Reproductive seasonality and sperm cryopreservation in the male tufted deer

(*Elaphodus cephalophus*)

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Running title: Tufted deer sperm cryopreservation

Abstract

The tufted deer is a small deer, listed as near threatened on the IUCN Red List, and there is no information on the fundamental reproductive biology of this species. In this study, we report for the first-time, characterization of male reproductive traits and cryopreservation of semen in this species. Males were subjected to electroejaculation during each season (autumn, winter, spring, and summer), and ejaculates were assessed for motility and quality traits. Fecal samples were collected 3 – 5 times weekly for 2 years and analyzed for androgen metabolites using enzyme immunoassay. Ejaculates with >70% motility were cryopreserved using Beltsville extender (BF5F) or Triladyl[®]. Straws were thawed and assessed subjectively as well as swim-up processed to isolate motile spermatozoa for CASA analysis and acrosome integrity at hourly interval. Tufted deer male reproductive and semen traits peaked in autumn. Mean fecal androgen concentrations were highest in the summer compared to baseline values during rest of the year. Sperm motility and acrosome integrity were lower immediately after thawing in both cryodiluents compared with raw ejaculates. Motility characteristics following swim-up were higher in BF5F compared with Triladyl. Four hour after thawing, both percent sperm motility and progression declined further and were similar between BF5F and Triladyl. However, the proportion of spermatozoa with intact acrosomal membranes was higher in BF5F than Triladyl. Results indicate that tufted deer exhibit seasonal variations in reproductive traits and that BF5F better preserves sperm motility and acrosomal integrity after cryopreservation compared with Triladyl.

Keywords: cervid, testosterone, semen, cryodiluents, CASA

1. Introduction

The tufted deer (*Elaphodus cephalophus*) is listed as ‘near threatened’ on the IUCN Red List [1]. Morphologically, the tufted deer is small deer, and all four subspecies (*E.c.cephalophus*, *E.c.michianus*, *E.c.ichangensis*, and *E.c.forciensus*) inhabit large areas of southern China and some areas in northern Myanmar [2]. Although tufted deer is not included in the list of the Convention on International Trade in Endangered Species (CITES), the species is listed as Vulnerable in the Chinese Red List [3], highlighting current threats to their survival. Their habitat overlaps with the giant panda (*Ailuropoda melanoleuca*), and are found in forested regions at 300 – 4,500 meters above sea level in proximity to water sources [4]. Within Asia, extensive habitat destruction and over hunting have led to a rapid decline in their population numbers [5]. Historically, studies on tufted deer have focused primarily on population distribution and habitat in the wild [2, 6-8] and a small number of studies have examined population genetics [9-12].

In North America, tufted deer were first exhibited at the San Diego Zoo. The founding population was comprised of three males (from China) and two females (one each from China and Germany) imported in 1985. Currently there are 38 males and 28 females distributed among 18 institutions in the United States. Similar to many other ungulate species, tufted deer populations in captivity also have been slowly declining due to a lack of interest in this species. The calculated current gene diversity of the extant population is ~70% and classified as a Yellow Species Survival Plan (SSP) program by the Association of Zoos and Aquariums, meaning that the population cannot retain 90% gene diversity for 10 generations [13]. Population modeling predicts that without a further infusion of founder animals, the *ex situ* populations gene diversity would decline to ~57% in 100 years. Therefore, the tufted deer SSP

plans to increase the number of exhibit spaces and ensure animals are provided an opportunity to breed to better manage this species *ex situ*. Long term management of small populations involve an interplay between maintaining gene diversity and minimizing inbreeding in subsequent generations. At present the *ex situ* breeding program has relied on natural breeding; however, when natural reproduction fails, it is important to implement reproductive technologies including semen cryopreservation and artificial insemination to facilitate reproduction between behaviorally incompatible animals and efficiently move germplasm among geographically isolated populations. Development of artificial insemination technologies warrants a thorough understanding of species-specific reproductive biology. However, there is a lack of fundamental information on the reproductive biology of tufted deer as well as the impact of season on male reproduction.

Seasonal changes in semen and reproductive parameters have previously been reported in many mammalian species, for example the ram [14, 15], Eld's deer (*Rucervus eldi thamin*, [16]), roe deer (*Capreolus capreolus*, [17]), red deer (*Cervus elaphus*, [18]), (pampas deer (*Ozotocerus bezoarticus*, [19]), and American bison (*Bison bison bison*, [20]). Tufted deer typically breed from September through December and fawn in April to July [4]. Males also exhibit seasonal antler growth and rutting behavior – barking by males [4]. In other cervids, additional physical/morphometric changes such as changes in neck girth, scrotal circumference and ejaculate quality also have been reported [18-20]. Hence it is also important to examine the influence of season on tufted deer reproductive.

Spermatozoa from several species of deer including the Eld's deer, fallow deer (*Dama dama*), spotted deer (*Axis axis*), and red deer (*Cervus elaphus*) have been successfully cryopreserved and live offspring produced via artificial insemination [21-25]. However, sperm

cryopreservation remains a challenge in numerous species. Major challenges to sperm cryopreservation include minimizing the toxic effects of cryoprotectants, limiting intracellular ice crystal formation and osmotic stress during freezing and thawing [26, 27]. Selection of an appropriate cryoprotectant relies on sperm membrane permeability properties as well as choice of usage temperature. To our knowledge, there is no information on semen cryopreservation in tufted deer.

Therefore, the overall objectives of this study were to i) assess seasonal changes in reproductive traits including physical characteristics, hormonal and ejaculate traits and ii) examine the cryosensitivity of tufted deer sperm to two different cryodiluents.

2. Materials and Methods

All chemicals used in the present study were purchased from Sigma-Aldrich Chemical Company (Sigma, St. Louis, MO, USA), unless stated otherwise. Animal procedures were reviewed and approved by the Animal Care and Use Committee at the Smithsonian Conservation Biology Institute (SCBI). This study was carried out from July 2008 to June 2010 at SCBI near Front Royal, Virginia (78.17° W, 38.88° N) and the Wildlife Conservation Society, Bronx Zoo, Bronx, New York (73.88° W, 40.85° N).

2.1 Animals

Five mature males (age range, 1 – 15 y; body weight, 16.8 – 21.8 kg; 1-2 ejaculate per male per season; 24 ejaculates) were examined over the three year study period. Animals were housed individually in roofed and open-sided shelter (approximately 10 × 11 m) under natural environmental conditions (Front Royal, VA). Animal were maintained on 300 g/day/animal of

17% protein herbivore diet (Mazuri[®] ADF-16, Land O'Lakes, Inc., Arden Hills, MN), 10 high fiber biscuits (Marion Leaf Eater Biscuits, Gorilla Sized, Marion Zoological Foods, Plymouth, MN), 100 g/day/animal of mixed produce (fresh sweet potatoes, turnips, carrots and kale; 25 g each) for three times a week, and ad libitum water.

Animals at the Bronx Zoo (n = 2 males; used only for sperm cryopreservation studies) were housed with one or two females with an offspring in a 10 × 7 m corral with a 7 × 3 m three-sided heated shelter or in a 4,000 m² wooded exhibit with a 9 × 3 m three-sided heated shelter. Each animal received 300 g/day/animal of 14% protein and 23% fiber herbivore diet (ADF-25 Herbivore, Mazuri), alfalfa/timothy mixed hay ad libitum, 170 g of legume hay, 25 g kale, 40 g yam, and water ad libitum.

All animals were provided routine preventive and clinical veterinary care.

2.2 Anesthesia and Morphometric Measurements

Males were anesthetized by intramuscular injections using a combination of azaperone (0.15 – 0.3 mg/kg; ZooPharm, Laramie, WY), butorphanol tartrate (0.15 – 0.2 mg/kg; Equanox; Vedco Inc, St. Joseph, MO) and medetomidine hydrochloride (0.025 – 0.03 mg/kg; ZooPharm, Laramie, WY) with ketamine hydrochloride (1 – 2 mg/kg; Vedco Inc, St. Joseph, MO). After recumbency, endotracheal intubation was performed to facilitate positive pressure ventilation, and a light plane of anesthesia was maintained with intravenous boluses of propofol (0.2 – 2.0 mg/kg per bolus, slowly administered to effect into the cephalic vein at an average rate of 0.029 mg/kg/min; Propofol; Abbott Animal Health, Abbott Park, IL). Upon completion of procedures, anesthesia was antagonized with intramuscular injections of

atipamezole (0.125 – 0.15 mg/kg; Pfizer Animal Health / Zoetis, New York, NY) and naltrexone (1.5 – 2.0 mg/kg; ZooPharm, Laramie, WY).

Testicular length and width were determined using scientific calipers (Scienceware; Sci-Art Product, Pequannock, NJ). Testicular volume was calculated using the formula

$$(Volume = \frac{4}{3}\pi \left(\frac{Testicular\ length}{2}\right) \left(\frac{Testicular\ width}{2}\right)^2; [28]).$$
 Scrotal circumference and neck girth were measured using a measuring tape [18].

2.3 Semen Collection and Evaluation

For semen collection, the rectum was evacuated using lubricated gloved fingers and the prepuce was cleaned using 0.9% saline solution and wiped dry with cotton gauze. Then a sine-wave electrostimulator (AC 60 Hz; P.T. Electronics, Boring, OR) and a lubricated Teflon probe (2.0 cm diameter) was used to administer 80 – 100 electrical stimulations in three series while incrementally increasing the stimulation voltage (range, 2 – 6 V) as previously described by Howard [29]. Electrical stimulations consisted of three series: 2, 3, 4V (10 stimulations each); 3, 4, 5V (10 stimulations each); and 3, 4, 5V (10 stimulations each), respectively. Between series, animals were rested and ejaculates examined for volume, pH (pH – indicator strips; EMD Millipore Corporation, Billerica, MA), osmolality (Wescor Inc, Logan UT), sperm motility and forward progressive status (scale 0 – 5; 5 = best; [29]). Each ejaculate was then transferred into sterile 15 mL screw-capped centrifuge tubes (Fisher Scientific) and maintained at 37°C in a water-bath. Upon completion of semen collection, raw semen with similar motility characteristics were pooled into a single tube, then a 20 – 30 µL aliquot was added to 500 µL of 0.3% glutaraldehyde in phosphate-buffered saline (pH 7.4; 340 mOsm) for sperm morphology assessment (Supplemental Material S1). Twenty

microliters of semen also was fixed in 500 μ L of 4% paraformaldehyde (USB Corporation, Cleveland, OH) for acrosomal membrane assessment using Coomassie Brilliant Blue staining (Supplemental Material S2).

2.4 Semen Cryopreservation

Ejaculated semen samples with >70% initial motility were used in this study.

Ejaculates from each donor was subdivided into two aliquots for cryopreservation in Beltsville extender (BF5F; [30]) versus Triladyl[®] (Minitüb GmbH, Tiefenbach, Germany). BF5F diluent consisted of 20% (vol/vol) egg yolk, 1.6% (wt/vol) glucose, 1.6% (wt/vol) fructose, 1.2% (wt/vol) Tes (N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid), 0.2% (wt/vol) tris (Tris[hydroxymethyl]aminomethane), 1,000 i.u. penicillin G per mL, 1,000 μ g streptomycin sulfate per mL, and 0.5% (vol/vol) surfactant mixture of sodium and triethanolamine lauryl sulfate (Orvus ES Paste; Equex, Nova Chemical Sales Inc., Scituate, MA). Triladyl consisted of a proprietary mixture of tris, citric acid, fructose, glycerol, antibiotics, and 20% egg yolk. For cryopreservation in BF5F, semen samples were diluted with BF5F (without glycerol) to $300 - 400 \times 10^6$ sperm per mL in 15-mL centrifuge tubes. The tubes were placed in a water-bath (500 mL; 25°C) that was transferred to a walk-in cold room. When the water bath reached 5°C, an equal volume of pre-cooled BF5F containing 8% (vol/vol) glycerol was added in three steps (added $\frac{1}{4}$ volume and incubated for 15 min, added another $\frac{1}{4}$ volume and incubated for 15 min; and then added the remaining diluent) to achieve a final concentration of 4% glycerol (vol/vol). Extended semen samples were then aspirated into labeled 0.5 mL French straws (AgTech Inc, Manhattan, KS) and heat-sealed. Straws were cryopreserved using a two-step method. Briefly, straws were placed 8 cm above liquid nitrogen for 1 min in

a styrofoam cooler, then moved to 3 cm above liquid nitrogen for 1 min and plunged into liquid nitrogen.

For cryopreservation in Triladyl, egg yolk (20% vol/vol) was added to Fraction A per manufacturer's instruction and the ejaculates were diluted 3:1 (semen:diluent) at ambient temperature (25°C) with Triladyl containing 12% glycerol to achieve 4% (vol/vol) glycerol concentration. Diluted sperm suspension was loaded into labeled 0.5 mL French straws, heat sealed, and placed inside a zip-loc plastic bag that was then placed inside a water jacket (500 mL; 25°C), cooled to 5°C (over a period of 3 h) and cryopreserved as described above.

2.5 Semen Thawing, Assessment and Swim-Up Processing

After at least 12 mo of storage, two straws per extender per ejaculate were randomly selected and thawed (30 s, 37°C). Following thawing, sperm total motility was assessed subjectively using a phase-contrast microscope (200x; Olympus BX41; Olympus, Japan). Sperm morphology and acrosomal membrane integrity were examined as described earlier. In order to assess sperm motility characteristics using Computer-Assisted Sperm Analysis (CASA; Hamilton Thorne Research, Beverly, MA), thawed aliquots were subjected to swim-up processing. Briefly, immediately after thawing the sperm suspension was transferred to a 1.5 mL centrifuge tube and centrifuged ($300 \times g$ for 10 min) to pellet the sperm. The supernatant was carefully removed and discarded. One-hundred μL of warm TALP (Tyrodes Albumin Lactate Pyruvate) comprising of NaCl (6.2 g/L), KCl (0.23 g/L), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.39 g/L), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.01 g/L) NaHCO_3 (2.1 g/L), Na_2HPO_4 (0.05 g/L), Na lactate (0.13%, vol/vol), Na pyruvate (0.02 g/L) and BSA (6 g/L); osmolarity 295 mOsm and pH 7.5 [31] was carefully layered over the sperm pellet and incubated (37°C; 30 min) to allow motile sperm to

swim up into the medium. The supernatant was then removed and transferred into a fresh centrifuge tube. Sperm suspensions were evaluated for motility characteristics using CASA at 0 (within 5 min after swim-up processing), 1, 2, 3, and 4 h. Briefly, two μL of sperm suspension was placed on a prewarmed 4X – CELL chamber slide (Cytonix, Beltsville, MD; 20 μm depth). At least 10 microscopic fields were evaluated using CASA to analyze sperm total motility, forward progressive motility and sperm motion characteristics including straight-line velocity (VSL, $\mu\text{m/s}$); curvilinear velocity (VCL, $\mu\text{m/s}$); average path velocity (VAP, $\mu\text{m/s}$); straightness ($\text{STR} = \text{VSL}/\text{VAP}$, %), linearity ($\text{LIN} = \text{VSL}/\text{VCL}$, %); amplitude of lateral head displacement (ALH), and beat cross frequency (BCF). CASA established setup parameters were as follows: frame capture speed rate, 60 Hz; cell size (min/max), 21/33 μm^2 ; threshold straightness, 80%; VAP cut off, 20 $\mu\text{m/s}$; and VSL cut off, 30 $\mu\text{m/s}$. Sperm acrosomal integrity was assessed at hourly interval (for up to 4 h) as described earlier.

2.6 Fecal Sample Collection and Analysis of Fecal Androgen Metabolites

Fecal samples were collected three to five times per wk for at least 18 mo. Fecal pellets were processed and hormone metabolites extracted as previously described by Brown et al.([32]; Supplement Material S3).

2.7 Statistical Analyses

All statistical analyses were carried out using Statistical Analysis System (SAS) package version 9.2 (SAS 9.2, SAS Institute Inc., Cary, NC). The year was divided into four seasons: autumn (September – November), winter (December – February), spring (March – May), and summer (June – August) per the National Oceanic and Atmospheric Administration

(USA). Data on seasonal effects on semen, hormonal and reproductive parameters were analyzed using GLM procedures. Season was considered as a fixed – crossed effect, whereas the male (donor) was set as a random effect. PROC Univariate of SAS was used to test normal distribution of the data. Non-normal data (concentration, total number of sperm, progressive motility, pH, volume, acrosomal integrity and BCF) were subjected to log transformation, confirmed for normal distribution and analyzed using GLM. Progressive motility, pH, ejaculate volume, and acrosomal integrity could not be normalized and hence analyzed using the Wilcoxon rank – sum test (PROC NPAR1WAY of SAS).

For post – thaw analyses, data were compared by two – way ANOVA with Tukey multiple comparisons test to detect difference across treatments. All *P* values lower than 0.05 were considered statistically significant.

Data presented represents mean $\pm$ SEM unless specified otherwise.

3. Results

3.1 Seasonal changes in gross morphometry and seminal traits

Mean neck girth was highest ($P < 0.05$) in the autumn (Q1 (median) Q3; 42.0 (44.0) 44.0 cm) compared to that during other seasons (Q1 (median) Q3; 37.0 (39.0) 43.0 cm), as was testicular length ($P < 0.05$; Table 1). The average ratio between testicular length and width also was the highest in autumn. Scrotal circumference, testicular width and testicular volume were unaffected ($P > 0.05$) by season.

Table 2 summarizes seasonal changes in fresh ejaculated semen. Spermic ejaculates were obtained from all males via electroejaculation. Sperm total motility, forward progressive motility and the percentage of spermatozoa with an intact acrosome were higher ($P < 0.05$) in

autumn (September – November) but decreased through the winter and spring. Ejaculate volume also was highest in autumn (Q1 (median) Q3; 0.5 (0.9) 1.6 mL) compared to other seasons ($P < 0.05$). Mean ejaculate osmolality was 316.6 ± 7.6 mOsm (Q1 (median) Q3; 301.0 (315.5) 340.0 mOsm; Table 2) with a pH of 7.6 (Q1 (median) Q3; 7.3 (7.9) 8.1; Table 2) and not affected by season.

There was no effect of season on percentages of morphologically normal sperm ($P > 0.05$; Table 2). The predominant sperm defect in raw ejaculates was a bent midpiece with a cytoplasmic droplet (approximately 15.0%), and the incidence was higher ($P < 0.05$) in summer compared to spring (Table 2).

3.2 Fecal androgen patterns

Overall mean concentration of fecal androgens was 1.04 ± 0.09 $\mu\text{g/g}$ (mean $\pm$ SD). Seasonal changes in mean fecal androgen concentrations for individual animals, as well as all combined, are shown in Figure 1. Mean androgen concentrations reached peak values in summer (1.21 ± 0.04 $\mu\text{g/g}$) and gradually declined through rest of the year (autumn, 1.16 ± 0.04 $\mu\text{g/g}$; winter, 1.03 ± 0.02 $\mu\text{g/g}$; and spring, 1.06 ± 0.02 $\mu\text{g/g}$).

3.3 Semen cryopreservation

Over half of ejaculates ($n = 14/25$; 56%) exhibited $<70\%$ total sperm motility, <250 million total spermatozoa or urine contamination and were not used for cryopreservation in this study. The influence of cryodiluents on sperm motility and acrosomal integrity is depicted in Table 3. Freeze-thawing resulted in a sharp ($P < 0.05$) decline in sperm motility (BF5F, 54.9%; Triladyl, 70.0%) compared with raw semen (76.7%). However, there were no

differences in sperm total motility, proportion of morphologically normal sperm or the percentage of spermatozoa with intact acrosomal membranes immediately after thawing. Swim-up processing improved overall sperm motility in both BF5F ($P < 0.05$) and Triladyl ($P < 0.01$) extenders compared with post-thawed semen suspensions (Table 3). Although there was no difference in the proportion of morphologically normal sperm between cryodiluents following swim-up, ejaculates cryopreserved in BF5F exhibited a higher ($P < 0.05$) proportion of spermatozoa with an intact acrosomal membrane compared with Triladyl. Swim-up processing of spermatozoa cryopreserved in Triladyl also yielded the lowest proportion of spermatozoa with a bent neck compared to immediately after thawing ($P < 0.05$; Table 3).

Sperm kinetics parameters (CASA analysis) are summarized in Table 4. After swim-up processing, recovered sperm in both groups were incubated (37°C) and evaluated for sperm traits (motility and forward progression) and kinetic parameters (VAP, VCL, VSL, LIN, STR, ALH, and BCF) by CASA. Aliquots also were analyzed for acrosomal integrity using Coomassie Brilliant Blue staining. At 0 h of incubation following swim-up processing, spermatozoa cryopreserved in BF5F exhibited a higher ($P < 0.05$) %motility, %forward progression, %intact acrosome, VSL, LIN, and STR when compared with Triladyl (Figure 2A-C and Table 4). Sperm motility and forward progression increased in spermatozoa cryopreserved in BF5F after 1 h compared with 0 h at 37°C and then declined with time (2, 3, and 4 h). At 4 h, sperm motility and forward progression declined ($P < 0.05$) rapidly in aliquots cryopreserved in BF5F compared with 1 and 2 h time intervals. Acrosomal membrane integrity in both diluents (BF5F and Triladyl) was reduced immediately after thawing. Moreover, cryopreservation in BF5F resulted in a significantly higher ($P < 0.05$) proportion of

spermatozoa with an intact acrosomal membrane compared with Triladyl at all-time points examined (Figure 2C).

4. Discussion

As the first systematic characterization of male reproductive biology of the tufted deer, we characterized ejaculate traits, fecal androgen metabolite patterns, and identified a cryodiluent that consistently yielded >35% post-thaw sperm motility after cryopreservation. Similar to a number of other deer species, season exerted a significant effect on physical morphometry, semen traits and fecal androgen metabolite concentrations. Amongst the two cryodiluents evaluated, spermatozoa cryopreserved in BF5F consistently yielded better sperm motility, forward progression, sperm kinetic parameters, and acrosomal integrity. Overall, this 1) represents novel information on the male reproductive biology of the tribe Muntiacini, which consists of two genera, *Elaphodus* and *Muntiacus* [33] and 2) denotes the first steps toward establishment of sperm banks and development of assisted reproductive technologies for improved genetic management of the tufted deer.

Seasonal changes in gross morphometry, ejaculate characteristics and androgen concentrations have been reported in the red deer [34], axis deer [35], fallow deer [36] and Eld's deer [16]. In the tufted deer, testicular length, scrotal circumference, and neck girth were greatest in the autumn however, there was no influence of season on total testicular volume or scrotal circumference. Likewise, the average ratio between testicular length and width was the highest in autumn. In the domestic bull, males with greater testicular length:width ratio and higher scrotal circumference have been reported to produce more sperm in their ejaculates [37]. Moreover, testicular length also is positively correlated with better semen traits and

breeding season in the ram [38], stallion [39] and human [40, 41]. A positive correlation between testicular size and neck girth was reported in the axis deer [35], fallow deer [36, 42], and the Eld's deer [16]. Previous reports in fallow and red deer also have suggested that neck girth is a reliable indicator of male reproductive performance [18, 36, 43, 44]. Thus, our results are in agreement with previous findings in other species, and may be useful in monitoring reproductive performance of male tufted deer.

Collection of spermic ejaculates from cervids is routinely achieved via electroejaculation [15, 25, 45], and in few cases using an artificial vagina [46]. Tufted deer males are highly fractious, so collection via the latter approach is likely impractical. In the present study, we demonstrated that electroejaculation is a safe and effective method for semen collection under anesthesia. Spermic ejaculates were obtained from all males evaluated, but the ejaculate quality was highly variable. Tufted deer males yield ejaculates characterized by small volume (1.2 ± 0.2 mL), high sperm concentration ($396.5 \pm 160.7 \times 10^6$ spermatozoa/mL), and a moderate percentage of motile spermatozoa ($57.0 \pm 3.8\%$). These results are comparable to previous reports on sambar deer [47], fallow deer [48] and Iberian red deer [45, 49]. The influence of exposure to females on overall ejaculate quality has previously been reported in the pampas deer [19]. Since males in the present study were housed with females (Bronx Zoo) or separate from female but with olfactory communication (SCBI), the effect of females on overall ejaculate quality cannot be ruled out. Furthermore, these differences in management also could have contributed to the observed variation in ejaculate quality. Mean semen pH and osmolality were 7.6 ± 0.1 and 316.6 ± 7.6 mOsm, respectively, and was influenced by male-male variation. The variation among males observed in this study may be attributed to differences in contributions from the accessory sex glands or

the extent of relaxation achieved with the anesthetic protocols described above and warrants further study. Season also exerted a strong influence on tufted deer seminal traits.

Electroejaculates contained higher proportions of motile and forward progressive spermatozoa in autumn compared with winter, spring or summer. A similar pattern also has previously been reported in several other mammals including the ram [50], stallions [51] and deer [23].

Although spermic ejaculates were collected during all seasons, there was a high incidence of sperm pleiomorphisms, particularly spermatozoa with a bent midpiece and a cytoplasmic droplet and spermatozoa with a proximal cytoplasmic droplet. Ejaculates also contained a higher (not significant) proportion of detached heads during winter and spring. Presence of cytoplasmic droplets in ejaculates is often considered an evidence of dysfunction in epididymal maturation or accessory sex glands resulting in abnormal spermatogenesis [52]. Seasonal variations in sperm pleiomorphisms also has been reported in other seasonally breeding mammals, especially during testicular regression in the non-breeding season (Eld's deer [21], fallow deer [48], and ram [50]). Lincoln [18] reported that presence of morphologically abnormal spermatozoa in ejaculates in rams could be an indirect consequence of seasonal fluctuations in gonadotrophin and testosterone secretions. In the present study, ejaculates contained a slightly higher proportion of abnormal spermatozoa in the non-breeding season coincident with lower fecal androgen concentrations but was not statistically significant. However, the percentage of abnormal spermatozoa did not differ among seasons. In seasonal breeding cervids, initiation of spermatogenesis occurs several months prior to peak breeding season [53, 54]. This pattern was most closely mirrored in Iberian red deer and Cantabrian chamois [55, 56]. Based on behavioral observations (Dolores Reed, personal

communication), hormone concentrations, and seminal traits, it is clear that autumn represents the peak breeding season for the tufted deer.

Abnormalities in sperm morphology also have been correlated with reduced fertility or infertility in various species, including humans [57], bulls [58], and boars [59]. Among the various structural compartments of a spermatozoon, the acrosome is considered vital in part due to its involvement in sperm-oocyte binding and fertilization [60]. In the present study, the proportion of spermatozoa with an intact acrosome reached a nadir during the winter compared to the other three seasons. Acrosomal abnormalities have been implicated in compromised fertility in various species [61-64], the impact of high proportions of abnormal acrosomes as well as other abnormalities on tufted deer fertility warrants further studies.

Semen cryopreservation is possibly the most useful tool available to preserve the male genome of rare and endangered species [23, 65-69]. When combined with other assisted reproductive technologies (ART; i.e. artificial insemination, AI; *in vitro* embryo production, IVP; semen sexing; intracytoplasmic sperm injection, ICSI), sperm cryopreservation permits effective genetic management of non-domestic species managed in captivity [70-72]. To our knowledge this is the first report of semen cryopreservation in the tufted deer or any other species in the tribe Muntiacini. In the present study, ejaculates were processed for cryopreservation without seminal plasma removal, an approach often used in cervids. A limitation of our study was that there was no information on the actual composition of Triladyl since it was a commercial product. Between BF5F and Triladyl, several components including egg yolk, fructose, and tris were similar although their proportions could have been different except for egg yolk (20% vol/vol). In contrast, BF5F contained an additional buffering agent, TES (N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid) with a pKa of 7.4 (closer to

physiological pH). During cryopreservation, spermatozoa experience sharp changes in pH and TES included in BF5F could have provided improved protection against pH fluctuations and thereby preserved a higher proportion of viable sperm after freeze-thawing. Furthermore, citric acid was only present in Triladyl. An earlier study on buffalo sperm cryopreservation reported that tris-based extenders improved freezability of buffalo spermatozoa including improved post-thaw motility, acrosomal integrity, and in vivo fertility compared with citric acid based extenders [73, 74]. Therefore, in the present study, our results could have been influenced by a number of factors either alone or acting synergistically and warrant further analyses of individual components on post-thaw sperm viability, motility and acrosomal integrity.

Although there were some differences in the composition of the cryodiluents used in the present study, glycerol was the sole permeating cryoprotectant. Glycerol is one of the most commonly used cryoprotectants for preserving sperm from domestic and non-domestic species [75-78]. Immediately after thawing, both post-thaw semen groups induced a decline in the proportion of motile spermatozoa (subjective motility assessment; 54.9% and 70.0% loss in BF5F and Triladyl, respectively, when compared with freshly ejaculated semen). Since most of the samples were above the threshold concentration permissible for CASA analysis, thawed aliquots were centrifuged and swim-up processed in TALP. Centrifugation and swim-up processing was not detrimental to tufted deer sperm motility. Following swim-up, BF5F consistently yielded a higher proportion of spermatozoa with good motility and forward progression. Swim-up processing failed to improve the overall proportion of morphologically normal spermatozoa, but the proportion of spermatozoa with a bent neck was significantly reduced in aliquots cryopreserved in Triladyl. Despite the decline in sperm motility,

spermatozoa from tufted deer appear to be relatively resistant to freeze-thawing induced damage. Swim-up sperm progressive motility and longevity are influenced by a number of factors including media composition (relative concentrations of energy substrates), incubation temperature, osmolality, and type and concentration of cryoprotectant [79]. The proportion of subjectively motile spermatozoa was higher after swim up processing in both BF5F and Triladyl. Similar to previous studies, swim-up processing reduced the number of sperm recovered in the supernant [31, 80]. It is plausible that other sperm separation methods, including density gradient centrifugation, may improve the proportion of motile sperm, the number of sperm recovered, as well as sperm morphology [81, 82]. CASA motility, forward progression, and kinetic parameters of swim-up spermatozoa were higher in BF5F than Triladyl at all time intervals evaluated. However, sperm motility, progressive motility, and velocity declined sharply over 4 h of *in vitro* incubation in TALP. These results may have resulted from depletion of energy substrates to support sperm motility because the only energy substrate in TALP was pyruvate. Pyruvate and lactate (also a component of TALP) also have been shown to induce capacitation and acrosome reaction in spermatozoa [83, 84]. These factors could have contributed to hyperactivation changes (high motility and forward progression) as well as motility parameters including VSL, LIN and STR) observed in aliquots immediately after swim-up processing. This was also accompanied by a concomitant sharp decline in the proportion of spermatozoa with an intact acrosomal membrane. Although TALP has been used for *in vitro* incubation of spermatozoa from various species, our results suggest that further studies are warranted to optimize *in vitro* culture conditions for tufted deer spermatozoa. These improvement also could facilitate the development of *in vitro* embryo production protocols for the tufted deer.

In conclusion, the present study is the first detailed analysis of male reproductive characteristics, androgen profiles, and semen cryopreservation in the tufted deer. Our findings reveal that it is possible to collect spermic ejaculates from captive tufted deer using electroejaculation, and cryodiluents used for cryopreserving spermatozoa in other cervids also protects tufted deer sperm from freeze-thawing induced damage. This new knowledge has immense practical value with respect to developing assisted reproductive technologies including establishment of a genome resource bank and artificial insemination for enhanced genetic management of this near threatened species [85, 86].

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Figure Legends:

Figure 1. Mean $\pm$ (SEM) fecal androgen concentrations in male tufted deer (*Elaphadus cephalophus*) on the basis of season (Autumn, September – November; Winter, December – February; Spring, March– May; and Summer, June – August). Values are mean $\pm$ standard error of mean (SEM) for all males. Bars with different superscripts are significantly different across seasons ($P < 0.05$). The dashed line represents the overall mean androgen concentration.

Figure 2. Effect of swim-up processing tufted deer (*Elaphadus cephalophus*) semen cryopreserved in two different extenders, BF5F (-■-) and Triladyl (···◆···). (A) Sperm motility by CASA; (B) progressive motility; (C) intact acrosome. Values are mean $\pm$ standard error of mean (SEM). Bars with capital letters indicate significant differences among time intervals ($P < 0.05$) and small letters indicate the significant differences between extender within a time interval ($P < 0.05$).

Table 1. Reproductive traits across seasons in male tufted deer (*Elaphadus cephalophus*, N = 5).

Parameters	Autumn ¹	Winter ¹	Spring ¹	Summer ¹
Testicular size				
Right				
Length (cm)	4.74 ± 0.2 ^a	4.33 ± 0.2 ^b	4.41 ± 0.3 ^b	4.43 ± 0.3 ^{ab}
Width (cm)	2.4 ± 0.3	2.3 ± 0.1	2.4 ± 0.4	2.5 ± 0.4
Volume (cm ³)	14.3 ± 3.4	12.2 ± 1.8	13.1 ± 3.9	14.6 ± 4.5
Left				
Length (cm)	4.9 ± 0.2 ^a	4.6 ± 0.3 ^{ab}	4.4 ± 0.4 ^b	4.4 ± 0.5 ^b
Width (cm)	2.3 ± 0.2	2.2 ± 0.2	2.4 ± 0.4	2.5 ± 0.5
Volume (cm ³)	13.6 ± 3.0	12.1 ± 2.3	13.8 ± 5.5	14.8 ± 6.3
Total testicular volume (cm ³)	27.9 ± 6.2	24.3 ± 4.0	26.9 ± 9.2	29.4 ± 10.8
Scrotal circumference (cm)	11.9 ± 1.2	11.5 ± 0.8	11.7 ± 1.4	11.3 ± 1.0
Neck girth (cm)	43.2 ± 2.9 ^a	39.0 ± 1.6 ^{bc}	41.6 ± 2.3 ^{ab}	35.7 ± 2.7 ^c

Values represent mean ± SD.

^{a, b, c} Significant differences among groups ($P < 0.05$).

¹ Autumn (September – November), Winter (December – February), Spring (March– May),

Summer (June – August)

Table 2. Comparison of semen parameters among seasons in male tufted deer (*Elaphadus cephalophus*, N = 5).

Parameters	Autumn ^{1, 2}	Winter ^{1, 2}	Spring ^{1, 3}	Summer ^{1, 2}
Semen qualities				
Motility (%)	76.0 ± 5.5 ^a	47.7 ± 21.8 ^b	50.6 ± 15.2 ^b	57.6 ± 15.0 ^{ab}
Progressive motility ⁴	3.5 ± 0.0 ^a	3.2 ± 0.3 ^{ab}	2.9 ± 0.4 ^b	3.0 ± 0.4 ^b
pH	7.6 ± 0.3	7.5 ± 0.7	7.8 ± 0.7	7.6 ± 0.9
Osmolarity (mOsm/kg)	310.8 ± 24.7	296.2 ± 34.2	333.4 ± 25.8	337.5 ± 16.3
Volume (ml)	2.0 ± 1.0	1.6 ± 1.7	0.7 ± 0.4	0.6 ± 0.5
Concentration (x 10 ⁶ spermatozoa/ml)	207.6 ± 216.4	350.9 ± 371.3	591.1 ± 1,223.0	331.3 ± 264.9
Total sperm (x 10 ⁶ spermatozoa)	529.6 ± 821.2	715.2 ± 809.6	677.0 ± 1,525.7	259.0 ± 226.9
Sperm morphology				
Normal sperm (%)	34.2 ± 10.2	19.8 ± 7.6	31.8 ± 9.6	16.2 ± 5.3
Abnormal sperm (%)	65.8 ± 10.2	80.2 ± 7.6	68.2 ± 9.6	83.8 ± 5.3
Microcephalic	2.2 ± 1.1	5.8 ± 3.0	2.3 ± 1.1	6.3 ± 2.7
Tightly coiled tail	6.4 ± 0.9	6.9 ± 1.4	8.0 ± 3.4	9.2 ± 2.8
Bent midpiece with droplet	16.2 ± 5.6 ^{ab}	17.7 ± 4.7 ^{ab}	7.7 ± 2.4 ^a	24.2 ± 7.8 ^b
Bent midpiece	4.0 ± 1.3	4.8 ± 1.8	3.7 ± 1.5	4.7 ± 2.7
Proximal droplet	11.2 ± 2.5	12.9 ± 4.5	7.8 ± 3.3	14.7 ± 5.1
Distal droplet	7.4 ± 4.5	6.1 ± 3.8	6.8 ± 4.0	3.6 ± 3.0
Detached head	1.4 ± 0.8	17.8 ± 10.2	17.5 ± 9.3	7.9 ± 2.8
Others	17.0 ± 4.7	8.2 ± 2.6	14.4 ± 5.3	13.2 ± 4.2
Acrosomal integrity				
Intact acrosome (%)	82.3 ± 4.7 ^a	58.6 ± 10.2 ^b	76.2 ± 2.9 ^{ab}	77.8 ± 2.9 ^{ab}
Damaged acrosome (%)	6.7 ± 1.3	22.6 ± 10.6	7.6 ± 1.1	7.6 ± 1.1
Non-intact acrosome (%)	11.0 ± 4.3	18.8 ± 4.3	14.6 ± 2.0	14.6 ± 2.0

Values represent mean $\pm$ SD.

^{a, b, c} Significant differences among groups ($P < 0.05$).

¹ Autumn (September – November), Winter (December – February), Spring (March– May),
Summer (June – August)

² N = 5 males; 1 ejaculate/male/season

³ N = 5 males; total number of ejaculates = 9

⁴ Based on a scale 0 to 5; 5 = best

Table 3. Comparisons of post- thawed semen traits of tufted deer (*Elaphadus cephalophus*) between two freezing extenders (BF5F vs. Triladyl) before and after swim-up processing.

Group	Processing	Subjective motility (%)	Normal morphology (%)	Abnormal morphology (%)	Intact acrosome (%)	Damaged acrosome (%)	Non-intact acrosome (%)
Pre-freeze		76.7 ± 2.0	49.5 ± 9.1	50.5 ± 9.1	74.3 ± 4.0	11.1 ± 3.3	14.6 ± 2.0
BF5F	Post – thawed	34.6 ± 6.6 ^{A*}	52.0 ± 6.4	48.0 ± 6.4	34.7 ± 2.9*	50.5 ± 2.2*	14.8 ± 2.2
	Swim – up	53.9 ± 7.4 ^{B*}	57.0 ± 7.3	43.0 ± 7.3	41.2 ± 2.9 ^{a*}	52.2 ± 2.5*	6.6 ± 1.3 ^{a*}
Triladyl	Post – thawed	23.1 ± 5.3 ^{A*}	52.2 ± 5.7	47.8 ± 5.7	29.1 ± 2.4*	50.2 ± 3.4*	20.8 ± 2.5*
	Swim – up	47.8 ± 6.5 ^{B*}	60.1 ± 6.5	39.9 ± 6.5	31.4 ± 1.6 ^{b*}	47.0 ± 7.7*	21.6 ± 7.4 ^b

Values represent mean ± SEM.

^{A, B} Significant difference between processing and same treatment ($P < 0.05$).

^{a, b} Significant differences between treatment and same processing ($P < 0.05$).

* Significant differences when compared with pre-freeze sample ($P < 0.05$).

Table 4. Mean ($\pm$ SEM) of sperm kinetic parameters (VAP, VCL, VSL, LIN, STR, ALH, and BCF) in swim-up processed tufted deer (*Elaphadus cephalophus*) semen cryopreserved in BF5F and Triladyl at 0, 1, 2, 3, and 4 h of *in vitro* incubation.

Parameters	Treatments	Storage time (h)				
		0	1	2	3	4
VAP ($\mu\text{m/s}$)	BF5F	77.1 $\pm$ 4.2 ^A	77.0 $\pm$ 5.1 ^A	72.5 $\pm$ 3.5 ^{AB}	69.6 $\pm$ 4.6 ^{AB}	51.7 $\pm$ 2.0 ^{B, a}
	Triladyl	67.5 $\pm$ 2.9 ^{AB}	78.7 $\pm$ 3.6 ^A	75.5 $\pm$ 1.9 ^A	70.9 $\pm$ 3.8 ^A	64.4 $\pm$ 5.8 ^{B, b}
VCL ($\mu\text{m/s}$)	BF5F	124.9 $\pm$ 4.6 ^A	121.9 $\pm$ 5.1 ^A	120.6 $\pm$ 3.5 ^A	118.4 $\pm$ 5.2 ^A	94.4 $\pm$ 1.5 ^{B, a}
	Triladyl	119.0 $\pm$ 4.3 ^A	132.4 $\pm$ 3.3 ^B	123.1 $\pm$ 3.7 ^{AB}	123.0 $\pm$ 3.8 ^{AB}	117.3 $\pm$ 5.1 ^{AB, b}
VSL ($\mu\text{m/s}$)	BF5F	68.0 $\pm$ 5.3 ^{A, b}	70.0 $\pm$ 5.7 ^A	64.5 $\pm$ 4.3 ^A	62.3 $\pm$ 4.8 ^A	44.8 $\pm$ 2.1 ^B
	Triladyl	53.2 $\pm$ 4.5 ^{A, a}	68.4 $\pm$ 5.2 ^B	66.3 $\pm$ 3.1 ^{AB}	62.0 $\pm$ 5.1 ^{AB}	53.9 $\pm$ 6.7 ^{AB}
LIN (%)	BF5F	54.3 $\pm$ 3.2 ^{AB, a}	57.7 $\pm$ 3.0 ^A	54.2 $\pm$ 3.1 ^{AB}	52.6 $\pm$ 2.3 ^{AB}	47.7 $\pm$ 1.7 ^B
	Triladyl	45.0 $\pm$ 2.9 ^{A, b}	52.2 $\pm$ 4.2 ^{AB}	55.0 $\pm$ 3.7 ^B	51.2 $\pm$ 4.0 ^{AB}	46.6 $\pm$ 3.7 ^{AB}
STR (%)	BF5F	85.1 $\pm$ 2.6 ^a	88.0 $\pm$ 1.9	86.4 $\pm$ 2.6	87.2 $\pm$ 1.6	84.2 $\pm$ 1.4
	Triladyl	75.1 $\pm$ 4.0 ^{A, b}	84.4 $\pm$ 3.5 ^B	86.3 $\pm$ 2.9 ^B	84.9 $\pm$ 3.4 ^B	81.3 $\pm$ 3.1 ^{AB}
ALH (μm)	BF5F	5.1 $\pm$ 0.2 ^A	4.8 $\pm$ 0.2 ^{AB}	4.9 $\pm$ 0.3 ^{AB}	4.7 $\pm$ 0.1 ^{AB}	4.2 $\pm$ 0.2 ^{B, a}
	Triladyl	5.8 $\pm$ 0.2 ^A	5.6 $\pm$ 0.5 ^{AB}	4.9 $\pm$ 0.4 ^B	5.0 $\pm$ 0.4 ^{AB}	5.2 $\pm$ 0.3 ^{AB, b}
BCF (Hz)	BF5F	29.7 $\pm$ 1.6	32.2 $\pm$ 1.2	32.8 $\pm$ 2.2	31.7 $\pm$ 0.7	32.8 $\pm$ 1.4
	Triladyl	37.4 $\pm$ 11.8	29.3 $\pm$ 1.4	29.2 $\pm$ 1.5	32.8 $\pm$ 1.6	30.4 $\pm$ 1.3

Values represent mean $\pm$ SEM.

^{A, B} Significant differences within a cryodiluent ($P < 0.05$).

^{a, b} Significant differences between cryodiluents ($P < 0.05$).

Figure 1

Figure 1.

Figure 2

Figure 2.