THE DEVELOPMENT OF ASSISTED REPRODUCTIVE TECHNIQUES FOR MANAGING MANED WOLVES (*CHRYSOCYON BRACHYURUS*) *EX SITU*

by

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DEDICATION

This thesis is dedicated to my remarkable parents, Moya and Rick Johnson, who have never failed to tell me or show me how proud they are. For my mother, your feistiness and boundless energy towards making a difference, both locally and provincially, has taught me that even little voices can make big changes. You accept every challenge with a smile and you never turn down an opportunity to learn new things. I will undoubtedly take the examples you have set for me to make changes for conservation. For my father, your passion for animals and the outdoors has inspired me to enjoy the simple things in nature, and to make sure it is there for future generations. Furthermore, your extreme patience and unparalleled work ethic has shown me that hard work always pays off, as long as you do what you love. I will never go into work without a smile because of you.

Oh, and there's no crying in baseball…

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Finally, to Eric. Thank you for being my home away from home.

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LIST OF ABBREVIATIONS

Artificial Insemination……...AI
Assisted Reproductive Techniques…….……...ART
Coefficient of Variation……CV
Cryoprotective Agent……...……..CPA
Cyclodextrin Loaded with Cholesterol……...……...CLC
Days……...…..d
Dilution Buffer…….…DB
Dimethyl Formamide……...……DMF
Dimethyl Sulfoxide……...…….DMSO
Enzyme Immuno Assay……..EIA
Equine Chorionic Gonadotropin……...……..eCG
Ethylene Glycol……...……EG
Fecal Progesterone Metabolites…….…FPM
Follicle Stimulating Hormone……..……..FSH
Forward Progressive Status…….FPS
Glycerol……...G
Gonadotropin-releasing Hormone……...……GnRH
Human Menopausal Gonadotropin……...……HMG
Hypothalamic Pituitary Axis……...……...……HPA
Immunochromatographic……ICG
Institutional Animal Care and Use Committee…….……IACUC
Liquid Nitrogen……..LN_2
Luteinizing Hormone…… ……LH
Methyl Formamide……..……MF
Milliosmoles……mOsm
Minutes……min
Optical Density…….OD
Recombinant Luteinizing Hormone……..……reLH
Seconds…….s
Smithsonian Conservation Biology Institute……SCBI
Sodium Chloride……NaCl
Standard Deviation……...……SD
Standard Error of the Mean……..……S.E.M.
Test Yolk Buffer……TYB
Years of Age……..yr

ABSTRACT

THE DEVELOPMENT OF ASSISTED REPRODUCTIVE TECHNIQUES FOR MANAGING MANED WOLVES *(CHRYSOCYON BRACHYURUS) EX SITU*

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George Mason University, 2012

Thesis Director: Dr. Elizabeth Freeman

The maned wolf, a neotropical canid, is currently threatened due to habitat loss, road mortality and human-wolf conflict. The global captive population serves as an 'ark' for retaining genetic diversity in the event of severe stochastic changes to the wild population. However, the captive population is declining in gene diversity due to a small and aging population and poor reproductive output. The development of assisted reproductive techniques, such as sperm cryopreservation and artificial insemination (AI), has the potential to enhance genetic management but a lack of basic knowledge pertaining to the reproductive biology of the maned wolf is delaying this process. Specific objectives of the study were to 1) investigate susceptibility of maned wolf spermatozoa to osmotic stress by examining sperm viability after exposure to hypertonic solutions and dilution back into isotonic medium; 2) determine the effects of cryoprotectants, cooling and warming rates on cryosurvival of maned wolf sperm through

evaluating post-thaw survival of samples cooled and warmed at various rates in the presence of different cryoprotectants; and 3) investigate the influence of management conditions on female's response to hormonal induction of ovarian activity and ovulation by utilizing fecal gonadal steroid monitoring. The results obtained from this study suggest that maned wolf spermatozoa are highly susceptible to dehydration but are tolerant of exposure to cryoprotectants (1 M glycerol and 1 M DMSO). Additionally, optimal cryosurvival of maned wolf spermatozoa can be achieved by using 1 M DMSO as a cryoprotectant along with a rapid warming rate (50 °C for 10 s). Furthermore, a field-friendly dry shipper approach achieves similar post-thaw motility to the other cooling methods used in this study (7 cm above LN_2 or 3 cm above LN_2). Finally, a 2.1 mg deslorelin implant consistently induces ovarian activity and ovulation in paired, but not singleton, female maned wolves. However, exogenous recombinant luteinizing hormone (reLH; 0.0375 mg) induces ovulation in singleton females after the induction of ovarian activity with deslorelin. The findings obtained from this research greatly improve our understanding of maned wolf physiology, specifically sperm cryosensitivity and female reproductive biology, while also providing insights into improved comparative approaches for sperm cryopreservation and induction of ovarian activity for timed AI.

CHAPTER 1: LITERATURE REVIEW

The Maned Wolf

The maned wolf (*Chrysocyon brachyurus*), a monotypic canid, is endemic to the Cerrado, Chaco and Pampas regions of South America (1). Named for the presence of long black hairs along their neck and shoulders, their long legs and unusual lateral gait are likely anatomical adaptations to conserve energy and facilitate travel through the tall vegetation that thrives throughout their home range (2). Although maned wolves are the tallest of all canid species, they are only half the weight of a gray wolf (*Canis lupus*) (30 kg *vs*. 80 kg, respectively) due to their slender build and thin coat (3). Large, erect ears likely aid in the detection and capture of small prey in tall grass; however, more than half of their diet consists of plant matter (4). Solitary, yet monogamous, breeding pairs defend a shared territory (20-120 km^2) throughout the year but only stay together for a short period during the breeding season (2).

Maned wolf habitat is comprised of vast grasslands, swamplands, savannahs and scrub forests (1). Today, the most stable populations inhabit central Brazil, Paraguay, northern and eastern Bolivia and southeastern Peru (1). More notably, there have been major declines in the southern ranges of this species including northeastern, central and eastern Argentina and Uruguay (1). Maned wolves are currently listed as 'Near Threatened' by the IUCN Red List of Threatened species (5) with approximately 20, 000

maned wolves remaining in Brazil, the predominant region for this species. The most significant threat to wild populations is habitat loss, especially due to agricultural conversion to grazing and croplands (5). Other main threats include road mortality (6) and human-wolf conflict in addition to issues associated with domestic dogs including disease transfer as well as interspecific competition and aggression (7). Currently, there are a number of field conservation initiatives in maned wolf range countries geared towards sustaining viable populations of this threatened species. Recent projects include population assessments (8–12), health evaluations (13–15), genetic studies (16,17), nutritional research (4), adrenal responses of wild wolves to environmental factors (18) and educational outreach efforts (19).

While field studies (20) are crucial to understanding the ecology and behavior of free-ranging maned wolves, research conducted *ex situ* also provides base knowledge required to maintain viable self-sustaining populations, both in nature and captivity (19). Such research also helps to enhance our understanding of basic wolf biology that cannot be obtained in the field. According to the 2010 International Register and Studbook for the Maned Wolf (21), there are currently 436 maned wolves residing in 153 institutions worldwide. In North America, there are 92 individuals kept in 30 zoos with 92.1% genetic diversity (22). Because low heterozygosity has been shown to impact reproduction (e.g., low birth weight and poor sperm production) and health (e.g., neonatal mortality and birth defect) (23), the Maned Wolf Species Survival Plan (MWSSP) has set a goal of retaining 90% genetic diversity for the next 100 years. However, under the current situation this goal is not achievable as it is expected that the North American

population can retain genetic diversity more than 90% for the next 5 years, but only 35.6% for the next 100 years (22). As a result, the MWSSP has placed priority on *ex situ* research examining the causes of poor reproduction and developing assisted reproduction techniques.

Assisted Reproduction for *Ex situ* Species Management

Reproductive sciences have contributed significantly to the study, management and recovery of rare species populations (24). Rapidly developing reproductive technologies have made it possible to characterize the uniqueness of species, populations and genotypes (25–27). This scholarly information is crucial to formulating protocols for enhanced animal welfare and natural breeding to ensure that valuable genotypes can be produced in perpetuity.

In zoo situations, almost all breeding recommendations are made to mate individuals that have highly desirable genotypes (to maintain genetic diversity within a small population). However, the designated mates may live in geographically disparate locations and even when moved at great expense and placed together may be sexually incompatible (27,28). To overcome these hurdles and maintain genetic diversity, artificial insemination (AI) has been used to successfully manage several threatened and endangered species maintained *ex situ*, including the whooping crane (*Grus Americana*) (29), black footed ferret (*Mustela nigripes*) (30), and giant panda (*Ailuropoda melanoleuca*) (31). Artificial insemination can eliminate the risk of transporting individuals to different locations for breeding and failed reproduction due to sexual incompatibility. And, when used in combination with semen cryopreservation, AI can

enable the re-infusion of valuable genes even after death of the sperm donor. Lastly, AI offers an additional animal welfare advantage for species such as the maned wolf that establish strong pair bonds. This characteristic normally introduces complexity into genetic management because individuals in an established pair may eventually need to be bred to another individual to maintain heterozygosity. Rather than psychologically 'stressing' the pair by forcing separation, AI effectively allows the pair to retain the bond while infusing new genes into the population 'artificially'. However, none of these significant advantages can ever be realized for the maned wolf without understanding the basic reproductive biology of this species.

Reproductive Biology of the Maned Wolf

Maned wolf reproductive biology is similar to that of other wild canids. For example, the maned wolf is monoestrus with a well-known and distinctive breeding season that is controlled by photoperiod (3). Proestrus, which can be characterized by vaginal swelling and secretions, can last for 2 wk (32), while estrus lasts anywhere from 1 to 10 d (33). Also, gestation is approximately 65 d; however, litters are unusually small with a mean of 2.6 pups (range: 1 to 7) (34). There are also strong similarities between the reproductive hormones of domestic dogs and maned wolves (32,35). During proestrus, estrogen concentrations increase for approximately 2 wk and decline at copulation. In late proestrus, progestagen begins to increase and remains elevated throughout estrus and the pregnant or pseudopregnant luteal phase of approximately 65 d (35).

Perhaps the most intriguing attribute unique to maned wolves is that ovulation occurs only in the presence of a male (35), although the regulator(s) of this phenomenon is not yet known. The maned wolf also has relatively low sperm output (61.2 ± 14.8 million sperm/ejaculate) compared to other canid species (i.e., coyote (*Canis latrans*) (36), red wolf (*Canis rufus*) (37) and gray wolf (38) usually produce >100 million sperm/ejaculate). Additionally, they have an unusually high percentage of structurally malformed sperm, even at the peak of breeding season (39). Also, while some canids produce sperm throughout an entire breeding season, maned wolves produce sperm only 6-8 wk annually, and spermatogenesis ceases soon after mating (19). This narrow window of spermatogenesis combined with low sperm output and evidence of induced ovulation make developing assisted reproductive techniques in this species particularly challenging. Thus far, AI has not been attempted in maned wolves, and there have been no reports on the feasibility of manipulating the female's reproductive cycle or semen cryopreservation methods.

Endocrinology of Reproductive Function

Reproductive function involves a number of hormones that are essential for regulating the processes that control ovarian activity in females. Endocrine secretion from the hypothalamic pituitary axis (HPA) is primarily responsible for ovarian activity; however, there are several feedback mechanisms that also modulate hormone production in the central nervous system (Figure 1.1) (40). Gonadotropin releasing hormone (GnRH) stimulates the release of two gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), from the anterior lobe of the pituitary which are vital to

reproductive function (41). Under the influence of internal (endocrine and paracrine) or external stimuli (e.g., photoperiod), the hypothalamus secrets GnRH that, in turns stimulates the release of FSH and LH from the pituitary. Follicle stimulating hormone promotes follicle growth and estrogen production whereas LH triggers ovulation, aids in the formation of a corpus luteum and maintains luteal function (41). Estrogen produced from the growing follicles upregulate the production of GnRH, resulting in increased gonadotropin secretion and preovulatory follicle(s) growth. Growing follicles also produce inhibin which, at high levels, can suppress FSH production. Large amounts of estrogen from prevolulatory follicles stimulate the release of an LH surge that results in ovulation and formation of the corpus luteum. The corpus luteum releases progesterone that exerts a negative feedback effect on gonadotropin production (42). The mechanisms of endocrine and paracrine relationships that regulate ovarian activities have been widely investigated in domestic species (42–48) but are still novel in lesser-studied, non-domesticated species (49).

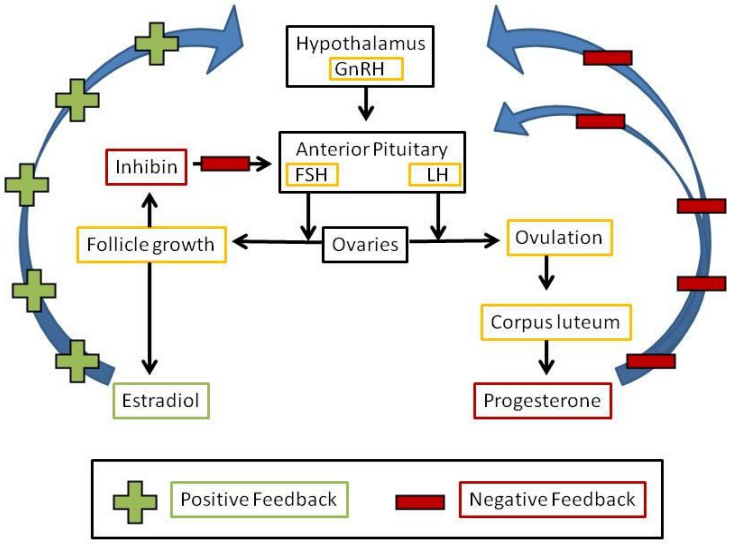


Figure 1.1 Interactions among female reproductive hormones

Reproductive cyclicity is easily identified in domestic species by evaluating either circulating hormones via enzyme- or radio-immunoassay or ovarian structure (i.e., the presence of preovulatory follicles or corpora lutea (50)) via rectal palpations (51,52), or ultrasound examinations (50). However, in non-domestic species these techniques are not always feasible. Therefore, investigating reproductive function-endocrine relationships through non-invasive hormone monitoring is a practical alternative (53).

The first reports of fecal steroid analysis for reproductive assessment were for pregnancy detection in women (54). Today, fecal steroid analysis is commonly used for diagnostics, as well as for studying reproductive endocrinology of domestic (55–58) and non-domestic species (35,53,59–62). The first report of fecal steroid analysis of maned wolves was in 1995 (63). To-date, non-invasive hormone monitoring has been applied to characterize reproductive endocrinology (32,35) and examine adrenal responses to

environmental enrichment *ex situ* (64) as well as the impact of anthropogenic factors on free ranging individuals in this species (18). However, this technology has not been used to explore the feasibility of ovarian activity manipulation for artificial insemination.

Manipulation of Ovarian Activity

Stimulation of ovarian activity and ovulation has significantly improved AI success in rare species such as the cheetah *(Acinonyx jubatus)* (65), Eld's deer *(Panolia eldii)* (66), and black-footed ferret *(Mustela nigripes)* (30). Knowledge gained through research on domestic counterparts of endangered species has assisted in developing these techniques with minimal use of genetically valuable animals for experimental procedures (65,67–69). Therefore, to facilitate successful stimulation of ovarian activity in maned wolves, it is advantageous to consider the most current techniques in domestic dog assisted reproduction.

There are various methods in use for manipulating ovarian activity in the domestic bitch, all varying in feasibility and success. Some protocols recommend the use of exogenous estrogen to induce proestrus (70–72) or utilize additional gonadotropins to further induce a fertile estrus (73–75). Similarly, exogenous gonadotropic hormones such as FSH, human chorionic gonadotropin (hCG) (76) and human menopausal gonadotropin (HMG) (77) have been used to stimulate the onset of proestrus followed by estrus and either spontaneous (73) or induced ovulation by additional gonadotropins such as LH (78). Equine chorionic gonadotropin (eCG), one of the most widely studied gonadotropins for estrus induction in dogs (79), shows promise but can cause premature luteal failure with subsequent use due to its long half-life interfering with normal

feedback mechanisms (80–82). Equine chorionic gonadotropin has shown more consistency than exogenous estrogens but like the former, requires daily injections for up to 14 d, which makes both techniques impractical for nondomestic species.

Other research has focused on the use of dopamine agonists (ergot derivatives) (83) that influence hypothalamic or pituitary responses by inducing natural estrus (84–86). Specifically, dopamine agonists stimulate the secretion of dopamine, which then inhibits the secretion of prolactin, a peptide hormone produced from the anterior pituitary. High levels of circulating prolactin are observed during anestrus and are believed to suppress gonadotropin secretion and/or ovarian responsiveness to gonadotropins (87,88). Therefore, suppressing prolactin using dopamine agonists activates gonadotropin secretion and stimulates cyclicity by reducing the length of anestrus. Not only is this treatment costly and extensive (may require >30 d of treatment before proestus occurs), but some centrally acting dopamine agonists induce recurrent vomiting in domestic bitches (79) which makes this treatment inappropriate for species with digestive issues, such as the maned wolf (89).

Gonadotropin releasing hormone -agonists are used in domestic bitches to elicit endogenous gonadotropins (LH and FSH) from the pituitary to provoke ovarian activity for proestrus and eventually estrus with spontaneous ovulation (72). Pulsatile administration of GnRH for estrus induction requires the use of infusion pumps for 7-14 d (90) and is therefore not appropriate for non-domesticated species. However, deslorelin (2.1 mg Ovuplant®, Peptech Animal Health, NSW, Australia), a short-acting GnRH agonist manufactured to induce ovulation in mares, has shown great potential for a

practical and consistent method of estrus induction in both dogs (91) and wild canids (92). Kutzler (79) reported 100% proestrus within 6-10 d of implant placement with 100 % ovulation. Volkmann et al (91) confirmed these results achieving 70% pregnancy after AI in females implanted with 2.1mg deslorelin. Asa et al. (92) applied this method to gray wolves and successfully induced fertile estrus, resulting in 100% pregnancy rate from natural breeding and 33% pregnancy after AI. Deslorelin has also been used to stimulate follicle growth prior to ovariectomy for oocyte retrieval and vitrification in endangered Mexican wolves (*Canis lupus baileyi*) (20), although there is no record of it being used to induce estrus for the purpose of AI in this subspecies.

Induction of Ovulation

With the island fox (*Urocyon littoralis*) being the only other canid species demonstrating induced ovulation (93), there are no records of successful estrus and ovulation induction in canids suspected of induced ovulation. Although domestic dogs are spontaneous ovulators, gonadotropins, including hCG and LH, have been supplemented as ovulatory agents in some estrus induction protocols with varying success (73,75,78,86,94). For example, Volkmann et al (94) found that administration of hCG to induce ovulation only suppressed progesterone secretion following a short-lived increase. Cirit et al (86) also reported no positive effects of hCG on ovulation rates. However, Wright (95) reported that ovulation was induced by hCG within 26-30 h of administration.

In felids, which are historically known to be induced ovulators, a combination of eCG and hCG are usually used to stimulate ovarian activity and induce ovulation (65).

However, evidence suggests that these protocols can cause hyperstimulation of follicles (96,97) and estrogen production (62) which can ultimately be detrimental to fertilization, embryogenesis and implantation (65). Continued use of eCG with hCG can also result in refractoriness (98,99) and can trigger an immune response (100). Crichton et al. (101) used porcine FSH and porcine LH to test ovarian responsiveness to gonadotropins in tigers (*Panthera tigris*) and found no evidence of ovarian hyperstimulation. Pituitary gonadotropin extracts such as FSH and LH can contain pathogens and the concentrations can fluctuate with each lot, making dosage inconsistent (102). Yoon et al. (103) used a recombinant form of equine LH (reLH; Equi-pur, AspenBio Pharma Inc., Castle Rock, CO) for ovulation induction in mares and obtained a 90% ovulation rate within 48 h of administration with a dosage of 0.75 mg. This product is free of contaminants and other pituitary hormones that could alter ovarian response (102). To our knowledge, this form of reLH has never been explored for ovulation induction in canids but may have enormous potential for improving current AI success.

Spermatozoal Response to Changes in Temperature and Osmotic Stress

Advancing methods for semen cryopreservation is vital for understanding the potential benefits of AI for the genetic management of endangered species. Although cryopreservation methods have been widely studied in 'traditional' species (49) and, more specifically, in the dog (104–109), red fox *(Vulpes vulpes)* (110,111), gray wolf and red wolf (37,38), optimal methods for cryopreserving maned wolf spermatozoa have yet to be established. Therefore, identifying factors impacting cryosurvival is crucial for establishing a practical yet standardized cryopreservation method in maned wolves.

During cryopreservation, there are two factors that are the main cause of cryoinjury in all cell types (112). The first is intracellular ice formation (112) and is a result of cooling too rapidly. If a cell is cooled too quickly, it is unable to transfer the appropriate amount of water across its membrane into the extracellular space before reaching the ice-nucleation temperature (-5 °C to -40 °C), resulting in intracellular ice formation and membrane damage (113). To prevent intracellular freezing, the cell must reach equilibrium with the extracellular osmolality before being frozen (114). The second factor is known as the solution effect (112) and it is a result of cooling too slowly. If the cell is cooled too slowly, the spermatozoon is exposed to harmful solute concentrations, which may cause excessive dehydration (113). Thus, it is crucial to establish an intermediate optimal cooling rate to achieve maximum cell survival.

Differences in water permeability between cell types account for a magnitude of difference in optimal cooling rates (114). Mammalian embryos have a low permeability and optimal cooling rates range from 0.3 °C to 0.6 °C/min (115). On the contrary, mammalian spermatozoa have extremely high water permeability, and thus, cooling rates range from 1 °C to 170 °C/min (114). More specifically, domestic dog spermatozoa show optimal cryosurvival at cooling rates between 10 °C and 30 °C/min (105) depending on the freezing techniques utilized. Some protocols utilize computerized freezing systems (108) (-20 °C/min) for cryopreservation while others freeze semen in liquid nitrogen vapor (109,116–118) (11 °C/min) or as pellets on dry ice (37,38). However, for application in wild canids, an ideal sperm cryopreservation protocol should include techniques that are practical for field use. Stoops et al (119) have been successful

at cryopreserving ocelot (*Leopardus pardalis)* sperm in a field setting using dry shippers. This technique has also played a valuable role in developing sperm cryopreservation methods for endangered fish (120).

While optimal cooling rates are necessary to prevent cryoinjury, they are not independently sufficient to maintain cell survival during cryopreservation. Most cells require additional protection against changes in cell volume associated with freezing and thawing during cryopreservation and this can be achieved by the addition of cryoprotective agents (CPA's). Permeating CPA's, such as glycerol or dimethyl sulphoxide (DMSO), lower the temperature at which ice forms and the concentration of intracellular electrolytes as well as decrease the extent of cell dehydration. Glycerol is the most commonly used permeating cryoprotectant for freezing canine semen (118) and recommended concentrations have varied between 4 and 11% (121). Studies testing alternatives to glycerol such as ethylene glycol (EG) or DMSO have also been carried out but have inconsistent results. No significant differences were found in fertility of frozen-thawed dog semen cryopreserved with 5% glycerol or 5% EG (117), or when comparing glycerol with DMSO (105). On the contrary, other studies have had minimal success with DMSO (122,123) and EG (124) in comparison to glycerol as a cryoprotectant. This variation in results could be due to differences in diluents composition among studies as some cryopreservation media include non-permeating CPA's to mediate the detrimental effects, such as toxicity, that are associated with permeating cryoprotectants (114). Non-permeating CPA's such as proteins, sugars and high molecular compounds, can permit the use of lower concentrations of permeating CPA's (114) by dehydrating and therefore

shrinking the cell before freezing. Non-permeating CPA's are also able to increase membrane fluidity by interacting with cell membrane phospholipids, and thus, decreasing the likelihood of membrane fusion associated with excessive dehydration during cooling (125). However, non-permeating CPA's in high concentrations can create a hyperosmotic environment for cells which can negatively affect cell viability as membrane integrity is severely compromised during recovery from dehydration (126). Gao et al (126) reported a sharp drop in sperm survival when cells lost at least 65% of their intracellular water during the dehydration process following exposure to hypertonic NaCl or sucrose solutions. Although severe shrinkage causes serious membrane damage, this study also suggested that the effects can be minimized by reducing the time that the cells are exposed to a hypertonic solution.

Spermatozoa from the bull (*Bos primigenius*) (127), ram (*Ovis aries*) (128), boar (*Sus scrofa domesticus*) (129), mouse (*Mus musculus)* (130), cat (*Felis domesticus*) (131) and stallion (*Equus ferus caballus*) (132) are sensitive to osmotic stress. Furthermore, spermatozoa from the teratospermic cat (>60% of sperm produced are pleiomorphic cells) are more sensitive to osmotic perturbations than normospermic counterparts (131). Although these previous studies are valuable for identifying similarities in responses to osmotic response, it is important to note that response of spermatozoa from different species varies with changes in the osmolality of the surrounding media (133). Domestic dog spermatozoa are much more sensitive to osmotic stress than human and avian spermatozoa. When exposed to solutions ≥ 700 mOsm, dog spermatozoa have only retained 30 – 40% membrane integrity (134). In contrast, human sperm only lost > 50%

membrane integrity when the osmolality of hypertonic solutions exceeded 2000 mOsm (126). Some eagle species have exhibited tolerance to extremely hypertonic solutions (3000 mOsm) and have maintained up to 98% viability post-exposure (135). However, dogs show similar osmotic sensitivity to that of feline spermatozoa (131) whereby changes in osmolality affect motility significantly more than membrane integrity. While 30 – 40% of dog spermatozoa remained intact, all spermatozoa had lost their motility. Therefore, it is important to assess cell membrane damage in addition to motility while assessing osmotic tolerance. To date, no studies have been conducted on maned wolves, or wild canids to determine spermatozoa sensitivity to osmotic alterations typical of the cryopreservation process.

Hypotheses and Objectives

The overall aim of my thesis was to develop techniques for successful AI in maned wolves by understanding the intricacies of sperm cryosensitivity and ovarian responsiveness to ovulation induction. My central questions were to (1) systematically examine how cryobiological factors, including osmotic stress, types of cryoprotectant, cooling and warming rates, influenced survival maned wolf spermatozoa and (2) examine how approaches for manipulating the reproductive cycle in other canids were applicable to maned wolves to facilitate AI; with the understanding that exogenous LH may be required to induce ovulation in singleton wolves. Objective 1 tested the hypothesis that maned wolf spermatozoa were highly susceptible to osmotic stress by examining sperm viability after exposure to hypertonic solutions and dilution back into isotonic medium (Chapter 2). Objective 2 tested the hypothesis that cooling and warming rates impacted

cryosurvival of maned wolf sperm through evaluating post-thawed survival of samples cooled and warmed at various rates (Chapter 3). Objective 3 utilized fecal gonadal steroid monitoring to test the hypothesis that management conditions influenced female response to hormonal induction of ovarian activity (Chapter 4).

The findings obtained from this research could provide important insight into the role of male presence on the female's ability to ovulate. This information is essential to the establishment of an effective protocol that will consistently control ovulation and allow sperm to be deposited at an optimal time. By concurrently developing maned wolf sperm cryopreservation methods while advancing our capability for effectively controlling the female reproductive cycle, we can begin to apply AI to genetically manage the captive maned wolf population. For example, a female can be inseminated with sperm from (1) a male living in a zoo within or outside the United States, (2) a wild individual or (3) an under-represented deceased animal. Artificial insemination can greatly enhance genetic diversity of maned wolves in North America, especially when genes of under-represented or non-represented founders living in other regions (especially, Brazil) can be disseminated into the population.

CHAPTER 2: MANED WOLF (*CHRYSOCYON BRACHYURUS*) SPERMATOZOA ARE HIGHLY SENSTIVE TO EXPOSURE TO HYPERTONIC SOLUTIONS OF NON-PERMEATING COMPOUNDS, BUT NOT TO PERMEATING CRYOPROTECTANTS

Abstract

Fundamental knowledge of sperm cryosensitivity is required to establish sperm cryopreservation techniques for the genetic management of a threatened canid, the maned wolf (*Chrysocyon brachyurus*). The objective of this study was to determine the osmotic tolerance of maned wolf spermatozoa. Semen was electroejaculated from 12 male maned wolves (n = 13 ejaculates) during the North American breeding season (October - February) and analyzed for motility, forward progressive status, morphology, viability, osmolality, pH, volume, and total sperm. Suitable samples (n = 7; motility > 40%, total sperm > 0.3 x 10^6) were washed, re-suspended in Ham's F10 medium supplemented with bovine serum albumin (BSA) and exposed to hypertonic solutions of NaCl (350, 400, 600, 800 and 1000 mOsm) and cryoprotectants (CPA's; 0.1 M sucrose, 0.2 M sucrose, 1 M glycerol and 1 M dimethyl sulfoxide (DMSO) in addition to a control (300mOsm) for 10 min. After dilution back into isotonic medium (300 mOsm, 37 °C), samples were assessed for motility and viability. Compared to the raw ejaculates, motility was affected ($P < 0.05$) by NaCl solutions $\geq$ 350 mOsm and by both solutions of sucrose. However, motility of sperm exposed to glycerol or DMSO was comparable ($P > 0.05$) to that of the raw ejaculate. Exposure of maned wolf spermatozoa to solutions of NaCl $\geq$ 600 mOsm

caused a significant reduction in motility compared to the control ($P < 0.05$) but did not affect viability. Motility and viability of maned wolf spermatozoa were not affected by exposure to hypertonic solutions of cryoprotectants ($P > 0.05$) compared to the control. In conclusion, maned wolf spermatozoa are sensitive to dehydration but are tolerant of exposure to cryoprotectants.

Introduction

Captive breeding programs are crucial for the study, management and recovery of rare species populations (24). Assisted reproductive techniques (ART), such as artificial insemination (AI) and sperm cryopreservation, have been used to successfully manage several threatened and endangered species maintained *ex situ*, including the whooping crane (*Grus Americana*) (29), black footed ferret (*Mustela nigripes*) (30) and giant panda (*Ailuropoda melanoleuca*) (31). Artificial insemination improves reproductive management of rare species by eliminating the risk of transporting individuals to different locations for breeding and failed reproduction due to sexual incompatibility. Semen cryopreservation is vital for gamete rescue and long-term storage of valuable genetics. When used simultaneously, AI with cryopreserved sperm can enable the re-infusion of valuable genes even after death of the sperm donor. Although AI has played a pivotal role in species recovery, its success with frozen semen in wild canids has been scarce. This is likely due to a lack of basic knowledge regarding species-specific reproductive physiology, including cryobiology, as well as the difficulties associated with managing species that have such complex social structures. Fortunately, the fur industry has increased demands for AI and sperm cryopreservation in the fox, thus, providing new

information about cryobiology in canids. For example, Farstad (110) found significant species differences in the osmotic tolerance, cryosurvival and fertility of spermatozoa from the blue fox *(Alopex lagopus)* in comparison to the silver fox *(Vulpus vulpus)*, which is more similar to the domestic dog (*Canis lupus familiaris*) with respect to post-thaw fertility. These species-specific differences emphasize the need to develop an understanding of the cryobiological properties of spermatozoa associated with each species.

Cryopreservation of spermatozoa requires cells to tolerate osmotically induced volume excursions during cooling and warming as well as cryoprotectant addition and removal (113). As temperatures drop to subzero, cells shrink due to an efflux of intracellular water due to the increasing chemical potential created by extracellular ice formation (114). If cells are cooled too quickly, intracellular water will not flow out of the cells in time to prevent intracellular ice formation. However, if cells are cooled too slowly, they are subjected to severe shrinkage, dehydration and exposure to high solute concentrations (114). Similar challenges are faced again upon thawing but can depend on whether cooling rate has induced intracellular ice formation or dehydration. For example, if a cell is cooled rapidly, a more rapid warming rate may prevent damage from the recrystallization of intracellular ice (114).

Permeating cryoprotecting agents (CPA) are commonly added and removed during the freezing and thawing process to help prevent cell damage; however, they also cause volume excursions (114). As permeating cryoprotectant is added to cryopreservation medium, cells initially shrink as water permeates out of the cell and

subsequently swell as the CPA flows in (136). Post-thaw removal of CPA's also cause volume excursions and can induce further damage to the cell than the initial addition (126). Therefore, cell survival depends almost entirely on the ability to tolerate these volumetric changes during freezing and thawing.

Although there are a variety of cryoprotectants currently in use to protect sperm from the negative effects of cryopreservation, most protocols favor glycerol (137). As a penetrating cryoprotectant, glycerol alters the arrangement of membrane lipids and proteins allowing increased membrane fluidity and enhanced cryosurvival (138). Other CPA's, such as dimethyl sulfoxide (DMSO) or ethylene glycol (EG), are also commonly used due to their ability to penetrate spermatozoa more rapidly than glycerol (139). However, there are species differences in tolerance to cryoprotectant toxicity (140) and thus, optimal cryoprotectants should be investigated on a species-specific level. Nonpermeating cryoprotectants, such as sucrose or raffinose, can act as osmotic buffers and are often included in cryopreservation media to to reduce CPA concentrations (114). Although, the hypertonicity of these sugars should be considered when cryopreserving spermatozoa from species that are sensitive to osmotic stress (114).

Spermatozoa from different species do not respond the same to changes in the osmolality of the surrounding media (133,141). Therefore, it is important to understand the differences and similarities in osmotic sensitivity not only within a population (131,142) but also within a taxonomic group. Within the canid family, osmotic sensitivity has only been studied in the domestic dog; sperm from this species are highly sensitive to osmotic stress (134,143). Specifically, exposure of dog spermatozoa to $\geq$ 500

mOsm of hypertonic NaCl or monosaccharides results in a significant reduction of sperm motility. However, dog spermatozoa are able to tolerate hypertonic solutions of cryoprotectants, such as glycerol and EG. To date, no studies have been conducted on any other canid species to determine spermatozoa sensitivity to osmotic alterations typical of the cryopreservation process.

The goal of the present study was to gain a better understanding of the basic biology of maned wolf spermatozoa so that we can further develop assisted breeding in this species. The objective was to examine the osmotic sensitivity of maned wolf spermatozoa to hypertonic solutions of non-permeating compounds (NaCl and sucrose) and permeating cryoprotectants (glycerol and DMSO). We hypothesized that maned wolf spermatozoa are highly susceptible to excessive dehydration induced by the high osmolality of NaCl, but not to shrinking and swelling caused by the presence of permeating cryoprotectants, as observed in domestic dogs (134,143).

Materials and Methods

Animals

Twelve adult male maned wolves housed in various zoological institutions across the United States were included in the study (Table 2.1).

Table 2.1 Demographic information about male maned wolves (n = 12) electroejaculated in 2009 and 2010.
Collections are listed in chronological order; one male was collected more than once.

Wolf ID	Facility and Location	Collection Date(s)	Age at Collection (yr)
2611	Smithsonian Conservation Biology Institute, VA	11.25.09	7.0
2374	Smithsonian Conservation Biology Institute, VA	11.25.09	9.5
2815	Smithsonian Conservation Biology Institute, VA	11.30.09	5.0
2161	White Oak Conservation Center, FL	12.08.09	11.0
1890	Dickerson Park Zoo, MO	12.15.09	13.0
3023	Dickerson Park Zoo, MO	12.16.09	3.0
2810	Dickerson Park Zoo, MO	12.16.09	5.0
2592	Louisville Zoo, KY	10.06.10	8.0
2844	Smithsonian Conservation Biology Institute, VA	10.12.10	5.0
2815	Smithsonian Conservation Biology Institute, VA	11.09.10	6.0
2814	Connecticut's Beardsley Zoo, CT	11.15.10	6.0
2461	Houston Zoological Park, TX	11.30.10	8.0
2954	Sedgwick County Zoo, KS	12.06.10	4.0

Animals were housed according to the Maned Wolf Species Survival Plan (MWSSP) and guidelines of the Association of Zoos and Aquariums. All animal procedures were approved by the Smithsonian Conservation Biology Institute's (SCBI) Institutional Animal Care and Use Committee (IACUC), the White Oak Conservation Center's (WOCC) IACUC and Houston Zoological Park's (HZP) IACUC.

Semen Collection, Evaluation and Processing

Semen collections were performed during two consecutive breeding seasons (2009-2010; Table 2.1). A surgical plane of anesthesia was induced at the discretion of the staff veterinarian at each institution using combinations of dissociatives, benzodiazepines, opiates and alpha-2 agonists, supplemented with isoflurane as needed.

The urinary bladder was emptied by catheterization of the urethra using an 8 Fr catheter (Kendall Sovereign Feeding Tube and Urethral Catheter 8 FR, 22"; Tyco Healthcare Group LP, Mansfield MA), and then flushed with sterile saline solution to minimize urine contamination in the semen samples. Electroejaculation was performed using a rectal probe of 1.9 cm in diameter with 3 longitudinal electrodes and an electrostimulator (P.T. Electronics, Boring, Oregon. 2-5 volts). The lubricated probe was placed in the rectum and low voltage (2-5 V) applied to the prostate region. A total of 60 - 90 electrical stimulations were delivered over a 30 min period consisting of two to three stimulation series with rest periods of 5-7 min in between series. Ejaculates were collected into pre-warmed, sterile collection cups and placed on a slide warmer set at 35-37 °C until assessment. Following the collection, animals were allowed to recover and monitored closely by keeper staff and veterinarians according to protocols enforced at each institution.

Raw ejaculates (n = 13) from each male (n = 12) were pooled and immediately assessed for volume, pH (EMD colorpHast pH Strips; EMD Chemicals, Darmstadt, Germany) and osmolality (Vapro 5520, Wescor, Inc. Logan, UT). A semen sample (4 µl) was placed on a pre-warmed slide (37 °C) with a cover glass and assessed for percent total motility and forward progressive status (FPS; scale, 0 – 4 with a 4 rating equivalent to rapid, straightforward progression (144) using phase contrast microscopy. A 20 µl aliquot of semen was fixed in 100 µl 0.3% glutaraldehyde in phosphate buffered saline (PBS) and stored at 4 °C for sperm morphological analysis. Another 10 µl of raw ejaculate was added to 10 µl eosin-nigrosin (E-N) stain, incubated for 30 s, smeared onto

three slides and air dried, all at room temperature. E-N stained slides were permanently preserved by placing a cover glass over a drop of mounting medium (Permount, Fisher Scientific, Pittsburg, PA) to set for subsequent assessment of sperm plasma membrane integrity (145). Finally, 5 µl was fixed with 95 µl of water and assessed for sperm cell concentration using a standard haemocytometer.

The remainder of each sample was immediately diluted with an equal volume of Ham's F-10 culture medium (Irvine Scientific, Santa Ana, CA) supplemented with 4 mg/mL BSA (A7906, Sigma, St. Louis, MO). Diluted samples were centrifuged (Eppendorf Mini-Spin, Hamburg, Germany) at 300 *g* for 10 min and the supernatant removed. Samples exhibiting < 40% motility or < 0.3 x 10^6 total spermatozoa per ejaculate were considered unsuitable for experimental use and were discarded.

Evaluation of Sperm Morphology

Sperm morphology was assessed by a single observer using phase-contrast microscopy (magnification: x 1,000). A total of 100 spermatozoa were counted and classified as either normal or as having primary or secondary abnormalities, with final values expressed as % normal. Primary abnormalities, which occur during spermatogenesis process (146), include macrocephalic, microcephalic, bicephalic, detached head, spermatid, abnormal acrosome or midpiece, no midpiece, tightly coiled tail or biflagellate. Secondary abnormalities which occur during epididymal storage or preparation of the sample (146) consist of a bent midpiece with no droplet, a bent midpiece with a retained droplet, a bent flagellum with no droplet, a bent flagellum with a retained droplet, retained proximal droplet, retained distal droplet and bent neck.

Evaluation of Sperm Plasma Membrane Integrity

Sperm plasma membrane integrity was assessed by a single observer using phase-contrast microscopy (magnification: x 1,000). A total of 200 sperm were counted per slide, categorized as either viable or nonviable and averaged; three slides per ejaculate were assessed and the average percentage of viable sperm calculated. Nonviable spermatozoa had increased permeability to the E-N stain and appeared pink or purple whereas viable gametes excluded the stain and remained white.

Experimental Design

All hypertonic treatments were prepared by supplementing Ham's F10 with: (1) permeating cryoprotectants (1 M glycerol (G5516; Sigma, St. Louis, MO) or 1 M DMSO (D8418; Sigma, St. Louis, MO)); (2) non-permeating cryoprotectants (0.1 M sucrose, 0.2 M sucrose (S0389; Sigma, St. Louis, MO)); or (3) hypertonic sodium chloride solutions (NaCl; 350, 400, 600, 800, and 1,000 mOsm with accuracy within ± 5.0 mOsm (S5886; Sigma, St. Louis, MO). Osmolalities were determined using a vapor pressure osmometer (Wescor, Inc. Logan, UT). To assess the osmotic sensitivity of maned wolf spermatozoa, sperm pellets were resuspended in Ham's F10 containing BSA (osmolality, 300.0 ± 5.0 mOsm) to a total volume of 200 µl and then exposed to one of the nine hypertonic treatments by aliquoting 20 µl of diluted spermsample in 80 µl of the respective solutions (Table 2.2). A control was exposed only to 80 µl Ham's F10 and BSA (300.0 ± 5.0 mOsm). After a 10-min equilibration at room temperature, a 20 µl sperm sample from each treatment was transferred back into isotonic medium (40 µl Ham's F10 containing BSA adjusted to 300.0 ± 5.0 mOsm). All samples and the control were then immediately assessed for osmolality, sperm motility and plasma membrane integrity.

Table 2.2 Osmolalities of prepared treatments before and after addition of sperm sample (1:5 sperm suspension to tested medium) for a 10 min incubation period.
Values are mean ± S.E.M. with the exception of the "before" value for the control and all "before" NaCl groups which were prepared to accuracy within ± 5.0 mOsm.

Treatment	Prepared osmolality (mOsm)	Osmolality after sperm added (mOsm)
Control	300.0 ± 5.0	301.7 ± 6.8
1 M Glycerol	1263.7 ± 8.4	1086.0 ± 12.5
1 M DMSO	1400.3 ± 9.6	1151.3 ± 8.6
0.1 M Sucrose	364.7 ± 5.2	369.0 ± 5.6
0.2 M Sucrose	483.0 ± 6.1	470.3 ± 4.7
NaCl 350 mOsm	350.0 ± 5.0	350.3 ± 5.9
NaCl 400 mOsm	400.0 ± 5.0	395.0 ± 4.8
NaCl 600 mOsm	600.0 ± 5.0	545.4 ± 3.8
NaCl 800 mOsm	800.0 ± 5.0	716.1 ± 5.6
NaCl 1000 mOsm	1000.0 ± 5.0	883.4 ± 7.8

Statistical analysis

All statistical analyses were conducted using SYSTAT 12 (Version 12.00.08, SYSTAT Software, Inc.). Percentage data for motility and viability was subjected to arcsine transformation to normalize the data. A Shapiro-Wilk test was then used to test for normality. Mean comparisons were performed using analysis of variance (ANOVA). A non-parametric Kruskel-Wallis test was employed to assess the effects of treatments on sperm motility. When significant differences among treatments were discovered, post-hoc, pairwise analyses were conducted by the Tukey Range Test. All values were reported as mean ± standard error of the mean (S.E.M.) and statistical significance was set at a $P < 0.05$.

Results

There was high variation among ejaculates both within and among males (Table 2.3.). Occurrence of oligospermia was high, as 85% of electroejaculates (11/13 electroejaculations) had concentrations < 50 $x10^6$ spermatozoa/mL (147) and one individual (13 yr) was classified as azoospermic (1/13 electroejaculations). Animals > 8 yr had lower total sperm output (1.0 ± 0.6 $x10^6$) than those of prime breeding age (3 - 8 yr; 69.4 ± 27.0 $x10^6$). Older maned wolves also exhibited lower total motility (35.0 ± 0.0%) than breeding age males (59.1 ± 7.2%). Presence of abnormal sperm was high, with 80% of electroejaculates containing >70% abnormal cells. The most abundant abnormalities were coiled tails (17.7 ± 4.4%; Figure 2.1.b), bent midpieces with (17.9 ± 3.2%; Figure 2.1.c) or without (6.5 ± 2.2%; Figure 2.1.d) droplets, bent tails with (8.3 ± 3.8%; Figure 2.1.e) or without (2.9 ± 0.7%) droplets and proximal droplets (11.9 ± 2.6%).

Table 2.3 Seminal parameters of fresh ejaculates (n = 13) from 12 maned wolves.

Seminal parameters	Mean ± S.E.M.	Range (min – max)
Semen pH	7.4 ± 0.2	6.0 - 9.0
Osmolality (mOsm)	335.7 ± 12.1	280.0 - 400.0
Ejaculate volume (mL)	2.3 ± 0.9	0.1 - 12.5
Sperm concentration (x 10^6 spermatozoa mL^{-1})	47.0 ± 29.2	0.3 - 339.0
Total spermatozoa per ejaculate (x 10^6)	3.8 ± 26.3	0.1 - 244.0
Motility (%)	60.0 ± 5.9	30.0 - 90.0
Forward progressive status (1-4)	2.6 ± 0.2	1 - 4
Viability (%)	71.1 ± 4.9	39.5 - 89.5
Morphologically normal sperm (%)	28.6 ± 6.5	14.0 - 53.0

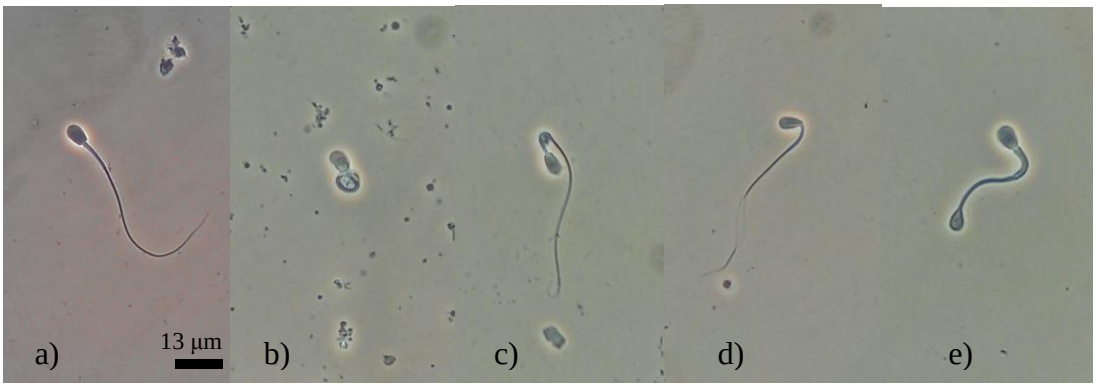


Figure 2.1 Micrographs of maned wolf spermatozoa:
a) normal sperm, b) coiled tail, c) bent midpiece with a cytoplasmic droplet, d) bent midpiece without a cytoplasmic droplet and e) bent tail with a cytoplasmic droplet

Of 13 electroejaculates, only seven were suitable for experimental use. Three of the discarded ejaculates had < 40% motility and three ejaculates had low total sperm outputs (< 0.3 x 10^6 spermatozoa per ejaculate). Of the six discarded ejaculates, three were from males > 8 yr, one was from a cryptorchid male and the other two were from males at end of their prime breeding age (7 and 8 yr). Treatments containing glycerol and DMSO had no effect on viability or motility of maned wolf spermatozoa (Figures 2.2a and b) compared to the control and raw ejaculate ($P > 0.05$). Although there was no significant reduction in sperm motility and viability compared to the control after exposure to 0.1 M and 0.2 M sucrose solution (350 mOsm and 460 mOsm, respectively), there was a reduction in motility compared to the raw ejaculate ($P < 0.05$). A significant decrease in sperm motility was observed when sperm were exposed to $\geq$ 600 mOsm of NaCl solution compared to the control and $\geq$ 350 mOsm compared to the raw ejaculate (Figure 2.2a; $P < 0.05$), although viability was not affected (Figure 2.2b).

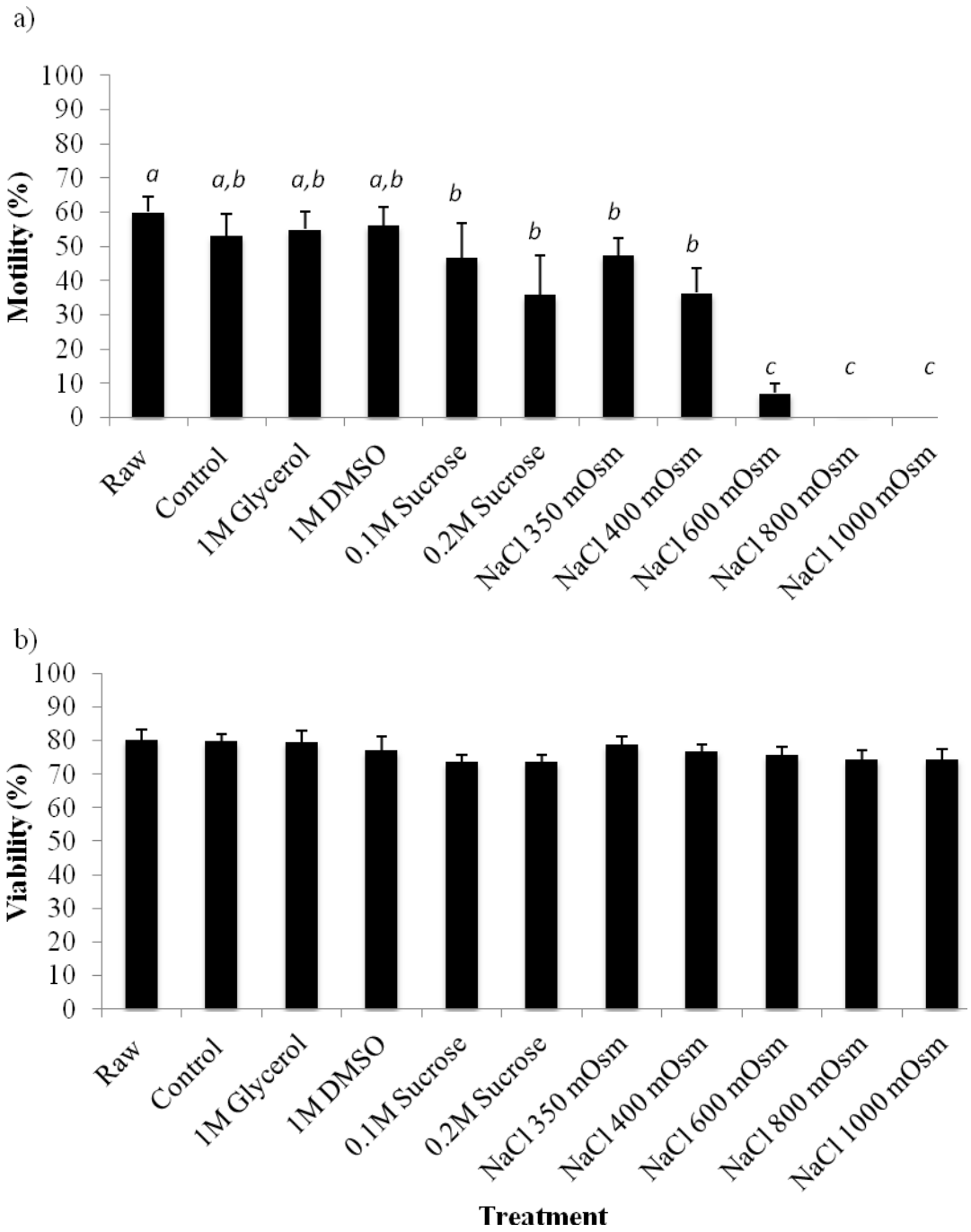


Figure 2.2 Differences in mean ± S.E.M of motility (a) and viability (b) of freshly ejaculated (raw) maned wolf spermatozoa (n = 7) after exposure to permeating-, non-permeating cryoprotectant or varying osmolalities of NaCl solution followed by dilution back into isotonic medium.
Superscripts indicate a significant difference among treatments (P < 0.05).

Discussion

For successful semen cryopreservation, it is essential to understand the species-specific intricacies of sperm responses to different steps of the cryopreservation process. The present study is the first to investigate osmotic sensitivity of spermatozoa from a wild canid. We learned that maned wolf spermatozoa are highly susceptible to osmotic stress but are tolerant of exposure to cryoprotectants (glycerol, DMSO and sucrose). We also learned that sperm quality is significantly compromised in wolves aged > 8 yr.

To date, seminal characteristics have been reported in several other canid species including the generic and Mexican gray wolf (148), red wolf (149), red and arctic *(Vulpes lagopus)* fox (150), bush dog (*Speothos venaticus*) (150), African wild dog *(Lycaon pictus)*(151) and coyote (152). The total number of spermatozoa collected by electroejaculation in the present study ($58.9 \pm 22.3 \times 10^6$) was consistent to those previously reported in the maned wolf (range: $22.1 – 44.4 \pm 10.1 \times 10^6$ spermatozoa/ejaculate (39,67,150). However, maned wolf sperm output is consistently low compared to previous findings in other canid species. For example, total sperm outputs were 756.2×10^6 spermatozoa in the Mexican gray wolf (148), 349.4×10^6 spermatozoa in the red wolf (147), $1{,}597.4 \times 10^6$ spermatozoa in the generic gray wolf (148) and 720.0×10^6 spermatozoa in the domestic dog (148).

With the exception of the bush dog (150), maned wolf seminal samples exhibit on average, lower motility (60% vs. 70 - 90%) and higher proportion of structurally abnormal spermatozoa (60% vs. 0 – 30%) than those of other wild canids (37,150,153). Poor semen quality has been associated with several factors such as disease, infections,

environmental toxins, malnutrition and genetic defects (154). More specifically, previous studies in humans (*Homo sapiens*) (155,156) and rats (*Rattus norvegicus*) (157) have associated poor semen quality with nutrient deficiencies. Suboptimal nutritional management is an ongoing concern within the captive maned wolf population (158) and may contributing to the poor seminal quality observed in the present study. However, wild maned wolves have also exhibited reduced motility (50.0 ± 20.0%) and high proportion of structurally abnormal spermatozoa (40.6 ± 10.4% normal) (39). Therefore, it is unlikely that a captive diet is the underlying cause of poor sperm quality in zoo-managed individuals.

One challenge in obtaining semen from wild canids is urine contamination into semen samples (159). Contamination of urine results in altered osmolality and pH which, in turn, leads to reduced motility and an increased incidence of abnormal tail morphology (159). Therefore, the high proportion of sperm abnormalities in maned wolf seminal samples (74.9 ± 4.4% abnormal), including bent (11.2 ± 3.8%) and coiled tails (17.7 ± 4.4%) is probably due to the presence of urine in the ejaculates, despite our attempt in flushing the bladder prior to each electroejaculation procedure. Thus, other methods that minimize urine contamination need to be implemented, such as more consistent anesthesia protocols (160) amongst institutions and collecting raw ejaculates directly into isotonic buffered solutions (132).

Low genetic diversity within the entire maned wolf populace ($\pi = 0.0013$), resulting from a genetic bottleneck during the last glacial period, is also a likely explanation for reduced sperm quality in both wild and captive populations (17).

Population bottlenecks occurring within small populations can lead to inbreeding depression and often result in reduced sperm quality (16,23,161,162). Inference of low genetic diversity on sperm quality has been shown in the black-footed ferret (163), rabbit (*Oryctolagus cuniculus*) (164) and several felid species (165). This element increases the importance of further developing assisted reproductive techniques, such as semen cryopreservation, for enhancing genetic management of populations lacking genetic diversity (67). With the development of these techniques, gene flow can be greatly enhanced between animals housed at different institutions, between wild and captive populations and even from deceased individuals.

Osmotic stress is one of the major limiting factors for successful semen cryopreservation as it leads to (1) mechanical damage in the sperm plasma membrane, (2) oxidative damage to membrane phospholipids and chromatin and (3) altered sperm metabolism (166). It has been shown that spermatozoa of many mammalian species, including the bull (*Bos primigenius*) (127), ram (*Ovis aries*) (128), boar (*Sus scrofa domesticus*) (28), cat (*Felis domesticus*) (131), stallion (*Equus ferus caballus*) (132) and dog (134,143) are sensitive to osmotic stress whereas some avian species are not as susceptible to osmotic damage (135). Domestic dog spermatozoa exposed to solutions ≥ 500 mOsm only retain 30 – 40% membrane integrity (134). In contrast, the Bonelli's eagle (*Hieraaetus fasciatus*) maintained up to 97 % viability post-exposure to 3000 mOsm NaCl solution (135). In the present study we showed that, similar to the domestic dog, maned wolf spermatozoa are highly susceptible to exposure to hypertonic solutions of NaCl as exhibited by a significant reduction in motility when exposed to solutions ≥

600 mOsm compared to the control. However, unlike the domestic dog where exposure to hypertonic NaCl solutions significantly decreases membrane integrity, the viability of maned wolf sperm remains intact. This finding is similar to that reported in the mouse (*Mus musculus)* (136), black-footed ferret (167), ram (128), bull (168) and cat (131). This difference in osmotic sensitivity between species could be a result of species-specific lipid composition of the spermatozoal membrane (166). Miller et al. (169) reported that there are structural differences in membrane fatty acids between fox species with different cryosurvival potential. Silver fox spermatozoa, which are consistently cryopreserved with much success, have a significantly higher ratio of unsaturated/saturated membrane fatty acids within the membranes as well as higher levels of membrane desmosterol and cholesterol in comparison to blue fox spermatozoa, which exhibits lower tolerance to osmotic stress and poor cryosurvival. Other species sensitive to osmotic stress such as the boar (170), ram (171) and bull (171) also exhibit low cholesterol content within the sperm plasma membrane. Increasing the cholesterol content of sperm membranes using cyclodextrins loaded with cholesterol (CLC) has been shown to increase the osmotic tolerance limits of boar (172), stallion (173), ram (174), bull (175) and rabbit spermatozoa (176). To our knowledge, the use of CLCs has not been explored in canids. Thus, future studies should explore the influence of CLC treatment on osmotic sensitivity of wild canids, including the maned wolf.

The low percentage of morphologically normal sperm found in maned wolf semen may also be a contributing factor to the high osmotic sensitivity exhibited by this species. Pukazhenthi et al. (141) compared osmotic sensitivity of spermatozoa from

teratospermic (< 40% morphologically normal sperm) versus normospermic individuals in both domestic and wild (serval *[Leptailurus serval]* and clouded leopard *[Neofelis nebulosa])* counterparts and showed that gametes recovered from teratospermic individuals were more sensitive to osmotic perturbations than normospermic. Mechanisms may be altering volume regulation in spermatozoa exhibiting flagellar angulation, causing increased sensitivity to osmotic stress in teratospermic males (165). Therefore, the high incidence of flagellar angulation exhibited in the present study could have contributed to the susceptibility of maned wolf spermatozoa to hypertonic solutions.

In the present study, exposure of maned wolf sperm to 0.1 M (364.7 ± 5.2 mOsm) or 0.2 M sucrose (483.0 ± 6.1 mOsm) followed by dilution back to an isotonic medium did not alter motility and viability compared to the control. The lack of an adverse effect on sperm survival after exposure to 0.2 M sucrose is surprising. Domestic dog sperm exposed to 500 mOsm solution of monosaccharides (i.e., glucose, fructose and galactose) followed by dilution back into isotonic medium exhibit significant reduction in sperm motility compared to the control (134). The difference in the findings between the two studies may be due to (1) specific variation in osmotic sensitivity (140,177) or (2) the differences in the impact of disaccharide (sucrose) and monosaccharide (glucose, galactose and fructose) on the plasma membrane (178).

Cryoprotectant agents (CPA) play an important role in minimizing the deleterious effects of cryopreservation on spermatozoa (114). However, the hypertonicity of CPAs can cause cell damage due to extensive volume changes during addition and removal (179) or due to inherent toxic properties of a given compound (180). While serving as an

effective cryoprotectant for avian (181), rabbit (182) and domestic goat sperm (*Capra aegagrus hircus*) (183), DMSO has been found to be ineffective for cryopreserving spermatozoa of other species such as warthogs (*Phacochoerus africanus*) (184), domestic boars (185) and Markhoz goats (*Capra falconeri*) (138). In the domestic dog, exposure of sperm to 0.5 M DMSO resulted in a significant reduction of motility (134). Earlier studies also demonstrated minimal success with DMSO as a cryoprotectant for domestic dog semen (122,123). Our finding that maned wolf sperm tolerate exposure to DMSO confirms that there are species variations among canids in response to cryoprotectant exposure. Due to its lower molecular weight, DMSO permeates plasma membrane more rapidly than glycerol (49), which potentially reduces the likelihood of osmotic damage. Therefore, the potential of this CPA in cryopreserving maned wolf spermatozoa requires further investigation.

In conclusion, maned wolf spermatozoa are extremely vulnerable to cell dehydration and therefore, successful cryopreservation may require (1) the use of permeating cryoprotectants and (2) a relatively high cooling rate to minimize water loss during cooling. Although successful cryopreservation methods have been long established for the domestic dog, this study reiterates the importance of identifying species-specific differences in canid spermatozoa so that optimal cryoprotectants can be identified. The findings from the present study lay an important foundation toward the development of improved semen cryopreservation protocols to further enhance the genetic management of this threatened species.

CHAPTER 3: INTERACTIONS OF CRYOPROTECTANT, COOLING RATE AND WARMING RATE ON THE CRYOSURVIVAL OF MANED WOLF *(CHRYSOCYON BRACHYURUS)* SPERMATOZOA

Abstract

Factors affecting sperm cryopreservation were assessed in the maned wolf (*Chrysocyon brachyurus*), a threatened neotropical canid. The objective of this study was to determine the interactions among cryoprotectant (CPA), cooling (CM) and warming rate (WR) on post-thaw survival of sperm. Spermatozoa was electroejaculated from seven male maned wolves (n = 12 ejaculates) during the North American breeding season (October - February) and analyzed for motility, forward progressive status, morphology, viability, acrosomal integrity, osmolality, pH, volume and total sperm output. Suitable samples (n = 6; motility > 30%, total sperm per ejaculate > 2.0 x 10^6) were washed and re-suspended in TEST yolk buffer (TYB) containing 0.075 M sucrose and either 1 M glycerol or 1 M DMSO. Samples were cooled for 5 min by one of three methods (1) Top-rack (in 7.5 cm LN_2 vapor with a cooling rate of -57.8 °C/min, from 4 °C to -120 °C), (2) Bottom rack (in 3 cm LN_2 vapor with a cooling rate of -124.2 °C/min from 4 °C to -120 °C), or (3) in a fully charged dry shipper (cooling rate of -67.0 °C/min from -10 °C to -120 °C) before being plunged into LN_2. Samples were thawed by one of two methods (1) 50 °C for 10 s or (2) 37 °C for 30 s and post thaw motility, viability and acrosomal integrity was assessed before dilution back into isotonic medium (300 mOsm),

after dilution into isotonic medium (0 h) and 1 h following dilution and incubation at 37 °C (1 h). Before dilution back into isotonic medium, DMSO showed superior motility to glycerol ($P < 0.05$) as did the more rapid warming rate of 50 °C for 10 s ($P < 0.05$). At 0 h, motility was higher in samples cooled on the top rack compared to the bottom rack and dry shipper methods ($P < 0.05$). Also at time 0 h samples cryopreserved in glycerol and cooled on the top rack exhibited higher ($P < 0.05$) motility than those in other treatments except for sperm cryopreserved in 1 M DMSO and cooled on the bottom rack. Samples cooled on the top rack and warmed for 30 s at 37 ºC had ($P < 0.05$) higher motility than samples cooled on either the bottom or top racks and thawed for 10 s at 50 ºC, and samples cooled in a dry shipper and thawed for 30 s at 37 ºC. At 0 h, viability was significantly higher in samples frozen in 1 M DMSO ($P < 0.05$) compared to glycerol. At 1 h, samples cooled on the bottom rack with DMSO exhibited higher motility than those in other treatments (glycerol and bottom rack, glycerol and top rack), and samples thawed for 30 s at 37 ºC had higher viability and acrosomal integrity than those thawed for 10 s at 50 ºC. In conclusion, DMSO may be a superior cryoprotectant to glycerol for the cryopreservation of maned wolf spermatozoa, especially when combined with a rapid cooling and warming rate. In addition, the dry shipper approach, although not optimal, delivers comparable post-thaw results to other cooling methods, and thus, requires further investigation as a possible field-friendly cryopreservation procedure for maned wolf spermatozoa.

Introduction

The maned wolf (*Chrysocyon brachyrus*) is a unique canid endemic to the Cerrado, Chaco and Pampas regions of South America (3). Being the only member of the genus *Chrysocyon*, its distinctiveness sets this species apart, both socially and biologically, from other large canids such as the gray wolf (*Canis lupus*) or African wild dog (*Lycaon pictus*). For example, mostly solitary in the wild (186), the maned wolf has a diet rich in fruits and vegetables with less than 50% of its nutrients obtained from live prey (4,187,188) and is thus, not reliant on a pack. The maned wolf also appears to be facultatively monogamous, as there have been reports of pairs resting, hunting and travelling together during the breeding season but not commonly seen together year-round (3,186). With an estimated 20,000 remaining in the wild, maned wolves are currently listed as 'Near Threatened' by the International Union for Conservation of Nature and are likely to experience a continuing decline due to ongoing habitat loss and degradation (5). Therefore, captive breeding programs have been established worldwide to create a hedge population against catastrophic events, such as a disease outbreak or further decline from habitat loss. However the captive population, especially in North America, is currently not self-sustaining due to poor reproduction, high neonatal mortality (19), as well as a small and aging population (22).

Currently in North America, there are 92 maned wolves, with 92.1% genetic diversity, kept in 30 zoos (22). Because low heterozygosity has been shown to impact reproduction (e.g., low birth weight and poor sperm production) and health (e.g., neonatal mortality and birth defects) (23), the Maned Wolf Species Survival Plan (MWSSP) has

set a goal of retaining 90% genetic diversity for the next 100 years (22). Under the current situation, it is expected that the North American population can only retain genetic diversity > 90% for the next 5 years, but is likely to fall to 35.6% over the next 100 years (22). For these reasons, the MWSSP has emphasized the importance of enhancing *ex situ* reproduction by establishing assisted reproductive techniques (ART), such as artificial insemination (AI) and sperm cryopreservation. Artificial insemination can eliminate the risk of transporting individuals to different locations for breeding and failed reproduction due to sexual incompatibility. When used in combination with semen cryopreservation, AI can enable the dissemination of valuable genes even after death of the sperm donor. Lastly, AI offers animal welfare advantage for species that establish strong pair bonds such as the maned wolf. This characteristic normally introduces complexity into genetic management because individuals in an established pair may eventually need to be bred to another individual to maintain heterozygosity. Rather than psychologically 'stressing' the pair by forcing separation, AI effectively allows the pair to retain their bond while infusing new genes into the population 'artificially'. However, none of these significant advantages can ever be realized without understanding the species-specific cryobiological properties of maned wolf spermatozoa.

During cryopreservation, there are two main factors affecting the survival of the cell (112). The first is intracellular ice formation (112) that normally occurs during rapid cooling. As the temperatures decline below ice nucleation temperature, extracellular ice forms. Cells respond to this change by moving water across the plasma membrane to maintain equilibrium with the extracellular solute. Cooling cells too quickly does not

allow sufficient time to lose adequate amounts of water which, in turn, results in the formation of intracellular ice crystals during cryopreservation (114). However, if cells are cooled too slowly, they are subjected to harmful concentrations of electrolytes and undergo severe dehydration in response to changes in osmolarity of the extracellular solute (114). Therefore, successful cryopreservation requires cells to be cooled at an optimal rate to avoid damage associated with intracellular ice formation and solution effects. Optimal cooling varies greatly among cell types depending on their water permeability. For example, the optimal cooling rate of red blood cells, which are highly permeable to water, is 10,000 °C/min, while that of embryos is < 1 °C/min (113). Furthermore, optimal cooling rates for spermatozoa vary among species. The optimal cooling rate for boar (*Sus scrofa domesticus*) semen is 30 °C/min (189) while the ram (*Ovis aries*) requires 50 – 60 °C/min (190) and the human *(Homo sapiens)* 1 – 10 °C/min (191). Domestic dog (*Canis lupus familiaris*) spermatozoa show optimal cryosurvival at cooling rates 10°C – 30 °C/min (105).

While optimal cooling rates are necessary to prevent cryoinjury, they are not sufficient to maintain cell survival independently during cryopreservation. Most cells require additional protection against biophysical and biochemical changes during freezing and thawing, which is achieved by the addition of cryoprotective agents (CPA's) (114). Cryoprotectants prevent cryoinjury by lowering the freezing point of intracellular solutes, reducing electrolyte concentrations and also by decreasing the extent of cell dehydration (114). Glycerol is the most commonly used permeating cryoprotectant for freezing sperm of many mammalian species, including the dog (123). Other CPA's,

including ethylene glycol (EG) and dimethyl sulfoxide (DMSO) also have been used to cryopreserve dog spermatozoa with varying success. Fertility of frozen-thawed dog semen cryopreserved with 5% glycerol or 5% EG (117), and glycerol with DMSO (105) are similar. Whereas other studies have had minimal success with DMSO (122,123) and EG (124) in comparison to glycerol for cryopreserving dog sperm.

Dog spermatozoa have been cryopreserved using various techniques, including controlled-rate freezers (108), liquid nitrogen vapor and dry ice (109,116–118) or as pellets on dry ice (37,38). However, for application in wild canids, an ideal sperm cryopreservation protocol should include 'field friendly' techniques that can be performed in a remote area where liquid nitrogen or dry ice is not available. Dry shippers are designed to maintain stable temperatures below – 150°C for more than 10 d after being charged with liquid nitrogen (LN_2). Therefore, dry shippers would be an ideal substitute for cryopreserving sperm in a field setting where resources are limited. Stoops et al. (119) successfully cryopreserved ocelot *(Leopardus pardalis)* sperm in a field setting using dry shippers. Specifically, ocelot spermatozoa cooled in dry shippers show a capacity to bind, penetrate and fertilize domestic cat oocytes comparable to spermatozoa cooled over liquid nitrogen vapor. Continued success using dry shippers to cool and store fish spermatozoa in the field have made this technique common practice in Brazil for hatchery programs as well as for the genetic enhancement, mitigation or conservation breeding of endangered fish species (120,192). To our knowledge, dry shippers have not been used to cryopreserve spermatozoa from any canid species.

To date, there have not been any studies on the cryopreservation of maned wolf spermatozoa. Our previous study (Chapter 2) has demonstrated that maned wolf spermatozoa were highly susceptible to osmotic stress, especially when exposed to high osmolalities of non-permeating compounds. However, maned wolf spermatozoa maintain motility and viability after exposure to 1 M concentrations of glycerol or DMSO. Therefore, as a continuation of our previous work, the objectives of the present study were to determine the effects of various cooling methods, warming rates and cryoprotectants on survival of frozen-thawed maned wolf spermatozoa. We hypothesized that cryosurvival of maned wolf spermatozoa depends on cooling and warming rate. Because DMSO has lower molecular weight than glycerol, we predicted that the former was superior to the latter in maintaining motility, viability and acrosomal integrity of cryopreserved maned wolf sperm.

Materials and Methods

Animals

Seven adult male maned wolves housed in various zoological institutions across the United States were included in the study (Table 3.1).

Table 3.1 Demographic information about male maned wolves (n = 7) electroejaculated in 2010 and 2011.
Collections are listed in chronological order; some males were collected more than once.

Wolf ID	Facility and Location	Collection Date	Age at Collection (yr)
2815	Smithsonian Conservation Biology Institute, VA	12.22.10	6.0
2844	Smithsonian Conservation Biology Institute, VA	12.22.10	5.0
2592	Louisville Zoo, KY	10.24.11	9.0
2844	Smithsonian Conservation Biology Institute, VA	11.01.11	6.0
3120	Smithsonian Conservation Biology Institute, VA	11.14.11	4.0
2810	Dickerson Park Zoo, MO	11.21.11	6.0
2815	Smithsonian Conservation Biology Institute, VA	10.31.11	7.0
2954	Smithsonian Conservation Biology Institute, VA	10.19.11	5.0
3121	Natural Science Center of Greensboro, NC	11.08.11	4.0
2954	Smithsonian Conservation Biology Institute, VA	12.19.11	5.0
2815	Smithsonian Conservation Biology Institute, VA	12.19.11	6.0
2844	Smithsonian Conservation Biology Institute, VA	12.20.11	5.0

Animals were housed according to the guideline of the Association of Zoo and Aquarium's Maned Wolf Species Survival Plan (MWSSP). All animal procedures were approved by the Smithsonian Conservation Biology Institute's (SCBI) Institutional Animal Care and Use Committee (IACUC).

Semen Collection, Evaluation and Processing

Semen collections were performed during two consecutive breeding seasons (2010 and 2011) and one to two collections were performed per animal per season. A surgical plane of anesthesia was induced at the discretion of the staff veterinarian at each institution using combinations of dissociatives, benzodiazepines, opiates and alpha-2 agonists, supplemented with isoflurane as needed. The urinary bladder was emptied by

catheterization of the urethra using an 8 Fr catheter (Kendall Sovereign Feeding Tube and Urethral Catheter 8 FR, 22"; Tyco Healthcare Group LP, Mansfield MA), and then flushed with sterile saline solution to minimize urine contamination in the semen samples. Electroejaculation was performed using a rectal probe of 1.9 cm in diameter with 3 longitudinal electrodes and an electrostimulator (P.T. Electronics, Boring, Oregon.). The lubricated probe was placed in the rectum and low voltage (2-5 V) applied to the prostate region. A total of 60 - 90 electrical stimulations were delivered over a 30 min period consisting of two to three stimulation series with rest periods of 5-7 min in between series. Ejaculates were collected into pre-warmed, sterile collection cups and placed on a slide warmer set at 35-37 °C until assessment. Following the collection, animals were allowed to recover and monitored closely by keeper staff and veterinarians according to protocols enforced at each institution.

Raw ejaculates (n = 12) from each male were pooled and immediately assessed for volume, pH (EMD colorpHast pH Strips; EMD Chemicals, Darmstadt, Germany) and osmolality (Vapro 5520, Wescor, Inc. Logan, UT). A semen sample (4 µl) was placed on a pre-warmed slide (37 °C), covered with a cover glass and assessed for percent total motility and forward progressive status (FPS; scale, 0–4 with a 4 rating equivalent to rapid, straightforward progression,(144)) using phase contrast microscopy. Twenty µl aliquots of semen were fixed in 100 µl 0.3% glutaraldehyde in phosphate buffered saline (PBS) and 100 µl 4% paraformaldehyde in PBS and stored at 4 °C for sperm morphological and acrosomal integrity analysis, respectively. Another 10 µl of raw ejaculate was added to 10 µl eosin-nigrosin (E-N) stain, incubated for 30 s, smeared onto

three slides and dried, all at room temperature. E-N stained slides were permanently preserved by placing a cover glass over a drop of mounting medium (Permount, Fisher Scientific, Pittsburg, PA) to set for subsequent assessment of sperm plasma membrane integrity (145). Finally, 5 µl was fixed with 95 µl of water and assessed for sperm cell concentration using a standard haemocytometer.

The remainder of each sample was immediately diluted with an equal volume of TEST Yolk buffer (TYB, Irvine Scientific, Santa Ana, CA). Diluted samples were centrifuged (Eppendorf Mini-Spin, Hamburg, Germany) at 300 *g* for 10 min and the supernatant removed. Samples exhibiting <40% motility or $<0.6 \times 10^6$ total spermatozoa per ejaculate were considered unsuitable for experimental use and were discarded (n = 6).

Evaluation of Sperm Morphology

Sperm morphology was assessed by a single observer using phase-contrast microscopy (magnification: x 1,000). A total of 100 spermatozoa were counted and classified as either normal or as having primary or secondary abnormalities, with final values expressed as % normal. Primary abnormalities, which occur during spermatogenesis process (146), include macrocephalic, microcephalic, bicephalic, detached head, spermatid, abnormal acrosome or midpiece, no midpiece, tightly coiled tail or biflagellate. Secondary abnormalities, which occur during epididymal storage or preparation of the sample (146), consist of a bent midpiece with no droplet, a bent midpiece with a retained droplet, a bent flagellum with no droplet, a bent flagellum with a retained droplet, retained proximal droplet, retained distal droplet and bent neck.

Evaluation of Acrosomal Integrity

For assessment of sperm acrosomal integrity, fixed samples were centrifuged for 5 min at 3000 *g* and the supernatant removed. Pellets were washed twice with 0.1 M ammonium acetate (pH 9.0) and resuspended in 20 -30 μl of the ammonium acetate solution. A 10 μl aliquot of this suspension was pipetted onto a microscope slide and allowed to dry at room temperature. Slides were then flooded with Coomassie stain (0.22 % Coomassie Blue [G-250, Fisher Biotech, Springfield, NJ] in 50% methanol, 10% glacial acetic acid and 40% deionized water; (193)) for 90 s, rinsed with deionized water, dried at room temperature and permanently preserved by placing a cover glass over a drop of mounting medium (Permount, Fisher Scientific, Pittsburg, PA). Acrosomal integrity was assessed by counting 200 sperm per slide using phase-contrast microscopy (magnification: x 1,000), categorized as intact or damaged/non-intact, and averaged per ejaculate (Figure 3.1). Sperm with intact acrosomal membranes exhibited blue staining throughout the acrosomal region. Non-intact or damaged sperm displayed a clear acrosomal region, patchy staining, or irregular membrane structures.

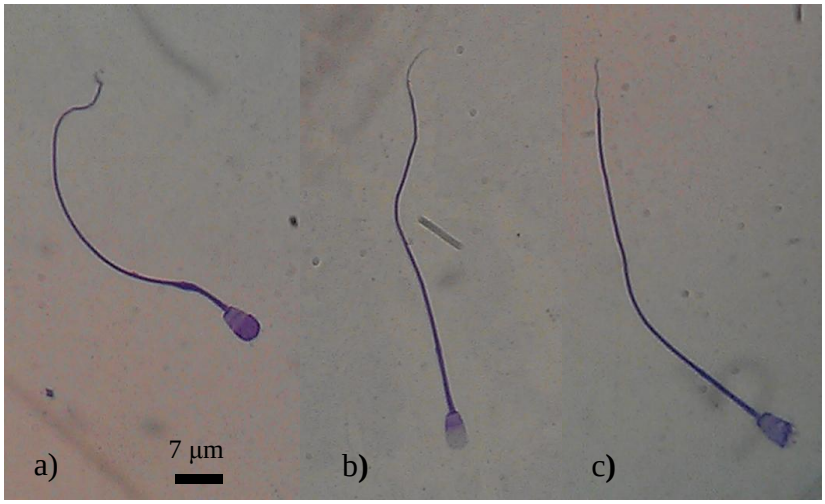


Figure 3.1 Maned wolf spermatozoa stained with Coomassie for assessment of acrosomal integrity.
Spermatozoa shown are: a) intact b) non-intact c) damaged.

Evaluation of Sperm Plasma Membrane Integrity

Sperm plasma membrane integrity was assessed by a single observer using phase-contrast microscopy (magnification: x 1,000). A total of 200 sperm were counted per slide, categorized as either viable or nonviable; three slides per ejaculate were assessed and the average percentage of viable sperm calculated. Nonviable spermatozoa had increased permeability to the E-N stain and appeared pink or purple whereas viable gametes excluded the stain and remained white.

Experimental Design

Electroejaculates (n = 12, 1-2 ejaculates/male) were processed as described above. Six of the 12 ejaculates were excluded due to low motility (< 40%, n = 2) low total sperm output (< 0.6 x 10^6 million sperm per ejaculate, n = 2) or were classified as azoospermic (n = 2). The remaining ejaculates were resuspended in TYB supplemented with 0.075 M sucrose (S0389; Sigma, St. Louis, MO) and either 1 M glycerol (G5516; Sigma, St.

Louis, MO) or 1 M DMSO (D8418; Sigma, St. Louis, MO) to a final volume of 320 µl. Aliquots (50 µl) were aspirated into ¼ cc straws (Veterinary Concepts, Spring Valley, WI) and sealed with an impulse heat sealer (MP-8, Midwest Pacific, Taiwan). Straws were placed in a Ziploc bag and submerged in approximately 250 ml of water contained in a plastic beaker prepared at room temperature. The beaker was then placed in a cooled water bath (5 °C) within a Styrofoam box (Outside measurements: 38 x 35 x 25 cm; Inside measurements: 32 x 28 x 16 cm; Thickness: 4 cm) and cooled to 5 °C (0.08 - 0.1 °C/min) for approximately 2 h. Before being plunged into LN_2, sperm was cooled in the vapor for 5 min by one of three methods: 1) Top-rack, 2) Bottom rack and 3) dry-shipper. For the first two methods a Styrofoam box (outside measurements: 38 x 35 x 25 cm; inside measurements: 32 x 28 x 16 cm; thickness: 4 cm) was filled with LN_2 to a depth of 3 cm. Straws were placed horizontally on a metal rack either 7.5 cm (Top-rack; -cooling rate of -57.8 °C/min, from 4 °C to -120 °C) or 3 cm (Bottom-rack; -cooling rate of -124.2 °C/min from 4 °C to -120 °C) above the surface of the LN_2. For the dry-shipper method, straws were placed directly into pre-cooled goblets and canes and plunged into a fully charged dry-shipper (Dry shipper; cooling rate of -67.0 °C/min from -10 °C to -120 °C, after the 'plateau' of dissipation of heat of fusion). Freezing curves (Figure 3.2) for each method were determined by inserting a thermocouple into straws containing TYB (five trials for each freezing curve, plotted values are the mean).

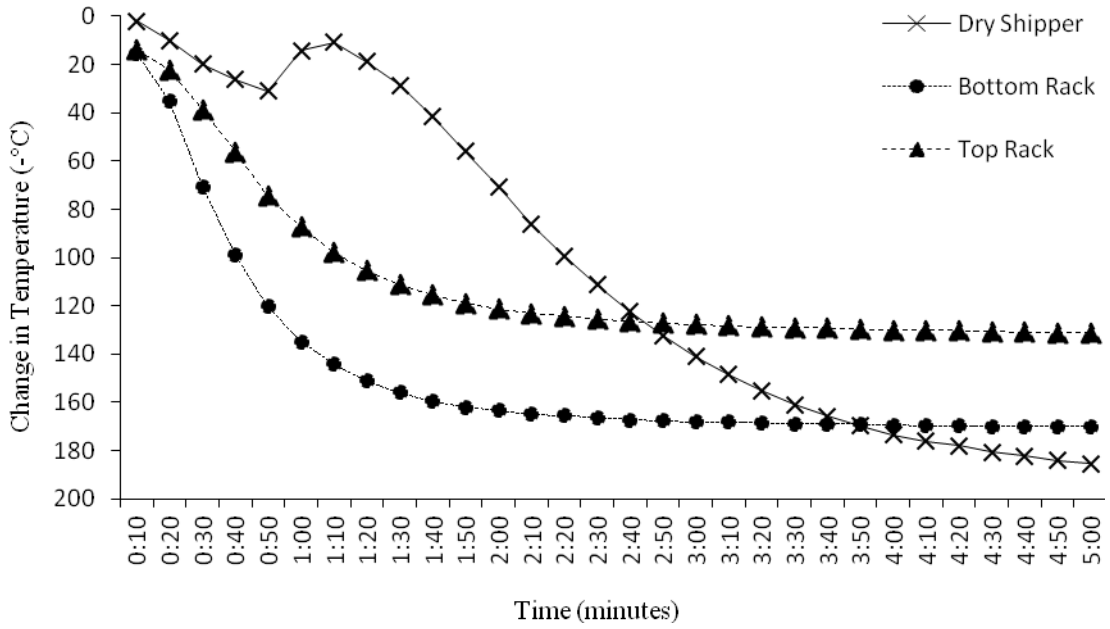


Figure 3.2 Freezing curves (n = 5) of top rack, bottom rack and dry-shipper methods for the cryopreservation of maned wolf spermatozoa.

After at least 24 h of cryopreservation, frozen samples were thawed by swirling straws vigorously in a water bath (Precision 180 series, Jouan, Inc, Winchester, VA) for either (1) 30 s at 37 ºC or (2) 10 s at 50 ºC. Thawed aliquots were expelled from the straw into a plastic 1.5 mL microcentrifuge tube (T9661; Eppendorf Safe-Lock, Sigma-Aldrich, St. Louis, MO) and analyzed for post-thaw motility parameters. Five µl aliquots were removed for post-thaw acrosomal and plasma membrane integrity analysis as described above. Thereafter, the remaining sample was slowly diluted with 80 µl Ham's F10 supplemented with bovine serum albumin (BSA; 4 mg/mL) and motility parameters, acrosomal integrity and plasma membrane integrity were assessed at time 0 and 1 h post-

incubation at 38.5 ºC in a CO_2 controlled incubator (Series II water injected, Thermo Forma, Marietta, OH).

Statistical analysis

All statistical analyses were conducted using SYSTAT 12 (Version 12.00.08, SYSTAT Software, Inc.). Percentage data for motility, viability and acrosomal integrity was subjected to arcsine transformation to normalize the data. A Shapiro-Wilk test was then used to test for normality. Mean comparisons were performed using analysis of variance (ANOVA). When significant differences among treatments were discovered, post-hoc, pairwise analyses were conducted by the Tukey Range Test. All values were reported as mean ± standard error of the mean (S.E.M.) and statistical significance was set at a $P < 0.05$.

Results

Table 3.2 illustrates the seminal characteristics of all raw ejaculates obtained in the present study. Mean motility, viability and acrosomal integrity of raw ejaculates included in the study (n = 6) was 72.5 ± 2.5%, 82.4 ± 1.9% and 76.1 ± 2.5% respectively. Overall, cryopreservation resulted in a reduction of mean sperm motility (16.8 ± 1.9%, range 0 – 50%), viability (58.3 ± 1.8%, range 14 – 88%) and acrosomal integrity (54.0 ± 1.6%, range 26 – 87%) in comparison to raw ejaculates.

Table 3.2 Seminal characteristics of raw maned wolf electroejaculates (n=12)

Seminal Parameters	Mean ± S.E.M.	Range (min. - max.)
Semen pH	7.7 ± 0.2	6.5 − 9.0
Osmolality (mOsm)	341.2 ± 7.5	294.0 − 395.0
Ejaculate volume (mL)	1.5 ± 0.6	0.2 − 6.7
Sperm concentration (x 10^6 spermatozoa mL^{-1})	36.2 ± 17.8	1.0 − 185.0
Total spermatozoa per ejaculate (x 10^6)	86.9 ± 64.0	0.4 − 715.0
Motility (%)	58.6 ± 7.3	0.0 − 80.0
Forward progressive status (0-4)	2.3 ± 0.3	0.0 − 3.0
Viability (%)	76.7 ± 2.3	75.2 − 89.7
Membrane integrity (%)	73.6 ± 1.9	64.5 − 80.5
Morphologically normal sperm (%)	25.6 ± 5.9	6.0 − 65.0

Motility

Cooling methods did not affect ($P > 0.05$) sperm motility immediately post-thaw before dilution back into isotonic medium (Table 3.3). However, samples cryopreserved in 1 M DMSO exhibited superior motility to those cryopreserved in 1 M glycerol (20.0 ± 1.9% *vs*. 13.5 ± 2.1%) as did samples thawed for 10 s at 50 ºC compared to those thawed for 30 s at 37 ºC (19.5 ± 2.2% *vs*. 14.0 ± 1.8%). Incubating sperm at 38.5 °C for 1 h resulted in decreased ($P = 0.000$) motility compared with that observed before dilution (Table 3.4). There was no significant effect of interactions among variables on motility before dilution.

After dilution into isotonic medium (0 h), post-thaw motility was affected by cooling method as samples cooled on the top rack had higher motility (20.3 ± 2.9 %) than those cooled using the bottom rack (12.8 ± 2.0%, $P = 0.047$) and dry shipper methods

(13.6 ± 1.7 %, P = 0.046). There were also significant interactions of CPA and cooling method as well as cooling and warming on sperm motility after dilution (Table 3.3). At 0 h, samples cryopreserved in glycerol and cooled on the top rack exhibited higher ($P < 0.05$) motility than those in other treatments except for sperm cryopreserved in 1 M DMSO and cooled on the bottom rack. Samples cooled on the top rack and warmed for 30 s at 37 ºC had ($P < 0.05$) higher motility than samples cooled on the bottom rack and thawed for 10 s at 50 ºC, samples cooled in a dry shipper and thawed for 30 s at 37 ºC and those samples cooled on the top rack and thawed for 10 s at 50 ºC (Table 3.4). There was also an interaction of CPA and cooling method at 1 h post dilution and incubation. Samples cooled on the bottom rack with DMSO exhibited higher motility than those in other treatments (glycerol and bottom rack, glycerol and top rack). No other differences in motility were observed among the various treatments at 1 h post dilution (Table 3.3).

Table 3.3 Statistical results (P-values and F-ratios) for the effects of cryoprotectant, cooling method and warming rate on post-thaw motility before dilution, after dilution and after 1 h of incubation.

		Motility					
		Before Dilution		After dilution (0 h)		After incubation (1 h)	
Effects	D.F.	F-ratio	P-value	F-ratio	P-value	F-ratio	P-value
Cryoprotectant (CPA)	1	5.779	0.019	0.229	0.634	2.261	0.138
Cooling Method (CM)	2	3.049	0.055	3.933	0.025	0.624	0.539
Warming Rate (WR)	1	4.234	0.044	3.559	0.064	0.635	0.429
CPA x CM	2	0.601	0.552	9.033	0.000	5.975	0.004
CPA x WR	1	0.249	0.620	0.641	0.427	0.006	0.936
CM x WR	2	1.189	0.312	3.514	0.036	0.524	0.595
CPA x CM x WR	2	0.134	0.875	0.644	0.529	0.056	0.945
Error		59		58		59	

Table 3.4 Mean ± SEM Post-thaw motility of sperm cryopreserved with various cryoprotectants, cooling and warming treatments.

			% Motility		
CPA	Cooling Method	Warming Method	Before dilution	After dilution (0 h)	After incubation (1 h)
DMSO	Top Rack	30 s at 37°C	20.0 ± 5.3	17.0 ± 4.0	8.2 ± 3.6
	Bottom Rack		15.0 ± 2.9	20.8 ± 4.5	15.0 ± 3.2
	Dry Shipper		19.2 ± 4.4	11.7 ± 2.1	8.3 ± 1.1
Glycerol	Top Rack		14.7 ± 6.0	35.0 ± 5.0	12.8 ± 4.2
	Bottom Rack		5.3 ± 2.2	10.0 ± 2.7	5.0 ± 2.4
	Dry Shipper		9.8 ± 3.7	12.7 ± 4.3	6.3 ± 2.2
DMSO	Top Rack	10 s at 50°C	26.0 ± 6.4	9.5 ± 2.3	7.7 ± 3.2
	Bottom Rack		24.2 ± 5.4	15.3 ± 2.9	11.8 ± 3.7
	Dry Shipper		16.7 ± 3.8	17.3 ± 3.1	8.3 ± 3.3
Glycerol	Top Rack		25.8 ± 6.0	19.2 ± 6.2	11.0 ± 2.6
	Bottom Rack		13.5 ± 4.1	5.0 ± 2.5	1.0 ± 0.5
	Dry Shipper		11.7 ± 4.9	12.7 ± 3.9	7.7 ± 3.3

Membrane Integrity

There was no effect of cryoprotectant, cooling method or warming method on sperm membrane integrity before dilution (Table 3.5). However there was an overall decline in membrane integrity from before dilution to 0 h ($P = 0.004$) and again from 0 h to 1 h ($P = 0.000$) (Table 3.6). At 0 h, viability was significantly higher in samples frozen in 1 M DMSO compared with those cryopreserved in 1 M glycerol. At 1 h, cryoprotecant no longer had any effect of viability. However, samples thawed for 30 s at 37 ºC had higher viability than those thawed for 10 s at 50 ºC at 1 h.

Table 3.5 Statistical results (P-values and F-ratios) for the effects of cryoprotectant, cooling method and warming rate on post-thaw viability before dilution, after dilution and after 1 h of incubation.

		Viability					
		Before Dilution		After dilution (0 h)		After incubation (1 h)	
Effects	D.F.	F-ratio	P-value	F-ratio	P-value	F-ratio	P-value
Cryoprotectant (CPA)	1	1.950	0.168	6.672	0.013	3.907	0.054
Cooling Method (CM)	2	0.354	0.703	0.326	0.724	0.898	0.414
Warming Rate (WR)	1	0.000	0.986	0.252	0.618	4.993	0.030
CPA x CM	2	0.134	0.875	0.455	0.637	0.696	0.504
CPA x WR	1	0.294	0.590	3.462	0.069	0.883	0.352
CM x WR	2	0.101	0.905	0.037	0.964	0.920	0.405
CPA x CM x WR	2	0.273	0.762	1.506	0.232	1.073	0.350
Error		54		48		48	

Table 3.6 Mean ± SEM post-thaw viability of sperm cryopreserved with various cryoprotectants, cooling and warming treatments.

			% Viability		
CPA	Cooling Method	Warming Method	Before dilution	After dilution (0 h)	After incubation (1 h)
DMSO	Top Rack	30 s at 37°C	60.6 ± 7.7	49.2 ± 4.7	45.3 ± 5.0
	Bottom Rack		64.9 ± 4.3	63.5 ± 3.5	45.0 ± 3.8
	Dry Shipper		60.5 ± 8.4	65.1 ± 2.3	41.1 ± 7.1
Glycerol	Top Rack		50.7 ± 6.4	42.4 ± 8.2	37.0 ± 5.6
	Bottom Rack		66.9 ± 5.9	37.2 ± 9.0	35.7 ± 4.6
	Dry Shipper		54.6 ± 10.0	33.9 ± 8.9	25.5 ± 6.7
DMSO	Top Rack	10 s at 50°C	59.8 ± 8.5	50.6 ± 4.3	34.1 ± 3.4
	Bottom Rack		64.6 ± 5.6	45.6 ± 4.4	32.1 ± 4.4
	Dry Shipper		61.9 ± 8.7	48.8 ± 7.5	29.4 ± 4.4
Glycerol	Top Rack		47.1 ± 9.9	37.6 ± 9.8	16.2 ± 1.0
	Bottom Rack		50.9 ± 11.7	47.8 ± 11.8	35.9 ± 11.1
	Dry Shipper		57.2 ± 13.3	41.6 ± 9.5	28.7 ± 6.7

Acrosomal Integrity

There was no effect of cryoprotectant, cooling method, or warming method on acrosomal integrity before or at 0 h (Table 3.7). However, at 1 h samples thawed for 30 s at 37 ºC had higher acrosomal integrity than those thawed for 10 s at 50 ºC (Table 3.8). There was an overall decline in acrosomal integrity from post-thaw to 0 h (P = 0.020) and again from 0 h to 1 h (P = 0.000).

Table 3.7 Statistical results (P-values and F-ratios) for the effects of cryoprotectant, cooling method and warming rate on post-thaw acrosomal integrity before dilution, after dilution and after 1 h of incubation.

		Acrosomal Integrity					
		Before Dilution		After dilution (0 h)		After incubation (1 h)	
Effects	D.F.	F-ratio	P-value	F-ratio	P-value	F-ratio	P-value
Cryoprotectant (CPA)	1	0.330	0.568	0.228	0.635	0.039	0.845
Cooling Method (CM)	2	0.483	0.620	0.625	0.985	0.181	0.835
Warming Rate (WR)	1	2.225	0.142	0.165	0.686	4.062	0.049
CPA x CM	2	0.301	0.742	1.093	0.343	0.020	0.980
CPA x WR	1	0.239	0.627	0.139	0.711	0.019	0.890
CM x WR	2	0.239	0.788	0.625	0.540	0.031	0.970
CPA x CM x WR	2	1.465	0.241	0.126	0.882	0.102	0.903
Error		47		47		48	

Table 3.8 Mean ± SEM post-thaw acrosomal integrity of sperm cryopreserved with various cryoprotectant, cooling and warming treatments.

			% Acrosomal Integrity		
CPA	Cooling Method	Warming Method	Before dilution	After dilution (0 h)	After incubation (1 h)
DMSO	Top Rack	30 s at 37°C	62.5 ± 4.8	48.1 ± 3.1	35.5 ± 8.1
	Bottom Rack		52.8 ± 7.0	58.0 ± 3.6	22.9 ± 5.2
	Dry Shipper		58.3 ± 3.6	50.6 ± 7.6	26.7 ± 6.9
Glycerol	Top Rack		55.8 ± 5.9	52.9 ± 5.8	26.4 ± 9.7
	Bottom Rack		49.3 ± 7.0	45.8 ± 6.0	24.9 ± 7.2
	Dry Shipper		56.6 ± 7.1	35.1 ± 7.2	26.9 ± 7.6
DMSO	Top Rack	10 s at 50°C	48.4 ± 5.4	43.1 ± 8.3	19.9 ± 5.8
	Bottom Rack		55.3 ± 4.4	49.0 ± 4.5	16.5 ± 4.3
	Dry Shipper		53.6 ± 7.6	53.9 ± 5.5	19.5 ± 3.1
Glycerol	Top Rack		61.3 ± 4.9	48.1 ± 8.2	20.7 ± 4.0
	Bottom Rack		50.5 ± 7.7	44.2 ± 5.6	17.2 ± 3.1
	Dry Shipper		43.3 ± 6.5	47.9 ± 7.2	18.3 ± 5.4

Discussion

Sperm cryopreservation plays an important role in enhancing genetic management of threatened species (25,26,69). However, the success of this technique depends entirely on understanding species-specific sensitivities of spermatozoa to various stages of cryopreservation (140). The present study represents the first effort to cryopreserve ejaculates from the maned wolf. Sperm survival was significantly influenced by cryoprotectant as well as cooling and warming methods but the effects were dependent upon cryoprotectant removal (i.e., dilution into isotonic medium). Interactions among variables were also significant, especially after cryoprotectant removal. In general, DMSO was a superior cryoprotectant to glycerol, particularly when combined with a

more rapid cooling method. As expected, the more rapid warming rate of 10 s at 50 °C was superior to 30 s at 37 °C for maintaining motility post-thaw. However, after one hour of incubation in isotonic medium at 38.5 °C, viability was better maintained in samples thawed at the slower rate. This could suggest that membrane damages incurred during rapid thawing weaken the cells' tolerance to the osmotic stress endured during cryoprotectant removal. Independent of cryoprotectants and warming rates, there were no differences in post-thawed sperm quality among cooling methods.

Permeating cryoprotectants protect spermatozoa during cryopreservation by delaying intracellular freezing and minimizing solution effects caused by cell dehydration (138). Glycerol is the most commonly used permeating cryoprotectant in sperm cryopreservation but other additives such as (but not limited to) EG, DMSO, methylformamide (MF), and dimethylformamide (DMF) have also been investigated. However, the efficacy of these cryoprotectants appears to vary among species. For example, although EG is the most appropriate cryoprotectant for rhesus monkey *(Macaca mulatta)* spermatozoa (194), yet it significantly reduces viability of honey bee *(Apis mellifera)* spermatozoa (195). Dimethyl formamide has recently become one of the more common cryoprotectants for stallion (*Equus ferus caballus*) spermatozoa (196) but it has no benefits for freezing goat *(Capra aegagrus hircus)* semen (197). The present study showed significantly improved post-thaw motility in maned wolf spermatozoa cryopreserved in DMSO compared to glycerol before dilution into isotonic medium. This was expected as our previous study reported a high tolerance of maned wolf spermatozoa to DMSO (Chapter 2) despite its assumed toxicity to dog spermatozoa (134)

further corroborating varying tolerance levels to cryoprotectants among species even within the same taxa. Tolerance of maned wolf spermatozoa to cryopreservation in DMSO in the present study suggests (1) interspecific differences in sperm membrane structure and function between maned wolves and dogs (140) or (2) species variation in osmotic tolerance limits to CPA's (198).

Like cryoprotectants, optimal cell cryosurvival requires cooling and warming methods specific to each species (199). For optimal cryosurvival in dogs, it is generally assumed that rapid cooling of sperm requires rapid thawing whereas slow cooling requires slow thawing (109,116). If a cell is cooled quickly, a more rapid warming rate will prevent re-crystallization of small (innocuous) intracellular ice crystals during thawing (200). If cooled slowly enough to preclude intracellular ice formation, a slower warming rate prevents osmotic shock and minimizes dehydration by allowing sufficient time for solutes to diffuse out of the cell (while water moves in) as the extracellular ice melts (200). In the present study, there was no significant interaction of cooling and warming rates before dilution into isotonic medium. However, consistent with those previous reports in dogs, it was evident that a more rapid cooling method combined with a rapid thawing rate enhanced cryosurvival following dilution into isotonic medium, as did a slow cooling treatment combined with a slower warming rate.

At 1 h, motility, but not viability or acrosomal integrity, was affected by an interaction of cryoprotectant and cooling rate. Spermatozoa cryopreserved in DMSO had better survival when cooled at a faster rate (bottom rack) whereas spermatozoa cryopreserved in glycerol survived best with the slower cooling method (top rack).

Interestingly, neither combination shows better survival than the other. These distinctions are likely due to differences in molecular weight between the two cryoprotectants. Due to its lower molecular weight, DMSO permeates the sperm plasma membrane more quickly than glycerol (139), thus requiring less time to equilibrate before and after freezing. Therefore, the slower cooling method (top rack) paired with glycerol likely reduced intracellular ice formation compared to the faster cooling method by allowing more equilibration time before reaching the ice nucleation temperature. This speculation is supported by the higher motility seen in samples thawed at a more rapid warming rate, as rapid warming prevents the re-crystallization of any intracellular ice that formed during cooling.

The results of this study showed that the deleterious effects of cryopreservation on maned wolf spermatozoa became more significant after dilution into isotonic medium. This is likely due to the severe damages induced by extreme volumetric changes as the cells are returned to isotonic conditions (141). This theory is supported by a significant decrease in motility, viability and acrosomal integrity exhibited by all treatment groups from post-thaw to 1 h and especially by a significant reduction in viability and acrosomal integrity from post-thaw to 0 h. A stepwise dilution approach for the removal of cryoprotectants could potentially aid in minimizing these damages. Removing glycerol in eight or more steps can significantly improve motility and membrane integrity in human (179), felid (141), and stallion (202) spermatozoa.

Independent of interactions with cryoprotectants and warming rates, there were no significant differences in motility, viability or acrosomal integrity among cooling

methods. This suggests that the use of dry shippers to cool maned wolf spermatozoa does not compromise post-thaw sperm quality in comparison to the other cooling methods. These results are encouraging as this cooling technique can be easily adopted in a field situation where liquid nitrogen or dry ice is not accessible. Future research focused on improving cryosurvival of sperm cryopreserved in a dry-shipper is vital as this technique facilitates the diffusion of new genetics from wild individuals into the *ex situ* population without removing wolves from their native habitat. Dry shippers have been investigated as a method for the cryopreservation of spermatozoa from several threatened species with considerable success (scimitar horned oryx (*Oryx dammah)*(203), ocelot (119), giant panda *(Ailuropoda melanoleuca)* (204) and the Sumatran rhino *(Dicerorhinus sumatrensis)* (205)).

Of the three semen parameters reviewed in the present study, motility was the most significantly affected; CPA, cooling method and warming rate all compromised motility either before or after dilution into isotonic medium. This is likely due to the increased resistance of the sperm plasma membrane to osmotic volume excursions compared to other cellular structures directly involved with motility, such as the mitochondria (114). Thus, motility can potentially act as an indicator of the extent to which a cell is undergoing osmotic stress. Viability was affected by CPA and warming rates but only after 1 h post-dilution while acrosomal integrity was only affected by warming rates at 1 h. Thus, membrane damages incurred during freezing and thawing may enhance the deleterious effects of osmotic stress induced during dilution back into isotonic medium, therefore only becoming apparent after 1 h incubation.

The present study demonstrates the first successful attempt to freeze maned wolf spermatozoa. Our study provides fundamental information for future studies aiming to optimize sperm cryopreservation method for this species. The findings suggest that DMSO is a superior cryoprotectant to glycerol, especially when combined with rapid cooling and warming methods. Our findings also revealed a 'field friendly' cryopreservation technique that exhibits post-thaw qualities comparable to those attained in a lab setting. These findings are an important step towards improving the genetic management of the *ex situ* maned wolf population, further contributing to the preservation of this unique canid.

CHAPTER 4: INDUCTION OF OVARIAN ACTIVITY AND OVULATION IN AN INDUCED OVULATOR, THE MANED WOLF (*CHRYSOCYON BRACHYURUS),* USING GNRH AGONIST AND RECOMBINANT LH

Abstract

The development of assisted reproductive techniques, such as ovarian manipulation and artificial insemination, are important for facilitating the genetic management of the maned wolf (*Chrysocyon brachyurus*), a threatened canid. The objectives of the present study were to investigate female maned wolf response to: (1) induction of ovarian activity with deslorelin both in the presence and absence of a male and (2) ovulation induction in singleton females using recombinant luteinizing hormone (reLH) through non-invasive fecal hormone monitoring. Females were either (1) paired with a male (n = 4) or unpaired (n = 7). Implants (2.1 mg Ovuplant®) were placed during the North American breeding season (Oct and November; 0 d) and were removed 7 (n = 3), 9 (n = 5) or 11 d (n = 3) post-implantation. Three of seven singleton females were injected with reLH (0.0375 mg Equi-pur) on the day of implant removal, while the remaining females (n = 4) did not receive the additional treatment. Fecal samples were collected 5-7 d/wk starting 30 d prior to hormone insertion until at least 70 d post implant removal for a total of 11 trials. Fecal progesterone metabolites (FPM) were extracted and analyzed by enzyme immunoassay. Evidence of ovulation, demonstrated by a rise in FPM at least 2 standard deviations (SD) above baseline for at least 3 consecutive days, occurred in 100%

of paired females. Singleton females that did not received reLH treatment exhibited no rise in FPM. The only exception was one female who exhibited increased FPM concentrations for 12 d before the hormone declined back to baseline levels. All singleton females treated with reLH exhibited a rise in FPM following injection, indicating 100% ovulation. In conclusion, deslorelin is effective at inducing ovarian activity and ovulation in paired female maned wolves but exogenous reLH is required to induce ovulation in singleton females, though these findings require further investigation due to a small sample size. This research further corroborates earlier studies that speculate this species to be an induced ovulator.

Introduction

The maned wolf (*Chrysocyon brachyurus*), a neotropical canid, is listed as 'Near Threatened' by the IUCN (5). The main threats to this species are road mortality, human-wolf conflict, disease risks from domestic dogs and most significantly, agricultural conversion of habitat throughout its home range in the grasslands of South America (5). It is expected that the wild population (currently 20, 000) will continue to decline due to ongoing habitat loss and degradation (5). Therefore, it is imperative that scholarly information on maned wolf husbandry, nutrition and reproductive biology be studied *ex situ* for the purpose of maintaining a hedge population against extinction.

The benefits of assisted reproductive techniques (ART) in endangered species management have been well documented (24–26,29–31,49,66,67,92,96,206–208). However, the successes of these techniques are highly dependent on the extent to which species-specific reproductive mechanisms have been elucidated. Previous studies have

shown that some aspects of maned wolf reproductive biology are similar to that of other wild canids. For example, the maned wolf is monoestrus with a well-known and distinctive breeding season (October - February) that appears to be controlled by photoperiod (3). Proestrus, which can be characterized by vaginal swelling and secretions, can last for 2 wk (32), while estrus lasts anywhere from 1 to 10 d (209). Also, like other canid species, gestation is approximately 65 d. Furthermore, there are strong similarities between the reproductive hormone profiles of domestic dogs *(Canis lupus familiaris)* and maned wolves (32,35). During proestrus, estrogen concentrations increase for approximately 2 wk and decline at copulation. In late proestrus, progestagen begins to increase and remain elevated throughout estrus and the pregnant luteal phase. Non-pregnant females display a similar gonadal hormone pattern by exhibiting a pseudopregnancy for approximately 65 d post-ovulation (35) as demonstrated by other canids.

Recently, our laboratory has discovered a unique reproductive characteristic of female maned wolves. Specifically, it appears that ovulation occurs only in the presence of a male (35), although the regulator(s) of this is not yet known. Induced ovulation has only recently been described in one other canid, the island fox (*Urocyon littoralis*) (93). The unique reproductive biology of maned wolves may present an additional challenge in controlling ovarian activity for timed AI.

Stimulation of ovarian activity and ovulation has significantly improved AI success in rare species such as the cheetah *(Acinonyx jubatus)* (65), Eld's deer *(Panolia eldii)* (66), and black-footed ferret *(Mustela nigripes)* (30). This success can be partially

attributed to the knowledge gained through research on domestic counterparts of these species (30). There are various methods that have been used to manage ovarian activity in the domestic bitch, all varying in feasibility and success. For example, some protocols recommend the use of exogenous estrogen to induce proestrus (70–72) or utilize additional gonadotropins to further induce a fertile estrus (73–75). Similarly, exogenous gonadotropins such as follicle stimulating hormone (FSH), human chorionic gonadotropin (hCG) (76) and human menopausal gonadotropin (HMG) (77) have been used to stimulate the onset of proestrus followed by estrus and either spontaneous (73) or induced ovulation by luteinizing hormone (LH) (78). However, several approaches are impractical for use in wild canids because the methods are costly and extensive (i.e., dopamine agonists require > 30 d of treatment) (79) requiring daily injections or even pulsatile administration of hormones for up to 14 d (90).

Deslorelin (2.1mg Ovuplant®, Peptech Animal Health, NSW, Australia), a short-acting gonadotropin releasing hormone (GnRH) agonist manufactured to induce ovulation in mares, has shown great potential as a practical and consistent method of estrus induction in both dogs (79,94) and wild canids (92). In domestic dogs, deslorelin implants achieve 100% proestrus within 6-10 d of implant placement with 100% ovulation (79) and can achieve 70% pregnancy rates after AI (91). Similarly, use of deslorelin in gray wolves *(Canis lupus)* successfully induces fertile estrus, resulting in 100 % pregnancy from natural breeding and 33% pregnancy after AI (92). Deslorelin has also been used to stimulate follicle growth prior to ovariectomy for oocyte retrieval and

vitrification in endangered Mexican wolves *(Canis lupus baileyi)* (20), although there is no record of it being used to induce estrus for the purpose of AI.

Ovulatory agents, including hCG and LH, have been used to induced ovulation in the domestic dog with varying success. For example, administration of hCG to induce ovulation only suppresses progesterone secretion following a short-lived increase (94) and has no positive effects on ovulation rates (86). However, Wright (95) reported that ovulation in domestic dogs is induced by hCG within 26-30 h of administration. These ovulatory agents have not been tested in maned wolves. The combination of equine chorionic gonadotropin (eCG) and hCG has been commonly used to stimulate ovarian activity and induce ovulation in felids (65). However, evidence suggests that these protocols can cause hyperstimulation of follicles (96,97) and estrogen production (62), which can ultimately be detrimental to fertilization, embryogenesis and implantation (65). Continued use of eCG with hCG can also result in refractoriness (98,99) and can trigger an immune response (100). Furthermore, pituitary gonadotropin extracts such as FSH and LH can contain pathogens and the concentrations can fluctuate with each lot, making dosage inconsistent (102). A recombinant form of equine LH (reLH; Equi-Pur, AspenBio Pharma Inc., Castle Rock, CO) that is free of contaminants and other pituitary hormones that could alter ovarian response obtained a 90% ovulation induction rate in mares (*Equus ferus caballus*) within 48 h of administration with a dosage of 0.75 mg (103). To our knowledge, reLH has never been explored for ovulation induction in canids but has enormous potential for improving AI success.

The objectives of the present study were to investigate female response to: 1) induction of ovarian activity with deslorelin both in the presence and absence of a male and 2) ovulation induction in singleton females using reLH. We used non-invasive fecal gonadal steroid monitoring to test the hypothesis that deslorelin would induce ovarian activity and ovulation in paired females but exogenous reLH may be required to induce ovulation in singleton females.

Material and Methods

Animals

Eight adult female maned wolves housed in various zoological institutions across the United States were included in the study (Table 4.1).

Table 4.1 Demographic information about female maned wolves (n = 8) participating in an ovulation induction study from 2007 – 2011.
Females are listed in chronological order of treatment; 3 wolves were treated more than once.

Wolf ID	Facility and location	Age at time of Implant (yr)	Management	reLH	Date Implanted	Implant Site	Duration of Implant (d)
1891	Houston Zoological Park, TX	11.0	Paired	no	10.07.07	Vulva	11
3015	Dickerson Park Zoo, MO	4.0	Paired	no	10.21.10	Ear	11
2539	Smithsonian Conservation Biology Institute, VA	7.0	Paired	no	10.08.10	Ear	9
2539	Smithsonian Conservation Biology Institute, VA	6.0	Paired	no	11.24.09	Ear	7
2257	Houston Zoological Park, TX	9.0	Unpaired	no	10.07.07	Vulva	11
2926	Fossil Rim Wildlife Center, TX	5.0	Unpaired	no	11.02.10	Ear	9
2612	Smithsonian Conservation Biology Institute, VA	4.0	Unpaired	no	11.24.09	Ear	7
2613	Smithsonian Conservation Biology Institute, VA	4.0	Unpaired	no	11.24.09	Ear	7
2612	Smithsonian Conservation Biology Institute, VA	5.0	Unpaired	yes	10.13.10	Ear	9
2613	Smithsonian Conservation Biology Institute, VA	5.0	Unpaired	yes	10.13.10	Ear	9
2945	White Oak Conservation Center, FL	6.0	Unpaired	yes	11.01.11	Ear	9

Animals were housed according to the Maned Wolf Species Survival Plan (MWSSP) and guidelines of the Association of Zoos and Aquariums. All animal procedures were approved by the Smithsonian Conservation Biology Institute's (SCBI) Institutional Animal Care and Use Committee (IACUC), the White Oak Conservation Center's (WOCC) IACUC, and Houston Zoological Park's (HZP) IACUC.

Ovarian Stimulation

Ovarian stimulation occurred over four nonconsecutive breeding seasons (2007-2011) totaling 11 stimulations (Paired females: n = 4; Unpaired females: n = 7). A surgical plane of anesthesia was induced at the discretion of the staff veterinarian at each institution using combinations of dissociatives, benzodiazepines, opiates and alpha-2 agonists, supplemented with isoflurane as needed. Females were implanted (0 d) with a GnRH agonist, deslorelin (2.1 mg, Ovuplant®, Peptech Animal Health, NSW, Australia) in the vulva mucosa (n = 2) as originally described in the dog (210) or in the subcutaneous layer of the ear (n = 9) to test female response to the implant at an alternative site. Implants were placed during October and November, which falls within North American breeding season (October – February) yet is early enough to avoid implanting females after they have already cycled. Implants were removed on d 7 (n = 3), 9 (n = 5) or 11 (n = 3) to identify whether time of removal had an effect on female response to the treatment. On the day of implant removal, females were anesthetized and the implant removed by creating a small incision at the implant site, debriding it of all hormone residue by flushing with sterile saline and the incision site was sutured. At the time of implant removal, unpaired females were injected intramuscularly with either

0.0375 mg (93.75 IU in 0.1 ml) reLH (n = 3; Equi-pur, AspenBio Pharma Inc., Castle Rock, CO), 0.1 mL sterile saline (n = 1) or left untreated (n = 3).

Fecal Collection and Hormone Extraction

Fresh fecal samples (within 12 h of defecation) were collected from females 5-7 d/wk starting approximately 1 month prior to hormone insertion, to establish baseline values, and continued until 2 months following the removal of the implant, to account for gestation or pseudopregnancy. Food coloring was added to the diet of paired females to distinguish experimental feces from those of their mates. Fecal samples were placed in individual Ziploc bags, labeled with the animal's identification, location and the date and stored at -20 ˚C until being transported to SCBI for hormonal analysis.

Fecal hormone extraction was performed as previously described (211). Briefly, fecal samples were freeze-dried in a lyophilizer (Labconco, Kansas City, MO), crushed in sample bags and sifted to separate fecal powder from undigested matter. Powdered feces were stored frozen (-20 ˚C) in 12 x 75 mm plastic tubes until extraction at which time a 0.2 g aliquot of well-mixed powder was shaken for 30 min in 90% ethanol: 10% distilled water (Large capacity mixer, Glas-Col, Terre Haute, IN). Each sample was then centrifuged at 2200 rpm (Centra CL2, Thermo IEC, Marietta, OH) for 20 min and the supernatant collected. The pellet was resuspended in 5 mL of 90% ethanol and re-centrifuged at 2200 rpm for 20 min. The supernatant was recovered and pooled with the previous one, dried overnight and re-suspended in 1 mL ethanol (100%). Each sample was then vortexed, sonicated (Ultrasonic cleaner, Cole-Parmer, Vernon Hills, IL), dried down again and resuspended in 1mL dilution buffer (DB; 0.2 M NaH_2PO_4, 0.2 M

Na_2HPO_4, 0.2 M NaCl, pH 7.0) to a final dilution of 1:10 for storage. The mean extraction efficiency was 75.6 ± 0.3%. Samples were further diluted in DB for enzyme immunoassay analysis (1:1000-50,000).

Endocrine Analysis

Fecal progesterone metabolites (FPM) were quantified using a single antibody pregnane enzyme immunoassay (EIA) (212). Microtiter plates (96-well, Nunc-Immuno, Maxisorp; Fisher Scientific, PA) were coated with pregnane antibody (monoclonal pregnane CL425 1:10,000 dilution; C. Munro, University of California) for 18 – 24 h (4 ºC) before the addition of 50 µl duplicate aliquots of fecal extract and triplicates of pregnane standard (0.78 – 200 pg; Sigma-Aldrich, MO). Conjugated pregnane (progesterone-3CMO horseradish peroxidase; 1:40,000; C. Munro, University of California) was added (50 µl) to all wells containing sample or standard and allowed to incubate at room temperature for 2 h before plate washing (0.16 M NaCl, 0.05% tween 20). Substrate solution (ABTS in citrate buffer; Sigma Chemical Co., St. Louis, MO) was immediately added to all wells (100 µl) and absorbance read on a microtiter plate reader (reading filter, 405 nm; reference filter, 540 nm; Dynex MRX; Dynex Tehnologies, VA) when the maximum binding wells reached an optical density (OD) of 1.00. Inter-assay coefficients of variation (CV) for two internal controls (n = 58 assays) were 10.9% (mean binding, 29.1%) and 6.2% (mean binding, 69.2%). Intra-assay CV was <10%.

Data Analysis

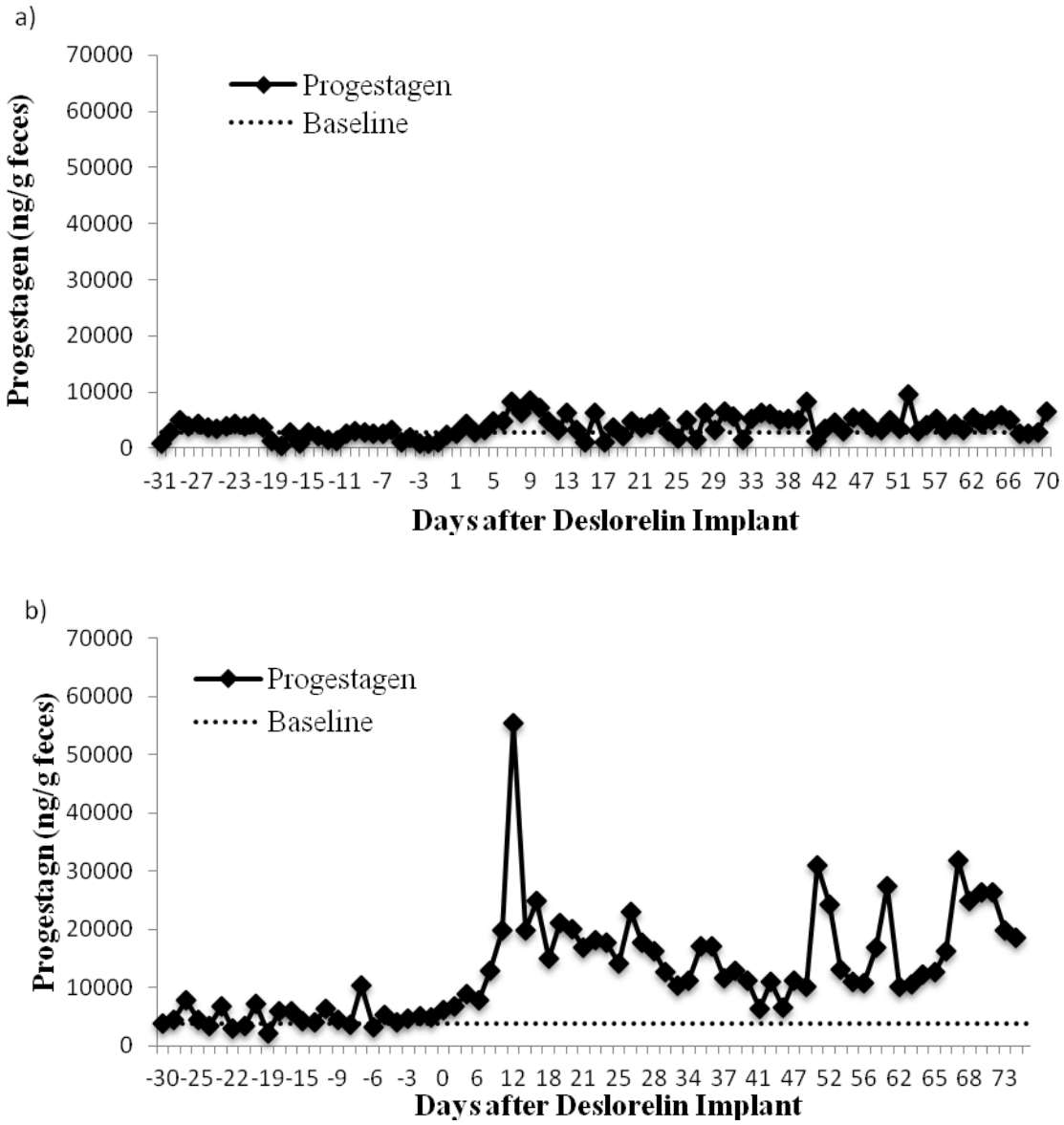
Longitudinal profiles of steroid metabolites were aligned to the day of deslorelin treatment (0 d). Baseline concentrations of fecal progestagens were determined by an iterative process whereby progestagen values in excess of 1.5 standard deviations (SD) of the baseline were removed from the data set until no values exceeded 1.5 SD of the baseline mean (213). Baseline progestagens were reported as the mean of the remaining values and this process was repeated each year for each female. Ovulation was designated by an increase in progestagen values at least 2 SD above baseline for at least 3 consecutive days after implant placement.

Results

Fecal progestagen metabolite concentrations for three out of the four singleton females receiving a deslorelin implant with no ovulatory agent remained at the baseline level throughout the study period, indicating that ovulation did not occur (Figure 4.1.a). However, one female (implanted in the ear for 7 d) exhibited a rise in FPM concentrations for 12 d following implant placement before decreasing back down to baseline levels (data not shown).

Induction of ovarian activity and subsequent ovulation was successful in 100% of the paired female maned wolves (n = 4 out of 4) (Figure 4.1.b). All of these females exhibited a rise in FPM above baseline starting on day 2.2 ± 0.2 (range 2 - 3 d) with the first peak occurring on day 12.5 ± 1.4 (range 9 – 16 d) and remaining elevated for a minimum of 65 d (Figure 4.1.b). All singleton females receiving a deslorelin implant with a subsequent reLH injection exhibited a rise in FPM above baseline beginning on

day 6.3 ± 1.7 (range 3 - 8 d). The first peak occurred 12.3 ± 1.6 day (range 10 – 14 d) post-implant and hormone levels remained elevated for at least 65 d (Figure 4.1.c). All of these females were implanted in the ear for 9 d.



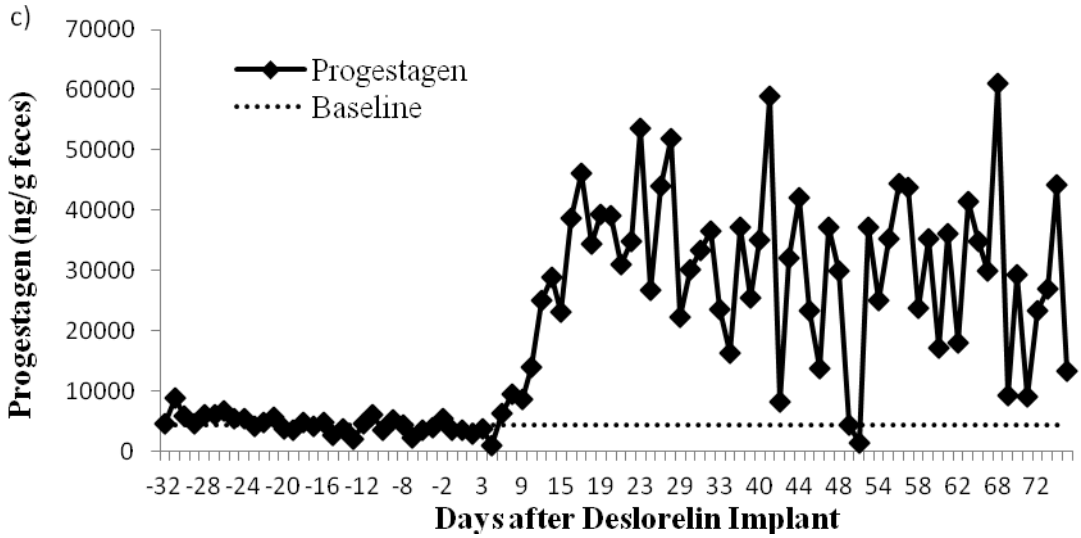


Figure 4.1 Fecal progestagen profiles of individual female maned wolves that were implanted with 2.1mg deslorelin on d 0 and either a) unpaired with no ovulation induction, b) paired with a male, or c) unpaired with ovulation induction upon removal of deslorelin implant.

Discussion

Understanding ovarian function and responsiveness to exogenous hormones is essential for successful AI. Induction of ovarian activity and ovulation is especially important for the reproductive management of monoestrus species, such as the maned wolf, as females will cycle just once during a lengthy breeding season (October – February) making it essential to develop a means to predict ovulation for timed AI. The present study is the first to investigate the influence of a GnRH agonist on ovarian activity in the maned wolf and is also the first to investigate ovulation induction in this species. Management condition (housed with or without a male) had significant influence on response to deslorelin and therefore, exogenous ovulation induction was required for singleton females to ovulate. Induction of ovarian activity was successful in

all paired females as well as in singleton females receiving an ovulatory agent, as indicated by a rise in FPM concentrations above baseline for at least 3 d. However, singleton females not receiving an ovulatory agent exhibited no signs of ovulation, with the exception of one female who may have ovulated.

In the present study, a 2.1mg deslorelin implant was 100% effective at inducing ovarian activity in paired female maned wolves. Ovulation was estimated to occur between 9 – 16 d (mean 12.5 ± 1.4 d) after implant insertion as projected by the first surge of fecal progestagen concentrations. This window is consistent with previous reports in bitches implanted with deslorelin for estrus induction (11-15 d (210); 11-19 d (94); 12 ± 2 d (214)). In gray wolves implanted with deslorelin, serum progesterone levels surged on 16 to 18 d and mating was seen on 11 to 17 d post-implant placement, though timing of ovulation was not estimated (92).

The finding that singleton females failed to ovulate after deslorelin treatment corroborated earlier reports that this species requires the presence of a male to ovulate, and thus, is an induced ovulator (35,215). The only other canid reported to demonstrate induced ovulation is the island fox *(Urocyon littoralis)* (93). In that study, 11 of 13 paired island foxes ovulated while 10 of 10 females housed singly did not ovulate. However, one island fox housed with another female might have ovulated suggesting that basic social stimulation may be sufficient to induce ovulation in this species. Two of the singleton maned wolves included in the present study were housed side by side and both failed to display fertile estrus as exhibited by a rise in FPM for the length of a pseudopregnangy (~ 65 d). However, one of the two exhibited a rise in progesterone for

12 d at levels (57 465.0 ng/g feces) comparable to those observed in females of prime breeding age post-ovulation (60 600.0 ± 4, 300.0 ng/g feces; (35). This suggests that ovulation may have occurred in this female, but the corpus luteum regressed prematurely.

Induced ovulation is most commonly reported in mammals among the Order Carnivora (93), and predominantly occurs in solitary species that exhibit multimale mating systems, such as felids (216). Although the maned wolf is solitary in the wild it is also monogamous (186) and thus, would not benefit from postcopulatory mate choice that felids exhibit (216). Because of this species' solitary nature and large home range, it is likely that the maned wolf benefits from induced ovulation by avoiding the risks of spontaneous ovulation occurring in the absence of a male (216).

Luteinizing hormone, in combination with FSH, has been investigated as an ovulatory agent in dogs but has not stimulated ovulation (72,74,217). Nonetheless, LH has been consistently successful at inducing ovulation in induced ovulatory species (30). In the present study, all of the female maned wolves receiving a deslorelin implant and subsequent reLH injection ovulated as exhibited by a rise in FPM above baseline for 3 d and FPM remained elevated for the length of a psuedopregnancy (~ 65 d). These results are extremely encouraging for the utilization of timed AI to preserve the genetics of valuable females that cannot be paired with a male due to reasons such as location, space limitations or aggression. Furthermore, our findings provide an opportunity for application to the reproductive management of another induced ovulator, the island fox, which is currently on the brink of extinction (218).

With a small captive population and very limited access to female maned wolves for research, the numbers available for participation in this study were minimal. For this reason, this study requires further investigation to increase the sample size and identify a narrower window of ovulation for timed AI. In the present study, FPM concentrations of ovulating females (both paired and unpaired) surged, on average, on day 12 with a range of 9 to 16 d. Thus, it is currently difficult to identify precisely when ovulation occurs using only FPM data for the purpose of timed AI. However, determining the timing of an LH surge would narrow this window (219) as ovulation in the dog occurs 48-60 h after the LH surge (220). A recent study in bottlenose dolphins (*Tursiops truncates*) has established methods to monitor urinary LH for timed AI utilizing immunochromatographic (ICG) assays originally designed to detect canine serum LH (221). Brown et al. (219) attempted to achieve the same success with urinary LH using immunoassays validated for elephant *(Elephas maximus)* serum but were unsuccessful. Thus, future research should explore the benefits of urinary LH in predicting the onset of ovulation for timed AI in maned wolves.

In a study conducted in the gray wolf, all females that received deslorelin became pregnant either by natural breeding or AI (92). However, none of the paired maned wolves in the present study became pregnant. Although age may have been a factor for one pair (11 yr female paired with a 12 yr male), the other three females were of typical breeding age (4, 6 and 7 yr). However, two of these females were housed with young males (2 and 3 yr) and one female was paired with a hand-reared male that had social anomalies, all of which could have prevented successful breeding attempts. Previous

work in domestic dogs has shown that induction of ovarian activity is successful initially but some females do not conceive or carry full term (222,223). This could be due to the time at which the implant is placed as females implanted in the late anestrous phase have shown better conception rates (224). Therefore, it is generally recommended that the implant be placed 200 d after the last estrus in the domestic dog (224). However, this recommendation is not pertinent to maned wolves as they are seasonal breeders (35) and therefore is not a possible explanation for the lack of pregnancies in the present study. While female maned wolves cycle anytime between October and February in North America, males do not exhibit their peak sperm concentration until December and January (39). In the present study, all implants in paired females were placed in either October (n = 3) or November (n = 1) and therefore, the lack of pregnancies could have been partly due to low sperm concentration in the males.

Previous work with GnRH agonists have revealed some concern over uterine health (225,226), premature luteal failure (227), and the individuals' ability to conceive post-treatment (228). For example, long-acting deslorelin implants (4.7 mg, Suprelorin®, Peptech Animal Health, NSW, Australia) that were used to suppress reproduction in sea otters *(Enhydra lutris)* lasted 2-3 yr longer than expected (228). In contrast, one female that had previously been implanted with Ovuplant in the present study conceived in the following season, showing that the 2.1 mg deslorelin implant has no impact on reproduction in subsequent breeding seasons for this maned wolf.

In conclusion, the present study demonstrates that management conditions (housed with or without a male) influence response to induction of ovarian activity

treatment in female maned wolves and exogenous LH is required to induce ovulation in singleton females. The findings obtained from this research further confirm the uniqueness of maned wolf reproductive biology and are the first steps towards the application of AI to better manage this threatened species *ex situ*.

CHAPTER 5: RESEARCH IMPACT AND SIGNIFICANCE

The research presented in this thesis filled gaps in our knowledge of the reproductive biology of the maned wolf (*Chrysocyon brachyurus*) while emphasizing the striking differences in reproductive biology between this unique canid and its domestic counterpart, the dog. The overall goal of this study was to study the intracacies of sperm cryosensivity and ovarian manipulation in maned wolves to aid in the development of AI in this species. I hypothesized that maned wolf spermatozoa were highly susceptible to osmotic stress, and that the type of cryoprotectant and differences in cooling and warming rates impacted cryosurvival. I also hypothesized that management conditions influenced female response to hormonal induction of ovarian activity and that an ovulatory agent would be required to induce ovulation in singleton females. The findings of this work provide a foundation for the continuation of research towards the establishment of assisted reproductive techniques for the genetic management of this species both *ex situ* and *in situ*.

The first study investigating tolerance of maned wolf spermatozoa to hypertonic solutions laid an important baseline for future research on sperm cryopreservation. We learned that maned wolf spermatozoa were highly intolerant of cell dehydration and volumetric changes but were able to withstand the high osmolalities of various cryoprotectants. From this, we can manipulate current cryopreservation protocols to

minimize volume excursions and dehydration by adding permeating cryoprotectants that enter the cell quickly enough to prevent significant volume changes. We identified two permeating cryoprotectants, glycerol and DMSO, that have the potential to protect cells in this manner without compromising cell function. Our results also showed that sucrose is an appropriate buffer for minimizing the effects of osmotic shock. These findings are an important first-step in developing an appropriate freezing medium for the successful cryopreservation of maned wolf spermatozoa. The results obtained from this research also emphasize the importance of recognizing sperm characteristics specific to different species even within the same taxonomic group. Nevertheless, similar to the domestic dog *(Canis lupus familiaris)*, maned wolf sperm are highly susceptible to osmotic stress associated with dehydration. Therefore, future studies should focus on overcoming the detrimental impact of osmotic stress on sperm viability. One potential strategy includes the addition of CLC to increase the fluidity of sperm plasma membrane.

The second study of this thesis focused on advancing knowledge about the impact of three key factors, cryoprotectants, cooling and warming rates, on cell cryosurvival of maned wolf spermatozoa. This study resulted in the first establishment of methods for cryopreserving maned wolf spermatozoa, which potentially achieve optimal cryosurvival with the use of DMSO as a cryoprotectant combined with a rapid cooling and warming rate. Furthermore, the field-friendly dry shipper approach, though not optimal, showed promising results as overall survival of sperm cryopreserved in this device exhibited comparable motility, plasma membrane- and acrosome integrity to spermatozoa frozen and thawed in laboratory conditions. Future research on optimizing the dry shipper

approach will widen the utilization of sperm cryopreservation for genetic management of this species. This technique can be carried out in zoos that lack the facilities and equipment required with traditional cryopreservation methods. Furthermore, this technique has application for the collection, preservation and transport of sperm from wild individuals to infuse new genetic diversity in the captive populations. Future research priorities with the goal of recovering at least 50% viable, fertile sperm after cryopreservation include (1) investigating a stepwise dilution approach for the removal of cryoprotectants, (2) testing the effects of a more rapid cooling rate for use with DMSO and (3) incorporating alternative cryoprotectants, such as EG, that may potentially optimize the dry shipper approach.

The third study of this thesis investigated how management conditions affect the hormonal induction of ovarian activity and subsequent ovulation. The results of this study demonstrated that the presence of a male is required to induce ovulation, but not estrus, in the maned wolf. However, ovulation can be induced using an exogenous hormone, recombinant LH, if needed. We can now predict ovulation for timed AI down to the week, which has never been done in this species; however further research on urinary LH may narrow this window down to a matter of hours. We can also potentially use this method, in an alternative approach, as a contraceptive. Females can be separated from their mates for a narrow window of time while ovarian activity and ovulation is induced. Being a monoestrus species, they theoretically should not re-cycle until the following breeding season. However, this approach requires further research before being recommended in practice. The findings from this study also have important

application to the genetic management of this species. With timed AI, females can potentially be inseminated with semen from males housed in other locations locally or globally. When used in combination with the progress made in studies 1 and 2, genetics from wild or deceased individuals can also be introduced into the population through AI with frozen semen.

Further investigation and application of the methods discussed in this thesis have the potential to improve the overall reproductive success of the *ex situ* maned wolf population by increasing genetic diversity. To achieve full genetic potential, it is essential that gene flow between the wild and captive population be optimized. Thus, further development of the research discussed herein is essential for maintaining a healthy and viable maned wolf population indefinitely.

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