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Reproductive seasonality and sperm cryopreservation in the male tufted deer (*Elaphodus cephalophus*)

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ABSTRACT

The tufted deer is a small deer, listed as near threatened on the International Union for Conservation of Nature 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 to 5 times weekly for 2 years and analyzed for androgen metabolites using enzyme immunoassay. Ejaculates with greater than 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 computer-assisted sperm 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 with 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 after swim-up were higher in BF5F compared with Triladyl. Four hours after thawing, both percent sperm motility and progression decreased 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.

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1. Introduction

The tufted deer (*Elaphodus cephalophus*) is listed as "near threatened" on the International Union for Conservation of Nature Red List [1]. Morphologically, the tufted deer is a small deer, and all four subspecies (*E cephalophus cephalophus*, *E cephalophus michianus*, *E cephalophus ichangensis*, and *E cephalophus 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, 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 to 4500 meters above sea level in proximity to water sources [4]. Within Asia,

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extensive habitat destruction and over hunting have led to a rapid decrease 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 comprised 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 decreasing 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 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* population's gene diversity would decrease to approximately 57% in 100 years. Therefore, the tufted deer Species Survival Plan 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 involves 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 and 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 sheep [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 reproduciton.

Spermatozoa from several species of deer including the Eld's deer, fallow deer (*Dama dama*), spotted deer (*Axis axis*), and red deer have been successfully cryopreserved and live offspring produced through 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 and 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 and temperature of cryoprotectant addition. 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 and 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 years; body weight, 16.8–21.8 kg; 1–2 ejaculate per male per season; 24 ejaculates) were examined over the 2-year study period. Animals were housed individually in roofed and open-sided shelter (ca. 10 × 11 m) under natural environmental conditions (Front Royal, VA). Animals were maintained on 300 g/day/animal of 17% protein herbivore diet (Mazuri ADF-16; Land O'Lakes, Inc., Arden Hills, MN, USA), 10 high fiber biscuits (Marion Leaf Eater Biscuits, gorilla sized; Marion Zoological Foods, Plymouth, MN, USA), 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 4000 m^2 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, USA), butorphanol tartrate (0.15–0.2 mg/kg; Equanol; Vedco Inc., St. Joseph, MO, USA), and medetomidine hydrochloride (0.025–0.03 mg/kg; ZooPharm) with ketamine hydrochloride (1–2 mg/kg; Vedco Inc.). After recumbency, endotracheal intubation was performed to facilitate positive pressure ventilation, and the plane of anesthesia was maintained with intravenous boluses of