos taurus*) semen proteome. J Anim Sci 2015;93:5240–53. doi:10.2527/jas.2015-9237.
- [168] Lima FS, Risco CA, Thatcher MJ, Benzaquen ME, Archbald LF, Santos JEP, et al. Comparison of reproductive performance in lactating dairy cows bred by natural service or timed artificial insemination. J Dairy Sci 2009;92:5456–66. doi:10.3168/jds.2009-2197.
- [169] Dalton JC, Nadir S, Bame JH, Noftsinger M, Nebel RL, Saacke RG. Effect of time of insemination on number of accessory sperm, fertilization rate, and embryo quality in nonlactating dairy cattle. J Dairy Sci 2001;84:2413–8. doi:10.3168/jds.S0022-0302(01)74690-5.
- [170] Pelissier CL. Dairy cattle breeding problems and their consequences. Theriogenology 1976;6:575–83. doi:10.1016/0093-691X(76)90125-4.
- [171] Li C, Sun Y, Yi K, Ma Y, Sun Y, Zhang W, et al. Detection of nerve growth factor (NGF) and its specific receptor (TrkA) in ejaculated bovine sperm, and the effects of NGF on sperm function. Theriogenology 2010;74:1615–22. doi:10.1016/j.theriogenology.2010.06.033.
- [172] Weng Q, Shi Z, Watanabe G, Taya K. Immunolocalization of NGF and its receptors trkA and p75 in the oviducts of golden hamsters during the estrous cycle. Exp Anim 2009;58:543–6. doi:10.1538/expanim.58.543.
- [173] Jin W, Tanaka A, Watanabe G, Matsuda H, Taya K. Effect of NGF on the motility and acrosome reaction of golden hamster spermatozoa *in vitro*. J Reprod Dev 2010;56:437–43. doi:10.1262/jrd.09-219N.
- [174] Li C, Ma Y, Yi K, Wang C, Li W, Liu Z, et al. The interactions between nerve growth factor and gonadotrophins in bovine oviduct. Anim Reprod Sci 2014;149:117–23. doi:10.1016/j.anireprosci.2014.06.030.
- [175] Shilpa M, Selvaraju S, Girishkumar V, Parthipan S, Binsila KB, Arangasamy A, et al. Novel insights into the role of cell-free seminal mRNAs on semen quality and cryotolerance of spermatozoa in bulls (*Bos taurus*). Reprod Fertil Dev 2017;29:2446–56. doi:10.1071/RD16290.
- [176] Parthipan S, Selvaraju S, Somashekar L, Arangasamy A, Sivaram M, Ravindra JP. Spermatozoal transcripts expression levels are predictive of semen quality and conception rate in bulls (*Bos taurus*). Theriogenology 2017;98:41–9. doi:10.1016/j.theriogenology.2017.04.042.
- [177] Saeednia S, Bahadoran H, Amidi F, Asadi MH, Naji M, Fallahi P, et al. Nerve growth factor in human semen: effect of nerve growth factor on the normozoospermic men during cryopreservation process. Iran J Basic Med Sci 2015;18:292–9.
- [178] Saeednia S, Shabani Nashtaei M, Bahadoran H, Aleyasin A, Amidi F. Effect of nerve growth factor on sperm quality in asthenozoospermic men during cryopreservation. Reprod Biol Endocrinol 2016;14:1–8. doi:10.1186/s12958-016-0163-z.
- [179] Niswender GD, Juengel JL, McGuire WJ, Belfiore CJ, Wiltbank MC. Luteal function: the estrous cycle and early pregnancy. Biol Reprod 1994;50:239–47. doi:10.1095/biolreprod50.2.239.

Chapter 3:

Nerve Growth Factor- β Improves Corpus Luteum Formation and Enhances Conceptus Development in Cows¹

3.1 Abstract

The objective of the current study was to determine if Nerve growth factor- β (NGF), purified from bovine seminal plasma, would improve corpus luteum function and enhance conceptus development when administered to cows at artificial insemination. Angus cows ($n = 60$) were synchronized using a GnRH-prostaglandin and intravaginal progesterone protocol (7-day CO-Synch/CIDR) and randomly allocated to 1 of 2 treatment groups: (1) CONT- 12 mL PBS; or (2) NGF- 296 μ g purified NGF in 12 mL PBS administered intramuscularly at insemination (day 0). Blood collections were performed to measure plasma concentrations of progesterone (days 0, 3, 7, 10, 14, 19) and pregnancy-specific protein B (day 24) using immunoassays. Expression of interferon-stimulated genes (*Isg15*, *Mx1*, *Mx2*, *Rtp4*) were assessed in peripheral blood leukocytes on day 19. Transrectal ultrasound was performed for measuring corpus luteum size (days 0, 3, 7, 10, 14, 19) and pregnancy diagnosis (days 28, 45, 66). Statistical analysis was performed using analysis of variance with repeated measures (SAS 9.4, Cary NC). Corpus luteum volume and diameter increased over time ($P < 0.001$), but did not differ between treatment groups ($P = 0.46$). Cows treated with NGF had increased plasma progesterone over CONT cows from days 10 to 19 ($P = 0.04$). Pregnancy rates at day 28 were 75% in NGF cows versus 59% in CONT cows ($P = 0.13$). In pregnant cows, pregnancy-specific protein B concentrations at day 24 were greater in NGF than CONT cows ($P < 0.05$). Additionally, fold-change expression of *Isg15* and *Mx2* at day

¹ Reprinted, with permission, from J. Stewart et al. Nerve Growth Factor- Beta, purified from bull seminal plasma, enhances corpus luteum formation and conceptus development in *Bos taurus* cows. Theriogenology 2018; 106: 30-38.

19 were greater in pregnant NGF cows than in pregnant CONT cows ($P < 0.05$), but no differences for *Mx1* and *Rtp4* were present. Here we demonstrate that NGF administration to cows at insemination improved corpus luteum function, which translated to improved early conceptus development as determined by upregulation of interferon-stimulated genes and increased pregnancy-specific protein B concentrations. These results suggest that seminal plasma NGF could play a role in conceptus development and may be important to improve fertility in cattle.

3.2 Introduction

Many studies have demonstrated that seminal plasma proteins may play a pivotal role in enhancing fertility [1–4]. This is of special relevance to the cattle industry where artificial insemination (AI) has advanced genetic selection of desirable traits such as milk production and meat quality [5]. Historically, bull semen studies have focused on improving semen freezing techniques and determining the minimum effective breeding dose, while overlooking the effects that semen processing may have on seminal plasma protein concentrations [6]. When processing semen for cryopreservation, samples are extended, which may dilute seminal plasma proteins to sub-physiologic levels and contribute to observed lower fertility rates when compared to natural mating [7]. While the direct effects of seminal plasma proteins on sperm motility and survivability are well-defined [4,8,9], the roles they play within the female reproductive system following insemination are less understood [10–13].

Nerve growth factor- β (NGF) has been identified in the seminal plasma of most domestic species and induces ovulation in camelids [14–16]. In camelids, NGF also has a dose-dependent luteotrophic effect that is attributed to its action on the hypothalamus [17,18], resulting in prolonged release of luteinizing hormone (LH) from the anterior pituitary gland [14]. Likewise,

NGF signaling in the ovary is critical for the first ovulation to occur in spontaneous ovulators [19], though we have only recently begun to truly understand its effects when introduced into the female through insemination. Recent studies have shown that intramuscular administration of purified llama NGF to cyclic heifers resulted in larger corpus luteum (CL) size and greater concentrations of plasma progesterone following ovulation [20]. In this previous study, heifers were not inseminated and, therefore, the translation of the observed luteotrophic effects to a developing embryo remains to be determined.

Enhancement of CL development and increased progesterone production may help to reduce early embryonic death in cattle [21]. The majority of bovine embryo losses occur before day 16 of pregnancy and may be attributed to the downstream effects of inadequate progesterone concentrations on histotroph secretion into the uterine lumen [21]. Histotroph stimulates embryonic growth, which positively correlates with production of interferon-tau [21]. A threshold interferon-tau concentration is required to effectively block luteolysis and achieve maternal recognition of pregnancy in ruminants [22]. Therefore, if fertilization is achieved, but progesterone and interferon-tau concentrations are inadequate, luteolysis and early embryonic death will occur.

The objectives of the current study were to purify NGF from bull seminal plasma and determine its effects on CL function and conceptus development when administered systemically to cows at AI. Exogenous progesterone supplementation has been shown to increase interferon-tau production previously in cows [23], and therefore we hypothesized that NGF administration would increase plasma progesterone production, leading to increased expression of interferon-stimulated genes (ISGs) and increased pregnancy-specific protein B (PSPB) production in pregnant cows bred by AI.

3.3 Materials and Methods

3.3.1 Ethics

All experimental procedures carried out in the present study were approved by the Institutional Animal Care and Use Committees of the University of Illinois at Urbana-Champaign (Protocol #15-201) and Virginia Polytechnic Institute and State University (Protocol #16-031).

3.3.2 Isolation and purification of NGF from bull seminal plasma

Semen samples were collected from University of Illinois-owned Angus or Angus \times Simmental bulls ($n = 40$) using electroejaculation. While restrained in a chute, a 60 mm upright weighted bull probe (Lane Manufacturing, Inc.) was placed rectally, and the programmed cycle on the electroejaculator was run (Pulsator IV, Lane Manufacturing, Inc.). Ejaculates were pooled, and protease inhibitor tablets (Complete, Mini, EDTA-free, Sigma Aldrich, St. Louis, MO) were added at 1 tablet per 10 mL of ejaculate. The pooled ejaculates were centrifuged at $1200 \times g$ for 30 min to separate seminal plasma from spermatozoa and then passed through a $0.8 \mu\text{M}$ filter. Pooled seminal plasma was stored at -80°C until purification.

Purification of NGF from pooled seminal plasma was performed using ion exchange chromatography to fractionate proteins based on their effective charges [24]. Upon thawing, the seminal plasma was diluted with 20 mM Sodium Phosphate buffer (pH 7.5) and then subjected to anion exchange chromatography (Q-Sepharose). The samples obtained from the Q-Sepharose column were lyophilized with subsequent dialysis against 0.1 M Na Citrate (pH 3.0) and 400 mM NaCl. Next, cation exchange chromatography (SP Sepharose) was performed using 0.1 M Na Citrate (pH 3.0) and 400 mM NaCl, 0.1M Na Citrate (pH 3.0) with no NaCl, and gradient elution

using 50 mM Tris HCl (pH 9.0) and 50 mM Tris HCl pH 9.0 + 500 mM NaCl. The fractions from SP Sepharose were collected, and a salt/buffer cleanup was performed on the samples. Trypsin digestion of the protein fractions was performed, followed by liquid chromatography-mass spectrometry to assess purity. The purity of NGF was measured to be 59.35%, with only minor contaminants present (Table 1; Fig. 4).

3.3.3 Administration of NGF to cows at artificial insemination

Angus cows were stratified by body condition score and days post-partum at AI and then randomly assigned to a control (CONT, n = 30) or treated (NGF, n = 30) group. The body condition was scored at day -10 using a standard 9-point scale based on visual estimations of fatness and muscling according to Eversole et al. [25]. Timed-artificial insemination was carried out using a standard estrus synchronization program (7-day Co-Synch + CIDR program) [26]. On day -10, all cows received 2 mL (100 µg) of GnRH agonist (Cystorelin, Merial Inc.) intramuscularly and an intravaginal device containing 1.38 g of progesterone (Eazi-Breed CIDR, Zoetis). At day -3, the CIDR device was removed, and 5 mL of prostaglandin F2α analog (25 mg dinoprost tromethamine, Lutalyse, Zoetis) was administered intramuscularly. At 60 to 66 hours after CIDR removal and prostaglandin administration (day 0), all cows received 2 mL of GnRH agonist IM and were artificially inseminated using commercially-obtained frozen semen from three proven bulls randomly distributed across treatments. At this time, cows assigned to the NGF group received an intramuscular injection of 296 µg purified NGF reconstituted in 12 mL phosphate buffered saline (PBS; pH 7.4). Cows assigned to the CONT group received 12 mL of PBS intramuscularly as a placebo. This NGF dosage was chosen based on previous work by Tribulo et al. [27], where 12

mL of whole seminal plasma was administered to heifers and found to contain a total dosage of 250 µg of NGF.

3.3.4 Assessment of ovarian structures, pregnancy diagnosis, and embryonic/fetal crown-rump measurement

Transrectal ultrasonography examination of ovarian, uterine, and fetal structures were carried out using a portable ultrasound with a 7.5 MHz linear-array transducer (Aloka SSD-500V; Hitachi Aloka Medical, Ltd.). Ovaries were evaluated at days 0, 3, 7, 10, 14, and 19 to measure pre-ovulatory follicle (day 0) and CL size. Each CL had vertical and perpendicular horizontal diameters measured and recorded. Corpus luteum volume was calculated using the formula $\text{volume} = \frac{4}{3} \times 3.14 \times r^3$, where $r = \frac{1}{2}$ the average value of the vertical and horizontal diameters [28]. If a fluid-filled central cavity was detected within the CL, volume of the cavity was determined similarly and subtracted from the total CL volume to represent the actual volume of luteal tissue present in the CL [29]. Pregnancy diagnosis and measurement of embryonic crown-rump length (CRL) were performed at day 28 post-AI. A cow was determined to be pregnant by visualization of an embryo with a heartbeat. Cows diagnosed as pregnant were reexamined at days 45 and 66 after AI to reconfirm the presence of a fetus with visible heartbeat and to measure fetal crown-rump length.

3.3.5 Determination of plasma progesterone and pregnancy-specific protein B concentrations

Blood samples from each cow were collected via venipuncture from the coccygeal vein at days 0, 3, 7, 10, 14, 19, and 24 after treatment and AI. Blood samples were immediately placed on ice and then centrifuged at $2,000 \times g$ for 15 min for plasma separation. Two aliquots of plasma

were transferred into polypropylene vials and stored at -20°C until further analyses. Progesterone concentrations, used as means to assess CL function, were analyzed on plasma collected from days 0 to 19 using a chemiluminescence assay (Immulite 2000 XPi platform; Siemens Medical Solutions USA, Inc.). Concentrations of PSPB, used as an indirect measure of early embryonic development [30], were analyzed on plasma collected from day 24 using a commercially available quantitative immunoassay (BioPRYN; Bovine Answers LLC.). The interassay coefficient of variation for the PSPB assay was reported to be $< 10\%$, whereas the intraassay coefficient of variation for progesterone was measured at 2.7% .

3.3.6 Quantitative real-time PCR analyses of interferon-stimulated genes

Whole blood was obtained from all cows on day 19 after AI to target the expression of interferon stimulated genes (ISGs) known to be critical for maternal recognition of pregnancy [30–32]. Peripheral blood leukocytes (PBL) were isolated, and the PBL pellet was suspended with 0.8 mL of Trizol (Molecular Research Center, Inc., Cincinnati, OH), transferred to 1.5-mL microtubes, and stored at -80°C until analyses. Extraction of RNA was conducted according to the manufacturer's recommendations (PureLink RNA Mini Kit, Invitrogen). Isolated RNA was evaluated for concentration and purity using a NanoDrop 2000 Spectrophotometer (Thermo Scientific). A total of $1\text{ }\mu\text{g}$ of mRNA was used to synthesize complementary DNA using a commercial kit (High-capacity cDNA Reverse Transcription Kit, Cat. No. 4368814, Applied Biosystems) following manufacturer's instructions. Complementary DNA was used for quantitative real-time reverse transcription PCR using an iCycler IQTM Real-Time PCR Detection System with iTaqTM Universal SYBR® Green Supermix (Bio-Rad Laboratories, Inc.). Differential expression of four target genes, interferon-stimulated gene 15 (*Isg15*), receptor

transporter protein 4 (*Rtp4*), myxovirus 1 (*Mx1*), and myxovirus 2 (*Mx2*), were compared in contrast to the two housekeeping genes, beta-actin (*Actb*) and ribosomal protein L19 (*Rpl19*), constitutively expressed in peripheral leukocytes of cows (Table 2) [33].

Data are presented using non-pregnant cows that were excluded from analyses as the reference for comparisons as described previously [32]. Briefly, relative expression values were obtained by determining the PCR amplification efficiency ($E = 2$) to the power of the delta-delta threshold cycle ($\Delta \Delta C_T$) obtained from the ΔC_T least square mean differences of pairwise comparisons between pregnant and non-pregnant NGF and CONT groups.

3.3.7 Statistical analyses

All responses were analyzed using the MIXED procedure of SAS version 9.4 (SAS/STAT; SAS Institute Inc., Cary, NC). Progesterone and CL size were analyzed using an ANOVA model with repeated measures to test for treatment or treatment \times day interactions. Binary responses were analyzed by logistic regression fitting a binary data distribution. The models tested included the effects of treatment (CONT vs. NGF) and the covariates BCS at enrollment, pre-ovulatory follicle size at AI, sires, and technicians, which all were found to have no effect on the outcomes and were removed from the final model. Presence and size of dominant follicle at AI and presence of progesterone >1 ng/mL at AI were evaluated and did not differ between treatments. Therefore, cows that either failed to ovulate (progesterone <1 ng/mL by day 10, $n = 3$) or had high progesterone (>1 ng/mL, $n = 2$) at the time of AI were not included in the final models. Continuous data were analyzed using models that were fitting a Gaussian or a logarithmic distribution, and residuals were assessed for normality. The final models used included all predictors of binary responses. For analyses of mRNA expression of ISGs and concentrations of PSPB in plasma, the

models included the effects of treatment, sire, and pregnancy status (pregnant vs. non-pregnant on day 28 post-AI). The covariance structure that resulted in the smallest Bayesian information criterion was selected from the mixed models. Pearson's correlation coefficients were calculated to determine associations between CL size and plasma progesterone concentrations. Embryonic and fetal CRL were compared between treatment groups using a one-tailed t-test. Treatment differences with $P \leq 0.05$ were considered as significant, and $0.05 < P \leq 0.10$ were deemed as a tendency.

3.4 Results

3.4.1 Ovarian dynamics at AI (Day 0)

The presence and size of the pre-ovulatory follicle at AI (day 0) did not differ between treatment groups ($P = 0.98$ and $P = 0.96$, respectively; Fig. 5A and B). The occurrence of progesterone concentrations >1.0 ng/mL at day 0 and ovulation in response to AI also did not differ between treatment groups ($P = 0.99$ and $P = 0.93$, respectively; Fig. 5C and D). These results demonstrated that the cows within each treatment group responded similarly to the implemented synchronization protocol.

3.4.2 Corpus luteum size and function

The effect of NGF administration on CL size were assessed as described elsewhere [20,28]. As expected, both CL diameter and volume changed over time in all cows ($P < 0.001$), increasing during the period of luteal development from days 0 to 10. However, there were no treatment ($P = 0.88$) or treatment \times time interactions in regard to either parameter ($P = 0.46$; Fig. 6A and B), indicating that NGF administration did not increase CL size in cows. Both CL diameter

and volume were strongly and positively correlated ($P \leq 0.02$) with plasma progesterone concentrations during the period of luteal development (days 3 and 7), with no correlation ($P \geq 0.31$) observed during luteal stasis at days 10 or 14 (Table 3). On the contrary, CL function appeared to be improved in NGF-treated cows, with greater plasma progesterone concentrations observed during the period of luteal stasis at days 10 to 19 when compared to CONT cows ($P \leq 0.05$; Fig. 6C).

3.4.3 Pregnancy classification, pregnancy-specific protein B production, and crown-rump length

Pregnancy per AI (P/AI) at day 28 were numerically, but not significantly ($P = 0.13$), higher in NGF cows (75.0%) than in CONT cows (58.6%), as depicted in Table 4. Likewise, at days 45 and 66 post-AI, P/AI were similar ($P = 0.12$), with 71.4% NGF cows and 55.2% CONT cows diagnosed as pregnant (Table 4). Pregnant NGF cows had higher PSPB concentrations than pregnant CONT cows at day 24 post-AI ($P \leq 0.05$; Fig. 7), whereas there were no differences in PSPB concentrations between non-pregnant NGF or CONT cows ($P > 0.05$; Fig. 7). While the embryonic and fetal CRL did not differ at days 28 or 45 post-AI ($P > 0.20$), there was a tendency for increased fetal CRL ($P = 0.06$) at day 66 in NGF versus CONT cows (Table 4).

3.4.4 Expression of interferon-stimulated genes

As expected, cows diagnosed as pregnant on day 28 had greater ($P \leq 0.05$) mRNA expression of *Isg15*, *Mx1*, *Mx2*, and *Rtp4* on day 19 than did non-pregnant cows, with average increases of 64-, 4.6-, 10-, and 3.2-fold, respectively (Fig. 8). Messenger RNA abundance of *Isg15* and *Mx2* were also affected by treatment ($P \leq 0.05$). Pregnant cows that were treated with NGF had a 28- and 4.3-fold increase in mRNA expression of *Isg15* and *Mx2*, respectively, over pregnant

CONT cows (Fig. 8A and C), suggesting upregulation due to increased interferon-tau production from the developing conceptus. On the contrary, there were no differences ($P > 0.05$) in mRNA expression of *Mx1* or *Rtp4* between pregnant NGF and CONT cows (Fig. 8B and D).

3.5 Discussion

As anticipated, NGF purified from bull seminal plasma played an important role in luteal development and its downstream effects on maternal recognition of pregnancy. The first objective of this study was to determine if NGF could be purified from bull seminal plasma so that its potential role in cows at insemination could be evaluated. The detection of NGF in the seminal plasma of bulls dates back to 1982, where 100 µg of pure NGF was isolated per mL of seminal plasma [34], which is similar to the yield in the current study. While not a novel concept, more sensitive techniques, such as mass spectrometry, exist now that allow us to more critically assess purity of the isolated protein [35]. Initial attempts to purify NGF using High Performance Liquid Chromatography, as has been described in camelids [36], yielded major contamination with Caltrin, whereas a single-step ion exchange (Mono-S) presented contamination with vasoactive intestinal peptide (data not shown). Purification of NGF from bull seminal plasma in the current study therefore required a more elaborate combination of anion and cation exchange chromatography, similar to that reported previously [34]. Species-specific differences in types and abundance of seminal plasma proteins likely account for the different protocol required to purify NGF from bull versus camelid seminal plasma.

Upon successful NGF purification, our second objective was to determine its effects on CL function and conceptus development when administered systemically to cows at AI. Consistent with a previous study where llama-derived NGF exhibited a luteotrophic effect in heifers [20], we

found increased concentrations of plasma progesterone beginning around day 10 after insemination in cows treated with NGF from bull seminal plasma. This similarity suggests that the function of NGF in seminal plasma may be conserved across species. In the current study, progesterone concentrations in cows treated with NGF were increased up to 19 days post-insemination. This is in contrast with the previous study, where heifers were not inseminated and progesterone started to decrease in heifers potentially undergoing early luteolysis by day 13 [20]. Differences in experimental designs (e.g., NGF source, animal breeds and ages, synchronization protocols used) may also have contributed to these differing outcomes. Another key difference in the current study is that all cows were treated with GnRH at AI as per normal estrus synchronization protocols so that we could strictly evaluate the luteotrophic effects of NGF. In the previous study, heifers were treated with NGF, GnRH (positive-control), or nothing (negative-control), with no differences in plasma progesterone observed between GnRH or NGF groups [20]. The enhanced luteal function in the current study, therefore, may represent a potential synergistic relationship between NGF and GnRH when administered at the time of AI and should be further explored.

A surprising finding in the current study was that there were no differences in CL sizes between treatments despite the improved progesterone production by day 10 in cows treated with NGF. This finding is consistent with those observed by Tribulo et al. [27], where administration of whole seminal plasma containing 250 µg NGF to heifers within 4 hours after ovulation resulted in no change in CL diameter and a tendency for elevated systemic progesterone by day 7. Perhaps the most interesting finding in this previous study was the significantly less variation in the interval to ovulation [27]. Since whole seminal plasma was administered, it cannot be confirmed that NGF alone contributed to the improved synchrony observed since other active constituents (such as

estradiol) were present. Though interval-to-ovulation was not evaluated in the current study, it is tempting to speculate that enhanced progesterone and conceptus development could be related to hastened onset of ovulation as previously observed [27]. However, we would expect to see a hastened onset of CL development and earlier differences in progesterone if that were the case. Nevertheless, future studies using the purified NGF developed herein may be necessary to make that distinction.

While CL diameter increased in heifers treated with llama NGF previously [20], another study showed that luteal growth in heifers occurred until day 7, after which the CL remained static in size, while progesterone still increased until day 14 [37]. In that study, the use of Doppler revealed that luteal blood flow doubled during this static phase, reaching maximal values on days 14 and 16 [37]. Similar to the current study, CL size and plasma progesterone concentrations were positively correlated only during the growth stage (days 4 to 7), whereas there was no correlation during the static phase (days 8 to 16) [37]. On the contrary, luteal blood flow was determined to be consistently correlated with plasma progesterone concentrations during both growth and static phases of the CL [37]. This suggests that the degree of luteal vascularity may affect progesterone production in the early diestrus period. Interestingly, an *in vitro* study demonstrated that isolated bovine thecal cells treated with recombinant NGF experienced increased Prostaglandin E₂ (PGE) production [38]. Prostaglandin E₂ acts as a pro-angiogenic molecule in vascular endothelium by recruiting the paracrine-autocrine mechanism characteristic of endothelium cells, resulting in vascular remodeling [39]. Increased vascularity of the CL improves the ability for signaling molecules (such as LH) to reach the ovary and stimulate progesterone production. In addition to being an angiogenic factor, PGE has also been found have a direct stimulatory effect on progesterone secretion in both small and large bovine luteal cells *in vitro* [40]. While a promising

mechanism for the role of NGF in the observed increase in progesterone production, further studies evaluating PGE production and luteal blood flow *in vivo* are necessary to investigate this possibility.

A second proposed mechanism for the effect of NGF on progesterone production is increased efficiency of LH binding in the CL. An *in vitro* study found that recombinant NGF increased the proliferative activity of thecal compartment cells in bovine ovarian tissues [38]. After ovulation, theca cells differentiate into small luteal cells that produce progesterone in response to LH [41,42]. Despite the previous belief that large luteal cells produce the most progesterone due to uninhibited levels of protein kinase A within the cell, other data suggests that small luteal cells may provide an earlier, yet crucial, surge in progesterone in response to LH that is important for early embryonic growth and maternal recognition of pregnancy [41,42]. Further, *in vitro* data showed that NGF receptor (TrkA) expression increased in bovine theca cells in response to LH treatment [38]. These findings support the idea of an upregulation mechanism since NGF has also been shown to increase LH production in bovine and llama pituitary cell cultures [43]. It is unclear whether an increase in number of small luteal cells would result in a measurable change in CL size, therefore an *ex vivo* model using gross and histological analyses would be required to accurately assess this proposed mechanism.

While studies previously demonstrated the luteotrophic effects of NGF from llama seminal plasma in cattle [20], the current study is the first to demonstrate that bovine-derived NGF also performs this function. Additionally, this study is the first to demonstrate how the luteotrophic effects of NGF can translate into improved maternal recognition of pregnancy. While interferon-tau, the signal for maternal recognition of pregnancy, is not secreted in high enough concentrations

to measure peripherally, expression of interferon-stimulated genes in leukocytes around days 18 to 20 has been used successfully for evaluating bovine conceptus development [30–32]. Additionally, pregnancy-associated glycoproteins secreted by binucleated cells in early placentation, such as PSPB, have been used as peripheral markers for pregnancy in cattle [30,32]. The upregulation of *Isg15* and *Mx2* mRNA at day 19 and increased plasma PSPB concentrations at day 24 are suggestive of improved conceptus development, presumably a result of increased progesterone production and uterine histotroph production [21]. These markers are consistent with the finding of a tendency for increased fetal CRL measured at day 66. Although it is unclear why CRL measurements at days 28 and 45 were not significantly different, it is likely that the changes were small enough early in pregnancy that they exceeded the sensitivity of transrectal ultrasound [30]. Additionally, though the 16% difference in pregnancy rates between NGF and CONT cows were not statistically significant in the current study, it is tantalizing to speculate that a study designed to measure fertility outcomes may demonstrate potential of NGF to improve reproductive performance in cattle.

Although other studies have shown the benefits of using supplemental progesterone to improve pregnancy per AI [23,44], timing of administration appears to be crucial for enhancing embryonic development, with inappropriate timing actually being detrimental [45]. In fact, a recent study found that initiating progesterone supplementation on day 4 post-estrus resulted in significantly greater embryo length compared to control heifers with experimentally compromised CL function [46]. Conversely, embryo length was similar to controls in heifers supplemented with progesterone beginning day 7 post-estrus [46]. In the current study, while there were no differences in progesterone concentrations at days 3 or 7, the next sample collection at day 10 demonstrated rising progesterone concentrations until day 19. Therefore, administration of a product that would

allow for a natural increase in progesterone production that occurs with CL formation would eliminate the concern about timing of administration and be more beneficial for use in the cattle industry.

In conclusion, this study demonstrated that NGF administration to cows at insemination improved corpus luteum function and early conceptus development as determined by upregulation of interferon-stimulated genes and increased pregnancy-specific protein B concentrations. These results suggest that NGF from bull seminal plasma plays an important role in early embryonic development in cows. NGF-treated cows had greater progesterone concentrations around the time of maternal recognition of pregnancy (days 10 to 19). Treatment with NGF also led to increased expression of interferon-stimulated genes *Isg15* and *Mx2* in peripheral blood leukocytes at day 19 and higher PSPB production from the placenta at day 24 post-AI. Future studies would be necessary to determine if these effects would decrease early embryonic loss and improve early conception rates in cattle.

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3.7 Tables and Figures

Table 1. Minor contaminants following protein purification.

Contaminant	Percentage present
C-type natriuretic peptide	16.41%
Serine protease inhibitor	11.26%
Cystatin-c	5.53%
Tubulin polymerization-promoting protein family 2	5.53%
Type II cytoskeletal keratin	4.77%
Polymeric immunoglobulin receptor	0.95%

Table 2. Primer reference and sequences for genes investigated by quantitative real-time PCR.

Target gene	Gene name	NCBI sequence	Primer	Primer sequence
<i>Isg15</i>	Interferon stimulated gene 15	NM_174366	Forward	5'-GGTATGAGCTGAAGCAGTT-3'
			Reverse	5'-ACCTCCCTGCTGTCAAGGT-3'
<i>Rtp4</i>	Receptor transporter protein 4	BC105539	Forward	5'-CACATGTACCTGGAGAACCAGA-3'
			Reverse	5'-AGGTAGCTCTGAAACCTTCCTG-3'
<i>Mx1</i>	Myxovirus 1	AF047692	Forward	5'-GTACGAGCCGAGTTCTCCAA-3'
			Reverse	5'-ATGTCCACAGCAGGCTCTTC-3'
<i>Mx2</i>	Myxovirus 2	NM_173941	Forward	5'-CTTCAGAGACGCCTCAGTCG-3'
			Reverse	5'-TGAAGCAGCCAGGAATAGTG-3'
<i>Actb</i>	β -actin	AY141970	Forward	5'-CTGGACTTCGAGCAGGAGAT-3'
			Reverse	5'-GGATGTCGACGTCACACTTC-3'
<i>Rpl19</i>	Ribosomal protein L 19	NM_001040516	Forward	5'-ATCGATCGCCACATGTATCA-3'
			Reverse	5'-GCGTGCTTCCTTGGTCTTAG-3'

Table 3. Correlation between CL size and plasma progesterone concentrations from days 3 to 19 after artificial insemination.
P* = 0.01-0.05, *P* ≤ 0.01

	Plasma Progesterone				
	Day 3	Day 7	Day 10	Day 14	Day 19
CL Diameter	0.34**	0.53**	0.14	0.05	0.33*
CL Volume	0.37**	0.58**	0.10	0.04	0.31*

Table 4. Effect of NGF treatment on pregnancy rates (%) and embryonic/fetal crown-rump length measurements (mean \pm SEM, mm) at 28, 45, and 66 days after artificial insemination (AI).

Days post-AI	Pregnancy/AI (%)			Crown-Rump Length (mm)		
	CONT	NGF	<i>P</i>-value	CONT	NGF	<i>P</i>-value
28	58.6	75.0	0.13	9.4 \pm 0.4	9.5 \pm 0.4	0.47
45	55.2	71.4	0.12	26.0 \pm 1.1	27.1 \pm 0.6	0.20
66	55.2	71.4	0.12	32.6 \pm 0.6	33.9 \pm 0.6	0.06

Figure 4. MS/MS spectra results from purified NGF protein that correspond to a peptide theoretical match for query number 23682.

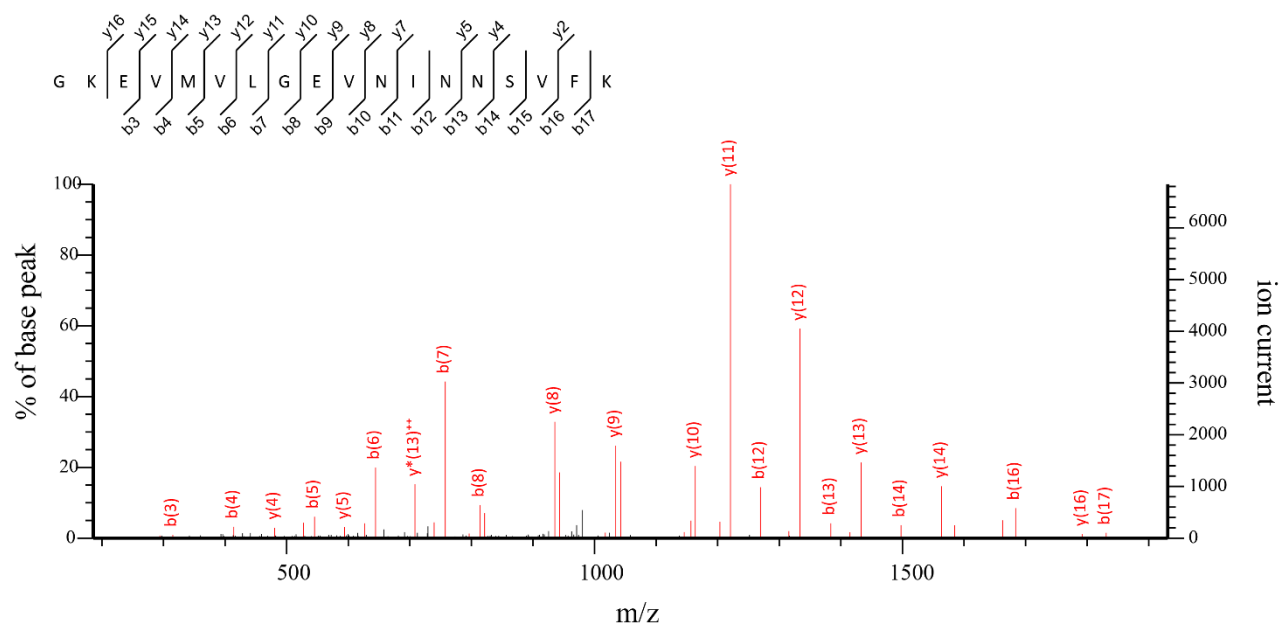


Figure 5. (A) The percentage of NGF vs. CONT cows that presented with a dominant follicle, and (B) the size of the dominant follicle (mm) at artificial insemination (AI). (C) The percentage of cows that had a functional CL (progesterone >1.0 ng/mL) at AI, and (D) the percentage of cows that ovulated in response to the synchronization protocol.

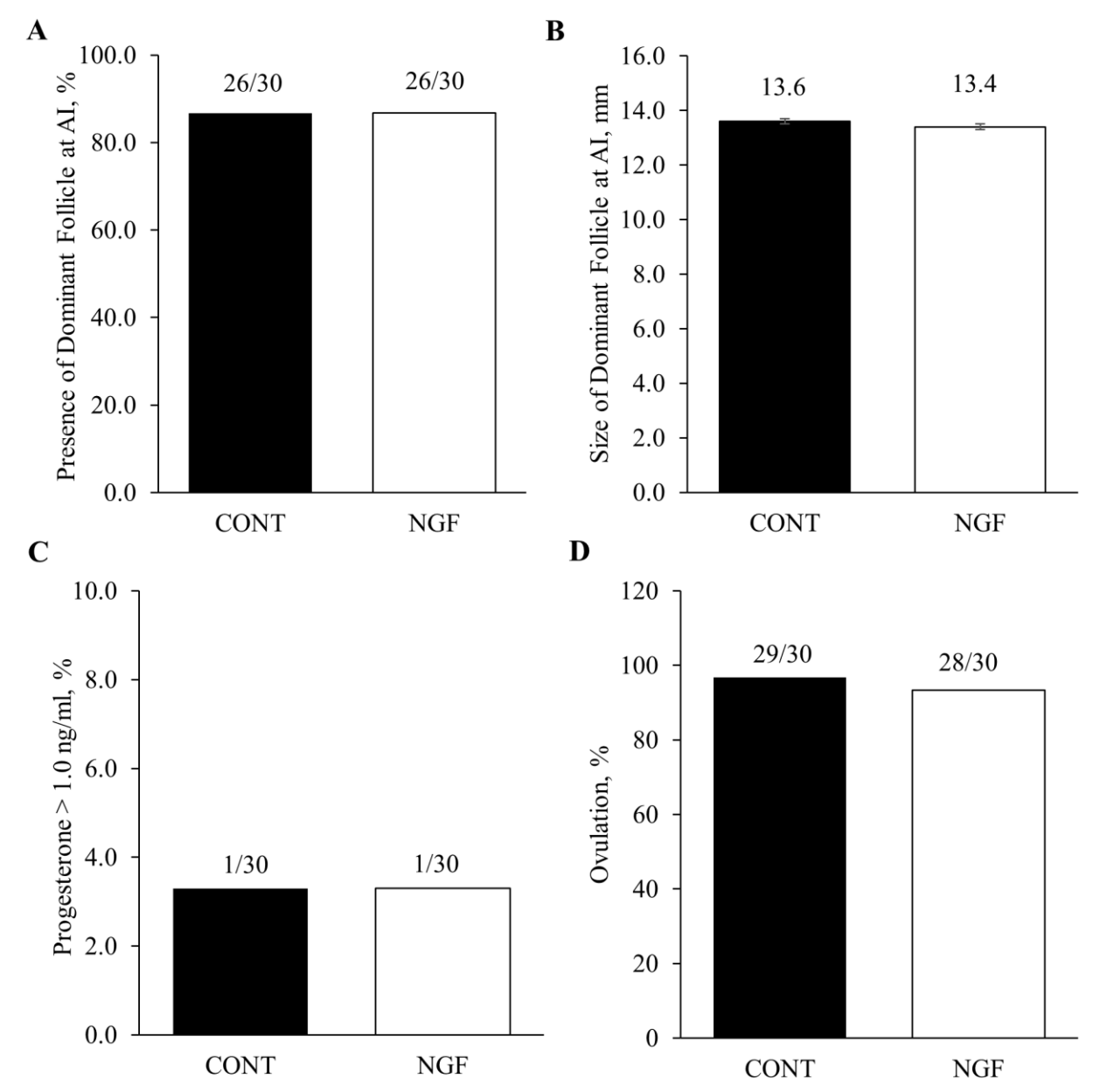


Figure 6. (A) Corpus luteum (CL) diameter, (B) CL volume, and (C) plasma progesterone (P4) concentrations during diestrus in NGF vs. CONT cows after artificial insemination (AI).
* $P = 0.06$, ** $P < 0.05$

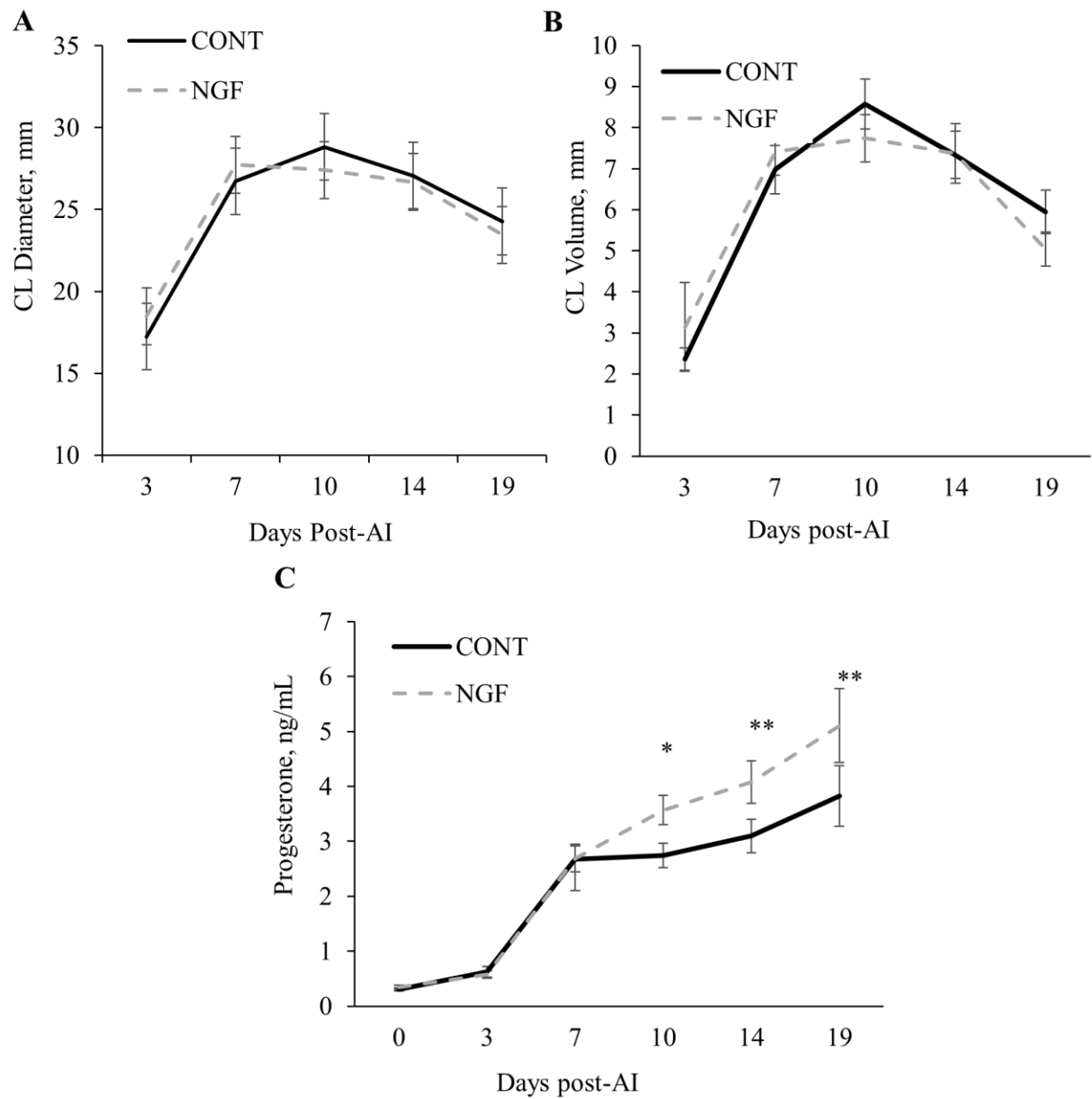


Figure 7. Plasma concentrations of pregnancy-specific protein B (PSPB) at day 24 after artificial insemination (AI) in NGF vs. CONT cows based on pregnancy status at day 28. Different letters (a, b, c) represent different ($P < 0.05$) least square means. Error bars represent the 95% confidence intervals for group means.

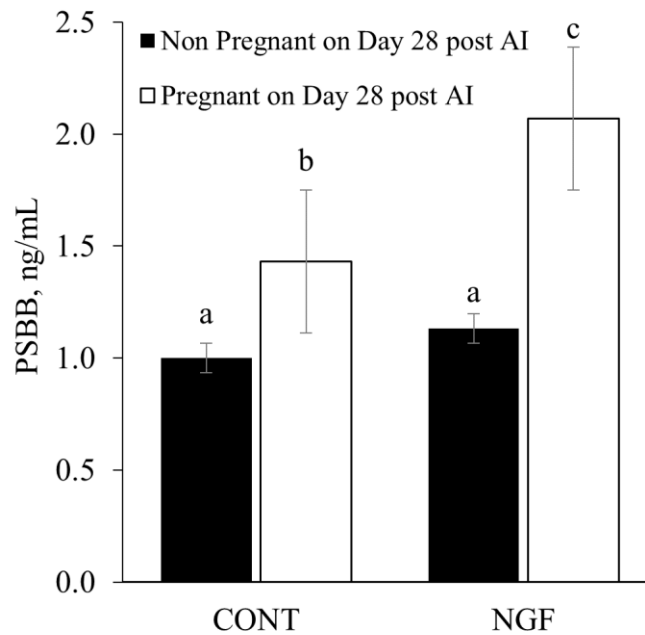
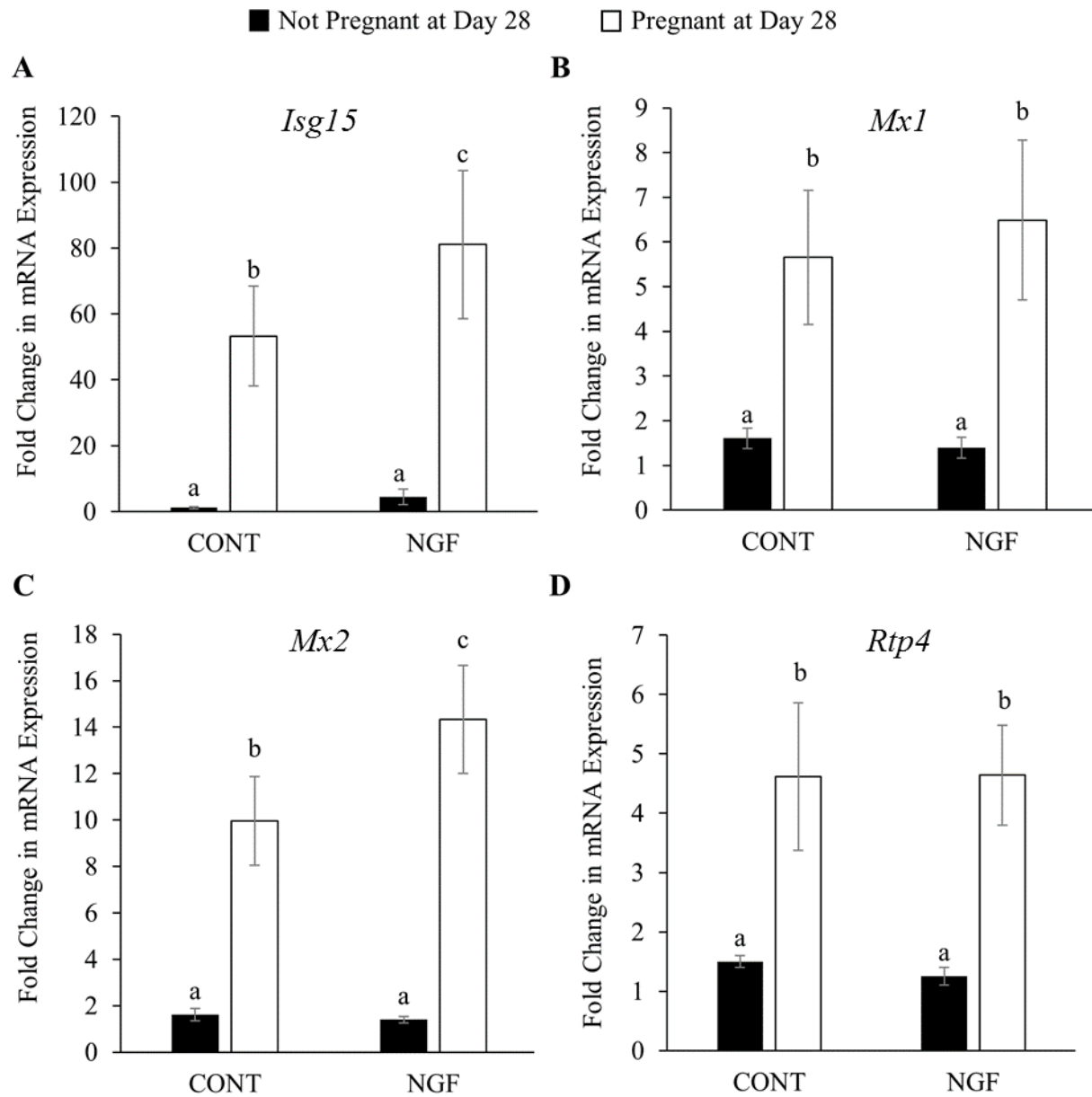


Figure 8. Fold-change in mRNA expression of four interferon-stimulated genes, (A) *Isg15*, (B) *Mx2*, (C) *Mx1*, and (D) *Rtp4*, in NGF vs. CONT cows based on pregnancy status at day 28 after artificial insemination. Different letters (a, b, c) represent different ($P < 0.05$) least square means. Error bars represent the 95% confidence intervals for group means.



3.8 References

- [1] Killian GJ, Chapman DA, Rogowski LA. Fertility-associated proteins in Holstein bull seminal plasma. *Biol Reprod* 1993;49:1202–7. doi:10.1095/biolreprod49.6.1202.
- [2] Gwathmey TM, Ignatz GG, Mueller JL, Manjunath P, Suarez SS. Bovine seminal plasma proteins PDC-109, BSP-A3, and BSP-30-kDa share functional roles in storing sperm in the oviduct. *Biol Reprod* 2006;75:501–7. doi:10.1095/biolreprod.106.053306.
- [3] Bromfield JJ. A role for seminal plasma in modulating pregnancy outcomes in domestic species. *Reproduction* 2016;152:R223–32. doi:10.1530/REP-16-0313.
- [4] Moura AA, Chapman DA, Koc H, Killian GJ. A comprehensive proteomic analysis of the accessory sex gland fluid from mature Holstein bulls. *Anim Reprod Sci* 2007;98:169–88. doi:10.1016/j.anireprosci.2006.03.012.
- [5] Hayes BJ, Chamberlain AJ, Maceachern S, Savin K, McPartlan H, MacLeod I, et al. A genome map of divergent artificial selection between *Bos taurus* dairy cattle and *Bos taurus* beef cattle. *Anim Genet* 2009;40:176–84. doi:10.1111/j.1365-2052.2008.01815.x.
- [6] Schjenken JE, Robertson SA. Seminal fluid and immune adaptation for pregnancy - comparative biology in mammalian species. *Reprod Domest Anim* 2014;49:27–36. doi:10.1111/rda.12383.
- [7] Lima FS, Risco CA, Thatcher MJ, Benzaquen ME, Archbald LF, Santos JEP, et al. Comparison of reproductive performance in lactating dairy cows bred by natural service or timed artificial insemination. *J Dairy Sci* 2009;92:5456–66. doi:10.3168/jds.2009-2197.
- [8] Holden SA, Fernandez-Fuertes B, Murphy EM, Lonergan P, Fair S. Effect of seminal plasma from high- and low-fertility bulls on cauda epididymal sperm function. *Reprod Fertil Dev* 2017. doi:10.1071/RD17136.
- [9] Li C, Sun Y, Yi K, Ma Y, Sun Y, Zhang W, et al. Detection of nerve growth factor (NGF) and its specific receptor (TrkA) in ejaculated bovine sperm, and the effects of NGF on sperm function. *Theriogenology* 2010;74:1615–22. doi:10.1016/j.theriogenology.2010.06.033.
- [10] Bromfield JJ, Schjenken JE, Chin PY, Care AS, Jasper MJ, Robertson SA. Maternal tract factors contribute to paternal seminal fluid impact on metabolic phenotype in offspring. *Proc Natl Acad Sci USA* 2014;111:2200–5. doi:10.1073/pnas.1305609111.
- [11] Robertson SA, Mau VJ, Tremellen KP, Seamark RF. Role of high molecular weight seminal vesicle proteins in eliciting the uterine inflammatory response to semen in mice. *J Reprod Fertil* 1996;107:265–77. doi:10.1530/JRF.0.1070265.
- [12] Sharkey DJ, Tremellen KP, Jasper MJ, Gemzell-Danielsson K, Robertson SA. Seminal fluid induces leukocyte recruitment and cytokine and chemokine mRNA expression in the human cervix after coitus. *J Immunol* 2012;188:2445–54. doi:10.4049/jimmunol.1102736.
- [13] Adams GP, Ratto MH, Silva ME, Carrasco RA. Ovulation-inducing factor (OIF/NGF) in seminal plasma: a review and update. *Reprod Domest Anim* 2016;51:4–17. doi:10.1111/rda.12795.
- [14] Adams GP, Ratto MH, Huanca W, Singh J. Ovulation-inducing factor in the seminal plasma of alpacas and llamas. *Biol Reprod* 2005;73:452–7.

doi:10.1095/biolreprod.105.040097.

- [15] Ratto MH, Leduc YA, Valderrama XP, van Straaten KE, Delbaere LTJ, Pierson RA, et al. The nerve of ovulation-inducing factor in semen. *Proc Natl Acad Sci U S A* 2012;109:15042–7. doi:10.1073/pnas.1206273109.
- [16] Kershaw-Young CM, Druart X, Vaughan J, Maxwell WMC. β -Nerve growth factor is a major component of alpaca seminal plasma and induces ovulation in female alpacas. *Reprod Fertil Dev* 2012;24:1093–7. doi:10.1071/RD12039.
- [17] Silva ME, Smulders JP, Guerra M, Valderrama XP, Letelier C, Adams GP, et al. Cetrorelix suppresses the preovulatory LH surge and ovulation induced by ovulation-inducing factor (OIF) present in llama seminal plasma. *Reprod Biol Endocrinol* 2011;9:74. doi:10.1186/1477-7827-9-74.
- [18] Silva ME, Recabarren MP, Recabarren SE, Adams GP, Ratto MH. Ovarian estradiol modulates the stimulatory effect of ovulation-inducing factor (OIF) on pituitary LH secretion in llamas. *Theriogenology* 2012;77:1873–82. doi:10.1016/j.theriogenology.2012.01.004.
- [19] Dissen GA, Hill DF, Costa ME, Dees W Les, Lara HE, Ojeda SR. A role for TrkA Nerve Growth Factor receptors in mammalian ovulation. *Endocrinology* 1996;137:198–209. doi:10.1210/en.137.1.198.
- [20] Tanco VM, Van Steelandt MD, Ratto MH, Adams GP. Effect of purified llama ovulation-inducing factor (OIF) on ovarian function in cattle. *Theriogenology* 2012;78:1030–9. doi:10.1016/j.theriogenology.2012.03.036.
- [21] Lonergan P. Influence of progesterone on oocyte quality and embryo development in cows. *Theriogenology* 2011;76:1594–601. doi:10.1016/j.theriogenology.2011.06.012.
- [22] Mann GE, Lamming GE. Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. *Reproduction* 2001;121:175–80.
- [23] Mann GE, Fray MD, Lamming GE. Effects of time of progesterone supplementation on embryo development and interferon-tau production in the cow. *Vet J* 2006;171:500–3. doi:10.1016/j.tvjl.2004.12.005.
- [24] Issaq HJ, Conrads TP, Janini GM, Veenstra TD. Methods for fractionation, separation and profiling of proteins and peptides. *Electrophoresis* 2002;23:3048–61. doi:10.1002/1522-2683(200209)23:17<3048::AID-ELPS3048>3.0.CO;2-L.
- [25] Eversole DE, Browne MF, Hall JB, Dietz RE. Body condition scoring beef cows. *Virginia Coop Ext* 2009:1–6. <https://pubs.ext.vt.edu/400/400-795/400-795.html> (accessed March 11, 2016).
- [26] Whittier WD, Currin JF, Schramm H, Holland S, Kasimanickam RK. Fertility in Angus cross beef cows following 5-day CO-Synch + CIDR or 7-day CO-Synch + CIDR estrus synchronization and timed artificial insemination. *Theriogenology* 2013;80:963–9. doi:10.1016/j.theriogenology.2013.07.019.
- [27] Tribulo P, Bogle OA, Mapletoft RJ, Adams GP. Bioactivity of ovulation inducing factor (or nerve growth factor) in bovine seminal plasma and its effects on ovarian function in cattle. *Theriogenology* 2015;83:1394–401. doi:10.1016/j.theriogenology.2014.12.014.

- [28] Mercadante VRG, Kozicki LE, Ciriaco FM, Henry DD, Dahlen CR, Crosswhite MR, et al. Effects of administration of prostaglandin F at initiation of the seven-day CO-Synch+controlled internal drug release ovulation synchronization protocol for suckled beef cows and replacement beef heifers. *J Anim Sci* 2015;93:5204–13. doi:10.2527/jas.2015-8967.
- [29] Kastelic JP, Bergfelt DR, Ginther OJ. Relationship between ultrasonic assessment of the corpus luteum and plasma progesterone concentration in heifers. *Theriogenology* 1990;33:1269–78. doi:10.1016/0093-691X(90)90045-U.
- [30] Carvalho PD, Consentini CC, Weaver SR, Barleta R V., Hernandez LL, Fricke PM. Temporarily decreasing progesterone after timed artificial insemination decreased expression of interferon-tau stimulated gene 15 in blood leukocytes, serum pregnancy-specific protein B concentrations, and embryo size in lactating Holstein cows. *J Dairy Sci* 2017;100:1–10. doi:10.3168/jds.2016-11996.
- [31] Gifford CA, Racicot K, Clark DS, Austin KJ, Hansen TR, Lucy MC, et al. Regulation of interferon-stimulated genes in peripheral blood leukocytes in pregnant and bred, nonpregnant dairy cows. *J Dairy Sci* 2007;90:274–80. doi:10.3168/jds.S0022-0302(07)72628-0.
- [32] Ribeiro ES, Bruno RGS, Farias AM, Hernández-Rivera JA, Gomes GC, Surjus R, et al. Low doses of bovine somatotropin enhance conceptus development and fertility in lactating dairy cows. *Biol Reprod* 2014;90:10. doi:10.1095/biolreprod.113.114694.
- [33] Robinson TL, Sutherland IA, Sutherland J. Validation of candidate bovine reference genes for use with real-time PCR. *Vet Immunol Immunopathol* 2007;115:160–5. doi:10.1016/j.vetimm.2006.09.012.
- [34] Harper GP, Glanville RW, Thoenen H. The purification of nerve growth factor from bovine seminal plasma. Biochemical characterization and partial amino acid sequence. *J Biol Chem* 1982;257:8541–8.
- [35] Yerlekar A, Dudhe P. A review on study and comparison between 2D gel electrophoresis and mass spectrometry. *IOSR J Comput Eng* 2014;16:97–104.
- [36] Pan G, Chen Z, Liu X, Li D, Xie Q, Ling F, et al. Isolation and purification of the ovulation-inducing factor from seminal plasma in the bactrian camel (*Camelus bactrianus*). *Theriogenology* 2001;55:1863–79. doi:10.1016/S0093-691X(01)00528-3.
- [37] Herzog K, Brockhan-Lüdemann M, Kaske M, Beindorff N, Paul V, Niemann H, et al. Luteal blood flow is a more appropriate indicator for luteal function during the bovine estrous cycle than luteal size. *Theriogenology* 2010;73:691–7. doi:10.1016/j.theriogenology.2009.11.016.
- [38] Dissen GA, Parrott JA, Skinner MK, Hill DF, Costa ME, Ojeda SR. Direct effects of nerve growth factor on thecal cells from antral ovarian follicles. *Endocrinology* 2000;141:4736–50. doi:10.1210/endo.141.12.7850.
- [39] Finetti F, Solito R, Morbidelli L, Giachetti A, Ziche M, Donnini S. Prostaglandin E2 regulates angiogenesis via activation of fibroblast growth factor receptor-1. *J Biol Chem* 2008;283:2139–46. doi:10.1074/jbc.M703090200.
- [40] Alila HW, Hansel W. A comparison of the effects of cyclooxygenase prostanoids on

- progesterone production by small and large bovine luteal cells. *Prostaglandins* 1988;36. doi:10.1016/0090-6980(88)90312-7.
- [41] Harrison LM, Kenny N, Niswender GD. Progesterone production, LH receptors, and oxytocin secretion by ovine luteal cell types on days 6, 10 and 15 of the oestrus cycle and day 25 of pregnancy. *J Reprod Fert* 1987;79:539–48. doi:10.1530/jrf.0.0790539.
- [42] Niswender GD, Juengel JL, McGuire WJ, Belfiore CJ, Wiltbank MC. Luteal function: the estrous cycle and early pregnancy. *Biol Reprod* 1994;50:239–47. doi:10.1095/biolreprod50.2.239.
- [43] Bogle OA, Ratto MH, Adams GP. Ovulation-inducing factor (OIF) induces LH secretion from pituitary cells. *Anim Reprod Sci* 2012;133:117–22. doi:10.1016/j.anireprosci.2012.06.006.
- [44] Monteiro Jr PLJ, Ribeiro ES, Maciel RP, Dias ALG, Solé Jr E, Lima FS, et al. Effects of supplemental progesterone after artificial insemination on expression of interferon-stimulated genes and fertility in dairy cows. *J Dairy Sci* 2014;97:4907–21. doi:http://dx.doi.org/10.3168/jds.2013-7802.
- [45] O'Hara L, Forde N, Carter F, Rizos D, Maillo V, Ealy AD, et al. Paradoxical effect of supplementary progesterone between day 3 and day 7 on corpus luteum function and conceptus development in cattle. *Reprod Fertil Dev* 2014;26:328–36. doi:10.1071/RD12370.
- [46] Parr MH, Scully S, Lonergan P, Evans ACO, Crowe MA, Diskin MG. Establishment of critical timing of progesterone supplementation on corpus luteum and embryo development in beef heifers. *Anim Reprod Sci* 2017. doi:10.1016/j.anireprosci.2017.02.005.

Chapter 4:

Direct Effects of Nerve Growth Factor- β on Steroidogenesis and Angiogenic Markers of the Bovine Pre-Ovulatory Follicle

4.1 Abstract

Nerve growth factor- β (NGF) is a seminal plasma protein that induces ovulation in camelids and has a luteotrophic effect. In spontaneously ovulating species, NGF signaling in the ovary is critical for the first ovulation to occur, but little is known regarding the interactions of seminal plasma derived NGF on the pre-ovulatory follicle. This study aimed to assess the direct effects of NGF on steroidogenesis and angiogenic markers in the bovine pre-ovulatory follicle. Heifers were synchronized using a GnRH-prostaglandin and intravaginal progesterone protocol (5-d CIDR-Synch). Ovariectomy was performed 48 hours after the second PGF injection when a pre-ovulatory follicle >12 mm was present. The pre-ovulatory follicle was excised and dissected into quarters. The theca interna with adherent granulosa cells was peeled from the theca externa and surrounding stromal tissue and cut into smaller pieces. The follicle wall pieces were incubated in 0.5 mL of culture media supplemented with LH (4 ng/mL) and FSH (4 ng/mL) and divided into one of two treatments: (1) NGF (n = 12): purified NGF (100 ng/mL) or (2) CONT (n = 12): phosphate-buffered saline negative control. Media was withdrawn and replaced completely with fresh media at 3, 6, 12, 24, 48, and 72 h of culture and frozen for subsequent steroid assays. At the end of the 72 h, follicle tissue pieces were flash frozen to assess steroidogenic and angiogenic gene expression. Treatment with NGF upregulated gene expression of steroidogenic enzyme 17 β -hydroxysteroid dehydrogenase in the follicle ($P = 0.04$). Consistently, NGF treatment increased testosterone production ($P < 0.01$), but did not alter production of progesterone ($P = 0.81$) and estradiol-17 β ($P = 0.14$) or expression of steroidogenic acute regulatory protein, side chain

cleavage enzyme, cytochrome P450 17 alpha-hydroxylase, and aromatase cytochrome P450, luteinizing hormone receptor, vascular endothelial growth factor A isoform 121, estrogen receptor alpha, or prostaglandin E synthase ($P \geq 0.31$). These results demonstrate a shift towards increased androgen production with NGF treatment, which may be secondary to theca cell proliferation. Additionally, treatment with NGF downregulated gene expression of the angiogenic enzyme fibroblast growth factor 2 ($P = 0.02$). Given the dynamics of fibroblast growth factor activity during the follicular-to-luteal transition, this finding suggests a possible hastened onset of cell remodeling that occurs during early corpus luteum development.

4.2 Introduction

Nerve growth factor- β (NGF) is a member of the neurotrophin family that has a critical role in mammalian follicle development and ovulation [1,2]. Expression of NGF has been localized to ovarian granulosa cells prior to formation of the first primordial follicles in neonatal mice [1] and rats [3] and is involved initiating folliculogenesis [4]. Though present during late fetal development, expression of NGF and its receptor, TrkA, in the ovary decreases postnatally between 24 and 48 h after birth and remains low until puberty in rats [3]. At the time of the first pre-ovulatory luteinizing hormone (LH) surge, a transient activation of NGF/TrkA occurs and is integral to the process of follicular cytodifferentiation proceeding the first ovulation [2]. In bovine theca cells, NGF induced prostaglandin E₂ synthesis [5], which facilitates follicular rupture at ovulation [6]. Additionally, NGF/TrkA signaling in theca cells contributes to the loss of gap junctions within the follicular wall that precedes ovulation [7]. The presence of both LH and follicle stimulating hormone (FSH) *in vitro* were necessary to stimulate NGF secretion from medium to large follicles in ewes, suggesting a synergistic role with gonadotropins during the pre-

ovulatory cascade [8]. Consistently, follicular NGF concentrations increased following the gonadotropin peaks in ewes, and *in vitro* treatment with NGF induced marked cumulus expansion and progressive cumulus-oocyte uncoupling [9], which functions to reinitiate meiotic progression of the oocyte at ovulation.

Our understanding of the role of NGF in promoting mammalian ovulation became even more complex when it was identified as the ovulation inducing factor in camelid seminal plasma [10,11]. Though the ability of the ovary to produce NGF in spontaneously ovulating species is well documented, few studies have evaluated whether the introduction of NGF from seminal plasma at time of breeding may have a role within the bovine hypothalamic-pituitary-ovarian axis [12–14]. In camelids, intrauterine absorption of seminal plasma NGF into systemic circulation occurs within 15 min of copulation, after which it stimulates the preovulatory LH peak from the anterior pituitary gland and exerts a dose-dependent luteotropic effect on the developing corpus luteum (CL) [15]. Studies have shown that NGF retains its luteotropic properties when administered systemically to cattle [12,14]. While the luteotropic effect in camelids is attributed to the prolonged LH secretion from the pituitary, in cattle there is evidence that NGF from the seminal plasma may act directly on the ovary [5].

Similar to camelids, bull seminal plasma contains NGF, which is concentrated into the sperm-rich fraction of the ejaculate, facilitating its transit into the cow reproductive tract [16]. The bovine uterus maintains a local countercurrent exchange between the uterine venous drainage and the ovarian artery that allows for direct transport of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the uterus to the ovary during luteolysis [17,18]. This anatomical mechanism could also provide a potential route for NGF to travel to and interact directly with the ovary [19]. A previous study demonstrated

that treating bovine theca cells with recombinant NGF *in vitro* resulted in increased androstenedione and progesterone release, prostaglandin E₂ production, and theca cell proliferation when compared to hCG-treated controls [5]. However, the signaling cascade within the ovary involves a complex interaction between the theca and granulosa cells, which has yet to be elucidated. This study aimed to assess the direct effects of NGF, purified from bull seminal plasma, on steroidogenesis and angiogenic markers in theca and granulosa cells of the bovine pre-ovulatory follicle. We hypothesize that NGF administration would stimulate steroidogenesis and angiogenic markers in thecal and granulosa cells from the bovine pre-ovulatory follicle.

4.3 Materials and Methods

4.3.1 Ethics

All experimental procedures carried out in the present study were approved by the Institutional Animal Care and Use Committees of the University of Illinois at Urbana-Champaign, USA (Protocol #15-201).

4.3.2 Heifer synchronization and ovariectomy

The 24 follicular tissue pieces composed of theca and granulosa cells used in the current study were extracted from two cyclic post-pubertal Holstein heifers. The heifers received an intravaginal progesterone-releasing device (1.38 g progesterone; Eazi-Breed CIDR, Zoetis, Parsippany-Troy Hills, NJ, USA) and an injection of GnRH agonist (100 µg Factrel®, Zoetis, Parsippany-Troy Hills, NJ, USA) intramuscularly. The intravaginal device was removed 5 days later, and both heifers were given PGF_{2α} analog (25 mg dinoprost tromethamine, Lutalyse, Zoetis, Parsippany-Troy Hills, NJ, USA) intramuscularly at the time of the intravaginal device removal.

and again 24 h later. The heifers were examined daily by transrectal ultrasonography to measure follicular growth, and an ovariectomy was performed at 48 h after the second PGF_{2α} injection when the dominant follicles reached at least 12 mm in diameter. Ovariectomy was performed as described elsewhere [20] using colpotomy in the standing position under sedation with 15 mg intramuscular xylazine (AnaSed® Injection, Lloyd, Inc., Shenandoah, IA, USA) and caudal epidural anesthesia with 5 mL of 2% (w/v) lidocaine HCl (VetOne®, Boise, Idaho, USA). Heifers were treated pre-operatively with ceftiofur crystalline free acid (6.6 mg/kg; Excede®, Zoetis, Parsippany-Troy Hills, NJ, USA) injected subcutaneously in the base of the ear and flunixin meglumine (2.2 mg/kg; Norbrook® Inc., Overland Park, KS, USA) intravenously. An incision was made in the dorsolateral aspect of the vaginal fornix, and the peritoneum was manually punctured after blunt dissection through the adventitia. The mesovarium was manually compressed, and the ovary containing the dominant follicle was removed using a chain Écraseur. Ovaries were placed in ice-cold phosphate buffered saline solution containing 2% antibiotic-antimycotic mixture (25 µg/mL amphotericin B, 10,000 units/mL penicillin, 10,000 µg/mL streptomycin; Gibco, Gaithersburg, MD, USA) for transport to the laboratory.

4.3.3 Isolation of follicular wall

The pre-ovulatory follicle was identified and excised from each ovary for use in the tissue culture system (Fig. 9). Follicular fluid was aspirated and frozen at -80°C. The follicles were dissected into quarters, and the theca interna with adherent granulosa cells was peeled from the theca externa and surrounding stromal tissue. The remaining follicle wall preparations (theca interna and granulosa cells) were cut into 26 pieces (average weight: 5.3 ± 0.7 mg), 24 of which were transferred to a costar 24-well plate (1 piece/well; Cambridge, MA, USA) for tissue culture,

as previously described [21,22]. The remainder of the tissue was flash frozen in liquid nitrogen and maintained at -80°C for further analyses.

4.3.4 Treatment of follicular wall tissue with NGF

The follicle wall pieces were cultured in 0.5 mL of medium consisting of Eagle's MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 1% L-glutamine (Gibco, Gaithersburg, MD, USA), 1% nonessential amino acids (Sigma-Aldrich, St. Louis, MO, USA), 1% penicillin-streptomycin (Sigma-Aldrich), 1% ITS (10 ng/ml insulin, 5.5 ng/ml transferrin, 5.5 ng/ml selenium, Sigma-Aldrich), 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA), 40 ng/mL cortisol (Sigma-Aldrich), 4 ng/mL human recombinant LH (Dr. A. F. Parlow, National Hormone and Peptide Program, Harbor- UCLA Medical Center, Torrance, CA, USA), and 4 ng/mL human recombinant FSH (Dr. A. F. Parlow, National Hormone and Peptide Program). Culture medium was either supplemented with purified bovine NGF (100 ng/mL, $n = 12$) or left untreated (control, $n = 12$). The single plate was incubated at 37°C in a humidified incubator gassed with 5% CO_2 :95% air for 72 h. The NGF used was purified from bovine seminal plasma as described elsewhere in a parallel study [14].

4.3.5 Hormone assays

The aliquots (0.5 mL) of culture medium at 3, 6, 12, 24, 48, and 72 h of culture were collected and preserved at -20°C for subsequent steroid assays. Progesterone, testosterone, and estradiol- 17β secretion by granulosa and theca cells into the culture media were measured using chemiluminescence assay (Immulite 2000 XPi platform; Siemens Medical Solutions, Malvern, PA, USA, Inc.). Total hormone production for each well was calculated by multiplying the

measured concentration by the volume of media (0.5 mL) and then dividing by tissue weight (mg). Intra-assay coefficient of variations were 4.0% (testosterone), 2.4% (progesterone), and 3.1% (estradiol-17 β). Inter-assay coefficient of variations were 12% (testosterone), 19% (progesterone), and 15% (estradiol-17 β). The progesterone assay had a detection range of 0.2 to 40 ng/mL and a sensitivity of 0.1 ng/mL. The testosterone assay had a detection range of 20 to 1600 ng/mL and a sensitivity of 15 ng/dL. The estradiol-17 β assay had a detection range of 20-2000 pg/mL and a sensitivity of 15 pg/mL.

4.3.6 Quantitative real-time PCR analyses

At the end of the 72-h culture period, follicle wall pieces were weighed and flash-frozen. Follicular tissue mRNA expression was determined for LH/ choriogonadotropin receptor (*Lhcgr*), FSH receptor (*Fshr*), prostaglandin E synthase (*Pges*), vascular endothelial growth factor A isoform 121 (*Vegfa₁₂₁*), vascular endothelial growth factor receptor 2 (*Vegfr2*), fibroblast growth factor 2 (*Fgf2*), estrogen receptor alpha (*Esr1*), steroidogenic acute regulatory protein (*Star*), side-chain cleavage enzyme (*Cyp11a1*), 17 α -hydroxylase/17-20 lyase (*Cyp17a1*), aromatase (*Cyp19a1*), 3 beta-hydroxysteroid dehydrogenase (*3 β -hsd*), and 17 β -hydroxysteroid dehydrogenase (*17 β -hsd*). Primer sequences are summarized in Table 5 [23,24].

Follicle tissue lysis and RNA extraction were conducted according to the manufacturer's recommendations (PureLink RNA Mini Kit, Invitrogen, Carlsbad, CA, USA). Isolated RNA was evaluated for concentration and purity using a NanoDrop One Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). A maximum of 2 μ g of mRNA was used to synthesize complementary DNA using a commercial kit (High-capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA, USA) supplemented with RNase inhibitor (RNase Inhibitor,

human placenta, New England BioLabs, Ipswich, MA, USA). Complementary DNA was used for quantitative real-time reverse transcription PCR using a 7500 Real-Time PCR Detection System (Applied Biosciences) with PowerUpTM SYBRTM Green Master Mix (2X; Applied Biosciences). All assays were carried out in triplicate for each target mRNA. The amplification conditions were as follows: 50°C for 2 min, 95°C for 2 min, and 40 cycles at 95°C for 15 s and 60°C for 60 s. Primers were designed for the constitutively expressed mRNAs, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), ribosomal protein L 15 (*Rpl-15*), and ribosomal protein L 19 (*Rpl-19*), with the expression value of each gene normalized to mean of these genes (Table 1). Relative expression values were obtained by determining the PCR amplification efficiency ($E = 2$) to the power of the delta-delta threshold cycle ($\Delta\Delta Ct$) obtained from the ΔCt least square mean differences of pairwise comparisons between initial and cultured tissue [25].

4.3.7 Statistical analyses

Data are presented as percentage mean \pm SEM. All statistical analyses were performed using R Version 3.4.3 (<https://www.r-project.org/>). Normality was confirmed using a Shapiro-Wilk test of the residuals. Non-normal data were transformed using Tukey's Ladder of Powers. If transformation did not result in a normalized population, a Kruskal-Wallis rank sum test was performed. Analysis of variance was applied to parametric data using a general linear mixed model with repeated measures applied for hormone data. The covariance structure that resulted in the smallest Bayesian information criterion was selected from the mixed models. Significance was declared at $P \leq 0.05$.

4.4 Results

4.4.1 NGF treatment increases androgen production in the bovine pre-ovulatory follicle

As previously reported, treatment of bovine theca cells with recombinant NGF increased androstenedione and progesterone production [5]. Given the two-cell, two-gonadotropin theory, where androgens produced by theca cells are aromatized to estrogens in the granulosa cells [26,27], we anticipated that purified NGF treatment would increase estradiol production secondarily through increased androgen precursor. On the contrary, NGF treatment did not alter follicular production of progesterone ($P = 0.81$; Fig. 10A) or estradiol-17 β ($P = 0.14$; Fig. 10C), but did increase testosterone production compared to untreated controls ($P < 0.01$; Fig. 10B). Testosterone concentrations in treated wells were consistently higher than untreated wells until completion of the culture at 72 h.

At completion of culture, follicle wall pieces were also flash frozen and subjected to gene quantification to evaluate steroidogenic enzyme expression. Consistent with increased testosterone production, follicle wall preparations treated with NGF experienced upregulated expression of the enzyme *17 β -hsd* ($P = 0.04$; Fig. 11A), which converts androstenedione to testosterone in the theca cell and estrone to estradiol in the granulosa cell. There was no effect of NGF on expression of the aromatase enzyme *Cyp19a1* that converts androstenedione to estrone and testosterone to estradiol in the granulosa cells, which explains the lack of difference in estradiol production from NGF-treated tissue. Treatment with NGF also did not alter expression of the steroidogenic enzymes responsible for the conversion of cholesterol to pregnenolone (*Star*, *Cyp11a1*), pregnenolone to progesterone (*3 β -hsd*), or progesterone to androstenedione (*Cyp17a1*) ($P \geq 0.31$; Table 6).

4.4.2 NGF treatment does not alter expression of gonadotropin or estrogen receptors

The cultured tissue was derived from follicles destined to ovulate within 24 h based on the synchronization protocol used. Therefore, by 72 h, we expected to see changes consistent with the post-ovulatory follicular to luteal transition. Following ovulation, the LH-receptor-bearing theca cells luteinize and become small luteal cells that secrete progesterone in response to LH signaling [28,29]. On the other hand, FSH-receptor-bearing granulosa cells luteinize and become large luteal cells, losing their FSH receptor [30]. Consistent with transition to luteal tissue, we observed an unaltered change in expression of *Lhcgr* after 72 h culture, whereas *Fshr* was undetectable by the assay (Table 6). Interestingly, despite evidence of NGF-induced theca cell proliferation in steroidogenesis, we found that thecal cell *Lhcgr* expression was not affected by NGF treatment ($P = 0.41$; Table 6). Another change commonly found during this transitional period is increased expression of *Esr1* during follicular growth and early luteal phase (d 1 through 4 of estrous cycle) [31]. The results herein described no significant increase in expression of *Esr1* following the 72 h culture period in either treatment group ($P = 0.77$; Table 6).

4.4.3 NGF treatment does not increase angiogenic markers in the bovine pre-ovulatory follicle

In llamas, systemic NGF treatment increased vascularity of the pre-ovulatory follicle [32,33]. While this angiogenic effect was speculated to be modulated through increased LH production, we were interested in evaluating the direct effects of NGF on angiogenic markers in the bovine ovary [24]. In cattle, ovarian function depends on a complex remodeling of the vascular system between ovulation and CL development that involves temporal expression of vascular endothelial growth factor A (VEGFA) and fibroblast growth factor 2 (FGF2) [34]. Another factor crucial to ovulation and CL formation is prostaglandin E₂, which can be stimulated from bovine

theca cells with NGF treatment and is thought to be derived from prostaglandin E synthase (PGES) activity [5,35]. The results herein demonstrated that NGF treatment down-regulated expression of *Fgf2* ($P = 0.02$; Fig. 11B), but did not alter *Pges* or *Vegfa*₁₂₁ expression in bovine follicle tissue ($P \geq 0.44$; Table 6).

4.5 Discussion

The current study evaluated the direct effects of NGF, purified from bull seminal plasma, on bovine pre-ovulatory follicle tissue. In a previous study, theca cells from bovine antral follicles treated with recombinant NGF displayed an increase in androstenedione and progesterone production [5]. Androstenedione is converted to testosterone in the theca cells by the enzyme 17 β -hydroxysteroid dehydrogenase (17 β -HSD) [21]. Consistently, in the current study, *17 β -hsd* expression was upregulated and testosterone production increased in the NGF-treated follicle tissue. Testosterone is produced by theca cells and converted to estradiol in the granulosa cell via the aromatase enzyme (CYP19A1) [21]. Despite the increased production of its testosterone precursor, estradiol concentrations and *Cyp19A1* expression were unaffected by NGF treatment. It was previously reported that NGF stimulated proliferation of theca cells from bovine pre-ovulatory follicles [5]. Therefore, the increase in testosterone production observed herein could be due to an increase in theca cell number, since estradiol production by the granulosa cells was unaffected.

After ovulation, theca cells differentiate into small luteal cells that, in response to LH binding, produce an early rise in progesterone that is essential for supporting initial embryonic growth [36]. Though we found no differences in expression of *Lhr* in the current study, it would be worthwhile to evaluate its expression *in vivo* to determine if there are downstream effects on the presence of small luteal cells in the mature bovine CL. This finding may explain how systemic

administration of NGF can improve CL development and function in cattle [12–14]. Additionally, one study demonstrated that 17 β -HSD-knockout female mice were able to cycle normally, but failed to initiate pseudopregnancy after being mated by sterile males [37], suggesting a critical role of this enzyme in promoting CL formation. Another study observed increasing expression of *Esr1* in the theca interna cells with the growing pre-ovulatory follicle [31]. It was speculated that this upregulation plays an important role in providing feedback to further stimulate theca cell production of androgens [31]. Though the current study did not detect an effect of NGF treatment of *Esr1* expression, further studies should evaluate its *in vivo* effect on the mature bovine CL since it exhibits temporal expression and has been associated with luteal blood flow [24].

Herein, treatment of follicle tissue with NGF did not alter expression of other steroidogenic enzymes or progesterone production, which serves as a precursor for androstenedione production in the pre-ovulatory follicle [21]. This is contradictory to the previous observation of increased theca cell progesterone production in response to NGF treatment [5]. One crucial distinction between studies is that our culture media contained gonadotropins (LH/FSH) in both control and NGF wells attempting to emulate the pre-ovulatory cascade, whereas the previous study treated only the control wells with gonadotropins [5]. Supplementing media with LH and FSH *in vitro* stimulated endogenous NGF secretion from medium to large follicles in ewes [8]. Therefore, the inclusion of gonadotropins in the control medium, but not in the NGF-treated medium, may confound the outcomes of exogenous NGF supplementation since the ovary is capable of endogenous NGF production in response to gonadotropin signaling. It is also worth noting that human chorionic gonadotropin (hCG) was used to treat control samples previously rather than LH [5]. While hCG shares the same receptor with LH, hCG also stimulates different intracellular signaling pathways [38], which could alter its downstream effects. Though studies have

demonstrated increased blood progesterone concentrations in cattle treated parentally with NGF [12–14], these effects were not evident until day 10 of the luteal phase and were likely secondary to the interactions of NGF with the pre-ovulatory follicle. However, infusion of both immature and mature bovine CL with NGF for 30 min using a microdialysis system stimulated progesterone release [39], suggesting a direct luteotrophic role of NGF. Future studies using a whole animal model may be beneficial to determine the putative role of NGF on CL function in cattle throughout the luteal phase to better understand its luteotrophic effects.

Systemic administration of purified llama NGF resulted in greater vascularity of the pre-ovulatory follicle of llamas at 4 h after treatment when compared to GnRH-treated llamas [32]. Previously, thecal cells extracted from bovine pre-ovulatory follicles treated with NGF stimulated prostaglandin E₂ release for up to 8 h [5]. Prostaglandin E₂ is synthesized by PGES and acts as a pro-angiogenic molecule in vascular endothelium by recruiting the paracrine-autocrine mechanism characteristic of endothelium cells, resulting in vascular remodeling [40]. Prostaglandin E₂ also supports luteal progesterone production in cattle [41], potentially through increased CL vascularity [42]. Consistently, one study observed higher *Pges* mRNA and PGES protein levels in the CL of early pregnancy (days 20 to 30) than in the luteal phase (days 8 to 12 of the estrous cycle) or after 40 days gestation of artificially inseminated cows [23]. To our surprise, NGF treatment did not enhance follicular expression of *Pges* in the current study. Interestingly, 17 β -HSD enzymes have been found to play a role in the synthesis of arachidonic acid and its downstream eicosanoid metabolites, such as prostaglandin E₂ [43]. Therefore, it is possible that NGF may induce prostaglandin E₂ synthesis through the observed upregulation of *17 β -hsd* presented herein, but further work is needed to confirm this hypothesis.

Contrary to our hypothesis, there was a downregulation in gene expression of *Fgf2* and no change in gene expression of *Vegfa*₁₂₁ in follicle tissue treated with NGF. Both VEGFA and FGF2 promote vascular supply growth during follicular to luteal transition in the cow ovary, with resulting changes in their localization patterns [44,45]. Follicular *Fgf2* mRNA and FGF2 protein increased around 4 h after GnRH administration in cows, corresponding with the LH surge [46]. Immediately following the LH surge, FGF2 stimulates the migration and proliferation of endothelial cells that help to establish luteal blood flow [34]. During early CL formation, FGF2 concentrations decrease while the capillary beds are reconstructed to establish blood flow [34]. In contrast, VEGFA concentrations remain high throughout ovulation and CL development to support endothelial cell survival [34]. Given that the assays were performed after 72 h in culture, it is possible that the timing to this reconstruction phase was hastened by NGF treatment, which may account for the observed decrease in *Fgf2* expression. Future studies assessing the temporal expression of these angiogenic enzymes are warranted to clarify exactly how NGF could alter the follicular to luteal transition.

In conclusion, the results of the current study demonstrated that NGF, purified from bull seminal plasma, can act directly on the theca and granulosa cells of the bovine pre-ovulatory follicle to stimulate testosterone, but not progesterone or estradiol production. Increased testosterone production appeared to be mediated through upregulation of the steroidogenic enzyme *17 β -hsd*, which may be secondary to theca cell proliferation and could play a role in CL formation. Additionally, decreased *Fgf2* expression in NGF-treated tissue may be due to the hastened onset of tissue remodeling that occurs during early CL development. Further, *in vivo* studies are warranted to determine the effects of NGF within the complex hypothalamic-pituitary-ovarian axis to unravel its putative roles in ovulation and CL formation in cattle.

4.6 Acknowledgments

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4.7 Tables and Figures

Table 5. List of genes and primers used for quantitative real-time PCR

Target genes and abbreviations	NCBI Sequence	Primer	Primer Sequence
Glyceraldehyde-3-phosphate dehydrogenase (<i>Gapdh</i>)	NM_001034034	Forward Reverse	5'-GGCGCCAAGAGGGTCAT-3' 5'-ACGCCCATCACAAACATGG-3'
Ribosomal protein L 15 (<i>Rpl-15</i>)	AY786141	Forward Reverse	5'-TGGAGAGTATTGCGCCTTCTC-3' 5'-CACAAGTTCCACCACACTATTGG-3'
Ribosomal protein L 19 (<i>Rpl-19</i>)	NM_001040515	Forward Reverse	5'-CAGACGATACCTGAATCTAAGAAGA-3' 5'-TGAGAATCCGCTTGTTTTTGAA-3'
Steroidogenic acute regulatory protein (<i>Star</i>)	XR_083945	Forward Reverse	5'-GGATTAACCAGGTTTCGGCG-3' 5'-CTCTCCTTCTTCCAGCCCTC-3'
Side-chain cleavage enzyme (<i>Cyp11A1</i>)	NM_176644	Forward Reverse	5'-GCCACATCGAGAACTTCCAGAAG-3' 5'-CTGGTGTGGAACATCTTGTAGACG-3'
3-beta-hydroxysteroid dehydrogenase (<i>3β-hsd</i>)	NM_174343	Forward Reverse	5'-TGTTGGTGGAGGAGAAGGATCTG-3' 5'-TGGGTACCTTTACATTGACGTTC-3'
17 β-hydroxysteroid dehydrogenase (<i>17β-hsd</i>)	NM_001102365	Forward Reverse	5'-TTGTGCGAGAGTCTGGCGATTCT-3' 5'-AGGAATCGCTCGGTGGTGAAGTA-3'
17α-hydroxylase/17-20 lyase (<i>Cyp17a1</i>)	NM_174304	Forward Reverse	5'-TGTGGCCCCCTACGCTGAT-3' 5'-CGCCAATGCTGGAGTCAAT-3'
Aromatase (<i>Cyp19a1</i>)	NM_174305	Forward Reverse	5'-GTCCGAAGTTGTGCCTATTGCCAGC-3' 5'-CCTCCAGCCTGTCCAGATGCTTGG-3'
Luteinizing hormone receptor (<i>Lhcgr</i>)	NM_174381	Forward Reverse	5'-CAGTCCCCCGCTTTCTCAT-3' 5'-GTAGAGCCCCATGCAGAAGTCT-3'
Follicular stimulating hormone receptor (<i>Fshr</i>)	NM_174061	Forward Reverse	5'-CGACTCTGTCACTGCTCTAACGG-3' 5'-CGTCAATTCCTTTGGCATAGGTGG-3'
Estrogen receptor alpha (<i>Esr1</i>)	NM_001001443	Forward Reverse	5'-AGGGAAGCTCCTATTTGCTCC-3' 5'-CGGTGGATGTGGTCCTTCTCT-3'
Prostaglandin E synthase (<i>Pges</i>)	NM_174443	Forward Reverse	5'-AGGACGCTCAGAGACATGGA-3' 5'-TTCGGTCCGAGGAAAGAGTA-3'
Vascular endothelial growth factor A isoform 121 (<i>Vegfa121</i>)	NM_174216	Forward Reverse	5'-CCGTCCCATTGAGACCCTG-3' 5'-CGGCTTGTCACAATTTTCTTGTC-3'
Fibroblast growth factor 2 (<i>Fgf2</i>)	NM_174056	Forward Reverse	5'-GAACGGGGGCTTCTTCCT-3' 5'-CCCAGTTCGTTTCAGTGCC-3'

Table 6. Fold change in mRNA expression of gonadotropin receptors, angiogenic enzymes, and steroidogenic enzymes in bovine follicle wall tissue preparations treated with 100 ng/mL NGF vs. untreated (Control) for 72 h. Data are presented as mean \pm SEM.

Gene names and abbreviations	Control	NGF	P-value
Steroidogenic acute regulatory protein (<i>Star</i>)	0.17 \pm 0.02	0.29 \pm 0.1	0.34
Side-chain cleavage enzyme (<i>Cyp11a1</i>)	17.81 \pm 9.2	8.67 \pm 5.0	0.40
3-beta-hydroxysteroid dehydrogenase (<i>3β-hsd</i>)	0.67 \pm 0.19	2.55 \pm 1.9	0.60
17 α -hydroxylase/17-20 lyase (<i>Cyp17a1</i>)	0.01 \pm 0.00	0.01 \pm 0.00	0.31
Aromatase (<i>Cyp19a1</i>)	0.02 \pm 0.01	0.01 \pm 0.01	0.53
Luteinizing hormone receptor (<i>Lhcgr</i>)	0.07 \pm 0.01	0.09 \pm 0.03	0.41
Follicular stimulating hormone receptor (<i>Fshr</i>)	Undetected	Undetected	N/A
Estrogen receptor alpha (<i>Esr1</i>)	0.04 \pm 0.01	0.05 \pm 0.02	0.77
Prostaglandin E synthase (<i>Pges</i>)	13.27 \pm 2.0	11.21 \pm 1.4	0.63
Vascular endothelial growth factor A isoform 121 (<i>Vegfa</i> ₁₂₁)	13.99 \pm 1.6	12.71 \pm 1.8	0.44

Figure 9. Graphical depiction of the methodology used to assess the effects of NGF, purified from bull seminal plasma, on the bovine pre-ovulatory follicle.

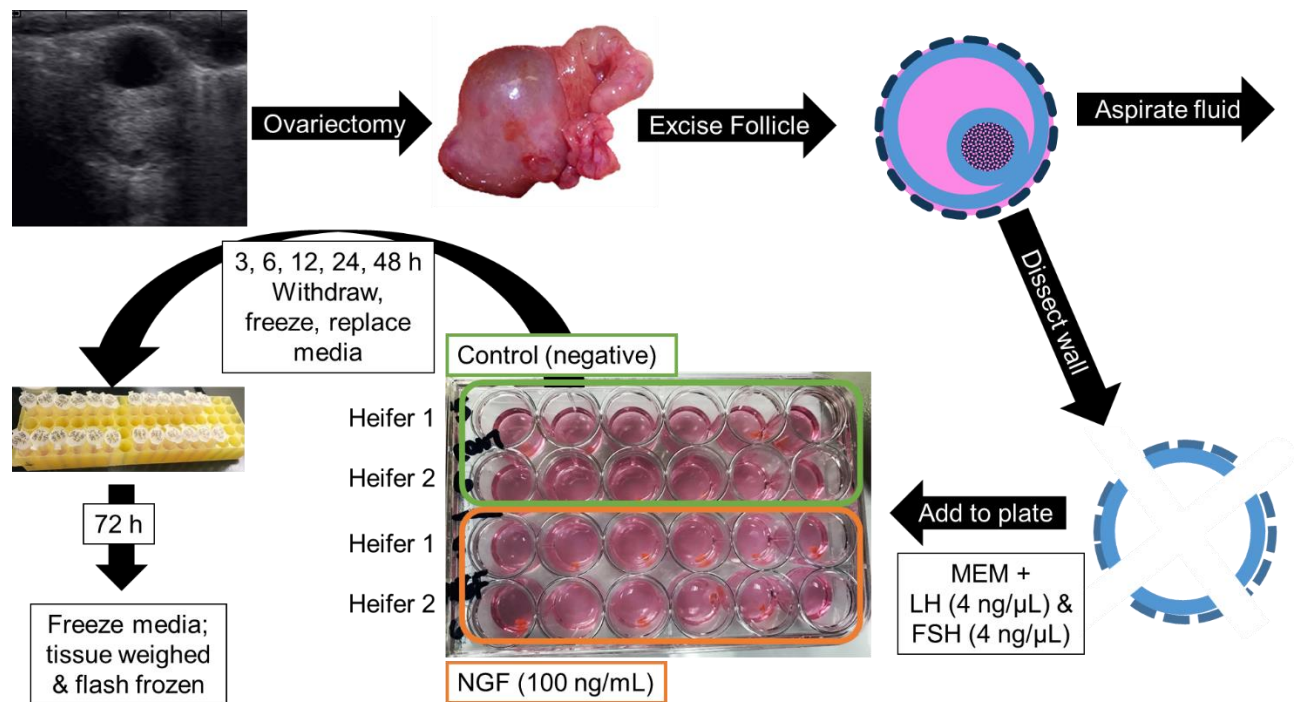


Figure 10. Steroid hormone production in follicle tissue preparations treated with 100 ng/mL NGF vs untreated (control) during 72 h. (A) Progesterone production did not differ between treatment groups ($P > 0.05$). (B) Testosterone production was increased in NGF-treated follicle tissue ($P < 0.05$). (C) Estradiol-17 β production did not differ between treatment groups ($P > 0.05$). Data is presented as mean \pm SEM.

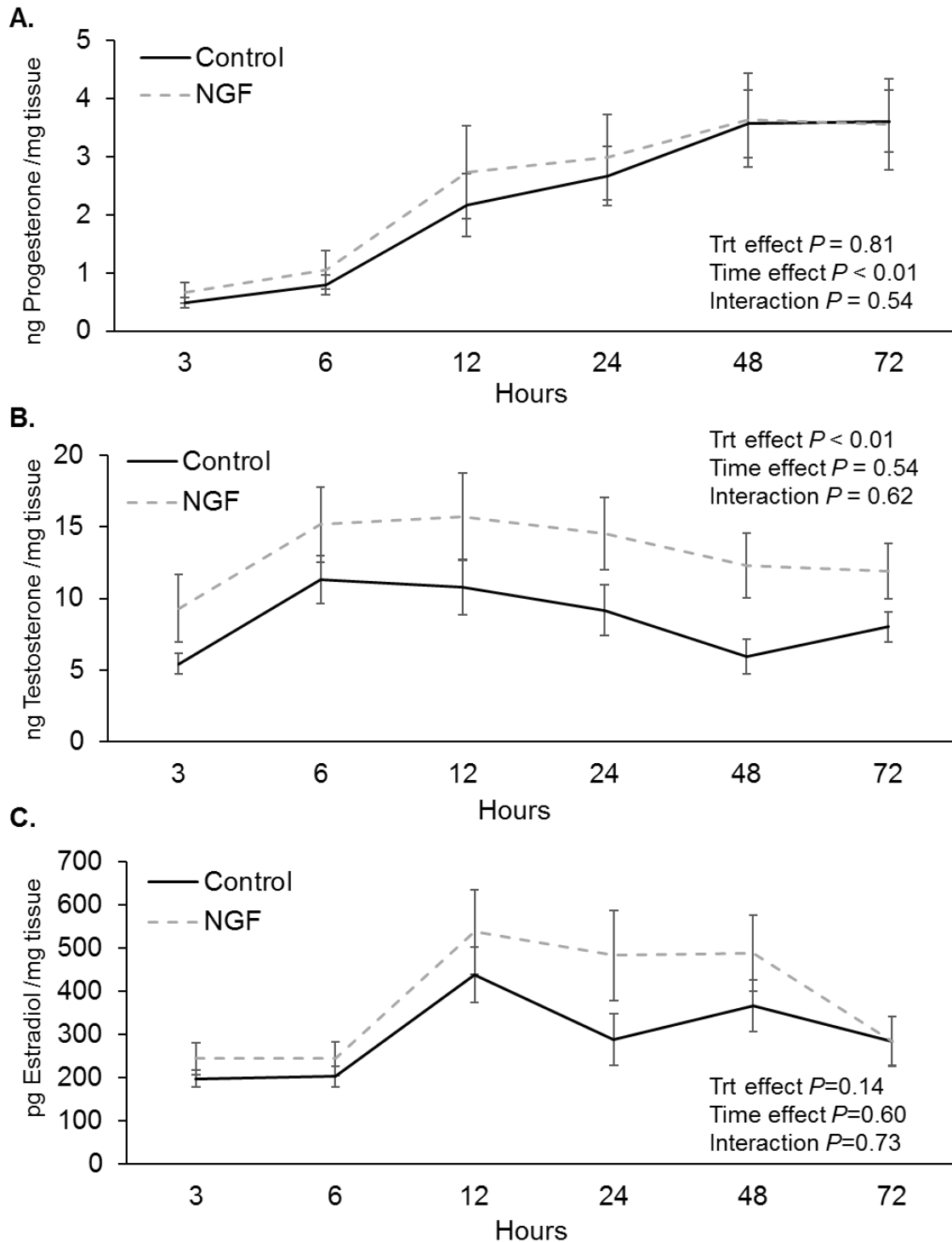
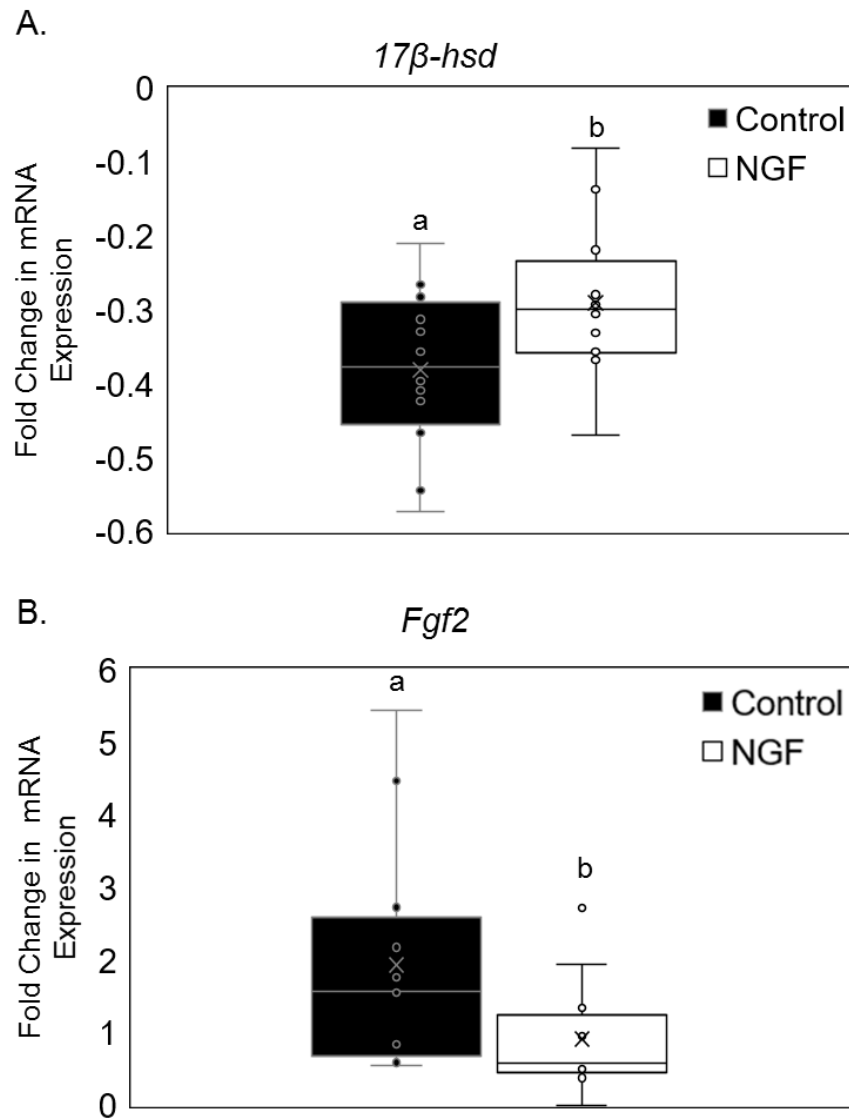


Figure 11. Box-and-whisker plots demonstrating fold change mRNA expression of enzymes in follicle tissue preparations treated with 100 ng/mL NGF vs untreated (control) for 72 h. (A) NGF treatment upregulated expression of steroidogenic enzyme 17 β -hydroxysteroid dehydrogenase (*17 β -hsd*). Transformed data is presented. (B) NGF treatment resulted in a down-regulation of angiogenic enzyme fibroblast growth factor 2 (*Fgf2*). Differing letters denote differences between treated vs. untreated ($P < 0.05$).



4.8 References

- [1] Dissen GA, Romero C, Hirshfield AN, Ojeda SR. Nerve growth factor is required for early follicular development in the mammalian ovary. *Endocrinology* 2001;142:2078–86. doi:10.1210/endo.142.5.8126.
- [2] Dissen GA, Hill DF, Costa ME, Dees W Les, Lara HE, Ojeda SR. A role for TrkA Nerve Growth Factor receptors in mammalian ovulation. *Endocrinology* 1996;137:198–209. doi:10.1210/en.137.1.198.
- [3] Dissen GA, Hirshfield AN, Malamed S, Ojeda SR. Expression of neurotrophins and their receptors in the mammalian ovary is developmentally regulated: changes at the time of folliculogenesis. *Endocrinology* 1995;136:4681–92. doi:10.1210/en.136.10.4681.
- [4] Ojeda SR, Romero C, Tapia V, Dissen GA. Neurotrophic and cell–cell dependent control of early follicular development. *Mol Cell Endocrinol* 2000;163:67–71. doi:10.1016/S0303-7207(99)00242-7.
- [5] Dissen GA, Parrott JA, Skinner MK, Hill DF, Costa ME, Ojeda SR. Direct effects of nerve growth factor on thecal cells from antral ovarian follicles. *Endocrinology* 2000;141:4736–50. doi:10.1210/endo.141.12.7850.
- [6] Tsafiriri A, Lindner HR, Zor U, Lamprecht SA. Physiological role of prostaglandins in the induction of ovulation. *Prostaglandins* 1972;2:1–10. doi:10.1016/0090-6980(72)90024-X.
- [7] Mayerhofer A, Dissen GA, Parrott JA, Hill DF, Mayerhofer D, Garfield RE, et al. Involvement of nerve growth factor in the ovulatory cascade: *trkA* receptor activation inhibits gap junctional communication between thecal cells. *Endocrinology* 1996;137:5662–70. doi:10.1210/endo.137.12.8940397.
- [8] Mattioli M, Barboni B, Gioia L, Lucidi P. Nerve growth factor production in sheep antral follicles. *Domest Anim Endocrinol* 1999;17:361–71. doi:10.1016/S0739-7240(99)00056-9.
- [9] Barboni B, Mattioli M, Gioia L, Turriani M, Capacchietti G, Berardinelli P, et al. Preovulatory rise of NGF in ovine follicular fluid: possible involvement in the control of oocyte maturation. *Microsc Res Tech* 2002;59:516–21. doi:10.1002/jemt.10230.
- [10] Ratto MH, Leduc YA, Valderrama XP, van Straaten KE, Delbaere LTJ, Pierson RA, et al. The nerve of ovulation-inducing factor in semen. *Proc Natl Acad Sci U S A* 2012;109:15042–7. doi:10.1073/pnas.1206273109.
- [11] Kershaw-Young CM, Druart X, Vaughan J, Maxwell WMC. β -Nerve growth factor is a major component of alpaca seminal plasma and induces ovulation in female alpacas. *Reprod Fertil Dev* 2012;24:1093–7. doi:10.1071/RD12039.
- [12] Tanco VM, Van Steelandt MD, Ratto MH, Adams GP. Effect of purified llama ovulation-inducing factor (OIF) on ovarian function in cattle. *Theriogenology* 2012;78:1030–9. doi:10.1016/j.theriogenology.2012.03.036.
- [13] Tribulo P, Bogle OA, Mapletoft RJ, Adams GP. Bioactivity of ovulation inducing factor (or nerve growth factor) in bovine seminal plasma and its effects on ovarian function in cattle. *Theriogenology* 2015;83:1394–401. doi:10.1016/j.theriogenology.2014.12.014.
- [14] Stewart JL, Mercadante VRG, Dias NW, Canisso IF, Yau P, Imai B, et al. Nerve Growth

- Factor-Beta, purified from bull seminal plasma, enhances corpus luteum formation and conceptus development in *Bos taurus* cows. *Theriogenology* 2018;106:30–8. doi:10.1016/j.theriogenology.2017.10.007.
- [15] Berland MA, Ulloa-Leal C, Barria M, Wright H, Dissen GA, Silva ME, et al. Seminal plasma induces ovulation in llamas in the absence of a copulatory stimulus: role of nerve growth factor as an ovulation-inducing factor. *Endocrinology* 2016;157:3224–32. doi:10.1210/en.2016-1310.
 - [16] Stewart JL, Canisso IF, Ellerbrock RE, Mercadante VRG, Lima FS. Nerve Growth Factor- β production in the bull: Gene expression, immunolocalization, seminal plasma constitution, and association with sire conception rates. *Anim Reprod Sci* 2018;197:335–42. doi:10.1016/j.anireprosci.2018.09.006.
 - [17] Knickerbocker JJ, Thatcher WW, Foster DB, Wolfenson D, Bartol FF, Caton D. Uterine prostaglandin and blood flow responses to estradiol-17 β in cyclic cattle. *Prostaglandins* 1986;31:757–76. doi:10.1016/0090-6980(86)90179-6.
 - [18] Krzymowski T, Stefańczyk-Krzymowska S, Koziorowski M. Counter current transfer of PGF2 alpha in the mesometrial vessels as a mechanism for prevention of luteal regression in early pregnancy. *Acta Physiol Pol* 1989;40:23–34.
 - [19] Schjenken JE, Robertson SA. Seminal fluid signalling in the female reproductive tract: Implications for reproductive success and offspring health. In: Bronson R., editor. *Male Role Pregnancy Loss Embryo Implant. Fail. Adv. Exp. Med. Biol.* vol 868, Springer, Cham; 2015, p. 127–58. doi:10.1007/978-3-319-18881-2_6.
 - [20] Carrasco R, Singh J, Adams GP. The dynamics of *trkA* expression in the bovine ovary are associated with a luteotrophic effect of ovulation-inducing factor/nerve growth factor (OIF/NGF). *Reprod Biol Endocrinol* 2016;14:47. doi:10.1186/s12958-016-0182-9.
 - [21] Fortune JE. Bovine theca and granulosa cells interact to promote androgen production. *Biol Reprod* 1986;35:292–9. doi:10.1095/biolreprod35.2.292.
 - [22] Komar CM, Berndtson AK, Evans ACO, Fortune JE. Decline in circulating estradiol during the periovulatory period is correlated with decreases in estradiol and androgen, and in messenger RNA for p450 aromatase and p450 17 α -hydroxylase, in bovine preovulatory follicles. *Biol Reprod* 2001;64:1797–805. doi:10.1095/biolreprod64.6.1797.
 - [23] Sakumoto R, Hayashi KG, Takahashi T. Different expression of PGE synthase, PGF receptor, TNF, Fas and oxytocin in the bovine corpus luteum of the estrous cycle and pregnancy. *Reprod Biol* 2014;14:115–21. doi:10.1016/j.repbio.2013.12.003.
 - [24] Lüttgenau J, Ulbrich SE, Beindorff N, Honnens A, Herzog K, Bollwein H. Plasma progesterone concentrations in the mid-luteal phase are dependent on luteal size, but independent of luteal blood flow and gene expression in lactating dairy cows. *Anim Reprod Sci* 2011;125:20–9. doi:10.1016/j.anireprosci.2011.02.002.
 - [25] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta$ Ct method. *Methods* 2001;25:402–8. doi:10.1006/METH.2001.1262.
 - [26] Hillier SG, Van Den Boogaard AMJ, Reichert LE, Van Hall E V. Intraovarian sex steroid hormone interactions and the regulation of follicular maturation: Aromatization of

- androgens by human granulosa cells in vitro. *J Clin Endocrinol Metab* 1980;50:640–7. doi:10.1210/jcem-50-4-640.
- [27] Fortune JE, Quirk SM. Regulation of steroidogenesis in bovine preovulatory follicles. *J Anim Sci* 1988;66:1–8. doi:10.1093/ansci/66.suppl_2.1.
- [28] Mamluk R, Wolfenson D, Meidan R. LH receptor mRNA and cytochrome P450 side-chain cleavage expression in bovine theca and granulosa cells luteinized by LH or forskolin. *Domest Anim Endocrinol* 1998;15:103–14. doi:10.1016/S0739-7240(97)00085-4.
- [29] Mamluk R, Chen D, Greber Y, Davis JS, Meidan R. Characterization of messenger ribonucleic acid expression for prostaglandin F2 α and luteinizing hormone receptors in various bovine luteal cell types. *Biol Reprod* 1998;58:849–56. doi:10.1095/biolreprod58.3.849.
- [30] Rajapaksha WRAKJS, Robertson L, O'Shaughnessy PJ. Expression of follicle-stimulating hormone-receptor mRNA alternate transcripts in bovine granulosa cells during luteinization in vivo and in vitro. *Mol Cell Endocrinol* 1996;120:25–30. doi:10.1016/0303-7207(96)03816-6.
- [31] Berisha B, Pfaffl MW, Schams D. Expression of estrogen and progesterone receptors in the bovine ovary during estrous cycle and pregnancy. *Endocrine* 2002;17:207–14. doi:10.1385/endo:17:3:207.
- [32] Ulloa-Leal C, Bogle OA, Adams GP, Ratto MH. Luteotrophic effect of ovulation-inducing factor/nerve growth factor present in the seminal plasma of llamas. *Theriogenology* 2014;81:1101–1107.e1. doi:10.1016/j.theriogenology.2014.01.038.
- [33] Silva M, Ulloa-Leal C, Valderrama XP, Bogle OA, Adams GP, Ratto MH. Nerve growth factor from seminal plasma origin (sp β -NGF) increases CL vascularization and level of mRNA expression of steroidogenic enzymes during the early stage of corpus luteum development in llamas. *Theriogenology* 2017;103:69–75. doi:10.1016/j.theriogenology.2017.07.041.
- [34] Robinson RS, Woad KJ, Hammond AJ, Laird M, Hunter MG, Mann GE. Angiogenesis and vascular function in the ovary. *Reproduction* 2009;138:869–81. doi:10.1530/REP-09-0283.
- [35] Nuttinck F, Marquant-Le Guienne B, Clément L, Reinaud P, Charpigny G, Grimard B. Expression of genes involved in prostaglandin E2 and progesterone production in bovine cumulus-oocyte complexes during in vitro maturation and fertilization. *Reproduction* 2008;135:593–603. doi:10.1530/REP-07-0453.
- [36] Harrison LM, Kenny N, Niswender GD. Progesterone production, LH receptors, and oxytocin secretion by ovine luteal cell types on days 6, 10 and 15 of the oestrus cycle and day 25 of pregnancy. *J Reprod Fert* 1987;79:539–48. doi:10.1530/jrf.0.0790539.
- [37] Hakkarainen J, Jokela H, Pakarinen P, Heikelä H, Kätänkänaho L, Vandenput L, et al. Hydroxysteroid (17 β)-dehydrogenase 1-deficient female mice present with normal puberty onset but are severely subfertile due to a defect in luteinization and progesterone production. *FASEB J* 2015;29:3806–16. doi:10.1096/fj.14-269035.
- [38] Casarini L, Lispi M, Longobardi S, Milosa F, La Marca A, Tagliasacchi D, et al. LH and

- hCG action on the same receptor results in quantitatively and qualitatively different intracellular signalling. PLoS One 2012;7:e46682. doi:10.1371/journal.pone.0046682.
- [39] Miyamoto A, Okuda K, Schweigert FJ, Schams D. Effects of basic fibroblast growth factor, transforming growth factor- β and nerve growth factor on the secretory function of the bovine corpus luteum *in vitro*. J Endocrinol 1992;135:103–14. doi:10.1677/joe.0.1350103.
 - [40] Finetti F, Solito R, Morbidelli L, Giachetti A, Ziche M, Donnini S. Prostaglandin E2 regulates angiogenesis via activation of fibroblast growth factor receptor-1. J Biol Chem 2008;283:2139–46. doi:10.1074/jbc.M703090200.
 - [41] Miyamoto A, Lützow H v., Schams D. Acute actions of prostaglandin F2 α , E2, and 12 in microdialyzed bovine corpus luteum *in vitro*. Biol Reprod 1993;49:423–30. doi:10.1095/biolreprod49.2.423.
 - [42] Herzog K, Brockhan-Lüdemann M, Kaske M, Beindorff N, Paul V, Niemann H, et al. Luteal blood flow is a more appropriate indicator for luteal function during the bovine estrous cycle than luteal size. Theriogenology 2010;73:691–7. doi:10.1016/j.theriogenology.2009.11.016.
 - [43] Kemiläinen H, Adam M, Mäki-Jouppila J, Damdimopoulou P, Damdimopoulos AE, Kere J, et al. The hydroxysteroid (17 β) dehydrogenase family gene HSD17B12 is involved in the prostaglandin synthesis pathway, the ovarian function, and regulation of fertility. Endocrinology 2016;157:3719–30. doi:10.1210/en.2016-1252.
 - [44] Laird M, Woad KJ, Hunter MG, Mann GE, Robinson RS. Fibroblast growth factor 2 induces the precocious development of endothelial cell networks in bovine luteinising follicular cells. Reprod Fertil Dev 2013;25:372. doi:10.1071/RD12182.
 - [45] Tamanini C, De Ambrogi M. Angiogenesis in developing follicle and corpus luteum. Reprod Domest Anim 2004;39:206–16. doi:10.1111/j.1439-0531.2004.00505.x.
 - [46] Berisha B, Steffl M, Amselgruber W, Schams D. Changes in fibroblast growth factor 2 and its receptors in bovine follicles before and after GnRH application and after ovulation. Reproduction 2006;131:319–29. doi:10.1530/rep.1.00798.

Chapter 5:

Effects of Nerve Growth Factor- β on Peri-Ovulatory Dynamics and Corpus Luteum Formation in Heifers

5.1 Abstract

Nerve growth factor- β (NGF) is a seminal plasma protein that stimulates bovine theca cell proliferation and steroidogenesis *in vitro* and is luteotrophic *in vivo*, yet there is little information on how it alters the complex dynamics of the hypothalamic-pituitary-ovarian axis throughout ovulation in cattle. The objectives of this study were to evaluate the effects of systemically administered purified bovine NGF on vascularity and steroidogenic function of the pre-ovulatory follicle (POF) and subsequent corpus luteum (CL) in cattle. Heifers ($n = 12$) were synchronized using a GnRH-prostaglandin and intravaginal progesterone protocol (5-d CIDR-Synch) and randomly allocated to 1 of 2 treatment groups: (1) Control- 12 mL phosphate-buffered saline; or (2) NGF- 250 μ g purified NGF in 12 mL phosphate-buffered saline administered intramuscularly at day 0. Using a crossover design, a second replicate was performed using at approximately 1 month after completion of the first replicate. Transrectal ultrasonography and blood collections were performed every 4 h from 0 to 32 h to evaluate POF size and vascularity, time to ovulation, and serum estradiol-17 β concentrations. Transrectal ultrasonography was performed daily to assess CL size and vascularity, and blood was obtained every 2 d to measure serum progesterone concentrations. On days 9 and 14, a CL biopsy was performed on a subset of heifers to assess steroidogenic and angiogenic gene expressions and measure the ratio of small to large luteal cells. There was a main effect of NGF treatment on POF diameter ($P = 0.02$), but no effect on serum estradiol-17 β levels ($P = 0.95$) or time to ovulation ($P = 0.42$). Treatment with NGF tended to increase CL diameter ($P = 0.10$) and increased serum progesterone levels from days 10 to 12 ($P =$

0.04). Consistently, there was a higher percentage of small luteal cells in the CL of NGF heifers ($P < 0.01$) and increased gene expression of steroidogenic acute regulatory protein ($P = 0.04$), 3- β -hydroxysteroid dehydrogenase ($P = 0.05$), and luteinizing hormone receptor ($P = 0.09$). There were no differences in vascularity of the POF or CL ($P \geq 0.16$), with decreased expression of the angiogenic enzyme PGES in the CL of NGF versus control heifers ($P = 0.03$). This study concluded that systemic administration of purified NGF to heifers has a luteotrophic affect attributed to increased small luteal cell number and steroidogenic enzyme activity within the CL.

5.2 Introduction

Nerve growth factor- β (NGF) is a seminal plasma protein found in a variety of species, such as camelids and bulls, and is positively associated with bull fertility [1,2]. It has been reported that NGF functions within the female reproductive tract to exert a luteotrophic effect in both cattle [3–5] and camelids [6–8]. Dose-dependent luteotrophic effects of NGF in camelids have been attributed to prolonged luteinizing hormone (LH) secretion [6], but this mechanism has yet to be observed in cattle. Though NGF stimulated LH release in bovine pituitary cultures [9], no change in LH pulsatility was detected in pre-pubertal heifers treated with purified llama NGF [3]. In mature heifers, NGF treatment was associated with a rise in circulating follicle stimulating hormone (FSH) concentrations, demonstrating potential interactions at the level of the anterior pituitary [3]. *In vitro* NGF treatment of theca cells from bovine pre-ovulatory follicles (POF) induced cell proliferation and stimulated prostaglandin E₂ (PGE), androgen, and progesterone release [10]. Through the two-cell, two-gonadotropin theory, it seems likely that increased androgen production from bovine theca cells could stimulate increased granulosa cell estradiol production [11]. Estradiol signaling within the hypothalamus stimulates gonadotropin secretion

from the anterior pituitary, promoting further growth of the dominant follicle and, eventually, ovulation [12]. In the human POF, NGF stimulated estradiol secretion directly and through upregulation of ovarian FSH receptors [13]. There is, therefore, a need to evaluate the effects of NGF within this complex hypothalamic-pituitary-gonadal axis to determine its role in ovulatory dynamics of cattle.

Llamas treated with NGF experienced an increase in vascularity of both the POF and subsequent corpus luteum (CL) [14,15]. Increased PGE production from the POF, stimulated by NGF treatment, could play a role in increasing vascularity within the ovary [16]. Vascularity of the CL is significantly correlated with progesterone production in the early luteal phase of cattle [17] and could account for observations of improved luteal function [3–5]. Alternatively, NGF-induced theca cell proliferation could increase the number of small luteal cells (SLC) present, which have the ability to secrete high quantities of progesterone when stimulated by LH signaling [18]. Though the proportion of SLC and large luteal cells (LLC) did not differ between NGF-treated and control llamas, there was an upregulation in expression of steroidogenic enzymes CYP11A1 and StAR [15], consistent with improved steroidogenic function. However, the luteotrophic mechanism of NGF in cattle, a spontaneously ovulating species, has yet to be assessed. Therefore, the objectives of the current study were to evaluate the effects of systemically administered purified bovine NGF on vascularity and steroidogenic function of the POF and subsequent CL in heifers.

5.3 Materials and Methods

5.3.1 Estrus synchronization and NGF treatment

Cycling, Holstein heifers at 12 to 14 months of age with no history of reproductive or health disorder were eligible for enrollment in the study. The study was performed in two replicates using a cross-over design, with the second replicate beginning approximately 1 month after completion of the first replicate. Enrolled heifers (n = 12 per replicate) received an intravaginal progesterone-releasing device (1.38 g progesterone; Eazi-Breed CIDR, Zoetis, Parsippany-Troy Hills, NJ) and 2 mL (100 µg) of gonadotropin releasing hormone (GnRH) agonist (Factrel®, Zoetis) intramuscularly. The CIDR was removed 5 days later, and heifers were given 5 mL of prostaglandin F2α (PGF) analog (25 mg dinoprost tromethamine, Lutalyse, Zoetis) intramuscularly at CIDR removal and again 24 hours later. Transrectal ultrasonography (Ibex Evo, EI Medical Imaging, Loveland, CO) was performed at 48 hours after the second PGF injection to ensure that a follicle, but no CL, was present. At this time, a second dose of 100 µg GnRH agonist was administered, and heifers were randomly assigned to one of two treatments: (1) NGF (n = 6 per replicate) – intramuscular administration of 250 µg purified bovine NGF reconstituted in 12 mL phosphate-buffered saline (pH 7.4); or (2) Control – intramuscular administration of 12 mL phosphate-buffered saline (n = 6 per replicate). The NGF used was purified from bovine seminal plasma and used previously for an *in vivo* study in cows [4]. Time 0 was defined as the time of treatment.

5.3.2 Determination of hormone concentrations

Blood samples were collected from the coccygeal or jugular veins throughout the study. At -1 h, blood was collected from all heifers for pre-treatment quantification of serum estradiol-17 β and progesterone, and then every 4 h for measuring estradiol-17 β concentrations until 32 h. Following ovulation, blood was collected every 2 days until day 14 for serum progesterone concentration analyses. Hormones were analyzed using chemiluminescence assay (Immulin 2000 Xpi platform; Siemens Medical Solutions USA, Inc.). Intra-assay coefficient of variations were 2.4% (progesterone) and 3.1% (estradiol-17 β). Inter-assay coefficient of variations were 19% (progesterone) and 15% (estradiol-17 β). The progesterone assay had a detection range of 0.2 to 40 ng/mL and a sensitivity of 0.1 ng/mL. The estradiol-17 β assay had a detection range of 20-2000 pg/mL and a sensitivity of 15 pg/mL.

5.3.3 Ultrasonographic examination of ovarian structures

The ovaries of each heifer were examined by transrectal ultrasonography equipped with a 7.5 MHz linear array-transducer and Doppler (Ibex Evo, EI Medical Imaging, Loveland, CO) immediately prior to treatment and every 4 h up to 32 h after treatment to measure the POF diameter and vascularity (Fig. 12A) and determine time to ovulation [14]. After ovulation, ultrasonography was performed daily to measure CL size and vascularity (Fig. 12B). Cineloops (8 seconds in length) of the POF/CL were recorded during power Doppler imaging and downloaded into VLC media player (www.videolan.org, Boston, MA, USA). Cineloops was examined frame-by-frame to select three images that represent the maximum vascular signal near the maximum cross-sectional area of the POF/CL as previously described [14]. Images were saved in JPG format with minimal compression and analyzed using ImageJ software (National Institute of Health,

Bethesda, MD, USA) to determine the degree of POF/CL vascularization. The degree of vascularization was estimated by measuring the area (cm²) of the vascular flow signals (power Doppler) overlaying the B-mode image of the POF/CL. The average of the three images was used to represent the value of each heifer at each time point.

5.3.4 Corpus luteum biopsy

A transvaginal ultrasound-guided biopsy of the CL was performed on a subset of heifers on days 9 (n = 6 per treatment) and 14 (n = 6 per treatment). The technique for biopsy has been described and validated for use on bovine CL previously [19–21]. Repeated biopsies did not affect CL size or function (progesterone production), and thus, these parameters can continue to be measured [19]. However, the effects of serial biopsies on CL vascularity have not been evaluated, therefore CL vascularity was not assessed in heifers after biopsies were performed. A MC 8.0MHz transducer was used for the transvaginal placement of the biopsy needle (Fig. 13A; EI Medical Imaging). The biopsy needle was 60 cm long, with a specimen notch (20 × 1 × 0.5 mm) covered by an 18-gauge cutting cannula triggered with an automated spring-loaded device (Fig. 13B; Cook Medical, Bloomington, IN).

Caudal epidural anesthesia was performed using 5 mL of lidocaine hydrochloride injectable 2%. The perineal area of each heifer was scrubbed with dilute betadine and alcohol. The transvaginal probe was inserted such that the transducer faced the wall of the vaginal fornix. The biopsy needle was fed into the needle channel of the probe, and the ovary was positioned transrectally against the vaginal wall. When the ovary was in position against the vaginal wall and transducer face, the inner needle was advanced into the CL (Fig. 13C). Once the needle was visualized on the ultrasound and determined to be in the correct position, the spring-locked cutting

cannula was fired, cutting off a section of tissue within the specimen notch and enclosing it within the covering of the outer cannula. The set was withdrawn and the tissue specimen was visually evaluated for quality. This procedure was repeated once within each heifer to obtain a total of two tissue specimens. One specimen was fixed in 10% formalin solution and the other was flash-frozen in liquid nitrogen for mRNA isolation and quantitative PCR analysis.

5.3.5 Histological analyses of CL tissue

Formalin-fixed CL sections from each heifer were embedded in paraffin, sectioned at a thickness of 3 μm , and mounted on several poly-L-lysine coated glass slides. These sections were stained with hematoxylin and eosin for examination under a light microscope for determining luteal cell number as previously described [15]. Large (LLC) and small luteal cells (SLC) were identified by their respective sizes of $< 20 \mu\text{m}$ and $> 35 \mu\text{m}$ [22] and counted in 10 fields/slide/animal at 400 \times for each time point. Measurements were analyzed using ImageJ software (National Institute of Health, Maryland, and Washington D.C., USA). Percentage of SLC were calculated as the percentage SLC over total cells counted (SLC + LLC).

5.3.6 Quantitative real-time PCR analyses of CL tissue

Luteal tissue mRNA expression was determined for luteinizing hormone/choriogonadotropin receptor (*Lhcgr*), oxytocin (*Ot*), prostaglandin E synthase (*Pges*), prostaglandin E receptor (*Pger*), vascular endothelial growth factor A isoform 121 (*Vegfa₁₂₁*), vascular endothelial growth factor receptor 2 (*Vegfr2*), fibroblast growth factor 2 (*Fgf2*), estrogen receptor alpha (*Esr1*), steroidogenic acute regulatory protein (*Star*), side-chain cleavage enzyme (*Cyp11a1*), 17 α -hydroxylase/17-20 lyase (*Cyp17a1*), aromatase (*Cyp19a1*), 3 beta-hydroxysteroid

dehydrogenase (3β -*hsd*), and 17 β -hydroxysteroid dehydrogenase (17β -*hsd*). Primer sequences are summarized in Table 7 [21,23].

Tissue lysis and RNA extraction were conducted according to the manufacturer's recommendations (PureLink RNA Mini Kit, Invitrogen, Carlsbad, CA). Isolated RNA was evaluated for concentration and purity using a NanoDrop One Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). A maximum of 2 μ g of mRNA was used to synthesize complementary DNA using a commercial kit (High-capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA). Complementary DNA was used for quantitative real-time reverse transcription PCR using a 7500 Real-Time PCR Detection System (Applied Biosciences) with PowerUpTM SYBRTM Green Master Mix (2X; Applied Biosciences.). Primers were designed for the constitutively expressed mRNAs, glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and ubiquitin (*Ubq*), with the expression value of each gene was normalized to mean of these genes. The expression value of each gene in a WT control was arbitrarily defined as 1 U. All assays were carried out in triplicate for each target mRNA.

5.3.7 Statistical analyses

Data are presented as percentage mean \pm SEM. All statistical analyses were performed using R Version 3.4.3 (<https://www.r-project.org/>). Normality was confirmed using a Shapiro-Wilk test of the residuals. Non-normal data was transformed using Tukey's Ladder of Powers. If transformation did not result in a normalized population, a Kruskal-Wallis rank sum test was performed. Analysis of variance was applied to data using a general linear mixed model with repeated measures using heifer ID as a random variable. The covariance structure that resulted in

the smallest Bayesian information criterion was selected from the mixed models. Significance was declared at $P \leq 0.05$ whereas tendency was discussed at $0.05 < P < 0.10$.

5.4 Results

5.4.1 Pre-ovulatory effects of NGF treatment

There was no difference in time to ovulation between NGF (13.78 ± 3 h) and control heifers (17.45 ± 3 h; $P = 0.42$). There was a main effect of NGF treatment on the size of the pre-ovulatory follicle ($P \leq 0.02$), with a tendency towards a treatment by time interaction ($P = 0.10$; Fig. 14A). Treatment with NGF did not affect follicle vascularity ($P = 0.16$; Fig. 14B) or serum estradiol-17 β concentrations ($P = 0.95$; Fig. 14C).

5.4.2 Post-ovulatory effects of NGF treatment

As expected, CL diameter increased over time ($P < 0.01$), with a tendency be higher in NGF over control heifers ($P = 0.10$; Fig. 15A). There was no treatment or treatment by time interaction on CL vascularity ($P = 0.20$; Fig. 15B). Serum progesterone concentrations increased over time in both groups ($P < 0.01$; Fig. 15C). There was an interaction of treatment by time, with higher serum progesterone concentrations observed in NGF heifers compared to control heifers from days 10 through 12 ($P = 0.04$; Fig. 15C). Percentage of small luteal cells was increased in the CL of NGF heifers when compared with control heifers ($P < 0.01$; Fig. 16).

There was a tendency for increased *Lhcgr* expression in the CL of NGF heifers compared to control heifers ($P = 0.09$; Fig. 17A), but no differences in *Ot* expression ($P=0.15$; Fig. 17B). Gene expression of steroidogenic enzymes *Star* and *3 β -hsd* were also upregulated in the CL of

NGF heifers ($P \leq 0.05$; Fig. 18A and C). There was a tendency for *17 β -hsd* to be decreased on day 9 and increased on day 14 in NGF heifers compared to control heifers ($P = 0.06$; Fig. 18D). There was no effect of NGF treatment on expression of steroidogenic enzymes *Cyp11a1* ($P = 0.12$; Fig. 18B) or *Cyp17a1* ($P = 0.28$; Table 8). Gene expression of angiogenic enzyme *Pges* was decreased in NGF heifers ($P = 0.03$), with a tendency for decreased gene expression of its receptor, *Pger* ($P = 0.07$; Table 8). There was no effect of NGF treatment on gene expression of angiogenic enzymes *Esr1* or *Fgf2* ($P \geq 0.41$; Table 8). Gene expression of *Vegfa₁₂₁*, *Vegfr2*, and *Cyp19a1* were undetectable in both treatment groups (Table 8).

5.5 Discussion

The current study aimed to evaluate the effects of systemically administered NGF on peri- and post-ovulatory dynamics in heifers. The luteotrophic effect of NGF observed in the current study is consistent with previous findings in cattle [3–5]. In one study, purified llama NGF was administered systemically to mature Hereford crossbred heifers with a dominant follicle size of ~12 to 14 mm [3]. Similar to the current study, plasma progesterone concentrations were increased on day 11 after ovulation in NGF-treated heifers compared to untreated control heifers [3]. Interestingly, plasma progesterone concentrations did not differ between NGF and GnRH treated heifers in this previous study [3]. In the current study, GnRH was given to both control and NGF-treated heifers to maintain a routine synchronization schedule and evaluate the effects of NGF specifically. These differences suggest that there may be a synergistic mechanism between GnRH and NGF signaling that promotes CL formation following ovulation in cattle. Pre-treatment with a GnRH antagonist (cetorelix) blocked the ability of both GnRH and NGF to invoke the pre-ovulatory LH surge in llamas [24]. The authors of this study suggested that NGF may act directly

at the level of the hypothalamus, yet only a low proportion of GnRH neurons (2.5%) in llamas have been found to contain the NGF receptor, TrkA [25]. Based on these findings, it has been suggested that NGF may interact with GnRH neurons through an intermediate cell type, such as kisspeptin [25,26]. Given previous studies in cattle that have demonstrated upregulated ovarian theca cell *Trka* mRNA expression in response to LH treatment *in vitro* [10] and increased TrkA protein in the developing CL *in vivo* [27], it seems more likely that GnRH-induced LH secretion may act directly on the ovary to enhance NGF signaling in cattle. Additionally, endogenous follicular NGF production increased following the gonadotropin surge in ewes [28], suggesting another potential synergistic role of NGF introduced from seminal plasma at copulation. Further work is needed in spontaneously ovulating species, such as ruminants, to better discern these complex interactions between GnRH and NGF signaling within the hypothalamic-pituitary-ovarian axis.

A previous study by our research group reported increased serum progesterone concentrations beginning around day 10 in cows treated with purified bovine NGF [4], consistent with findings in the current study. Increased progesterone in NGF-treated animals persisted to days 14 and 19 previously [4], whereas it only persisted through day 12 in the current study. However, the previous study used beef cows instead of dairy heifers, and artificial insemination was performed concurrently [4]. In that study, enhanced conceptus development was detected in pregnant NGF-treated cows, which was attributed to the role of progesterone on uterine histotroph production [29–31]. The timing of increased progesterone production may play a role in promoting embryonic growth and maternal recognition of pregnancy in cattle. Supplementing progesterone with a controlled internal drug release device from days 4 to 7 and 4 to 10 post-estrus in heifers with experimentally induced subluteal function resulted in significantly greater embryo length

compared to untreated sublethal control heifers [32]. On the contrary, supplementing progesterone from days 7 to 10 did not increase embryo length [32]. These findings suggest that there is a critical period between 4 and 7 days gestation in which increased progesterone may influence embryonic growth, yet NGF-induced luteotrophic effects are not apparent systemically until day 10. It is possible that either there are undetectable changes in local progesterone production earlier or that NGF can act directly on the ovulated oocyte. Incubation of sheep oocytes with NGF during *in vitro* fertilization induced early cleavage and improved embryo development [33]. More work is needed to evaluate the effects of NGF on uterine histotroph production, oocyte maturation, and *in vitro* fertilization in cattle to better distinguish the direct and indirect effects of NGF on conception.

In llamas, administration of purified NGF exerted its luteotrophic effect by changing the pattern of LH release and enhancing POF vascularity at 4 hours after treatment [14]. Contradictory to this study, we found no difference in POF vascularity of NGF-treated heifers. Treatment of bovine theca cells *in vitro* with recombinant NGF increased androstenedione production [10], which is a precursor for estradiol synthesis. Follicular estradiol production regulates the hypothalamic events that lead to ovulation [34] and modulates the stimulatory effect of NGF on pituitary LH secretion in llamas [35]. Therefore, we expected to see increased systemic estradiol concentrations and a hastened onset of ovulation in NGF-treated heifers. On the contrary, we found that NGF treatment did not affect serum estradiol concentrations. Interestingly, there was a treatment effect on follicle size, suggesting an alternative interaction between NGF and the POF, potentially through increased PGE synthesis and antrum formation [10]. While the mean time to ovulation was less in NGF-treated heifers (~14 h) compared to control heifers (~17 h), there was a large variation in both groups that rendered this finding not significant. Though improved synchrony was detected in cows administered seminal plasma systemically [5], the findings from

the current study suggest that other factors may be involved. However, future studies with a larger sample size would be necessary to better determine the significance of this finding.

Similarly, we found no changes in CL vascularity between NGF and control heifers. Gene expression of *Fgf2* and *Esr1* in the CL were previously associated with luteal blood flow in cattle [21] and did not differ between treatment groups in this study. Interestingly, gene expression of *Vegfa*₁₂₁ and *Vegfr2* in the CL were also associated with luteal blood flow in cattle previously [21], but were undetectable in our study. Highest expression of these genes in the bovine CL were detected during early luteal phase (days 1 to 4), followed by a significant decrease during mid to late luteal phases [36,37]. It is possible that these transcripts decrease much more significantly in heifers than in cows, rendering them undetectable in the current study. Another unexpected finding was downregulated *Pges* gene expression in the CL of NGF versus control heifers. Expression of *Pges* in the CL is stimulated by interferon tau and upregulated in the CL of early pregnancy when compared to non-pregnant animals [23,38]. Interestingly, prostaglandin E synthase is localized exclusively in LLC and is activated during luteal maintenance, which explains its role in early pregnancy [39]. The observed decrease in *Pges* expression could potentially be secondary to increased SLC number and function in NGF heifers, but follow-up studies are necessary to understand the relevance of these findings.

In addition to increasing CL vascularity, NGF treatment enhanced steroidogenesis through upregulated *Cyp11a1* and *Star* transcripts in the CL of llamas [15]. While we did not detect any differences in the gene expression of *Cyp11a1*, whose enzyme facilitates the conversion of cholesterol to pregnenolone [40], we did observe an upregulation of *Star* gene expression in the CL of NGF heifers. The steroidogenic acute regulatory protein functions to translocate cholesterol

from the outer to the inner mitochondrial membrane and is the rate-limiting step in steroid hormone production [41]. There was also an upregulation in the gene expression of *3 β -hsd*, whose enzyme converts pregnenolone to progesterone [40] and is associated with increased progesterone secretion from luteal tissue [42]. Gene expression of *17 β -hsd*, whose enzyme converts androstenedione to testosterone and estrone to estradiol, tended to be down-regulated on day 9 and upregulated on day 14 in the CL of NGF heifers. While *17 β -hsd* gene expression was relatively low overall, the initial decrease may be secondary to improved progesterone productivity, whereas the subsequent increase may be related to onset of prostaglandin production and luteolysis [43]. The combination of these findings provide a rational explanation for the enhanced progesterone production in NGF heifers from days 10 to 12 of the current study.

The increased percentage of SLC over LLC observed in the CL of NGF heifers seems to best explain the luteotrophic effect of NGF in cattle and is in contrast to llamas, where no changes in proportion of SLC to LLC were detected with NGF treatment [15]. Consistently, NGF treatment tended to increase *Lhcgr* gene expression, which is localized to the SLC [44], but did not alter *Ot* gene expression, which is localized to the LLC [45]. These findings indicate that the observed changes are likely due to increased total quantity of SLC, rather than a decrease in LLC. In the bovine POF, *in vitro* NGF treatment induced proliferation of theca cells [10] that luteinize to become SLC [44], providing a logical explanation for the observed increase in SLC number. Consistently, LH signaling is required to maintain normal expression of *Star* and *3 β -hsd* genes and progesterone synthesis in the ruminant CL [42,46]. Treatment with NGF tended to increase CL diameter, which may be explained by increased SLC number and is consistent with another report in heifers [3]. In contrast, CL diameter did not change in beef cows treated with purified NGF [4].

It is likely that ultrasound may not be sensitive enough to pick up subtle changes in CL size, and perhaps the changes are more evident in heifers than in cows.

In conclusion, the current study confirmed the finding that NGF, purified from bull seminal plasma, is luteotrophic in Holstein heifers. In contrast to llamas, the luteotrophic effect in heifers is not attributed to changes in vascularity of the POF and CL. On the contrary, it appears that NGF interacts directly with the pre-ovulatory follicle to increase theca cell proliferation, leading to an increased number of SLC in the subsequent CL. The increased SLC population enhanced systemic progesterone levels in heifers from days 10 to 12 through increased *Star* and *3 β -hsd* expression in the CL. Future work is needed to evaluate whether NGF interacts with the hypothalamus or anterior pituitary in cattle to better understand its luteotrophic properties in spontaneously ovulating species.

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5.7 Tables and Figures

Table 7. List of genes and primers used for quantitative real-time PCR

Target genes and abbreviations	NCBI Sequence	Primer	Primer Sequence
Glyceraldehyde-3-phosphate dehydrogenase (<i>Gapdh</i>)	NM_001034034	Forward	5'-GGCGCCAAGAGGGTCAT-3'
		Reverse	5'-ACGCCCATCACAAACATGG-3'
Polyubiquitin (<i>Ubq3</i>)	NM_174133	Forward	5'-AGATCCAGGATAAGGAAGGCAT-3'
		Reverse	5'-GCTCCACCTCCAGGGTGAT-3'
Luteinizing hormone receptor (<i>Lhcgr</i>)	NM_174381	Forward	5'-CAGTCCCCCGCTTTCTCAT-3'
		Reverse	5'-GTAGAGCCCCATGCAGAAAGTCT-3'
Oxytocin (<i>Ot</i>)	NM_176855	Forward	5'-GACCTCCGCCTGCTACATTG-3'
		Reverse	5'-TAGTTCTCCTCTTGGCAGCG-3'
Steroidogenic acute regulatory protein (<i>Star</i>)	XR_083945	Forward	5'-GGATTAACCAGGTTTCGGCG-3'
		Reverse	5'-CTCTCCTTCTTCCAGCCCTC-3'
Side-chain cleavage enzyme (<i>Cyp11A1</i>)	NM_176644	Forward	5'-GCCACATCGAGAACTTCCAGAAG-3'
		Reverse	5'-CTGGTGTGGAACATCTTGTAGACG-3'
3-beta-hydroxysteroid dehydrogenase (<i>3β-hsd</i>)	NM_174343	Forward	5'-TGTTGGTGGAGGAGAAGGATCTG-3'
		Reverse	5'-TGGGTACCTTTACATTGACGTTG-3'
17 β-hydroxysteroid dehydrogenase (<i>17β-hsd</i>)	NM_001102365	Forward	5'-TTGTGCGAGAGTCTGGCGATTCT-3'
		Reverse	5'-AGGAATCGCTCGGTGGTGAAGTA-3'
17α-hydroxylase/17-20 lyase (<i>Cyp17a1</i>)	NM_174304	Forward	5'-TGTGGCCCCCTACGCTGAT-3'
		Reverse	5'-CGCCAATGCTGGAGTCAAT-3'
Aromatase (<i>Cyp19a1</i>)	NM_174305	Forward	5'-GTCCGAAGTTGTGCCTATTGCCAGC-3'
		Reverse	5'-CCTCCAGCCTGTCCAGATGCTTGG-3'
Estrogen receptor alpha (<i>Esr1</i>)	NM_001001443	Forward	5'-AGGGAAGCTCCTATTTGCTCC-3'
		Reverse	5'-CGGTGGATGTGGTCTTCTCT-3'
Fibroblast growth factor 2 (<i>Fgf2</i>)	NM_174056	Forward	5'-GAACGGGGGCTTCTTCCT-3'
		Reverse	5'-CCCAGTTCGTTTCAGTGCC-3'
Prostaglandin E synthase (<i>Pges</i>)	NM_174443	Forward	5'-AGGACGCTCAGAGACATGGA-3'
		Reverse	5'-TTCGGTCCGAGGAAAGAGTA-3'
Prostaglandin E receptor (<i>Pger</i>)	XM_584035	Forward	5'-CCCTGGGTGTACATCTTGCT-3'
		Reverse	5'-CCTCCCAAGTGCTCTTGTTT-3'
Vascular endothelial growth factor A isoform 121 (<i>Vegfa₁₂₁</i>)	NM_174216	Forward	5'-CCGTCCCATTGAGACCCTG-3'
		Reverse	5'-CGGCTTGTCAACAATTTTCTTGTC-3'
Vascular endothelial growth factor receptor 2 (<i>Vegfr2</i>)	NM_001110000	Forward	5'-AGACTGGTTCTGGCCCAAC-3'
		Reverse	5'-GAAGCCTTTCTGGCTGTC-3'

Table 8. Relative mRNA expression of angiogenic and steroidogenic enzymes in the corpus luteum of heifers treated systemically with NGF (250 µg) or phosphate-buffered saline (control). Data are presented as mean ± SEM.

Gene names and abbreviations	Control		NGF		<i>P</i> -value
	Day 9	Day 14	Day 9	Day 14	
17 α -hydroxylase/17-20 lyase (<i>Cyp17a1</i>)	0.06 ± 0.02	0.04 ± 0.02	0.02 ± 0.01	0.01 ± 0.00	0.28
Aromatase (<i>Cyp19a1</i>)	Undetected		Undetected		N/A
Estrogen receptor alpha (<i>Esr1</i>)	0.30 ± 0.15	0.35 ± 0.13	0.32 ± 0.09	0.20 ± 0.10	0.65
Fibroblast growth factor 2 (<i>Fgf2</i>)	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.41
Prostaglandin E synthase (<i>Pges</i>)	0.22 ± 0.06	0.18 ± 0.07	0.18 ± 0.09	0.07 ± 0.02	0.03
Prostaglandin E receptor (<i>Pger</i>)	0.09 ± 0.03	0.06 ± 0.03	0.08 ± 0.06	0.01 ± 0.00	0.07
Vascular endothelial growth factor A isoform 121 (<i>Vegfa</i> ₁₂₁)	Undetected		Undetected		N/A
Vascular endothelial growth factor receptor 2 (<i>Vegfr2</i>)	Undetected		Undetected		N/A

Figure 12. Power Doppler ultrasonography image of the pre-ovulatory follicle (A) and corpus luteum (B) in heifers treated with purified bovine NGF (250 μ g) or phosphate-buffered saline (control), which did not differ between treatments ($P \geq 0.16$).

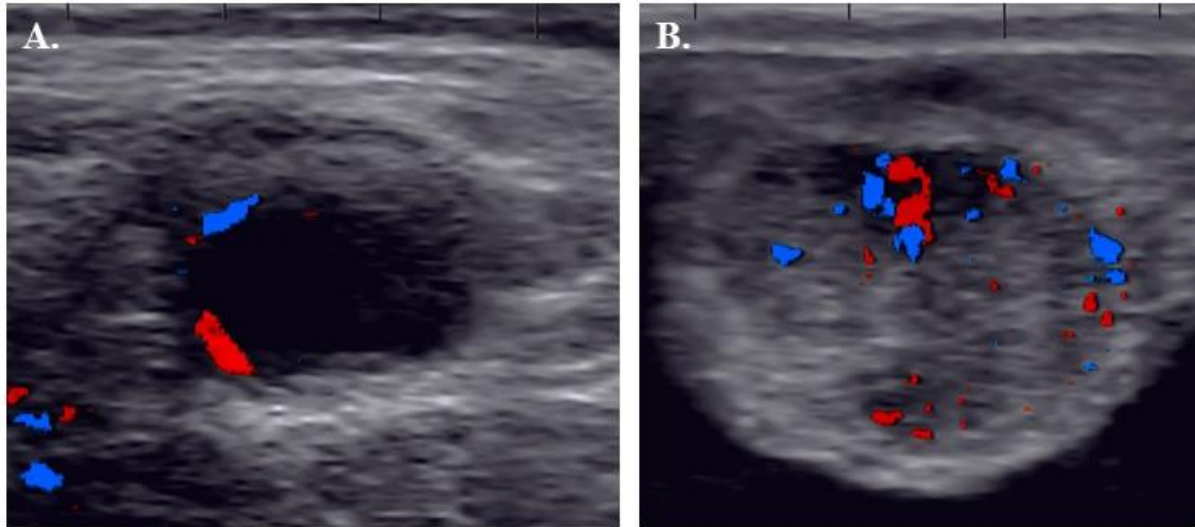


Figure 13. (A) A MC8.0MHz transducer was used for the transvaginal placement of the 60 cm long biopsy needle. (B) The biopsy needle contained a specimen notch ($20 \times 1 \times 0.5$ mm) covered by an 18-gauge cutting cannula triggered with an automated spring-loaded device. (C) When the ovary was positioned transrectally against the vaginal wall and transducer face, the inner needle was advanced into the corpus luteum. Once the needle was visualized on the ultrasound and determined to be in the correct position, the spring-locked cutting cannula was fired, cutting off a section of tissue within the specimen notch and enclosing it within the covering of the outer cannula.

A.



B.



C.



Figure 14. Line graphs demonstrating changes over time in pre-ovulatory dynamics of heifers treated with NGF (250 µg) or phosphate-buffered saline (control). (A) There was a main effect of treatment on follicle diameter ($P = 0.02$). (B) Percentage of follicle vascular area was not affected by NGF treatment ($P = 0.16$). (C) Serum estradiol concentrations were also not affected by NGF treatment ($P = 0.95$). Data are presented as mean \pm SEM.

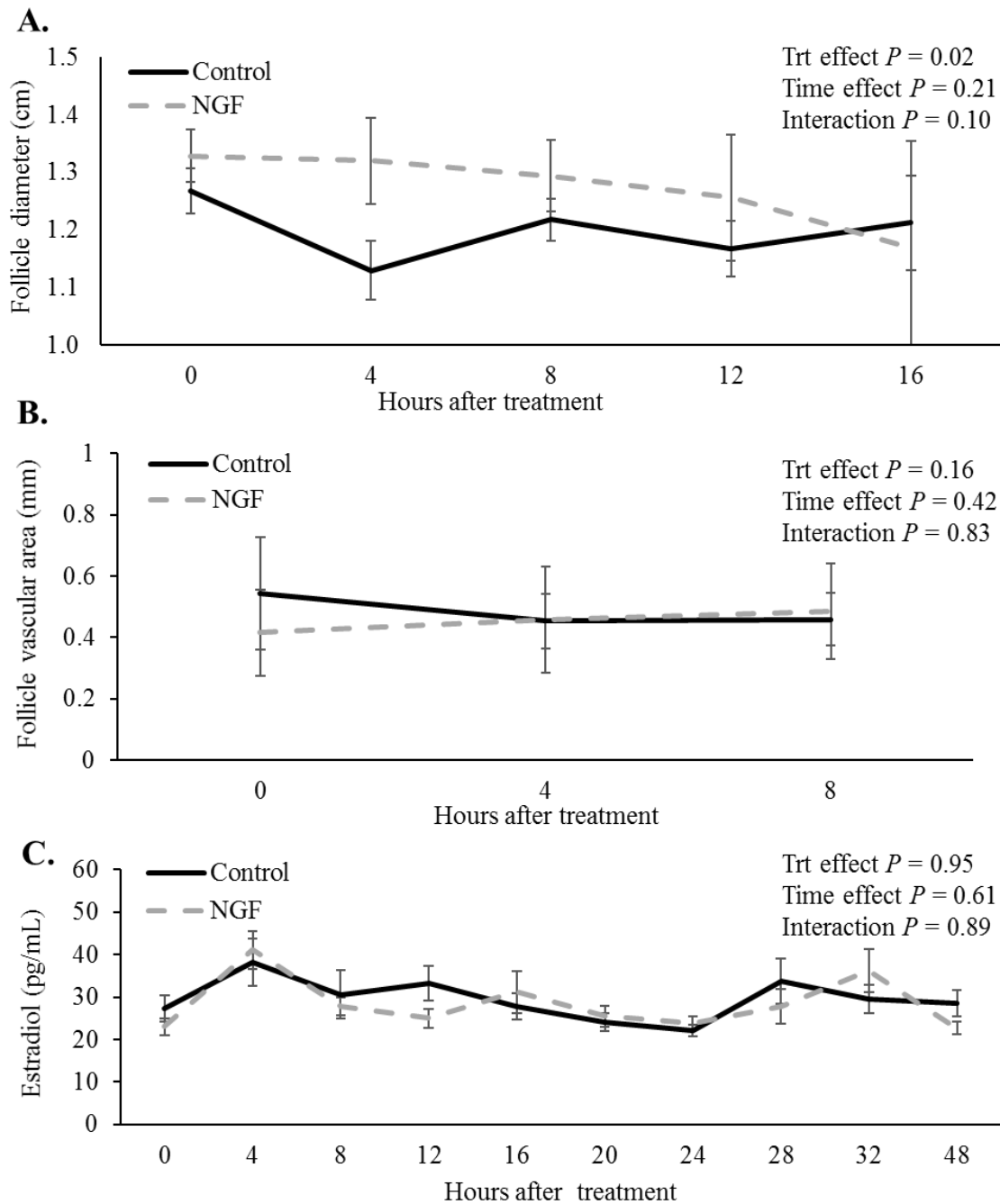


Figure 15. Graphs demonstrating changes over time in post-ovulatory dynamics of heifers treated with NGF (250 μ g) or phosphate-buffered saline (control). (A) There a tendency towards a treatment effect on corpus luteum (CL) diameter ($P = 0.10$). (B) Percentage of CL vascular area was not affected by NGF treatment ($P = 0.20$). (C) Serum progesterone concentrations increased over time ($P < 0.01$), with increased concentrations observed in NGF-treated over control heifers from days 10 to 12 ($P = 0.04$), denoted by asterisks (*). Data are presented as mean \pm SEM.

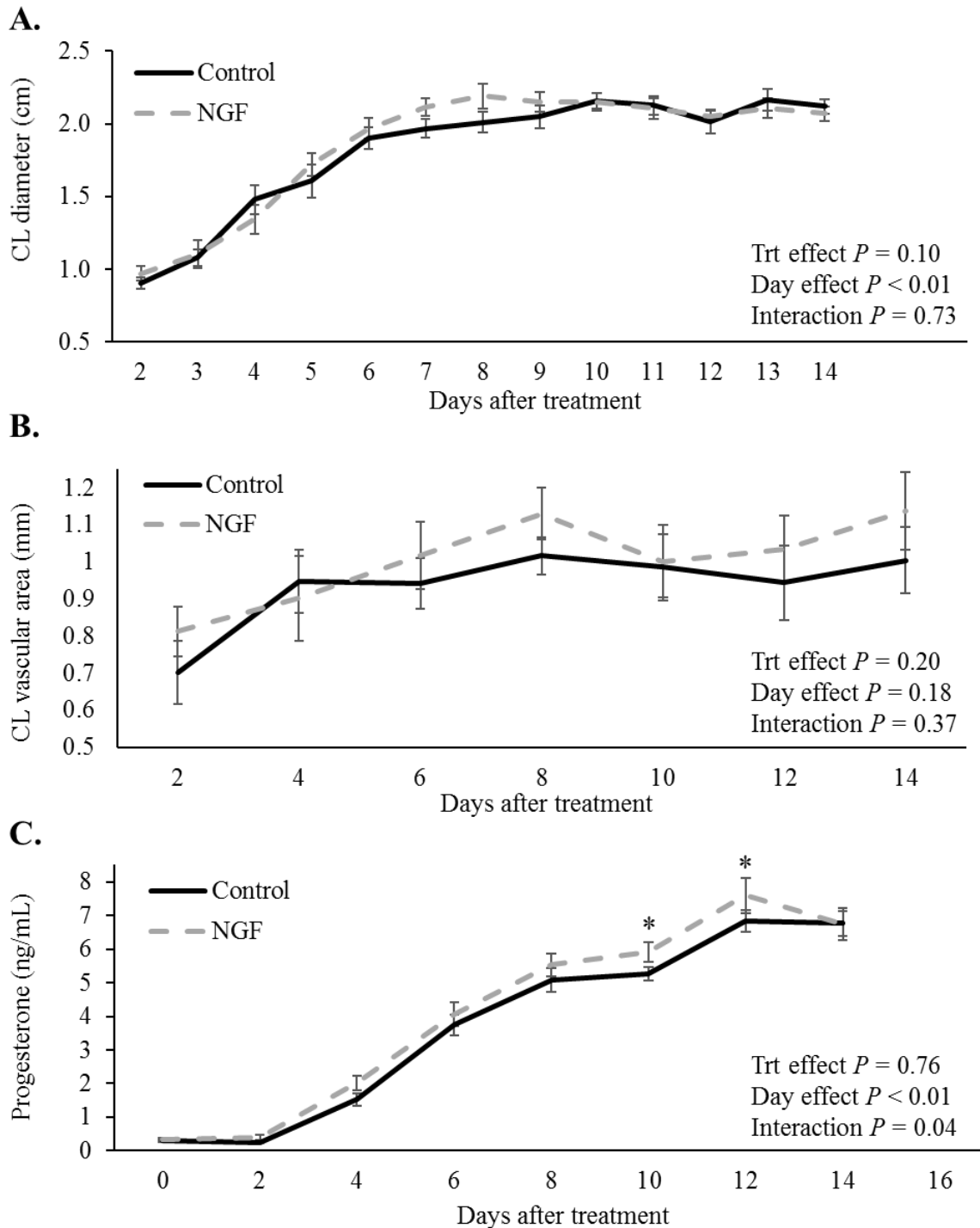
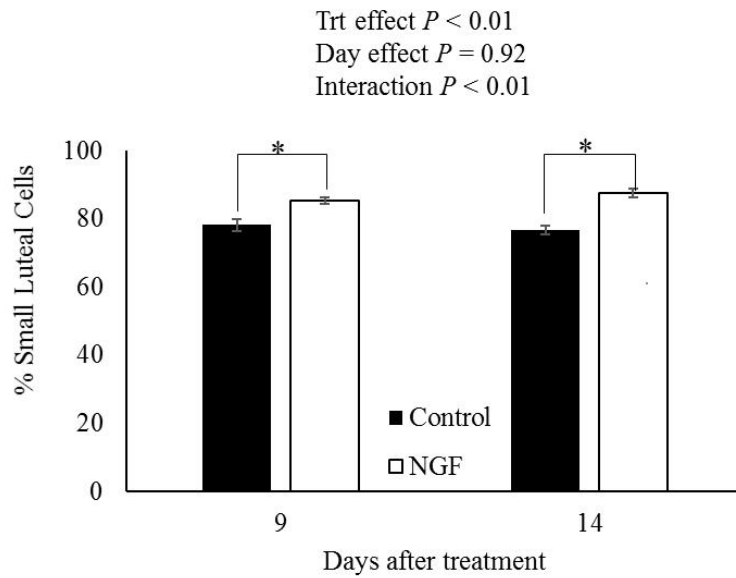
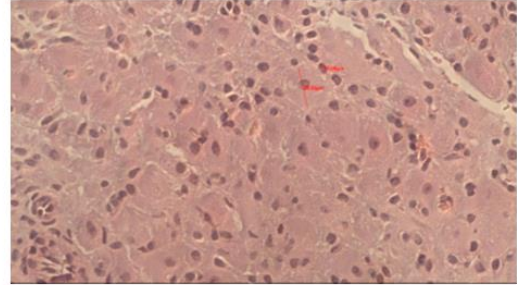


Figure 16. Bar graphs and representative hematoxylin & eosin stained histological slides demonstrating increased percentages of small luteal cells over large luteal cells in the corpus luteum of heifers treated with 250 μ g NGF versus phosphate-buffered saline (control; $P < 0.01$). Data are presented as mean \pm SEM.



Control



NGF

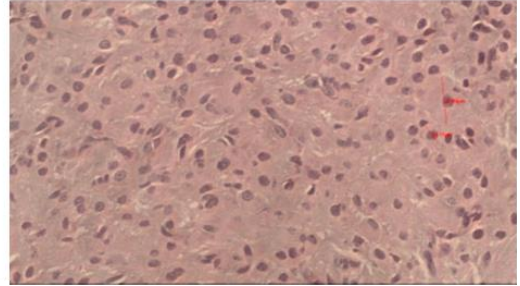


Figure 17. Relative abundance of LH receptor (*Lhcgr*) and oxytocin (*Ot*) gene expression from the corpus luteum of heifers treated with NGF (250 µg) or phosphate-buffered saline (control). (A) There was a tendency for *Lhcgr* to be increased on both days 9 and 14 in NGF-treated heifers ($P = 0.09$), suggesting increased small luteal cell numbers. (B) There were no differences between treatment groups on *Ot* expression ($P = 0.15$), suggesting no changes in large luteal cell number between treatments. Data are presented as mean \pm SEM.

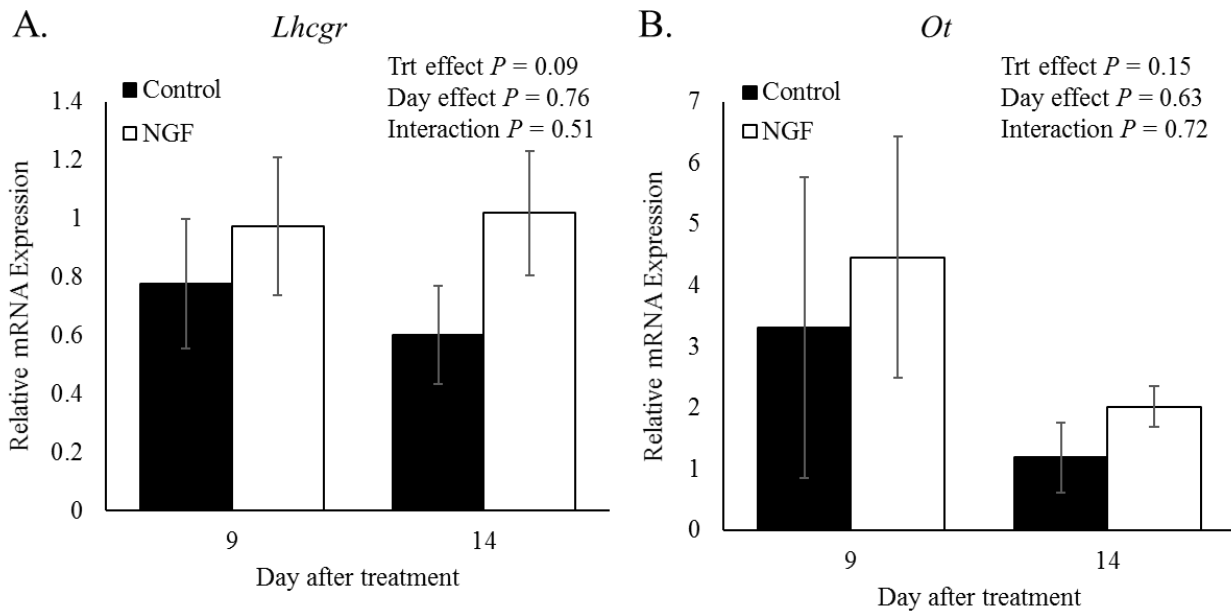
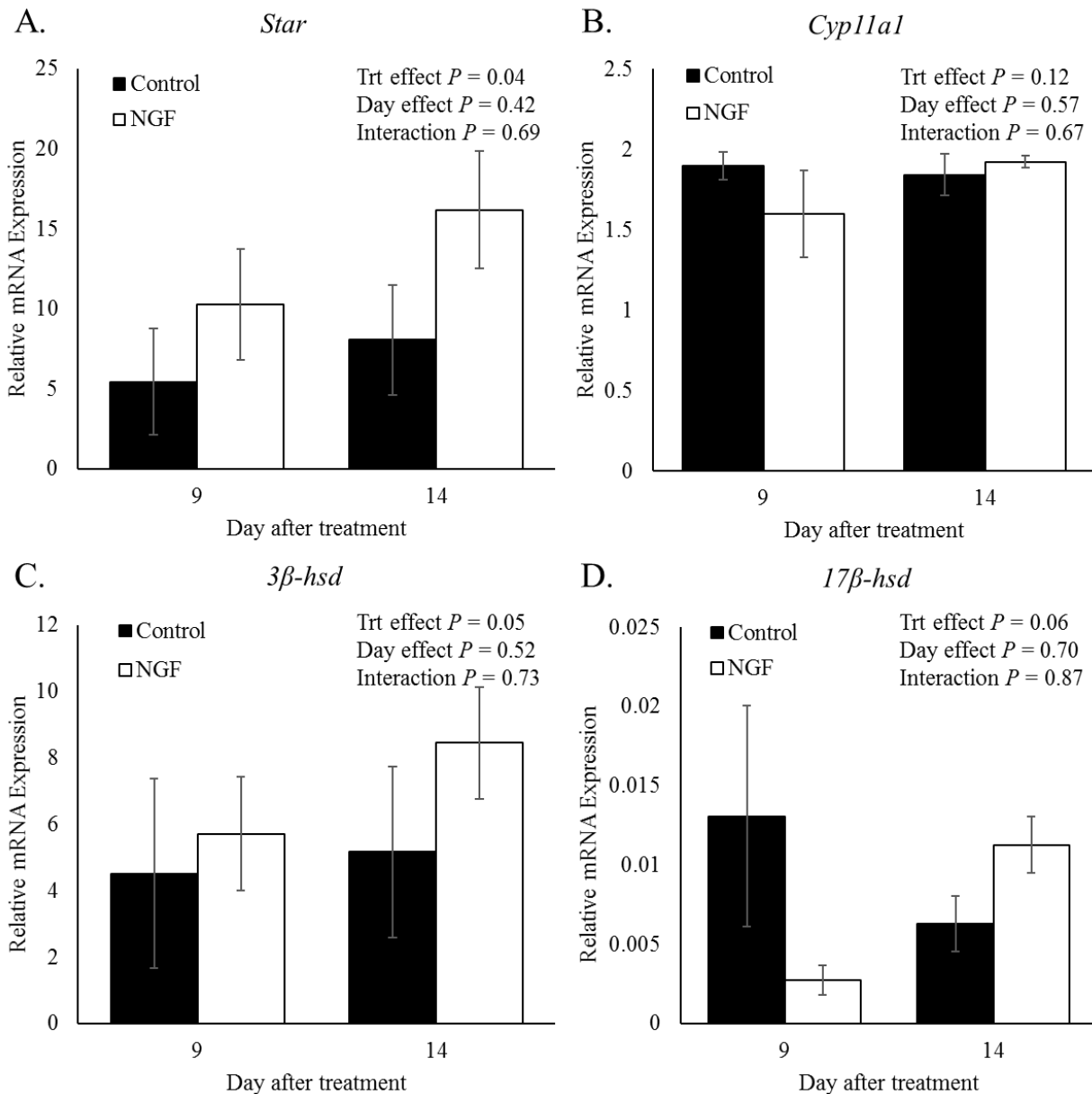


Figure 18. Relative abundance of steroidogenic enzyme gene expression from the corpus luteum of heifers treated systemically with NGF (250 μ g) or phosphate-buffered saline (control). (A) NGF-treated heifers had increased *Star* expression compared to control heifers ($P = 0.04$). (B) There was no difference in *Cyp11a1* expression between treatment groups ($P = 0.12$). (C) NGF-treated heifers had increased expression of *3 β -hsd* over control heifers ($P = 0.05$). (D) There was a tendency for *17 β -hsd* expression to be decreased at day 9 and increased at day 14 in NGF-treated heifers ($P = 0.06$). Data are presented as mean \pm SEM.



5.8 References

- [1] Stewart JL, Canisso IF, Ellerbrock RE, Mercadante VRG, Lima FS. Nerve Growth Factor- β production in the bull: Gene expression, immunolocalization, seminal plasma constitution, and association with sire conception rates. *Anim Reprod Sci* 2018;197:335–42. doi:10.1016/j.anireprosci.2018.09.006.
- [2] Bogle OA, Carrasco RA, Ratto MH, Singh J, Adams GP. Source and localization of ovulation-inducing factor/nerve growth factor in male reproductive tissues among mammalian species. *Biol Reprod* 2018. doi:10.1093/biolre/ioy149.
- [3] Tanco VM, Van Steelandt MD, Ratto MH, Adams GP. Effect of purified llama ovulation-inducing factor (OIF) on ovarian function in cattle. *Theriogenology* 2012;78:1030–9. doi:10.1016/j.theriogenology.2012.03.036.
- [4] Stewart JL, Mercadante VRG, Dias NW, Canisso IF, Yau P, Imai B, et al. Nerve Growth Factor-Beta, purified from bull seminal plasma, enhances corpus luteum formation and conceptus development in *Bos taurus* cows. *Theriogenology* 2018;106:30–8. doi:10.1016/j.theriogenology.2017.10.007.
- [5] Tribulo P, Bogle OA, Mapletoft RJ, Adams GP. Bioactivity of ovulation inducing factor (or nerve growth factor) in bovine seminal plasma and its effects on ovarian function in cattle. *Theriogenology* 2015;83:1394–401. doi:10.1016/j.theriogenology.2014.12.014.
- [6] Tanco VM, Ratto MH, Lazzarotto M, Adams GP. Dose-response of female llamas to ovulation-inducing factor from seminal plasma. *Biol Reprod* 2011;85:452–6. doi:10.1095/biolreprod.111.091876.
- [7] Adams GP, Ratto MH, Huanca W, Singh J. Ovulation-inducing factor in the seminal plasma of alpacas and llamas. *Biol Reprod* 2005;73:452–7. doi:10.1095/biolreprod.105.040097.
- [8] Ratto MH, Delbaere LT, Leduc YA, Pierson RA, Adams GP. Biochemical isolation and purification of ovulation-inducing factor (OIF) in seminal plasma of llamas. *Reprod Biol Endocrinol* 2011;9:24. doi:10.1186/1477-7827-9-24.
- [9] Bogle OA, Ratto MH, Adams GP. Ovulation-inducing factor (OIF) induces LH secretion from pituitary cells. *Anim Reprod Sci* 2012;133:117–22. doi:10.1016/j.anireprosci.2012.06.006.
- [10] Dissen GA, Parrott JA, Skinner MK, Hill DF, Costa ME, Ojeda SR. Direct effects of nerve growth factor on thecal cells from antral ovarian follicles. *Endocrinology* 2000;141:4736–50. doi:10.1210/endo.141.12.7850.
- [11] Bao B, Garverick HA. Expression of steroidogenic enzyme and gonadotropin receptor genes in bovine follicles during ovarian follicular waves: a review. *J Anim Sci* 1998;76:1903–21. doi:10.2527/1998.7671903x.
- [12] Walters DL, Schallenberger E. Pulsatile secretion of gonadotrophins, ovarian steroids and ovarian oxytocin during the periovulatory phase of the oestrous cycle in the cow. *J Reprod Fertil* 1984;71:503–12. doi:10.1530/jrf.0.0710503.
- [13] Salas C, Julio-Pieper M, Valladares M, Pommer R, Vega M, Mastronardi C, et al. Nerve growth factor-dependent activation of trkA receptors in the human ovary results in

- synthesis of follicle-stimulating hormone receptors and estrogen secretion. *J Clin Endocrinol Metab* 2006;91:2396–403. doi:10.1210/jc.2005-1925.
- [14] Ulloa-Leal C, Bogle OA, Adams GP, Ratto MH. Luteotrophic effect of ovulation-inducing factor/nerve growth factor present in the seminal plasma of llamas. *Theriogenology* 2014;81:1101–1107.e1. doi:10.1016/j.theriogenology.2014.01.038.
 - [15] Silva M, Ulloa-Leal C, Valderrama XP, Bogle OA, Adams GP, Ratto MH. Nerve growth factor from seminal plasma origin (sp β -NGF) increases CL vascularization and level of mRNA expression of steroidogenic enzymes during the early stage of corpus luteum development in llamas. *Theriogenology* 2017;103:69–75. doi:10.1016/j.theriogenology.2017.07.041.
 - [16] Finetti F, Solito R, Morbidelli L, Giachetti A, Ziche M, Donnini S. Prostaglandin E2 regulates angiogenesis via activation of fibroblast growth factor receptor-1. *J Biol Chem* 2008;283:2139–46. doi:10.1074/jbc.M703090200.
 - [17] Herzog K, Brockhan-Lüdemann M, Kaske M, Beindorff N, Paul V, Niemann H, et al. Luteal blood flow is a more appropriate indicator for luteal function during the bovine estrous cycle than luteal size. *Theriogenology* 2010;73:691–7. doi:10.1016/j.theriogenology.2009.11.016.
 - [18] Alila HW, Hansel W. A comparison of the effects of cyclooxygenase prostanoids on progesterone production by small and large bovine luteal cells. *Prostaglandins* 1988;36. doi:10.1016/0090-6980(88)90312-7.
 - [19] Kot K, Anderson LE, Tsai SJ, Wiltbank MC, Ginther OJ. Transvaginal, ultrasound-guided biopsy of the corpus luteum in cattle. *Theriogenology* 1999;52:987–93. doi:10.1016/S0093-691X(99)00188-0.
 - [20] Miyamoto A, Shirasuna K, Wijayagunawardane MPB, Watanabe S, Hayashi M, Yamamoto D, et al. Blood flow: A key regulatory component of corpus luteum function in the cow. *Domest Anim Endocrinol* 2005;29:329–39. doi:10.1016/j.domaniend.2005.03.011.
 - [21] Lüttgenau J, Ulbrich SE, Beindorff N, Honnens A, Herzog K, Bollwein H. Plasma progesterone concentrations in the mid-luteal phase are dependent on luteal size, but independent of luteal blood flow and gene expression in lactating dairy cows. *Anim Reprod Sci* 2011;125:20–9. doi:10.1016/j.anireprosci.2011.02.002.
 - [22] Yoshioka S, Abe H, Sakumoto R, Okuda K. Proliferation of luteal steroidogenic cells in cattle. *PLoS One* 2013;8:1–12. doi:10.1371/journal.pone.0084186.
 - [23] Sakumoto R, Hayashi KG, Takahashi T. Different expression of PGE synthase, PGF receptor, TNF, Fas and oxytocin in the bovine corpus luteum of the estrous cycle and pregnancy. *Reprod Biol* 2014;14:115–21. doi:10.1016/j.repbio.2013.12.003.
 - [24] Silva ME, Smulders JP, Guerra M, Valderrama XP, Letelier C, Adams GP, et al. Cetrorelix suppresses the preovulatory LH surge and ovulation induced by ovulation-inducing factor (OIF) present in llama seminal plasma. *Reprod Biol Endocrinol* 2011;9:74. doi:10.1186/1477-7827-9-74.
 - [25] Carrasco RA, Singh J, Adams GP. The relationship between gonadotropin releasing hormone and ovulation inducing factor/nerve growth factor receptors in the hypothalamus

- of the llama. *Reprod Biol Endocrinol* 2018;16:83. doi:10.1186/s12958-018-0402-6.
- [26] El Allali K, El Bousmaki N, Ainani H, Simonneaux V. Effect of the camelid's seminal plasma ovulation-inducing factor/ β -NGF: a kisspeptin target hypothesis. *Front Vet Sci* 2017;4. doi:10.3389/fvets.2017.00099.
- [27] Carrasco R, Singh J, Adams GP. The dynamics of *trkA* expression in the bovine ovary are associated with a luteotrophic effect of ovulation-inducing factor/nerve growth factor (OIF/NGF). *Reprod Biol Endocrinol* 2016;14:47. doi:10.1186/s12958-016-0182-9.
- [28] Barboni B, Mattioli M, Gioia L, Turriani M, Capacchietti G, Berardinelli P, et al. Preovulatory rise of NGF in ovine follicular fluid: possible involvement in the control of oocyte maturation. *Microsc Res Tech* 2002;59:516–21. doi:10.1002/jemt.10230.
- [29] Lonergan P. Influence of progesterone on oocyte quality and embryo development in cows. *Theriogenology* 2011;76:1594–601. doi:10.1016/j.theriogenology.2011.06.012.
- [30] Mullen MP, Bazer FW, Wu G, Parr MH, Evans ACO, Crowe MA, et al. Effects of systemic progesterone during the early luteal phase on the availabilities of amino acids and glucose in the bovine uterine lumen. *Reprod Fertil Dev* 2014;26:282–92. doi:10.1071/RD12319.
- [31] Mann GE, Fray MD, Lamming GE. Effects of time of progesterone supplementation on embryo development and interferon-tau production in the cow. *Vet J* 2006;171:500–3. doi:10.1016/j.tvjl.2004.12.005.
- [32] Parr MH, Scully S, Lonergan P, Evans ACO, Crowe MA, Diskin MG. Establishment of critical timing of progesterone supplementation on corpus luteum and embryo development in beef heifers. *Anim Reprod Sci* 2017. doi:10.1016/j.anireprosci.2017.02.005.
- [33] Crispo M, dos Santos-Neto PC, Vilariño M, Mulet AP, de León A, Barbeito L, et al. RAPID COMMUNICATION: Nerve growth factor influences cleavage rate and embryo development in sheep. *J Anim Sci* 2016;94:4447–51. doi:10.2527/jas.2016-0736.
- [34] Pinilla L, Aguilar E, Dieguez C, Millar RP, Tena-Sempere M. Kisspeptins and reproduction: physiological roles and regulatory mechanisms. *Physiol Rev* 2012;92:1235–316. doi:10.1152/physrev.00037.2010.
- [35] Silva ME, Recabarren MP, Recabarren SE, Adams GP, Ratto MH. Ovarian estradiol modulates the stimulatory effect of ovulation-inducing factor (OIF) on pituitary LH secretion in llamas. *Theriogenology* 2012;77:1873–82. doi:10.1016/j.theriogenology.2012.01.004.
- [36] Schams D, Berisha B. Angiogenic factors (VEGF, FGF and IGF) in the bovine corpus luteum. *J Reprod Dev* 2002;48:233–42. doi:10.1262/jrd.48.233.
- [37] Berisha B, Schams D, Kosmann M, Amselgruber W, Einspanier R. Expression and tissue concentration of vascular endothelial growth factor, its receptors, and localization in the bovine corpus luteum during estrous cycle and pregnancy. *Biol Reprod* 2000;63:1106–14. doi:10.1095/biolreprod63.4.1106.
- [38] Arosh JA, Banu SK, Kimmins S, Chapdelaine P, MacLaren LA, Fortier MA. Effect of interferon- τ on prostaglandin biosynthesis, transport, and signaling at the time of maternal recognition of pregnancy in cattle: evidence of polycrine actions of prostaglandin E 2.

- Endocrinology 2004;145:5280–93. doi:10.1210/en.2004-0587.
- [39] Arosh JA, Banu SK, Chapdelaine P, Madore E, Sirois J, Fortier MA. Prostaglandin biosynthesis, transport, and signaling in corpus luteum: a basis for autoregulation of luteal function. *Endocrinology* 2004;145:2551–60. doi:10.1210/en.2003-1607.
 - [40] Niswender GD. Molecular control of luteal secretion of progesterone. *Reproduction* 2002;123:333–9. doi:10.1530/rep.0.1230333.
 - [41] Stocco DM. StAR protein and the regulation of steroid hormone biosynthesis. *Annu Rev Physiol* 2001;63:193–213. doi:10.1146/annurev.physiol.63.1.193.
 - [42] Rekawiecki R, Nowik M, Kotwica J. Stimulatory effect of LH, PGE2 and progesterone on StAR protein, cytochrome P450 cholesterol side chain cleavage and 3 β hydroxysteroid dehydrogenase gene expression in bovine luteal cells. *Prostaglandins Other Lipid Mediat* 2005;78:169–84. doi:10.1016/j.prostaglandins.2005.06.009.
 - [43] Kemiläinen H, Adam M, Mäki-Jouppila J, Damdimopoulou P, Damdimopoulos AE, Kere J, et al. The hydroxysteroid (17 β) dehydrogenase family gene HSD17B12 is involved in the prostaglandin synthesis pathway, the ovarian function, and regulation of fertility. *Endocrinology* 2016;157:3719–30. doi:10.1210/en.2016-1252.
 - [44] Harrison LM, Kenny N, Niswender GD. Progesterone production, LH receptors, and oxytocin secretion by ovine luteal cell types on days 6, 10 and 15 of the oestrus cycle and day 25 of pregnancy. *J Reprod Fert* 1987;79:539–48. doi:10.1530/jrf.0.0790539.
 - [45] Fehr S, Ivell R, Koll R, Schams D, Fields M, Richter D. Expression of the oxytocin gene in the large cells of the bovine corpus luteum. *FEBS Lett* 1987;210:45–50. doi:10.1016/0014-5793(87)81295-4.
 - [46] Niswender GD, Juengel JL, Silva PJ, Rollyson MK, McIntush EW. Mechanisms controlling the function and life span of the corpus luteum. *Physiol Rev* 2000;80:1–29. doi:10.1152/physrev.2000.80.1.1.

Chapter 6:

Biology of Nerve Growth Factor- β Production in the Bull¹

6.1 Abstract

Nerve growth factor- β (NGF) is a seminal plasma protein that regulates sperm physiology in bulls, yet its production and association with fertility are not fully understood. The objective of this study was to describe NGF distribution in the bull accessory sex glands, quantify its seminal plasma concentrations, and determine its association with sire conception rates. Accessory sex glands (ampulla, bulbourethral, prostate, vesicular glands) were harvested post-mortem from mature bulls ($n = 3$). Tissue samples were frozen for quantitative PCR and formalin-fixed for immunohistochemistry. Seminal plasma was collected in fractions (pre-ejaculate vs. sperm-rich) by electroejaculation from Angus cross ($n = 42$) and Holstein (sperm-rich fraction only; $n = 10$) bulls. Holstein ($n = 76$) and Jersey ($n = 14$) bulls were collected by artificial vagina, and those with available sire conception rate scores were allocated to two categories: (1) negative/0 deviations ($n = 15$); or (2) positive deviations ($n = 45$). Enzyme-linked immunoassay was used to measure seminal plasma NGF concentrations. Relative *Ngf* gene expression was highest in the vesicular, intermediate in the ampulla, and lowest in the prostate and bulbourethral glands ($P \leq 0.03$). Highest intensity of NGF staining was detected in the ampulla and vesicular glands ($P < 0.05$). Concentrations of NGF were higher in the sperm-rich fraction than in the pre-ejaculate and higher in samples collected by artificial vagina ($P < 0.01$). Seminal plasma NGF concentrations were higher in bulls with positive sire conception rate deviations than those with negative/0 deviations ($P = 0.05$). This study determined that NGF is produced in the ampulla and vesicular glands of

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bulls and is secreted into the sperm-rich fraction of the ejaculate. The positive association of NGF with bull fertility warrants further investigation to determine its potential uses in advanced reproductive techniques.

6.2 Introduction

Seminal plasma proteins play an important role in enhancing bull fertility [1–3]. Nerve growth factor- β (NGF) is a protein present in bull seminal plasma that regulates sperm physiology [4]. Interestingly, NGF also alters ovarian function [5,6], improves corpus luteum development [5,7], and enhances markers of conceptus development [7] when administered systemically to cows or heifers. Collectively, these findings suggest that NGF may have several mechanisms by which it can affect reproductive success. The role of seminal plasma proteins, such as NGF, in regulating fertility is of special relevance to the cattle industry where artificial insemination has been extensively used to advance genetic selection of desirable traits such as milk production and meat quality [8]. Historically, bull semen cryopreservation studies have focused on developing the most suitable semen freezing methods and extenders and determining the minimal effective breeding dose, while overlooking the effects that semen processing may have on seminal plasma protein concentrations [9]. When processing semen for cryopreservation, samples are extended, which may dilute out seminal plasma proteins to sub-physiologic levels and contribute to lower fertility [10–12]. Therefore, there is a need to obtain data regarding the role of NGF in the bull reproductive tract.

Ejaculated bovine sperm have NGF-immunoreactivity in the head and tail, and its tyrosine kinase A receptor (TrkA) is localized in the acrosomal cap, nucleus, and tail regions [4]. In addition to several other proteins, sperm *Ngf* mRNA abundance in bulls was strongly associated with

positive sire conception rates [13]. More recently, seminal plasma *Ngf* mRNA expression was found to be positively associated with the maintenance of post-thaw functional membrane integrity in bull sperm, suggesting that it could be used to assess cryotolerance [14]. Another study reported higher spermatozoa *Ngf* expression in good semen-producing (<25% discarded ejaculates) versus poor semen-producing (>40% discarded ejaculates) bulls, though no correlation with field conception rates was detected [15]. Relative spermatozoa *Ngf* expression levels were also positively associated with pre-freeze mitochondrial membrane potential and post-thaw sperm velocity parameters in *Bos taurus* bulls [15]. Based on these findings, it seems that NGF might be a suitable protein for improving sperm cryopreservation in bulls. Interestingly, abundance of some seminal plasma proteins differ when semen is collected by electroejaculation versus artificial vagina, which are both commonly used collection methods in bulls (Rego et al., 2015). Therefore, more data are needed regarding normal production and secretion of NGF into the seminal plasma and its association with bull fertility traits.

There is limited information available regarding the production of NGF in bull seminal plasma. Early studies using biological assays reported moderate levels of NGF in the prostate [16,17] and high levels in the vesicular glands [18] in bulls. In llamas, where seminal plasma NGF is highly concentrated and essential for inducing ovulation [19,20], its distribution was strongly localized to the mucosal prostatic cells [21]. Concentrations of NGF in the seminal plasma of bulls is reportedly less than in llamas [6], indicating that there are significant species variations that need to be investigated. The objectives of the current study were to (1) describe the distribution of NGF production in bovine accessory sex glands; (2) quantify NGF secretion into the seminal plasma; and (3) determine if NGF concentrations are associated with sire conception rates or breeding soundness parameters in bulls.

6.3 Materials and Methods

6.3.1 *Animal care and use*

All experimental procedures carried out in the present study were approved by the Institutional Animal Care and Use Committees of the University of Illinois at Urbana-Champaign (Protocol #15201).

6.3.2 *Accessory sex gland harvest*

Accessory sex glands (ampulla, bulbourethral, prostate, and vesicular glands) were harvested opportunistically from three bulls euthanized at the University of Illinois Veterinary Teaching Hospital. Bull 1 was a 4-year-old Holstein bull euthanized after diagnosis of cecal dilation and torsion on exploratory surgery. Bull 2 was a 4-year-old Angus cross bull euthanized due to lameness from tenosynovitis and arthritis. Bull 3 was a 3.5-year-old Angus bull euthanized due to lameness from suspected radial nerve damage. All tissues were obtained within an hour of euthanasia. Tissue samples from each gland were flash-frozen in liquid nitrogen, submerged in RNA-later (Invitrogen) and frozen the next day, or fixed in 10% formalin. Frozen samples were maintained at -80°C until analyses.

6.3.3 *Extraction of RNA and RT-PCR*

Total RNA extraction from accessory sex glands was carried out using TRIzol (Molecular Research Center, Inc.) as previously described [1]. Extraction of RNA was conducted according to the manufacturer's recommendations (PureLink RNA Mini Kit, Invitrogen). Isolated RNA was evaluated for concentration and purity using a NanoDrop One Spectrophotometer (Thermo Fisher

Scientific). A total of 1 mg of mRNA was used to synthesize complementary DNA using a commercial kit (High-capacity cDNA Reverse Transcription Kit, Cat. No. 4368814, Applied Biosystems). Complementary DNA was used for quantitative real-time reverse transcription PCR using a 7500 Real-Time PCR Detection System (Applied Biosciences) with iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories, Inc.). The expression value of each gene, nerve growth factor- β (*Ngf*) and tyrosine kinase A (*Trka*), was normalized to the amount of an internal control gene (glyceraldehyde-3-phosphate dehydrogenase, *Gapdh*) to calculate a relative amount of RNA in each sample. The expression value of each gene in a WT control was arbitrarily defined as 1 U. All assays were carried out in duplicate for each target mRNA. A relative quantitative fold change was determined using the $\Delta\Delta C_t$ method. Primer sequences are summarized in Table 9 [22].

6.3.4 Immunohistochemistry

Distribution of NGF were assessed in accessory sex gland tissues using standard immunohistochemistry staining. Each of the tissues samples was embedded in paraffin, cut in 4- μ m sections, and mounted on slides. After deparaffinization, the slides were pretreated with a commercial heat induced antigen retrieval reagent (Diva decloaker DV 2004; Biocare Medical). After rinsing with buffer (TBS Auto Wash Buffer TWB945M; Biocare Medical), the slides were then placed on the Biocare IntelliPath automated staining platform and incubated with a stable hydrogen peroxide solution for 5 minutes at room temperature to block endogenous peroxidase (Peroxidazed 1, PX968). Following washing, tissues were incubated with a universal blocking reagent (Background Punisher, BP974) for 10 minutes to reduce nonspecific antibody binding. After blocking, a primary anti-NGF rabbit polyclonal antibody (NBP2-19536; Novus Biologicals LLC) was applied at 1:500 dilution for 30 minutes. Negative controls were incubated without

primary antibody. Slides were then washed with buffer and incubated with a secondary antibody conjugated to a HRP polymer (Rabbit-on-FARMA HRP-Polymer, BRR4009) for another 30 minutes. Following washing, slides were incubated with a 3, 3' Diaminobenzidine (DAB) peroxidase chromogen solution for 5 minutes, which causes conversion from colorless substrate to a brown product in areas where the antibody bound the target protein (NGF). Slides were subsequently counterstained with hematoxylin for 5 minutes to stain the nucleus of the cells. Bovine cerebellum was used as a positive control [23].

Slides were evaluated with a phase contrast microscope, and four images from each gland from each bull were saved at 200× magnification. Images were obtained such that a representative portion of the duct was included in each image. Percentage and intensity of positive staining were measured from each image using the IHC Profiler plugin in ImageJ [24]. This software describes the percentage contribution of high, moderate, and low intensity positive and negative DAB staining within an image. The average scores for the four images within each gland and bull were calculated.

6.3.5 Semen collection and breeding soundness evaluation

University of Illinois-owned Angus cross bulls ($n = 42$; mean age: 3.25 ± 0.3 y) and privately-owned Holstein bulls ($n = 10$; mean age: 4.2 ± 0.5 y) were subjected to breeding soundness examinations with semen collected by electroejaculation. All bulls were in adequate body condition. The Angus cross bulls were collected in October of 2017, and the Holstein bulls were collected in April of 2018. A 60 or 75 mm upright weighted bull probe was placed rectally to allow for stimulation using a commercial electroejaculation unit (Pulsator IV, Lane Manufacturing, Inc.). For the Angus cross bulls, both the pre-ejaculate and sperm-rich fractions of

the ejaculate were collected separately as determined by visual opacity of the sample (clear = pre-ejaculate; cloudy = sperm-rich fraction). Only the sperm-rich fraction was collected in the Holstein bulls. At time of collection, aliquots of each fraction were immediately plunged in liquid nitrogen and stored at -80°C until analysis. An aliquot of the sperm-rich fraction from each bull was evaluated on site for mass motility using light microscopy at $100\times$ and classified as very good (rapid swirling), good (slow swirling), fair (movement, but no swirling), or poor (no movement). An aliquot of the sperm-rich fraction from each bull was also fixed in 10% formalin for morphological analysis. One clinician evaluated 100 sperm cells from each sample under $1000\times$ magnification with a phase contrast microscope, classified each sperm cell as normal or abnormal, and further defined abnormalities as primary or secondary defects [25,26]. Bulls were classified as satisfactory or unsatisfactory using parameters defined by the Society for Theriogenology [26].

6.3.6 Semen collection and sire conception rate

An aliquot (200 μL) of semen was obtained from 90 dairy bulls ($n = 76$ Holstein, mean age: 1.8 ± 0.1 y; $n = 14$ Jersey, mean age: 1.7 ± 0.3 y) collected via artificial vagina at a commercial bull stud facility in February of 2016 (Select Sires, Plain City, Ohio). Samples were immediately plunged in liquid nitrogen and stored at -80°C until analysis. Sire conception rates (SCR) scores were available for 53 Holstein and 7 Jersey bulls. The SCR is an estimate of AI service-sire fertility from outcome (pregnant or not) after ~ 300 services (mean $3,311 \pm 565$ services) and is reported as a sire's fertility deviation (-4 to $+4$) from the average fertility of a population [27]. Based on the scores obtained, bulls from the current study were allocated to two categories: (1) negative/0 SCR deviations (NEG); or (2) positive SCR deviations (POS).

6.3.7 Enzyme-linked immunoassay

Upon thawing, all ejaculates were centrifuged at $5,000 \times g$ for 10 minutes to separate the seminal plasma from sperm pellet. The supernatant was harvested and diluted to an appropriate concentration with phosphate buffered saline. An enzyme-linked immunoassay kit (ELISA Duoset, #DY256; R&D Systems, Inc) developed to detect human β -NGF, which has ~93% homology with bovine NGF [28], was used to measure the concentrations of NGF in the seminal plasma of each bull as previously described [29]. The lower limit of detection of this assay was 31.3 pg/mL. Samples were measured in duplicate according to the manufacturer. The assay was validated for bovine by assessing the ability of serial (1:1) seminal plasma dilutions to generate displacement curves parallel to that of ELISA standard recombinant human β -NGF. Intra-assay CV was measured to be ~7%.

6.3.8 Statistical analysis

Data are presented as percentage mean \pm SEM. All statistical analyses were performed using R Version 3.4.3 (<https://www.r-project.org/>). Normality was confirmed using a Shapiro-Wilk test of the residuals. Non-normal data was transformed using Tukey's Ladder of Powers. If transformation did not result in a normalized population, a Kruskal-Wallis rank sum test was performed. Analysis of variance was applied to parametric data using a general linear mixed model, and a Tukey HSD test was performed as needed for post-hoc analysis. Pearson's correlation coefficient test was used to evaluate associations. Coefficient of correlations were defined as strong ($r \geq 0.60$), moderate ($0.35 \leq r < 0.60$), and low correlation ($r < 0.35$). Significance was declared at $P \leq 0.05$.

6.4 Results

6.4.1 Localization of NGF and its receptor in the accessory sex glands

Relative *Trka* mRNA expression did not differ between glands ($P = 0.96$; Fig. 19B). On the contrary, relative *Ngf* mRNA expression was highest in the vesicular gland, intermediate in the ampulla, and lowest in the prostate and bulbourethral glands ($P \leq 0.03$; Fig. 19A). On immunohistochemistry staining for NGF, total percentage of negative stained area at 200 \times did not differ between glands ($P = 0.19$; Table 10). Total percentages of moderate and high intensity positive stained areas were highest in the ampulla and vesicular glands, whereas the total percentage of low intensity positive stained area was highest in the prostate gland ($P < 0.01$; Table 10). There was a strong correlation between positive NGF staining intensity and relative *Ngf* gene expression ($r = 0.62$; $P = 0.03$). Immunostaining, regardless of intensity, appeared to be localized mostly to the apical side of glandular cells in all glands, with some staining observed in the lumen of the vesicular glands (Fig. 20).

6.4.2 NGF concentrations and breeding soundness evaluation

The pre-ejaculate in the Angus cross bulls contained lower NGF concentrations ($0.06 \pm 0.03 \mu\text{g/mL}$) compared to the sperm-rich fraction ($7.2 \pm 0.8 \mu\text{g/mL}$; $P < 0.01$). Based on this data, we elected to evaluate the sperm-rich fraction only in the Holstein bulls collected by electroejaculation. The NGF concentrations in the sperm-rich fraction were higher in the Angus cross than in the Holstein bulls collected by electroejaculation ($P < 0.01$; Table 11). There was no correlation between age and seminal plasma NGF concentrations ($r < 0.2$; $P > 0.05$). All Holstein (10/10) and 66% (27/41) Angus cross bulls were classified as satisfactory potential breeders based

on criteria for breeding soundness established by the Society for Theriogenology (>70% normal sperm morphology AND >30% motility) [26]. Bulls not classified as satisfactory failed due to high percentages of sperm morphology defects. Due to the differences between breeds, breeding soundness parameters were assessed in the Angus cross bulls only. Satisfactory bulls had lower NGF concentrations in their pre-ejaculate (37 ± 16 ng/mL) than unsatisfactory bulls (105 ± 72 ng/mL; $P < 0.01$). There were no differences in NGF concentrations of the sperm-rich fraction between satisfactory (7.0 ± 9.3 µg/mL) and unsatisfactory bulls (7.8 ± 14 µg/mL; $P=0.90$). Additionally, there were no differences in NGF concentrations of pre-ejaculate or sperm-rich fractions based on motility classification ($P \geq 0.53$). However, the percentage of primary sperm defects was strongly correlated with NGF concentrations in the pre-ejaculate ($r = 0.60$; $P < 0.01$) and had low correlation with NGF concentrations in the sperm-rich fraction ($r = 0.28$; $P = 0.07$).

6.4.3 NGF concentrations and sire conception rates

Holstein bulls had similar seminal plasma NGF concentrations as Jersey bulls when collected by artificial vagina ($P = 0.99$; Table 11). Holstein bulls collected by electroejaculation had lower seminal plasma NGF concentrations than those collected by artificial vagina ($P < 0.01$; Table 11). For sire conception rate deviations, bulls in the NEG category ($n = 15$; 4.1 ± 0.5 µg/mL) had lower seminal plasma NGF concentrations than those in the POS category ($n = 45$; 5.3 ± 0.4 µg/mL; $P = 0.05$).

6.5 Discussion

The current study is the first to compare NGF distribution amongst the accessory sex glands of bulls using both quantitative gene expression and immunohistochemistry. Relative gene

expression in the current study indicated that *Ngf* mRNA is highest in the vesicular gland and intermediate in the ampulla, with low expression in the bulbourethral and prostate glands. Our immunohistochemistry findings are consistent with others that observed highest NGF staining intensity in the ampulla and vesicular glands in the bull [30,31]. Consistently, there were low to moderate amounts of NGF staining intensity in the prostate and bulbourethral glands [31]. These findings are also in agreement with previous studies in bulls that reported moderate levels of NGF in the prostate [16,17] and high levels in the vesicular glands [18] using biological assays. Interestingly, NGF is strongly localized to the prostate gland in llamas [21,31] and rabbits [32], both of which are induced ovulators. Since llamas lack a vesicular gland, the observation that vesicular and ampulla glands, rather than the prostate, are the major contributor of this protein in bulls is likely a manifestation of differing anatomy across species [19,20].

The seminal plasma in the sperm-rich fraction of the bull ejaculate is the most abundant source of NGF when compared to the pre-ejaculate fraction. It is worth mentioning that since the seminal plasma was flash-frozen prior to removing spermatozoa, the lysed cells may have contributed additional sperm-bound NGF to the sperm-rich fraction. Nevertheless, this finding suggests that the vesicular gland and ampulla are major contributors of NGF compared to the prostate and bulbourethral glands. An earlier study found that the pre-ejaculate fluid produced during sexual preparation is mostly prostatic and bulbourethral in origin, whereas the sperm-rich fraction contains fluid primarily from the vesicular gland, ampulla, and epididymides [33]. Though we did not evaluate the contribution from the epididymis, previous work reported little to no NGF immunoreactivity in the epithelium or lumen of the bull epididymis, suggesting minimal contribution to the seminal plasma [31]. The secretion of NGF into the sperm-rich fraction of the bull ejaculate is likely another species-specific manifestation related to its function. While bulls

copulate within a matter of seconds, copulation lasts around 30 minutes in llamas, with sperm ejection not occurring until after 5 to 10 minutes [34]. Since llamas undergo multiple emissions and ejaculations during this prolonged copulation period, continuous secretion into any portion of the ejaculate will allow for NGF to exert its ovulation-inducing role within the female reproductive tract [19,29,35]. On the contrary, concentration of NGF into the sperm-rich fraction of the bull allows for it to enter the female reproductive tract and influence ovulation and corpus luteum formation [5–7].

It is interesting that higher pre-ejaculate NGF concentrations were found in bulls deemed unsatisfactory breeders than those classified as satisfactory in this study. The positive association between NGF concentrations in the pre-ejaculate and the percentage of primary sperm defects seems to have contributed to this association. Since primary sperm defects occur from insults to spermatogenesis, it is unlikely that the higher NGF concentrations directly caused the sperm abnormalities observed. It is possible that the time to ejaculation may have been prolonged in these bulls, increasing the amount of pre-ejaculate produced, though the procedures were not timed to confirm this notion. Additionally, the cause of the spermatogenesis insult could also have caused a dysregulation in NGF secretion into the pre-ejaculate. Primary sperm defects can result from either prolonged environmental stressors or disruption in testosterone production [36]. Similarly, the secretory activity of accessory sex glands are largely influenced by testosterone in bulls [37,38]. Interestingly, there was only a low correlation between primary sperm defects and NGF concentrations in the sperm-rich fraction, suggesting that a potential dysregulation primarily occurs during the pre-ejaculatory phase. Further studies are needed to determine if testosterone or other factors may be involved in influencing NGF production and secretion in the bull and how it relates to semen quality.

Seminal plasma concentrations of NGF were lower in Holstein bulls collected by electroejaculation than those collected by artificial vagina. This finding may be related to the stimulation of more prostatic and bulbourethral gland secretions by the electrical stimulus, causing a dilution effect. In contrast, electroejaculation increased the abundance of several seminal plasma proteins of accessory sex gland origin in Brahman bulls [39]. Many of the proteins evaluated in this previous study were suspected to be prostatic in origin and may explain the discrepancy observed. Interestingly, the seminal plasma concentrations of NGF in the Angus cross bulls collected by electroejaculation were highest out of all of the other groups. However, this was the only group of bulls collected in the fall, which may have contributed to the difference. Seasonal changes in *Ngf* mRNA expression has been reported in the reproductive tract of both male and female ground squirrels [40,41]. Though bulls are not considered seasonal species, studies have reported seasonal fluctuations in testosterone production [42–44], which could correspond with changes in seminal plasma composition. Interestingly, in three separate proteomic studies using Holsteins, *Bos taurus*, and *Bos indicus* bulls, only 22 out of hundreds of proteins identified in bull seminal plasma overlapped [45–47]. Collectively, these findings all suggest that there could be variation in seminal plasma NGF concentrations based on species, breed, age, season, and or sampling techniques. While there were differences in age between the collection groups in the current study, we found no correlation with seminal plasma NGF concentrations. There is, therefore, a distinct need to evaluate each of these factors separately to better understand the biology of NGF production within the bull.

Low relative gene expression of the NGF receptor, *Trka*, throughout the four accessory sex glands suggests that NGF has minimal direct influence on accessory sex gland function in the bull. Instead, NGF likely exhibits most of its effects on the spermatozoa or within the female

reproductive tract. Ejaculated bovine sperm have NGF-immunoreactivity localized to the sperm head and tail and its receptor (TrkA) in the acrosomal cap, nucleus, and tail regions [4]. In addition to several other proteins, sperm *Ngf* mRNA abundance in bulls was strongly associated with positive sire conception rates [13], which is attributed to its direct effects on sperm membrane integrity [4]. Seminal plasma *Ngf* mRNA expression was found to be positively associated with the maintenance of post-thaw functional membrane integrity in bull sperm, suggesting a role in improving fertility in bulls collected for cryopreservation and artificial insemination [14]. Consistently, we observed an association between seminal plasma NGF concentrations and positive sire conception rates in bulls collected by artificial vagina. Further identified roles for NGF include increasing expression of FSH and LH receptors in bovine oviduct epithelial cells [22] and enhancing CL function in heifers and cows [5,7]. Collectively, these studies suggest that the beneficial role of NGF on sire conception rates acts through both its effects in the male and female reproductive tracts and warrants further investigation.

In conclusion, this study confirms that NGF production and secretion occurs primarily in the ampulla and vesicular glands of the bull. Consistently, NGF is secreted primarily into the sperm-rich fraction of the ejaculate and is positively associated with sire conception rates. Pre-ejaculate NGF concentrations are positively correlated with percentage of primary sperm defects and may be a potential marker for dysregulation of spermatogenesis. Further studies are needed to determine what factors (season, testosterone production, stressors) may influence both spermatogenesis and accessory sex gland function to better understand this relationship. Likewise, future studies aimed at determining the roles of NGF on both cryopreservation of semen and within the female reproductive tract may help us to determine its usefulness in improving bovine reproductive efficiency.

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6.7 Tables and Figures

Table 9. Primer reference and sequences for gene expression of nerve growth factor- β (*Ngf*) and its receptor, tyrosine kinase A (*Trka*), investigated by quantitative PCR in bull accessory sex glands.

Gene	Accession Number	Primer Sequence	Length
Glyceraldehyde-3-phosphate dehydrogenase (<i>Gapdh</i>)	NM_001034034.2	5'-GGCGCCAAGAGGGTCAT-3' 5'-GGTGGTGCAGGAGGCATT-3'	120
Nerve growth factor- β (<i>Ngf</i>)	NM_001099362.1	5'-TCAACAGGACTCACAGGAGCAA-3' 5'-ACCTCTCCCAGCACCATCAC -3'	151
Tyrosine kinase A (<i>Trka</i>)	XM_613650.9	5'-CTGGGTGAGGGTGCCTTT-3' 5'-CGCTCTCAGACACCTCCTTCA-3'	112

Table 10. Percentage of high, moderate, and low intensity positive and negative NGF IHC staining (mean \pm SEM) in the ampulla, bulbourethral (BBG), prostate, and vesicular (VG) glands at 200 \times . Differing letters denote differences between glands ($P < 0.05$).

	% High Positive	% Moderate Positive	% Low Positive	% Negative
Ampulla	0.27 \pm 0.13 ^a	30 \pm 10 ^a	36 \pm 4.6 ^a	34 \pm 5.8
BBG	0.07 \pm 0.00 ^b	13 \pm 3.3 ^{ab}	46 \pm 2.6 ^b	39 \pm 2.0
Prostate	0.08 \pm 0.00 ^b	6.1 \pm 1.3 ^b	59 \pm 2.2 ^c	35 \pm 3.3
VG	0.17 \pm 0.02 ^a	28 \pm 1.4 ^a	43 \pm 1.5 ^{ab}	29 \pm 2.0

Table 11. Differences in seminal plasma NGF concentrations (mean \pm SEM) based on bull breed and semen collection method. Differing letters denote differences between groups ($P < 0.05$).

Bulls	Age (y)	Collection method	Season	NGF concentration ($\mu\text{g/mL}$)
Angus cross (n = 42)	3.3 \pm 0.3	Electroejaculation	Fall	7.19 \pm 0.78 ^a
Holstein (n = 10)	4.2 \pm 0.5	Electroejaculation	Spring	0.73 \pm 0.10 ^b
Holstein (n = 76)	1.8 \pm 0.1	Artificial vagina	Spring	5.19 \pm 0.27 ^c
Jersey (n = 14)	1.7 \pm 0.3	Artificial vagina	Spring	5.27 \pm 0.77 ^{ac}

Figure 19. Relative mRNA expression of nerve growth factor- β (*Ngf*) and its receptor, tyrosine kinase A (*Trka*), in the ampulla, bulbourethral (BBG), prostate, and vesicular (VG) glands. Differing letters denote differences between glands ($P < 0.05$).

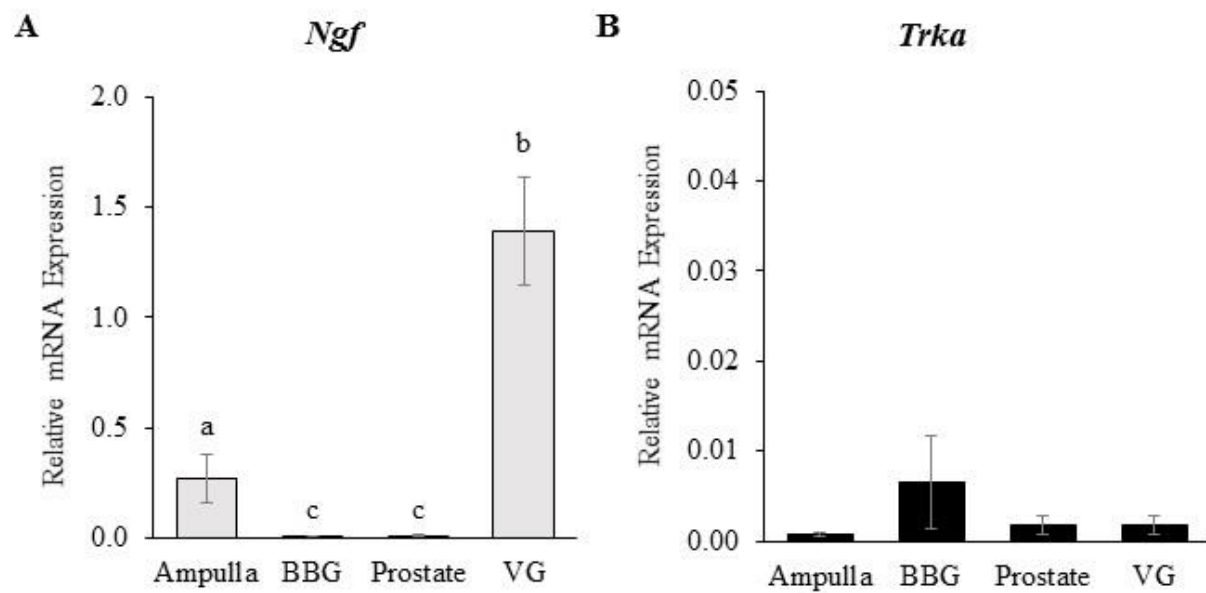
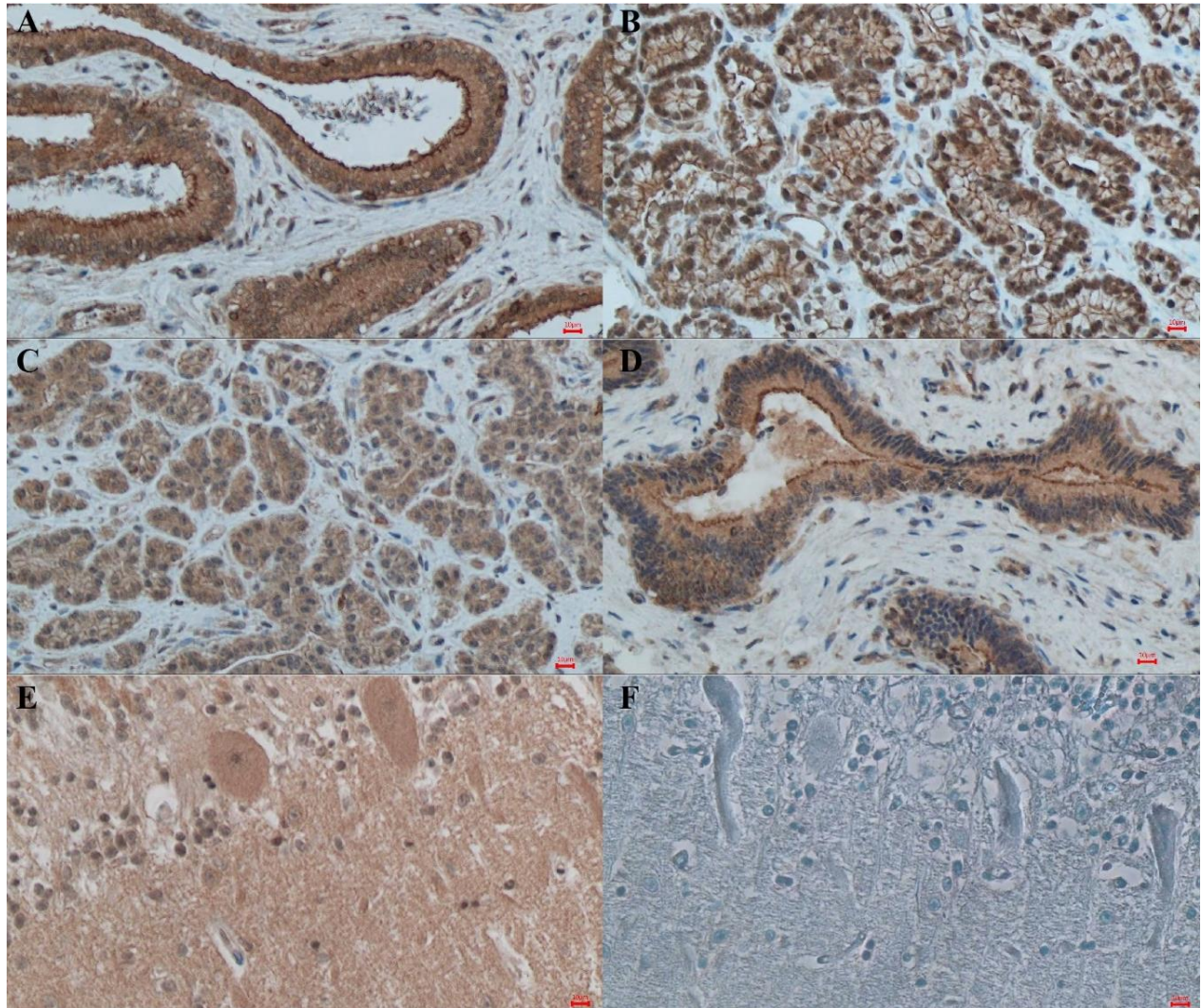


Figure 20. Representative immunolocalization of NGF in bull reproductive organs evaluated at 20×. A) Strong immunoreaction is localized on the apical side of the glandular cells of the ampulla. B) Low to moderate positive immunostaining localized in the bulbourethral glandular cells. C) Low to moderate positive immunostaining localized in the prostate glandular cells. D) Strong immunoreaction is localized on the apical side of the glandular cells of the vesicular gland. Positive staining also appears within the lumen. E) NGF stained cells in Purkinje cells of cerebellum used as positive control. F) Negative control, in which the primary antibody was omitted, showing lack of reactivity in the cerebellum. Scale bars = 10 μ m.



6.8 References

- [1] Erikson DW, Way AL, Chapman DA, Killian GJ. Detection of osteopontin on Holstein bull spermatozoa, in cauda epididymal fluid and testis homogenates, and its potential role in bovine fertilization. *Reproduction* 2007;133:909–17. doi:10.1530/REP-06-0228.
- [2] Gwathmey TM, Ignatz GG, Mueller JL, Manjunath P, Suarez SS. Bovine seminal plasma proteins PDC-109, BSP-A3, and BSP-30-kDa share functional roles in storing sperm in the oviduct. *Biol Reprod* 2006;75:501–7. doi:10.1095/biolreprod.106.053306.
- [3] Killian GJ, Chapman DA, Rogowski LA. Fertility-associated proteins in Holstein bull seminal plasma. *Biol Reprod* 1993;49:1202–7. doi:10.1095/biolreprod.49.6.1202.
- [4] Li C, Sun Y, Yi K, Ma Y, Sun Y, Zhang W, et al. Detection of nerve growth factor (NGF) and its specific receptor (TrkA) in ejaculated bovine sperm, and the effects of NGF on sperm function. *Theriogenology* 2010;74:1615–22. doi:10.1016/j.theriogenology.2010.06.033.
- [5] Tanco VM, Van Steelandt MD, Ratto MH, Adams GP. Effect of purified llama ovulation-inducing factor (OIF) on ovarian function in cattle. *Theriogenology* 2012;78:1030–9. doi:10.1016/j.theriogenology.2012.03.036.
- [6] Tribulo P, Bogle OA, Mapletoft RJ, Adams GP. Bioactivity of ovulation inducing factor (or nerve growth factor) in bovine seminal plasma and its effects on ovarian function in cattle. *Theriogenology* 2015;83:1394–401. doi:10.1016/j.theriogenology.2014.12.014.
- [7] Stewart JL, Mercadante VRG, Dias NW, Canisso IF, Yau P, Imai B, et al. Nerve Growth Factor-Beta, purified from bull seminal plasma, enhances corpus luteum formation and conceptus development in *Bos taurus* cows. *Theriogenology* 2018;106:30–8. doi:10.1016/j.theriogenology.2017.10.007.
- [8] Hayes BJ, Chamberlain AJ, Maceachern S, Savin K, McPartlan H, MacLeod I, et al. A genome map of divergent artificial selection between *Bos taurus* dairy cattle and *Bos taurus* beef cattle. *Anim Genet* 2009;40:176–84. doi:10.1111/j.1365-2052.2008.01815.x.
- [9] Schjenken JE, Robertson SA. Seminal fluid and immune adaptation for pregnancy - comparative biology in mammalian species. *Reprod Domest Anim* 2014;49:27–36. doi:10.1111/rda.12383.
- [10] Lima FS, Risco CA, Thatcher MJ, Benzaquen ME, Archbald LF, Santos JEP, et al. Comparison of reproductive performance in lactating dairy cows bred by natural service or timed artificial insemination. *J Dairy Sci* 2009;92:5456–66. doi:10.3168/jds.2009-2197.
- [11] Dalton JC, Nadir S, Bame JH, Noftsinger M, Nebel RL, Saacke RG. Effect of time of insemination on number of accessory sperm, fertilization rate, and embryo quality in nonlactating dairy cattle. *J Dairy Sci* 2001;84:2413–8. doi:10.3168/jds.S0022-0302(01)74690-5.
- [12] Pelissier CL. Dairy cattle breeding problems and their consequences. *Theriogenology* 1976;6:575–83. doi:10.1016/0093-691X(76)90125-4.
- [13] Kasimanickam V, Kasimanickam R, Arangasamy A, Saberivand A, Stevenson JS, Kastelic JP. Association between mRNA abundance of functional sperm function proteins and fertility of Holstein bulls. *Theriogenology* 2012;78:2007–19.

- doi:10.1016/j.theriogenology.2012.07.016.
- [14] Shilpa M, Selvaraju S, Girishkumar V, Parthipan S, Binsila KB, Arangasamy A, et al. Novel insights into the role of cell-free seminal mRNAs on semen quality and cryotolerance of spermatozoa in bulls (*Bos taurus*). *Reprod Fertil Dev* 2017;29:2446–56. doi:10.1071/RD16290.
 - [15] Parthipan S, Selvaraju S, Somashekar L, Arangasamy A, Sivaram M, Ravindra JP. Spermatozoal transcripts expression levels are predictive of semen quality and conception rate in bulls (*Bos taurus*). *Theriogenology* 2017;98:41–9. doi:10.1016/j.theriogenology.2017.04.042.
 - [16] Harper GP, Thoenen H. The distribution of nerve growth factor in the male sex organs of mammals. *J Neurochem* 1980;34:893–903. doi:10.1111/j.1471-4159.1980.tb09663.x.
 - [17] Harper GP, Glanville RW, Thoenen H. The purification of nerve growth factor from bovine seminal plasma. Biochemical characterization and partial amino acid sequence. *J Biol Chem* 1982;257:8541–8.
 - [18] Hofmann H-D, Unsicker K. The seminal vesicle of the bull: a new and very rich source of nerve growth factor. *Eur J Biochem* 1982;128:421–6. doi:10.1111/j.1432-1033.1982.tb06981.x.
 - [19] Adams GP, Ratto MH, Huanca W, Singh J. Ovulation-inducing factor in the seminal plasma of alpacas and llamas. *Biol Reprod* 2005;73:452–7. doi:10.1095/biolreprod.105.040097.
 - [20] Ratto MH, Leduc YA, Valderrama XP, van Straaten KE, Delbaere LTJ, Pierson RA, et al. The nerve of ovulation-inducing factor in semen. *Proc Natl Acad Sci U S A* 2012;109:15042–7. doi:10.1073/pnas.1206273109.
 - [21] Bogle OA, Singh J, Adams GP. 248 Distribution of ovulation-inducing factor in male reproductive tissues of llamas. *Reprod Fertil Dev* 2012;25:272. doi:10.1071/RD03082.
 - [22] Li C, Ma Y, Yi K, Wang C, Li W, Liu Z, et al. The interactions between nerve growth factor and gonadotrophins in bovine oviduct. *Anim Reprod Sci* 2014;149:117–23. doi:10.1016/j.anireprosci.2014.06.030.
 - [23] Colitti M, Loor JJ, Stefanon B. Expression of NGF, BDNF and their receptors in subcutaneous adipose tissue of lactating cows. *Res Vet Sci* 2015;102:196–9. doi:10.1016/j.rvsc.2015.08.016.
 - [24] Varghese F, Bukhari AB, Malhotra R, De A. IHC profiler: An open source plugin for the quantitative evaluation and automated scoring of immunohistochemistry images of human tissue samples. *PLoS One* 2014;9. doi:10.1371/journal.pone.0096801.
 - [25] Kuster CE, Singer RS, Althouse GC. Determining sample size for the morphological assessment of sperm. *Theriogenology* 2004;61:691–703. doi:10.1016/S0093-691X(03)00240-1.
 - [26] Chenoweth PJ, Hopkins FM, Spitzer JC, Larsen RE. Guidelines for using the bull breeding soundness evaluation form. *Clin Theriogenology* 2010;2:43–50.
 - [27] Norman HD, Hutchison JL, Wright JR. Sire conception rate: new national AI bull fertility evaluation. *USDA AIPL Res Rep SCR1* 2008. <https://aipl.arsusda.gov/reference/arr->

scr1.htm.

- [28] Meier R, Becker-André M, Götz R, Heumann R, Shaw A, Thoenen H. Molecular cloning of bovine and chick nerve growth factor (NGF): delineation of conserved and unconserved domains and their relationship to the biological activity and antigenicity of NGF. *EMBO J* 1986;5:1489–93. doi:10.1002/j.1460-2075.1986.tb04387.x.
- [29] Berland MA, Ulloa-Leal C, Barria M, Wright H, Dissen GA, Silva ME, et al. Seminal plasma induces ovulation in llamas in the absence of a copulatory stimulus: role of nerve growth factor as an ovulation-inducing factor. *Endocrinology* 2016;157:3224–32. doi:10.1210/en.2016-1310.
- [30] Adams GP, Ratto MH, Silva ME, Carrasco RA. Ovulation-inducing factor (OIF/NGF) in seminal plasma: a review and update. *Reprod Domest Anim* 2016;51:4–17. doi:10.1111/rda.12795.
- [31] Bogle OA. Nerve growth factor: its role in male fertility as an ovulation inducer. University of Saskatchewan, 2015.
- [32] Maranesi M, Zerani M, Leonardi L, Pistilli A, Arruda-Alencar J, Stabile AM, et al. Gene expression and localization of NGF and its cognate receptors NTRK1 and NGFR in the sex organs of male rabbits. *Reprod Domest Anim* 2015;50:918–25. doi:10.1111/rda.12609.
- [33] Seidel GEJ, Foote RH. Compartmental analysis of sources of the bovine ejaculate. *Biol Reprod* 1970;2:189–96. doi:10.1095/biolreprod2.2.189.
- [34] Lichtenwalner ABB, Woods GLL, Weber JAA. Seminal collection, seminal characteristics and pattern of ejaculation in llamas. *Theriogenology* 1996;46:293–305. doi:10.1016/0093-691X(96)00186-0.
- [35] Tanco VM, Ratto MH, Lazzarotto M, Adams GP. Dose-response of female llamas to ovulation-inducing factor from seminal plasma. *Biol Reprod* 2011;85:452–6. doi:10.1095/biolreprod.111.091876.
- [36] Barth AD, Bowman PA. The sequential appearance of sperm abnormalities after scrotal insulation or dexamethasone treatment in bulls. *Can Vet J* 1994;35:93–102.
- [37] Lindner HR, Mann T. Relationship between the content of androgenic steroids in the testes and the secretory activity of the seminal vesicles in the bull. *J Endocrinol* 1960;21:341–NP. doi:10.1677/joe.0.0210341.
- [38] Nass SJ, Miller DJ, Winer MA, Ax RL. Male accessory sex glands produce heparin-binding proteins that bind to cauda epididymal spermatozoa and are testosterone dependent. *Mol Reprod Dev* 1990;25:237–46. doi:10.1002/mrd.1080250305.
- [39] Rego JPA, Moura AA, Nouwens AS, McGowan MR, Boe-Hansen GB. Seminal plasma protein profiles of ejaculates obtained by internal artificial vagina and electroejaculation in Brahman bulls. *Anim Reprod Sci* 2015;160:126–37. doi:10.1016/J.ANIREPROSCI.2015.07.015.
- [40] Li B, Sheng X, Bao L, Huang S, Li Q, Liu Y, et al. Seasonal changes in expression of nerve growth factor and its receptors TrkA and p75 in the ovary of wild ground squirrel (*Citellus dauricus* Brandt). *J Ovarian Res* 2014;7:1–9. doi:10.1186/1757-2215-7-3.

- [41] Zhang H, Wang Y, Zhan J, Wang L, Li Q, Sheng X, et al. Testicular expression of NGF, TrkA and p75 during seasonal spermatogenesis of the wild ground squirrel (*Citellus dauricus* Brandt). *Eur J Histochem* 2015;59:16–22. doi:10.4081/ejh.2015.2522.
- [42] Chacur MGM, Mizusaki KT, Filho LRAG, Oba E, Ramos AA. Seasonal effects on semen and testosterone in zebu and taurine bulls. *Acta Sci Vet* 2013;41:1–5.
- [43] Jiménez-Severiano H, Quintal-Franco J, Vega-Murillo V, Zanella E, Wehrman ME, Lindsey BR, et al. Season of the year influences testosterone secretion in bulls administered luteinizing hormone. *J Anim Sci* 2003;81:1023–9. doi:10.2527/2003.8141023x.
- [44] Stewart JL, Shipley CF, Ireland FA, Jarrell VL, Timlin CL, Shike DW, et al. Long-term effects of pyrethrin and cyfluthrin, a type II synthetic pyrethroid, insecticide applications on bull reproductive parameters. *Reprod Domest Anim* 2016;51. doi:10.1111/rda.12729.
- [45] Westfalewicz B, Dietrich MA, Mostek A, Partyka A, Bielas W, Niżański W, et al. Analysis of bull (*Bos taurus*) seminal vesicle fluid proteome in relation to seminal plasma proteome. *J Dairy Sci* 2016;100:1–17. doi:10.3168/jds.2016-11866.
- [46] Kelly VC, Kuy S, Palmer DJ, Xu Z, Davis SR, Cooper GJ. Characterization of bovine seminal plasma by proteomics. *Proteomics* 2006;6:5826–33. doi:10.1002/pmic.200500830.
- [47] Rego JPA, Crisp JM, Moura AA, Nouwens AS, Li Y, Venus B, et al. Seminal plasma proteome of electroejaculated *Bos indicus* bulls. *Anim Reprod Sci* 2014;148:1–17. doi:10.1016/j.anireprosci.2014.04.016.

Chapter 7:

Effects of Nerve Growth Factor- β Supplementation on Cryopreservation of Electro-ejaculated and Epididymal Harvested Bull Semen

7.1 Abstract

Nerve growth factor- β (NGF) is a seminal plasma protein associated with improved sperm membrane integrity and motility in mammalian species. The objective of this study was to compare post-thaw semen quality from ejaculated and epididymal-harvested bull sperm incubated with purified NGF prior to cryopreservation. Semen was obtained from Angus \times Simmental crossbred bulls ($n = 10$) collected by electroejaculation, followed by castration and epididymal sperm harvest 3 days later. Samples were pre-extended with a commercial extender, and sperm motility was assessed using computer-aided sperm analysis. Each sample was divided into one of four treatments to achieve a final NGF concentration of 0 ng/mL (CONT), 0.5 ng/mL (LOW), 5 ng/mL (MED), or 50 ng/mL (HIGH). Semen was frozen manually in 0.5-mL straws. Frozen straws were thawed and incubated at 37°C for post-thaw motility assessment in 30 min intervals for 4 h. Samples were stained with fluorescent probes for evaluation of sperm viability (SYBR-14/PI), acrosomal integrity (FITC-PNA/PI), and chromatin stability (acridine orange) using flow cytometry. Kruskal-Wallis rank sum test and ANOVA were used for statistical analyses, with significance set at $P < 0.05$. Post-thaw sperm motility and velocity parameters were decreased, while linearity (LIN) was increased in HIGH versus CONT ejaculated samples ($P < 0.01$), but no differences were observed in epididymal samples ($P = 0.22$). HIGH ejaculated samples also exhibited a lower amplitude of lateral head displacement (ALH) at 2.5 and 3 h post-thaw ($P < 0.01$). Post-thaw viability, acrosome integrity, and DNA fragmentation index were not affected by NGF treatment in either ejaculated or epididymal sperm ($P \geq 0.15$). In conclusion, treatment with

NGF did not significantly improve cryotolerance of sperm collected by electroejaculation or epididymal harvest in bulls. Supplementation of extender with high concentrations of NGF decreased post-thaw curvilinear velocity and ALH and increased LIN, which may suggest a role in preventing premature sperm hyperactivation and capacitation. Though the current study does not support a role of NGF in improving sperm cryotolerance, further studies should address the effects it may have on fertility within the female reproductive tract.

7.2 Introduction

Several studies have demonstrated an association between seminal plasma proteins and bull fertility [1–4]. The role of seminal plasma proteins in regulating fertility is of special relevance to the cattle industry where artificial insemination has advanced genetic selection of desirable traits such as milk production and meat quality [5]. Historically, bull semen studies have focused on improving semen freezing techniques and determining the minimum effective breeding dose for artificial insemination, while overlooking the effects that semen processing and freezing may have on seminal plasma protein functions [6]. Though early studies identified nerve growth factor- β (NGF) in the seminal plasma of bulls [7,8], little is known regarding its function in bovine reproduction. Seminal plasma NGF has been implicated as a signaling molecule in the female reproductive tract with potential roles in increasing expression of gonadotropin receptors in bovine oviduct epithelial cells [9] and enhancing corpus luteum function in heifers [10] and cows [11,12].

In addition to several other proteins, sperm *Ngf* mRNA abundance in bulls was strongly associated with positive sire conception rates [13], which was attributed to beneficial effects of NGF on sperm membrane integrity [14]. An immunolabelling study of ejaculated bovine sperm revealed that NGF was localized to the sperm head and tail, while its tyrosine kinase A receptor

(TrkA) was present in the acrosomal cap, nucleus, and tail regions [14], suggesting that NGF may play a physiological role on sperm function. Consistently, another study found that seminal plasma concentrations of NGF were also positively associated with sire conception rates [15]. Seminal plasma *Ngf* mRNA expression was also found to be positively associated with the maintenance of post-thaw functional membrane integrity in bull spermatozoa, suggesting that it could be used to assess cryotolerance [16]. Another study reported higher spermatozoal *Ngf* expression in good semen-producing (<25% discarded ejaculates) versus poor semen-producing (>40% discarded ejaculates) bulls, though no correlation between *Ngf* expression and field conception rates was detected [17]. Consistently, relative *Ngf* expression levels were also positively associated with pre-freeze mitochondrial membrane potential and post-thaw sperm velocity parameters in *Bos taurus* bulls [17]. In both normozoospermic and asthenozoospermic men, supplementation of freezing extender with NGF at 0.5 ng/mL significantly improved sperm viability and motility and decreased DNA fragmentation [18,19]. Consistently, treatment of frozen-thawed bovine sperm cells with 40 to 80 ng/mL NGF increased both leptin secretion and sperm membrane integrity [14].

Recent studies assessing the distribution of NGF in the bull reproductive tract determined that it is mainly expressed in the accessory sex glands [15,20]. Since these glands are the main source of the seminal plasma [2] and epididymal-harvested sperm is deprived of the secretions of these glands, this type of semen allows for some insights on the putative role of NGF of non-ejaculated sperm. Freezing of epididymal semen is not typically performed in bulls, but in select cases of sudden death or injury of a genetically valuable bull, harvesting epididymal sperm for cryopreservation can be useful [21]. Based on all the aforementioned findings, it seems reasonable to suggest that NGF may be an optimal candidate protein to enhance sperm cryopreservation techniques in bulls. Thus, the objective of the current study was to compare post-thaw semen

quality from both ejaculated and epididymal-harvested bull sperm extended with final concentrations of 0 ng/mL (CONT), 0.5 ng/mL (LOW), 5 ng/mL (MED), or 50 ng/mL (HIGH) of NGF prior to cryopreservation.

7.3 Materials and Methods

7.3.1 Animal Care and Use

Animals in this trial were cared for in accordance with guidelines in the Guide for the Care and Use of Agricultural Animals in Agriculture Research and Teaching [22]. All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Illinois in Urbana-Champaign.

7.3.2 Animals

Angus × Simmental crossbred bulls (n=10; age: 15.1 ± 0.05 m) were housed at the University of Illinois Beef Farm in Urbana, IL. Bulls weighed 585 ± 17 kg and had a body condition score of 5.8 ± 0.2 out of 9 using a previously validated scoring system [23]. A complete breeding soundness examination performed 30 days before enrollment in the experiment was unremarkable. Scrotal circumference was measured in cm using a scrotal tape measure placed around the widest portion on the scrotum. The average scrotal circumference was 40.5 ± 0.6 cm (range: 36 to 42 cm), which is in accordance with the criterion determined by the Society for Theriogenology to classify a bull as a satisfactory potential breeder [24].

7.3.3 Semen collection by electroejaculation

All bulls were collected by electroejaculation. To obtain semen samples, a 60 mm upright weighted bull probe (Lane Manufacturing, Inc., Denver, CO) was placed rectally, and the programmed cycle on the electroejaculator (Pulsator IV, Lane Manufacturing, Inc.,) was used. If an appropriate ejaculate was not obtained, the electroejaculator cycle was run manually by an experienced clinician. Semen was collected into 15 mL conical tubes using collecting handles coupled with a disposal plastic cone. An aliquot of each ejaculate was formalin-fixed for morphological later analysis. The remainder of the sample was diluted with pre-warmed (37°C) Optixcell extender (IMV Technologies, Maple Grove, MN) at a 1:1 ratio and transported approximately 30 min later to the investigators' laboratory in a 37°C water bath, where further processing and analyses were performed as described below.

7.3.4 Epididymal sperm harvest

Three days after semen collection, all the bulls were castrated using a Newberry knife, an emasculator and ligature of the spermatic cord. A pudendal block and local anesthesia were performed with 40 and 20 ml of 2% lidocaine, respectively. To prevent pain post-castration, the bulls were administered flunixin meglumine (3.3mg/kg transdermal pour-on, Merck Animal Health, Madison, NJ) and then repeated as needed based on bull's demeanor (i.e., depressed, off feed, laying down). Bull demeanor, food intake, rectal temperature, and surgical incision were monitored daily by the investigators with the help of the farm personnel. All the testicles were transported to the investigators' laboratory within 30 min after castration. One testicle from each bull was subjected to sperm harvest from the cauda epididymis. Briefly, the cauda epididymis was excised and removed from the testicle and body of the epididymis using a #10 scalpel. The cauda

epididymis was rinsed with saline and placed in a petri dish. A #10 scalpel was used to dissect the cauda epididymis, and ~10 mL Optixcell extender was used to flush the semen from the tubules. Once the cauda epididymis was determined to be thoroughly flushed, an aliquot of the pre-diluted semen was formalin-fixed for morphological analysis, while the remaining semen underwent further processing for motility analyses and cryopreservation. One bull was excluded from data analyses in the epididymal sperm harvest group due to extremely poor semen quality before cryopreservation (~4% overall motility).

7.3.5 Semen analyses and cryopreservation

Sperm concentration from each sample was measured using a NucleoCounter (ChemoMetec Inc., Bohemia, NY). Semen from each bull and collection were divided into one of four treatments to achieve a final sperm concentration of 40 million spermatozoa per mL and a final NGF concentration of 0 ng/mL (CONT), 0.5 ng/mL (LOW), 5 ng/mL (MED), or 50 ng/mL (HIGH). The NGF used was purified from bovine seminal plasma and used previously for *in vivo* studies in cows [11].

Computer-aided sperm analysis (SpermVision, MiniTube of America, Inc., Verona, WI) was used to measure overall and progressive motilities in pre-freeze CONT samples using a phase-contrast microscope with a heated (37°C) stage (20×; Olympus BX41; Olympus Corporation, Center Valley, PA). Data were obtained by averaging motility measurements of a minimum of 500 cells or seven fields from various portions of the slide. Output measurements used to describe sperm motion included average path velocity (VAP, $\mu\text{m/s}$), straight line velocity (VSL, $\mu\text{m/s}$), curvilinear velocity (VCL, $\mu\text{m/s}$), straightness of the average path (STR, %), linearity of the curvilinear path (LIN, %), wobble (WOB, %), amplitude of lateral head displacement (ALH, μm),

and beat cross frequency (BCF, number per second) [25]. CASA-established setup parameters were as follows per manufacturer defaults for bovine sperm: frame capture speed rate, 60 Hz; cell size (min/max), 18/60 μm^2 ; threshold straightness, 50%; VAP cutoff, 56 $\mu\text{m/s}$; and VSL cutoff, 28 $\mu\text{m/s}$.

Immediately following the final dilution, the samples were manually loaded into 0.5-mL semen straws, sealed ultrasonically, and incubated in a cold room at 5°C for 3 to 5 h per manufacturer's recommendation. Following incubation, straws were placed at 4 cm above the level of liquid nitrogen for 15 min and then submerged and stored in liquid nitrogen until post-thaw analysis. Straws were thawed in a water bath at 37°C for 1 min. The contents of each straw were dispensed into a 1.8 mL Eppendorf tube, homogenized and then incubated at 37°C for 4 h, while motility parameters were assessed every 30 min intervals. The remainder of each thawed straw was formalin-fixed for post-thaw morphological analysis. One clinician evaluated 100 sperm cells from each sample under 100 \times magnification with a phase contrast microscope, classified each sperm cell as normal or abnormal, and further defined abnormalities as primary or secondary defects, as previously described [24,26].

7.3.6 Fluorescent probes for sperm evaluation

The flow cytometry assays used in the present study were performed according to protocols previously published [27–29]. Semen straws (40 million spermatozoa/mL) from each bull and treatment were thawed individually and subjected to each stain as described below. The LIVE/DEAD® Sperm Viability Kit (SYBR-14 and propidium iodide (PI) dyes; #L-7011) was purchased from Molecular Probes, Inc. (Eugene, Oregon). Fluorescein isothiocyanate-conjugated pea (*Pisum sativum*) agglutinin (FITC-PNA; #L-7381) was obtained from Sigma Chemical Co.

(Saint Louis, Missouri). Acridine orange, C.I. (#04539-500) was purchased from Polysciences, Inc. (Warrington, Pennsylvania). Dimethyl sulfoxide, anhydrous (DMSO; #D12345), triton X-100 surfact-amps detergent solution (#85111), and phosphate-buffered saline (PBS, pH 7.4) (#10010-023) were purchased from Thermo Fischer Scientific (Waltham, Massachusetts). All other chemicals used were reagent grade and purchased from Sigma Aldrich (St. Louis, Missouri).

7.3.7 Assessment of sperm viability

Sperm viability was assessed using a combination of SYBR-14 and propidium iodide (PI) based on the integrity of the sperm plasma membrane [29]. SYBR-14 was used as a membrane-permeable DNA intercalating agent with maximum emission of 516 nm (green) and readily stains all nuclei. Propidium iodide was also used as an intercalating agent with maximum emission of 617 nm (red), but it only stains the nucleus if the sperm plasma membrane is damaged which makes it a useful counterstain for dead cells. Briefly, stock solutions of SYBR-14 (0.02 mM in DMSO) and PI (2.4 mM in water) were prepared and frozen in aliquots at -20°C for subsequent use. Staining solution was prepared on the day of flow cytometry analysis by combining 5 μL SYBR-14 (0.02 mM) and 5 μL PI (2.4 mM) per mL of HEPES buffer (10 mM Hepes, 150 mM NaCl, 0.1% BSA, pH 7.4). Working concentrations of SYBR-14 and PI were 100 nM and 12 μM , respectively. Cryopreserved semen was thawed in a 37°C water bath for 1 min, and 25 μL of thawed semen was added to 0.5 mL staining solution to create a final concentration of 2 million spermatozoa per mL. The samples were mixed by reverse pipetting and subsequently incubated in the dark at 37°C for 10 to 15 min prior to flow cytometric analysis.

Samples were analyzed using a Cytex Aurora Flow Cytometer (CytexTM Biosciences Inc., Fremont, CA). The two dyes were excited in the flow cytometer using a 488-nm argon excitation

laser. Fluorescent data of all events was collected until 10,000 gated events were recorded. Two-dimensional plots of SYBR-14 (green) versus PI (red) fluorescence events were drawn, and debris was gated out based on those events that emitted minimal fluorescence (Fig. 21A). As previously described [29], three sperm populations in the SYBR-14/PI stained sperm preparations were present. Presumptively live cells with intact cell membranes made up population 1, with only green fluorescent signal (SYBR-14 positive) detected. Population 2 exhibited a mixed green (SYBR-14 positive) and red (PI positive) fluorescence and were considered to be moribund sperm, with early or minor membrane damage. Population 3 comprised cells with only red fluorescent signal (PI positive) detected and were considered dead. The percentage of cells in each of the three populations was calculated.

7.3.8 Assessment of acrosome integrity

Acrosome integrity was assessed using a combination of FITC-PNA (peanut agglutinin conjugated with fluorescein isothiocyanate) and PI as previously described [29]. FITC-PNA has a maximum emission of 521 nm (green) and targets the inner leaflet of the outer acrosomal membrane, identifying damaged acrosomes. Again, PI was used as a counterstain to allow for simultaneous assessment of sperm viability and condition of the acrosome. Briefly, stock solutions of FITC-PNA (1 mg/mL in PBS) were prepared and frozen in aliquots at -20°C for subsequent use [29]. Staining solution was prepared by combining 0.625 μL PI (2.4 mM) and 1 μL FITC-PNA (1 mg/mL) per mL of PBS. Final concentrations of the stains were 1.5 μM PI and 1 $\mu\text{g/mL}$ FITC-PNA. Cryopreserved semen was thawed in a 37°C water bath for 1 min, and 25 μL of thawed semen was added to 0.5 mL staining solution to create a final concentration of 2 million

spermatozoa per mL. The samples were mixed by reverse pipetting and subsequently incubated in the dark at 37°C for 10 to 15 min prior to flow cytometric analyses.

Flow cytometric analyses were carried out as described above with the exception that FITC-PNA fluorescence was detected at 515–545 nm fluorescence detector 1. Fluorescent data of all events was collected until 10,000 gated events were recorded. Non-sperm events were gated out of analyses as judged on scatter properties. Two-dimensional plots of FITC-PNA (green) versus PI (red) fluorescence events were drawn and divided into quadrants to determine the frequency of sperm in each of four populations (Fig. 21B). Population 1 included sperm that were live (PI negative) with an intact acrosome (FITC-PNA negative). Population 2 encompassed sperm that were live (PI negative) with a damaged acrosome (FITC-PNA positive). Populations 3 and 4 contained sperm that were dead (PI positive) with intact (FITC-PNA negative) or damaged (FITC-PNA positive) acrosomes, respectively.

7.3.9 Assessment of sperm chromatin stability

Chromatin stability was assessed by meta-chromatic staining with acridine orange based on the susceptibility of the sperm DNA to acid-induced denaturation in situ [27]. This is a cell-permeant nucleic acid binding dye that emits green fluorescence when bound to double stranded DNA (dsDNA) and red fluorescence when bound to single stranded DNA (ssDNA) or RNA. Cryopreserved semen was thawed in a 37°C water bath for 1 min, and 90 µL of thawed semen was added to 110 µL PBS to create a final sperm concentration of approximately 18 million spermatozoa per mL. Acid-induced denaturation of DNA in situ was attained by adding 0.4 ml of an acid-detergent solution (0.17% Triton X-100, 0.15 M sodium chloride, and 0.08 N hydrogen chloride; pH 1.4) to 0.2 mL diluted semen. After 30 sec, the cells were stained by adding 1.2 mL

of a citric phosphate solution (0.1 M citric acid, 0.2 M sodium phosphate dibasic, 1 mM ethylenediaminetetraacetic acid, 0.15 M sodium chloride; pH 6.0) containing 6 µg/ml acridine orange, to achieve a working concentration of 2 million spermatozoa per mL, and mixed by reverse pipetting. The stained samples were subsequently incubated in the dark at 37°C for 3 min prior to flow cytometric analyses.

Flow cytometry analysis was carried out as above using a 488-nm argon excitation laser. Fluorescent data of all events was collected until 10,000 gated events were recorded. Debris was discarded electronically and based on low fluorescence values. Two populations were discerned by drawing a diagonal line between the groups of cells with stable chromatin (intact DNA) to the left of the line (medium to high green fluorescence) and the group of cells with loose chromatin (damaged DNA) to the right of the line (medium to high red fluorescence) as demonstrated in Fig. 21C. The frequency of each of these populations was quantified. DNA fragmentation index was calculated as the number of cells with loose chromatin divided by the sum of both populations.

7.3.10 Statistical analyses

Data are presented as percentage mean \pm SEM. All statistical analyses were performed using R Version 3.4.3 (<https://www.r-project.org/>). Normality was confirmed using a Shapiro-Wilk test of the residuals. Non-normal data was transformed using Tukey's Ladder of Powers. A Kruskal-Wallis rank sum test was performed on non-parametric data. Analysis of variance was applied to data using a general linear mixed model with bull ID as a random variable, and a Tukey HSD test was performed as needed for post-hoc analysis. Significance was declared at $P \leq 0.05$.

7.4 Results

7.4.1 Ejaculated sperm

The mean normal sperm morphology in ejaculated samples before cryopreservation was $63 \pm 6\%$, ranging from 31 to 89%. The most common sperm abnormality present was proximal cytoplasmic droplets ($13 \pm 5\%$), followed by simple bent tails ($7.8 \pm 2.5\%$), distal cytoplasmic droplets ($7.2 \pm 2.7\%$), and detached heads ($6 \pm 2.9\%$). Percentage of normal sperm, primary sperm defects, and secondary sperm defects did not differ at post-thaw between treatments with a similar distribution in specific defects ($P \geq 0.15$; Table 12).

Overall total and progressive sperm motilities in ejaculated samples before treatment and cryopreservation were $72 \pm 6.5\%$ and $61 \pm 7.1\%$, respectively. Post-thaw overall and progressive motilities decreased over time in all samples ($P < 0.01$) but were lower in HIGH and LOW than in CONT or MED samples ($P < 0.01$; Fig. 22A and B). Sperm velocity parameters, VAP and VCL, were lowest in HIGH, intermediate in LOW and CONT, and highest in MED samples ($P \leq 0.04$; Fig. 22C and D). Amplitude of lateral head displacement was decreased in HIGH samples at 2.5 and 3.0 h post-thaw ($P = 0.03$; Fig. 22E). Linearity was lowest in LOW samples, intermediate in CONT and MED, and highest in HIGH samples ($P \leq 0.05$; Fig. 22F).

Post-thaw flow cytometric sperm analyses revealed no differences in percentages of live ($P = 0.76$), dead ($P = 0.66$), or moribund ($P = 0.98$) spermatozoa amongst treatments (Fig. 23A). Similarly, there were no differences in the percentages of live spermatozoa with intact acrosomes between treatments ($P = 0.95$; Fig. 23C). The DNA fragmentation index also did not differ between treatments ($P = 0.77$; Fig. 23E).

7.4.2 Epididymal sperm

Average normal sperm morphology in epididymal samples before cryopreservation was $16 \pm 2.3\%$, ranging from 6 to 33%. The most common sperm abnormality present was distal cytoplasmic droplets ($55 \pm 7.5\%$), followed by proximal cytoplasmic droplets ($11 \pm 3.6\%$), detached heads ($8.4 \pm 3.6\%$), and simple bent tails ($7.6 \pm 2.3\%$). At post-thaw evaluation, there was a higher percentage of normal sperm cells in LOW and HIGH samples than in CONT samples ($P \leq 0.05$; Table 12). Despite this difference, there were no differences in the percentages of primary or secondary sperm defects amongst treatments with a similar distribution of specific defects ($P \geq 0.23$; Table 12).

Average overall and progressive sperm motilities in epididymal samples before treatment and cryopreservation were $75 \pm 3.7\%$ and $57 \pm 3.7\%$, respectively. Post-thaw overall and progressive motilities decreased over time in all samples ($P < 0.01$), but did not differ treatments ($P \geq 0.22$; Fig. 24A and B). Similarly, treatment did not alter post-thaw sperm motion parameters ($P \geq 0.10$; Fig. 24C-F).

Post-thaw flow cytometric sperm analyses revealed no differences in percentages of live ($P = 0.15$), dead ($P = 0.15$), or moribund ($P = 0.10$) spermatozoa amongst treatments (Fig. 23B). Similarly, there were no differences in the percentages of live spermatozoa with intact acrosomes between treatments ($P = 0.14$; Fig. 23D). The DNA fragmentation index also did not differ between treatments ($P = 0.68$; Fig. 23F).

7.5 Discussion

This study was set to assess the post-thaw semen quality from both ejaculated and epididymal-harvested bull sperm extended with final concentrations of 0 ng/mL (CONT), 0.5 ng/mL (LOW), 5 ng/mL (MED), or 50 ng/mL (HIGH) of NGF prior to cryopreservation. Since ejaculated bovine sperm have been found to have NGF-immunoreactivity localized to the sperm head and tail and its receptor (TrkA) in the acrosomal cap, nucleus, and tail regions [14], it seems logical to hypothesize that seminal plasma NGF should have a direct effect on spermatozoa function and based on other studies in man and bulls it would improve semen cryopreservation. However, under the present conditions the effects of NGF on bull sperm cryotolerance were not as beneficial as we hypothesized.

Production of NGF occurs predominantly in the ampulla and vesicular glands of bulls, secreting this protein almost exclusively into the sperm-rich fraction of the ejaculate [15]. In the current study, purified NGF was added to both ejaculated and epididymal-derived bull sperm. While NGF immunoreactivity has also been detected in the myoid epithelial cells around the seminiferous tubules of the testis, the lumen and spermatids were NGF negative, suggesting no testicular contribution of NGF to the seminal plasma [20]. Therefore, the current study aimed to evaluate the effects of adding purified NGF to spermatozoa exposed to accessory sex gland fluids (ejaculated) versus spermatozoa with no exposure to accessory sex gland fluids (epididymal). As expected, the percentage of normal spermatozoa was higher in ejaculated samples (~63%) than in epididymal-derived samples (~16%), with the latter having a higher percentage of secondary defects (21% vs. 71%, respectively). The most common secondary defect detected in epididymal samples were distal cytoplasmic droplets, which did not differ by treatment. It appears, therefore,

that NGF signaling does not play a pivotal role in final sperm maturation at ejaculation and other factors need to be assessed for improving epididymal sperm quality [30].

One study reported higher *Ngf* expression in spermatozoa of good semen-producing (<25% discarded ejaculates) versus poor semen-producing (>40% discarded ejaculates) bulls [17]. In that study, *Ngf* expression levels exerted a positive influence on frozen-thawed sperm velocity parameters, LIN, VAP, STR, and VSL [17], which have been strongly and positively correlated to bull fertility [31]. Consistently, both sperm *Ngf* mRNA abundance and seminal plasma NGF concentrations have been associated with positive sire conception rates in bulls [13,15]. However, the current study found minimal changes in motility and velocity parameters of both ejaculated and epididymal-derived sperm treated with NGF before cryopreservation. Interestingly, ejaculated sperm incubated with high concentrations of NGF exhibited an overall decrease in motility and velocity parameters and increase in LIN. Additionally, ALH was decreased at 2.5 and 3 h post-thaw in ejaculates supplemented with high NGF concentrations. Though the decrease in motility may suggest a detrimental effect of NGF on sperm viability, the combination of decreased ALH and increased LIN suggest that the NGF could be protective against premature hyperactivation and capacitation of sperm [32–34]. These findings were not observed in epididymal sperm, suggesting that interactions with other seminal plasma components secreted by the accessory sex glands may be necessary. Consistently, spermatozoa *Ngf* expression levels were found to have significant positive correlation with *Bmp2*, *Casp3*, and *Tradd* transcripts, which all have roles in maintaining sperm function and fertility [17]. This data suggests that there is a complex synergistic interaction of seminal plasma proteins that may influence sperm cryotolerance and survivability, and future studies should evaluate combinations of these factors to determine the effects on sperm motility and motion parameters.

A previous study found no effect of NGF on the rate of the acrosome reaction [14], which is consistent with findings in the current study that indicate no change in acrosome integrity amongst NGF treatments in bull spermatozoa. The current study also found no effect of NGF treatment on DNA fragmentation index, which were within the recommended 10 to 20% threshold for predicting adequate bull fertility [35,36]. Seminal plasma *Ngf* mRNA expression in bulls was positively associated with maintenance of post-thaw functional membrane integrity in spermatozoa previously [16]. Additionally, exogenous NGF added to frozen-thawed bull spermatozoa at 20, 80, or 120 ng/mL improved sperm viability after a 2 h incubation period [14]. Leptin secretion from bovine sperm was also increased in samples incubated with 40 and 80 ng/mL NGF for 2 h [14]. Leptin signaling in spermatozoa has been implicated as a potential regulator of sperm capacitation and survival in pigs [37]. In contrast to these findings, supplementing freezing extender with purified NGF to semen before cryopreservation did not affect sperm membrane integrity immediately after thawing in either ejaculated or epididymal-derived sperm in the current study. On the contrary, we found that high and low NGF concentrations decreased overall and progressive sperm motilities in ejaculated samples over a 4 h period post-thaw compared to control samples. Further studies are needed to assess the relevance of these findings in regard to ensuring sperm survivability and transit within the female reproductive tract and overall fertilizing capability. A previous study found that supplementing *in vitro* fertilization media with 100 ng/mL NGF induced early cleavage and improved embryo development in sheep [38]. Therefore, fertility studies, both *in vitro* and *in vivo*, are necessary to determine the relevance of these findings in cattle.

In conclusion, treatment with NGF did not significantly improve cryotolerance of sperm collected by electroejaculation or epididymal harvest in bulls. Supplementation of freezing

extender with high concentrations of NGF decreased post-thaw VCL and ALH and increased LIN, which may suggest a role in preventing premature sperm hyperactivation and capacitation. Though the current study does not support a role of NGF in improving sperm cryotolerance, further studies should address the effects it may have within the female reproductive tract that could influence fertility.

7.6 Acknowledgments

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7.7 Tables and Figures

Table 12. Morphological analysis of sperm samples collected by electroejaculation and epididymal sperm harvest prior to freezing (PRE) and at post-thaw after incubation with 0 ng/mL NGF (CONT), 0.5 ng/mL NGF (LOW), 5 ng/mL NGF (MED), or 50 ng/mL NGF (HIGH). Data are presented as mean \pm SEM. Differing superscripts denote differences between groups ($P \leq 0.05$).

Treatment	Ejaculated			Epididymal		
	% Normal	% Primary	% Secondary	% Normal	% Primary	% Secondary
PRE	63 \pm 6.1	16 \pm 5.4	21 \pm 4.6	16 \pm 2.3 ^{ab}	13 \pm 3.5	71 \pm 5.6
CONT	66 \pm 7.0	15 \pm 4.7	19 \pm 4.1	15 \pm 2.1 ^b	16 \pm 4.7	69 \pm 6.5
LOW	63 \pm 6.4	17 \pm 6.2	20 \pm 4.4	18 \pm 2.5 ^a	14 \pm 4.5	68 \pm 6.8
MED	65 \pm 6.4	16 \pm 5.4	19 \pm 5.2	17 \pm 2.5 ^{ab}	14 \pm 5.0	69 \pm 7.2
HIGH	62 \pm 6.0	18 \pm 5.8	21 \pm 4.0	18 \pm 2.9 ^a	14 \pm 5.0	68 \pm 7.1

Figure 21. Representative density plots for flow cytometric analyses performed on post-thaw bull semen. (A) Sperm viability assay using SYBR-14 (green fluorescence, X-axis) and propidium iodide (PI; red fluorescence, Y-axis). The top left gate encloses PI positive events, corresponding to dead sperm. The bottom right gate encloses SYBR-14 positive events, corresponding to live sperm. The top right gate represents events with mixed fluorescence, indicating moribund sperm. (B) Acrosomal integrity assay using FITC-PNA (peanut agglutinin conjugated with fluorescein isothiocyanate; green fluorescence, X-axis) and PI (red fluorescence, Y-axis). The lower quadrants contain live sperm (low PI) with either intact acrosomes (low FITC-PNA) on the left or damaged acrosomes (high FITC-PNA) on the right. The upper quadrants encompass the populations of dead sperm (high PI), with damaged acrosomes (high FITC-PNA) on the right and intact acrosomes (low FITC-PNA) on the left. (C) Chromatin stability assay using acridine orange. Units indicate fluorescence intensity with binding to double stranded (intact) DNA producing green fluorescence (Y-axis) and binding to single stranded (damaged) DNA or RNA producing red fluorescence (X-axis). The elongated cloud on the left is the sperm population with good chromatin integrity (Intact DNA) emitting green fluorescence. Dots to the right of the diagonal line are cells outside of the main population that bear loose chromatin (Damaged DNA) emitting red fluorescence.

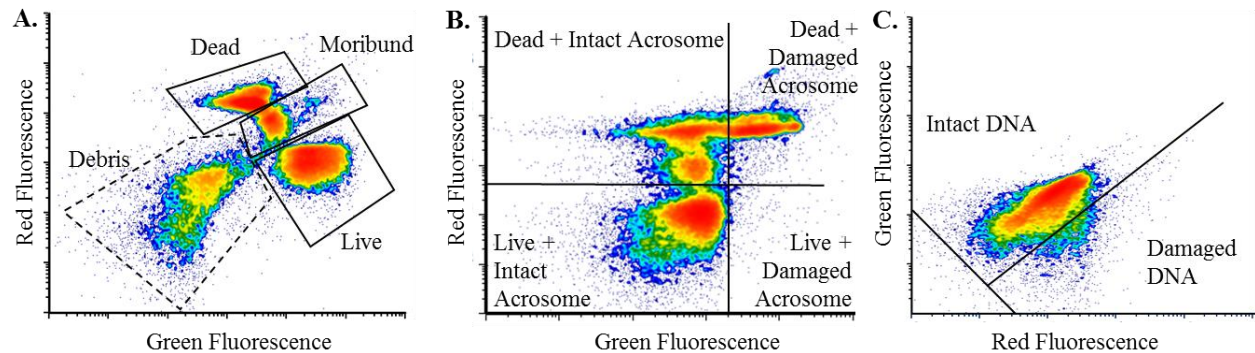


Figure 22. Line graphs demonstrating changes over time in the motility and motion parameters of frozen-thawed ejaculated bull spermatozoa supplemented with 0 ng/mL NGF (CONT), 0.5 ng/mL NGF (LOW), 5 ng/mL NGF (MED), or 50 ng/mL NGF (HIGH). Data are presented as mean \pm SEM. Asterisks denote treatment by time interactions ($P \leq 0.05$).

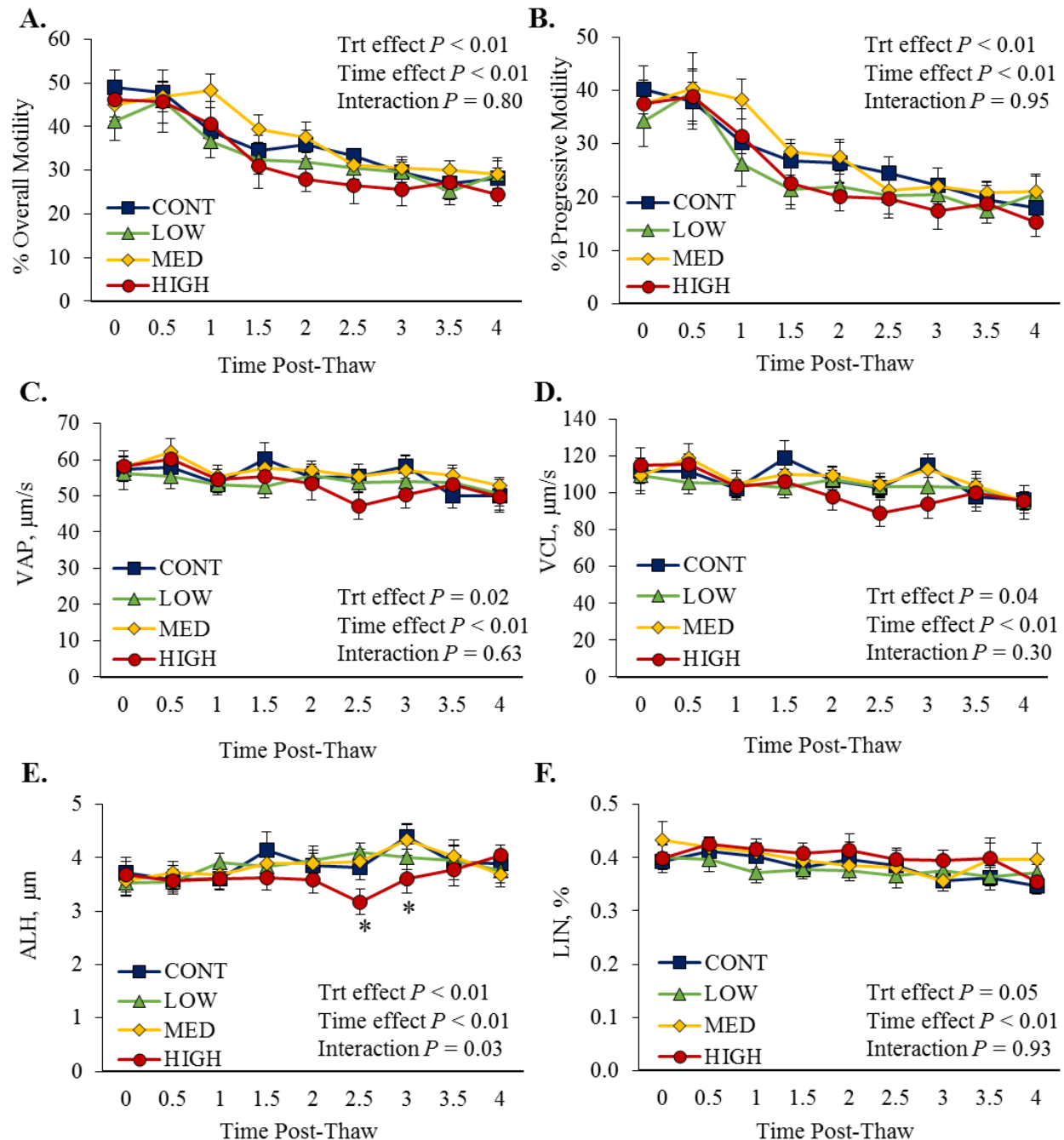


Figure 23. Results of post-thaw flow cytometric analyses from semen samples collected by electroejaculation (A, C, E) or epididymal sperm harvest (B, D, F) after incubation with 0 ng/mL NGF (CONT), 0.5 ng/mL NGF (LOW), 5 ng/mL NGF (MED), or 50 ng/mL NGF (HIGH) in bulls. There were no differences in sperm viability (A, B), acrosome integrity (C, D), or DNA stability (E, F) between treatments ($P > 0.05$).

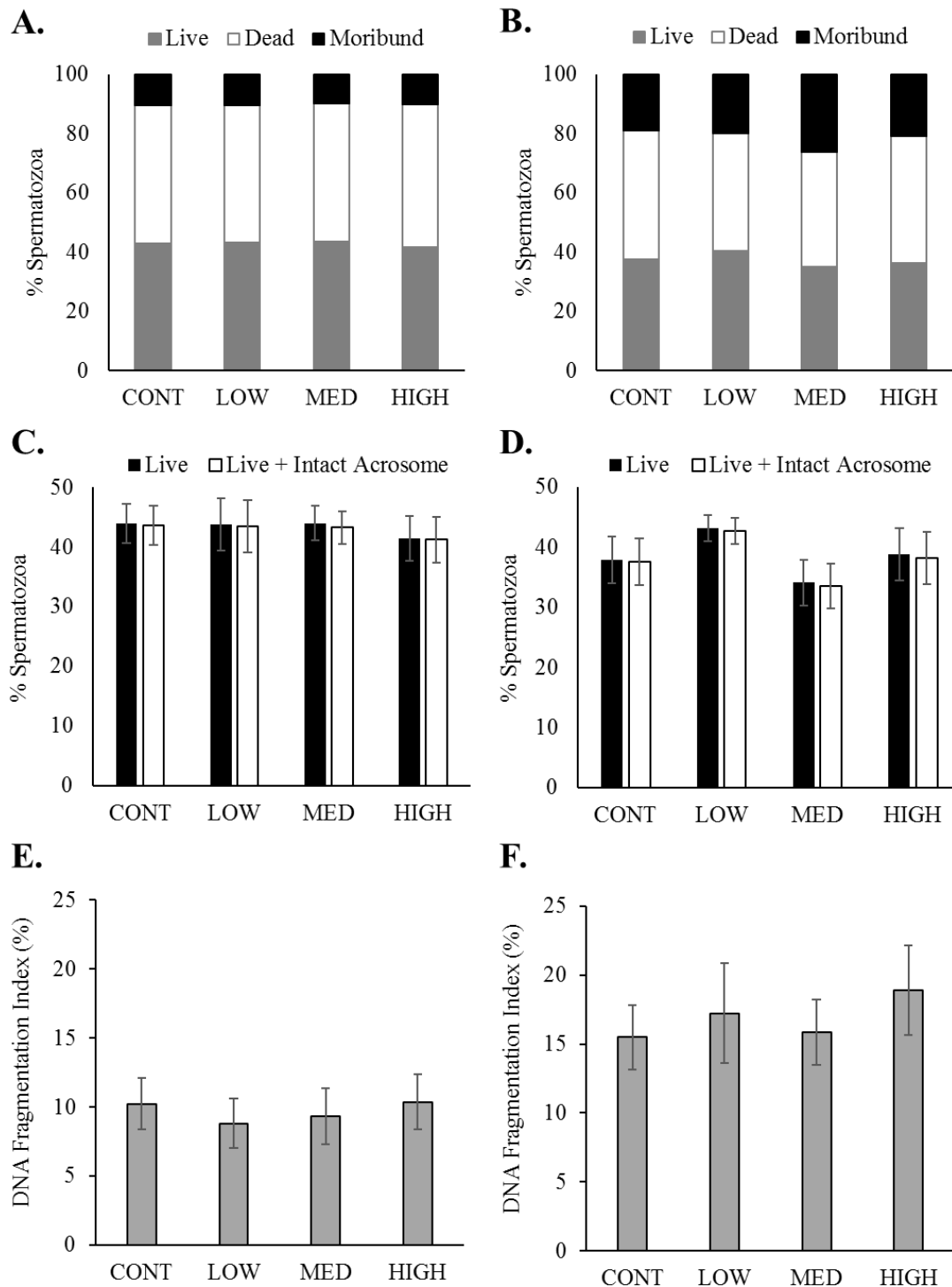
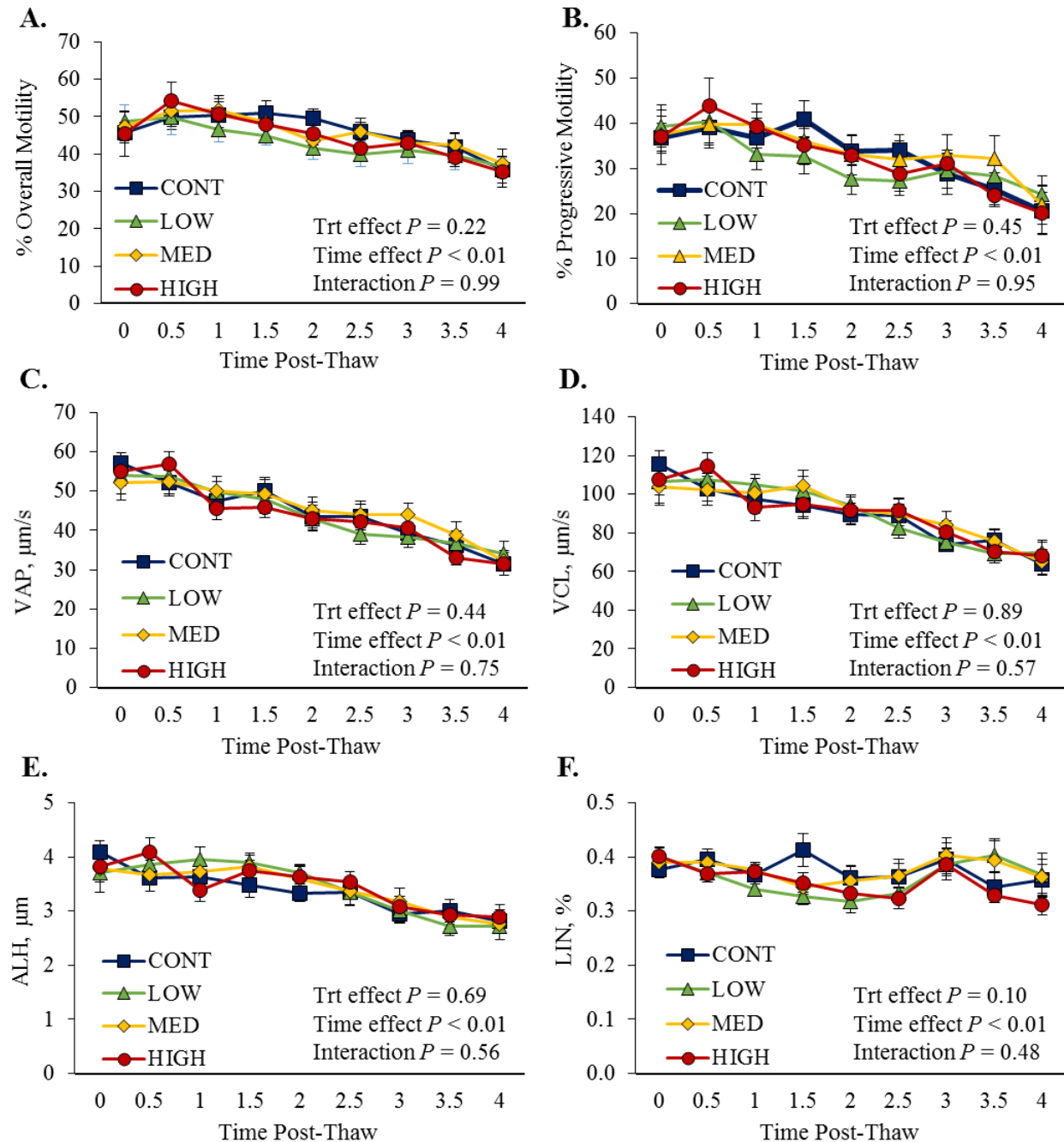


Figure 24. Line graphs demonstrating changes over time in the motility and motion parameters of frozen-thawed bull epididymal spermatozoa supplemented with 0 ng/mL NGF (CONT), 0.5 ng/mL NGF (LOW), 5 ng/mL NGF (MED), or 50 ng/mL NGF (HIGH). Data are presented as mean \pm SEM.



Abbreviations: VAP = average path velocity; $\mu\text{m/s}$ VCL = curvilinear velocity, $\mu\text{m/s}$; LIN = linearity of the curvilinear path, %; ALH = amplitude of lateral head displacement, μm .

7.8 References

- [1] Killian GJ, Chapman DA, Rogowski LA. Fertility-associated proteins in Holstein bull seminal plasma. *Biol Reprod* 1993;49:1202–7. doi:10.1095/biolreprod49.6.1202.
- [2] Moura AA, Koc H, Chapman DA, Killian GJ. Identification of proteins in the accessory sex gland fluid associated with fertility indexes of dairy bulls: a proteomic approach. *J Androl* 2006;27:201–11. doi:10.2164/jandrol.05089.
- [3] Henault MA, Killian GJ, Kavanaugh JF, Griel Jr LC. Effect of accessory sex gland fluid from bulls of differing fertilities on the ability of cauda epididymal sperm to penetrate zona-free bovine oocytes. *Biol Reprod* 1995;52:390–7. doi:10.1095/biolreprod52.2.390.
- [4] Viana AGA, Martins AMA, Pontes AH, Fontes W, Castro MS, Ricart CAO, et al. Proteomic landscape of seminal plasma associated with dairy bull fertility. *Sci Rep* 2018;8:16323. doi:10.1038/s41598-018-34152-w.
- [5] Hayes BJ, Chamberlain AJ, Maceachern S, Savin K, McPartlan H, MacLeod I, et al. A genome map of divergent artificial selection between *Bos taurus* dairy cattle and *Bos taurus* beef cattle. *Anim Genet* 2009;40:176–84. doi:10.1111/j.1365-2052.2008.01815.x.
- [6] Schjenken JE, Robertson SA. Seminal fluid and immune adaptation for pregnancy - comparative biology in mammalian species. *Reprod Domest Anim* 2014;49:27–36. doi:10.1111/rda.12383.
- [7] Harper GP, Thoenen H. The distribution of nerve growth factor in the male sex organs of mammals. *J Neurochem* 1980;34:893–903. doi:10.1111/j.1471-4159.1980.tb09663.x.
- [8] Harper GP, Glanville RW, Thoenen H. The purification of nerve growth factor from bovine seminal plasma. Biochemical characterization and partial amino acid sequence. *J Biol Chem* 1982;257:8541–8.
- [9] Li C, Ma Y, Yi K, Wang C, Li W, Liu Z, et al. The interactions between nerve growth factor and gonadotrophins in bovine oviduct. *Anim Reprod Sci* 2014;149:117–23. doi:10.1016/j.anireprosci.2014.06.030.
- [10] Tanco VM, Van Steelandt MD, Ratto MH, Adams GP. Effect of purified llama ovulation-inducing factor (OIF) on ovarian function in cattle. *Theriogenology* 2012;78:1030–9. doi:10.1016/j.theriogenology.2012.03.036.
- [11] Stewart JL, Mercadante VRG, Dias NW, Canisso IF, Yau P, Imai B, et al. Nerve Growth Factor-Beta, purified from bull seminal plasma, enhances corpus luteum formation and conceptus development in *Bos taurus* cows. *Theriogenology* 2018;106:30–8. doi:10.1016/j.theriogenology.2017.10.007.
- [12] Tribulo P, Bogle OA, Mapletoft RJ, Adams GP. Bioactivity of ovulation inducing factor (or nerve growth factor) in bovine seminal plasma and its effects on ovarian function in cattle. *Theriogenology* 2015;83:1394–401. doi:10.1016/j.theriogenology.2014.12.014.
- [13] Kasimanickam V, Kasimanickam R, Arangasamy A, Saberivand A, Stevenson JS, Kastelic JP. Association between mRNA abundance of functional sperm function proteins and fertility of Holstein bulls. *Theriogenology* 2012;78:2007–19. doi:10.1016/j.theriogenology.2012.07.016.
- [14] Li C, Sun Y, Yi K, Ma Y, Sun Y, Zhang W, et al. Detection of nerve growth factor (NGF)

- and its specific receptor (TrkA) in ejaculated bovine sperm, and the effects of NGF on sperm function. *Theriogenology* 2010;74:1615–22. doi:10.1016/j.theriogenology.2010.06.033.
- [15] Stewart JL, Canisso IF, Ellerbrock RE, Mercadante VRG, Lima FS. Nerve Growth Factor- β production in the bull: Gene expression, immunolocalization, seminal plasma constitution, and association with sire conception rates. *Anim Reprod Sci* 2018;197:335–42. doi:10.1016/j.anireprosci.2018.09.006.
 - [16] Shilpa M, Selvaraju S, Girishkumar V, Parthipan S, Binsila KB, Arangasamy A, et al. Novel insights into the role of cell-free seminal mRNAs on semen quality and cryotolerance of spermatozoa in bulls (*Bos taurus*). *Reprod Fertil Dev* 2017;29:2446–56. doi:10.1071/RD16290.
 - [17] Parthipan S, Selvaraju S, Somashekar L, Arangasamy A, Sivaram M, Ravindra JP. Spermatozoal transcripts expression levels are predictive of semen quality and conception rate in bulls (*Bos taurus*). *Theriogenology* 2017;98:41–9. doi:10.1016/j.theriogenology.2017.04.042.
 - [18] Saeednia S, Bahadoran H, Amidi F, Asadi MH, Naji M, Fallahi P, et al. Nerve growth factor in human semen: effect of nerve growth factor on the normozoospermic men during cryopreservation process. *Iran J Basic Med Sci* 2015;18:292–9.
 - [19] Saeednia S, Shabani Nashtaei M, Bahadoran H, Aleyasin A, Amidi F. Effect of nerve growth factor on sperm quality in asthenozoospermic men during cryopreservation. *Reprod Biol Endocrinol* 2016;14:1–8. doi:10.1186/s12958-016-0163-z.
 - [20] Bogle OA, Carrasco RA, Ratto MH, Singh J, Adams GP. Source and localization of ovulation-inducing factor/nerve growth factor in male reproductive tissues among mammalian species. *Biol Reprod* 2018. doi:10.1093/biolre/ioy149.
 - [21] Martins CF, Rumpf R, Pereira DC, Dode MN. Cryopreservation of epididymal bovine spermatozoa from dead animals and its uses in vitro embryo production. *Anim Reprod Sci* 2007;101:326–31. doi:10.1016/J.ANIREPROSCI.2007.01.018.
 - [22] FASS. Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching. 3rd ed. Consortium for Developing a Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching. Champaign, IL: 2010.
 - [23] Eversole DE, Browne MF, Hall JB, Dietz RE. Body condition scoring beef cows. *Virginia Coop Ext* 2009:1–6. <https://pubs.ext.vt.edu/400/400-795/400-795.html> (accessed March 11, 2016).
 - [24] Chenoweth PJ, Hopkins FM, Spitzer JC, Larsen RE. Guidelines for using the bull breeding soundness evaluation form. *Clin Theriogenology* 2010;2:43–50.
 - [25] Amann RP, Waberski D. Computer-assisted sperm analysis (CASA): Capabilities and potential developments. *Theriogenology* 2014;81:5–17. doi:10.1016/j.theriogenology.2013.09.004.
 - [26] Kuster CE, Singer RS, Althouse GC. Determining sample size for the morphological assessment of sperm. *Theriogenology* 2004;61:691–703. doi:10.1016/S0093-691X(03)00240-1.
 - [27] Martínez-Pastor F, Johannisson A, Gil J, Kaabi M, Anel L, Paz P, et al. Use of chromatin

- stability assay, mitochondrial stain JC-1, and fluorometric assessment of plasma membrane to evaluate frozen-thawed ram semen. *Anim Reprod Sci* 2004;84:121–33. doi:10.1016/j.anireprosci.2003.12.006.
- [28] Martínez-Pastor F, Mata-Campuzano M, Álvarez-Rodríguez M, Álvarez M, Anel L, de Paz P. Probes and techniques for sperm evaluation by flow cytometry. *Reprod Domest Anim* 2010;45:67–78. doi:10.1111/j.1439-0531.2010.01622.x.
- [29] Robles V, Martínez-Pastor F. Chapter 16: Flow cytometric methods for sperm assessment. In: Carrell DT, Aston KI, editors. *Spermatogenesis. Methods Protoc.*, vol. 927, New York City: Humana Press; Springer Science+Business Media, LLC; 2013, p. 175–86. doi:10.1007/978-1-62703-038-0.
- [30] Harayama H, Shibukawa T, Miyake M, Kannan Y, Kato S. Fructose stimulates shedding of cytoplasmic droplets from epididymal boar spermatozoa. *Reprod Fertil Dev* 1996;8:1039–43. doi:10.1071/RD9961039.
- [31] Farrell PB, Presicce GA, Brockett CC, Foote RH. Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (CASA) and the relationship to fertility. *Theriogenology* 1998;49:871–9. doi:10.1016/S0093-691X(98)00036-3.
- [32] Kathiravan P, Kalatharan J, Karthikeya G, Rengarajan K, Kadirvel G. Objective sperm motion analysis to assess dairy bull fertility using computer-aided system - a review. *Reprod Domest Anim* 2011;46:165–72. doi:10.1111/j.1439-0531.2010.01603.x.
- [33] Muiño R, Tamargo C, Hidalgo CO, Peña AI. Identification of sperm subpopulations with defined motility characteristics in ejaculates from Holstein bulls: Effects of cryopreservation and between-bull variation. *Anim Reprod Sci* 2008;109:27–39. doi:10.1016/j.anireprosci.2007.10.007.
- [34] Marquez B, Suarez SS. Different signaling pathways in bovine sperm regulate capacitation and hyperactivation. *Biol Reprod* 2004;70:1626–33. doi:10.1095/biolreprod.103.026476.
- [35] Kasimanickam R, Nebel RL, Peeler ID, Silvia WL, Wolf KT, McAllister AJ, et al. Breed differences in competitive indices of Holstein and Jersey bulls and their association with sperm DNA fragmentation index and plasma membrane integrity. *Theriogenology* 2006;66:1307–15. doi:10.1016/j.theriogenology.2006.04.025.
- [36] Evenson DP, Wixon R. Clinical aspects of sperm DNA fragmentation detection and male infertility. *Theriogenology* 2006;65:979–91. doi:10.1016/j.theriogenology.2005.09.011.
- [37] Aquila S, Rago V, Guido C, Casaburi I, Zupo S, Carpino A. Leptin and leptin receptor in pig spermatozoa: Evidence of their involvement in sperm capacitation and survival. *Reproduction* 2008;136:23–32. doi:10.1530/REP-07-0304.
- [38] Crispo M, dos Santos-Neto PC, Vilariño M, Mulet AP, de León A, Barbeito L, et al. RAPID COMMUNICATION: Nerve growth factor influences cleavage rate and embryo development in sheep. *J Anim Sci* 2016;94:4447–51. doi:10.2527/jas.2016-0736.

Chapter 8:

Summary, Conclusions, and Future Directions

8.1 Summary, Conclusions, and Future Directions

The objectives of my doctoral dissertation were to determine the role of nerve growth factor- β (NGF) on ovulation, corpus luteum (CL) function, and conceptus development in cows/heifers and understand its production and role in sperm cryotolerance in the bull. Nerve growth factor- β is a protein found in the seminal plasma of a variety of species, including the bull [1]. Though the initial roles of NGF were thought to be limited to regulating neuronal survival and differentiation, studies have since demonstrated its broader physiological implications, including those within the reproductive and endocrine systems [2]. One of the most profound roles of NGF discovered yet is its ability to elicit luteinizing hormone (LH) secretion from the anterior pituitary and induce ovulation in camelids [3–5]. In spontaneously ovulating species, NGF signaling in the ovary is critical for the first ovulation to occur [14], though we have only recently begun to truly understand its effects when introduced into the female reproductive tract from seminal plasma. Collectively, the studies presented herein have demonstrated that NGF is secreted primarily into the sperm-rich fraction of the bull ejaculate and may interact directly with the bovine pre-ovulatory follicle to stimulate thecal cell steroidogenesis, increase follicle size, and alter local factors associated with CL formation. The resulting luteotrophic effect of NGF signaling seems to have a role in enhancing conceptus growth and ensuring maternal recognition of pregnancy in cattle.

In Chapter 3, I hypothesized that systemic administration of purified NGF to cows at the time of artificial insemination would improve CL function and conceptus development. I found that, despite no change in CL size between groups, NGF-treated cows had increased plasma progesterone levels over CONT cows from days 10 to 19, within the window for maternal

recognition of pregnancy to occur. Interestingly, pregnancy rates were numerically higher in NGF-treated cows (75%) versus CONT cows (59%), though the study lacked enough power to detect a statistical significance. Regardless, expressions of interferon-stimulated gene 15 (*Isg15*) and myxovirus 2 (*Mx2*) were upregulated in peripheral blood leukocytes of pregnant NGF-treated cows compared to pregnant CONT cows, which are suggestive of increased interferon tau production from the developing conceptus. Consistently, plasma concentrations of pregnancy-specific protein B, which is produced from the binucleate cells of the placenta, were also increased in pregnant NGF-treated cows, indicative of augmented placentation. At day 66 of gestation, fetal crown rump length also tended to be increased in pregnant NGF cows, which may be a sequela to the initial enhancement in conceptus growth. Though we suspect enhanced conceptus growth is related to improved luteal function, further studies using *in vitro* maturation and *in vitro* fertilization are necessary to determine if there is a direct effect of NGF on the developing conceptus. Additionally, larger field studies are needed to evaluate effects of NGF administration on cow fertility and pregnancy rates.

In Chapter 4, I hypothesized that NGF could interact directly with the pre-ovulatory follicle *in vitro* to upregulate steroidogenic and angiogenic gene expressions and increase steroid production. Contrary to my hypothesis, NGF treatment did not alter expression of any of the angiogenic genes except fibroblast growth factor 2 (*Fgf2*), which was down-regulated. Interestingly, fibroblast growth factor 2 plays an important role in promoting vascular supply growth during the follicular to luteal transition in the cow ovary [6,7]. This eventually leads to a decrease in its concentrations that allows for reconstruction of the capillary beds to establish luteal blood flow [8]. I suspect that given the time frame of culture (72 hours), the decrease in *Fgf2* expression may be a result of hastened onset to tissue reconstruction that is critical during early

CL development. Another interesting finding was the upregulation in the expression of steroidogenic enzyme 17 β -hydroxysteroid dehydrogenase (*17 β -hsd*) in NGF-treated follicular tissue, which was consistent with increased testosterone production. Previous studies found that NGF stimulates prostaglandin E₂ (PGE) synthesis from the bovine theca cells [9]. Though I failed to detect any differences in expression of its precursor, PGE synthase (*Pges*), one study found that 17 β -hydroxysteroid dehydrogenase enzymes play a role in the synthesis of arachidonic acid and its downstream eicosanoid metabolites, such as PGE [10]. Therefore, the results presented herein suggest that NGF plays a role in enhancing androgen synthesis from the bovine pre-ovulatory follicle and hastening the onset of tissue remodeling during the follicular to luteal transition. Further studies are needed with differing lengths of NGF treatment *in vitro* to better characterize the changes that occur during the luteinization period that may alter subsequent CL function.

In Chapter 5, I hypothesized that administering NGF systemically to heifers with a pre-ovulatory follicle would improve vascularity and steroidogenesis of the follicle, hasten onset to ovulation, and improve subsequent CL function. Interestingly, NGF treatment had an effect on follicle size, though there were no differences in follicular vascularity or serum estradiol concentrations. Given the results of previous studies, I suspect that the increased follicular size may be due to follicular edema production stimulated by PGE [9,11]. While the average time to ovulation was 4 hours sooner in NGF-treated than in CONT heifers, there was no statistical difference, which may be attributed to a lack of statistical power in the study. Consistent with findings from Chapter 3, serum progesterone concentrations were higher in NGF-treated heifers from days 10 to 12. Results from the CL biopsies suggested that this luteotrophic effect is due to increased number of small luteal cells and upregulated expression of steroidogenic acute regulatory protein (*Star*) and 3 beta-hydroxysteroid dehydrogenase (*3 β -hsd*). The increase in small

luteal cell number is likely secondary to NGF-induced theca cell proliferation within the pre-ovulatory follicle [9], since small luteal cells are derived from the theca cells [12]. There was also tendency for increased gene expression of luteinizing hormone receptor (*Lhcgr*), which is localized to small luteal cells [12], and no difference in oxytocin (*Ot*) expression, which is localized to large luteal cells [13]. Consistently, LH signaling is required to maintain normal expression of *Star* and *3 β -hsd* genes and stimulate progesterone synthesis in the ruminant CL [14,15]. Though a previous study reported increased follicular and luteal vascularity with NGF treatment of llamas [16], we found no such changes in cattle. These results demonstrate a local effect of NGF on the ovulatory dynamics that help to establish luteal function in cattle. Future studies are necessary to uncover the potential synergistic effects between NGF and GnRH within the complex hypothalamic-pituitary-ovarian axis and better understand its role within the bovine reproductive system.

In Chapter 6, it was hypothesized that NGF is produced predominantly in the bull vesicular gland and is positively associated with sire conception rates. The findings from this study demonstrated that NGF production occurs in both the ampulla and vesicular glands of the bull, with secretion occurring predominantly into the sperm-rich fraction of the ejaculate. This finding is in contrast to llamas, where the prostate gland is the main source [17,18]. However, given the short duration of copulation in cattle compared to other species, concentration into the sperm-rich fraction ensures that protein deposition into the female reproductive tract will occur [19]. Seminal plasma concentrations of NGF were found to be higher in bulls with positive sire conception rate deviations compared to those that were below average. Additional identified roles for NGF include increased expression of gonadotropin receptors in bovine oviduct epithelial cells [20] and improved sperm viability in frozen-thawed bull spermatozoa [21]. Collectively, these studies

suggest that the beneficial role of NGF on sire conception rates acts through its effects in the male and female reproductive tracts and warrants further investigation.

In Chapter 7, it was hypothesized that NGF would improve cryotolerance of both ejaculated and epididymal derived spermatozoa in bulls. Contradictory to our hypothesis, this study demonstrated that supplementing the freezing extender with NGF did not improve post-thaw sperm morphology, motility, viability, acrosome integrity, or chromatin stability in either ejaculated or epididymal derived spermatozoa. While the results of this study did not support a role of NGF in improving sperm cryotolerance, further studies should address the effects it may have on sperm survivability that may influence its transport through the female reproductive tract.

Collectively, the results from my doctoral dissertation work demonstrate that seminal plasma derived NGF plays an important role in bovine reproduction. Though the concentration of NGF into the sperm-rich fraction of the bull ejaculate suggests a role in sperm functionality, the results presented herein suggests a more profound effect within female reproductive tract. Firstly, NGF increases thecal cell steroidogenic function within the pre-ovulatory follicle through increased enzyme activity, which may hasten onset to ovulation. Secondly, NGF may promote tissue reconstruction that is critical during the follicular to luteal transition following ovulation. Thirdly, NGF-induced thecal cell proliferation in the pre-ovulatory follicle increased small luteal cell numbers in the subsequent CL, resulting in increased progesterone production prior to maternal recognition of pregnancy. Lastly, NGF treatment enhanced conceptus development, a potential sequela of its luteotrophic function, and may be crucial to minimizing early embryonic losses in cattle. Future studies should be aimed at determining its effectiveness when utilized in assisted reproduction technologies to enhance reproductive success in cattle and other species.

8.2 References

- [1] Harper GP, Glanville RW, Thoenen H. The purification of nerve growth factor from bovine seminal plasma. Biochemical characterization and partial amino acid sequence. *J Biol Chem* 1982;257:8541–8.
- [2] Tessarollo L. Pleiotropic functions of neurotrophins in Development. *Cytokine Growth Factor Rev* 1998;9:125–37. doi:10.1016/S1359-6101(98)00003-3.
- [3] Adams GP, Ratto MH, Huanca W, Singh J. Ovulation-inducing factor in the seminal plasma of alpacas and llamas. *Biol Reprod* 2005;73:452–7. doi:10.1095/biolreprod.105.040097.
- [4] Ratto MH, Leduc YA, Valderrama XP, van Straaten KE, Delbaere LTJ, Pierson RA, et al. The nerve of ovulation-inducing factor in semen. *Proc Natl Acad Sci U S A* 2012;109:15042–7. doi:10.1073/pnas.1206273109.
- [5] Kershaw-Young CM, Druart X, Vaughan J, Maxwell WMC. β -Nerve growth factor is a major component of alpaca seminal plasma and induces ovulation in female alpacas. *Reprod Fertil Dev* 2012;24:1093–7. doi:10.1071/RD12039.
- [6] Laird M, Woad KJ, Hunter MG, Mann GE, Robinson RS. Fibroblast growth factor 2 induces the precocious development of endothelial cell networks in bovine luteinising follicular cells. *Reprod Fertil Dev* 2013;25:372. doi:10.1071/RD12182.
- [7] Tamanini C, De Ambrogi M. Angiogenesis in developing follicle and corpus luteum. *Reprod Domest Anim* 2004;39:206–16. doi:10.1111/j.1439-0531.2004.00505.x.
- [8] Robinson RS, Woad KJ, Hammond AJ, Laird M, Hunter MG, Mann GE. Angiogenesis and vascular function in the ovary. *Reproduction* 2009;138:869–81. doi:10.1530/REP-09-0283.
- [9] Dissen GA, Parrott JA, Skinner MK, Hill DF, Costa ME, Ojeda SR. Direct effects of nerve growth factor on thecal cells from antral ovarian follicles. *Endocrinology* 2000;141:4736–50. doi:10.1210/endo.141.12.7850.
- [10] Kemiläinen H, Adam M, Mäki-Jouppila J, Damdimopoulou P, Damdimopoulos AE, Kere J, et al. The hydroxysteroid (17 β) dehydrogenase family gene HSD17B12 is involved in the prostaglandin synthesis pathway, the ovarian function, and regulation of fertility. *Endocrinology* 2016;157:3719–30. doi:10.1210/en.2016-1252.
- [11] Murdoch WJ, Peterson TA, Van Kirk EA, Vincent DL, Inskeep EK. Interactive roles of progesterone, prostaglandins, and collagenase in the ovulatory mechanism of the ewe. *Biol Reprod* 1986;35:1187–94. doi:10.1095/biolreprod35.5.1187.
- [12] Harrison LM, Kenny N, Niswender GD. Progesterone production, LH receptors, and oxytocin secretion by ovine luteal cell types on days 6, 10 and 15 of the oestrus cycle and day 25 of pregnancy. *J Reprod Fert* 1987;79:539–48. doi:10.1530/jrf.0.0790539.
- [13] Fehr S, Ivell R, Koll R, Schams D, Fields M, Richter D. Expression of the oxytocin gene in the large cells of the bovine corpus luteum. *FEBS Lett* 1987;210:45–50. doi:10.1016/0014-5793(87)81295-4.
- [14] Niswender GD, Juengel JL, Silva PJ, Rollyson MK, McIntush EW. Mechanisms controlling the function and life span of the corpus luteum. *Physiol Rev* 2000;80:1–29.

doi:10.1152/physrev.2000.80.1.1.

- [15] Rekawiecki R, Nowik M, Kotwica J. Stimulatory effect of LH, PGE₂ and progesterone on StAR protein, cytochrome P450 cholesterol side chain cleavage and 3 β hydroxysteroid dehydrogenase gene expression in bovine luteal cells. *Prostaglandins Other Lipid Mediat* 2005;78:169–84. doi:10.1016/j.prostaglandins.2005.06.009.
- [16] Ulloa-Leal C, Bogle OA, Adams GP, Ratto MH. Luteotrophic effect of ovulation-inducing factor/nerve growth factor present in the seminal plasma of llamas. *Theriogenology* 2014;81:1101–1107.e1. doi:10.1016/j.theriogenology.2014.01.038.
- [17] Bogle OA, Singh J, Adams GP. 248 Distribution of ovulation-inducing factor in male reproductive tissues of llamas. *Reprod Fertil Dev* 2012;25:272. doi:10.1071/RD03082.
- [18] Bogle OA, Carrasco RA, Ratto MH, Singh J, Adams GP. Source and localization of ovulation-inducing factor/nerve growth factor in male reproductive tissues among mammalian species. *Biol Reprod* 2018. doi:10.1093/biolre/ioy149.
- [19] Lichtenwalner ABB, Woods GLL, Weber JAA. Seminal collection, seminal characteristics and pattern of ejaculation in llamas. *Theriogenology* 1996;46:293–305. doi:10.1016/0093-691X(96)00186-0.
- [20] Li C, Ma Y, Yi K, Wang C, Li W, Liu Z, et al. The interactions between nerve growth factor and gonadotrophins in bovine oviduct. *Anim Reprod Sci* 2014;149:117–23. doi:10.1016/j.anireprosci.2014.06.030.
- [21] Li C, Sun Y, Yi K, Ma Y, Sun Y, Zhang W, et al. Detection of nerve growth factor (NGF) and its specific receptor (TrkA) in ejaculated bovine sperm, and the effects of NGF on sperm function. *Theriogenology* 2010;74:1615–22. doi:10.1016/j.theriogenology.2010.06.033.

Appendix A:

Cryopreservation of white-tailed deer (*Odocoileus virginianus*) using soybean-, liposome-, and egg yolk-based extenders

The comparison of extenders for cryopreservation of white-tailed deer semen is a project I contributed to during my Ph.D. program, but it was not part of my dissertation work. The following paper is a result of work done on this project.

Cryopreservation of white-tailed deer (*Odocoileus virginianus*) semen using soybean-, liposome-, and egg yolk-based extenders¹

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Abstract

The objectives of the present study were to compare the use of soybean-based (Andromed), liposome-based (Optixcell), and egg yolk-based (Ovine Red, Triladyl, and Biladyl) extenders for cryopreservation of white-tailed deer semen. In experiment 1, ejaculates obtained from six bucks were aliquoted into the following extenders: Andromed, Ovine Red, Triladyl, and Biladyl (containing 4%, 6%, or 8% of glycerol). In experiment 2, ejaculates obtained from eight bucks were divided amongst Andromed, Ovine Red, and Optixcell extenders. Total and progressive sperm motility were assessed for each sample before and after cryopreservation using a computer-automated semen analyzer. In experiment 2, flow cytometry was used for post-thaw assessment of sperm viability (SYBR-14/PI), acrosome integrity (FITC-PNA/PI), and chromatin stability (acridine orange). In experiment 1, both Andromed and Ovine Red extenders exhibited higher post-thaw total motility than Biladyl containing 4% or 6% of glycerol ($p < 0.05$). Andromed also produced higher progressive motility than all other extenders ($p < 0.01$) before and after cryopreservation with no differences amongst the other extenders ($p \geq 0.11$). In experiment 2, there were no differences in total and progressive motility between Andromed, Ovine Red, or Optixcell extenders ($p \geq 0.39$). Additionally, there were no differences in sperm viability ($p = 0.18$), acrosome integrity in viable sperm ($p \geq 0.10$), or DNA fragmentation index ($p = 0.15$). These

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results demonstrated that soybean (Andromed) and liposome-based (Optixcell) extenders are equally as effective at cryopreserving white-tailed semen as egg yolk-based Ovine Red extender, but are superior to egg yolk-based Biladyl or Triladyl extenders.

Key words: semen freezing, cervid andrology, semen evaluation, fluorescent probes, semen biotechnology

1. Introduction

White-tailed deer farming is an economically important industry in North America. In the United States alone, this industry is estimated to generate approximately 30,000 jobs and \$3.0 billion annually (Anderson et al., 2007). The sustainability of deer farming relies heavily on the use of artificial insemination with frozen semen to effectively disseminate valuable genetics. However, there are limited controlled studies and clinical data assessing the most suitable extender(s) for deer semen cryopreservation (Ake-Lopez et al., 2010; Clemente-Sánchez et al., 2014). While egg yolk-based semen extenders have been used in clinical practice with apparent success in white-tailed deer (Asher et al., 2000), there have been growing concerns regarding the use of animal origin products for semen cryopreservation in mammals (Pillet et al., 2012). First, the use of animal products may put both technicians and recipient animals at an increased risk of cross contamination with pathogens. Additionally, endotoxin production secondary to microbial contamination of semen samples can damage the fertilizing capacity of spermatozoa (Bousseau et al., 1998). Moreover, to be effective, fresh eggs must be obtained and processing must be performed carefully, which imposes time and technical constraints for the use of egg yolk-based extenders. Finally, the phospholipid composition of egg yolk is extremely variable amongst

animals, making it challenging to standardize the use of egg yolk-based extenders (Aydin and Dogan, 2010).

Egg yolk phospholipids, such as phosphatidylcholine, provide protection to the sperm cell in the freezing-thawing process and can be derived from other sources (Pillet et al., 2012). Soy lecithin, a natural mixture of phosphatidylcholine and several fatty acids such as stearic, oleic, and palmitic acid, has been proposed as an alternative to egg yolk for cryoprotection of sperm during freezing (Kumar et al., 2015). Commercial soybean-based extenders have been used successfully in dairy bulls (Aires et al., 2003), rams (Fukui et al., 2008), and domestic bucks (Salmani et al., 2014). Additionally, synthetically derived liposomes composed of egg yolk phospholipids have also been effective in protecting spermatozoa during cryopreservation in stallions (Pillet et al., 2012) and bulls (Röpke et al. 2011), while allowing for a standardized composition in extender. Both of these products are available as commercial extenders, but their effectiveness for sperm cryopreservation in white-tailed deer have yet to be elucidated.

The objective of experiment 1 was to compare total and progressive sperm motility in white-tailed deer before and after cryopreservation when using a soybean-based extender (Andromed) versus different egg yolk-based extenders (Ovine Red; Triladyl; Biladyl containing 4%, 6% or 8% of glycerol). The objectives of experiment 2 were to compare a liposome-based extender (Optixcell) with soybean-based (Andromed) and egg yolk-based (Ovine Red) extenders to cryopreserve white-tailed deer semen, as judged by total and progressive sperm motility and by determination of sperm viability (SYBR-14/propidium iodide), acrosomal integrity (fluorescein isothiocyanate conjugated with peanut agglutinin/propidium iodide), and chromatin stability

(acridine orange) using flow cytometry. Our hypothesis was that Andromed or Optixcell extenders would be more effective than egg yolk-based extenders at cryopreserving white-tailed deer semen.

2. Materials and methods

2.1 Animal Care and Use

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (Protocol #13423) at the University of Illinois Urbana-Champaign. Animals were maintained at a commercial deer farm in east central Illinois.

2.1 Extender Preparation

The preparation of each extender was carried out according to individual manufacturer recommendations. In experiment 1, six extenders were evaluated: Andromed (Minitube of America, LLC, Verona, Wisconsin, USA), Ovine Red (IMV Technologies, Maple Grove, Minnesota, USA), Triladyl (Minitube of America, LLC, Verona, Wisconsin, USA), and Biladyl (Minitube of America, LLC, Verona, Wisconsin, USA) containing 4%, 6%, or 8% of glycerol. In experiment 2, three extenders were evaluated: Andromed, Ovine Red, and Optixcell (IMV Technologies, Maple Grove, Minnesota, USA). Extenders were prepared fresh on the morning of each semen collection and total volumes of extenders were prepared based on the anticipated number of bucks being sampled that day.

For egg yolk-based extenders (Ovine Red, Triladyl, and Biladyl), freshly laid eggs were obtained on the day of or day before semen collection. Shells were sterilized by briefly passing through an open flame. Each egg was opened carefully, and egg yolk was separated from the egg white by passing it three to four times from one half of the shell into the other. The yolk was then

laid down on filter paper and rolled until completely free of egg white. The egg yolk was then positioned at the edge of the paper and propped against a 15 mL polypropylene tube. The membrane was carefully ruptured, such that the yolk drained into the tube and the membrane remained in the filter. This was repeated for each egg until the necessary amount of egg yolk was obtained. Antibiotic solution used for Andromed, Triladyl, and Biladyl extenders was prepared by adding 22 mL distilled water to a bottle of antibiotic cocktail (Cocktail AB, Minitube of America, LLC, Verona, Wisconsin, USA) that contained 60 mg tylosin, 300 mg gentamycin sulfate, 360 mg spectinomycin, and 180 mg lincomycin.

Each Biladyl preparation was prepared as fractions A and B, with B containing the different concentrations of glycerol (4%, 6%, and 8%). Fraction A working solution was prepared by adding 1 mL fresh egg-yolk and 0.1 mL antibiotic solution per 3.9 mL Fraction A stock solution (double distilled sterile water 24.9 g, Tris 12.1 g, citric acid 6.9 g, fructose 5 g), and the solution was warmed to 37°C for pre-dilution of semen samples. Fraction B working solution was prepared as was Fraction A by adding 1 mL fresh egg yolk and 0.1 mL antibiotic solution per 3.9 mL Fraction B stock solution (double distilled sterile water 140 g, glycerol 86 g, Tris 12.1 g, citric acid 6.9 g, fructose 5 g). Once the processed samples were cooled to 5°C, Fraction B was added at a ratio of 1:1, 1:2, or 1:3 (v:v) per Fraction A to allow for differing glycerol concentrations (8%, 6%, and 4%, respectively) for incubation.

Triladyl stock solution was prepared by adding one bottle of Triladyl concentrate (200 g) to 580 mL distilled water and stored at 5°C until each use. On each day of collection, 1 mL egg yolk and 0.1 mL antibiotic solution were added per 3.9 mL Triladyl stock solution to create a working extender. The working extender was warmed to 37°C prior to use. Ovine Red extender was

prepared by adding 4 mL Ovine Red stock solution per 1 mL of fresh egg yolk at 5°C. After thorough mixing, the entire extender preparation was filtered using sterile gauze and subsequently warmed to 37°C prior to use.

Andromed extender was prepared by adding 1 mL Andromed concentrate and 0.1 mL antibiotic solution per 3.9 mL distilled water and subsequently warmed to 37°C prior to use. Optixcell extender was prepared by adding 1 mL Optixcell stock solution per 3 mL distilled water and subsequently warmed to 37°C prior to use.

2.2 Animals & anesthesia

White-tailed deer bucks included in this study were all captive born and raised. Two separate experiments were conducted in the current study that consisted of different populations of bucks. Bucks were included if classified as prospective breeders as determined by a scrotal circumference of greater than 12 cm and an estimated gross sperm motility of greater than 30% (Sathe and Shipley, 2014). Experiment 1 took place February of 2014 and included a total of six bucks with ages ranging from 1.5 to 4 yrs (average 2.4 ± 0.3 yrs). All bucks included in this experiment appeared to be in good reproductive health with a mean scrotal circumference of 15.3 ± 0.4 cm. Two bucks (ages 4 and 1.5 yr, both with scrotal circumference of ≥ 14 cm) were collected, but excluded from experiment 1 due to poor estimated gross sperm motility ($< 30\%$). Experiment 2 occurred in January of 2015 and included a total of eight bucks with ages ranging from 1.5 to 2.5 yrs (average 1.6 ± 0.1 yrs). All bucks included in this experiment appeared to be in good reproductive health with a mean scrotal circumference of 14.3 ± 0.6 cm. These bucks produced an average of 1.22 ± 0.13 mL of ejaculate with a concentration of 1.59 ± 0.38 billion spermatozoa per

milliliter. . Two bucks (both 1.5 yr old with scrotal circumferences of 10 and 13.5 cm) failed to ejaculate and, therefore, were excluded from experiment 2.

Anesthesia and animal handling were performed by a team of experienced veterinarians. Bucks were anesthetized using a combination of 0.88 mg/kg tiletamine-zolazepam (Telazol®, Zoetis, Florham Park, New Jersey, USA) and 2.2 mg/kg xylazine (Cervicine®, ZooPharm, Windsor, Colorado, USA) administered intramuscularly via dart gun. A 2.2 mg/kg dose of ketamine (Ketaset®, Zoetis, Florham Park, New Jersey, USA) was given intravenously if needed for additional anesthesia. Each buck was then transported to a heated room and placed on a table for semen collection. Following semen collection, anesthesia was reversed using an intramuscular injection of 4.4 mg/kg tolazoline (Tolazine®, Lloyd, Inc., Shenandoa, Iowa, USA).

2.3 Semen collection and processing

Once bucks were anesthetized, the penis was manually exteriorized by applying simultaneous gentle pressure at the sigmoid flexure and base of the prepuce and the penis was then held extended using a folded gauze. To obtain semen samples, a 31.75 mm diameter, three electrode ram probe (Lane Manufacturing, Inc., Denver, Colorado, USA) was placed rectally, and a manual cycle was run on the electroejaculator unit (Pulsator IV, Lane Manufacturing, Inc., Denver, Colorado, USA). Semen was collected into a 15 mL conical tube submerged in a 37°C water bath until processing. A small drop of sample was evaluated under the microscope to ensure that a viable and appropriately concentrated sample was obtained prior to processing. Once the semen was deemed appropriate, 10 µL was added to a 1:201 dilution tube (Repro-Pette™ system, Reproduction Provisions, LLC, Walworth, Wisconsin, USA) for manual concentration calculation using a hemocytometer. The remainder of the sample was divided into six tubes for experiment 1 and three

tubes for experiment 2 and diluted with pre-warmed extender (37°C) at a ratio of 1:1. Samples were then transported approximately 30 minutes to the laboratory in a 37°C water bath. Once the raw concentration for each sample was determined, additional extender was added in a one-step method for all extenders except Biladyl, which required a two-step method, to make a final concentration of 120 million spermatozoa per mL. Immediately following this final dilution step, the samples were slowly cooled to 5°C by placing tubes in a 37°C water bath and placing the immersed samples in a cold-room at 5°C. Samples were incubated at 5°C for 3 to 4 h, adhering to manufacturers' requirements for all extenders. The samples were loaded into 0.5 mL semen straws and frozen manually after incubation by placing the straws 4 cm above the level of liquid nitrogen for 15 min and then submerging and storing in liquid nitrogen until further analysis.

2.4 Semen evaluation

Evaluation of total and progressive sperm motility was performed using Computer Assisted Sperm Analysis (CASA) equipment (Spermvision II, Minitube of America, Inc. Verona, Wisconsin, USA). The parameter settings were adjusted for cervid sperm as recommended by the manufacturer. Briefly, ~3 µL semen (360,000 spermatozoa) was placed into 20 µM depth chamber slides (Vitrolife, Microcell Counting Chambers, San Diego, California, USA) that were pre-warmed to 37°C. Data were obtained by averaging motility measurements of three to seven readings from various portions of the chambered slide. Pre-freeze sperm motility parameters were obtained after extending samples to the final concentration of 120 million per mL prior to cooling to 5°C for incubation. Post-thaw sperm motility parameters were assessed by CASA after thawing cryopreserved semen straws in a 37°C water bath for a minimum of 30 sec.

2.5 Fluorescent probes for sperm evaluation

Fluorescent probes were used for post-thaw evaluation of sperm cryopreserved in 0.5 mL straws using Andromed (n = 5), Ovine Red (n = 4), and Optixcell (n = 4) extenders from experiment 2. The LIVE/DEAD® Sperm Viability Kit (SYBR-14 and propidium iodide (PI) dyes; #L-7011) was purchased from Molecular Probes, Inc. (Eugene, Oregon, USA). Fluorescein isothiocyanate-conjugated pea (*Pisum sativum*) agglutinin (FITC-PNA; #L-7381) was purchased from Sigma Chemical Co. (Saint Louis, Missouri, USA). Acridine orange, C.I. (#04539-500) was purchased from Polysciences, Inc. (Warrington, Pennsylvania, USA). Dimethyl sulfoxide, anhydrous (DMSO; #D12345), triton X-100 surfact-amps detergent solution (#85111), and phosphate-buffered saline (PBS, pH 7.4) (#10010-023) were purchased from Thermo Fischer Scientific (Waltham, Massachusetts, USA). All other chemicals used were reagent grade and purchased from Sigma Aldrich (St. Louis, Missouri, USA).

2.6 Assessment of sperm viability

Sperm viability was assessed using a combination of SYBR-14 and propidium iodide (PI) based on the integrity of the sperm plasma membrane as previously described by Robles and Martínez-Pastor (2013). SYBR-14 was used as a membrane-permeable DNA intercalating agent with maximum emission of 516 nm (green) and readily stains all nuclei. Propidium iodide was also used as an intercalating agent with maximum emission of 617 nm (red), but it only stains the nucleus if the sperm plasma membrane is damaged which makes it a useful counterstain for dead cells. Briefly, stock solutions of SYBR-14 (0.02 mM in DMSO) and PI (2.4 mM in water) were prepared and frozen in aliquots at -20°C for subsequent use (Robles and Martínez-Pastor, 2013). Staining solution was prepared on the day of flow cytometry analysis by combining 5 µL SYBR-

14 (0.02 mM) and 5 μ L PI (2.4 mM) per mL of HEPES buffer (10 mM Hepes, 150 mM NaCl, 0.1% BSA, pH 7.4). The working concentrations of SYBR-14 and PI were 100 nM and 12 μ M, respectively. Cryopreserved semen was thawed in a 37°C water bath for 1 min, and 8 μ L of thawed semen was added to 0.5 mL staining solution to create a final concentration of 1.92 million spermatozoa per mL. The samples were mixed by reverse pipetting five to ten times and were subsequently incubated in the dark at 37°C for 10 to 15 min prior to flow cytometric analysis.

Samples were analyzed using a BD LSR II Flow Cytometry Analyzer (BD Biosciences, San Jose, California, USA) at the Roy Carver Biotechnology Center of the University of Illinois Urbana-Champaign. The two dyes were excited in the flow cytometer using a 488-nm argon excitation laser. Fluorescent data of all events was collected until 10,000 gated events were recorded. Two-dimensional plots of SYBR-14 versus PI fluorescence events were drawn, and debris was gated out based on those events that emitted minimal red and green fluorescence. As previously described (Robles and Martínez-Pastor, 2013), three sperm populations in the SYBR-14/PI stained sperm preparations were present (Fig. 1). Presumptively viable cells made up population 1, with only green fluorescent signal (SYBR-14 positive) detected. Population 2 exhibited a mixed green (SYBR-14 positive) and red (PI positive) fluorescence and were considered to be moribund with early or minor membrane damage. Population 3 comprised cells with only red fluorescent signal (PI positive) detected and were considered dead. The percentage of cells in each of the three populations was calculated.

2.7 Assessment of acrosome integrity

Acrosome integrity was assessed using a combination of FITC-PNA (peanut agglutinin conjugated with fluorescein isothiocyanate) and PI as previously described by Robles and

Martínez-Pastor (2013). FITC-PNA has a maximum emission of 521 nm (green) and targets the inner leaflet of the outer acrosomal membrane, identifying damaged acrosomes. Again, PI was used as a counterstain to allow for simultaneous assessment of sperm viability and condition of the acrosome. Briefly, stock solutions of FITC-PNA (1 mg/mL in PBS) were prepared and frozen in aliquots at -20°C for subsequent use (Robles and Martínez-Pastor, 2013). Staining solution was prepared by combining 0.625 μL PI (2.4 mM) and 1 μL FITC-PNA (1 mg/mL) per mL of PBS. Final concentrations of the stains were 1.5 μM PI and 1 $\mu\text{g/mL}$ FITC-PNA. Cryopreserved semen was thawed in a 37°C water bath for 1 min, and 8 μL of thawed semen was added to 0.5 mL staining solution to create a final concentration of 1.92 million spermatozoa per mL. The samples were mixed by reverse pipetting five to ten times and were subsequently incubated in the dark at 37°C for 10 to 15 min prior to flow cytometric analyses.

Flow cytometric analyses were carried out as described above with the exception that FITC-PNA fluorescence was detected at 515–545 nm fluorescence detector 1. Fluorescent data of all events was collected until 10,000 gated events were recorded. Non-sperm events were gated out of analyses as judged on scatter properties. Debris was manually excluded in Ovine Red samples due to the presence of egg yolk. Two-dimensional plots of FITC-PNA versus PI fluorescence events were drawn. As previously described (Robles and Martínez-Pastor, 2013), four sperm populations in the FITC-PNA/PI stained sperm preparations were present (Fig. 2). Population 1 (Viable + IA) included sperm that were viable (PI negative) with an intact acrosome (FITC-PNA negative). Population 2 (Viable + DA) encompassed sperm that were viable (PI negative) with a damaged acrosome (FITC-PNA positive). Populations 3 (Dead + IA) and 4 (Dead + DA) contained sperm that were dead (PI positive) with intact (FITC-PNA negative) or damaged (FITC-PNA

positive) acrosomes, respectively. These populations were divided by quadrants, and the frequency of each population was quantified.

2.8 Assessment of sperm chromatin stability

Chromatin stability was assessed by meta-chromatic staining with acridine orange (AO) based on the susceptibility of the sperm DNA to acid-induced denaturation in situ (Martínez-Pastor et al., 2004). This is a cell-permeant nucleic acid binding dye that emits green fluorescence when bound to double stranded DNA (dsDNA) and red fluorescence when bound to single stranded DNA (ssDNA) or RNA. Cryopreserved semen was thawed in a 37°C water bath for 1 min, and 15 µL of thawed semen was added to 985 µL PBS to create a final sperm concentration of approximately 1.8 million spermatozoa per mL. Acid-induced denaturation of DNA in situ was attained by adding 0.4 ml of an acid-detergent solution (0.17% Triton X-100, 0.15 M sodium chloride, and 0.08 N hydrogen chloride; pH 1.4) to 0.2 mL diluted semen. After 30 sec, the cells were stained by adding 1.2 mL of a citric phosphate solution (0.1 M citric acid, 0.2 M sodium phosphate dibasic, 1 mM ethylenediaminetetraacetic acid, 0.15 M sodium chloride; pH 6.0) containing 6 µg/ml AO and mixed by reverse pipetting five to ten times. The stained samples were subsequently incubated in the dark at 37°C for 3 min prior to flow cytometric analyses.

Flow cytometry analysis was carried out as above using a 488-nm argon excitation laser. Fluorescent data of all events was collected until 10,000 gated events were recorded. Debris was discarded electronically and based on low fluorescence values. Cells were plotted depending on red and green fluorescence intensity. Two populations were discerned (Fig. 3) by drawing a diagonal line between the groups of cells with stable chromatin (intact DNA) to the left of the line (medium to high green fluorescence and low red fluorescence) and the group of cells with loose

chromatin (damaged DNA) to the right of the line (medium to high green fluorescence and medium to high red fluorescence). The frequency of each of these populations was quantified. DNA fragmentation index (DFI %) was calculated as the number of cells with loose chromatin divided by the sum of both populations.

2.6 Statistical Analysis

Data are presented as percentage mean \pm SEM. All statistical analyses were performed using R Version 3.2.2 (<https://www.r-project.org/>). Data for each extender and buck in experiment 1 were analyzed separately from data obtained in experiment 2 and are presented as such. Homogeneity of variances among samples was established using a Bartlett test, and normality was confirmed using a Shapiro-Wilk test of the residuals. General Linear Models were used for all analyses of variance with repeated measures for determination of treatment \times time interactions for total and progressive motility parameters. Post-thaw fluorescent probe data from experiment 2 were also analyzed using one-way analyses of variance. A Tukey HSD test was performed as needed for post-hoc analysis. Pearson's correlation test was used to evaluate the association between flow cytometry parameters and the corresponding post-thaw motility from each sample analyzed in experiment 2. Parameters with high correlation were defined at $r > 0.66$, moderate correlation at $0.55 < r \leq 0.65$, and low correlation at $0.45 < r \leq 0.55$. Significance was declared at $p \leq 0.05$ whereas tendency was discussed at $0.05 < p < 0.10$.

3. Results

3.1 Total and progressive sperm motility

As expected, cryopreservation was associated with a reduction in total ($p \leq 0.02$) and progressive motility ($p \leq 0.04$) for all extenders (Fig. 4 and 5). In experiment 1, there was a time by group interaction for total sperm motility with sperm cryopreserved in Andromed and Ovine Red extenders presenting a higher percent of total motility at post-thaw than those extended in Biladyl 4% or Biladyl 6% ($p < 0.05$) with no differences observed prior to cryopreservation ($p \geq 0.99$) (Fig. 4a). Additionally, sperm cryopreserved with Triladyl extender tended to have a higher total sperm motility at post-thaw than those extended in Biladyl 4% ($p = 0.08$) with no differences observed prior to cryopreservation ($p > 0.99$). There were no differences in total sperm motility between Andromed, Ovine Red, Triladyl, or Biladyl 8% extenders ($p \geq 0.65$), between Triladyl, Biladyl 8%, or Biladyl 6% extenders ($p \geq 0.15$), or between Biladyl 8%, Biladyl 6%, or Biladyl 4% extenders ($p \geq 0.89$) (Fig. 4a). There was a main effect of treatment for progressive sperm motility with sperm cryopreserved in Andromed extender exhibiting higher progressive motility than all other extenders ($p < 0.01$) (Fig. 4b). There were no differences in progressive sperm motility between Ovine Red, Triladyl, or Biladyl (containing 8%, 6%, or 4% of glycerol) extenders ($p \geq 0.11$) (Fig. 4a). In experiment 2, both total and progressive sperm motility did not differ between Andromed, Ovine Red, or Optixcell extenders ($p \geq 0.59$, Fig. 5a; and $p \geq 0.39$, Fig. 5b, respectively).

3.2 Fluorescent probes for sperm evaluation

The quantitative results of flow cytometric analyses are summarized in Table 1. There were no differences in the percentages of dead ($p = 0.17$), viable ($p = 0.18$), or moribund ($p = 0.52$) sperm

amongst Andromed, Ovine Red, and Optixcell extenders. There were also no differences in percentage of viable sperm cells with intact acrosome ($p = 0.53$), percentage of viable sperm cells with damaged acrosome ($p = 0.10$), or percentage of dead sperm cells with damaged acrosome ($p = 0.96$) between Andromed, Ovine Red, and Optixcell extenders. There was a tendency for percentage of dead sperm cells with intact acrosome in Optixcell to be less than in Andromed ($p = 0.07$), but no differences between Ovine Red and Andromed ($p = 0.24$) or between Ovine Red and Optixcell ($p = 0.73$) were identified. Finally, there was no difference in the DNA fragmentation index ($p = 0.15$) amongst Andromed, Ovine Red, and Optixcell extenders.

3.3 Correlation between motility and fluorescent probes for sperm evaluation

Andromed and Optixcell motility parameters were significantly and highly correlated with the percent of dead sperm with damaged acrosome (supplement). Whereas, semen cryopreserved with Ovine Red was significantly and highly correlated with progressive motility. Since the remaining results for staining with fluorescent probes were not significant, we combined all data for further analyses (Table 2). Post-thaw total and progressive sperm motility exhibited high and positive correlation with sperm viability ($p \leq 0.01$). Additionally, both post-thaw total and progressive sperm motility exhibited high negative correlation with percentage of dead sperm with damaged acrosome ($p \leq 0.01$) with a tendency to have a low but positive correlation to the percentage of viable sperm with intact acrosome and percentage of viable sperm with damaged acrosome ($p < 0.10$). Furthermore, post-thaw progressive motility was moderately and negatively correlated with DNA fragmentation index ($p = 0.04$).

4. Discussion

Recently, there has been much incentive to develop alternatives to traditional egg yolk-based extenders for cryopreservation of semen in domesticated species. Our present study is the first to demonstrate similar effectiveness of both soybean-based (Andromed) and liposome-based (Optixcell) extenders for cryopreservation of white-tailed deer semen compared to a traditional egg-yolk extender (Ovine Red). In addition, Andromed appears to be a more suitable extender than Triladyl and Biladyl in preserving sperm motility parameters after cryopreservation.

While the use of Andromed extender to cryopreserve white-tailed deer semen had not been previously reported, in one study a different commercial soybean-based extender (Bioxcell) presented similar post-thaw parameters to Triladyl and Biladyl 8% extenders (Clemente-Sánchez et al., 2014). Herein, high percent of post-thaw total motility in white-tailed deer sperm cryopreserved with Andromed extender was consistent with post-thaw motility results observed in bulls when Andromed was compared to a tris-based egg yolk extender (Aires et al., 2003). In that study, a subsequent fertility test revealed higher conception rates in Andromed cryopreserved bull semen (Aires et al., 2003). In rams, Andromed was reported to have similar post-thaw motility and higher percent of sperm with intact acrosome in comparison with Triladyl (Jerez et al., 2016). Andromed was also found to have similar post-thaw motility in goats (Salmani et al., 2014) and similar lambing rates and prolificacy in sheep (Fukui et al., 2008) when compared to a tris-based egg yolk extender. In red deer, Triladyl and Andromed performed better than soybean-based Bioxcell extender in regards to post-thaw total sperm motility with no significant differences in progressive motility (Martínez-Pastor et al., 2009). Altogether, these results indicate that soybean-based extenders seem to be superior or least equally effective as egg yolk-based extenders at

cryopreserving semen across species and might be advantageous when compared with egg yolk based extenders in white-tailed deer.

Contrary to our hypothesis, sperm cryopreserved with Optixcell, a liposome-based extender, resulted in similar post-thaw motility to Andromed and Ovine Red extenders. Interestingly, progressive sperm motility was also not different between Andromed and Ovine Red, which is conflicting with the results in experiment 1. While the cause of this discrepancy cannot be fully elucidated, it may be attributed to the different population of bucks used in experiments 1 and 2. Nevertheless, both of these findings are in contrast to a study in horses that showed higher post-thaw motility in egg yolk-based versus liposome-based extenders, but no differences in fertility rate or membrane integrity (Pillet et al., 2012). Our findings also differ from those observed in water buffalo (*Bubalus bubalis*), which reported better total and progressive motility in Optixcell versus soybean-based (Andromed and Bioxcell) and tris-based egg yolk extenders (Kumar et al., 2015). These variations highlight the importance of conducting our study with white-tailed deer, the species of interest, as limited extrapolations can be made from other species of mammals. The comparable motility parameters observed in the present study indicate that liposome-based extenders can also be a suitable substitute to egg yolk-based extenders for cryopreservation of semen in white-tailed deer.

Our study appears to be the first to employ fluorescent probes for evaluation of spermatozoa in white-tailed deer. Results obtained with flow cytometry to evaluate the sperm components are consistent with post-thaw motility parameters for Optixcell, Andromed, and Ovine Red extenders. Interestingly, a similar study in red deer found that a non-commercial, liposome-based extender produced a lower percentage of viable sperm than Triladyl extender, but did not differ from

soybean-based extenders, Andromed and Bioxcell (Martínez-Pastor et al., 2009). Though we did not test Triladyl in experiment 2, the lack of difference in sperm viability between soybean-based and liposome-based extenders in the current study was consistent with those results. Furthermore, sperm viability had a high correlation with post-thaw total and progressive motilities in the current study, which is consistent with other reports in stallions and bulls (Garner et al., 1997; Thomas et al., 1998, Love et al., 2003), suggesting that assessment of sperm motility may also be an indirect indicator of sperm viability in white-tailed deer.

Additionally, both total and progressive motilities had high, negative correlation with the percentage of dead sperm with damaged acrosomes and a low, positive correlation with viable sperm with both intact and damaged acrosomes, which is likely a result of the correlation with viability, as has been demonstrated previously in bulls (Thomas et al., 1997) and stallions (Kirk et al., 2005). In the present study, sperm cryopreserved in Andromed extender tended to have a higher percentage of dead sperm with intact acrosomes than Optixcell. These results are similar to findings in red deer (*Cervus elaphus*) where sperm cryopreserved with Andromed extender had higher percentages of sperm with intact acrosomes than a liposome-based extender (Martínez-Pastor et al., 2009). On the contrary, no differences were found in either plasma or acrosomal membrane integrity between Optixcell, Andromed, Bioxcell, and tris-based egg yolk extenders in water buffalo (Kumar et al., 2015). Nevertheless, *in vivo* or *in vitro* fertilization studies are warranted to determine the true relevance of these differences in regards to reproductive success.

Index of DNA fragmentation was not only similar between extenders but also was below 15%, which has been associated with normal fertility in men, bulls, boars, and stallions (Ballachey et al., 1988, Evenson et al., 1994, Kenney et al, 1995, Varghese et al., 2011). Interestingly, the DNA

fragmentation index was negatively correlated with progressive motility, but not with total motility. This suggests that assessment of progressive sperm motility is a useful parameter to determine substructural damage in sperm. As previously mentioned, the higher the DNA fragmentation index, the lower the fertility in various species. Further controlled studies are warranted to determine the reason for this association and determine its clinical relevance.

In summary, soybean-based (Andromed) and liposome-based (Optixcell) extenders are equally or more effective than traditional egg-yolk based extenders (Ovine Red, Triladyl, and Biladyl) for cryopreservation of white-tailed deer semen, as determined by *in vitro* sperm analyses, and appear to be a suitable replacement for use in clinical practice. It is important to note that direct fertility was not assessed in the current study, thus future studies should be performed to examine the association between different semen parameters observed in the current study and fertility.

Conflict of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality in conducting the experiment and publishing the manuscript.

Author contributions

All authors were involved in anesthesia, semen collection, and cryopreservation. The project was designed by C.F.S., A.S.K., and J.L.S. Extender preparation was done by A.S.K. and J.L.S. All CASA sperm analyses were performed by J.L.S. Flow cytometry was performed by J.L.S., R.E.E., E.P., and I.F.C. Statistical analyses was performed by J.L.S. and F.S.L. All authors contributed to the data interpretation and preparation of the manuscript.

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Tables

Table 1. Summary of fluorescent probe results for semen cryopreserved using Andromed (AM), Ovine Red (OR), and Optixcell (OC) extenders in experiment 2 (mean \pm SEM).

	% Viable	% Dead	% Moribund	% Viable + IA	% Viable + DA	% Dead + IA	% Dead + DA	DFI %
AM	80 \pm 4.5	18 \pm 3.9	2.8 \pm 0.8	44 \pm 8.0	11 \pm 3.1	23 \pm 5.3 ^a	22 \pm 6.3	7.2 \pm 1.5
OR	75 \pm 5.9	23 \pm 6.0	2.2 \pm 0.5	56 \pm 2.6	8.9 \pm 2.9	13 \pm 1.9 ^{a,b}	23 \pm 3.1	14 \pm 4.0
OC	89 \pm 3.3	9.6 \pm 2.9	1.7 \pm 0.5	50 \pm 8.7	21 \pm 5.1	8.5 \pm 2.7 ^b	21 \pm 5.9	5.8 \pm 2.2
<i>p</i>-value	0.18	0.17	0.52	0.53	0.10	0.07	0.96	0.15

IA: Intact acrosome; DA: damaged acrosome; DFI: DNA fragmentation index. There were no significant differences ($p \leq 0.05$). Data with differing superscripts tended to be different ($p < 0.10$).

Table 2. Correlation coefficients between total (TM) and progressive (PM) motility (%) and flow cytometry data in experiment 2.

	% Viable	% Dead	% Moribund	% Viable + IA	% Viable + DA	% Dead + IA	% Dead + DA	DFI %
TM	0.67***	-0.69***	-0.19	0.52*	0.53*	-0.27	-0.91***	-0.42
PM	0.75***	-0.76***	-0.31	0.52*	0.48*	-0.32	-0.82***	-0.57**

IA: Intact acrosome; DA: damaged acrosome; DFI: DNA fragmentation index. ***Data with high correlation ($p \leq 0.01$); **Data with moderate correlation ($p \leq 0.05$); * Data with low correlation ($p < 0.10$).

Figures

Fig. 1. Representative flow cytometry dot plots for the sperm viability assay, SYBR-14/propidium iodide (PI), for Andromed (a), Ovine Red (b), and Optixcell (c) extenders within one buck. Units indicate fluorescence intensity with high SYBR-14 producing green fluorescence (X-axis) and high PI producing red fluorescence (Y-axis). Based on staining pattern, three populations of cells and one unstained population/debris (bottom left quadrant) are observed. The top left quadrant encloses PI positive events (high PI, low SYBR-14), corresponding to dead sperm. The bottom right quadrant encloses SYBR-14 positive events (high SYBR-14, low PI), corresponding to viable sperm. The top right quadrant represents events with mixed fluorescence, indicating moribund sperm. All three extenders showed similar patterns of sperm viability.

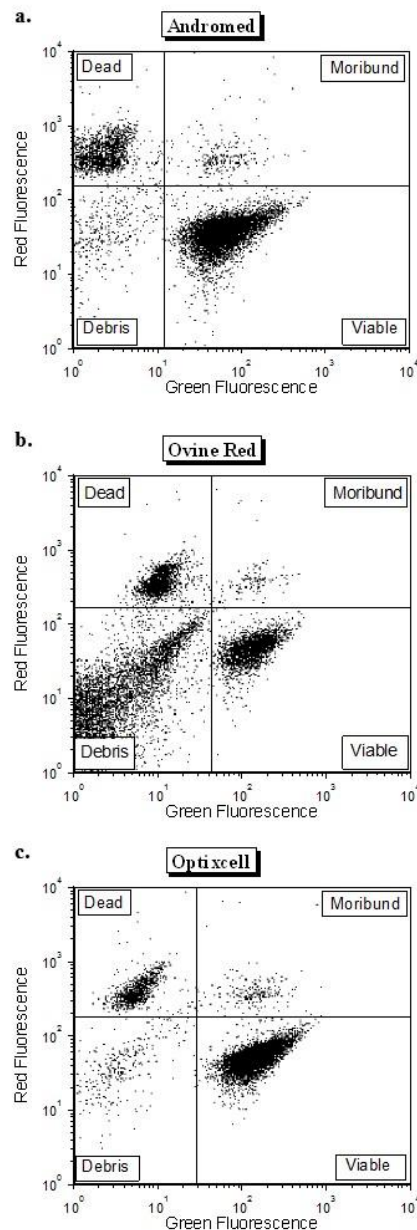


Fig. 2. Representative flow cytometry dot plots for acrosomal integrity assay, FITC-PNA (peanut agglutinin conjugated with fluorescein isothiocyanate) and propidium iodide (PI), for Andromed (a), Ovine Red (b), and Optixcell (c) extenders within one buck. Units indicate fluorescence intensity with high FITC-PNA producing green fluorescence (X-axis) and high PI producing red fluorescence (Y-axis). Four populations of sperm are recognized with this staining protocol. The first unstained population of viable sperm (low PI) with intact acrosomes (low FITC-PNA) is in the lower-left quadrant (Viable + IA). In (b), a population of debris was manually discarded due to the presence of egg yolk extender. The upper quadrants encompass the populations of dead sperm (high PI), with damaged acrosomes (high FITC-PNA) on the right (Dead + DA) and intact acrosomes (low FITC-PNA) on the left (Dead + IA). A fourth population of viable (low PI) and acrosome-damaged sperm (high FITC-PNA) are in the lower-right quadrant (Viable + DA). All three extenders show similar quality in regards to acrosome integrity.

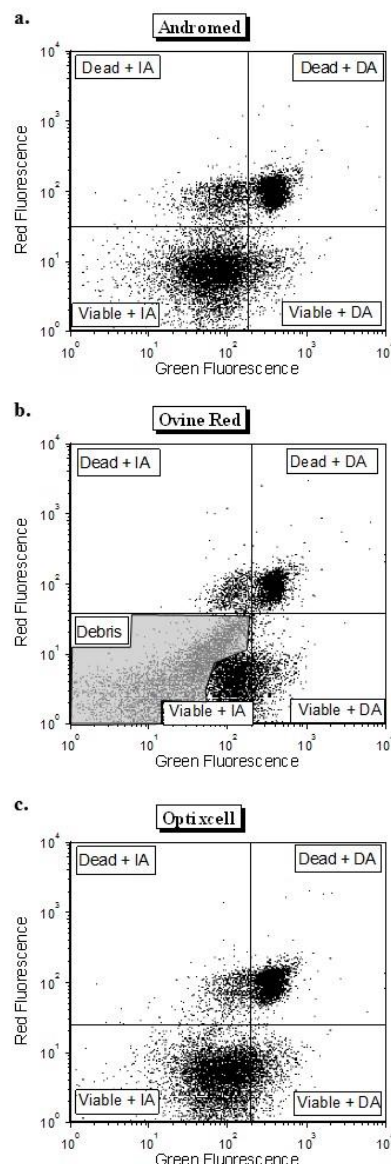


Fig. 3. Representative flow cytometry dot plots for the chromatin stability assay, acridine orange, for Andromed (a), Ovine Red (b), and Optixcell (c) extenders from one buck. Units indicate fluorescence intensity with binding to double stranded (intact) DNA producing green fluorescence (Y-axis) and binding to single stranded (damaged) DNA or RNA producing red fluorescence (X-axis). A population of unstained debris (lower left corner) was discarded. The elongated cloud on the left is the sperm population with good chromatin integrity (Intact DNA) emitting green fluorescence. Dots to the right of the diagonal line are cells outside of the main population that bear loose chromatin (Damaged DNA) emitting red fluorescence. There was no significant difference in in the DNA fragmentation index for the 3 extenders.

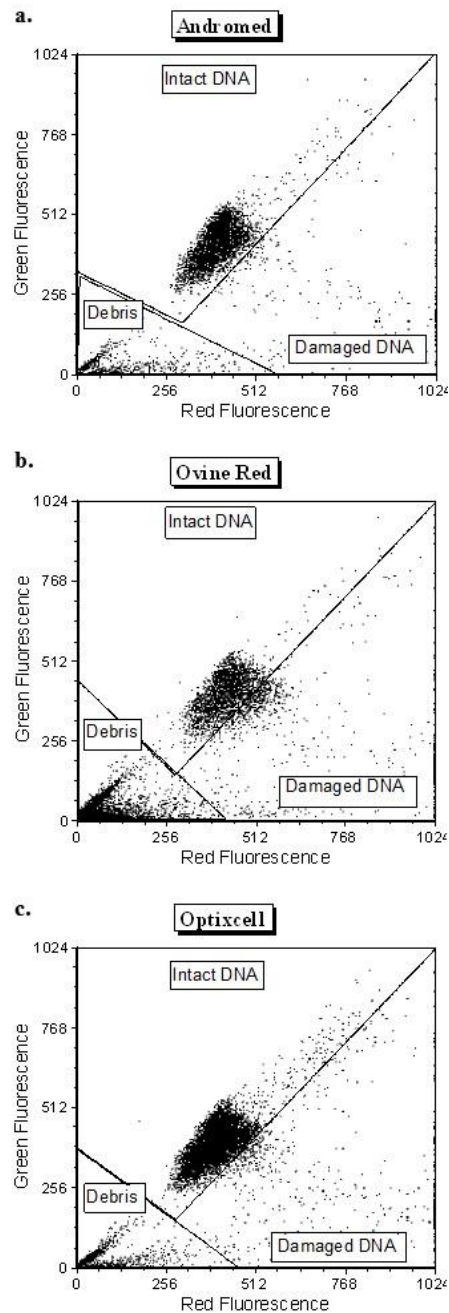


Fig. 4. Bar graph showing percent total (a) and progressive (b) sperm motility immediately before and after semen cryopreservation with Biladyl 4% (B4), Biladyl 6% (B6), Biladyl 8% (B8), Triladyl (TR), Ovine Red (OR), and Andromed (AM) extenders in experiment 1 (mean \pm SEM). Data points with differing superscripts were significantly different ($p \leq 0.05$). Superscripts denoted with an asterisk (*) represent data points with a tendency to be different ($p < 0.10$).

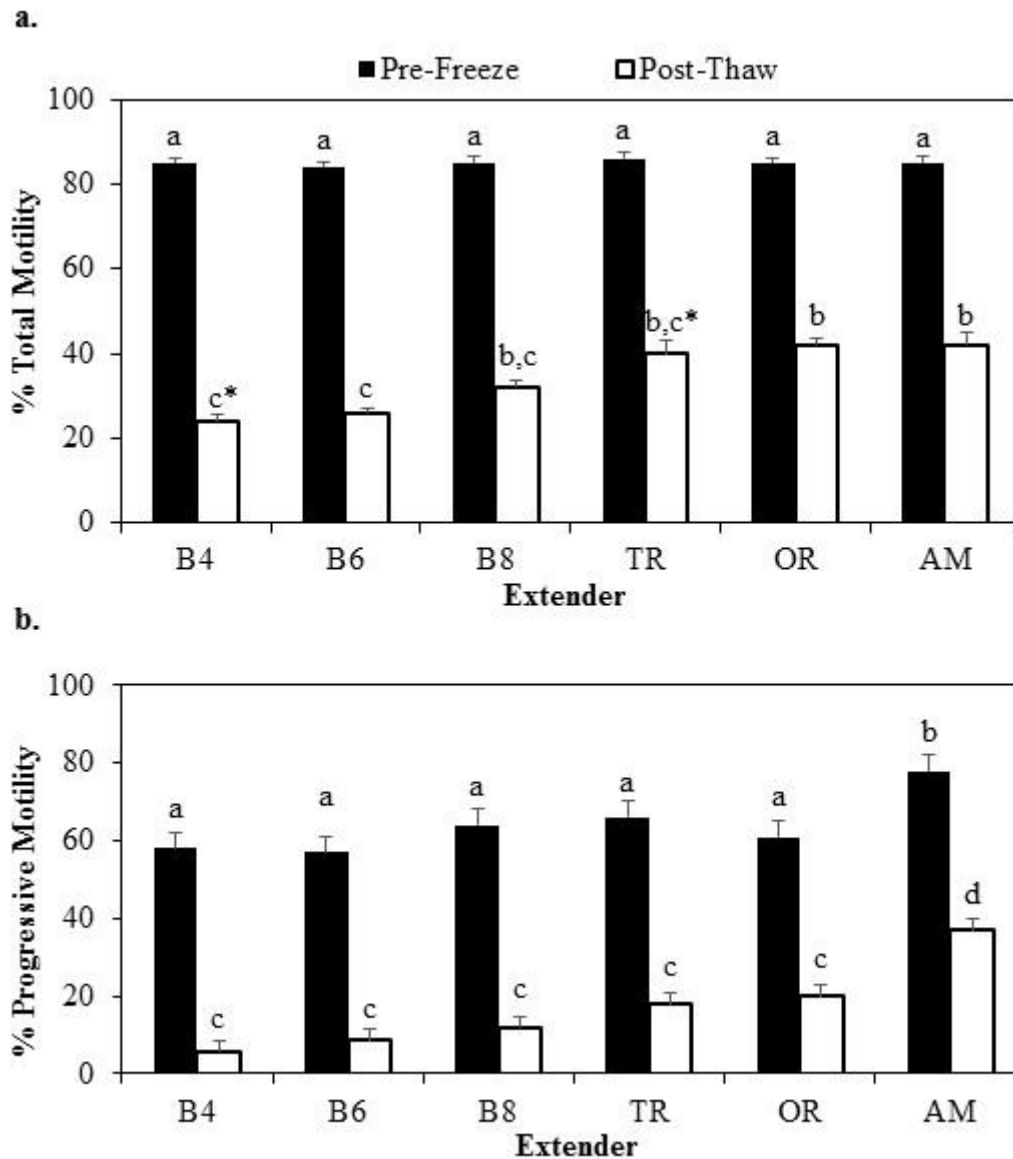
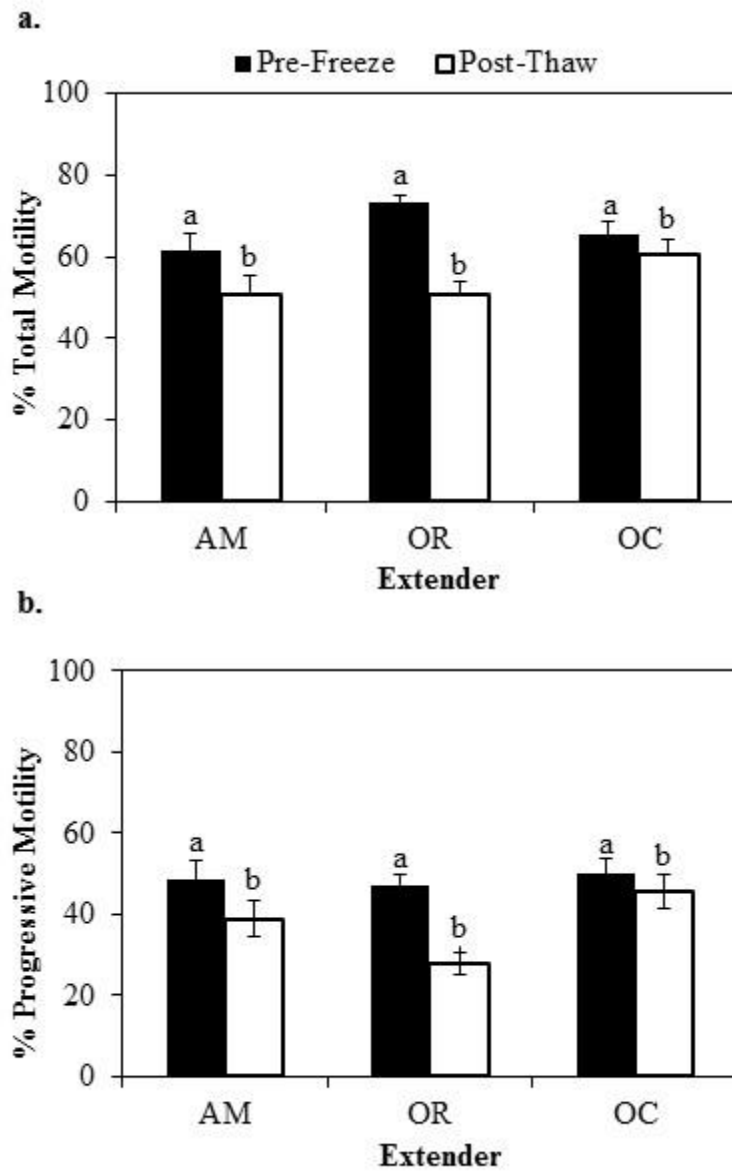


Fig. 5. Bar graph showing percent total (a) and progressive (b) sperm motility immediately before and after semen cryopreservation with Andromed (AM), Ovine Red (OR), and Optixcell (OC) extenders in experiment 2 (mean \pm SEM). Data points with differing superscripts were significantly different ($p \leq 0.05$).



References

- Aires, V.A., Hinsch, K.D., Mueller-Schloesser, F., Bogner, K., Mueller-Schloesser, S., Hinsch, E., 2003. In vitro and in vivo comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of bovine semen. *Theriogenology*. 60(2), 269-279.
- Ake-Lopez, J., Cavazos-Arizpe, E., Magana-Monforte, J.G., Centurion-Castro, F., Silva-Mena, C., 2010. Effect of age and postmortem time on some white-tailed deer (*Odocoileus virginianus texanus*) epididymal sperm characteristics and response of cryopreservation. *American Journal of Animal and Veterinary Sciences*. 5(3), 183-186.
- Anderson, D., Frosch, B.J., Outlaw, J.L., 2007. Economic impact of the United States cervid farming industry. Agricultural and Food Policy Center, Texas A&M University, College Station, TX. <https://www.afpc.tamu.edu/pubs/2/480/rr-2007-04.pdf> (accessed 16.02.12)
- Asher, G.W., Berg, D.K., Evans, G., 2000. Storage of semen and artificial insemination in deer. *Animal Reproduction Science*. 62(1), 195-211.
- Aydin, R., Dogan, I., 2010. Fatty acid profile and cholesterol content of egg yolk from chickens fed diets supplemented with purslane (*Portulaca oleracea* L.). *Journal of the Science of Food and Agriculture*. 90(10), 1759-1763.
- Ballachey, B.E., Evenson, D.P., Saacke, R.G., 1988. The sperm chromatin structure assay. Relationship with alternate tests of semen quality and heterospermic performance of bulls. *J Androl*. 9(2), 109-15.
- Bousseau, S., Brillard, J.P., Marquant-Le Guienne, B., Guerin, B., Camus, A., Lechat, M., 1998. Comparison of bacteriological qualities of various egg yolk sources and the in vitro and in vivo fertilizing potential of bovine semen frozen in egg yolk or lecithin based diluents. *Theriogenology*. 50(5), 699-706.
- Clemente-Sánchez, F., Cessa-Reyes, V., Cortez-Romero C, Tarango-Arambula L.A., Arenas-Baez, P., 2014. Commercial extenders and freezing curves for the preservation of sperm cells of white-tailed deer (*Odocoileus virginianus*). *Journal of Applied Animal Research*. 1. 1-6.
- Evenson, D.P., Thompson, L., Jost, L., 1994. Flow cytometric evaluation of boar semen by the sperm chromatin structure assay as related to cryopreservation and fertility. *Theriogenology*. 41(3), 637-651.
- Fukui, Y., Kohno, H., Togari, T., Hiwasa, M. and Okabe, K., 2008. Fertility after artificial insemination using a soybean-based semen extender in sheep. *Journal of Reproduction and Development*. 54(4), 286-289.
- Garner, D.L., Thomas, C.A., Joerg, H.W., Dejarnette, J.M., Marshall, C.E., 1997. Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. *Biology of reproduction*. 57(6), 1401-1406.

- Jerez, R., González, N., Olaciregui, M., Luño, V., de Blas, I., Gil, L., 2016. Use of soy milk combined with different cryoprotectants for the ram semen cryopreservation. *Small Ruminant Research*. 134, 34-38.
- Kenney, R.M., Evenson, D.P., Garcia, M.C., Love, C.C., 1995. Relationships between sperm chromatin structure, motility, and morphology of ejaculated sperm and seasonal pregnancy rate. *Society for the Study of Reproduction*. 4, 647-653.
- Kirk, E.S., Squires, E.L., Graham, J.K., 2005. Comparison of in vitro laboratory analyses with the fertility of cryopreserved stallion spermatozoa. *Theriogenology*, 64(6), 1422-1439.
- Kumar, P., Saini, M., Kumar, D., Balhara, A.K., Yadav, S.P., Singh, P., Yadav, P.S., 2015. Liposome-based semen extender is suitable alternative to egg yolk-based extender for cryopreservation of buffalo (*Bubalus bubalis*) semen. *Animal reproduction science*. 159, 38-45.
- Love, C.C., Thompson, J.A., Brinsko, S.P., Rigby, S.L., Blanchard, T.L., Lowry, V.K., Varner, D.D., 2003. Relationship between stallion sperm motility and viability as detected by two fluorescence staining techniques using flow cytometry. *Theriogenology*. 60(6), 1127-1138.
- Martinez-Pastor, F., Johannisson, A., Gil, J., Kaabi, M., Anel, L., Paz, P., Rodriguez-Martinez, H., 2004. Use of chromatin stability assay, mitochondrial stain JC-1, and fluorometric assessment of plasma membrane to evaluate frozen-thawed ram semen. *Animal reproduction science*. 84(1), 121-133.
- Martínez-Pastor, F., Martínez, F., Alvarez, M., Maroto-Morales, A., García-Alvarez, O., Soler, A.J., Garde, J.J., De Paz, P., Anel, L., 2009. Cryopreservation of Iberian red deer (*Cervus elaphus hispanicus*) spermatozoa obtained by electroejaculation. *Theriogenology*. 71(4), 628-638.
- Pillet, E., Labbe, C., Batellier, F., Duchamp, G., Beaumal, V., Anton, M., Desherces, S., Schmitt, E., Magistrini, M., 2012. Liposomes as an alternative to egg yolk in stallion freezing extender. *Theriogenology*. 77(2), 268-279.
- Robles, V., Martínez-Pastor, F., 2013. Flow cytometric methods for sperm assessment. *Spermatogenesis: Methods and Protocols*. 175-186.
- Röpke, T., Oldenhof, H., Leiding, C., Sieme, H., Bollwein, H., Wolkers, W.F., 2011. Liposomes for cryopreservation of bovine sperm. *Theriogenology*. 76(8), 1465-1472.
- Salmani, H., Towhidi, A., Zhandi, M., Bahreini, M., Sharafi, M., 2014. In vitro assessment of soybean lecithin and egg yolk based diluents for cryopreservation of goat semen. *Cryobiology*. 68(2), 276-280.
- Sathe, S., Shipley, C.F., 2014. Applied Andrology in Sheep, Goats and Selected Cervids, in: Chenoweth, P.J., Lorton, S. (Eds.), *Animal Andrology: Theories and Applications*, CAB International, Boston, Massachusetts, pp. 226-253.

Thomas, C.A., Garner, D.L., DeJarnette, J.M., Marshall, C.E., 1997. Fluorometric assessments of acrosomal integrity and viability in cryopreserved bovine spermatozoa. *Biology of reproduction*. 56(4), 991-998.

Thomas, C.A., Garner, D.L., DeJarnette, J.M., Marshall, C.E., 1998. Effect of cryopreservation of bovine sperm organelle function and viability as determined by flow cytometry. *Biology of reproduction*. 58(3), 786-793.

Varghese, A.C, Fischer-Hammadeh, C., Hammadeh, M.E., 2011. Acridine Orange Test for Assessment of Human Sperm DNA Integrity, in: Zini, A., Agarwal, A. (Eds.), *Sperm Chromatin: Biological and Clinical Applications in Male Infertility and Assisted Reproduction*, Springer Science + Business Media, LLC, New York, pp. 189-200.

Appendix B:

Physiological variations in reproductive and metabolic features of white-tailed deer (*Odocoileus virginianus*) bucks throughout the rutting season

The effects of seasonality on white-tailed deer reproduction is a project I contributed to during my Ph.D. program, but it was not part of my dissertation work. The following paper is a result of work done on this project.

Physiological variations in reproductive and metabolic features of white-tailed deer (*Odocoileus virginianus*) bucks throughout the rutting season¹

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Abstract

White-tailed deer farming is a growing industry in the United States, yet there is still a need to improve breeding practices in captive cervids. The objective of this study was to compare reproductive and somatic characteristics of white-tailed bucks early in the breeding season (September), at peak rut (December), and late breeding season (March). We hypothesized that reproductive parameters would improve at the peak of the breeding season. Young, mature bucks (n=7-11, ages 2 to 3 yr) were anesthetized via projector with tiletamine-zolazepam and xylazine. Semen was collected by electroejaculation and evaluated for sperm output, motility, and morphology. The bulbourethral, vesicular, and prostate glands were measured by transrectal ultrasound. Body condition score, thoracic circumference, rump fat thickness, scrotal circumference, and testicular measurements were also recorded. Serum testosterone, estradiol, and IGF-I concentrations were measured. Body condition score and rump fat thickness were highest in September, intermediate in December, and lowest in March ($p<0.01$), whereas thoracic circumference was lowest in September ($p=0.02$). In September, serum IGF-I was highest ($p<0.01$) and estradiol was lowest ($p\leq 0.02$). Serum testosterone levels were lowest in March and highest in December ($p<0.01$). Testicular volume and scrotal circumference were highest in September,

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intermediate in December, and lowest in March ($p<0.01$). Bulbourethral gland length was greatest in March ($p\leq 0.05$), whereas prostate length was greatest in September ($p\leq 0.02$). Vesicular gland length and width were highest in December, intermediate in September, and lowest in March ($p\leq 0.02$). Both overall and progressive sperm motilities were lowest in March ($p<0.01$), but did not differ between September and December ($p\geq 0.12$). In December, bucks had the highest percentage of normal sperm and lowest percentage of primary ($p\leq 0.01$) defects. Collectively, our results demonstrated variation in reproductive and metabolic characteristics of white-tailed deer bucks throughout the breeding season at 40°N. The physiological variations described here will be useful for veterinarians and researchers performing reproductive evaluations in white-tailed deer bucks.

Keywords: andrology, breeding soundness, cervid, metabolism, semen quality

1. Introduction

Deer farming is a growing industry with a total economic impact that has increased from \$3.0 billion to \$7.9 billion annually over the last 10 years and currently supports more than 56,000 jobs [1,2]. Deer breeding operations that sell stocker bucks to hunting facilities generate approximately \$935 million alone [2]. The majority of cervid species represented in these reports were white-tailed deer (WTD) (*Odocoileus virginianus*) [1,2]. However, there is surprisingly limited data on normal reproductive characteristics in this species [3]. As in other mammals, male reproductive soundness is crucial to ensuring a successful breeding season and optimal profit for the owner. Currently, veterinarians extrapolate from established bull andrology parameters when assessing the reproductive potential of WTD bucks, despite major physiological reproductive differences in these species [4,5].

White-tailed deer are exclusively short-day breeders, with bucks entering the rutting season in September, and does beginning follicular development by September or October [6]. At rut, rising blood testosterone concentrations coincide with mineralization of the antlers and rubbing off of velvet, thus coining the term “in hard antler”. Seasonal blood testosterone fluctuations have been well described in cervids, with peak concentrations from October to December in WTD bucks [7–11]. Testosterone enhances spermatogenesis and influences accessory sex gland functions; therefore, seasonal fluctuations of this hormone may affect deer sperm quality throughout rut [12]. Breeding soundness examinations are recommended early in the breeding season to allow ample time to find replacement males if needed. Conversely, assessments may be indicated late in the season to investigate the cause of poor conception rates in does. Normal physiological variations during the transitional periods (i.e., beginning or end of rut) may confound the evaluation of buck reproductive potential. Therefore, characterization of breeding soundness parameters at multiple time points throughout the breeding season may provide relevant reference ranges for other investigators and practicing veterinarians.

In addition to reproductive variations, cervids exhibit a distinctive appetite cycle throughout the year. Voluntary feed intake of WTD increases from March through August, during sexual inactivity, with concomitant increase in fat deposition [13,14]. Conversely, voluntary feed intake decreases throughout rut, which triggers mobilization of fat deposits [13,14]. Alterations in metabolic hormones, such as insulin-like growth factor-I (IGF-I), have been reported in response to changes in both season and nutrition [15]. It was previously reported that blood IGF-I concentrations are strongly and positively correlated with antler growth in WTD [16]. Additionally, IGF-I was positively associated with serum testosterone, suggesting a potential synergistic role in the reproductive function of WTD bucks [11]. Therefore, it is important to

evaluate the role of metabolic and steroid hormones on reproductive function throughout the breeding season.

Given the current economic importance of cervids in the hunting industry and scarce information in the literature, the establishment of the normal reproductive parameters of WTD at different stages of rut is needed. Therefore, the objective of this study was to evaluate reproductive and metabolic characteristics of WTD bucks early in the breeding season (September), at peak rut (December), and late breeding season (March) in central Illinois. We hypothesized that reproductive parameters would be superior at peak rut despite metabolic parameters being decreased.

2. Materials and methods

The Institutional Animal Care and Use Committees of the University of Illinois at Urbana-Champaign approved all experimental procedures carried out in the present study under the protocol #16-040. The animal component of this study was performed from September of 2016 to March of 2017 in central Illinois (40.1117°N, 88.0417°W).

2.1 Animals and anesthesia

White-tailed deer bucks included in this study ($n=7-11$, mean age 2.6 ± 0.3 yr) were captive-born and raised at a commercial deer farm in east central Illinois. Sample collection took place throughout the breeding season: (1) early (September 8, $n=11$ bucks); (2) middle (December 16, $n=8$); and (3) late (March 4, $n=7$ bucks). Bucks not included in later time points were either sold or died. Throughout the study period (September 8 through March 4), the average mean temperature was 8°C, with daily means ranging from -16°C minimum to 26°C maximum. The

average maximum temperature was 13°C, with daily maximums ranging from –12°C minimum to 33°C maximum. The average minimum temperature was 2°C, with daily minimums ranging from –20°C minimum to 19°C maximum. The total precipitation recorded during this time was 40 cm with ranges from 0 to 6.1 cm per day.

Bucks were fed a 14 % crude protein with approximately 5% fat diet in the winter and a 16% crude protein and approximately 5% fat diet in the spring, summer and fall. This diet is a grain mix with added vitamins and mineral to meet or exceed NRC requirements. This is fed in an ad libitum basis either in gravity flow feeders or in troughs. Deer are housed on pasture consisting of orchard grass, perennial ryegrass and white/ladino clover at a stocking density of approximately six to eight adults per acre. No other supplement is given except occasional "treats" such as apples, pears or other fruit and cookies (animal crackers) to get animals close for observation of health and antler status. Access to clean fresh water is year-round through a pressure system in automatic waterers that are energy free. Pastures are enclosed with eight foot net wiring and animals have access to shade (trees) or shade cloth areas. There is a handling facility with drop chute and box system and seven large pens on the farm with various age and production groups per pen.

Anesthesia and animal handling were performed by a team of experienced veterinarians. Bucks were anesthetized using a combination of 0.88 mg/kg tiletamine-zolazepam (Telazol®, Zoetis, Florham Park, New Jersey, USA) and 2.2 mg/kg xylazine (Cervicine®, ZooPharm, Windsor, Colorado, USA) administered intramuscularly via projector. A 2.2 mg/kg dose of ketamine (Ketaset®, Zoetis, Florham Park, New Jersey, USA) was given intravenously if needed for additional anesthesia. Following semen collection, anesthesia was reversed with an intramuscular injection of 4.4 mg/kg tolazoline (Tolazine®, Lloyd, Inc., Shenandoa, Iowa, USA). Mineralized

antlers were removed above the pedicle in September to minimize injury caused by inter-male aggression during rut.

2.2 Physical examination

Once anesthetized, bucks were moved to an enclosed facility for examination and sample collection. A body condition score (BCS; scale of 1 to 5) was assigned by three independent evaluators using guidelines described for red deer (*Cervus elaphus*) and averaged [17]. Thoracic circumference was measured at the level of the last rib and used to estimate body weight (kg) using the equation $Y = -74.84 + 0.15X$, where X represents the thoracic circumference in mm [18]. Lastly, rump fat thickness was measured with ultrasound at a line between the spine, at its closest point to the tuber coxae and the ischial tuberosity, and immediately adjacent to the cranial process of the ischial tuberosity [19]. Both sides were measured and averaged. The accessory sex glands were measured using transrectal ultrasound with a 7.5 MHz linear-array transducer (Ibex Evo, EI Medical Imaging, Loveland, CO). Length and width of the prostate, vesicular glands, and bulbourethral glands were measured as depicted in Fig 1. Anatomical location and ultrasonographic appearance of each gland were validated at the onset of the study by dissection and ultrasonography of the reproductive tracts post-mortem in three mature bucks.

Ultrasonography was also used to measure the length (L), width (W), and depth (D) of the testicles (Fig. 1) to estimate testicular volume ($TV = 0.5236 \times L \times W \times D$). Scrotal circumference was measured with a flexible tape by pulling it snugly against the greatest diameter, and testes and epididymides were palpated for abnormalities. Testicular shape was classified as long (ratio ≤ 0.5), long/moderate (ratio ≤ 0.65), long/ovoid (ratio ≤ 0.75), ovoid/spherical (ratio ≤ 0.86) and spherical (ratio > 0.86) when obtaining the ratio from the average width and length of each testes as

previously described [20]. The prepuce was inspected for lesions, and the penis was manually exteriorized by applying simultaneous gentle pressure at the sigmoid flexure and base of the prepuce. The penis remained extended using a folded gauze for semen collection.

2.3 Semen collection and analysis

Semen was obtained using an electroejaculator and three-electrode ram probe (31.75 mm diameter) under manual pulse stimulation (Pulsator IV Lane Manufacturing, Inc., Denver, Colorado, USA). Semen was collected into a 15 mL conical tube submerged in a 37°C water bath until processing. An aliquot (~200 µL) of the ejaculate was immediately diluted 1:60 in warm (37°C) Optixcell extender (IMV Technologies, Maple Grove, Minnesota, USA) and placed into a pre-warmed 20 µM chamber slide (Vitrolife, Microcell Counting Chambers, San Diego, CA). Computer-Assisted Sperm Analysis (Spermvision II, MiniTube of America, Inc., Verona, WI) was used to obtain overall and progressive sperm motilities and sample concentration. The parameter settings were adjusted for cervid sperm as recommended by the manufacturer. Data were obtained by averaging motility measurements of three to seven readings from various portions of the chambered slide. Another aliquot of ejaculate was fixed with warm buffered formalin for sperm morphological assessment. One blinded clinician evaluated 100 sperm cells from each sample under 100× magnification with a phase contrast microscope, classified each sperm cell as normal or abnormal, and further defined abnormalities as primary or secondary defects, as previously described [4,21].

2.4 Blood collection and hormone assays

At each time point (September, December, March), blood samples were obtained via jugular venipuncture while bucks were under anesthesia. Serum was collected and preserved at -80°C until analyses. Serum testosterone, estradiol, and IGF-I concentrations were determined with chemiluminescence assays (Immulite 2000 XPi platform; Siemens Medical Solutions USA, Inc.). Intra-assay coefficient of variations were 1.5% (testosterone), 3.9% (estradiol), and 3.3% (IGF-I), which were within reported manufacturer conventional ranges. The estradiol immunoassay had a detection range of 20 to 2000 pg/ mL and sensitivity of 15 pg/mL. The testosterone assay had a detection range of 20 to 1600 ng/mL and sensitivity of 15 ng/mL. The last assay detected unbounded IGF-I with a detection range of 20 to 1600 ng/mL and sensitivity of 20 ng/mL.

2.5 Statistical analyses

Data are presented as percentage mean \pm SEM. All statistical analyses were performed using R Version 3.2.2 (<https://www.r-project.org/>). Homogeneity of variances among samples was established using a Bartlett test, and normality was confirmed using a Shapiro-Wilk test of the residuals. A Kruskal-Wallis test with a Dunn post-hoc test was performed for non-parametric data (morphology). Analysis of variance was applied to parametric data using a general linear mixed model with buck ID as a random variable, and a Tukey HSD test was performed as needed for post-hoc analysis. Repeated measures were also used for motility and testicular/gland measurements to assess interactions of cryopreservation (motility) and symmetry (testes/glands) in addition to the main effect of month. Pearson's correlation coefficient test was used to evaluate the association between parameters within time points. Coefficient of correlations were defined as

strong ($r > 0.70$), moderate ($0.55 \leq r \leq 0.70$), and low correlation ($r < 0.55$). Significance was declared at $p \leq 0.05$.

3. Results

3.1 Body condition and hormone analyses

Average BCS was highest in September ($p < 0.01$), intermediate in December, and lowest in March ($p < 0.01$; Table 1). Thoracic circumference measurements were lower in September than December ($p = 0.02$), but did not differ between other time points ($p \geq 0.36$; Table 1). Measurements of rump fat thickness differed between months, being highest in September, intermediate in December, and lowest in March ($p < 0.01$; Table 1). Average BCS was strongly correlated ($r = 0.74$, $p = 0.05$) with rump fat thickness in September and moderately correlated with thoracic circumference ($r = 0.58$, $p = 0.06$) in September and March ($r = 0.68$, $p = 0.09$). There were no correlations between rump fat thickness and thoracic circumference at any time point, nor were there any correlations between any of these parameters in December ($p \geq 0.10$). Serum IGF-I was highest in September, intermediate in March, and lowest in December ($p < 0.01$; Table 1). In September, serum IGF-I was strongly and negatively correlated with serum testosterone ($r = -0.72$, $p = 0.03$). In December, serum IGF-I was moderately correlated with average BCS ($r = 0.64$, $p = 0.09$) and rump fat thickness ($r = 0.67$, $p = 0.07$) and strongly correlated with serum estradiol concentrations ($r = 0.71$, $p = 0.05$). No other correlations were detected.

All bucks (11/11) were in hard antler by the time of first sample collection in September, and most (6/7) had shed their antler buds within 2 weeks of the final sample collection in March (Fig. 2A and B). Serum estradiol levels were below the assay detection limit in all bucks in September. Therefore, data were categorized to allow for statistical analysis (1 = < 20 pg/mL; 2 = 20-40 pg/mL;

3 = 40-60 pg/mL; 4 = 60-80 pg/mL; 5 = 80-100 pg/mL) and are summarized in Table 2. When comparing categories, serum estradiol levels were lowest in September, intermediate in December, and highest in March ($p \leq 0.02$; Table 2). Mean concentrations in December and March did not differ when compared directly ($p = 0.17$; Suppl. Table 1). Serum testosterone levels were below the assay detection limit (0.2 ng/mL) in all bucks in March and in two out of 11 bucks in September. Thus, testosterone was also categorized to allow for statistical analysis (1 = <20 ng/mL; 2 = 20-40 ng/mL; 3 = 40-60 ng/mL; 4 = 60-80 ng/mL; 5 = 80-100 ng/mL; 6 = >100 ng/mL) and are summarized in Table 2. When comparing categories, serum testosterone levels were lowest in March, intermediate in September, and highest in December ($p \leq 0.02$; Table 2). Mean concentrations calculated in September and December did not differ when compared directly ($p = 0.75$; Suppl. Table 1); however, this calculation excluded the 18% that were below detection limits in September and should be interpreted cautiously.

3.2 Testicle and accessory sex gland evaluation

Palpation of testes and epididymides revealed no abnormalities at any time point. Testicular width, volume, and SC were highest in September, intermediate in December, and lowest in March ($p < 0.01$; Fig. 3A, C, D). However, testicle length did not differ between September and December ($p = 0.91$), but was lower in March ($p < 0.01$; Fig. 3B). Testicular shape was classified as long/moderate in September (ratio = 0.58), which differed ($p < 0.01$) from the long shape observed in December (ratio = 0.50) and March (ratio = 0.47). The 95% confidence interval for SC was 18.0 to 20.4 cm in September, 16.2 to 17.8 cm in December, and 13.0 to 14.4 cm in March. Scrotal circumference was positively associated with testicular volume in both September ($r = 0.74$; $p = 0.01$) and December ($r = 0.74$; $p = 0.04$), but had low correlation in March ($r = 0.38$; $p = 0.40$).

Scrotal circumference was also positively associated with sperm output in September ($R=0.62$; $p=0.04$) but had low correlation in December ($r=-0.12$; $p=0.80$) or March ($R=0.19$; $p=0.70$). The bulbourethral glands were greater in length in March versus September and December ($p\leq 0.05$), with no differences in width between months ($p=0.67$; Fig. 4A and B). Both VG length and width were highest in December, intermediate in September, and lowest in March ($p\leq 0.02$; Fig. 4C and D). The prostate length was greatest in September compared to December or March ($p\leq 0.02$), with no difference in width between months ($p=0.17$; Fig. 4E and F). Testes and accessory sex glands all exhibited appropriate symmetry.

3.2 Semen quality

Total sperm volume, concentration, and output did not differ between months ($p\geq 0.08$; Table 3). Interestingly, though most samples were subjectively white and cloudy in appearance in September and December, 57% (4/7) of the ejaculates obtained in March were pink-tinged (Fig. 2C and D). Both overall and progressive sperm motilities were lowest in March ($p<0.01$), but did not differ between September and December ($p\geq 0.12$; Table 3). The 95% confidence interval for overall motility was 61 to 81% in September, 60 to 100% in December, and 34 to 57% in March. The 95% confidence interval for progressive motility was 47 to 70% in September, 53 to 97% in December, and 19 to 45% in March.

The percentage of normal sperm cells was highest in December ($p<0.01$) with no differences between September and March ($p=0.39$; Table 3). Similarly, there were less primary morphologic sperm defects observed in December ($p<0.01$), with no differences between September and March ($p=0.47$; Table 3). Of the primary defects, there were less proximal droplets and strongly folded tail of sperm in December than in March or September ($p\leq 0.01$) and more

sperm cells with a knobbed acrosome in March versus September or December ($p \leq 0.01$). In December, there were less sperm cells with secondary morphologic defects than in March ($p=0.02$), but only a tendency for less than in September ($p=0.08$; Table 3). There were no differences in secondary sperm defects between March and September ($p=0.22$; Table 3). Of the secondary morphologic sperm defects, there was a tendency to have less sperm cells with distal droplets in December than in March or September ($p=0.07$).

4. Discussion

To our knowledge, this is the first study to characterize reproductive parameters of WTD bucks throughout the breeding season. With the increasing demand for stocker bucks in the WTD industry [1,2], breeding soundness examinations may become vital for ensuring a successful breeding season. Therefore, we foresee that our findings will be useful to practicing veterinarians performing breeding soundness in farm-raised WTD bucks.

Reproductive examinations typically include physical soundness, with BCS used to assess body energy reserves [4]. In an attempt to standardize the BCS, we used a 1 to 5 scale previously described for red deer [17] and compared it to rump fat thickness as a more objective indicator of fat reserves in body tissues [19]. Both BCS and rump fat thickness were highest in September, intermediate in December, and lowest in March, and exhibited a strong correlation in September. This is consistent with the behavioral variations throughout rut season (e.g., voluntary feeding intake, inter-male aggression, fence-side walking). Thus, BCS assessments taken in the beginning of the season reflected the reduced activity and increased voluntary food intake in spring and summer, whereas BCS at peak and end of rut reflected the increased buck activities and reduced food intake. Thoracic circumference has also been described as an estimator of body weight in

WTD [18]. However, the only difference observed in the current study was a decrease in September, suggesting limited value in determining physical soundness in WTD bucks. Based on these findings, it seems that BCS, but not thoracic circumference, is an appropriate tool for assessing nutritional status during a reproductive examination.

Insulin-like growth factor-I is influenced by both season and nutrition in male red deer [15]. Interestingly, serum IGF-I in the current study was highest in September, when both BCS and rump fat thickness were also at their highest. Since voluntary hypophagia in WTD is associated with rut [14], it is not surprising that blood levels of this metabolic hormone would be at its highest in September and the lowest in March, as previously observed [11]. While a positive relationship between blood IGF-I and testosterone levels have been reported during the breeding season in WTD bucks [11], we reported a strong negative correlation in September and no correlation in December. The negative correlation was expected, since IGF-I is strongly and positively associated with antler growth in WTD bucks [16]. Treatment with IGF-I has been demonstrated to increase proliferation of the mesenchymal and cartilaginous cells derived from the antler proliferation zone, whereas high testosterone concentrations impaired these mitogenic effects [22,23]. Therefore, it is likely that IGF-I and testosterone work in opposition to stimulate antler growth during velvet (IGF-I) and then to promote hardening of the antler during rut (testosterone). Controlled studies evaluating the regulation of these hormones during rut are necessary to better define these relationships and determine its usefulness in evaluating reproductive potential in WTD bucks.

Evaluation of the reproductive organs is another crucial part of the reproductive examination. In bulls, SC is routinely performed as a means to predict sperm output [4,5], yet we found a significant variation in SC throughout the breeding season. A previous study reported a

SC of 15.3 ± 0.4 cm in WTD bucks collected in February (mean age 2.4 ± 0.3 years) and 14.3 ± 0.6 cm in bucks collected in January (mean age 1.6 ± 0.1 years) [24]. This is comparable to the measurements obtained in March of the previous study. Additionally, the previous study reported failure of ejaculation in two of the bucks with SC of 10 and 13.5 cm [24], which suggests that 13.5 cm is an appropriate minimum SC when evaluating adult bucks late in the breeding season (January through March). Another author reported average SC measurements in WTD bucks that ranged from 18.04 to 20.25 cm, but when the measurements were obtained is unknown [3]. These SC values are similar to those observed in September of the current study, with the lowest measurement of 17 cm being a potential appropriate minimum SC for pre-breeding evaluation. Though we are somewhat limited by the variability of sperm concentrations that electroejaculation may produce, it is tempting to speculate that we may be able to use SC in our pre-breeding exam to predict sperm output and breeding efficiency in WTD bucks given the strong, positive correlation between SC and sperm output in September. The change in testicular shape from long/moderate in September to long in December and March may have influenced the ability to predict sperm output during these later times and should be further investigated.

Per-rectum evaluation of the accessory sex glands are routinely performed in the bull breeding soundness exam [4], with ultrasound being indicated for more in depth evaluation [25]. Since rectal palpation is not practical due to the small stature of most cervids, ultrasound is necessary for evaluating accessory sex glands [12]. In the current study, prostatic length was greatest in September, which is consistent with measurements of prostate volumes reported in roe deer (*Capreolus capreolus*) [12] and red deer (*Cervus elaphus*) [26], but differs from prostatic weights in fallow deer (*Dama dama*) that were highest mid-October [27]. Vesicular gland volume in roe deer (*Capreolus capreolus*) appeared to be highest in September, lowest in December, and

intermediate in March [12]. Combined vesicular gland weights were highest in October in both red deer (*Cervus elaphus*) [26] and fallow deer (*Dama dama*) [27]. These observations are in contrast to WTD bucks in the current study, where both vesicular gland length and width were intermediate in September, highest in December, and lowest in March. These differences are likely attributable to species-specific variations in reproductive seasonality, with roe deer (*Capreolus capreolus*) typically in rut from mid-July to mid-August [12] and red deer from mid-September to the end of October [26], highlighting the need to evaluate each cervid species separately. Another possible explanation would be operator variability, which we attempted to minimize by having the same individual performing the measurements at each time point.

The seasonal changes in testicular and accessory sex gland sizes have practical implications for semen quality. From February to May, the seminiferous tubules of WTD bucks are in a complete resting stage [28]. From June to September, WTD testes enter the primary development stage, where mostly spermatogonia and primary spermatocytes are present in the tubules [28]. Further development is initiated mid-September, with full spermatogenic production occurring within 3 weeks [28]. This timeline explains the higher number of morphologic sperm defects recorded in March and September when compared to December, when the process of spermatogenesis is at its full potential. There were higher numbers of knobbed acrosomes in March, which are usually associated with environmental stressors [29], but may be related to declining testosterone and spermatogenesis during this stage. A similar trend was observed in tufted deer (*Elaphodus cephalophus*), where higher numbers of spermatozoa with intact acrosomes were observed from September to November [30]. The high numbers of cytoplasmic droplets recorded in September are likely from the testes beginning to enter the primary development stage and are consistent with those observed in the early breeding season of wapiti (*Cervus elaphus ssp. canadensis*) [31]. Based

on these findings, we have determined that pre- or post-breeding morphological analyses may have minimal predictive value for determining semen quality during peak rut and should be interpreted cautiously based on the specific sperm cell defects observed.

The pink-tinged semen observed in 57% of the bucks in March was another interesting observation in the current study. We speculate that this finding is related to the regression of one or more of the accessory sex glands that may be driven by reduced serum testosterone [32]. Since vesicular glands were smallest in March and are a major contributor to seminal plasma in other ruminants [33], it seems likely that there is a change in its function and structure. Seminal plasma contains a variety of proteins, lipids, and enzymes that contribute to fertility in a variety of mammals [34–37]. Changes in seminal plasma composition based on season has been observed in red deer (*Cervus elaphus*) [38], and may explain why motility in September is better than that of March, despite similarities in morphological defects. This finding also highlights the importance of performing a thorough morphological analysis of semen quality rather than depending on motility alone.

In conclusion, the present study describes the changes in breeding soundness parameters in young, captive WTD bucks throughout the breeding season at 40°N. Our findings reveal that BCS is superior to thoracic circumference measurements for evaluation of physical soundness of WTD bucks. Between early and peak rut, average BCS dropped from 3.5 to 3.0 (5-point scale), with semen quality improving during this time. Therefore, this finding provides us with an optimum BCS to strive for in farmed WTD throughout rut to maintain sperm quality. Additionally, our data appears to support other authors' recommendations that a minimum SC of 17 cm is ideal when evaluating WTD bucks early in the breeding season (~September) versus a minimum of 13 cm for those being assessed late in the season (January through March). Lastly, there are significant

changes to testicular and accessory sex gland sizes throughout the breeding season that are associated with semen quality alterations. Further studies are warranted to define potential changes in seminal plasma constituents and elucidate their role as markers for predicting buck fertility throughout the breeding season.

Authors' contributions

JLS, CFS, and IFC participated in designing the study. JLS performed all data analysis and prepared the manuscript. All authors were involved with sample collection throughout the study and final manuscript revisions.

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Table 1. Summary of metabolic parameters of white-tailed bucks assessed in September, December, and March. Data are presented as mean \pm SEM. Data points with differing superscripts were significantly different ($p \leq 0.05$).

	September	December	March
Body condition score (scale 1-5)	3.5 ± 0.2^a	3.0 ± 0.1^b	2.5 ± 0.1^c
Thoracic circumference (cm)	99.8 ± 1.6^a	103 ± 1.2^b	101 ± 1.3^{ab}
Estimated body weight (kg)	75 ± 2.4	78 ± 1.8	77 ± 1.9
Rump fat thickness (mm)	9.4 ± 1.0^a	5.7 ± 0.6^b	2.4 ± 0.6^c
IGF-I (ng/mL)	289 ± 26^a	81 ± 5.2^b	155 ± 22^c

Table 2. Categorical distribution of serum estradiol and testosterone concentrations in White-tailed deer bucks in September, December, and March. Estradiol was lowest in September, intermediate in December, and highest in March ($p \leq 0.01$). Testosterone was lowest in March, intermediate in September, and highest in December ($p \leq 0.02$). Months with differing superscripts were significantly different ($p \leq 0.05$).

		Category					
		1 (<20)	2 (20-40)	3 (40-60)	4 (60-80)	5 (80-100)	6 (>100)
Estradiol (pg/mL)	September ^a	11/11 (100%)	0	0	0	0	0
	December ^b	0	1/8 (12%)	4/8 (50%)	3/8 (38%)	0	0
	March ^c	0	0	3/7 (43%)	1/7 (14%)	3/7 (43%)	0
Testosterone (ng/mL)	September ^a	2/11 (18%)	0	2/11 (18%)	2/11 (18%)	1/11 (9%)	4/11 (36%)
	December ^b	0	0	1/8 (13%)	0	1/8 (13%)	6/8 (75%)
	March ^c	7/7 (100%)	0	0	0	0	0

Suppl. Table 1. Mean concentrations of serum estradiol and testosterone in white-tailed deer bucks in September, December, and March. Serum estradiol was below detection limits in all bucks in September; but the calculated mean did not differ between December and March ($p=0.26$). Serum testosterone was below detection limits in all bucks in March, but the calculated mean did not differ between September and December ($p=0.80$); however, this calculation excluded the 18% that were below detection limits and should be interpreted cautiously.

	Estradiol (pg/mL)	Testosterone (ng/mL)
September	< 20 (100%)	135 ± 30 (82%)
December	54 ± 5.7	146 ± 24
March	66 ± 7.8	<20 (100%)

Table 3. Comparison of semen variables obtained by electroejaculation in WTD bucks in September, December, and March. Data are presented as mean \pm SEM. Differences between months are denoted by differing superscripts ($p \leq 0.05$).

	September	December	March
Volume (mL)	0.8 \pm 0.1	0.8 \pm 0.2	1.0 \pm 0.2
Concentration (x10⁹/mL)	1.2 \pm 0.3	4.4 \pm 2.3	1.6 \pm 0.3
Output (x10⁹/mL)	0.9 \pm 0.2	2.0 \pm 0.6	1.5 \pm 0.3
Overall motility (%)	71 \pm 4.4 ^a	80 \pm 8.0 ^a	45 \pm 4.4 ^b
Progressive motility (%)	58 \pm 5.0 ^a	75 \pm 8.7 ^a	32 \pm 4.9 ^b
Normal morphology (%)	62 \pm 8.2 ^a	89 \pm 3.4 ^b	60 \pm 7.3 ^a
Primary defects (%)	29 \pm 9.1 ^a	4.6 \pm 0.8 ^b	21 \pm 5.1 ^a
Secondary defects (%)	9.5 \pm 2.2 ^{ab}	6.7 \pm 3.3 ^a	18 \pm 7.1 ^b

Figure 1. Ultrasonography images of the reproductive tract from white-tailed deer bucks illustrating the assessments for size and appearance of the testes, prostate, bulbourethral, and vesicular glands.

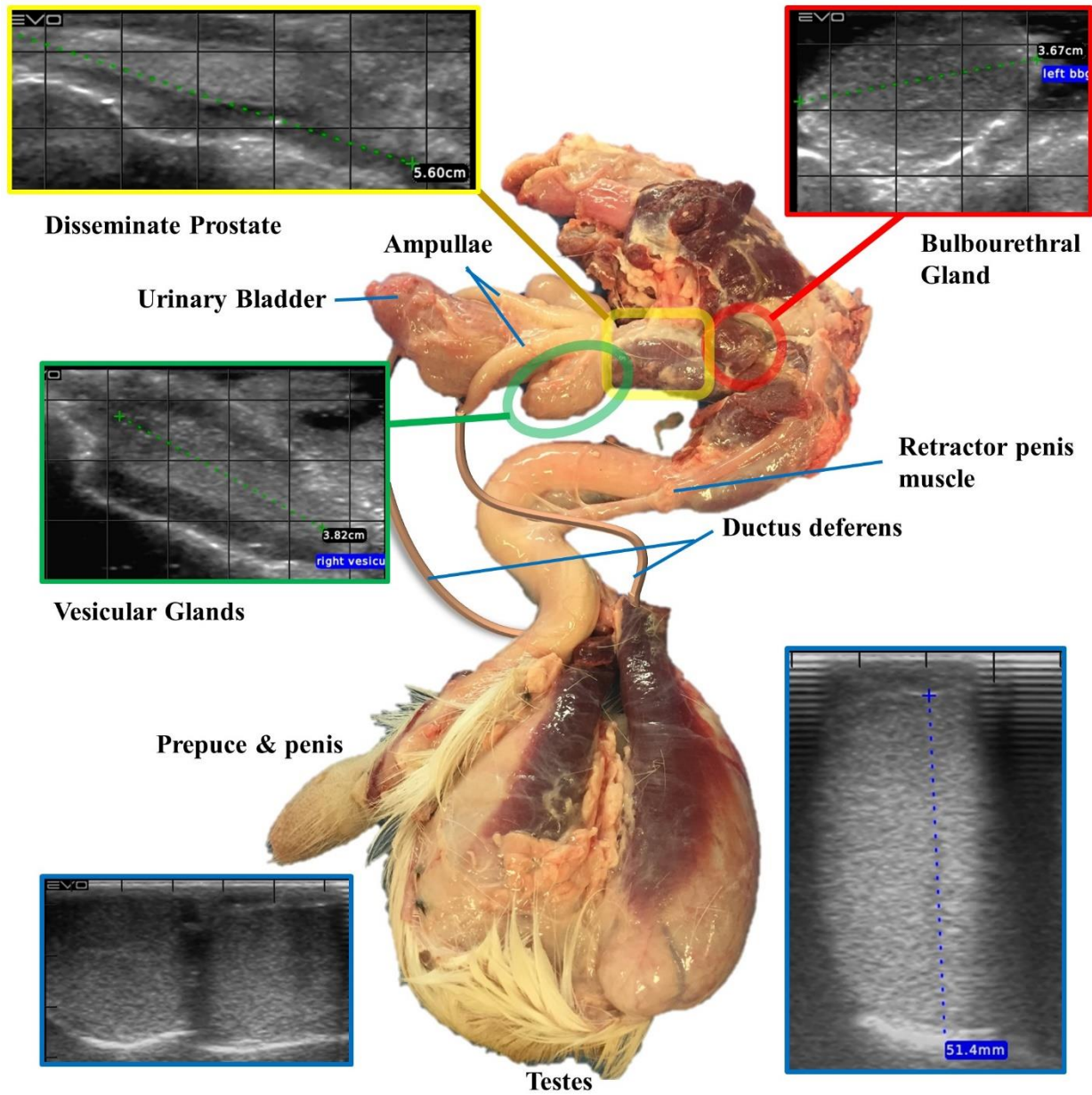


Figure 2. Physical differences between bucks evaluated in September, December, and March. All bucks (11/11) were in hard antler at the time of first collection in September (A), whereas most (6/7) had shed their antlers within two weeks of the final collection in March (B). Semen appeared cloudy and white in all samples collected in September and December (C); whereas in March, 4/7 ejaculates were pink-tinged (D).

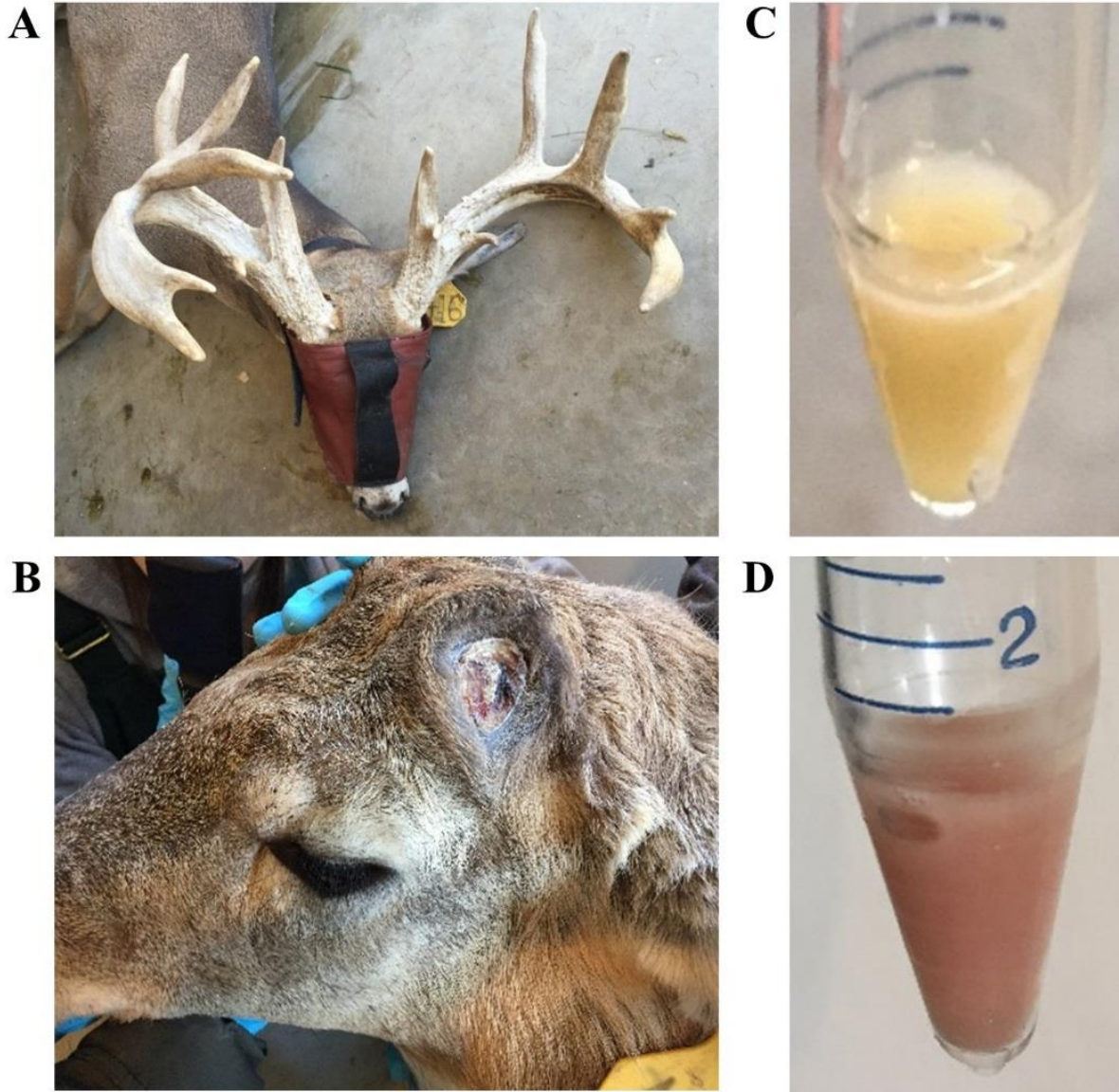


Figure 3. Changes in testicular size (width, length, volume) and scrotal circumference by month (September, SEPT; December, DEC; March, MAR). Data are presented as mean \pm SEM. All testicular measurements displayed appropriate symmetry with differences between months denoted by differing superscripts ($p \leq 0.05$).

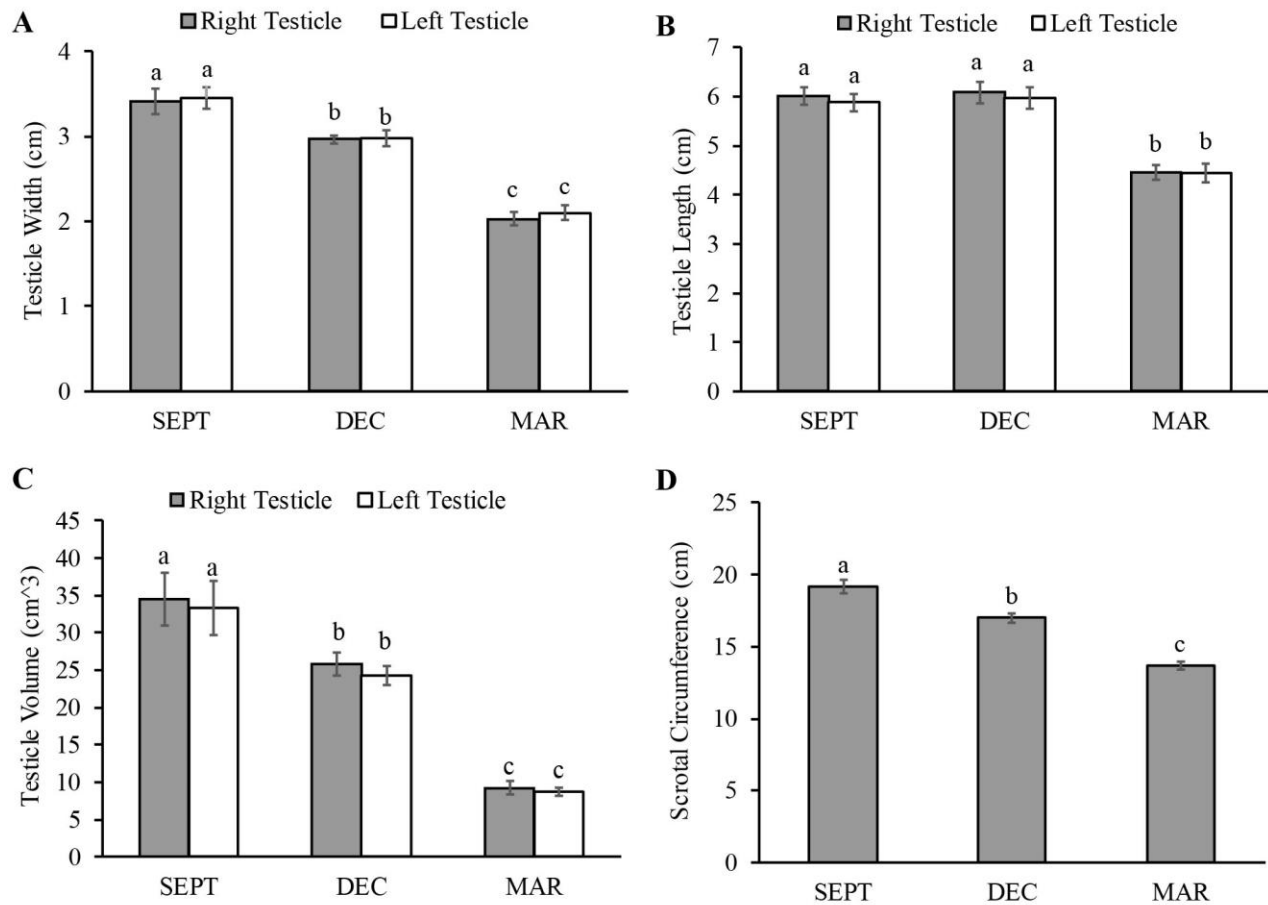
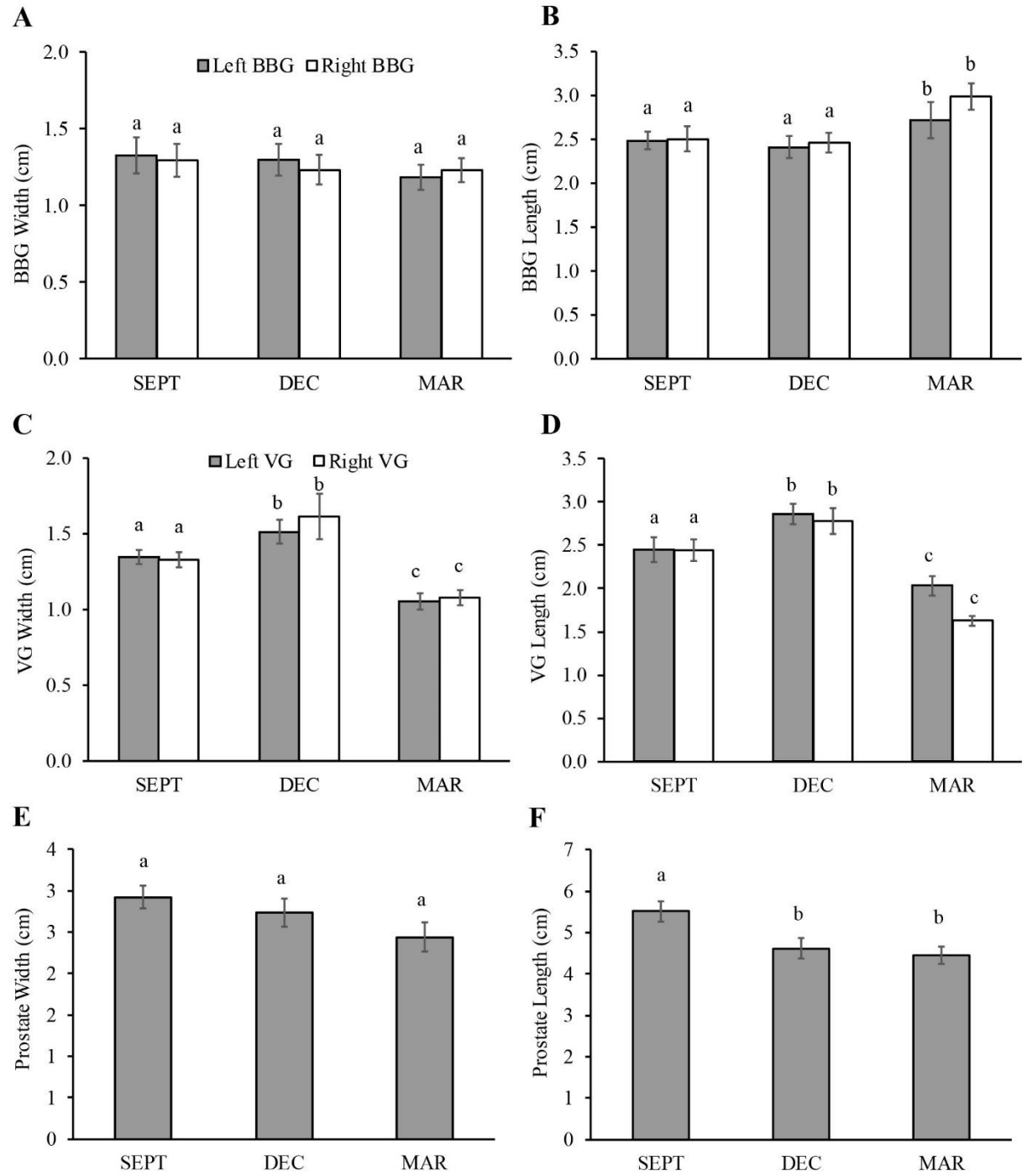


Figure 4. Changes in dimensions (width, length) of accessory sex glands by month (September, SEPT; December, DEC; March, MAR). Data are presented as mean \pm SEM. All glands displayed appropriate symmetry with differences between months denoted by differing superscripts ($p \leq 0.05$). BBG = bulbourethral gland; VG = vesicular glands



References

- [1] Anderson DP, Frosch BJ, Outlaw JL. Economic impact of the United States cervid farming industry. College Station, Texas: 2007.
- [2] Anderson DP, Outlaw JL, Earle ML, Richardson JW. Economic impact of U.S. deer breeding and hunting operations. College Station, Texas: 2017.
- [3] Sathe S, Shipley CF. Applied Andrology in Sheep, Goats and Selected Cervids. In: Chenoweth PJ, Lorton SP, editors. Anim. Androl. Theor. Appl., Boston, Massachusetts, Massachusetts: CAB International; 2014, p. 226–53.
- [4] Chenoweth PJ, Hopkins FM, Spitzer JC, Larsen RE. Guidelines for using the bull breeding soundness evaluation form. Clin Theriogenology 2010;2:43–50.
- [5] Gipson TA, Vogtl DW, Massey JW, Ellersiecke MR. Associations of scrotal circumference with semen traits in young beef bulls. Theriogenology 1985;24:217–25. doi:10.1016/0093-691X(85)90186-4.
- [6] Woolf A, Harder JD. Population dynamics of a captive white-tailed deer herd with emphasis on reproduction and mortality. Wildl Monogr 1979;67:3–53.
- [7] Mirarchi RE, Howland BE, Scanlon PF, Kirkpatrick RL, Sanford LM. Seasonal variation in plasma LH, FSH, prolactin, and testosterone concentrations in adult male white-tailed deer. Can J Zool 1978;56:121–7. doi:10.1139/z78-017.
- [8] Bubenik GA, Bubenik AB, Schams D, Leatherland JF. Circadian and circannual rhythms of LH, FSH, testosterone, prolactin, cortisol, T3 and T4 in plasma of mature, male white-tailed deer. Comp Biochem Physiol 1983;76A:37–45.
- [9] Bubenik GA, Morris JM, Schams D, Claus A. Photoperiodicity and circannual levels of LH, FSH, and testosterone in normal and castrated male, white-tailed deer. Can J Physiol Pharmacol 1982;60:788–93. doi:doi.org/10.1139/y82-110.
- [10] Bubenik GA, Schams D, Coenen G. The effect of artificial photoperiodicity and antiandrogen treatment on the antler growth and plasma levels of LH, FSH, testosterone, prolactin and alkaline phosphatase in the male white-tailed deer. Comp Biochem Physiol 1987;87A:551–9. doi:10.1016/0300-9629(87)90359-8.
- [11] Ditchkoff SS, Spicer LJ, Masters RE, Lochmiller RL. Concentrations of insulin-like growth factor-I in adult male white-tailed deer (*Odocoileus virginianus*): associations with serum testosterone, morphometrics and age during and after the breeding season. Comp Biochem Physiol - Part A Mol Integr Physiol 2001;129:887–95. doi:10.1016/S1095-6433(01)00351-8.
- [12] Goeritz F, Quest M, Wagener A, Fassbender M, Broich A, Hildebrandt TB, et al. Seasonal timing of sperm production in roe deer: Interrelationship among changes in ejaculate parameters, morphology and function of testis and accessory glands. Theriogenology 2003;59:1487–502. doi:10.1016/S0093-691X(02)01201-3.

- [13] Scott IC, Asher GW, Barrell GK, Juan J V. Voluntary food intake of pregnant and non-pregnant red deer hinds. *Livest Sci* 2013;158:230–9. doi:10.1016/j.livsci.2013.04.021.
- [14] Moen AN. Seasonal changes in heart rates, activity, metabolism, and forage intake of white-tailed deer. *J Wildl Manage* 1978;42:715–38.
- [15] Webster JR, Corson ID, Littlejohn RP, Stuart SK, Suttie JM. Effects of season and nutrition on growth hormone and insulin-like growth factor-I in male red deer. *Endocrinology* 1996;137:698–704.
- [16] Suttie JM, Gluckman PD, Butler JH, Fennessyi PF, Corsoni IAND, Laasi FJ. Plasma IGF-1 concentrations were measured throughout the first period of development of the the annual cycle of antler replacement has been development. *Endocrinology* 1985;116:846–8.
- [17] Audige L, Wilson PR, Morris RS. A body condition score system and its use for farmed red deer hinds. *New Zeal J Agric Res* 1998;41:545–53. doi:10.1080/00288233.1998.9513337.
- [18] Weckerly FW, Leberg PL, Van Den Bussche RA. Variation of weight and chest girth in white-tailed deer. *J Wildl Manage* 1987;51:334–7. doi:10.2307/3801012.
- [19] Cook RC, Cook JG, Stephenson TR, Myers WL, Mccorquodale SM, Vales DJ, et al. Revisions of rump fat and body scoring indices for deer, elk, and moose. *J Wildl Manage* 2010;74:880–96. doi:10.2193/2009-031.
- [20] Bailey TL, Monke D, Hudson RS, Wolfe DF, Carson RL, Riddell MG. Testicular shape and its relationship to sperm production in mature Holstein bulls. *Theriogenology* 1996;46:881–7. doi:10.1016/S0093-691X(96)00245-2.
- [21] Kuster CE, Singer RS, Althouse GC. Determining sample size for the morphological assessment of sperm. *Theriogenology* 2004;61:691–703. doi:10.1016/S0093-691X(03)00240-1.
- [22] Sadighi M, Haines SR, Skottner A, Harris AJ, Suttie JM. Effects of insulin-like growth factor-I (IGF-I) and IGF-II on the growth of antler cells *in vitro*. *J Endocrinol* 1994;143:461–9. doi:10.1677/joe.0.1430461.
- [23] Sadighi M, Li C, Littlejohn RP, Suttie JM. Effects of testosterone either alone or with IGF-I on growth of cells derived from the proliferation zone of regenerating antlers *in vitro*. *Growth Horm IGF Res* 2001;11:240–6. doi:10.1054/ghir.2001.0232.
- [24] Stewart JL, Shipley CF, Katich AS, Po E, Ellerbrock RE, Lima FS, et al. Cryopreservation of white-tailed deer (*Odocoileus virginianus*) semen using soybean-, liposome-, and egg yolk-based extenders. *Anim Reprod Sci* 2016;171. doi:10.1016/j.anireprosci.2016.05.006.
- [25] Weber JA, Hilt CJ, Woods GL. Ultrasonographic appearance of the bull accessory sex glands. *Theriogenology* 1988;29:1347–55.
- [26] Lincoln GA. The seasonal reproductive changes in the red deer stag (*Cervus elaphus*). *J*

- Zool 1971;163:105–23. doi:10.1111/j.1469-7998.1971.tb04527.x.
- [27] Chapman NG, Chapman DI. Seasonal changes in the male accessory glands of reproduction in adult Fallow deer (*Dama dama*). J Zool London 1979;189:259–73.
 - [28] Robinson RM, Thomas JW, Marburger RG. The reproductive cycle of male white-tailed deer in central Texas. J Wildl Manage 1965;29:53–9.
 - [29] Vogler CJ, Bame JH, DeJarnette JM, McGilliard ML, Saacke RG. Effects of elevated testicular temperature on morphology. Theriogenology 1993;40:1207–19. doi:10.1016/0093-691X(93)90291-C.
 - [30] Panyaboriban S, Singh RP, Songsasen N, Padilla L, Brown J, Reed D, et al. Reproductive seasonality and sperm cryopreservation in the male tufted deer (*Elaphodus cephalophus*). Theriogenology 2016;86:914–23. doi:10.1016/j.theriogenology.2016.03.014.
 - [31] Haigh JC, Cates WF, Glover GJ, Rawlings NC. Relationships between seasonal changes in serum testosterone concentrations, scrotal circumference and sperm morphology of male wapiti (*Cervus elaphus*). J Reprod Fertil 1984;70:413–8. doi:10.1530/jrf.0.0700413.
 - [32] Thompson DL, Pickett BW, Squires EL, Nett TM. Sexual behavior, seminal pH and accessory sex gland weights in geldings administered testosterone and (or) estradiol-17b. J Anim Sci 1980;51:1358–66.
 - [33] Seidel GEJ, Foote RH. Compartmental analysis of sources of the bovine ejaculate. Biol Reprod 1970;2:189–96.
 - [34] Killian GJ, Chapman DA, Rogowski LA. Fertility-associated proteins in Holstein bull seminal plasma. Biol Reprod 1993;49:1202–7. doi:10.1095/biolreprod49.6.1202.
 - [35] Moura AA, Koc H, Chapman DA, Killian GJ. Identification of proteins in the accessory sex gland fluid associated with fertility indexes of dairy bulls: a proteomic approach. J Androl 2006;27:201–11. doi:10.2164/jandrol.05089.
 - [36] Lewis S, Boyle P, McKinney K, Young I, Thompson W. Total antioxidant capacity of seminal plasma is different in fertile and infertile men. Fertil Steril 1995;64:868–70. doi:10.1016/S0015-0282(16)57870-4.
 - [37] Brandon CI, Heusner GL, Caudle AB, Fayrer-Hosken RA. Two-dimensional polyacrylamide gel electrophoresis of equine seminal plasma proteins and their correlation with fertility. Theriogenology 1999;52:863–73.
 - [38] Koziorowska-Gilun M, Szurnicka M, Dziekonska A, Kordan W, Gizejewski Z, Filipowicz K. Characteristics of antioxidant systems of yellow fraction of red deer's (*Cervus elaphus* L) semen during the rutting period. Reprod Domest Anim 2016;51:248–54. doi:10.1111/rda.12673.

Appendix C:

Variation in post-thaw sperm quality of white-tailed deer bucks (*Odocoileus virginianus*) during rut

Variation in post-thaw sperm quality of white-tailed deer is a project I contributed to during my Ph.D. program, but it was not part of my dissertation work. The following paper is a result of work done on this project.

Variation in post-thaw sperm quality of white-tailed deer bucks (*Odocoileus virginianus*) during rut¹

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Abstract

White-tailed deer farming is a growing industry in the United States, with breeding operations contributing significantly to the industry's economic impact. Artificial insemination with frozen semen allows for selection and dissemination of valuable genetics, yet surprisingly little is known regarding the best time throughout rut to perform semen cryopreservation. The objective of this study was to compare semen quality following cryopreservation of white-tailed deer bucks collected early in the breeding season (September, n=6), at peak rut (December, n=8), and late season (March, n=7). We hypothesized that post-thaw semen quality would be enhanced at peak rut. Mature bucks were anesthetized with tiletamine-zolazepam and xylazine administered intramuscularly via projector. Semen was collected by electroejaculation and cryopreserved using Optixcell extender. Overall and progressive sperm motility were assessed for each sample before and after cryopreservation using a computer-aided sperm analyzer. Flow cytometry was used for post-thaw assessment of sperm viability (SYBR-14/PI), acrosome integrity (FITC-PNA/PI), and DNA stability (acridine orange). Analysis of variance was applied to normalized data using a general linear mixed model with buck ID as a random variable, and a Tukey HSD test was performed as needed for post-hoc analysis. Pre-freeze overall and progressive sperm motilities were lowest in March, intermediate in September, and highest in December ($P \leq 0.04$). Post-thaw

¹ Reprinted, with permission, from J. Stewart et al. Variation in post-thaw sperm quality of white-tailed deer bucks (*Odocoileus virginianus*) during rut. *Animal Reproduction Science* 2018; 195: 121-130.

overall and progressive motilities were lowest in September ($P \leq 0.02$), but did not differ between December and March ($P \geq 0.12$). The DNA Fragmentation Index was lowest in December, intermediate in September, and highest in March ($P \leq 0.05$). The percentage of live spermatozoa was higher in December than September ($P < 0.01$), but the percentage of intact acrosomes per live sperm cell was highest in September ($P = 0.03$). This study confirms that post-thaw semen quality appears to be superior during peak rut (December) in bucks. Though semen collected early or late in rut may present acceptable motility, DNA stability is impaired, which could adversely affect fertility rates. This data suggests that semen cryopreservation during transitional periods should be avoided, though field studies evaluating the translation of these results into success rates of artificial insemination are needed to determine the effects on conception.

Keywords: andrology, cervid, fluorescent probes, semen cryopreservation

1. Introduction

White-tailed deer (WTD) farming is an economically important industry that generates approximately 56,000 jobs and \$7.9 billion annually in the United States (Anderson et al., 2017). These numbers have more than doubled throughout the past 10 years, indicating that the industry is rapidly growing (Anderson et al., 2007). Due to the increasing popularity of private hunting, the need to disseminate genetics to select for desirable traits (e.g., fast growth, well-branched and broad antlers) is crucial (Barnes et al., 2016; Flinn et al., 2015; Lukefahr and Jacobson, 1998; Wallingford et al., 2017). The sustainability of deer farming relies heavily on the use of artificial insemination with frozen semen to disseminate valuable genetics, yet surprisingly little research on white-tailed deer semen cryopreservation has been performed.

While our previous study showed that non-egg yolk semen extenders can be used successfully

in WTD (Stewart et al., 2016), the best time to collect and cryopreserve semen remains undetermined. Owners may choose to collect bucks for semen cryopreservation either early or late in the breeding season to facilitate natural cover of their own does. Early season collections also allow for pre-breeding examinations to be performed to ensure that a buck will be reproductively sound for the season. Semen quality at the beginning and end of the breeding season in cervids are influenced by the effects of increasing and decreasing testosterone production, respectively, on spermatogenesis (Haigh et al., 1984; Martínez-Pastor et al., 2005; Robinson et al., 1965). Higher quality semen in red deer (*Cervus elaphus*), at the peak of rut has also been attributed to increased antioxidant production, which protects spermatozoa from oxidative stress (Koziorowska-Gilun et al., 2016). Oxidative stress has been associated with increased chromatin damage and loss of acrosome integrity in red deer following cryopreservation (Castellanos et al., 2015) and has been shown to adversely affect fertility in a variety of species (Evenson et al., 1994; Kenney et al., 1995; Varghese et al., 2011). Since WTD experience seasonal reproductive variations similar to red deer, it is necessary to determine if there are comparable factors that adversely affect semen cryopreservation outcomes.

Improving sperm cryopreservation techniques in WTD will likely translate into better fertility and profitability for farmers selling semen for use in artificial insemination. The objective of this study was to compare post-thaw semen quality in WTD bucks collected early in the breeding season (September), at peak rut (December), and late breeding season (March) by assessing sperm motility, viability, acrosome integrity, and chromatin stability. We hypothesized that post-thaw semen quality would be enhanced at peak rut.

2. Materials and Methods

2.1 Animal care and use

All experimental procedures carried out in the present study were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign (Protocol #16-040). Animals were maintained at a commercial deer farm in east central Illinois (40.1117°N, 88.0417°W).

2.2 Animals and anesthesia

White-tailed deer bucks included in this study (mean age 2.6 ± 0.3 years old) were all captive born and raised. Sample collection took place at three different time points throughout the breeding season: (1) early (September n=6); (2) peak (December n=8); and (3) late (March n=7). Bucks not included in all time points either were unable to undergo cryopreservation due to sample handling error or were sold or died between collections. Anesthesia and animal handling were performed by a team of experienced veterinarians as previously described (Stewart et al., 2016). Bucks were anesthetized using a combination of 0.88 mg/kg tiletamine-zolazepam (Telazol®, Zoetis, Florham Park, New Jersey, USA) and 2.2 mg/kg xylazine (Cervicine®, ZooPharm, Windsor, Colorado, USA) administered intramuscularly via projector. A 2.2 mg/kg dose of ketamine (Ketaset®, Zoetis, Florham Park, New Jersey, USA) was given intravenously if needed for additional anesthesia. Each buck was transported to a covered room and placed on a table for semen collection. Following semen collection, anesthesia was reversed using an intramuscular injection of 4.4 mg/kg tolazoline (Tolazine®, Lloyd, Inc., Shenandoah, Iowa, USA).

2.3 Semen collection, analyses, and cryopreservation

Once bucks were anesthetized, the penis was manually exteriorized by applying simultaneous gentle pressure at the sigmoid flexure and base of the prepuce. The penis was then held extended using folded gauze. To obtain semen samples, a 31.75 mm diameter, three-electrode ram probe (Lane Manufacturing, Inc., Denver, Colorado, USA) was placed rectally, and a manual cycle was run on the electroejaculator unit (Pulsator IV, Lane Manufacturing, Inc., Denver, Colorado, USA). This device uses a sine wave pattern with maximum output at 25 Ω resistance of 45 volts and 1 amp. Stimulation began at the lowest setting and increased in a stepwise manner, where it was maintained for 4 to 5 s before returning to zero for 2 to 3 s. Approximately four stimuli were applied at each power setting, and no deer required stimulation above the fourth power level to achieve ejaculation. Semen was collected directly into a 15 mL conical tube submerged in a 37°C water bath until processing. Tubes were switched after each ~0.25 mL of ejaculate was produced to prevent urine contamination.

An aliquot (200 μ L) of the ejaculate was immediately diluted 1:60 in warm (37°C) Optixcell extender (IMV Technologies, Maple Grove, Minnesota, USA) and placed into a pre-warmed 20 μ M chamber slide (Vitrolife, Microcell Counting Chambers, San Diego, CA). Computer-aided sperm analysis (SpermVision, MiniTube of America, Inc., Verona, WI) was used to measure overall and progressive motilities and sample concentration using a phase-contrast microscope with a heated (37°C) stage (20 \times ; Olympus BX41; Olympus Corporation, Center Valley, PA). Data were obtained by averaging motility measurements of a minimum of 500 cells or seven fields from various portions of the chambered slide. Output measurements used to describe sperm motion included average path velocity (VAP, μ m/s), straight line velocity (VSL, μ m/s), curvilinear

velocity (VCL, $\mu\text{m/s}$), straightness of the average path (STR, %), linearity of the curvilinear path (LIN, %), wobble (WOB, %), amplitude of lateral head displacement (ALH, μm), and beat cross frequency (BCF, number per second) (Amann and Waberski, 2014).). The parameter settings were as follows per manufacturer defaults for cervid sperm: frame capture speed rate, 60 Hz; cell size (min/max), 22/60 μm^2 ; threshold straightness, 50%; VAP cutoff, 10 $\mu\text{m/s}$; and VSL cutoff, 52 $\mu\text{m/s}$.

The remainder of the sample was diluted with pre-warmed (37°C) Optixcell extender at a 1:1 ratio and transported approximately 30 min to the laboratory in a 37°C water bath, where further processing was performed. Based on individual sample concentration, additional extender was added in a one-step method to prepare a final concentration of 120 million spermatozoa per mL. Immediately following this final dilution, the samples were slowly cooled to 5°C by placing tubes in a 37°C water bath and placing the immersed samples in a cold-room at 5°C. Samples were loaded into 0.5-mL semen straws, sealed with a glass bead (Minitube USA, Inc., Delavan, WI), and incubated at 5°C for 3 to 5 h, per manufacturers' recommendations. Following incubation, straws were frozen manually by placing at 4 cm above the level of liquid nitrogen for 15 min and then submerging and storing in liquid nitrogen until further analysis. Post-thaw sperm motility parameters were assessed by computer-aided sperm analysis after thawing cryopreserved semen straws in a 37°C water bath for a minimum of 30 sec to allow the straw to thaw completely.

2.4 Fluorescent probes for sperm evaluation

The flow cytometry assays used in the present study were performed according to protocols previously published (Martínez-Pastor et al., 2010, 2004; Robles and Martínez-Pastor, 2013) and routinely used in our group to assess semen of WTD bucks (Stewart et al., 2016) and stallions

(Ellerbrock et al., 2017). Fluorescent probes were used for post-thaw evaluation of sperm cryopreserved during each time point in 0.5 mL straws using Optixcell extender. Semen straws from each buck and timepoint were thawed individually and subjected to each stain as described below. The LIVE/DEAD® Sperm Viability Kit (SYBR-14 and propidium iodide (PI) dyes; #L-7011) was purchased from Molecular Probes, Inc. (Eugene, Oregon, USA). Fluorescein isothiocyanate-conjugated pea (*Pisum sativum*) agglutinin (FITC-PNA; #L-7381) was obtained from Sigma Chemical Co. (Saint Louis, Missouri, USA). Acridine orange, C.I. (#04539-500) was purchased from Polysciences, Inc. (Warrington, Pennsylvania, USA). Dimethyl sulfoxide, anhydrous (DMSO; #D12345), triton X-100 surfact-amps detergent solution (#85111), and phosphate-buffered saline (PBS, pH 7.4) (#10010-023) were purchased from Thermo Fischer Scientific (Waltham, Massachusetts, USA). All other chemicals used were reagent grade and purchased from Sigma Aldrich (St. Louis, Missouri, USA).

2.5 Assessment of sperm viability

Sperm viability was assessed using a combination of SYBR-14 and propidium iodide (PI) based on the integrity of the sperm plasma membrane (Robles and Martínez-Pastor, 2013). Briefly, stock solutions of SYBR-14 (0.02 mM in DMSO) and PI (2.4 mM in water) were prepared and frozen in aliquots at -20°C for subsequent use (Robles and Martínez-Pastor, 2013). Staining solution was prepared on the day of flow cytometry analysis by combining 5 μL SYBR-14 (0.02 mM) and 5 μL PI (2.4 mM) per mL of HEPES buffer (10 mM Hepes, 150 mM NaCl, 0.1% BSA, pH 7.4). Working concentrations of SYBR-14 and PI were 100 nM and 12 μM , respectively. Cryopreserved semen was thawed in a 37°C water bath for 1 min, and 8 μL of thawed semen was added to 0.5 mL staining solution to create a final concentration of 2 million spermatozoa per mL.

The samples were mixed by reverse pipetting and subsequently incubated in the dark at 37°C for 10 to 15 min prior to flow cytometric analysis.

Samples were analyzed using a BD LSR II Flow Cytometry Analyzer (BD Biosciences, San Jose, California, USA) at the Roy Carver Biotechnology Center of the University of Illinois Urbana-Champaign. The two dyes were excited in the flow cytometer using a 488-nm argon excitation laser. Fluorescent data of all events was collected until 10,000 gated events were recorded. Two-dimensional plots of SYBR-14 versus PI fluorescence events were drawn, and debris was gated out based on those events that emitted minimal red and green fluorescence. As previously described (Robles and Martínez-Pastor, 2013), three sperm populations in the SYBR-14/PI stained sperm preparations were present (Fig. 1A-C). Presumptively live cells made up population 1, with only green fluorescent signal (SYBR-14 positive) detected. Population 2 exhibited a mixed green (SYBR-14 positive) and red (PI positive) fluorescence and were considered to be moribund with early or minor membrane damage. Population 3 comprised cells with only red fluorescent signal (PI positive) detected and were considered dead. The percentage of cells in each of the three populations was calculated.

2.6 Assessment of acrosome integrity

Acrosome integrity was assessed using a combination of FITC-PNA (peanut agglutinin conjugated with fluorescein isothiocyanate) and PI as previously described by Robles and Martínez-Pastor (2013). Briefly, stock solutions of FITC-PNA (1 mg/mL in PBS) were prepared and frozen in aliquots at -20°C for subsequent use (Robles and Martínez-Pastor, 2013). Staining solution was prepared by combining 0.625 μ L PI (2.4 mM) and 1 μ L FITC-PNA (1 mg/mL) per mL of PBS. Final concentrations of the stains were 1.5 μ M PI and 1 μ g/mL FITC-PNA.

Cryopreserved semen was thawed in a 37°C water bath for 1 min, and 8 µL of thawed semen was added to 0.5 mL staining solution to create a final concentration of 2 million spermatozoa per mL. The samples were mixed by reverse pipetting and subsequently incubated in the dark at 37°C for 10 to 15 min prior to flow cytometric analyses.

Flow cytometric analyses were carried out as described above with the exception that FITC-PNA fluorescence was detected at 515–545 nm fluorescence detector 1. Fluorescent data of all events was collected until 10,000 gated events were recorded. Non-sperm events were gated out of analyses as judged on scatter properties. Two-dimensional plots of FITC-PNA versus PI fluorescence events were drawn. As previously described (Robles and Martínez-Pastor, 2013), four sperm populations in the FITC-PNA/PI stained sperm preparations were present (Fig. 1D-F). Population 1 included sperm that were live (PI negative) with an intact acrosome (IA; FITC-PNA negative). Population 2 encompassed sperm that were live (PI negative) with a damaged acrosome (DA; FITC-PNA positive). Populations 3 and 4 contained sperm that were dead (PI positive) with intact (FITC-PNA negative) or damaged (FITC-PNA positive) acrosomes, respectively. These populations were divided into quadrants, and the frequency of each population was quantified.

2.7 Assessment of sperm chromatin stability

Chromatin stability was assessed by meta-chromatic staining with acridine orange (AO) based on the susceptibility of the sperm DNA to acid-induced denaturation in situ (Martínez-Pastor et al., 2004). Cryopreserved semen was thawed in a 37°C water bath for 1 min, and 15 µL of thawed semen was added to 985 µL PBS to create a final sperm concentration of approximately 2 million spermatozoa per mL. Acid-induced denaturation of DNA in situ was attained by adding 0.4 ml of an acid-detergent solution (0.17% Triton X-100, 0.15 M sodium chloride, and 0.08 N hydrogen

chloride; pH 1.4) to 0.2 mL diluted semen. After 30 sec, the cells were stained by adding 1.2 mL of a citric phosphate solution (0.1 M citric acid, 0.2 M sodium phosphate dibasic, 1 mM ethylenediaminetetraacetic acid, 0.15 M sodium chloride; pH 6.0) containing 6 µg/ml AO and mixed by reverse pipetting. The stained samples were subsequently incubated in the dark at 37°C for 3 min prior to flow cytometric analyses.

Flow cytometry analysis was carried out as above using a 488-nm argon excitation laser. Fluorescent data of all events was collected until 10,000 gated events were recorded. Debris was discarded electronically and based on low fluorescence values. Two populations were discerned (Fig. 1G-I) by drawing a diagonal line between the groups of cells with stable chromatin (intact DNA) to the left of the line (medium to high green fluorescence and low red fluorescence) and the group of cells with loose chromatin (damaged DNA) to the right of the line (medium to high green fluorescence and medium to high red fluorescence). The frequency of each of these populations was quantified. DNA fragmentation index (DFI) was calculated as the number of cells with loose chromatin divided by the sum of both populations.

2.8 Statistical analyses

Data are presented as percentage mean \pm SEM. All statistical analyses were performed using R Version 3.4.3 (<https://www.r-project.org/>). Homogeneity of variances among samples was established using a Bartlett test, and normality was confirmed using a Shapiro-Wilk test of the residuals. Values with non-normal distribution were transformed using a Box-Cox power transformation. Analysis of variance was applied to data using a general linear mixed model with buck ID as a random variable, and a Tukey HSD test was performed as needed for post-hoc analysis. Pearson's correlation test was used to evaluate the association between flow cytometry

parameters and the corresponding post-thaw motility from each sample analyzed. Parameters with strong correlation were defined at $r \geq 0.60$, moderate correlation at $0.60 > r \geq 0.40$, and low correlation at $0.40 > r \geq 0.20$. Significance was declared at $P \leq 0.05$ whereas tendency was discussed at $0.05 < P < 0.10$.

3. Results

3.1 Sperm motility

As reported in our concurrent study, semen volume and sperm concentration did not differ between months (Stewart et al., 2017). Pre-freeze overall and progressive sperm motilities were lowest in March, intermediate in September, and highest in December ($P \leq 0.04$; Fig. 2). Following cryopreservation, overall and progressive sperm motilities decreased in September and December ($P \leq 0.01$), but increased in March ($P \leq 0.04$; Fig. 2). Post-thaw overall and progressive motilities were lowest in September ($P \leq 0.02$), but did not differ between March and December ($P \geq 0.12$; Fig. 2).

Computer-aided sperm analysis outputs describing the motion of spermatozoa are summarized in Table 1. Pre-freeze VAP, VCL, VSL, STR, and BCF did not differ between months ($P > 0.05$). However, VSL, STR, and BCF decreased after cryopreservation at all three months ($P \leq 0.04$). Post-thaw VSL and BCF were higher in December than in March ($P \leq 0.02$), but post-thaw STR did not differ between months ($P = 0.62$). Though VAP and VCL only decreased after cryopreservation in September ($P \leq 0.02$), at post-thaw they were higher in December than in March ($P \leq 0.05$). Pre-freeze LIN was higher in December than in September ($P = 0.01$), decreased after cryopreservation in December and March ($P \leq 0.03$), and did not differ between months at post-thaw ($P = 0.98$). Pre-freeze WOB was highest in December ($P \leq 0.01$), decreased after cryopreservation in December

($P<0.01$), and did not differ between months at post-thaw ($P=0.69$). Pre-freeze ALH was lower in March than in September ($P=0.01$), increased after cryopreservation in both December and March ($P<0.01$), and did not differ between months at post-thaw ($P=0.13$).

Fluorescent probes for sperm evaluation

The post-thaw DFI was lowest in December, intermediate in September, and highest in March ($P\leq 0.05$; Table 2). The total percentage of live spermatozoa with intact plasma membrane at post-thaw was higher in December than September ($P=0.03$), but did not differ between March and December or September ($P\geq 0.15$; Table 2). Likewise, the percentage of dead spermatozoa at post-thaw was lower in December than September ($P=0.03$), with no differences between March and December or September ($P=0.30$; Table 2). There were no differences observed in the percentage of moribund spermatozoa post-thaw between months ($P=0.86$; Table 2). There were more total live spermatozoa with intact acrosomes post-thaw in December than in September ($P<0.01$; Table 2). However, the percentage of intact acrosomes per live spermatozoa post-thaw was highest in September ($P=0.03$; Table 2).

3.2 Correlation between motility and fluorescent probes for sperm evaluation

Correlation coefficients between overall and progressive motility (%) and flow cytometric assessments are summarized in Table 3. Post-thaw overall and progressive sperm motility exhibited strong, positive correlation with sperm viability and strong, negative correlation with percentage of dead sperm ($P<0.01$). The percentage of live sperm with intact acrosomes also had strong, positive correlation with both overall and progressive sperm motility ($P<0.01$). Furthermore, overall motility tended to exhibit low, negative correlation with DFI ($P=0.09$), whereas progressive motility had moderate, negative correlation with DFI ($P=0.03$).

4. Discussion

The present study appears to be the first to describe seasonal variations in post-thaw semen quality of young WTD bucks at 40°N. While a previous study evaluated different extenders in WTD for freezing semen at both late season (February, March) and peak rut (October, December), they did not specifically evaluate differences between the collection times (Clemente-Sánchez et al., 2015). White-tailed deer are exclusively short-day breeders, with bucks entering rut around September (Woolf and Harder, 1979). Recent studies have thoroughly described the seasonal variations in reproductive characteristics of WTD does (Green et al., 2017) and bucks (Stewart et al., 2017) throughout rut. One limitation of the current study was that we were unable to perform fluorescent probe assays on the semen prior to freezing, so it is unclear whether the results are due to decreased freezing ability or from poor quality initially. Our concurrent study demonstrated that early (September) or late (March) in the breeding season, bucks have increased percentages of primary sperm defects, suggesting the latter (Stewart et al., 2017). The present report expands upon that data by confirming the translation into poor semen quality following cryopreservation. Though our study contained predominantly young bucks, it was reported previously that age had no effect on epididymal sperm quality and freezing ability within 5 hr postmortem in WTD bucks (Ake-Lopez et al., 2010).

The minimal effective breeding dose for the various artificial insemination methods with frozen-thawed semen has not been defined for WTD. One study in Mississippi reported pregnancy rates of 65 to 100% when performing intravaginal insemination with semen doses ranging from 12 to 60 million progressively motile sperm per straw (Jacobson et al., 1989). Another study achieved an overall conception rate of 67% using frozen-thawed semen vials containing at least

100 million live normal cells with 60% or higher motility deposited intracervically (Magyar et al., 1989). In red deer, intrauterine insemination with breeding doses of 25 to 38 million motile sperm yielded pregnancy rates of 22-49% (Fennessy et al., 1990). Another study involving both red and fallow deer (*Dama dama*) reported higher conception rates (60 to 70%) using intrauterine doses of 10 to 40 million motile spermatozoa (Asher et al., 1993). Furthermore, doses of only 7.5 to 10 million motile sperm per uterine horn yielded a 45% pregnancy rate in Eld's deer (*Cervus eldi thamin*; Monfort et al., 1993) and a 50% pregnancy rate in sika deer (*Cervus nippon*; Willard et al., 1996). Anecdotally, one of the authors (CFS) uses breeding doses as low as 2 to 4 million motile sperm and has obtained satisfactory pregnancy rates (>70%) with the use of laparoscopic insemination in WTD. Using this criterion, semen processed in the current study contained 15 (September), 39 (December), and 29 (March) million progressively motile sperm per straw at post-thaw, which are similar to values reported previously in WTD (Stewart et al., 2016; Williams et al., 2004) and could potentially result in satisfactory pregnancy rates with proper breeding management and experienced inseminators.

Cryopreservation of semen seemed to affect sperm motility most prominently in September. The decline in VAP, VCL, and VSL in September likely attributed to the lower post-thaw overall and progressive motilities observed at this month. Interestingly, while pre-freeze motility was lowest in March, it increased following cryopreservation and did not differ from December at post-thaw. Despite similar post-thaw motilities in December and March, several sperm motion parameters (VAP, VCL, VSL, BCF) were higher in December than March at post-thaw, which may indicate subtle differences. The unexpected motility improvement in March could be related to changes in seminal plasma constituents that were alleviated by the extender composition. Pink-tinged ejaculates were produced in 57% of the bucks collected in March, suggestive of blood

contamination and associated with changes in accessory sex gland size (Stewart et al., 2017). Seasonal variation in accessory sex gland size has also been reported in other cervid species such as roe deer (*Capreolus capreolus*; Goeritz et al., 2003), red deer (Lincoln, 1971), and fallow deer (Chapman and Chapman, 1979). In addition to these physical changes, the seminal plasma of red deer experienced increased antioxidant content at the peak of rut, which may aid sperm freezability (Koziorowska-Gilun et al., 2016). In rams, motility was improved after incubating freeze-thawed sperm with seminal plasma harvested in autumn and winter, but not spring or summer, demonstrating seasonal variability in their composition (Domínguez et al., 2008).

While the improved motility in March makes a promising argument for freezing semen late in the breeding season, we also observed significantly worse sperm DNA stability in frozen-thawed samples from the March collection (16%). A previous study from our group reported a DFI of 6% in WTD semen collected and froze in Optixcell extender in January, which is higher than those reported in both September (2.5%) and December (1.2%) of the current study. This finding suggests that post-thaw DFI may start to increase with increasing daylength in WTD. Conversely, there was significantly less variation in DFI values of red deer in the non-breeding season versus pre-rut, rut, or post-rut (Garcia-Macias et al., 2006). While there are no guidelines for using DFI to determine fertility in cervids, studies have defined subfertility at DFI >30% for humans (Evenson et al., 2002), though lower values have been reported in small ruminants (López-Fernández et al., 2008). The changes observed in the current study between peak rut and late breeding season seem to provide evidence that fertility may be compromised when using semen frozen during this period, despite appropriate motility. The finding that post-thaw progressive motility is moderately correlated with DFI is consistent with a previous report (Stewart et al., 2016) and suggests that it may be a useful predictor of DNA stability in WTD. Additionally, the high

number of primary sperm defects previously reported in September and March in WTD may be noncompensable and could also adversely affect fertility outcomes (Stewart et al., 2017). However, it remains unknown if these alterations in semen quality result in poor conception rates after artificial insemination or if direct deposition of the semen into the uterus via laparoscopic insemination may compensate for reduced semen quality.

In December, there was a higher number of live sperm than in September or March. Given the higher viability in December, it is not surprising that a higher percentage of live spermatozoa with intact acrosomes in the entire sample (live + dead spermatozoa) was also observed. Similar to previous reports, motility was strongly correlated with viability (Stewart et al., 2016). However, when evaluating acrosome integrity in the live sperm population only, there was a higher percentage of intact acrosomes in September versus December or March. Surprisingly, acrosome integrity alone exhibited a weak correlation with motility, suggesting that acrosome status may be independently influenced by other seminal plasma factors. Similarly, tufted deer (*Elaphodus cephalophus*) semen had a higher percentage of intact acrosomes in autumn (September to November) when compared to winter (December to February) (Panyaboriban et al., 2016). In contrast, epididymal sperm in red deer exhibited higher viability and acrosomal integrity in the post-rut period compared to rut (Martínez-Pastor et al., 2005). However, it is crucial to note that epididymal sperm are not exposed to seminal plasma secretions, which may contribute to seasonal variations observed. Additionally, the samples in the current study were only evaluated after thawing, so it is unclear if differences in acrosome integrity were due to poor initial quality or reduced freezing ability.

Further studies are necessary to determine if there are interventions that can improve sperm quality early and late in the breeding season. In red deer, supplementing freezing extenders with

enzymatic antioxidants (i.e., catalase and superoxide dismutase) before cryopreservation improved post-thaw sperm motility, viability, acrosome integrity, and mitochondrial status, but had no effect on DNA stability (Fernandez-Santos et al., 2006). Nutritional strategies should also be considered, as it was found that under-fed rams experienced more DNA damage than well-fed rams (Guan et al., 2014). Nutritional supplementation with specific compounds that can act as antioxidants should be evaluated as a means to improve semen quality during transitional periods. Vitamin E supplementation has been shown to protect testicular cell membrane and mitochondria and improve semen quality in rams (Yue et al., 2010). Another report found that sorting WTD sperm via flow cytometry decreased the baseline amount of frozen-thawed sperm containing fragmented DNA and increased DNA longevity (Kjelland et al., 2011). Even more interesting was the discovery that frozen-thawed sex-sorted semen experienced even less DNA fragmentation over time than conventional-sorted semen in WTD (Kjelland et al., 2011). Though the use of conventional sorting to select superior sperm may be cost-prohibitive, the use of sex-sorted semen is becoming a useful option for WTD farmers (Sugai et al., 2017). Studies evaluating the usefulness and feasibility of these technologies for improving post-thaw semen quality during seasonal transitional periods are therefore needed.

In conclusion, this study demonstrates that there are variations in post-thaw semen quality between early breeding season (September), peak rut (December), and late breeding season (March) in WTD bucks. Motility was highest in December before freezing. However, motility appeared to improve in March, with no difference in post-thaw overall or progressive motilities between December and March collections. Despite this finding, DNA stability was superior in December, suggesting that fertility may be enhanced when using frozen semen collected during peak rut versus transitional periods (September or March). Acrosomal integrity in the live sperm

population was highest in September. One limitation of this study was the small number of bucks available; therefore, a field trial using artificial insemination with a larger number of ejaculates across several farms may help to validate these results. Future studies need to evaluate the different components of seminal plasma further and determine if they can be supplemented to extenders at cryopreservation to enhance freezability in WTD sperm throughout the rutting season.

Conflict of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality in conducting the experiment and publishing the manuscript.

Author contributions

All authors were involved in project design, anesthesia, semen collection, and cryopreservation. Sperm analyses were performed by J.L.S. and R.E.E. Statistical analyses and manuscript preparation were performed by J.L.S. All authors contributed to and approved the final revision of the manuscript.

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Table 1. Computer-aided sperm analysis outputs describing the motion of sperm samples collected and cryopreserved from WTD in September, December, and March. Data are presented as mean \pm SEM. Differing superscripts denote differences between months, whereas asterisks denote differences between pre-freeze and post-thaw ($P \leq 0.05$).

		VAP	VCL	VSL	STR	LIN	WOB	ALH	BCF
September	Pre-Freeze	76 \pm 12	153 \pm 26	56 \pm 9.5	72 \pm 4.4	37 \pm 3.9 ^a	50 \pm 2.2 ^b	5.2 \pm 0.5 ^a	29 \pm 3.4
	Post-Thaw	58 \pm 5.2 ^{*ab}	114 \pm 13 ^{*ab}	36 \pm 2.8 ^{*b}	62 \pm 1.4 [*]	32 \pm 1.7	51 \pm 1.6	5.0 \pm 0.2	21 \pm 1.4 ^{*ab}
December	Pre-Freeze	70 \pm 5.1	128 \pm 12	56 \pm 4.3	80 \pm 0.2	44 \pm 1.0 ^b	55 \pm 1.2 ^a	4.5 \pm 0.2 ^{ab}	29 \pm 0.8
	Post-Thaw	70 \pm 2.5 ^a	141 \pm 4.9 ^a	46 \pm 3.9 ^{*a}	66 \pm 2.8 [*]	33 \pm 1.3 [*]	50 \pm 0.7 [*]	5.5 \pm 0.3 [*]	25 \pm 1.1 ^{*a}
March	Pre-Freeze	60 \pm 5.5	119 \pm 14	45 \pm 3.6	76 \pm 1.9	39 \pm 2.3 ^{ab}	51 \pm 1.7 ^b	4.3 \pm 0.3 ^b	27 \pm 1.2
	Post-Thaw	56 \pm 4.0 ^b	112 \pm 9.1 ^b	37 \pm 2.0 ^{*b}	66 \pm 1.7 [*]	33 \pm 1.3 [*]	50 \pm 0.9	5.3 \pm 0.3 [*]	21 \pm 0.6 ^{*b}

Abbreviations: VAP = average path velocity, $\mu\text{m/s}$; VCL = curvilinear velocity, $\mu\text{m/s}$; VSL = straight line velocity, $\mu\text{m/s}$; STR = straightness of the average path, %; LIN = linearity of the curvilinear path, %; WOB = Wobble, %; ALH = amplitude of lateral head displacement, μm ; BCF= beat-cross frequency, number per second. Data expressed as mean \pm SEM.

Table 2. Summary of fluorescent probe analyses for WTD semen cryopreserved in September, December, and March. Data are presented as mean \pm SEM. Differing superscripts denote differences between months ($P \leq 0.05$).

Post-Thaw Fluorescent Probe Analyses						
	Live (%)	Dead (%)	Moribund (%)	DFI (%)	Total live sperm with IA (%)	IA/ live sperm (%)
September	59 \pm 8.7 ^a	39 \pm 8.5 ^a	2.6 \pm 0.6	2.5 \pm 0.8 ^a	40 \pm 5.5 ^a	96 \pm 1.3 ^a
December	75 \pm 6.6 ^b	23 \pm 6.7 ^b	2.9 \pm 1.1	1.2 \pm 0.3 ^b	62 \pm 7.5 ^b	91 \pm 2.4 ^b
March	71 \pm 4.4 ^{a,b}	27 \pm 4.4 ^{a,b}	1.8 \pm 0.2	16 \pm 5.9 ^c	52 \pm 5.9 ^{a,b}	91 \pm 1.3 ^b
<i>p</i>-value	0.03	0.03	0.86	0.05	<0.01	0.03

IA = Intact acrosome.

Table 3. Correlation coefficients between overall and progressive motility (%) and flow cytometric assessments.

	% Live	% Dead	% Moribund	%Live sperm with IA	% IA/ live sperm	DFI%
Overall motility	0.60***	-0.61***	-0.03	0.74***	-0.16	-0.39*
Progressive motility	0.66***	-0.66***	0.01	0.80***	-0.10	-0.50**

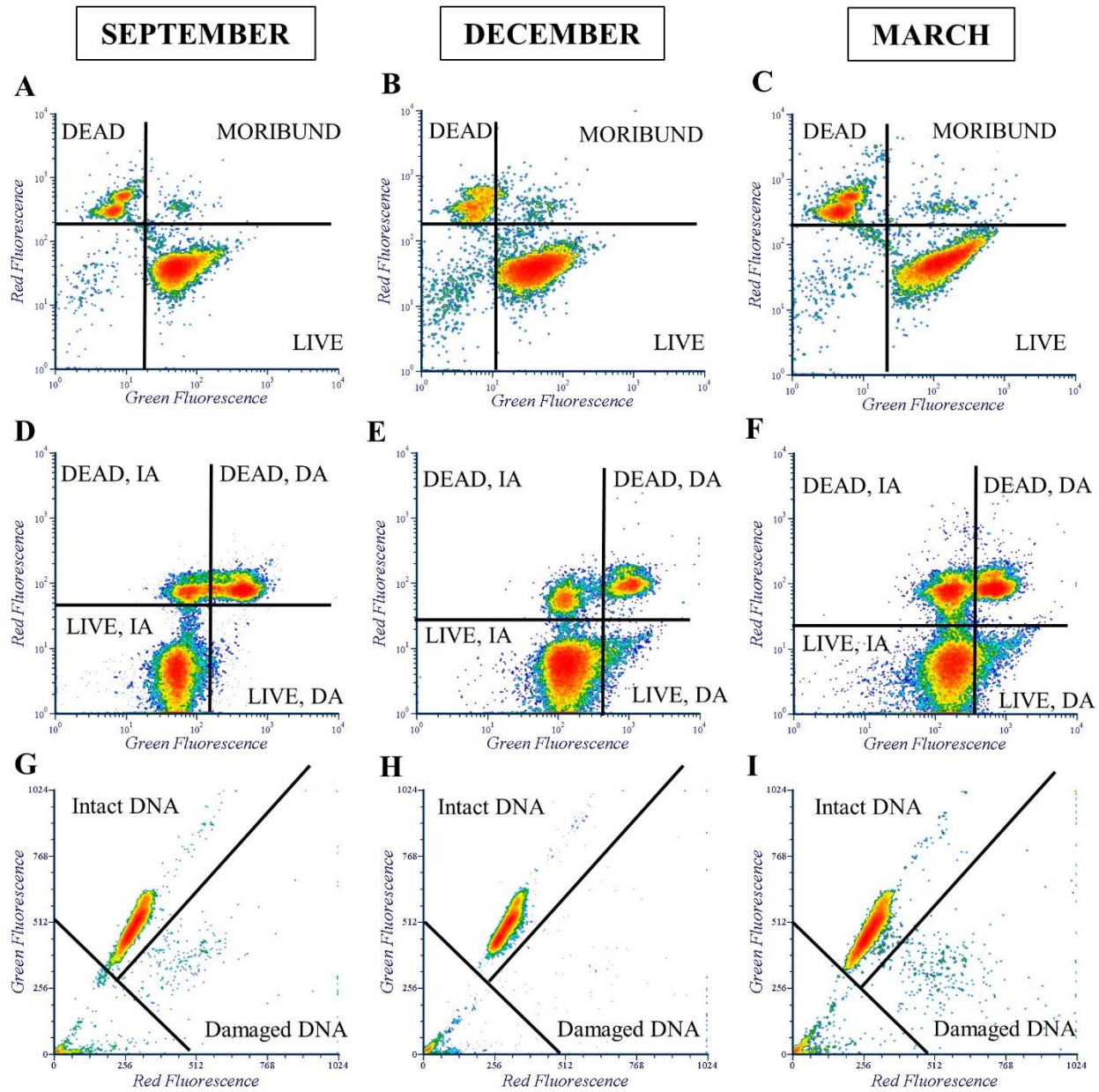
DFI: DNA Fragmentation Index; IA: intact acrosome.

*Data with low correlation ($P < 0.10$).

**Data with moderate correlation ($P \leq 0.05$).

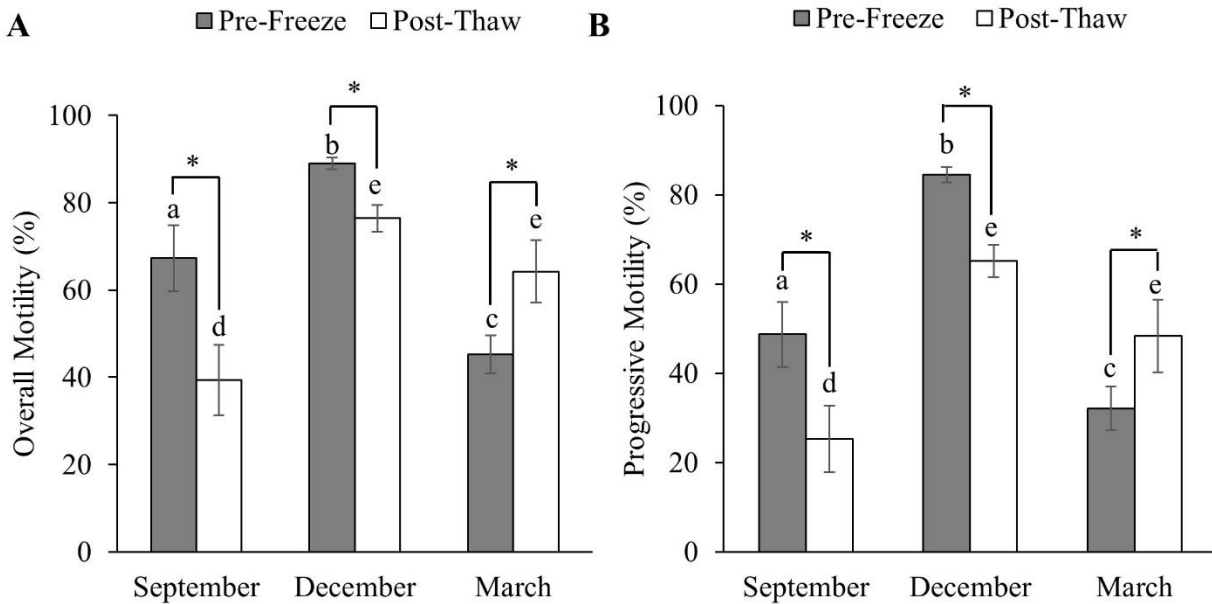
***Data with strong correlation ($P \leq 0.01$).

Figure 1. Representative flow cytometry dot plots for the sperm viability assay (A-C), acrosomal integrity assay (D-F), and chromatin stability assay (G-I) for a buck collected in September (A, D, G), December (B, E, H), and March (C, F, I).



IA: intact acrosome; DA: damaged acrosome.

Figure 2. Bar graph showing percent (A) overall and (B) progressive sperm motility immediately before and after semen cryopreservation in September, December, and March. Differing superscripts denote differences between months, whereas asterisks denote differences between pre-freeze and post-thaw ($P \leq 0.05$).



References

- Ake-Lopez, J., Cavazos-Arizpe, E., Magana-Monforte, J.G., Centurion-Castro, F., Silva-Mena, C., 2010. Effect of age and postmortem time on some white-tailed deer (*Odocoileus virginianus texanus*) epididymal sperm characteristics and response of cryopreservation. *Am. J. Anim. Vet. Sci.* 5, 183–186.
- Amann, R.P., Waberski, D., 2014. Computer-assisted sperm analysis (CASA): Capabilities and potential developments. *Theriogenology* 81, 5–17.
<https://doi.org/10.1016/j.theriogenology.2013.09.004>
- Anderson, D.P., Frosch, B.J., Outlaw, J.L., 2007. Economic impact of the United States cervid farming industry. Agricultural and Food Policy Center, Texas A&M University, College Station, Texas. <https://www.afpc.tamu.edu/pubs/2/480/rr-2007-04.pdf>
- Anderson, D.P., Outlaw, J.L., Earle, M.L., Richardson, J.W., 2017. Economic impact of U.S. deer breeding and hunting operations. Agricultural and Food Policy Center, Texas A&M University, College Station, Texas.
- Asher, G.W., Fisher, M.W., Fennessy, P.F., Mackintosh, C.G., Jabbour, H.N., Morrow, C.J., 1993. Oestrous synchronization, semen collection and artificial insemination of farmed red deer (*Cervus elaphus*) and fallow deer (*Dama dama*). *Anim. Reprod. Sci.* 33, 241–265.
[https://doi.org/10.1016/0378-4320\(93\)90118-B](https://doi.org/10.1016/0378-4320(93)90118-B)
- Barnes, K.R., Mills Jr., F.D., Wolfskill, L.A., Nair, S.S., R., F.J., Ullrich, D.R., 2016. White-tailed deer semen auction data: a hedonic analysis. *Texas J. Agric. Nat. Resour.* 29, 39–51.
- Castellanos, P., del Olmo, E., Fernández-Santos, M.R., Rodríguez-Estival, J., Garde, J.J., Mateo, R., 2015. Increased chromatin fragmentation and reduced acrosome integrity in spermatozoa of red deer from lead polluted sites. *Sci. Total Environ.* 505, 32–38.
<https://doi.org/10.1016/j.scitotenv.2014.09.087>
- Chapman, N.G., Chapman, D.I., 1979. Seasonal changes in the male accessory glands of reproduction in adult Fallow deer (*Dama dama*). *J. Zool. London* 189, 259–273.
<https://doi.org/10.1111/j.1469-7998.1979.tb03962.x>
- Clemente-Sánchez, F., Cessa-Reyes, V., Cortez-Romero, C., Tarango-Arambula, L.A., Arenas-Baez, P., 2015. Commercial extenders and freezing curves for the preservation of sperm cells of white-tailed deer (*Odocoileus virginianus*). *J. Appl. Anim. Res.* 43, 468–473.
<https://doi.org/10.1080/09712119.2014.980422>
- Domínguez, M.P., Falcinelli, A., Hozbor, F., Sánchez, E., Cesari, A., Alberio, R.H., 2008. Seasonal variations in the composition of ram seminal plasma and its effect on frozen-thawed ram sperm. *Theriogenology* 69, 564–573.
<https://doi.org/10.1016/j.theriogenology.2007.11.010>

- Ellerbrock, R.E., Prell, M.J., Stewart, J.L., Bojko, M.S., Lima, F.S., Canisso, I.F., 2017. Comparison of centrifugation and noncentrifugation methods to cryopreserve stallion epididymal semen. *J. Equine Vet. Sci.* 50, 27–32. <https://doi.org/10.1016/j.jevs.2016.11.005>
- Evenson, D.P., Larson, K.L., Jost, L.K., 2002. Sperm chromatin structure assay: its clinical use for detecting sperm DNA fragmentation in male infertility and comparisons with other techniques. *J. Androl.* 23, 25–43. <https://doi.org/10.1002/j.1939-4640.2002.tb02599.x>
- Evenson, D.P., Thompson, L., Jost, L., 1994. Flow cytometric evaluation of boar semen by the sperm chromatin structure assay as related to cryopreservation and fertility. *Theriogenology* 41, 637–651. [https://doi.org/10.1016/0093-691X\(94\)90174-H](https://doi.org/10.1016/0093-691X(94)90174-H)
- Fennessy, P.F., Mackintosh, C.G., Shackell, G.H., Fennessy, P.F., Mackintosh, C.G., 1990. Artificial insemination of farmed red deer (*Cervus elaphus*). *Anim. Prod.* 51, 613–621. <https://doi.org/10.1017/S0003356100012654>
- Fernandez-Santos, M.R., Martinez-Pastor, F., Garcia-Macias, V., Estes, M.C., Soler, A.J., Paz, P., Anel, L., Garde, J.J., 2006. Sperm characteristics and DNA integrity of Iberian red deer (*Cervus elaphus hispanicus*) epididymal spermatozoa frozen in the presence of enzymatic and nonenzymatic antioxidants. *J. Androl.* 28, 294–305. <https://doi.org/10.2164/jandrol.106.000935>
- Flinn, J.J., Demaris, S., Strickland, B.K., Gee, K.L., Webb, S.L., Jones, P.D., Jacobson, H.A., 2015. Estimating age and antler traits of photographed male white-tailed deer. *J. Southeast. Assoc. Fish Wildl.* 2, 135–143.
- Garcia-Macias, V., Martinez-Pastor, F., Alvarez, M., Borragan, S., Chamorro, C. a., Soler, a. J., Anel, L., de Paz, P., 2006. Seasonal changes in sperm chromatin condensation in ram (*Ovis aries*), Iberian red deer (*Cervus elaphus hispanicus*), and brown bear (*Ursus arctos*). *J. Androl.* 27, 837–846. <https://doi.org/10.2164/jandrol.106.000315>
- Goeritz, F., Quest, M., Wagener, A., Fassbender, M., Broich, A., Hildebrandt, T.B., Hofmann, R.R., Blottner, S., 2003. Seasonal timing of sperm production in roe deer: Interrelationship among changes in ejaculate parameters, morphology and function of testis and accessory glands. *Theriogenology* 59, 1487–1502. [https://doi.org/10.1016/S0093-691X\(02\)01201-3](https://doi.org/10.1016/S0093-691X(02)01201-3)
- Green, M.L., Kelly, A.C., Satterthwaite-Phillips, D., Manjerovic, M.B., Shelton, P., Novakofski, J., Mateus-Pinilla, N., 2017. Reproductive characteristics of female white-tailed deer (*Odocoileus virginianus*) in the Midwestern USA. *Theriogenology* 94, 71–78. <https://doi.org/10.1016/j.theriogenology.2017.02.010>
- Guan, Y., Malecki, I.A., Hawken, P.A.R., Linden, M.D., Martin, G.B., 2014. Under-nutrition reduces spermatogenic efficiency and sperm velocity, and increases sperm DNA damage in sexually mature male sheep. *Anim. Reprod. Sci.* 149, 163–172. <https://doi.org/10.1016/j.anireprosci.2014.07.014>

- Haigh, J.C., Cates, W.F., Glover, G.J., Rawlings, N.C., 1984. Relationships between seasonal changes in serum testosterone concentrations, scrotal circumference and sperm morphology of male wapiti (*Cervus elaphus*). J. Reprod. Fertil. 70, 413–8. <https://doi.org/10.1530/jrf.0.0700413>
- Jacobson, H.A., Bearden, H.J., Whitehouse, D.B., 1989. Artificial insemination trials with white-tailed deer. J. Wildl. Manage. 53, 224–227. <https://doi.org/10.2307/3801338>
- Kenney, R.M., Evenson, D.P., Garcia, M.C., Love, C.C., 1995. Relationships between sperm chromatin structure, motility, and morphology of ejaculated sperm, and seasonal pregnancy rate, in: Equine Reproduction VI, Biol Reprod Mono 1. pp. 647–653.
- Kjelland, M.E., González-Marín, C., Gosálvez, J., López-Fernández, C., Lenz, R.W., Evans, K.M., Moreno, J.F., 2011. DNA fragmentation kinetics and postthaw motility of flow cytometric-sorted white-tailed deer sperm. J. Anim. Sci. 89, 3996–4006. <https://doi.org/10.2527/jas.2011-4014>
- Koziorowska-Gilun, M., Szurnicka, M., Dziekonska, A., Kordan, W., Gizejewski, Z., Filipowicz, K., 2016. Characteristics of antioxidant systems of yellow fraction of red deer's (*Cervus elaphus* L) semen during the rutting period. Reprod. Domest. Anim. 51, 248–254. <https://doi.org/10.1111/rda.12673>
- Lincoln, G.A., 1971. The seasonal reproductive changes in the red deer stag (*Cervus elaphus*). J. Zool. 163, 105–123. <https://doi.org/10.1111/j.1469-7998.1971.tb04527.x>
- López-Fernández, C., Fernández, J.L., Gosálbez, A., Arroyo, F., Vázquez, J.M., Holt, W. V., Gosálvez, J., 2008. Dynamics of sperm DNA fragmentation in domestic animals. III. Ram. Theriogenology 70, 898–908. <https://doi.org/10.1016/j.theriogenology.2008.04.055>
- Lukefahr, S.D., Jacobson, H.A., 1998. Variance component analysis and heritability of antler traits in white-tailed deer. J. Wildl. Manage. 62, 262–268. <https://doi.org/10.2307/3802287>
- Magyar, S.J., Biediger, T., Hodges, C., Kraemer, D.C., Seager, S.W.J., 1989. A method of artificial insemination in captive white-tailed deer (*Odocoileus virginianus*). Theriogenology 31, 1075–1079. [https://doi.org/10.1016/0093-691X\(89\)90491-3](https://doi.org/10.1016/0093-691X(89)90491-3)
- Martínez-Pastor, F., Guerra, C., Kaabi, M., Garcia-Macias, V., De Paz, P., Alvarez, M., Herraiez, P., Anel, L., 2005. Season effect on genitalia and epididymal sperm from Iberian red deer, roe deer and Cantabrian chamois. Theriogenology 63, 1857–1875. <https://doi.org/10.1016/j.theriogenology.2004.08.006>
- Martínez-Pastor, F., Johannisson, A., Gil, J., Kaabi, M., Anel, L., Paz, P., Rodriguez-Martinez, H., 2004. Use of chromatin stability assay, mitochondrial stain JC-1, and fluorometric assessment of plasma membrane to evaluate frozen-thawed ram semen. Anim. Reprod. Sci. 84, 121–133. <https://doi.org/10.1016/j.anireprosci.2003.12.006>

- Martínez-Pastor, F., Mata-Campuzano, M., Álvarez-Rodríguez, M., Álvarez, M., Anel, L., de Paz, P., 2010. Probes and techniques for sperm evaluation by flow cytometry. *Reprod. Domest. Anim.* 45, 67–78. <https://doi.org/10.1111/j.1439-0531.2010.01622.x>
- Monfort, S.L., Asher, G.W., Wildt, D.E., Wood, T.C., Schiewe, M.C., Williamson, L.R., Bush, M., Rall, W.F., 1993. Successful intrauterine insemination of Eld's deer (*Cervus eldi thamin*) with frozen-thawed spermatozoa. *J. Reprod. Fertil.* 99, 459–465. <https://doi.org/10.1530/jrf.0.0990459>
- Panyaboriban, S., Singh, R.P., Songsasen, N., Padilla, L., Brown, J., Reed, D., Techakumphu, M., Pukazhenth, B., 2016. Reproductive seasonality and sperm cryopreservation in the male tufted deer (*Elaphodus cephalophus*). *Theriogenology* 86, 914–923. <https://doi.org/10.1016/j.theriogenology.2016.03.014>
- Robinson, R.M., Thomas, J.W., Marburger, R.G., 1965. The reproductive cycle of male white-tailed deer in central Texas. *J. Wildl. Manage.* 29, 53–59. <https://doi.org/10.2307/3798631>
- Robles, V., Martínez-Pastor, F., 2013. Chapter 16: Flow cytometric methods for sperm assessment, in: Carrell, D.T., Aston, K.I. (Eds.), *Spermatogenesis: Methods and Protocols*. Humana Press; Springer Science+Business Media, LLC, New York City, pp. 175–186. <https://doi.org/10.1007/978-1-62703-038-0>
- Stewart, J.L., Schmidt, L., Ellerbrock, R.E., Scholz, S.S., Lima, F.S., Canisso, I.F., Shipley, C.F., 2017. Effects of seasonality on male white-tailed deer (*Odocoileus virginianus*) reproductive characteristics, in: American Association of Small Ruminant Practitioners Research Summary. Omaha, NE. http://www.aabp.org/meeting/display_research.asp?recnum=135
- Stewart, J.L., Shipley, C.F., Katich, A.S., Po, E., Ellerbrock, R.E., Lima, F.S., Canisso, I.F., 2016. Cryopreservation of white-tailed deer (*Odocoileus virginianus*) semen using soybean-, liposome-, and egg yolk-based extenders. *Anim. Reprod. Sci.* 171. <https://doi.org/10.1016/j.anireprosci.2016.05.006>
- Sugai, N.J., Stewart, J.L., Lima, F.S., Canisso, I.F., Shipley, C.F., 2017. Efficacy of using sex-sorted semen technologies for laparoscopic artificial insemination in a commercial white-tailed deer farm (*Odocoileus virginianus*), in: American Association of Small Ruminant Practitioners Research Summary. Omaha, NE. http://www.aabp.org/meeting/display_research.asp?recnum=142
- Varghese, A.C., Fischer-Hammadeh, C., Hammadeh, M.E., 2011. Chapter 13: Acridine Orange Test for Assessment of Human Sperm DNA Integrity, in: Zini, A., Agarwal, A. (Eds.), *Sperm Chromatin: Biological and Clinical Applications in Male Infertility and Assisted Reproduction*. Humana Press; Springer Science+Business Media, LLC, New York City, pp. 189–199. <https://doi.org/10.1007/978-1-4419-6857-9>
- Wallingford, B.D., Diefenbach, D.R., Long, E.S., Rosenberry, C.S., Alt, G.L., 2017. Biological

- and social outcomes of antler point restriction harvest regulations for white-tailed deer. Wildl. Monogr. 196, 1–26. <https://doi.org/10.1002/wmon.1022>
- Willard, S.T., Hughes Jr., D.M., Bringans, M., Sasser, R.G., White, D.R., Jaques, J.T., Godfrey, R.W., Welsh Jr., T.H., Randel, R.D., 1996. Artificial insemination, hybridization, and pregnancy detection in sika deer (*Cervus nippon*). Theriogenology 46, 779–789. [https://doi.org/10.1016/S0093-691X\(96\)00236-1](https://doi.org/10.1016/S0093-691X(96)00236-1)
- Williams, B., Flores-Foxworth, G. Chapman, S., Romano, J., Kidd, B., Fuchs, G., Westhusin, M., Kraemer, D., Frels, D., 2004. 124 Cryopreservation of white-tailed deer (*Odocoileus virginianus*) semen. Reprod. Fertil. Dev. 16, 184–185. <https://doi.org/doi.org/10.1071/RDv16n1Ab124>
- Woolf, A., Harder, J.D., 1979. Population dynamics of a captive white-tailed deer herd with emphasis on reproduction and mortality. Wildl. Monogr. 67, 3–53.
- Yue, D., Yan, L., Luo, H., Xu, X., Jin, X., 2010. Effect of Vitamin E supplementation on semen quality and the testicular cell membranal and mitochondrial antioxidant abilities in Aohan fine-wool sheep. Anim. Reprod. Sci. 118, 217–222. <https://doi.org/10.1016/j.anireprosci.2009.08.004>