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**POPULATION GENETICS AND SPERM PHYSIOLOGY ASSOCIATED WITH
GENOME RESOURCE BANKING IN THE ELD'S DEER**

by

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A thesis submitted to the Faculty of Graduate Studies
and Research of McGill University in partial fulfillment of
the requirements for the degree of Master of Science

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ABSTRACT

Genome resource banking (collection, storage and use of germ plasm) has emerged as a potentially powerful tool to assist in managing and conserving endangered species. The development of genome resource banks is multi-faceted. This thesis focused on aspects of population genetics and the phenomenon of sperm capacitation, a fundamental biological process crucial to the use of germ plasm for assisted breeding (i.e., artificial insemination, *in vitro* fertilization). Computerized simulation modeling, using two endangered species models, the Eld's deer (*Cervus eldi thamin*) and Przewalski's horse (*Equus przewalskii*) clearly demonstrated the value of certain sperm storage and use strategies for maintaining genetic diversity in captive populations. Laboratory studies with Eld's deer sperm examining a host of conventional strategies (developed in livestock) demonstrated clear challenges in provoking capacitation and the acrosome reaction in the Eld's deer. This finding reaffirmed the importance of species-specific studies in wildlife research.

RÉSUMÉ

La création de banques génomiques (collection, entreposage et utilisation de plasmides) s'est avérée être un outil potentiel puissant pour l'aide à la gestion et à la conservation des espèces menacées d'extinction. Le développement de banques génomiques comporte plusieurs facettes. Cette thèse se concentre sur les aspects de la génétique des populations et sur les phénomènes de la capacitation des spermatozoïdes, un processus biologique fondamental et crucial pour l'utilisation de plasmides pour la reproduction assistée (c'est à dire l'insémination artificielle, la fertilisation *in vitro*). L'application de systèmes de modélisation informatique à deux espèces modèles en voie d'extinction soit, le cerf Eld (*Cervus eldi thamin*) et le cheval Przewalski (*Equus przewalskii*) a clairement démontré l'importance d'entreposer certains spermatozoïdes ainsi que celle de l'utilisation de stratégies pour maintenir la diversité génétique des populations captives. Les études de laboratoire du sperme du cerf Eld examinant une série de stratégies conventionnelles (développées pour la gestion du bétail) ont démontré les défis rencontrés lors de la provocation de la capacitation ainsi que la réaction acrosomique du cerf Eld. Ce résultat affirme l'importance des études propres aux espèces dans la recherche dans le domaine de la faune.

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PREFACE

This thesis consists of two separate but interrelated subjects. I am responsible for all data collection, statistical analyses, and writing of the manuscripts. The computer simulations for section 2 were performed with the guidance of Dr. Jon Ballou, Population Manager at the Smithsonian Institution's Department of Zoological Research. The sperm collection trials were performed at the Conservation & Research Center under the guidance of Dr. Steven Monfort and Dr. David Wildt, senior scientists of the Reproductive Physiology Unit. These three supervisors, in addition to my supervisor at McGill University, Dr. David Bird, provided editing.

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SECTION 1:

LITERATURE REVIEW

General Introduction

The extinction of animal species continues at an alarming rate. The long-term preservation of the earth's remaining biodiversity is becoming increasingly dependent upon populations maintained in "artificial environments" (i.e., captive management programs). Despite consensus among conservationists that ecosystem preservation is our highest priority, human encroachment continues to limit habitat needed to ensure the maintenance of genetically-viable animal populations (Shonewald-Cox, 1983). As Conway (1986) eloquently stated "...the preservation of a segment of grizzly bear habitat without grizzly bears, condor country without condors, or the Mountains of the Moon without Mountain gorillas seems like saving the husk without the kernel."

Maintenance of genetically viable wildlife populations in captivity requires pre-emptive biological research designed to optimize propagation (Wildt, 1992, 1997; Wildt *et al.*, 1997). Results derived from such studies often provide useful insights that can be extrapolated to wild populations. In fact, captive populations often serve as the only available resource for conducting systematic wildlife research. Wild animals in appropriate captive conditions also can be powerful ambassadors for enhancing public awareness of the plight of endangered species. Additionally, captive populations can serve as valuable genetic reservoirs for species being reintroduced into native habitats.

Beginning in 1981, the American Association of Zoological Parks and Aquariums (AAZPA) (now known as the American Zoo and Aquarium Association [AZA]) pioneered the concept of small population genetic management for certain zoo-held species. Species Survival Plans (SSPs) were established for a few high-profile, endangered species to maximize genetic variation through selective breeding based on the mean kinship (MK) concept (Hutchins & Wiese, 1991; Ballou & Lacy, 1995; Robinson & Challinor, 1995). The MK of an animal is a measure of its average

relatedness to all animals within the population (Johnston & Lacy, 1991; Ballou & Lacy, 1995). More precisely, the MK of an individual is the average value of its coefficients of kinship to all living animals (including itself) within the population. Animals having high MKs have many relatives within a population, and therefore, are deemed to be less genetically-valuable than counterparts with low MKs. Animals with the latter trait have few relatives within a population and are likely to be considered critical by the species managers for sustaining a genetically vigorous population.

According to fundamental SSP principles, optimal pairings are those that are among individuals bearing similar, but low, MKs (Wiese & Willis, 1993). It is considered undesirable to combine genes from a valuable individual with those of a low-priority member of the managed population. Such a strategy makes it impossible to increase the frequency of rare alleles through future breedings without simultaneously augmenting the frequency of less desirable, common alleles. Although the goal is always to minimize the inbreeding coefficients (f) of resulting offspring, SSPs also must consider a variety of practical considerations such as (1) ages of potential mates, (2) geographic proximity of potential mates, and (3) costs and logistics associated with transporting animals between cooperating zoos (Wiese & Willis, 1993). In addition to maintaining genetic variation in zoo-maintained species, SSPs also seek to facilitate reintroduction of species into nature, such as has been done for the Przewalski's horse (*Equus przewalskii*), golden lion tamarin (*Leontopithecus rosalia*), scimitar-horned oryx (*Oryx dammah*), and black-footed ferret (*Mustela nigripes*) (Robinson & Challinor, 1995). Finally, SSPs also promote basic and applied research, especially investigations (such as the one here) that may ultimately serve to enhance the genetic management and conservation of rare species.

Basic research often advances new ideas, and this is precisely what led to considering the value of establishing genome resource banks (GRBs). GRBs rely firstly on the science of cryobiology for the purpose of storing biomaterials in a safe,

organized and viable fashion, often for many years (Wildt, 1997). Cryopreserving biomaterials to benefit management and conservation is a powerful tool, especially for germ plasm used in conjunction with assisted reproductive techniques. Cryopreservation complements reproductive technologies such as artificial insemination (AI), *in vitro* fertilization (IVF), and embryo transfer (ET) which now are commonly used to enhance domestic livestock production and to overcome some types of human infertility. These techniques also are becoming increasingly recognized as potential tools for augmenting the captive propagation of wildlife species (Wildt, 1992, 1997; Wildt *et al.*, 1997). The establishment of GRBs would provide the potential to conserve a greater number of total species because some of the required genetic variability (i.e., genes) could be maintained in liquid nitrogen, thus freeing animal enclosure space for other at-risk species.

I. Genome Resource Banks and Population Management

Benefits of Cryopreservation and Genome Resource Banks

Using cryopreserved gametes and embryos has potential for facilitating cost-effective movement of genetic material between managed populations (Wildt, 1992, 1997; Wildt *et al.*, 1997). Such a strategy benefits conservation managers by obviating the need for translocating animals from one location to another. This alternative permits simplified transfer of sperm and embryos that can be used for offspring production via assisted reproductive techniques. Advanced approaches now also permit transferring genetic material between wild and zoo populations, thereby eliminating the need to remove animals from the wild while bolstering genetic variation in captive populations (Wildt *et al.*, 1997). GRBs used in concert with reproductive technologies also have the potential for overcoming behavioral incompatibilities among genetically desirable mating pairs.

The storage of viable germ plasm permits generation length to be extended

indefinitely. Generation length is defined as the average time required for an individual to replace itself in the population. The longer the animal reproduces, the greater its contribution to the population's generation length (Ballou, 1992). Because genetic diversity is lost with each generation, extending the length reduces the number of generations within a given time interval, lessening the rate of losing genetic diversity. Prolonging generation interval is advantageous to captive propagation programs because this strategy permits small populations to maintain levels of genetic variation comparable to larger populations having shorter generation intervals.

Space-related problems always seriously limit the ability of institutions to effectively protect and manage endangered species. Costs of building and maintaining facilities that will accommodate viable populations can be prohibitive. Frozen storage of viable gametes and embryos from genetically-valuable individuals preserves genes indefinitely, making them available for future use. The capability of maintaining smaller populations without sacrificing genetic diversity frees up enclosure space for other species requiring conservation attention (Ballou, 1992). GRBs also serve to insure endangered species against unforeseeable emergencies, such as environmental catastrophes, including epizootics (Wildt, 1992, 1997; Wildt *et al.*, 1997).

GRBs also provide potential economic benefits, especially in the context of food animal production (Wildt, 1997; Wildt *et al.*, 1997). For example, hybridizations between domestic and wild species (e.g., domestic cattle bred with wild cattle sperm) may result in offspring that are parasite- and heat-resistant and/or provide more meat or milk. Finally, it is worth emphasizing that GRBs should contain tissues, blood products, and DNA (as well as gametes and embryos). Thus, these reservoirs should contain biomaterials available to conservation and non-conservation scientists alike useful for many purposes beyond only propagation, including measuring levels of genetic variation, identifying parentage and conducting disease surveillance and forensics (Wildt, 1992, 1997; Wildt *et al.*, 1997).

A Model GRB: The Siberian and Sumatran Tiger

A comprehensive written Action Plan for establishing an effective GRB is critically important since numerous complex, interrelated factors must be addressed (Wildt, 1997). For example, it is essential to carefully describe standardized protocols for proper handling of biomaterials, especially since rare and valuable resources will be shared among institutions. In 1992, the IUCN's Conservation Breeding Specialist Group (CBSG) established guidelines for the development of GRB action plans. Ten main issue categories were identified as critically important to include in an action plan, ranging from species life history to the amount of germ plasm (semen) necessary for efficient cryopreservation (Wildt, 1997). A GRB action plan was written in 1995 (Wildt *et al.*, 1995) for the Siberian (*Panthera tigris altaica*) and Sumatran (*P. t. sumatrae*) tiger populations in North America. This document described the demographic and genetic criteria for selecting individuals to be cryobanked, and provided recommendations for enhancing global management of endangered tigers. The proposal identified several major challenges associated with establishing GRBs, including the requirement for relatively large quantities of cryopreserved germ plasm needed to meet genetic management goals.

A Model Species: The Eld's Deer

The Eld's deer is an endangered, subtropical species from Southeast Asia. There are three subspecies. The Burmese brow-antlered deer (*Cervus eldi thamin*) is scattered throughout central Myanmar (formerly known as Burma, 10-20°N latitude) (Wemmer & Grodinsky, 1988), and fewer than 2000 individuals probably remain in the wild (McShea *et al.*, 1999). The Manipur subspecies (*C. e. eldi*) exists in the wild, solely in Keibul Lamajo, a 40 sq km national park located in northeastern India (Singh, 1980). The other subspecies, *C. e. siamensis*, exists in small herds sparsely distributed between Thailand and Vietnam where it is threatened with extinction (Whitehead,

1993). The Eld's deer has become endangered as a result of habitat loss and over-hunting (Talbot, 1960). Approximately 120 Eld's deer (*C. e. thamin*) are maintained in North America zoos and breeding facilities, with the largest herds held at the San Diego Zoo (California), the Wildlife Conservation Society (New York), and the National Zoological Park's Conservation & Research Center (Virginia).

Most of what is known about Eld's deer biology is gleaned from intensive studies of captive populations. Reproductive-endocrine studies have revealed that the species is a seasonally polyestrous, spontaneous ovulator with estrus commencing in late winter or early spring (Wemmer & Grodinsky, 1988; Monfort *et al.*, 1990). Estrus is followed by a period of seasonal anestrus that commences in autumn and lasts for approximately 4 to 6 months. Females are able to conceive as yearlings, and the majority of births occur throughout the months of September and November, after an eight month gestation. Males can ejaculate viable sperm at one year of age, and indications of behavioral "rut" (e.g., increased aggression) are observed in late winter and early spring (Monfort *et al.*, 1993a).

Monfort *et al.* (1993a) examined seasonal variations in ejaculate quality of Eld's deer stags maintained in captivity in North America. Motile sperm were obtained from all males in all seasons, with the highest concentration occurring in winter and spring. The most structurally normal spermatozoa per ejaculate were produced in the winter and spring. Stags exhibited a circannual, hypothalamic-pituitary-gonadal cycle, with onset of pituitary activation in the autumn and winter. Peak gonadal activity occurred during the winter and spring as daylengths were increasing. It remains unknown if fertility is regulated by photoperiod or by endogenous regulatory rhythms. Interesting features of Eld's deer biology are the presence of a seasonal reproductive rhythm (unlike other tropical cervids that demonstrate reproductive cycles throughout the year), and that reproductive cycles are not altered upon relocation to temperate environments (Monfort *et al.*, 1993a,b; Hosack *et al.*, 1997). Additionally, Eld's deer

exhibit reproductive cycles that are shifted six months compared to temperate cervid species, such as white-tailed deer (*Odocoileus virginianus*) living at the same northern latitude (Hosack *et al.*, 1997).

Assisted reproduction (AI, IVF) has been proposed as a potentially viable tool for contributing to the management of the North American herd (Monfort *et al.*, 1993c). Studies with frozen-thawed sperm have been carried out and post-thaw sperm motilities of 60 to 70% have been achieved (Monfort *et al.*, 1993c). Morphology assessments of post-thaw ejaculates have revealed that 85 to 90% of thawed sperm appear morphologically normal. Multiple offspring also have been produced following AI with frozen-thawed spermatozoa (Monfort *et al.*, 1993c). Although frozen-thawed Eld's deer sperm has been used with some success in a few AI trials, recent attempts to produce multiple embryos using IVF have been unsuccessful (S. Monfort, pers. comm.). *In vitro* insemination of oocytes matured *in vivo* resulted in sperm binding to the zona pellucida, but no signs of penetration into the cytoplasm. IVF also has been attempted with oocytes matured *in vitro*. Only one 13-cell embryo was produced, which may have resulted from either genuine fertilization or parthenogenetic cleavage (R. Spindler, unpubl. data).

Assisted reproduction has enormous potential for enhancing the genetic management of Eld's deer by ensuring optimal pairings (regardless of geographic location of the animals) to maximize population genetic diversity. Fewer living individuals would be required in North American herds to maintain a genetically viable population because a portion of the gene pool would be cryopreserved. Genes (as sperm) could be maintained for decades before being reinfused into the population. A GRB also would eliminate the need to ship Eld's deer (a highly "stress-sensitive" species) for breeding purposes. Finally, systematic cryostorage of valuable genes (i.e., sperm) would "insure" the North American population from unexpected catastrophes.

Basic Population Genetics and Genetic Diversity

Living organisms have thousands of genes located on their chromosomes (Foose & Ballou, 1988; Ballou & Foose, 1996). The precise location of a gene on a chromosome is its locus, and the terms gene and locus are often used interchangeably. Genes may appear in varied forms, referred to as alleles, with different alleles producing different biological outcomes in a given organism (i.e., eye or hair colour). Most animal species are diploid, meaning that they have two copies of each gene. One copy is inherited from each parent, and each copy may occur in diverse forms. A locus may be identified as monomorphic (i.e., one allele per gene) or polymorphic (i.e., several alleles per gene). Genetic diversity arises when polymorphic loci are present. Both copies of a gene may designate the same allele; hence, an animal is homozygous for that locus, or different alleles may be represented resulting in a heterozygous locus. The sum of an individual's genes is its genome, and the total aggregate of alleles for all the genes within the population of a species makes up its gene pool.

Genetic diversity includes both allelic diversity and heterozygosity (Chesser *et al.*, 1980; Foose & Ballou, 1988; Willis & Wiese, 1993; Ballou & Foose, 1996). Allelic diversity designates the number of different alleles found at a given locus within a population. Conversely, heterozygosity is the percentage of loci that are heterozygous, either within a population or an individual. Genetic diversity is crucial for populations, as well as individuals. Allelic diversity allows populations to adapt to inconstant environments, whereas individual fitness is related to level of heterozygosity (Allendorf, 1986).

Genetic variation may be lost in small populations due to genetic drift (Chesser *et al.*, 1980; Foose & Ballou, 1988; Johnston & Lacy, 1991; Ballou & Foose, 1996). Alleles are passed randomly from parents to offspring and represent only a portion of the alleles present in the parental generation. If few progeny are produced, then it is inevitable that allelic frequencies will change from one generation to the next, with a

chance that some alleles will be lost completely from the population.

Loss of genetic diversity is both time- and population size-dependent (Ballou & Foose, 1996). For example, the smaller the population size, the more rapidly genetic diversity is lost. The more time that passes by, the greater the overall loss. Therefore, it is important for conservation managers to establish captive populations sufficiently large to maintain high levels of genetic diversity. Captive populations of endangered species are likely to require long-term management strategies for optimizing genetic diversity for hundreds of years.

One proposed strategy recommends that 90% of the heterozygosity present in founder populations be preserved for 200 years (Soulé *et al.*, 1986). This idea was based on the assumptions that current rates of habitat destruction would continue unabated, and that assisted reproductive technologies would eventually become effective tools for augmenting the genetic management of small, fragmented captive populations. Contemporary population management paradigms now use 100-year time spans rather than 200 years, which permits increased flexibility in maintaining smaller captive populations while still meeting prescribed genetic goals (Foose *et al.*, 1995).

Maintenance of genetic diversity in managed populations is critical for the following reasons. First, it is required by populations to allow adaptation to changing environments, a vital characteristic when dealing with species designated for reintroduction (Ballou, 1984; Foose & Ballou, 1988). Specific genetic factors are necessary for populations to endure and recover from habitat stresses and natural disasters (Mace, 1986). Second, genetic diversity relates to individual fitness and therefore, the ability to subsist and procreate (Ballou, 1984; Foose & Ballou, 1988). Increased levels of homozygosity also may enhance expression of deleterious recessive genes (Ballou, 1984). Decreased levels of fitness, due to inbreeding, have been observed in almost all captive species that have been examined, including ungulates, small mammals, and primates (Ballou & Ralls, 1982; Ralls & Ballou, 1983).

Captive Management Strategies

Inter-institutional cooperation is essential for ensuring the maintenance of genetically-viable populations. In contrast to the genetic management strategies used for domestic livestock (i.e., bovine, domestication or selection for a specific trait), endangered species programs strive to maximize genetic variation while minimizing selection (Frankham *et al.*, 1986). Endangered species programs also seek to provide stability, both in terms of population size and structure, which minimizes extinction risk (Mace, 1989). Thus, the hallmarks of effective endangered species captive breeding programs are to sustain genetic diversity and demographic stability (Mace, 1989; Ballou & Foose, 1996).

Frankham *et al.* (1986) have defined four types of captive management strategies:

- (1) select for individuals well-adapted to captive environments (i.e., display species);
- (2) manage the population to maximize genetic diversity while maintaining demographic stability (i.e., long-term support species, such as the ones studied in this thesis);
- (3) maximize reproduction to supply animals for reintroduction (i.e., breed and release species);
- (4) augment reproduction in species that breed poorly in captivity; these species may subsequently be managed under strategy #2 (above).

Although captive populations are naturally fragmented as a result of maintaining subpopulations in geographically disparate locations (i.e., zoos), wild populations also are commonly isolated due to habitat fragmentation. Thus, both wild and captive populations suffer similar adverse demographic and genetic consequences related to population fragmentation. For this reason, much of the knowledge gained by developing strategies to enhance the genetic management of captive populations should be useful for managing wild populations (Mace, 1989).

Genetic and Demographic Considerations

One of the first considerations when initiating a management program is the captive carrying capacity for the species of interest. As defined by Foose and Ballou (1988), the captive carrying capacity is "...the optimum number of individuals to be maintained over the long term and will represent a compromise between the minimum necessary for genetic and demographic viability and the maximum that can be accommodated without excluding other taxa from captive programs." The minimum viable population (MVP) size of a captive population represents the other end of the spectrum, meaning the lower limit of the carrying capacity for a population (Foose & Ballou, 1988; Ballou & Foose, 1996). When determining a MVP, it is important to consider the demographic and genetic goals of a program, along with the biological attributes of a population.

Information gained from genetic analyses provides one component of breeding management plans. The goal is to generate as much information as possible about the genetic characteristics of a population, including determining distribution of founder genes, how living animals are related to one another, and estimates of a population's ability to maintain its genetic variation (Ballou & Foose, 1996).

Demographic analyses are used (with genetic analyses) to formulate management plans by examining the dynamics of a population, including population growth rates, annual reproductive rates, fertility, survival rates, and age structure (Ballou & Foose, 1996). These analyses can also determine the carrying capacity of a population in the face of known genetic and resource constraints, and the number of annual births needed to achieve desired population growth rates.

Therefore, genetic and demographic analyses complement one another by combining information on the dynamics of a population with its genetic characteristics and goals.

There is a wide array of genetic and demographic factors to consider that are

essential to a population's integrity (Foose, 1980; Foose & Ballou, 1988; Ballou & Foose, 1996) including (among others):

(1) Pedigree analysis. Resolving the pedigree of each individual within the population permits one to determine if a population has experienced one or more genetic bottlenecks in its recent or ancient history, as well as the relative importance of all the ancestors.

(2) Determination of founders. Wild-born individuals, having no known relatives in a captive population generally are assumed to be unrelated to one another, and are designated as "founders." The genetic contribution of founders to each living animal within the population can be tracked, as can their genetic contribution to the entire population. The term "founder contribution" refers to "the percentage of an individual's or population's genes that have descended from each founder" (Ballou & Foose, 1996). Significant information also may be obtained by examining what proportion of a founder's genes have managed to survive to the present population. This phenomenon is referred to as "gene retention" or "gene survival." Overrepresentation of founders is avoided by limiting reproduction to "genetically important" individuals. MK often is used as an indicator for ranking individuals, whereas inbreeding coefficient is used to determine relatedness.

(3) Deduction of genetically effective population size (N_e). This factor provides an index of a population's ability to retain genetic diversity through time. Rate of loss of genetic diversity per generation is expressed as $1/2N_e$. Three main variables are useful in making this deduction: a) number of breeders in a population; b) their sex ratio; and c) number of offspring they have produced throughout their lives.

(4) Miscellaneous factors. Accurate information about current population size, as well as the names and locations of cooperating institutions, is essential, along with the age and sex of each individual in the population. Additional factors to consider include survivorship and fertility rate of both sexes, generation lengths (as described earlier),

and a population's growth rate (used to deduce MVP size and carrying capacity).

Studbooks

A studbook tracks a species through time and contains the following information: (1) identities of all animals registered (living and dead); (2) sexes; (3) parentage; (4) locations where an individual has been kept; and (5) dates of birth and death (Glatston, 1986; Ballou & Foose, 1996). Studbooks provide the essential data for demographic analyses, the calculation of inbreeding coefficients, and the determination of optimal genetic pairings, all of which are used to retain genetic diversity and prevent inbreeding. Studbook data generally are managed using computerized studbook management and analysis software packages, such as the Single Population Animal Record Keeping System (SPARKS).

II. Sperm Capacitation: A Prerequisite for Successful Fertilization

Research

The task of establishing a GRB for a given species is multi-faceted. Many types of expertise are required (Wildt, 1997) in population genetics and reproductive biology, including sperm physiology. The ability of sperm to survive a cryo-stress is species-specific (Howard *et al.*, 1986). Therefore, the development of a sperm freezing protocol for a given species requires thorough investigation into the basic characteristics (both form and function) of spermatozoa across a range of temperatures. Numerous factors must be considered to ensure that a significant proportion of the sperm within an ejaculate survives the extremes imposed by freezing to a temperature suitable for storing and thawing to body temperature.

Freshly ejaculated mammalian spermatozoa are unable to fertilize oocytes until undergoing a period of adjustment known as "capacitation" (Austin, 1951). The physiological changes that characterize capacitation were first observed nearly 50 years

ago in independent studies by Austin (1951) and Chang (1951) who studied rats and rabbits, respectively. Both researchers concluded that spermatozoa require time within the female reproductive tract to acquire the capacity to fertilize an oocyte. It has since been confirmed that capacitation usually occurs while sperm are moving through the female reproductive tract (Yanagimachi, 1981; Bedford, 1983; Katz *et al.*, 1989), and that capacitation is induced by epithelial cells bordering the reproductive tract or specific factors within their secretions.

Yanagimachi (1990) described capacitation as "a result of the evolutionary adaptation of mammalian spermatozoa to internal fertilization." In the case of mammals, spermatozoa must travel from one end of the female reproductive tract to the other to reach the site of sperm-oocyte interaction. Epididymal glycoproteins "coat" sperm to protect them during transit through the female reproductive tract (Yanagimachi, 1981). Although additional glycoprotein coats may be applied to sperm by seminal plasma at the moment of ejaculation, all of these layers are believed to be removed during the capacitation process (Yanagimachi, 1990). This effect is significant because it permits the interaction of specific sperm receptors sites with oocyte receptors so that the acrosome reaction (AR) may proceed (Piko, 1969).

Yanagimachi and Chang (1963) first demonstrated that sperm capacitation could be induced *in vitro* when epididymal hamster spermatozoa were incubated at 37°C in Tyrode's solution or Medium 199 in the presence of oocytes. Parrish *et al.* (1988) demonstrated that bovine sperm capacitation could be induced *in vitro* within 4 h by incorporating heparin into the incubation medium. Subsequently, Park *et al.* (1989) discovered that a medium containing caffeine, as well as heparin, induced capacitation of bovine spermatozoa within 1 h. Heparin also has been used to achieve sperm capacitation in bison (*Bos bison*, McHugh *et al.*, 1995), buffalo (*Bubalus bubalis*, Madan *et al.*, 1994), gaur (*B. gaurus*, Johnston *et al.*, 1994), and white-tailed deer (G. Killian, pers. comm.).

The AR is often used as an index of whether capacitation has occurred *in vitro*. Membrane changes associated with capacitation precede fusion of the plasma and outer acrosomal membranes necessary for the AR (Yanagimachi & Usui, 1974; Bedford, 1983). As described by Kopf (1999), the AR is "an exocytotic event that is an absolute prerequisite to successful fertilization and occurs following sperm binding to the zona pellucida of the egg; exocytosis involves the fusion and vesiculation of the plasma membrane overlying the acrosome and the outer acrosomal membrane." The AR ensues when spermatozoa interact with the zona pellucida (ZP), the extracellular glycoprotein layer surrounding the mammalian ovum (Wassarman, 1987; Yanagimachi, 1994; Kopf, 1999). The ZP has been shown to be responsible for promoting the AR in many species, both *in vivo* and *in vitro*, and is suspected to be the universal inducer in all mammals *in vivo*. Progesterone also has been found to induce the AR, especially in human sperm, and presumably by encouraging an influx of intracellular calcium (Ca^{2+}) (Kopf, 1999). Acrosome reacted sperm penetrate the ZP and fertilize the ovum by fusing with its plasma membrane. The AR has been induced *in vitro* in the guinea pig (Yanagimachi, 1975), bull (Byrd, 1981), human (Byrd & Wolf, 1986; Jaiswal *et al.*, 1998), boar (Peterson *et al.*, 1978), and domestic cat (Long *et al.*, 1996) by supplementing culture medium with calcium ionophore. Lysophosphatidylcholine (LC) has also been shown to induce the AR in the hamster (Ohzu & Yanagimachi, 1982; Llanos & Meizel, 1983), bull (Parrish *et al.*, 1988, 1989), human (Byrd & Wolf, 1986), and scimitar-horned oryx (Roth *et al.*, 1998). The effects of these agents on inducing the AR in capacitated sperm can be observed by preparing smears of diluted sperm on glass slides and then applying a stain that binds differentially to intact and non-intact acrosomes. However, the most reliable end-point for assessing capacitation is the ability of sperm to penetrate oocytes (Cross & Meizel, 1989; Leibfried-Rutledge *et al.*, 1997).

In addition to sperm membrane changes, capacitation also involves an alteration

in sperm motility termed "hyperactivation," which has been observed in the hamster and guinea pig (Katz & Yanagimachi, 1981; Cummins & Yanagimachi, 1986; Suarez *et al.*, 1991), rabbit (Suarez *et al.*, 1983), human (Mortimer & Mortimer, 1990), and mouse (Suarez & Osman, 1987). Hyperactivation involves a change in sperm motility from low amplitude, symmetrical flagellar beats (caudal epididymal or freshly ejaculated sperm) to an erratic "whiplash-like" flagellar motion. This form of motility was first observed by Yanagimachi (1969, 1970) in hamster sperm incubated in follicular fluid or serum.

When viewed in medium having low viscosity, hyperactivated sperm often swim in a circular pattern (Suarez *et al.*, 1991). However, highly viscous mucous is secreted within the oviduct, which shifts the motility pattern of hyperactivated sperm to a more progressive trajectory (Suarez *et al.*, 1991; Suarez & Dai, 1992). Therefore, it appears that the purpose of hyperactivated sperm motility is to contend with the mucoid oviductal secretions and the extracellular matrix of the cumulus oophorus surrounding the freshly ovulated ovum (Suarez & Dai, 1992).

Hypotheses have been proposed for the role of hyperactivation in the fertilization process. Hyperactivation may serve: (1) as a sperm propulsion method for locating the ovum (Katz *et al.*, 1978; Suarez *et al.*, 1983); (2) to increase thrust to penetrate the ovum's cumulus and zona pellucida (Katz *et al.*, 1978; Katz & Yanagimachi, 1980); and/or (3) to prevent sperm from becoming attached to the oviductal wall (Suarez *et al.*, 1983). Because hyperactivated sperm frequently change direction, they are often able to avoid pockets and grooves in the oviductal mucosa, thereby increasing the chance of discovering an ovum (Suarez & Osman, 1987).

Little is known about the biochemical pathways associated with hyperactivation, but it has been determined that Ca^{2+} generally is necessary to sustain hyperactivated motility *in vitro* (Fraser, 1987). Furthermore, hyperactivation is reversible and depends on the maintenance of intracellular Ca^{2+} at an elevated level

(Suarez, 1996). Inducers that initiate hyperactivation via a signal transduction pathway have been proposed to exert their effects in the peri-ovulatory period (Cooper *et al.*, 1979), thereby guaranteeing sperm presence in the oviduct near ovulation.

It should be noted that capacitation and hyperactivation are not necessarily linked (Suarez, 1996). The two events often occur concurrently, but also have been observed to occur independently. Thus, the two phenomena may well be regulated by different pathways.

Factors Regulating Sperm Capacitation and the AR

Capacitation has been achieved *in vitro* with cauda epididymal and/or ejaculated sperm exposed to an assortment of stimuli (Visconti & Kopf, 1998). Media used for these studies are usually similar in nature to oviductal fluid and contain energy substrates (pyruvate, lactate, and glucose; depending on species). The medium also usually contains a protein source (commonly serum albumin), sodium bicarbonate, and Ca^{2+} (Visconti & Kopf, 1998). The fact that capacitation may be induced *in vitro* in the absence of any biological fluids (i.e., oviductal fluid) suggests that spermatozoa are capable of initiating the capacitation process without oviductal stimulators (Visconti & Kopf, 1998). Despite lack of a universal capacitation medium, certain factors such as serum albumin, Ca^{2+} , and bicarbonate are known to be essential to *in vitro* inducement of capacitation (Visconti & Kopf, 1998).

Cations. Extracellular Ca^{2+} is suspected to be crucial for both sperm capacitation and the AR (Yanagimachi & Usui, 1974), and both events are distinguished by an intracellular Ca^{2+} rise (Singh *et al.*, 1978; Didion & Graves, 1989; Leclerc *et al.*, 1992; Bedford & Cross, 1999). In fact, evidence indicates that Ca^{2+} is the most critical ion in regulating sperm function (Fraser, 1995). At least three systems are believed to regulate intracellular Ca^{2+} concentration: (1) a Ca^{2+} -ATPase that pumps Ca^{2+} from the cell (Bradley & Forrester, 1982; Breitbart *et al.*, 1983, 1984); (2) a Na^{+} -

Ca^{2+} exchanger that pumps Ca^{2+} out and sodium in (Bradley & Forrester, 1980), or vice versa (Rufo *et al.*, 1984); and (3) voltage-operated channels (VOCC) that permit Ca^{2+} inflow (Babcock & Pfeiffer, 1987; Fraser & McIntyre, 1989; Florman *et al.*, 1992; Fraser, 1993; Arnoult *et al.*, 1996). More specifically, these channels appear to be T-type (VOCC_T) or low voltage-activated Ca^{2+} channels (Garcia & Meizel, 1999). Ca^{2+} channels do not appear critical to sperm capacitation, but do appear to be essential for the AR to occur (Fraser, 1995). This hypothesis is supported by experiments in which a "channel blocker" such as nifedipine inhibited the AR, but not capacitation (Fraser & McIntyre, 1989; Fraser *et al.*, 1993).

Because Ca^{2+} influx occurs during sperm capacitation and the AR, it is possible that different systems modulate intracellular Ca^{2+} levels at each phase (Fraser, 1995). Nonetheless, comparative importance of the three systems in sperm capacitation is unknown, but evidence has accumulated that supports the significance of a Ca^{2+} -ATPase. Drugs, such as quercetin and ethacrynic acid (known to inhibit the Ca^{2+} -ATPases of somatic cells), have been shown to hasten sperm capacitation and increase acrosomal exocytosis (Fraser, 1995). Molecules referred to as "decapacitation factors" (DFs) have been identified on the sperm cell surface (Chang, 1957; Bedford & Chang, 1962). These molecules can separate and reunite with sperm cells and, when added to capacitated sperm, can reverse capacitation--hence, the term "decapacitation." Mouse DF may be involved in regulating intracellular Ca^{2+} concentrations through a Ca^{2+} -ATPase (Adeoya-Osiguwa & Fraser, 1993, 1994). Presence of DF may result in expression of Ca^{2+} -ATPase and therefore, less intracellular Ca^{2+} . If DF is removed by *in vivo* or *in vitro* processes, intracellular Ca^{2+} rises and eventually attains a threshold where sperm can then acrosome react. Exposure of capacitated cells to DF stimulates the Ca^{2+} -ATPase causing intracellular Ca^{2+} to decline.

Leclerc *et al.* (1989, 1990, 1992) monitored concentrations of the intracellular Ca^{2+} -mediator calmodulin during the "heparin-induced" capacitation of bull

spermatozoa. Adding heparin to the medium decreased the sperm Ca^{2+} -mediator calmodulin concentrations in a dose-dependent fashion. This decline coincided with simultaneous increases in Ca^{2+} -mediator calmodulin in the incubation medium and *in vitro* fertilization success. Decreases in sperm Ca^{2+} -mediator calmodulin concentrations may reduce the activity of a Ca^{2+} -ATPase resulting in increased intracellular Ca^{2+} (Leclerc *et al.*, 1992). Somatic cells contain Na^+ - Ca^{2+} exchangers that usually function to move sodium (Na^+) in and Ca^{2+} out so that intracellular Ca^{2+} concentrations remain low. It is possible that, in the case of sperm cells, conditions arise that cause the exchanger to function in the opposite direction (Rufo *et al.*, 1984).

Although commonly found in the extracellular environment of sperm *in vivo*, knowledge of Na^+ 's effect on sperm capacitation and the AR is limited (Fraser, 1995). Studies reveal a slight rise in intracellular Na^+ affiliated with capacitation, and a substantially higher influx with the AR. Experiments indicate that mammalian spermatozoa have Na^+ , K^+ (potassium)-ATPases (Quinn & White, 1968; Gordon *et al.*, 1978) that bind the inhibitor ouabain (O'Donnell & Ellory, 1970). Incubation of mouse sperm with ouabain for 40 min increases successful capacitation (Fraser *et al.*, 1993), whereas incubation for 2 h hastens the AR (Harrison & Fraser, 1993).

It has also been proposed that a Na^+ - Ca^{2+} exchanger may function to regulate intracellular Na^+ . However, this seems unlikely since mammalian sperm require increases in both Na^+ and Ca^{2+} ions (Fraser, 1995). K^+ is another cation suspected to play a role in sperm function, but likely during the AR and not sperm capacitation (Fraser, 1995). Few studies have focused on the significance of K^+ to mammalian sperm function.

pH. The pH of the spermatozoon's environment may significantly influence the cell's function, including capacitation and the AR. Bicarbonate ions may be responsible for regulating pH within the female reproductive tract (Fraser, 1995). Bicarbonate ion concentrations are low in the epididymis and high in seminal plasma

and the oviduct (Brooks, 1983). The pH changes encountered by sperm while traversing the male and female reproductive tracts may be responsible for capacitation prevention in the epididymis and its stimulation in the oviduct. Increased intracellular pH stimulates sperm capacitation in the guinea pig (Hyne & Garbers, 1981) and bull (Wassarman, 1987; Parrish *et al.*, 1989). Furthermore, Parrish *et al.* (1989) demonstrated that altering the rate of intracellular alkalinization that normally occurs in heparin-treated bull sperm impedes capacitation.

Membrane proteins/lipids. Alterations in sperm plasma membrane proteins and lipids are likely to be an important feature of sperm capacitation (Bedford & Cross, 1999). Membrane proteins may be lost or transformed during capacitation, and a decline in the cholesterol/phospholipid ratio in sperm plasma membranes also may occur (Bedford & Cross, 1999). During *in vitro* capacitation, serum albumin [usually in the form of bovine serum albumin (BSA)] seems to be responsible for removing cholesterol from sperm plasma membranes (Go & Wolf, 1985; Langlais & Roberts, 1985; Cross, 1998; Visconti & Kopf, 1998). Changes in plasma membrane concentration *in vivo* occur as sperm pass through the epididymis and/or cholesterol is acquired from seminal plasma (Cross, 1998).

One hypothesis for how cholesterol regulates sperm function is that a loss of cholesterol from the sperm plasma membrane encourages the fusion of acrosomal and plasma membranes when sperm are exposed to AR inducers. Interestingly, no fusion is observed in sperm exposed to a cholesterol-inhibitor (Cross, 1998). A second hypothesis is that the loss of cholesterol uncovers receptors for mannose on the sperm surface (Benoff, 1993). Several reasons exist for the belief that this receptor is relevant to sperm-ZP interactions. First, mannose and mannosylated proteins hinder sperm from binding to the ZP. Secondly mannose and mannosylated proteins are capable of inducing the AR. Thirdly mannose is a component of the ZP. It also has been suggested that the removal of cholesterol from sperm plasma membranes results in the

rise in intracellular pH required for capacitation. In support of this hypothesis, the maintenance of the cholesterol/phospholipid ratio at a constant level precludes spermatozoa from responding to inducers of the AR (Bedford & Cross, 1999).

Sperm extenders/cryopreservation. Semen extenders and/or the process of cryopreservation are known to induce premature capacitation of bull sperm by disrupting specific sperm plasma membrane factors (Ijaz & Hunter, 1989; Ijaz *et al.*, 1989; Cormier *et al.*, 1997). This is undesirable because capacitated sperm are less likely than uncapacitated sperm to reach the site of fertilization within the female reproductive tract. Capacitated sperm are relatively short-lived and more likely to acrosome react prematurely (Cormier *et al.*, 1997). Thus, premature capacitation may explain why cryopreserved sperm are associated with reduced fertility compared to freshly ejaculated sperm (Cormier *et al.*, 1997).

Cyclic AMP. Cyclic AMP (cAMP) has been identified as a potential factor regulating sperm capacitation and the AR (Visconti & Kopf, 1998). The association of cAMP with capacitation and the AR is poorly understood; most studies have focused on cAMP's involvement with sperm motility. Protein kinase A (PK-A) activity is known to increase during mouse sperm capacitation, signifying heightened levels of intracellular cAMP and a potential role during capacitation (Visconti & Kopf, 1998). Changes in intracellular Ca^{2+} and bicarbonate during capacitation may be linked to regulating cAMP metabolism, since both of these ions enhance the activity of adenylate cyclase, an enzyme responsible for producing cAMP activity in mammalian sperm.

Mechanisms associated with capacitation. Capacitation has been associated with increases in protein tyrosine phosphorylation in mouse, human, bull (Visconti & Kopf, 1998), and cat sperm (Pukazhenthil *et al.*, 1996a, 1998a,b). Increases in protein tyrosine phosphorylation require the presence of BSA, Ca^{2+} and sodium bicarbonate in the incubation medium at appropriate concentrations (Visconti & Kopf, 1998). If any one of these components is absent, then protein tyrosine phosphorylation and

capacitation are inhibited. Furthermore, increases in protein tyrosine phosphorylation during incubation in capacitation-inducing media are not observed with caput epididymal sperm. Such sperm are unable to capacitate and fertilize; therefore, the capacity to (1) undergo capacitation and (2) for increased protein tyrosine phosphorylation to occur is acquired while sperm mature and traverse the epididymis since caudal sperm both capacitate and fertilize (Visconti & Kopf, 1998).

BSA appears to regulate sperm capacitation and protein tyrosine phosphorylation through its effect on cholesterol movement (Visconti & Kopf, 1998). Incubation in the presence of a cholesterol analogue prevents BSA from removing cholesterol from sperm plasma membranes, inhibiting both capacitation and protein tyrosine phosphorylation.

Thus, contemporary theory holds that extracellular Ca^{2+} and sodium bicarbonate are activators of the enzyme adenylate cyclase, which is associated with mammalian sperm (Visconti & Kopf, 1998). Because this enzyme is responsible for producing cAMP, it is believed that Ca^{2+} and sodium bicarbonate may modulate capacitation and protein tyrosine phosphorylation via a cAMP-mediated pathway. Studies have revealed that capacitation and protein tyrosine phosphorylation appear to be directed by a PK-A pathway (Visconti & Kopf, 1998, Pukazhenthil *et al.*, 1998a). This theory is supported by experiments in which PK-A inhibitors prevent both capacitation and protein tyrosine phosphorylation.

It has been suggested that interaction between capacitated sperm and ZP3, one of the zona glycoproteins, stimulates a Na^+/H^+ exchanger that subsequently triggers events leading to the AR (Fraser, 1993, 1994). Actions proceeding sperm/ZP3 interactions and causing activation of Ca^{2+} channels are unknown, but the following steps have been proposed. Activating a Na^+/H^+ exchanger increases intracellular Na^+ and decreases intracellular hydrogen ions (H^+), thereby increasing intracellular pH. The latter stimulates Ca^{2+} channels causing Ca^{2+} influx that, in turn, allows sperm to

undergo the AR.

Events following the stimulation of Ca^{2+} channels are unclear. In brief, the rise in intracellular Ca^{2+} activates a polyphosphoinositide-specific phospholipase C (PIC) in sperm membranes to hydrolyze phosphatidylinositol-4,5-bisphosphate (PIP_2) to produce inositol-1,4,5-trisphosphate (InP_3) and *sn*-1,2-diacylglycerol (DAG) (Fraser, 1995). Both of these molecules are known to be intracellular second messengers in somatic cells. InP_3 stimulates the release of stored intracellular Ca^{2+} , and DAG activates protein kinase C (involved in protein phosphorylation, most likely of important regulatory proteins) (Berridge, 1993).

Thesis Objectives

The overall aim of this thesis was to facilitate the development of an effective sperm GRB for an endangered species using both computational and physiological approaches.

Specific objectives:

- (1) Use historical demographic data from two mammalian species (Eld's deer, Przewalski's horse) to examine, by computer modeling, the effectiveness of various GRB strategies.
- (2) Examine a crucial physiological phenomenon (sperm capacitation) associated with sperm processing and cryopreservation in the endangered Eld's deer, a species targeted for GRB development.

These aims were accomplished in two individual, but interrelated projects. All results ultimately will be useful for developing a formal GRB Action Plan that, in turn, will lead to enhanced genetic management of Eld's deer.

SECTION 2:
**USE OF COMPUTER SIMULATION TO DETERMINE THE EFFICACY OF
DIFFERENT GRB STRATEGIES**

Abstract

Genome resource banks (GRBs) and assisted reproductive techniques are increasingly being recognized as tools useful in the management and conservation of biodiversity, including endangered species. Cryotechnology offers the ability to store valuable genetic material (including germ plasm) indefinitely. However, the actual application to endangered species management requires more than technical knowledge about sperm freezing and thawing. There is a need to systematically understand the quantitative impacts of various germ plasm storage and use scenarios. The most efficient GRB strategy must be identified to secure the genetic diversity of a given small population. Banking strategies were analyzed using the historical data of two managed populations with varied pedigrees. The following types of sperm banks were assessed: (1) a "Wild Bank" consisting of sperm (i.e., genes) from five to 10 males unrelated to the population and to each other; and (2) a "Best Male" bank containing sperm from only the most genetically-valuable males (during a particular time period) of a managed population. These different bank types were then used to evaluate the effectiveness of different bank usage frequencies (on a yearly basis). The efficiency of each scenario was assessed by examining the level of inbreeding and gene diversity in resulting hypothetical offspring, as well as in the entire managed population. A sperm usage frequency of five times per year was determined to significantly benefit the management of the populations of both species. "Wild banks" successfully strengthened genetic diversity, and a "Best Male" bank combined with a usage frequency of five times per year was also effective at enhancing genetic variability, but to a lesser degree than the "Wild Bank" approach. Finally, lengthening the interval

between storage and sperm usage as much as possible (i.e., 20-30 years) increased the value of the cryopreserved sperm in terms of genetic importance. In conclusion, these evaluations revealed that computer simulations can be used to provide further insight into which management schemes are most appropriate for different species possessing different pedigrees and genetic variabilities.

Introduction

A GRB is the organized collection, storage and use of biomaterials, including germ plasm (i.e., sperm). The establishment of GRBs in concert with assisted breeding techniques has the potential of improving the preservation of many endangered species, especially those managed genetically in captive breeding programs. Soulé *et al.* (1986) have estimated that nearly 815 mammalian species will require the assistance of captive propagation programs in the next 200 years. Unfortunately, zoos only have space for accommodating approximately 100 mammal species in sufficient numbers to ensure genetically- and demographically-viable populations for the long-term (Conway, 1987). Therefore, the organized cryopreservation and careful use of gametes would better allow maintaining genetic diversity, since fewer living animals of a given species would be required in zoos. A by-product would be increased space for other species requiring conservation attention (Johnston & Lacy, 1995). The systematic cryopreservation and use of sperm would also permit extending the generation length of a population or individual indefinitely, thereby diminishing the probability of losing genetic diversity by reducing generations numbers within a given time period (Ballou, 1992).

There are other reasons for establishing GRBs. Combined with the use of assisted breeding techniques (such as artificial insemination), GRBs could eliminate the need to translocate animals between breeding institutions, while surmounting the difficulties associated with behaviorally incompatible mating pairs (Wildt, 1992, 1997;

Wildt *et al.*, 1997). The availability of surplus germ plasm from wild, free-living animals and its importation into zoos also would obviate the need to ever again collect animals from nature to support zoo breeding programs (Wildt *et al.*, 1997).

The use of GRBs for species conservation and methods for selecting sperm donors have been examined (Johnston & Lacy, 1991, 1995). GRB strategies have been analyzed for effectiveness at eliminating or reducing the loss of genetic diversity as a consequence of genetic drift. Pedigree analysis software, such as GENES (Lacy, 1993), has been used to carry out theoretical scenarios with results that can eventually be extrapolated to managed populations. Johnston and Lacy (1995) assessed four strategies for selecting genetically-valuable individuals whose genes should be banked. Effectiveness was based on how each approach affected a population's gene and allelic diversity. Each strategy was tested using demographic data collected from four North American captive populations (okapi, *Okapia johnstoni*; golden-headed lion tamarin, *Leontopithecus rosalia chrysomelas*; Siberian tiger, *Panthera tigris altaica*; gaur, *Bos gaurus*) with distinctly different pedigree organizations. The following four GRB schemes were assessed: (1) all the males in the population; (2) only living founders and early generation progeny; (3) males left over after minimizing mean kinship (MK) (Johnston & Lacy, 1991; Ballou & Lacy, 1995) through computerized culling; and (4) males left over after culling to the same number of donors found in strategy #2. The two strategies that relied on MK did not include female specimens (i.e., only sperm banking was examined). These strategies used "iterative culling" (the selective removal of individuals from the population) of males having the highest MKs to determine which males represented the highest level of gene diversity within the population. This iterative process was required because culling a male changed the MKs of the remaining males. In the third strategy, males were eliminated from the population in succession until the removal of one more male resulted in a reduction in gene diversity. The last strategy was a continuation of the third, whereby males were

culled until the number of males that remained was equal to the number of males in the founder bank (strategy #2).

Comparisons were performed using the pedigree analysis software-GENES (Lacy, 1993). Following 100 theoretical matings, the four strategies were found to be ranked identically across all four of the populations as follows: $1 > 3 > 4 > 2$. The "All Male Bank" performed the best. However, such an extensive bank may not always be feasible, and a GRB incorporating only a portion of the most valuable males may achieve a level of genetic variation that is quite comparable. Therefore, a repetitive culling method can be used to select a group of males that are prime candidates for a GRB. The advantages are that less space needs to be allocated to each species designated for long-term gamete storage, resulting in the development of a more cost-effective sperm bank.

Johnston and Lacy (1991) also investigated the effectiveness of employing sperm banks to maintain the genetic diversity of the North American gaur herd. Management programs based on pedigree analysis were found to be closer to optimum than schemes based on random mating. Essentially, it becomes possible to produce more genetically valuable individuals using pedigree-based strategies.

The effectiveness of a GRB will ultimately rely on the selection of genes designated for cryobanking (Johnston & Lacy, 1995). Choosing a GRB strategy depends on each specific case study, and examination of a population's pedigree structure and demographics is required. Therefore, the overall objective of this study was to use computer simulations to determine the efficacy of different semen banking strategies. First, historical population dynamics were examined in two different species (Eld's deer, *Cervus eldi thamin*; Przewalski's horse, *Equus przewalskii*) to determine if GRBs established years in the past would have increased genetic diversity in current populations of the two target species. Second, the effectiveness of different semen banking strategies was tested to determine which approaches would be most effective

for maintaining genetically-viable, managed populations. Third, the frequency with which cryobanked gametes were infused into extant populations was varied to determine if frequent use of a GRB was superior for maintaining genetic diversity compared to less frequent use.

Methods

Managed Eld's deer and Przewalski's horse populations were used to assess the retrospective effectiveness of various GRB strategies. Information contained in the Single Population Animal Record Keeping System (SPARKS) computerized databases for each of these species was used for all genetic modeling.

Adequate historical records exist for the North American Eld's deer population from 1976 to the present (M. Rodden, pers. comm.). In Myanmar the free-ranging population of the Eld's deer is limited to central plains, predominantly on the Irrawaddy Plain and including the Pegu or Sittang Plain located in the east (Salter & Sayer, 1986). The North American Eld's deer population originates from 15 potential founders (M. Rodden, pers. comm.). Fewer than 2000 individuals are believed to remain in the wild (McShea *et al.*, 1999) and ~ 120 individuals are maintained in North American zoos and breeding facilities.

The Przewalski's horse originates from Asia and has been bred in captivity since the early 1900s. Because the contemporary population is derived from only 13 founders, it is highly inbred (Ryder, 1988). The species is extinct in the wild, and the captive population consists of more than 900 individuals. Although the Przewalski's horse is genetically distinct from the domestic horse (*E. caballus*), the two species are capable of interbreeding and producing fertile offspring (Short, 1975). Management of the Przewalski's horse can be difficult since domestic horse genes were incorporated into the population by a domestic mare in 1906 (Ballou, 1994). The managed population consists of two major lineages: (1) the Prague line that is comprised of

descendants originating from the domestic mare, and (2) the Munich line that does not contain any domestic horse genes (Ballou, 1994). The population is managed to maintain the purity of the Munich line (i.e., gene flow is permitted from the Munich line to the Prague line, but not in the opposite direction). For the purpose of the present study, the two lineages were combined.

GENES pedigree analysis software (Lacy, 1999) was used in association with SPARKS to identify genetically-desirable matings. Individuals having unknown ancestry were omitted from all scenarios. Hypothetical sperm banks were created at a particular time using the “best males” (ranked according to MK) in the managed population. Theoretical banks consisting of wild-born individuals (“wild banks”), completely unrelated to the managed population and to one another, were also created and incorporated into scenarios. Depending on the scenario being examined (below), females that actually gave birth at a given time were recognized and sorted by MK, allowing identification of the top-ranked breeding females. Some of these individuals (depending on the scenario) were re-paired with genetically-valuable males chosen from the existing population or from one of the previously created sperm banks.

Using the steps described above, comparisons were made between frequent and infrequent uses of a bank. For example, one comparison was the incorporation of banked semen (from five genetically unrelated males) into a population once per year versus incorporating banked semen five times per year over a defined time period [“Best Male 1” (BM1) and “Best Male 5” (BM5), respectively]. These two banks contained the genes from males that were considered to represent the maximum genetic diversity during a certain time period (1976 in the case of the Eld’s deer, and 1960 in the case of the Przewalski’s horse).

This same comparison was performed with both of the species using the creation of hypothetical wild banks. A wild bank consisting of five males was used to incorporate banked semen into a managed population once per year [“Wild Bank 1”

(WB1)], and a wild bank composed of 10 males was used to incorporate banked semen into a managed population five times per year ["Wild Bank 5" (WB5)]. The purpose of these four scenarios was to determine which GRB frequency use and strategy was most efficient for a particular species.

An additional scenario was carried out ("100 MK") to determine the relevant genetic importance of banked semen (usually from deceased males) versus that of semen from extant individuals. A simulated semen bank was created containing sperm from males alive in the population during a specific period (1976-1979 in the case of the Eld's deer, and 1960 in the case of the Przewalski's horse) that represented the maximum genetic diversity available. The GENES simulation program was used to carry out 100 MK-based pairings among members of the current managed population. One hundred pairings were performed a second time with the contemporary population, but "frozen sperm" was also used from the cryobanked males described above. Outcomes and frequencies with which banked versus non-banked males were selected for pairings (using GENES) were compared. This scenario was similar to the "Best Male" and "Wild Bank" scenarios because, in all cases, MK was the major criterion used to select optimal pairings. The "100 MK" scenario was different because the software, and not the program user, selected the pairings.

Results

Table 1 compares the resultant genetic characteristics for the strategies examined with those of the actual populations. Values refer to the levels of genetic variation present in the managed and actual populations during the last year examined for each respective scenario. The managed Eld's deer population was much smaller than the managed Przewalski's horse population (by approximately 10 fold), but contained more gene diversity and a much lower level of inbreeding. The two populations had very different pedigrees and, therefore, provided an interesting

comparison.

All scenarios improved the genetic variation of the actual populations. Separate examination of the "Best Male" and "Wild Bank" scenarios revealed that a sperm usage frequency of five times per year was more effective at strengthening genetic diversity in both managed populations than a once per year frequency. This was expected since higher yearly "dosages" of banked sperm provided the managed populations with a greater degree of "new" or rare genes. The WB5 strategy greatly enhanced gene diversity and reduced inbreeding in both the Eld's deer and Przewalski's horse populations (Figs. 1-4).

Concerning reduction in inbreeding proportions, the strategies were ranked equally across both populations: WB5 → BM5 → WB1 → BM1 → Actual. In the case of the Eld's deer, this same ranking was observed with respect to gene diversity acquired through optimal pairings. However, the BM5 and WB1 scenarios exchanged positions in the ranking of the strategies in relation to the level of gene diversity obtainable in the Przewalski's horse (i.e., WB5 → WB1 → BM5 → BM1 → Actual).

Contrasting the genetic importance of banked sperm with that of extant genes in living animals revealed that the value of banked sperm increased with time. When banked males were incorporated into the managed populations and GENES was instructed to perform 100-MK based pairings, the pedigree analysis software selected the banked males for suggested pairings much more frequently than extant males. With respect to the Przewalski's horse, two of the banked males were selected for 100% of the proposed 100 pairings. Banked males were selected for 85% of the 100 suggested pairings in the Eld's deer. The incorporation of the banked sperm into the managed populations also served to enhance gene diversity and reduce inbreeding of the descendant populations.

Discussion

Johnston and Lacy (1995) proposed that an ideal GRB would contain the following: (1) biomaterials from as few individual animals as possible; (2) the majority or all of the allelic diversity existing within the living population; (3) a considerable level of gene diversity; (4) genetic material available for the purpose of future genetic management; and (5) easily identified donors. Although the establishment of ideal GRBs may not always be feasible, it is critical that efficient GRBs are developed to counteract the loss of genetic diversity through genetic drift. Normally the maintenance of large breeding populations are required to regulate genetic drift (Lacy, 1987; Johnston & Lacy, 1991), but GRBs provide institutions with the option of housing smaller populations capable of retaining adequate levels of genetic diversity (Ballou, 1992). This is very significant, especially since the combined enclosure space of the world's zoos could easily fit into an area similar in size to Brooklyn, New York (Conway, 1986).

Through the use of computer simulations, it is possible to investigate the effects of different management strategies on the genetic composition of a population (Lacy, 1987). This study examined the effects of different GRB strategies on the genetic parameters of a given population. The hope is that these simulations will provide insight into which GRB approaches should be adopted for a particular species in the generations still to come. As expected, the WB5 (Wild Bank 5) scenario managed to greatly enhance the genetic stability of both the populations assessed. The effect of banking genetically-valuable males, originating from a managed population, was also examined. A usage frequency of five per year proved to be highly beneficial to both the Eld's deer and Przewalski's horse populations. The BM5 (Best Male 5) scenario required more time (years) to improve upon the genetic metrics of the Przewalski's horse population than the Eld's deer population. This is undoubtedly because the Przewalski's horse population is much larger, both presently and historically, in

addition to experiencing a higher degree of inbreeding. The Przewalski's horse has a more extensive pedigree, with the contemporary population originating from founders acquired in 1945. In contrast, the Eld's deer population appears to have procured founders as late as 1980.

An interesting result was the disparity between the two populations in the ranking of the GRB strategies with respect to genetic diversity achievable through pairings. The Eld's deer population's overall genetic variation is superior to that of the Przewalski's horse, and therefore, GRBs containing the most genetically valuable males will also be superior. Perhaps that is why the BM5 scheme was more effective at bolstering the gene diversity of the Eld's deer than the Przewalski's horse. It should also be noted that, overall, the BM5 scenario performed better than WB1 (Wild Bank 1). This reveals that a "wild bank" will not necessarily do better than a captive-generated bank.

The "100 MK" scenario showed that genes do become more valuable with time. Hence, it may be advantageous to store sperm from the most genetically-valuable males of an extant population and to reinfuse it into the population only after an extended period of time (i.e., 20-30 years). This scenario appeared to be slightly more effective for the Eld's deer than the highly inbred Przewalski's horse. Possibly a storage period longer than 30 years would prove to be more "potent" for a population, such as that of the Przewalski's horse, possessing a vast pedigree and stricken by elevated inbreeding.

When examining the results for the present study it should be kept in mind that the two Przewalski's horse lineages were united to evaluate the different GRB strategies. This population would not normally be managed in this manner, but still served as a good comparison for the Eld's deer population.

The formation of GRBs has the potential to greatly enhance the conservation of endangered species. A great deal of research is still required to establish protocols and

GRBs that ensure the storage of viable sperm. It must be taken into account that different populations will require different management strategies. One cannot simply assume that a GRB strategy that is efficient for one species, will also benefit the management of another. The pedigree of a population must be examined carefully before delineating a specific GRB plan. Breeding institutions will have to work together to develop global GRB plans that allow adequate transfer of genetic material among breeding facilities. Population geneticists must work in concert with reproductive physiologists to investigate the numerous details involved with the cryopreservation and storage of gametes. If executed precisely, GRBs provide the possibility to save a greater number of species for the long-term.

Table 1. Comparison of the current living populations of the Eld's deer and Przewalski's horse and the four genome resource bank strategies examined.

Bank	FGE	%GD	%MF
Eld's deer			
Actual	4.3916	88.61	12.82
BM1	4.5639	89.04	12.81
BM5	5.9865	91.65	9.06
WB1	4.6171	89.17	12.65
WB5	7.2122	93.07	9.56
Przewalski's horse			
Actual	3.2997	84.85	20.27
BM1	3.7534	86.68	18.03
BM5	3.9030	87.19	17.06
WB1	4.6200	89.18	16.18
WB5	6.5948	92.42	11.54

FGE = founder genome equivalent → “number of founders that would be required to obtain the levels of genetic diversity that are observed in the current population if the founders were all equally represented” (Ballou & Foose, 1996).

%GD = % gene diversity

%MF = % mean inbreeding

Fig. 1. Comparison of changes in gene diversity through time for the managed Eld's deer population as a result of the application of different GRB strategies.

Fig. 2. Comparison of changes in levels of inbreeding through time for the managed Eld's deer population as a result of the application of different GRB strategies.

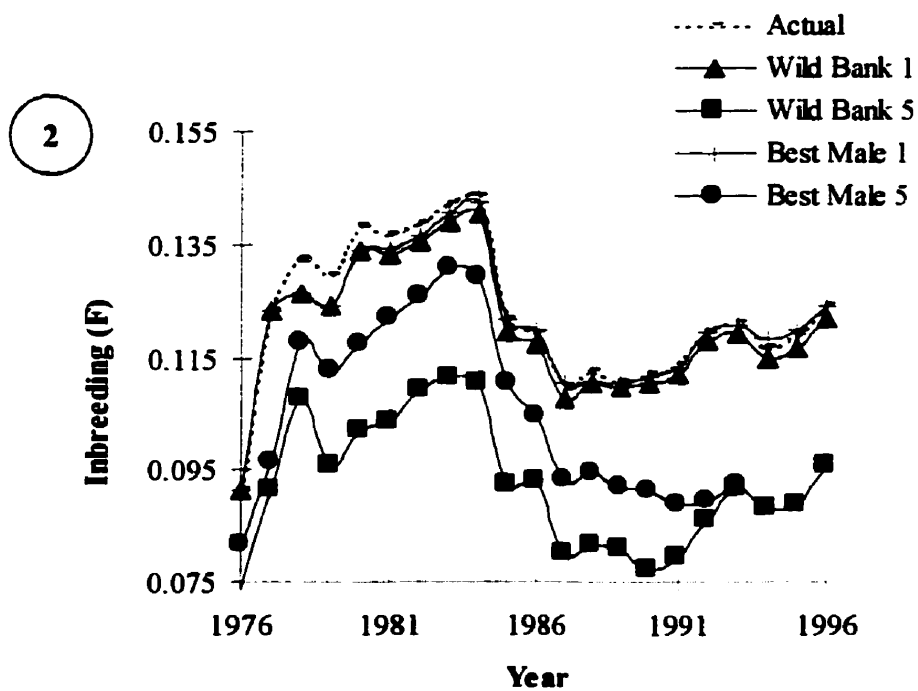
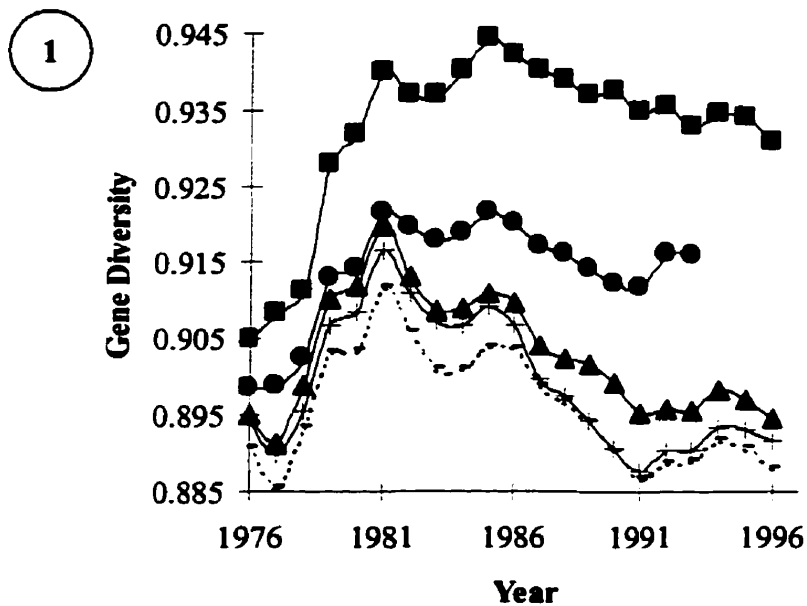
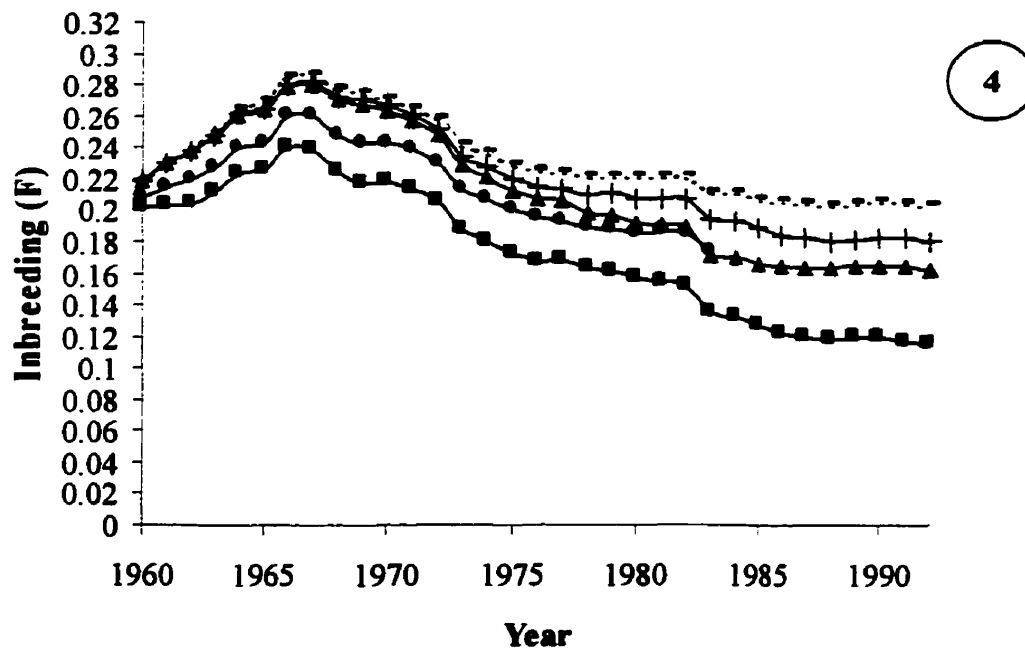
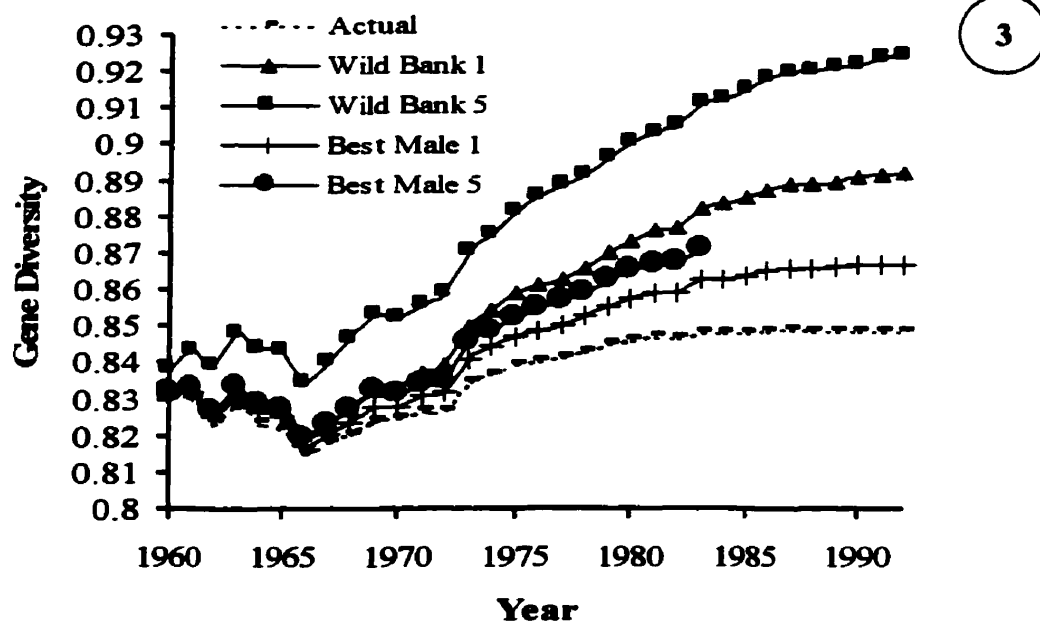


Fig. 3. Comparison of changes in gene diversity through time for the managed Przewalski's horse population as a result of the application of different GRB strategies.

Fig. 4. Comparison of changes in levels of inbreeding through time for the managed Przewalski's horse population as a result of the application of different GRB strategies.



Connecting Statement

Genome resource banks (GRBs) are used to preserve genes, such as sperm, in a viable state via the processes of cryobiology. As demonstrated in the previous section, there is a paucity of information on the optimal scenarios for banking sperm (from a population genetics perspective) to maximize genetic vigor in managed populations. But, for virtually all wildlife species, little effort also has been undertaken to understand species-specific cellular and physiological mechanisms that regulate the survival of sperm to a cryo-stress. Sperm must endure being frozen (potentially for decades) at a temperature optimum for long-term storage (usually -196°C) and restored to body temperature when required for assisted breeding programs.

An integral phenomenon associated with sperm function and fertilization is capacitation. Without sperm capacitation, assisted breeding techniques, such as artificial insemination (AI) and *in vitro* fertilization (IVF), are impossible. Assisted breeding techniques must often rely on frozen-thawed sperm, and capacitation ability is affected by the cryopreservation process. Consequently, it is important to examine the mechanics of capacitation in both fresh and frozen-thawed sperm. Sperm physiology factors are different for every species; thus, basic sperm function must be investigated in any species for which the establishment of a GRB has been deemed necessary. Therefore, in addition to studying banking strategies from a population genetics perspective, this Master's also delved into one of the most challenging aspects of understanding sperm function, the phenomenon of sperm capacitation. Knowledge from this study eventually will also contribute to the practical and effective development of GRBs for conserving endangered species.

SECTION 3:

INDUCTION OF SPERM CAPACITATION *IN VITRO* IN THE ELD'S DEER; CRUCIAL INFORMATION FOR ESTABLISHING A SUCCESSFUL GENOME RESOURCE BANK

Abstract

The potential of inducing sperm capacitation was examined in the endangered Eld's deer (*Cervus eldi thamin*). Sperm motility and viability (percentage of sperm cells with intact membranes) were assessed *in vitro* over time after attempting to provoke capacitation in (calcium) Ca^{2+} , dibutyryl cyclic AMP (dbcAMP), or fetal calf serum (FCS)-supplemented TALP. The ability to induce an acrosome reaction (AR) was tested using calcium ionophore A23187 (CI) versus lysophosphatidylcholine (LC) treatment. Both freshly ejaculated and frozen-thawed sperm were washed and resuspended in: (1) TALP (control); (2) TALP + 10 mM calcium chloride (CaCl_2); (3) TALP + 1 mM dbcAMP; and (4) TALP + 20% FCS. Sperm aliquots were evaluated at 0, 3, 6, 9, and 12 h for motility, viability, and ability to acrosome react after exposure to CI (10 μM) or LC (100 $\mu\text{g/ml}$). Fresh sperm aliquots in TALP + 10 mM CaCl_2 with CI had fewer ($p < 0.05$) intact acrosomes than the TALP control or dbcAMP and FCS treatments after 9 h. Mean (\pm SEM) percentage of intact acrosomes in CaCl_2 -treated sperm declined ($p < 0.05$) from $80.2 \pm 2.6\%$ (0 h) to $49.7 \pm 7.3\%$ after prolonged incubation (9 h). In contrast, percentage of intact acrosomes in both the CaCl_2 treatment control (TALP + 10 mM CaCl_2 + DMSO without CI; $76.2 \pm 3.3\%$) and TALP control group ($64.6 \pm 4.1\%$) exceeded ($p < 0.05$) values in the CaCl_2 treatment group at 9 h. The proportion of acrosome reacted fresh sperm was not influenced by LC treatment. Capacitation was not induced ($p > 0.05$) by any of the presumptive sperm capacitors after freeze-thawing. Likewise, neither CI nor LC induced the AR in these sperm, suggesting that the freeze-thawing process may have induced premature

capacitation. These data indicate that Eld's deer sperm are resilient to protocols that commonly induce capacitation in mammalian sperm, including those commonly effective in domesticated ungulates. Although CI induced the AR in fresh CaCl_2 -treated sperm, the concentration used appeared toxic to Eld's deer sperm. This was demonstrated by the reduced levels of motility consistently exhibited by sperm incubated in the presence of CI. These findings reaffirm the significance of species-specific processes regulating sperm capacitation in mammals. Capacitation impedance in the deer may be related to sensitivity or threshold resistance to conventional "capacitators" or to novel, as yet undiscovered, mechanisms that will require more detailed study.

Introduction

Detailed studies of gamete physiology are a prerequisite to developing effective assisted breeding programs for wildlife species (Howard *et al.*, 1986; Roth *et al.*, 1998). The Eld's deer is an endangered subtropical species from Southeast Asia. It exists in scattered herds throughout central Myanmar (formerly known as Burma, 10-20°N latitude) (Wemmer & Grodinsky, 1988), and fewer than 2000 individuals probably remain in the wild (McShea *et al.*, 1999). The Eld's deer has become endangered as a result of habitat loss and over-hunting (Talbot, 1960). Approximately 120 Eld's deer are maintained in North American zoos and breeding facilities, with the largest herds held at the San Diego Zoo (California), the Wildlife Conservation Society (New York), and the National Zoological Park's Conservation & Research Center (Virginia).

The captive management of the Eld's deer has benefited from the development of assisted breeding technology. Aspects of endocrinology and sperm physiology have been previously studied in the Eld's deer (Monfort *et al.*, 1990, 1993a,c). Furthermore, an artificial insemination (AI) protocol has been founded and used to consistently

produce young using frozen-thawed sperm (Monfort *et al.*, 1993c). We also have to consider the value of developing *in vitro* fertilization (IVF) as another tool for enhancing genetic management of Eld's deer. In this case, the ability to create embryos in the laboratory, including with cryopreserved gametes, would provide even more options to secure the species and to move germ plasm (and thus novel genes) among geographically disparate populations to enhance genetic heterozygosity. However, preliminary IVF studies using sperm collected and processed with methods similar to those in domestic cattle, have resulted in poor sperm/oocyte binding *in vitro* (S. Monfort, pers. comm.). One explanation for this deficiency was that Eld's deer sperm were failing to capacitate *in vitro*.

To date, there are no detailed studies of capacitation in this, or other cervid species. Modified Tyrode's medium supplemented with heparin has been used to successfully induce capacitation (within ~ 4 h) in frozen-thawed white-tailed deer (*Odocoileus virginianus*) sperm (G. Killian, pers. comm.). Additionally, the AR has been examined in red deer (*C. elaphus*) via the use of a triple-staining technique (Garde *et al.*, 1997). Sperm were incubated in tissue culture medium 199 (TCM 199) with CI, and maximal numbers of acrosome reacted sperm were detected after 9 h of incubation *in vitro*. Based on this limited work in deer and more extensive studies in cattle, the present study was designed to test three potential sperm capacitors for Eld's deer sperm: (1) CaCl_2 , (2) a cAMP analogue, and (3) fetal calf serum. The addition of surplus CaCl_2 was chosen as there is evidence that Ca^{2+} plays an important role during mammalian sperm capacitation and the AR (Handrow *et al.*, 1989; Fraser & McDermott, 1992; Yanagimachi, 1994). The role of cAMP during capacitation and the AR is unclear (Yanagimachi, 1994), but evidence suggests that it is central to a signaling pathway associated with mammalian sperm capacitation (Vandevoort *et al.*, 1994; Leclerc *et al.*, 1996; Galantino-Homer *et al.*, 1997; Visconti *et al.*, 1999ab). FCS was chosen based on its role in modulating cholesterol-binding on sperm membranes,

and its concentration was extrapolated from unpublished sperm capacitation studies conducted on farmed red deer (R. Spindler, pers. comm.).

Both CI and LC were chosen to induce the AR in Eld's deer sperm since both of these techniques have been used successfully in bull sperm (Byrd, 1981; Parrish *et al.*, 1988, 1989). Because assisted reproductive techniques, by necessity, often rely on the use of cryopreserved sperm, the mechanisms of sperm capacitation and the AR were examined in both fresh and frozen-thawed sperm.

The overall aim of this study was to examine essential aspects of sperm physiology, namely the mechanisms underlying sperm capacitation in the endangered Eld's deer. Our hypotheses were that (1) both fresh and frozen-thawed sperm could be induced to capacitate *in vitro* using approaches considered conventional in other ungulate species, especially domestic cattle; (2) frozen-thawed sperm could be induced to capacitate under the same conditions as fresh sperm, and (3) both fresh and frozen-thawed sperm would undergo the AR in the presence of either CI or LC.

Materials and Methods

Animals. Ejaculates were collected from six Eld's deer stags (five to 12 years of age) housed at the Conservation & Research Center, Front Royal, VA (38°N latitude). Stags were housed in indoor stalls (3.4 m x 4.6 m with skylights) with access to outdoor enclosures (3.6 m x 36.6 m) allowing exposure to natural photoperiodic changes. Diet included alfalfa hay and a pelleted ration daily and water *ad libitum*.

Semen collection. Eld's deer were anesthetized for electroejaculation using a combination of ketamine hydrochloride (Ketaset: Aveco Co., Fort Dodge IA, USA, 2 mg/kg) and xylazine hydrochloride (Rompun: Mobay Corp., Shawnee KA, USA, 0.25mg/kg) administered *i.m.* A standardized electroejaculation protocol (Howard *et al.*, 1986; Monfort *et al.*, 1993c) was used to collect semen from stags once animals had reached a satisfactory plane of anesthesia. Feces were removed from the rectum

before electroejaculation to facilitate stimulation. A sine-wave electrostimulator (AC, 60 Hz) and a Teflon rectal probe (2.6 cm diameter; P.T. Electronics, Boring OR, USA) were used to administer three series of stimuli given in a 3 sec on-off pattern with electrical stimulations ranging from 3 to 6 volts. Each series was separated by a 5 min rest interval and proceeded as follows:

- a) first series, 10 stimuli each at 3, 4, and 5 volts;
- b) 5 min rest;
- c) second series, 10 stimuli each at 4, 5, and 6 volts;
- d) 5 min rest;
- e) third series, 10 stimuli each at 5 and 6 volts.

The penis, protruding from the preputial sheath, was positioned above a clean plastic specimen container to ensure that the ejaculate did not become contaminated with debris. The risk of urine contamination was minimized by frequently switching specimen containers.

Semen handling and evaluation. Raw semen was assessed for percent sperm motility (x 250), speed of forward progression on a scale of 0 (no movement) to 5 (rapid and direct forward movement) (Howard *et al.*, 1986), and volume between consecutive stimuli series. Motility assessments were made by examining at least four separate microscopic fields, and only seminal aliquots with adequate sperm motility (70% or greater) were used. Semen was maintained at 37°C in a water-bath throughout the semen collection process. An aliquot (10 µl) of raw semen from the final sample was fixed in 0.3% glutaraldehyde, and 100 sperm were assessed for morphology (Howard *et al.*, 1986). The final volume of raw semen was diluted in a test-tube with an equal volume of warmed BF5F without glycerol. BF5F is modified BF5 diluent (Pursel & Johnston, 1972) containing 20% egg yolk, 1.6% glucose, 1.6% fructose, 1.2% tes-n-tris methyl-2-aminoethane sulfonic acid, 0.2% trisaminoethane, and 0.5% surfactant mixture of sodium and triethanolamine lauryl sulfate. Sperm concentration

was determined using a hemocytometer method (Howard *et al.*, 1986) and was adjusted to ~ 200 million motile sperm/ml by diluting appropriately with warmed (37°C) BF5F (0% glycerol).

Semen cryopreservation. Diluted semen was equilibrated in a water-bath (4°C) for ~ 1 h. A volume of chilled BF5F (8% glycerol, 4°C) was then added to achieve a final freezing concentration of ~ 100×10^6 motile sperm/ml (2-4% final glycerol concentration). Thirty min after adding glycerol, semen was loaded into pre-cooled, plastic, 0.25 ml straws that were heat-sealed using an alcohol burner and forceps. Approximately 60 min after adding glycerol, straws were frozen on a block of dry ice (-78°C, 2 min) and plunged into liquid nitrogen (-196°C) for long-term storage. Semen straws were thawed for 10 sec in air, followed by 30 sec in a 37°C water-bath.

Sperm viability evaluations. Sperm viability was assessed using the Live/Dead fluorescent viability stains propidium iodide (PI) and SYBR-14 (Molecular Probes, Eugene OR, USA). For viability evaluations, 1 µl of each stain (PI: 8 mg/ml DMSO; SYBR-14: 0.1 mg/ml DMSO) was added to ~ 50 µl of sperm solution that was incubated for 10 min. Percentage viable sperm was determined subjectively using fluorescence microscopy. Sperm that stained green by SYBR-14 were considered viable (membrane intact), whereas sperm that stained red were deemed non-viable.

Acrosomal integrity evaluations. Acrosomal integrity was evaluated by staining smears of sperm suspensions with fluorescein isothiocyanate-conjugated *Arachis hypogaea* agglutinin (FITC-PNA; Sigma Chemical Co., St. Louis MO, USA) (Long *et al.*, 1996). Smears were made by spreading 5 µl of sperm suspension across a glass slide and then air-drying. Smears were stained with 10-20 µl of FITC-PNA spread across slides using a coverslip. Slides were then incubated in a humidified container at 4°C for 15 min. After incubation, slides were rinsed twice with PBS and left to air-dry in the dark. Approximately 10 µl of mounting medium (Vector laboratories, Inc., Burlingame CA, USA) was applied to a dry slide along with a

coverslip. Acrosomal integrity was determined for 200 sperm per slide using fluorescence microscopy. Sperm were characterized as having intact acrosomes if the region overlaying the acrosome was evenly stained bright green (Fig. 1A). Sperm with mottled or patchy staining patterns, nicks, or ballooning acrosomes were classified as damaged or partially reacted (Fig. 1B). Sperm with little or no staining, a stained band at the equatorial region, or a loose acrosome was characterized as having undergone the AR (Fig. 1C).

Sperm capacitation and AR inducement. Three factors (added to TALP medium) were tested separately for effectiveness at inducing Eld's deer sperm capacitation *in vitro*: (1) 10 mM CaCl_2 (Sigma Chemical Co.); (2) 1 mM dbcAMP (Sigma Chemical Co.); and (3) 20% FCS (Hyclone Laboratories, Logan UT, USA). The CaCl_2 and cAMP concentrations were selected based on previous bovid studies (Handrow *et al.*, 1989; Galantino-Homer *et al.*, 1997; Roth *et al.*, 1998).

LC (Sigma Chemical Co.; Parrish *et al.*, 1988) and CI (Sigma Chemical Co.; Byrd, 1981) were tested separately for effectiveness at inducing the AR in capacitated sperm. Aliquots of each sperm treatment were added 1:1 to TALP (Parrish *et al.*, 1988), LC (100 $\mu\text{g}/\text{ml}$), DMSO (1 $\mu\text{l}/250 \mu\text{l}$ TALP), and CI (20 μM in 1 μl DMSO/250 μl TALP).

Experimental Design

Freshly ejaculated semen samples ($n = 6$) were evaluated for volume, sperm motility, sperm progressive motility, and sperm concentration (Fig. 2). All met minimal quality standards allowing use in the experiment. Ejaculates were diluted with an equal volume of BF5F (0% glycerol, 37°C), and a portion (~30% of the diluted ejaculate) of the diluted sample was subdivided into four equal portions (sperm concentration was $\sim 100 \times 10^6$ motile sperm/ml). Remaining ejaculate was frozen and stored as described previously. After all semen was further diluted with equal volumes

of TALP and centrifuged (300 x g for 8 min), the supernatant was discarded, and sperm pellets were resuspended in TALP containing one of the following four main treatments: (1) TALP (control); (2) TALP + 10 mM CaCl₂; (3) TALP + 1 mM dbcAMP; and (4) TALP + 20% FCS.

Each of the main treatments was further subdivided into four aliquots to test the efficacy of LC (100 µg/ml, incubated for 30 min) or CI (10 µM, incubated for 30 min) in combination with each main treatment. LC was dissolved in TALP and CI in DMSO and TALP. Therefore, TALP served as the control for LC, and a mixture of TALP and DMSO served as the control for CI. The controls were added separately to each of the main treatments. At each time-point (0, 3, 6, 9, and 12 h), 25 µl aliquots from each of the main treatments were diluted with equal volumes of TALP, TALP-LC, TALP-DMSO, or TALP-DMSO-CI, resulting in 16 total permutations. The main treatments were incubated (37°C; 5% CO₂; 20% O₂) for a total of 12 h, or until sperm motility dropped to 10% or less (this length of time was termed "longevity"). Sperm motility and progressive motility of the four main treatments were recorded at each time-point, as well as for the LC and CI sub-treatments (and their respective controls). A sperm motility index (SMI) value was calculated as the product of progressive motility multiplied by 20, plus the percentage sperm motility value, all divided by two (Howard & Wildt, 1990). Sperm smears were prepared for acrosomal integrity assessments as previously described.

Frozen semen was thawed four to six weeks after initial cryopreservation and subjected to the same protocol as described for fresh sperm.

Statistics

Means and standard errors were calculated for all sperm quality data. Data for percentage of intact acrosomes in the different control and treatment levels, and among males, were subjected to arcsine transformation and compared using repeated measures

analysis via multivariate analysis of variance (MANOVA). The SAS procedure PROC GLM was used, and means were compared using the Student-Newman-Keuls test with α at 0.05. Percentage viability data were analyzed similarly. SMI values did not require transformation but were also subjected to multivariate analysis. Statistical analyses were performed on fresh sperm through the 9 h time-point. For the frozen aliquots, analysis continued through 6 h, because thawed Eld's deer sperm generally did not survive beyond this time-point.

Results

Sperm quality for the six sperm donors was high (Table 1). Males produced semen volumes and sperm motility and morphology traits consistent with normal fertility after natural or assisted breeding (Monfort *et al.*, 1993c). One of the six sperm donors was a proven breeder. There were no differences ($p > 0.05$) in semen or sperm traits among males on the basis of age.

Mean (\pm SEM) SMI values for fresh sperm incubated in the presence of the main treatments ranged from 73.8 ± 1.9 to 76.7 ± 2.2 at 0 h, and 33.8 ± 3.2 to 47.5 ± 2.7 at 12 h incubation (Fig. 3). SMI values declined gradually throughout the incubation interval. Mean SMI values for fresh sperm combined with sub treatments (main treatments combined with either LC, CI, or their respective controls) ranged from 27.7 ± 3.3 to 72.5 ± 3.1 at 0 h, and 0.0 to 48.1 ± 8.9 at 12 h of incubation. Mean viability values for fresh sperm sub treatments ranged from $78.3 \pm 6.4\%$ to $95.0 \pm 0.0\%$ at 0 h, and $20.0 \pm 20.0\%$ to $61.3 \pm 6.6\%$ at 12 h of incubation. Poorest motility and viability values were consistently associated with the presence of CI in the medium.

There were no differences ($p > 0.05$) in acrosomal integrity in cAMP and FCS treatment groups compared to TALP controls. Acrosomal integrity after the addition of CI or LC to the cAMP and FCS treatments was similar ($p > 0.05$) to respective controls (Figs. 4 & 5). Furthermore, the TALP control did not have a measurable impact on

sperm capacitation ($p > 0.05$). In contrast, by 6 h of incubation, the proportion of intact acrosomes decreased ($p < 0.05$) after treatment with 10 mM CaCl_2 and CI (Fig. 6). Table 2 depicts individual male responses to the 10 mM CaCl_2 /CI combination. With the exception of a single male, this treatment induced a marked decline in intact acrosomes by 9 h. In contrast, LC failed to induce the AR in fresh, capacitated Eld's deer sperm ($p > 0.05$; data not shown).

Freeze-thawing reduced ($p < 0.05$) mean SMI and viability values compared to freshly collected counterparts. Mean SMI values for frozen-thawed sperm incubated in the presence of the main treatments ranged from 53.3 ± 1.7 to 57.5 ± 1.8 at 0 h, and 30.0 ± 0.0 to 33.8 ± 3.75 at 9 h (Fig. 7). Mean SMI values for frozen-thawed sperm combined with sub treatments ranged from 0.0 to 39.6 ± 2.4 at 0 h, and 0.0 to 30.0 ± 5.0 at 9 h. Mean sperm viability values for thawed sperm ranged from $28.3 \pm 1.7\%$ to $48.3 \pm 1.7\%$ at 0 h, and $3.0 \pm 2.0\%$ to $32.5 \pm 2.5\%$ at 9 h. Sperm incubated in the presence of CI consistently exhibited the lowest motility and viability values (Fig. 6.).

Frozen-thawed sperm experienced reduced levels of acrosomal integrity compared to fresh sperm (~ 20 to 30% less intact acrosomes), as well as reduced longevity (Table 1). Acrosomal integrity results for cryopreserved sperm were similar for all treatments assessed (Fig. 8). None of the main treatments were efficient at inducing capacitation in frozen-thawed sperm ($p > 0.05$), and neither LC nor CI induced the AR.

Discussion

These results demonstrated only limited success in artificially inducing *in vitro* capacitation of Eld's deer sperm using methods considered conventional in other ungulate species. This suggests that Eld's deer sperm may differ from the sperm of other cervid species, resulting in different fertilization mechanisms. These data confirm that recent failures to achieve effective sperm/oocyte interactions *in vitro* in

this species in our laboratory may be at least partially explained by low capacitation rates in Eld's deer sperm. Little effort has been directed at understanding sperm capacitation in cervids. The data suggest that there are as yet, undiscovered mechanisms regulating the functional ability of deer sperm to undergo the processes required to provoke sperm/oocyte interaction *in vitro*. These mechanisms need to be understood to achieve the goal of routinely manipulating reproduction, and allowing assisted breeding to consistently contribute to genetic management of captive populations.

The FITC-PNA staining method was determined to be effective for evaluating acrosomal integrity in Eld's deer sperm using fluorescence microscopy. The protocol required little processing time, and the staining patterns were easily discerned and similar to those observed in felid (Long *et al.*, 1996) and scimitar-horned oryx (*Oryx dammah*; Roth *et al.*, 1998) sperm. Domestic cat sperm express brightly labeled equatorial segments after the AR occurs (Long *et al.*, 1996), whereas oryx (Roth *et al.*, 1998) and Eld's deer sperm occasionally display a fine fluorescent line along the equatorial segment.

The data implied that Ca^{2+} supplementation increased capacitation of fresh Eld's deer sperm. The requirement for Ca^{2+} during sperm capacitation has been examined in the bull (Handrow *et al.*, 1989) and mouse (Visconti & Kopf, 1998). Furthermore, adding CI to medium supplemented with high Ca^{2+} (10 mM) induced the AR in fresh, capacitated Eld's deer sperm. Of considerable interest is that the only proven breeder among the six donors exhibited the greatest decline in intact acrosomes when sperm was exposed to Ca^{2+} and CI (90.2% at 0 h to 33.6% at 9 h). Although it is understood that an influx of Ca^{2+} is associated with the AR (Yanagimachi & Usui, 1974; Didion & Graves, 1989), its occurrence during capacitation is not well understood (Yanagimachi, 1994). The results suggested that adding Ca^{2+} to the incubation medium augmented the response to CI, but it was not clear if Ca^{2+} exerted

its effects at the time of capacitation or on the AR. Roth *et al.* (1998) also reported this difficulty in distinguishing when Ca^{2+} exerts its effects, but in the scimitar-horned oryx.

A cAMP analogue was not effective at inducing capacitation in fresh or frozen-thawed Eld's deer sperm. The role of cAMP during sperm capacitation and the AR, like Ca^{2+} , is not fully understood (Yanagimachi, 1994), but investigations have been made in several species using cAMP analogs such as dbcAMP. Supplementing media with dbcAMP and extra Ca^{2+} has been found to encourage sperm capacitation in the teratospermic clouded leopard (*Neofelis nebulosa*) (Pukazhenti *et al.*, 1996b). Combinations of dbcAMP and 1-methyl-3-isobutylxanthine (IBMX; a phosphodiesterase inhibitor) have been incubated with human sperm (Leclerc *et al.*, 1996) and hamster sperm (Visconti *et al.*, 1999a) to promote capacitation and the AR. Furthermore, a combination of dbcAMP and caffeine (another phosphodiesterase inhibitor) is required to induce hyperactivated motility in macaque (*Macaca fascicularis*) sperm (Vandevoort *et al.*, 1994). Perhaps the combination of a phosphodiesterase inhibitor and a cAMP analogue would prove to be more efficient at promoting capacitation and the AR in Eld's deer sperm, since the phosphodiesterase inhibitor would prevent the breakdown of excess cAMP and therefore the inhibition of protein tyrosine phosphorylation.

Based on a novel approach being used in New Zealand to capacitate red deer sperm (R. Spindler, pers. comm.), FCS was considered in our study as a cholesterol-binding protein. A protein source, usually bovine serum albumin (BSA), is incorporated into the incubation medium and acts as a sink for the extraction of cholesterol from the sperm plasma membrane (Go & Wolf, 1985; Langlais & Roberts, 1985; Cross, 1998). Visconti *et al.* (1999b) proposed that cholesterol efflux mediated by BSA results in an increase in the permeability of the sperm membrane to bicarbonate and Ca^{2+} ions. These ions then activate the sperm adenylate cyclase, resulting in increases in cAMP concentrations and protein kinase A (PK-A) activity,

consequently encouraging protein tyrosine phosphorylation.

Like cAMP, FCS was ineffective at inducing sperm capacitation in the Eld's deer. Since both of these potential capacitors are thought to be linked to the cAMP-mediated-PK-A pathway proposed to exist in mammalian sperm, it is possible that this pathway does not play a predominant role in the Eld's deer sperm capacitation process, and that Eld's deer sperm may possess some yet unknown unique fertilization mechanisms.

CI appeared toxic to Eld's deer sperm at the dosage tested since sperm motility and viability values were markedly lower ($p < 0.05$) in the presence of this AR inducer. CI has a long history of being useful for inducing the AR in multiple species (Table 3). We relied on a CI dosage consistent with that used for bull sperm (Byrd, 1981). Garde *et al.* (1997) recently used a triple-staining technique to simultaneously determine viability and acrosomal integrity in red deer sperm, and a CI concentration about 10 fold less than that used for the present study. Adequate levels of sperm motility and viability were achieved throughout the duration of a 9 h *in vitro* incubation interval. Further, maximum percentages of live acrosome reacted sperm ($24 \pm 1.10\%$) were also observed at 9 h. Thus, these authors claimed an ability to readily stimulate capacitation in red deer sperm. These data are obviously in conflict with our findings, where we assert that it was quite difficult to stimulate capacitation in Eld's deer. Indeed there may be species-specificity, but it is worth noting that Garde *et al.* (1997) tested a limited number of samples ($n = 3$). Furthermore, they examined epididymal sperm (compared to ejaculated sperm in the present study). Compared to epididymal counterparts, ejaculated sperm of most species studied to date appear to be more resistant to sperm capacitors (Yanagimachi, 1994). This is due to the addition of "stabilizers" on plasma membranes during the ejaculation process (Yanagimachi, 1994). Both studies also relied upon different incubation media. A modified Tyrode's medium (TALP; Parrish *et al.*, 1988) was used for the present study, whereas TCM 199

was used for red deer sperm. TCM 199 was designed for sustaining somatic cells *in vitro* and contains components that are detrimental to embryos, and, therefore, germ cell maintenance (Bavister, 1995). Thus, the tissue culture medium in the red deer study may have exerted a destabilizing effect, provoking spontaneous ARs. Therefore, the results obtained by Garde *et al.* (1997) may be misleading, and we predict that cervid sperm in general are difficult to stimulate to capacitate *in vitro*.

The data on frozen-thawed sperm indicated that the cryopreservation process may have disrupted the time course of Eld's deer sperm capacitation, resulting in premature capacitation. Acrosomal integrity percentages were considerably reduced for frozen-thawed sperm ($p < 0.05$), starting at the first time-point (0 h), and none of the main treatments or sub-treatment combinations seemed to encourage further increases in sperm capacitation and the AR. Evidence suggests that frozen-thawed sperm may be capable of undergoing the AR sooner than uncapacitated fresh sperm (Watson *et al.*, 1992). Holt and North (1986) reported dramatic modifications in the organization of membrane lipids in ram spermatozoa as a result of temperature-related phase transitions from the gel to liquid-crystalline state and vice versa. These transitions are associated with changes in membrane permeability, including the leakage of solutes, particularly the efflux of K^+ and the influx of Ca^{2+} (Drobnis *et al.*, 1993). Therefore, CI may have been able to permeate the plasma membrane of frozen-thawed sperm easier than that of the fresh sperm, resulting in accelerated sperm capacitation. It is also possible that the BF5F extender used in the present study may have caused an increase in spontaneous acrosome reactions as has been demonstrated with other egg yolk based extenders (Ijaz & Hunter, 1989; Ijaz *et al.*, 1989).

The most significant contribution we have made in this study is to illustrate the challenges to be met in artificially stimulating capacitation and the AR in Eld's deer sperm. As conventional approaches are either not effective or cause capacitation combined with sperm mortality, there is an obvious need for more research.

Supplementing TALP medium with high concentrations of Ca^{2+} (10 mM) to promote capacitation, and inducing the AR using the CI technique, seems to be the most promising strategy to pursue further. Additional research could also focus on the use of chlortetracycline (CTC; Saling & Storey, 1979; Cross & Meizel, 1989; Abeydeera *et al.*, 1997; Hewitt & England, 1998). This fluorescent probe allows distinguishing sperm that have capacitated but those that have not acrosome reacted, as well as those that have capacitated and acrosome reacted. Therefore, this approach permits identifying precise points in the process where mechanisms are proceeding or failing. Regardless of the approach for inducing capacitation and the AR, certainly more information could be secured by combining such studies with sperm/oocyte interaction studies *in vitro*. IVF trials are imperative since the ultimate test for sperm capacitation is to determine if sperm/oocyte binding/penetration is achievable *in vitro*.

It is crucial that sperm capacitation becomes well understood to insure that we have a comprehensive knowledge of sperm function that is key to achieving applied success using IVF. This information, in turn, is fundamental to the practical establishment and routine use of genome resource banks as a management and conservation tool. Transfer of genetically-valuable material between zoos and breeding facilities requires the development of efficient, standardized laboratory protocols. Capacitation of both fresh and frozen-thawed sperm must be investigated in every species designated for genome resource banking. Protocols will necessarily vary from species to species and it is fruitless to cryopreserve valuable genetic material, destined to aid in the long-term management of endangered species, if the viability of frozen-thawed sperm is doubtful.

Table 1. Ejaculate characteristics of six individual Eld's deer stags (19-24 May).

Male	Age (yr)	Ejaculate volume (ml)	Pre-freeze sperm motility (%)	Normal sperm forms (%)	^a Post-thaw Sperm motility at 0 h (%)	^b Longevity <i>in vitro</i> (h)	
						Fresh	Frozen-thawed
1	11	2.0	80	98.0	50	12	9
2	11	2.9	80	95.5	40	12	6
3	4	2.3	80	94.0	40	9	6
4	5	1.4	75	(-)	40	12	6
5	4	0.3	85	99.0	40	9	6
6	4	1.9	80	97.0	50	12	9

No differences ($p > 0.05$) were observed among stags for all parameters.

^aPost-thaw motilities are for washed sperm resuspended in TALP medium (control).

^bLength of time until sperm motility dropped to $\leq 10\%$. Sperm were observed for a maximum of 12 h.

(-)Missing value

Table 2. Intact acrosomes for fresh Eld's deer sperm incubated in the presence of 10 mM CaCl₂ and CI (10 µM).

Male	Intact acrosomes (%)	
	0 h	9 h
1	76.5	52.8
2	85.9	56.3
3	77.2	26.0
4	73.6	76.5
5	78.0	53.2
6	90.2	33.6

Table 3. Calcium ionophore concentrations previously used in different species to induce the acrosome reaction.

Species	μM	Reference
Bull	10	Byrd, 1981
Domestic cat	4	Long <i>et al.</i> , 1996; Pukazhenthil <i>et al.</i> , 1998
Red deer	1	Garde <i>et al.</i> , 1997
Eld's deer	10	Present study

Fig. 1. Acrosomal staining patterns observed in Eld's deer sperm using the FITC-PNA method: **A.** intact sperm; **B.** damaged sperm with a ballooning acrosome; and **C.** acrosome reacted sperm.

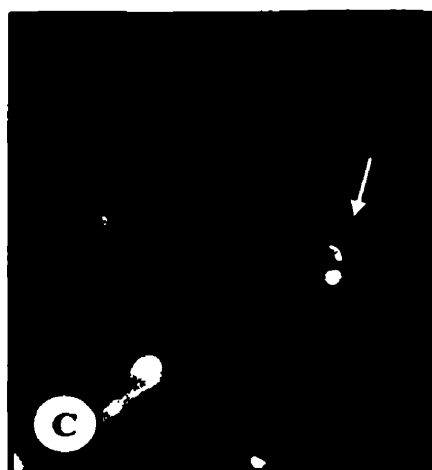
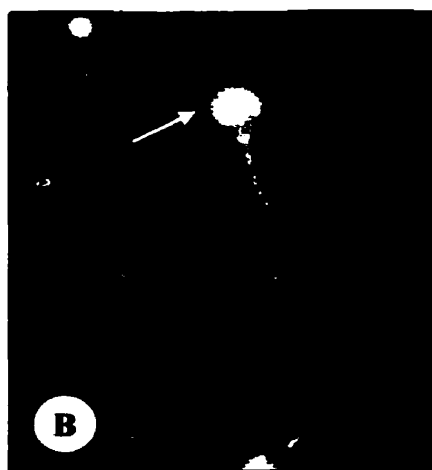
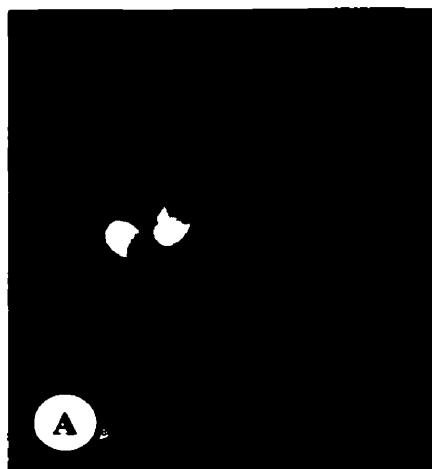


Fig. 2. Both CI and LC were evaluated for effectiveness at inducing the AR in Eld's deer sperm *in vitro*. Sperm from the main treatments also were incubated with the respective controls for CI and LC. The control for CI was a mixture of DMSO and TALP, and the control for LC was TALP.

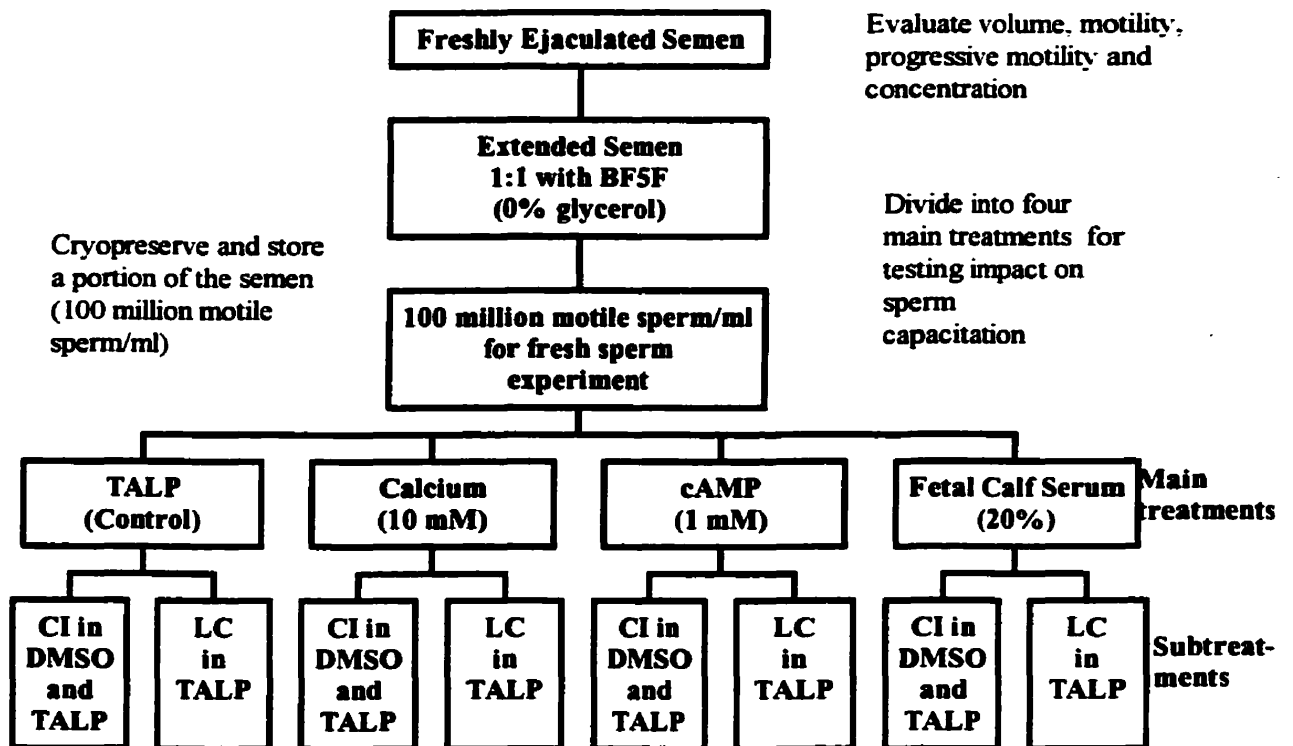


Fig. 3. Mean (\pm SEM) SMI values for fresh sperm ($n = 6$ ejaculates) incubated in TALP medium alone or in combination with either 10 mM CaCl_2 , cAMP or FCS. Within time periods, bars with an asterisk differ ($p < 0.05$).

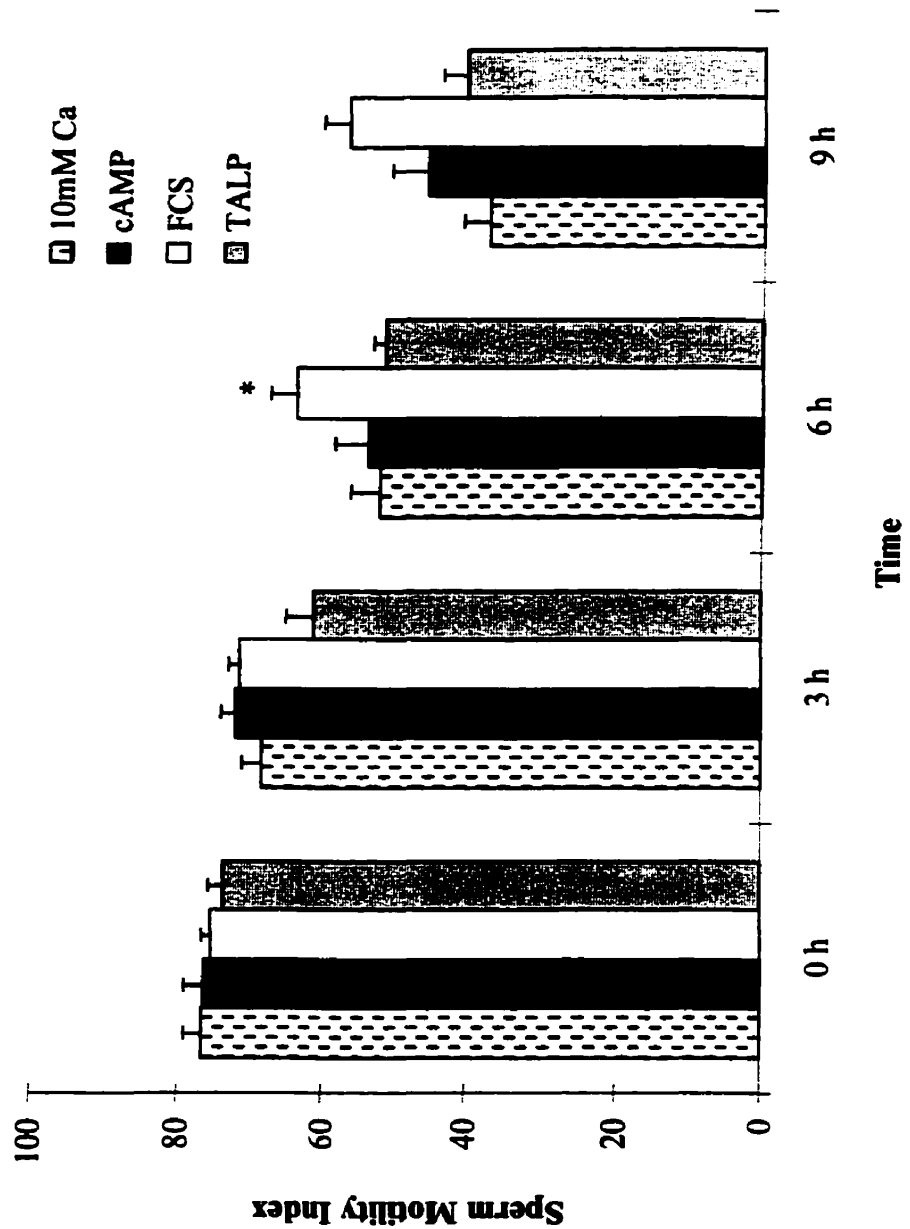


Fig. 4. Mean (\pm SEM) percentage of intact acrosomes for fresh sperm ($n = 6$ ejaculates) incubated with cAMP and DMSO, CI, TALP or LC.

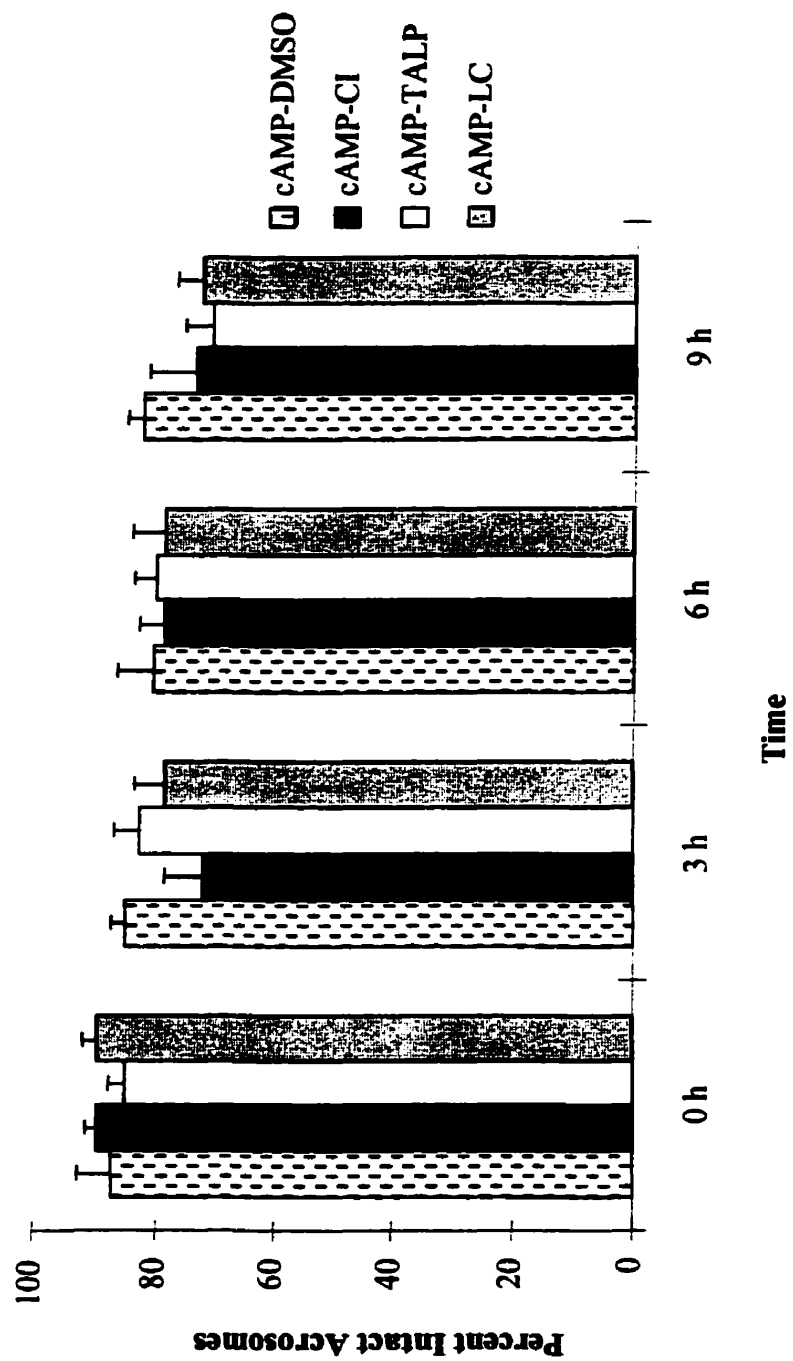


Fig. 5. Mean (\pm SEM) percentage of intact acrosomes for fresh sperm ($n = 6$ ejaculates) incubated in medium with FCS and DMSO, CI, TALP or LC.

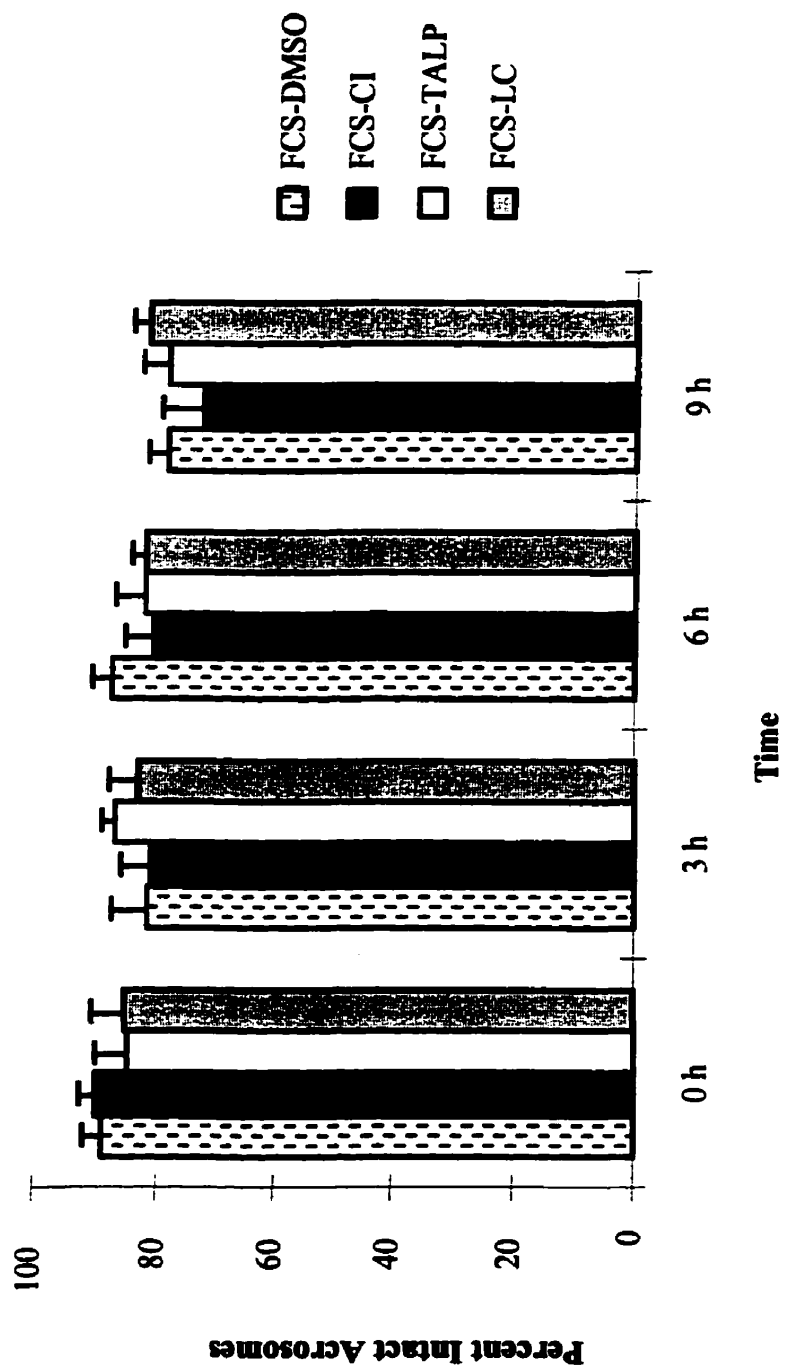


Fig. 6. Mean (\pm SEM) traits of fresh sperm ($n = 6$ ejaculates) incubated in TALP with 10 mM CaCl_2 and with and without CI (10 mM Ca-Cl). The control is 10 mM Ca-DMSO . Within time periods, bars with an asterisk differ ($p < 0.05$).

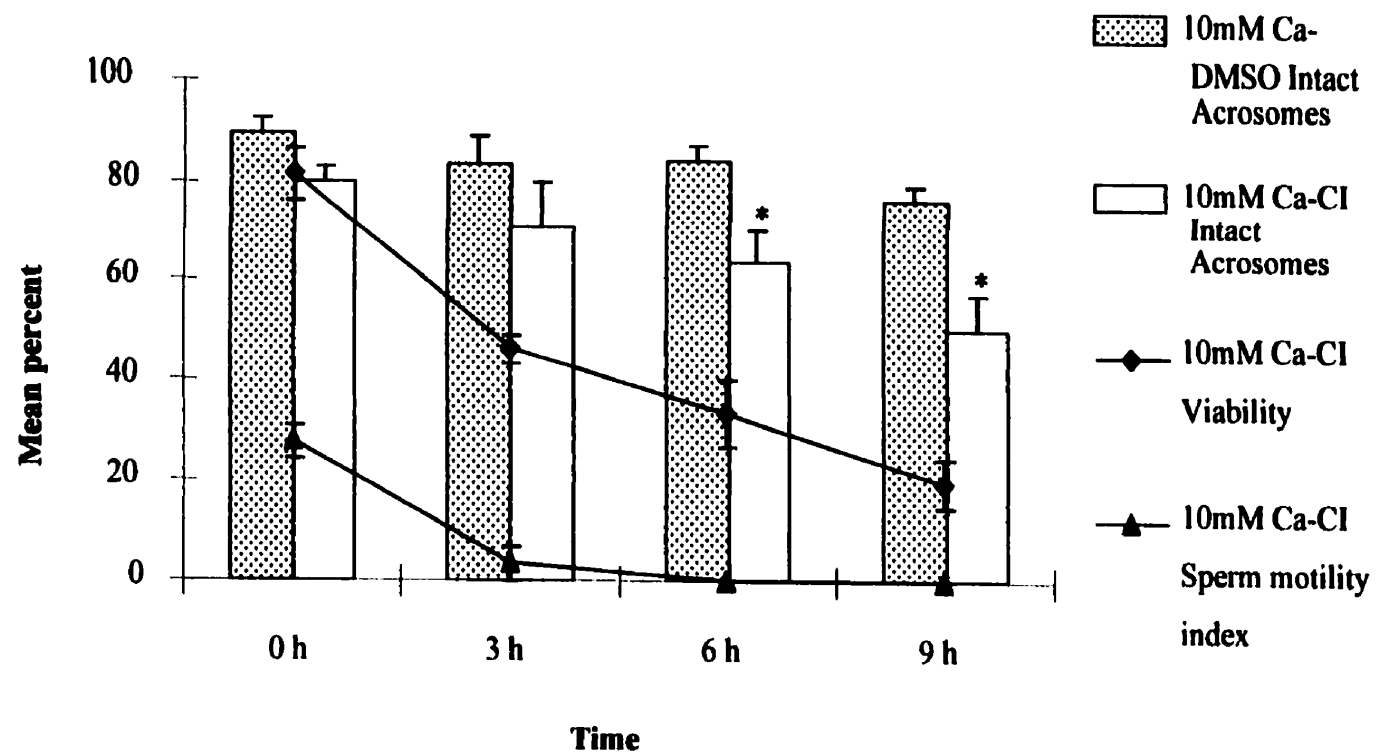


Fig. 7. Mean (\pm SEM) SMI values for frozen-thawed sperm ($n = 6$ ejaculates) incubated in TALP medium alone or in combination with either 10 mM CaCl_2 , cAMP or FCS. Within time periods, bars with an asterisk differ ($p < 0.05$).

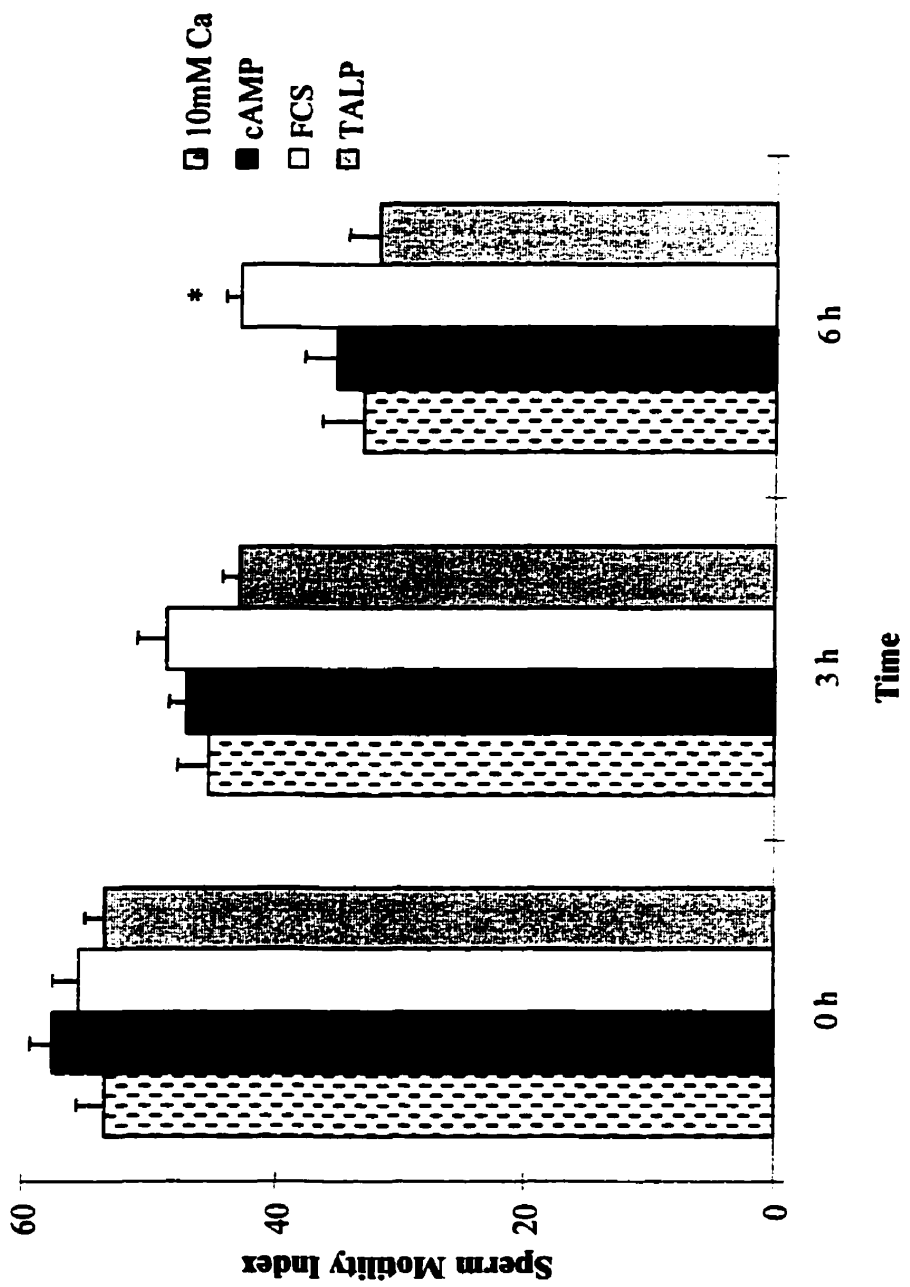
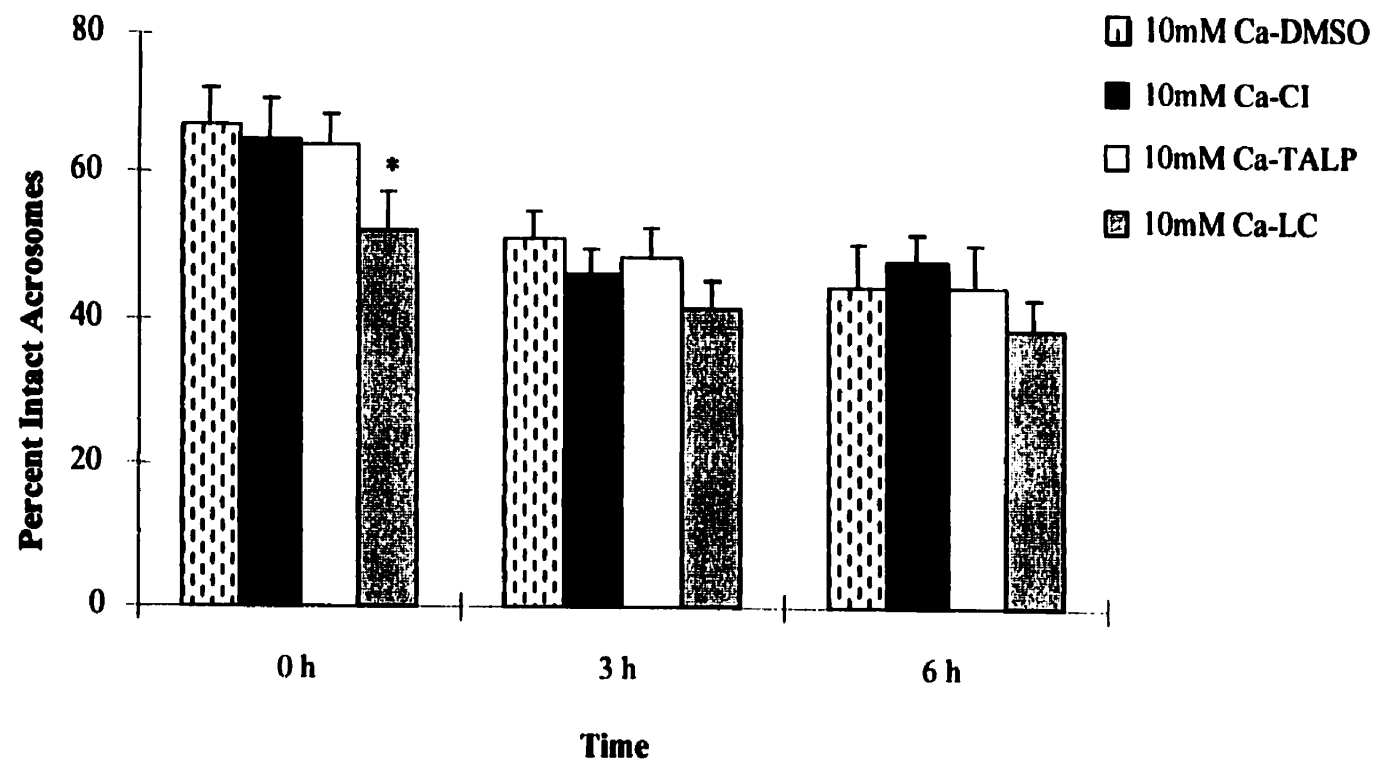


Fig. 8. Mean (\pm SEM) percentage of intact acrosomes for frozen-thawed sperm ($n = 6$ ejaculates) incubated in medium with 10 mM CaCl_2 and DMSO, CI, TALP or LC; FCS, cAMP and TALP demonstrated similar ($p > 0.05$) profiles for frozen-thawed sperm. Within time periods, bars with an asterisk differ ($p < 0.05$).



SECTION 4:

CONCLUSIONS

Additional research is required to ascertain which protocol is the most efficient at inducing sperm capacitation in the Eld's deer *in vitro*. Further sperm collection trials are necessary to analyze the effectiveness of other potential sperm capacitation inducers. Furthermore, re-examination of factors at different concentrations may also prove fruitful. Unfortunately, the often low numbers of individuals available to researchers compromises endangered species research. The investigation of sperm capacitation in the Eld's deer would be enhanced by having access to stags from other zoological parks and/or breeding facilities, allowing for a more thorough examination at the possibility of inter-male variation. Eld's deer *in vitro* fertilization trials may also provide further insight into fertilization mechanisms.

Various genome resource bank (GRB) strategies must be analyzed for every species that has been designated for banking. One cannot simply assume that one strategy will be effective for all pedigree structures. Both genetic and demographic factors must be taken into account to found functional GRBs. This Master's used historical data to create hypothetical GRBs, but the generation of a simulation program that could be used to project into the future would be very valuable. Additionally, the examination of hypothetical interactive sperm banks would be worthwhile. This would involve forming a bank that is updated with new/additional biomaterial at regular intervals. Overall, the use of simulation programs was found to be a useful method for gaining insight into the effectiveness of potential GRB strategies.

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