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Spermatozoa from the maned wolf (*Chrysocyon brachyurus*) display typical canid hyper-sensitivity to osmotic and freezing-induced injury, but respond favorably to dimethyl sulfoxide ☆,☆☆

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ABSTRACT

We assessed the influences of medium osmolality, cryoprotectant and cooling and warming rate on maned wolf (*Chrysocyon brachyurus*) spermatozoa. Ejaculates were exposed to Ham's F10 medium (isotonic control) or to this medium plus NaCl (350–1000 mOsm), sucrose (369 and 479 mOsm), 1 M glycerol (1086 mOsm) or dimethyl sulfoxide (Me_2SO, 1151 mOsm) for 10 min. Each sample then was diluted back into Ham's medium and assessed for sperm motility and plasma membrane integrity. Although glycerol and Me_2SO had no influence ($P > 0.05$), NaCl and sucrose solutions affected sperm motility ($P < 0.05$), but not membrane integrity. Motility of sperm exposed to <600 mOsm NaCl or sucrose was less ($P < 0.05$) than fresh ejaculate, but comparable ($P > 0.05$) to the control. As osmolality of the NaCl solution increased, motility decreased to <5%. In a separate study, ejaculates were diluted in Test Yolk Buffer containing 1 M glycerol or Me_2SO and cooled from 5 °C to −120 °C at −57.8 °C, −124.2 °C or −67.0 °C/min, frozen in LN_2, thawed in a water bath for 30 s at 37 °C or 10 s at 50 °C, and then assessed for motility, plasma- and acrosomal membrane integrity. Cryopreservation markedly ($P < 0.05$) reduced sperm motility by 70% compared to fresh samples. Higher ($P < 0.05$) post-thaw motility (20.0 ± 1.9% versus 13.5 ± 2.1%) and membrane integrity (51.2 ± 1.7% versus 41.5 ± 2.2%) were observed in samples cryopreserved in Me_2SO than in glycerol. Cooling rates influenced survival of sperm cryopreserved in glycerol with −57.8 °C/min being advantageous ($P < 0.05$). The findings demonstrate that although maned wolf spermatozoa are similar to domestic dog sperm in their sensitivity to osmotic-induced motility damage, the plasma membranes tolerate dehydration, and the cells respond favorably to Me_2SO as a cryoprotectant.

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Introduction

The maned wolf (*Chrysocyon brachyurus*) is a unique canid endemic to the Cerrado, Chaco and Pampas regions of South America, mostly in Brazil [46]. With an estimated 20,000 maned wolves remaining in the wild, this species is listed as 'Near Threatened' by the International Union for Conservation of Nature, but appears to be in marked decline due to habitat loss and degradation related to vastly expanded livestock and crop farming [28]. Because of its growing rarity, fascinating morphology and general interest by people in 'wolves', the maned wolf is managed in *ex situ* collections in North America, Latin America and Europe. These populations are important as a resource for public awareness programs in zoos and as a hedge in case of some catastrophic event (e.g., disease) occurring in nature [55]. Although the tallest of all canid species, the average maned wolf is only half the weight of a gray wolf (*Canis lupus*) (30 kg versus 80 kg, respectively) due to a slender build and thin coat [12]. Large, erect ears likely aid in the detection and capture of small prey in tall grass, even though plants comprise more than half of the diet [12]. Solitary, yet monogamous, breeding pairs defend a shared territory (20–120 km^2), but remain physically together for only a short period during the breeding season [12,46]. Maned wolves managed in zoological collections also appear to exhibit a very narrow window (1–10 d) of reproductive

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activity [55]. As occurs in Latin American and European zoos, the captive population of maned wolves in the USA has never been self-sustaining, largely due to poor reproduction and high neonatal mortality [54]. As a result, there are only 84 specimens in 31 zoos and breeding centers in the USA [47]. These institutions work together under the umbrella of a Maned Wolf Species Survival Plan (SSP), sharing and transporting animals between locations with a goal of retaining 90% of existing genetic diversity for the next 100 yr [55]. Sustaining heterozygosity is believed crucial for carnivores as even slight elevations in inbreeding are known to increase sperm pleiomorphisms [2,44], congenital abnormalities [48] and neonatal mortality [49]. The contemporary North American maned wolf population currently retains 92.1% of its original genetic diversity [55]. However, given low reproductive rates over the last 10 yr, computer simulations predict that this level of heterozygosity can be sustained for only the next 6 yr after which it will decrease precipitously for 8 decades to ~65%, likely facilitating population extinction [47].

One of the major challenges in meeting the genetic goals for the maned wolf are too few total animals distributed among too many holding sites. Thus, ultimate success depends on the frequent translocation of prime breeding (genetically appropriate) individuals over long distances to ensure ideal matings. While expense and compliance with recommendations are issues, it is not uncommon for transported animals to be sexually incompatible [43,64], thereby having wolves in the same enclosure that are aggressive or ambivalent to each other. The Maned Wolf SSP has advocated for using artificial insemination (AI) with thawed spermatozoa to help meet genetic management goals of the *ex situ* population. This tool has played a pivotal role in the 'conservation breeding' of certain species, including the black-footed ferret (*Mustela nigripes*) [26] and whooping crane (*Grus americana*) [5], where offspring produced by AI have been reintroduced into nature. Artificial insemination with thawed sperm has been applied commonly in the domestic dog since the mid-1970s [39] and is used commercially in the fur industry for the blue fox (*Alopex lagopus*) [14] and silver fox (*Vulpes vulpes*) [14]. However, the number of published articles on the details of sperm cryosensitivity among wild canids is few, with studies limited to the red wolf (*Canis rufus*) [23], blue fox [14] and silver fox [14]. These limited evaluations have suggested species-specificities, for example, with blue fox spermatozoa being more susceptible to cryo-damage than those of the silver fox or domestic dog [34]. If AI with thawed spermatozoa is ever to become useful for the genetic management of the maned wolf, then the first priority is understanding the sensitivity of these cells to differing cryoprotectants, osmolalities and cooling/warming rates.

The biophysical and biochemical changes accompanying the cryopreservation and thawing of 'generic' mammalian spermatozoa are well-known and related to tolerance to certain cryoprotective agents (CPAs) as well as cooling and warming effects [32]. Permeating CPAs (including glycerol, Me_2SO and ethylene glycol) are commonly used [16,35] and, although playing a safeguarding role, can induce cellular damage related to osmotic shock and chemical toxicity [4,16]. More recently, nonpermeating CPAs, including sucrose and raffinose, have been used as osmotic buffers to reduce required CPA concentrations [16]. As temperature decreases to subzero, an efflux of intracellular water causes the cell to shrink due to the increasing chemical potential created by extracellular ice formation [32]. When cooled too quickly, water remaining within the cell freezes and can cause lysis. When cooling is too slow, cells experience hyper-shrinkage and over-exposure to high solutes that compromise viability [32]. The reversal process via warming also influences cell survival. Rapid warming generally is preferred because it prevents intracellular ice recrystalization that is injurious [32,52]. It also is well established that optimal cooling rate varies among cell types and even for the same cell type among differing species [16,25,31]. For example, spermatozoa from the boar survive cryopreservation ideally when cooled at 30 °C/min [15] compared to that of the ram at 50–60 °C/min [13] and human at 1–10 °C/min [24]. Domestic dog spermatozoa exhibit optimal cryosurvival when cooled at rates of 10–30 °C/min [60,67].

Our laboratory is interested in understanding the reproductive biology of rare, wild canid species, including the maned wolf. To eventually allow AI with frozen-thawed spermatozoa to contribute to genetic management, the prerequisite is fully comprehending the cryo-sensitivity of spermatozoa. This mandates examining: (1) osmotic impacts related to permeating (glycerol and Me_2SO) versus non-permeating (NaCl and sucrose) agents; and (2) the influence of CPA use relative to different cooling and warming rates on post-thaw sperm survival. Within the Canidae family (comprised of 36 species [28]), osmotic sensitivity has only been studied in the domestic dog where sperm motility is rapidly compromised by exposure to hypertonic solutions of $\geqslant$500 mOsm of NaCl or monosaccharides (glucose, fructose and galactose) [53]. By contrast, these dog spermatozoa tolerate hypertonic solutions of the permeating CPAs glycerol and ethylene glycol [53]. Thus, for the present project, we posed and tested two hypotheses: (1) maned wolf spermatozoa are highly susceptible to excessive dehydration induced by the high osmolality of non-permeating solutes, but not to shrinking and swelling caused by the presence of permeating CPAs; and (2) the cryosurvival of spermatozoa from this species is CPA, cooling and warming-dependent.

Materials and methods

Animals

The project was conducted over 3 consecutive years during the breeding season (Oct–Dec) studying 14 adult, male maned wolves (3–13 yr old) managed in eight zoological institutions or breeding centers in the USA (Table 1). These captive-born animals were maintained according to guidelines of the Maned Wolf SSP and the Association of Zoos and Aquariums. In brief, wolves were housed individually or paired with a female in outdoor enclosures ranging from 300 m^2 to 1300 m^2 and with indoor (denning) access. Each animal was provided dry, commercial dog food (ProPlan Lamb & Rice) with supplemental fruits, vegetables and protein sources (i.e., mouse, rat, fish and chicken) and water ad libitum. All animal procedures were approved by the Institutional Animal Care and Use Committees of the Smithsonian Conservation Biology Institute, the White Oak Conservation Center, Houston Zoological Park and George Mason University.

Semen collection, evaluation and initial processing

Chemicals were purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise indicated. Before semen collection, each wolf was induced into a surgical plane of anesthesia using Telazol (3 mg/kg body weight) alone or a combination of Telazol (1 mg/kg) plus ketamine hydrochloride (1 mg/kg body weight) plus butorphanol (0.4 mg/kg). To eliminate urine contamination of semen, the urethra and bladder were catheterized using an 8 Fr catheter (Tyco Healthcare Group LP, Mansfield MA) followed by flushing with sterile saline.

Electroejaculation was conducted using a 1.9 cm in diameter rectal probe (with three longitudinal electrodes) and an electrostimulator (P.T. Electronics, Boring, OR). Each anesthetized donor was treated with 60–90 low level stimuli (2–5 V) over a 30 min period. Stimuli were delivered in sets of 20–30 per series, with two to three series per electroejaculation episode and a 5–7 min rest