THE EFFECT OF CONCENTRATION AND TIME OF INCUBATION OF GLYCEROL ON SEMEN CHARACTERISTICS IN FELIDS

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THE EFFECT OF CONCENTRATION AND TIME OF INCUBATION OF GLYCEROL ON SEMEN CHARACTERISTICS IN FELIDS

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I am greatly appreciative for my committee members, fellow students, family members, and friends.

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CHAPTER I

INTRODUCTION

Endangered Felid Populations

Populations of felids have become increasingly threatened around the world. According to the Small Cat Conservation Alliance, there are 36 recognized species of cats in the world, 23 of which are threatened with extinction. Twenty-two of the 36 species are classified as small cats. Many endangered species have become a part of an ever-shrinking genetic pool. A number of the threatened felid species have gone through population bottlenecking at one point or another (Russelo, Gladyshev, Miquelle, & Caccone, 2004). With the continued encroachment of urbanization into wildlife habitats, it is likely that some indigenous species may be unable to survive.

There is relatively little information about endangered small cats compared to other carnivores (Sanderson, 2012). Studies at the Audubon Center for the Research of Endangered Species in New Orleans has focused its conservation efforts on small African cats like the black footed and sand cat. The National Zoo Cat Conservation Project at the Smithsonian Conservation Biology Institute in Washington, DC has used modern reproductive technologies, sometimes originally developed for human infertility, in the fight against decreasing genetic diversity in endangered cats. However, because of endangerment, an increase in genetic diversity is necessary to avoid further losses from

disease susceptibility and poor fertility caused by inbreeding. Use of assisted reproductive technologies like artificial insemination, *in vitro* fertilization, embryo transfer and cryopreservation of sperm, oocytes and embryos may be required to prevent the complete annihilation of endangered small cats (Smithsonian Conservation Biology Institute).

Interest in controlling reproductive function dates back to biblical times. It is believed that Democritus initiated the first documentable study of fertility around 470 BC. Since then, scientists have continued to investigate ways of controlling fertility. In recent years, interest in the scientific community has focused on the cryopreservation of living cells as a means of enhancing reproductive rate. The science of cryopreservation encompasses processes such as temperature suppression, freezing, and vitrification of cells. The advancements in these technologies have played a major role in the conservation of countless species and are thought to be useful for future conservation efforts, particularly of endangered species.

Researchers around the globe are attempting to develop artificial reproductive technologies (ART) that will allow reproductive cells, including oocytes, sperm and embryos from endangered species to be preserved. Improving ART used in freezing sperm will likely increase cell integrity and most likely the effectiveness of those cells when they are finally used. Since techniques used to successfully freeze living cells and tissues is relatively new, researchers are now trying to "fine-tune" existing procedures to increase the success of cryopreservation. Improvements in applied research are imperative to push forward the field of cryobiology, possibly improving conservation efforts, particularly on endangered species, for the future. Investigations into the chemical

composition of feline sperm and the associated sensitivities of these cells to cryoprotectants during both freezing and thawing procedures are needed (Luvoni, 2006). There is a continuous need to assess genetic variation even in recovering populations and identify the important role that captive breeding programs may play in preserving remnant genetic diversity of endangered species (Russelo, Gladyshev, Miquelle, & Caccone, 2004).

Strategies of Species Preservation

Techniques such as artificial insemination, embryo transfer, inner cytoplasmic sperm injection, *in vitro* fertilization and nuclear transfer benefit greatly when advancements are made in cryobiology. Many of these strategies have been successfully used in felid species. One example is the black-footed cat at Audubon Center for the Research of Endangered Species, ACRES, born through *in vitro* fertilization and embryo transfer using frozen semen (Endangered Kittens are Born using In-Vitro Fertilization, 2011).

The use of many recently developed reproductive technologies has largely been the result of advances in cryopreservation. However, viable gametes are a requisite for the successful implementation of reproductive technologies such as IVF, ET, and other ART procedures. Therefore, perpetuation of endangered species using reproductive technologies will require an exquisite knowledge and understanding of gamete cryobiology.

Semen storage technology was revolutionized around 60 years ago when the cryoprotectant glycerol was first used to freeze sperm (Polge, Smith, & Parkes, 1949). Glycerol has since been used to freeze sperm in many species. Platz et al., the first

reported cryopreservation of ejaculated cat semen, recorded a conception rate of 11% after intravaginal insemination of queens with the frozen sample (Platz, Wildt, & Seager, 1978). The use of cryopreserved sperm has animal welfare implications. For example, shipping sperm from one location to another to facilitate breeding is less stressful than transporting animals (Smithsonian Conservation Biology Institute).

Problem in Cryopreservation of Gametes

The use of frozen semen in the domestic cat is mostly limited to research laboratories with assisted reproduction technologies primarily because of a lack of economic incentive (Luvoni, 2006). There are many challenges in developing and perfecting ART, including both biological and environmental factors such as slight changes in temperature, time, and/or chemical composition of media used. Hence, progress in improving ART will likely come thorough tedious research efforts. Because of the importance of ART in fields ranging from agriculture to the preservation of endangered species of animals and plants, it is imperative that the scientific community continues to push forward in developing ART. Studies reported in this document were designed to determine the effect of glycerol concentration and time of refrigeration prior to freezing on the post-thaw survival rate of feline spermatozoa.

CHAPTER II

REVIEW OF LITERATURE

Endangered Felids in the Wild

Smaller wild cats represent 80% of all cat species. Three quarters of the wildcat family weigh less than 50 lbs. Many are around the size of a domestic cat, and the smallest species weighs just 2 lb. as an adult (International Society for Endangered Cats Canada, 2012). The majority of cat species in the wild are now vulnerable, threatened, or endangered. While populations of some felid species have become stable with the help of the many organizations like Small Cat Conservation Alliance, ACRES, and National Zoo Cat Conservation Project, they are unlikely to increase in number in the wild. Many felids species are threatened by the loss of habitat. Cats are intelligent animals, and sensitive to degradation of their habitat or decline of prey species (International Society for Endangered Cats Canada, 2012). Habitat loss is the catalyst causing many of the problems that arise in endangered or threatened species such as disease, hunger, and predation. Perpetuation of species is further threatened by genetic bottlenecking (Sanderson, 2012). Reduction of genetic variation is often difficult to overcome. If the only animals able to be used are the individuals within a reasonable distance from where they are needed then the conservation efforts are greatly limited. It is for this reason that gamete preservation is so important. Scientists now have the ability to preserve and store gametes for future uses using ART procedures from both living and recently diseased

individuals. Without the drive to identify the best gamete preservation parameters the ability to fertilize or store embryos would be sub-par. It is for this reason that this research project was conducted with the focus of germ plasma preservation as opposed to embryo.

Cryopreservation of Cells from Reproductive Tissues

Species Variation

Cryopreservation is the process of cooling and storing cells, tissues, or organs at very low temperatures, while maintaining their future viability. Procedures of cryopreserving have been established that minimize cell damage when exposed to sub-zero temperatures by altering the osmolarity and concentration of cryoprotectants of media prior to freezing (Luvoni, 2006). Many of the procedures developed for sperm cryopreservation are species specific. Species differences are apparent in the movement of water within sperm cells, which could significantly impact sperm survival during cryopreservation. Therefore, cryopreservation procedures developed for one species may not be suitable for another species (Luvoni, 2006). In cats, poor post-thaw survival was observed when spermatozoa were frozen using media and procedures developed for cattle (Luvoni, 2006). Sperm from domestic cats have a different head shape than some other animals. They also have other unusual physical characteristics that make it more difficult to develop successful cryopreservation methods (Luvoni, 2006). One example of difference to cattle is their sensitivity to higher concentrations of glycerol. Due to the enhanced sensitivity, glycerol concentrations of about 5% are used in freezing cat spermatozoa (Nelson, Crichton, Doty, Volenec, Morato, & Pope, 1999). Variations

between species are likely genetically linked which provides a basis for genomic-based research in semen cryopreservation (Holt, 2000).

Cryopreservation Protocols

While procedures that minimize sperm loss during cryopreservation of domestic cat semen have been documented, optimal freezing or cooling rate for feline spermatozoa has yet to be determined (Luvoni, 2006). Many biological systems can endure ultra-cold temperature storage if they survive ice crystal formation during freezing and thawing (Hammerstedt, 1990). Recrystallization, the change in structure of an ice crystal without a concomitant change in chemical composition, is a major factor causing cell death during cryopreservation. Maintaining the integrity of the plasma, mitochondrial, and acrosomal membranes during freezing and thawing is critical for successful cryopreservation of sperm (Hammerstedt, 1990). There are multiple steps involved in successfully freezing and thawing sperm. Successful cryopreservation of sperm typically involves extension and cooling, penetrating of the cryoprotectant into the cell, selection of an ideal storage vessel, freezing, storage, and thawing.

Media and Cryoprotectants

Media

Identification of a suitable incubation media is critical for cryopreservation of sperm. The osmolarity of incubation medium is critical; the osmolality of feline semen ranges from 290mOsm/kg to 320 mOsm/kg (Luvoni, 2006). In cats, it has been shown that swelling of the membrane caused by a hypotonic solution is more detrimental than shrinking caused by a hypertonic solution (Pukazhenthi, Spindler, Wildt, Bush, & Howard, 2002). Most commonly, a TEST (trishydroxymethylaminomethane sulphonic

acid) buffered solution supplemented with glycerol and 20% egg yolk has been successfully used for felid sperm cryopreservation (Luvoni, 2006).

Types of Cryoprotectants

Numerous cryoprotectants have been used to preserve sperm. Cryoprotectants can be penetrating (glycerol and dimethylsulfoxide) or nonpenetrating (lactose and trehalose) depending upon their ability to pass through the membrane. Both types of cryoprotectants can cause dehydration of the cell by osmotically induced water egress, but they differ in their ability to enter the cell and reside in cytoplasm and membranes. The rate of cryoprotectant transport across cell membranes has not been well established; however, the process can be expected to proceed at a slower rate than water, with similar dependence on membrane structure (Hammerstedt, 1990). Understanding the physiological characteristics of membrane permeability is important in selecting the chemical composition of a cryoprotectant. Research has shown that glycerol is a suitable cryoprotectant for feline and numerous other species (Luvoni, 2006).

Glycerol

Glycerol is a simple polyol compound. It is a colorless, odorless, viscous liquid that has three hydroxyl groups that are responsible for its solubility in water and its hygroscopic nature. It is a viscous trihydric alcohol that can be phosophorylated and dephosphoryclated by ubiquitous glycerol kinase and general phosphatases (Izawa, M, Yokoigawa, & Inoue, 2004). Glycerol is a semipermeable solute with relatively small lipophilic molecules that are permitted to absorb to the membranes of internal organelle bodies (Batycky, 1997). Cell volume expansion or contraction depends largely on the internal absorption behavior of semipermeable solutes and membrane permeation

parameters (Batycky, 1997). Cellular response to osmotic stresses, such as the addition of glycerol, is understood from observing cell volume change following the applied osmotic stresses (Liu et al., 1995). Increasing glycerol concentration breaks apart clusters of water molecules found in water, ice, crystal lattices, and in bulk liquid water. Addition of a cryoprotectant to cell suspensions alters congregating properties of bulk water to lower the freezing point, which aids in the survival of the cells during freezing (Mazur, 1984).

Sperm Preservation

Membrane Reorganization

Cell membranes are considered to be a lipid bilayer with integral proteins dispersed throughout. Reorganization of lipids causes changes in membrane integrity, which can affect the kinetics of water and cryoprotectant transport (de Gier, 1989). The addition of glycerol to cells at either 37C or 4C may affect survival of sperm due to the large volume changes that are induced by a cryoprotectant (Fiser & Fairfull, 1989). The lipid bilayer of a membrane is altered by glycerol, as reflected in an increased interdigitation of the nonpolar regions of the two bilayers (Boggs & Rangaraj, 1985). Therefore, glycerol alters permeability rates of water across cell membranes, which is essential to survival from freeze-thaw treatments (Bashford, Alder, Menestrina, Micklem, Murphy, & Pasternak, 1986). The interactions of lipids and proteins caused by glycerol results in subtle, but important, alterations of membrane pores, enzymes, and receptors (Aloia, Curtin, & Gordon, 1988).

Hydrogen Bonds

Glycerol is a common component in nature; it reduces the water available to form extracellular ice in algae, insects, fish and reptiles (Towey & Dougan, 2012). Though it is

ubiquitous in nature and is extensively used in science, the molecular mechanism by which it stabilizes and protects cells is poorly understood (Towey & Dougan, 2012). Glycerol interacts favorably with water to strengthen the hydrogen bond network of the solvent (McDuffie, Quinn, & Litovitz, 1962). With increasing glycerol concentration in mixtures with high water content, the average number of hydrogen bonds in water from first hydration shell interactions grows at the expense of bulk water interactions. This means that when glycerol is added to water, the structure of hydrogen bond network changes, and the water molecules rotate, causing the hydrogen bonds to break, which are then given to the nearest neighbor molecule. The group of water molecules oriented directly around an ion is called the first hydration shell. Each ion has a hydration shell that prevents water molecules from forming the hexagonal structure of ice. As the concentration of glycerol increases, the average number of hydrogen bonds/first hydration shell water surrounding glycerol increases (Dashnau, Nucci, Sharp, & Vanderkooi, 2006). After the bulk water pool is depleted, hydrogen bonding in the first hydration shell becomes disrupted as glycerol molecules compensate for the lack of sufficient water molecules for solvation, association of solvent and solute molecules. Water, a stronger hydrogen bond acceptor, becomes less available to hydrogen bond with the alkyl backbone as glycerol concentration increases. High glycerol concentration mixtures mimic the strong hydrogen-bonding pattern seen in ice, yet crystallization does not occur. These results lend support to the belief that the combined effect of bulk water depletion, increase in alkyl backbone interactions, and hydrogen bond linearization contributes to the cryoprotective properties of higher concentration glycerol/water mixtures (Dashnau, Nucci, Sharp, & Vanderkooi, 2006).

In lower concentrations of glycerol/water mixtures, like the ones used to preserve the functionality of biological molecules during cooling and thawing processes, glycerol efficiently hydrogen bonds with the surrounding water molecules primarily in isolation (Towey & Dougan, 2012). Researcher has shown that many glycerol molecules exist as monomers in a dilute glycerol-water mixture, while a small number of large clusters exist. Since glycerol molecules efficiently take the place of water molecules in the hydrogen bonded network, an extended water network is impeded. It is thought that this glycerol preference to monomers prevents the disruption of biological systems at lower temperatures (Towey & Dougan, 2012).

Cooling of Sperm

Prior to freezing, sperm are diluted with a medium that provides essential nutrients required for survival as well as ions that maintain proper osmolarity. Addition of media to sperm extends lifespan/survivability and is called extended semen. Glycerol, used as a cryoprotectant, is typically added to extended sperm prior to freezing.

Once an understanding of the molecular function of glycerol is established, the processes of cryopreservation and their role in cell survival can be better appreciated. Numerous studies have demonstrated that too successfully freeze sperm, the extended semen must be combined with glycerol and cooled before freezing. Rate of cooling of extended semen is affected by the concentration glycerol. Prior to freezing, a cryoprotectant is added before cooling because temperature alters physical properties (Hammerstedt, 1990). When cooling sperm, within a period of 30 minutes, the suspension (diluted sperm including seminal plasma) passes through the critical temperature zone of 15-20 C where the physical properties of the membrane change

drastically. The rate of temperature change must be identified that allows movement of water and cryoprotectant without either intracellular ice formation or irreversible membrane change (Hammerstedt, 1990). Membrane related changes are slow at lowered temperatures because of the reduced mobility of the components in the medium, but increased time spent during the pre-freeze incubation period allows movement of ions through the lipid bilayer. The critical aspect of the cooling process is the passage of water across the cell membrane, which depends on the difference between extra- and intracellular osmolality among other things (Parks, 1997). The steps used in cryopreservation procedures usually includes gradual cooling of the sample, loading the sperm cells into straws, then lower the temperature to -80° C before immersion into liquid nitrogen (Pope, Yong, & Dresser, 1991). A suitable freezing rate for cat spermatozoa was reported to be -10° C/min from +5°C to -80°C before liquid nitrogen immersion (Pope, Yong, & Dresser, 1991). Instead of liquid nitrogen vapor, dry ice is used to reach -80°C more quickly.

Freezing

The temperature at which freezing (change from one state of matter to another) occurs is altered by the presents of the cryoprotectant. The ability of glycerol/water mixtures to inhibit ice crystallization is linked to the concentration of glycerol and the hydrogen bonding patterns formed by these solutions (Dashnau, Nucci, Sharp, & Vanderkooi, 2006). The effects of the percent glycerol on the integrity of a cell during cooling are not always clear until it has undergone further treatments such as freezing (Hammerstedt, 1990).

During the freezing process, volume adjustments of great magnitude will occur as the cell is exposed to hypertonic conditions during freezing because the solute concentrations will approach 3-5 Osm. Eternal crystallization of ice rapidly occurs, and the cell membrane begins its tortuous path to the dehydrated storage state (Hammerstedt, 1990). The super cold storing temperature brings a period of respite, since low temperatures result in minimal molecular motion (Hammerstedt, 1990). Upon freezing, and while frozen, cells remain at a rest state until they are thawed.

Thawing

During thaw, rate of egress of cryoprotectant and influx of water have to balance; otherwise sperm membrane integrity will be lost (Hammerstedt, 1990). It is well established that optimum cell survival requires that the rate chosen for freezing be paired with an appropriate rate of thawing. (Mazur, 1984)(Schneider, 1986). The melting of external ice crystals rapidly reduces solute concentrations and initiates an inward rush of water (Hammerstedt, 1990). During the final stage of a typical thawing cycle, cryoprotectant is removed from a thawed cell causing the initially equal salt concentrations to change. Removal of cryoprotectants in multiple steps with an isotonic solution minimizes the loss of sperm motility and reduces membrane disruption (Pukazhenthi, Spindler, Wildt, Bush, & Howard, 2002). A major volume change will occur within the cells when sperm are transferred from the glycerol containing extender to a glycerol free buffer solution. The cell expands because there is cryoprotectant within, while there is none in the isosmotic bath until the cryoprotectant escapes across the cell memebrane and into the bath, leaving the cell at its original size (Batycky, 1997). In an ideal situation, all of the cells would live and there would be little to no difference

between fresh and frozen sperm cells. Unfortunately, that is not the case but it is the goal. Getting closer to this goal translates into better success of cryopreservation.

Assisted Reproductive Technologies

Cryopreservation of gametes is important when long-term storage is necessary for in vitro fertilization (IVF) or artificial insemination that must be done at a future date (Luvoni, 2006). Applied research, like proposed herein, is designed to improve upon what is currently known about the cryopreservation of felid sperm. In one study, artificial insemination using frozen ejaculated cat semen resulted in approximately 30% motility after thawing, yielding a conception rate of 57% (Tsutsui, Wada, Anzai, & Hori, 2003). The use of ART is not limited to ejaculate sperm. Cryopreserved ejaculated and epididymal spermatozoa also have been successfully used in IVF of cat oocytes (Karja, Otoi, Murakami, Fahrudin, & Suzuki, 2002). Even with the loss of cell integrity, there are other options for preserving endangered animal species such as inner cytoplasmic sperm injection (ICSI). ICSI may partially overcome the functional limitations imposed on spermatozoa by cryopreservation (Luvoni, 2006). Assessments of these functions, like motility and cell membrane integrity, may assist in deciding how to preserve certain cells. All of these techniques are being used to further conservation efforts of felids.

Motility and Membrane Integrity

Sperm motility and acrosomal morphology are greatly affected by freeze-thaw procedures (Pope, Yong, & Dresser, 1991). A goal of cryopreservation is preventing capacitation-like reactions while maintaining motility and cellular integrity. Ejaculate sperm have a higher percentage of intact acrosomes than freshly collected epididymal spermatozoa, although motility rates of freshly collected epididymal spermatozoa is

similar to ejaculated sperm (Luvoni, 2006). The optimal procedure to preserve motility and integrity of cat sperm cells has not been developed; however, the site of deposit of the frozen semen in the genital tract greatly alters conception rate (Tsutsui, Wada, Anzai, & Hori, 2003). That an ideal procedure for cryopreserving felid sperm has not been developed is impetus for researchers described in this proposal.

Assessment of Sperm

There are two assessments used to determine sperm quality at the Audubon Center for the Research of Endangered Species in New Orleans and in this research; (i) Computer-Assisted Semen Analyzer, CASA, and (ii) LIVE/DEAD Sperm Viability Kit®. These methods have proven to be some of the best indicators of sperm cell membrans integrity. Live/Dead staining consists of a fluorescence-based assay for analyzing the viability and fertilizing potential of sperm using a fluorescence microscopy. Membrane-permeant SYBR® 14 nucleic acid stain labels live sperm with green fluorescence, and membrane-impermeant propidium iodide labels the nucleic acids of membrane-compromised sperm with red fluorescence. This viability kit can be used for either eukaryotic somatic or sperm cells (Life Technologies, 2012).

The second assessment, CASA, consists of five measurements that serve as indicators of sperm quality. Motility is one of the most important characteristics associated with the fertilizing ability of spermatozoa and is an expression of their viability and structural integrity. CASA provides precise and accurate information on different sperm motion characteristics (Kathiravan, Kalathran, Karthikeya, Rengarajan, & Kadirvel, 2011). CASA objectively measures motility, progressive, straightness, linearity,

and rapid cell among other things. These identifiers are thought to be some of the best information for estimating sperm quality:

1. Motility - Sperm motility is critical at the time of fertilization because it facilitates passage of the sperm through the zona pellucida. Without intervention, a non-motile or abnormally motile sperm is not going to fertilize. Assessing the quantity of a sperm population that is motile is perhaps the most widely used measure of semen quality (Rouge, 2003).

2. Progressive - A progressively motile sperm swims forward in an essentially straight line, whereas a non-progressively motile sperm swims, but with an abnormal path, such as in tight circles (Hinting, Comhaire, & Schoonjans, 1988).

3. Linearity – the quotient of linear velocity divided by velocity, multiplied by 100 (Hinting, Comhaire, & Schoonjans, 1988). It is the ratio of straight line velocity/track velocity (microns/sec).

4. Straightness – the shortest distance between the start and end point calculated per unit of time (Hinting, Comhaire, & Schoonjans, 1988). It is the ratio of straight line velocity/smoothed path velocity (microns/sec).

5. Rapid Cell - The speed at which the cells are moving

These assessments are thought to be the best indicators of fertility given by the computer assisted sperm analysis.

CHAPTER III

METHODS

Animals

Animals used as sperm donors were housed in the domestic cat facility at Audubon Center for the Research of Endangered Species in New Orleans, Louisiana. Two tom cats were used in this study and were trained to mount female cats in order to collect semen used in the study. This was all done after approval from the ACRES IACUC.

Animal Care

Toms were given free access to commercially available cat food and fresh water daily. Lights were programmed to a 12:12 dark-light cycle. Access to the cat colony was restricted to animal handlers.

Semen Collection

A “teaser” female was used to elicit mounting behavior from non-sedated tomcats. The teaser queen (female cat) was chosen based on her affectionate demeanor, presentation and lordotic behavior. Upon proper stimulation, the tom cats were permitted to mount, but not to enter the female. Two artificial vaginas (AV) were prepared before each collection. The AV was constructed from the top portion of a 1.5ml centrifuge tubes and the bottoms of small balloons. The balloon was stretched around the top of the

centrifuge tube and put into the glass vial. A minute amount of non-spermicidal lubricate was used to coat the AV. The AV was attached to two small glass serum bottles, which were filled with approximately 98-degree water.

Upon mounting of the queen, the AV was carefully placed over the glans penis of the tomcat. If the AV became contaminated with urine during semen collection, another AV was used. Upon completion of ejaculation, the AV was removed and the teaser and tom were carefully separated and returned to the colony. Afterward, the AV was immediately covered with a paper towel to minimize light exposure to the sample while being transferred to the laboratory located in the same building.

Semen Processing

The semen sample was immediately transported to a Laminar Flow hood for processing. Two microliter of original sample was placed on a slide and visualized under a microscope to assess the overall quality of the sample. The assessment was used to determine if the sample was of sufficient quality to warrant further study. The semen sample was stored at 38C until the concentration of the sample was determined.

An automated sperm diagnostic instrument, designated Sperma Cue, was used to measure concentration of the original sample. For analytical purposes, a 3µl aliquot of the semen sample was diluted with 27µl of formaline citrate. Twenty-seven microliters of the diluted semen was pipetted into a cuvette and placed into the Sperma Cue and measured six times. The average sperm concentration/ml was determined from these measurements. The concentration was used to calculate the volume of semen needed for each of the three treatments. Media needed for treatment in experiment II were also calculated so that there

were 10 million cells/ 180µl (placed into a 1/4cc straw). Sperm cells used in both experiment I and II were kept at 4C at a concentration of 10 million cells/ 75µl.

Extenders

Two different extenders (media), purchased from Irvine Scientific ®, were used in this study. One medium was used for incubation purposes, while the second was used for freezing purposes. Both media were supplemented with gentamicin and test yolk buffer. Only freezing media was supplemented with glycerol. Glycerol concentration in the freezing media was made into a stock solution of 12%. This media was used to make the 3% and 6% glycerol dilutions.

Semen Evaluation

Semen evaluations were conducted at different times throughout both experiment I and II. The two primary assessments used in both studies were (i) a live/dead stain, and (ii) the Computer Assisted Semen Analysis (CASA). There were five sets of assessments conducted per repetition for both studies.

CASA

A 1ul aliquot of semen was combined with 49ul of HEPES 199 and gently mixed in a .5ml centrifuge tube. A 2.6µl aliquot of extended semen was pipetted into a Leja® 20 micron 4 chamber slide. To minimize the adverse effects of cold shock, slides were pre-heated on the warming plate or incubator. Five random recording were captured by the CASA instrument and averaged for each of the five assessments. The CASA assessments focused on in this research included motility, progressive, straightness, linearity, and

rapid cell. Upon completion of the assessments, the information was recorded in an Excel® spreadsheet by the CASA instrument.

Live/Dead

LIVE/DEAD® sperm viability kits were purchased from Molecular Probes®, which consisted of a fluorescence-based assay for analyzing the viability and freezing potential of sperm (live/dead product information). The kits contained component A, SYBER 14 dye, and component B, Propidium iodide. First, 530µl of Hepes199 was mixed with 32µl of SYBER 14 dye. Next, .5ml centrifuge tubes were filled with 25µl of 5µl Propidium Iodide + 20µl diluted SYBER 14 solution. A 0.5µl semen sample was combined with 5µl formaline citrate and 5µl of prepared stain, and then mixed in a .5ml centrifuge tube. Five microliters from this solution was pipetted onto a microscope slide and covered with a slipcover and viewed under a fluorescence microscope equipped with the appropriate color filters. Propidium Iodide produces a red stain that is indicative of the dead cells, while SYBER 14 produces a green stain representative of the living cells. The data was recorded and images saved.

Procedures Experiment I

Experiment I

The experiment was designed to test the hypothesis that when incubated at 4C, survival rates of felid sperm will be affected by time of incubation and concentration of glycerol. The experiment was organized so that it could be repeated during any given five-day span at the ACRES facility. After establishing the quality of the original sample, and determining that the semen sample was satisfactory (described previously), the semen was used in experiments involving three concentrations of glycerol. The extended

semen samples were stored at 4C at a dilution of 10 million cells/75µl with 0%, 3%, and 6% glycerol respectively. The glycerol was added at 15%, 20%, 25%, and 40% of the total volume using a stock solution of 12% glycerol freezing medium at room temperature. The samples were immediately placed into 4C water bath in walk-in cooler where they remained for the subsequent five days. The samples were allowed to equilibrate to 4C for 4 hr and then assessed daily for four subsequent days using the CASA and Live/Dead procedures.

Statistical Analysis

The data were analyzed with a model that included the main effects of glycerol concentration and day/time, and included the interaction of glycerol concentration and day/time using the GLM procedures of SAS. Differences in treatments (% glycerol and day) were partitioned using the Least Significant Difference.

Procedures Experiment II

Experiment II

Experiment II was conducted simultaneously with experiment I and was designed to test if post-thaw survival is enhanced in felid sperm incubated in the presence of glycerol at 4C prior to freezing. Semen quality was assessed using CASA, and Live/Dead techniques. Semen samples were divided equally into three parts, which were used in the three treatments. Treatments included 0%, 3%, and 6% glycerol. Glycerol from the 12% stock solution was slowly added to the extended semen at room temperature, as previously described in experiment I to until the targeted concentration was achieved. The samples were then cooled to 4C and maintained in a water bath for 4 hr. The three treatments were analyzed using the CASA and Live/Dead assessments.

Prior to freezing, all treatments were brought to a final concentration of 6% glycerol in a concentration of 10 million cells/ 150µl, and immediately placed into 1/4cc straws. The addition of glycerol was incrementally increased at 15%, 20%, 25% and 40% of the total volume from the 12% glycerol stock solution. Straws were sealed and placed on a block of dry ice for 20 min. All dilutions were conducted in the walk-in cooler at 4C. At the end of 20 min, the straws were plunged into liquid nitrogen. The samples remained frozen until they were thawed and assessed. Liquid nitrogen maintained the cells between -196 and -210C.

To thaw, straws were removed from liquid nitrogen one by one, gently shook in the air for 5 sec, and then swirled in a water bath for 5 sec. The water was removed from the outside of the straw. The end of the straw was cut and the contents emptied into a 1.5ml centrifuge tube incubated in a water bath at 37 C under a Laminar Flow hood. Each straw was emptied into a separate tube. The volume of each tube was measured. To reduce the effects of glycerol on assessment parameters, an equal volume of medium (without glycerol) was added incrementally at 15%, 20%, 25%, and 40% of the total volume. Additional HEPES 199 was then added to the sample in 33%, 66%, and 200% of the sample volume. Each extended semen sample was centrifuged for 5 min at 800 x g. The supernate was carefully removed and 50µl of HEPES 199 was added. The samples were all placed in the 37 C incubator and assessed at 0hr, 1hr, and 3hrs using CASA and Live/Dead analysis.

Statistical Analysis

The data were analyzed with a model that included the main effects of glycerol concentration and day/time post-thaw, and included the interaction of glycerol

concentration and day/time post-thaw using the GLM procedures of SAS. Differences in treatments (% glycerol and day/time post-thaw) were partitioned using the Least Significant Difference.

CHAPTER IV

RESULTS

Experiment I

Semen collected from trained tomcats using an AV was used in this study. No contamination of urine was observed in the semen samples. Although there was considerable variation in the quality of semen from both tomcats, the overall quality used in the study was acceptable. The assessments made included number of live cells, motility, progressive cell movement, linearity, straightness, and rapid cell movement. Refrigeration (cooling from 27C to 4C) significantly decreased ($p<0.05$) the ratio of live sperm cells per sample over four days regardless of glycerol concentration, as shown in Figure 1. While there was no significant difference between day 2 and day 3 or day 3, 4, and 5, there was a significant difference between day 2 and day 4 and 5. There were no differences between treatments (0%, 3%, and 6%) with regard to concentrations of glycerol (Figure 2).

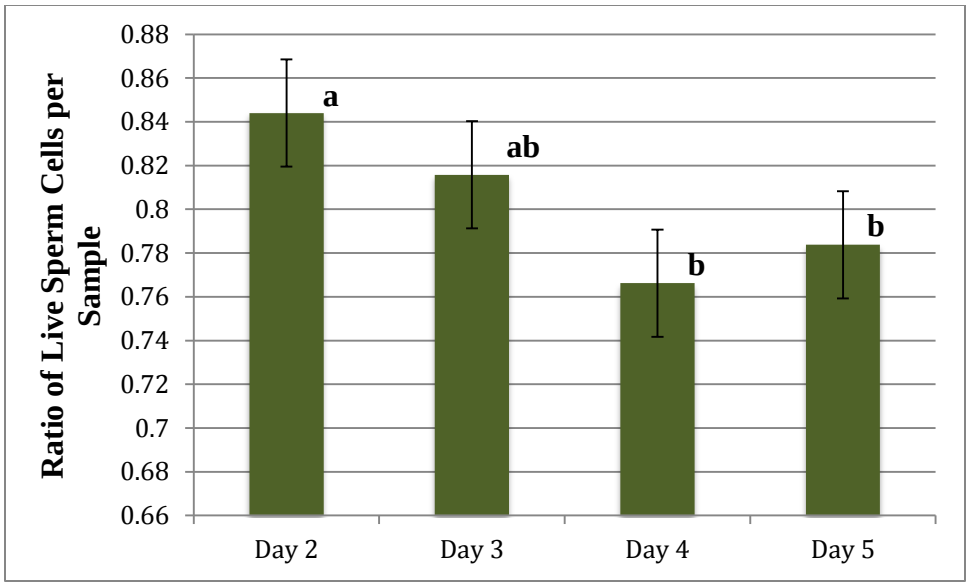

Figure 1. Effect of incubation time at 4C on sperm survival. Bars with different letters indicate statistical differences $p<0.05$. The values in this graph represent mean +- standard error.

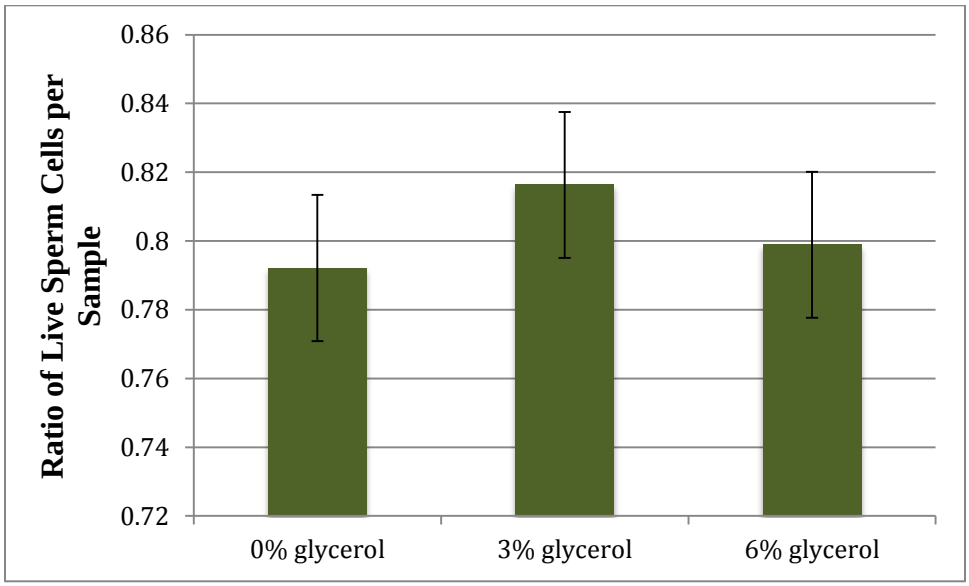

Figure 2. Effect of glycerol concentration on sperm survival at 4C. The values in this graph represent mean +- standard error. This graph shows no significant difference.

Depicted in figure 3 is the motility ratio expressed as a function of percent glycerol. The results, in general terms, indicate an overall decrease in the motility ratio with differences observed between day 1 and day 5.

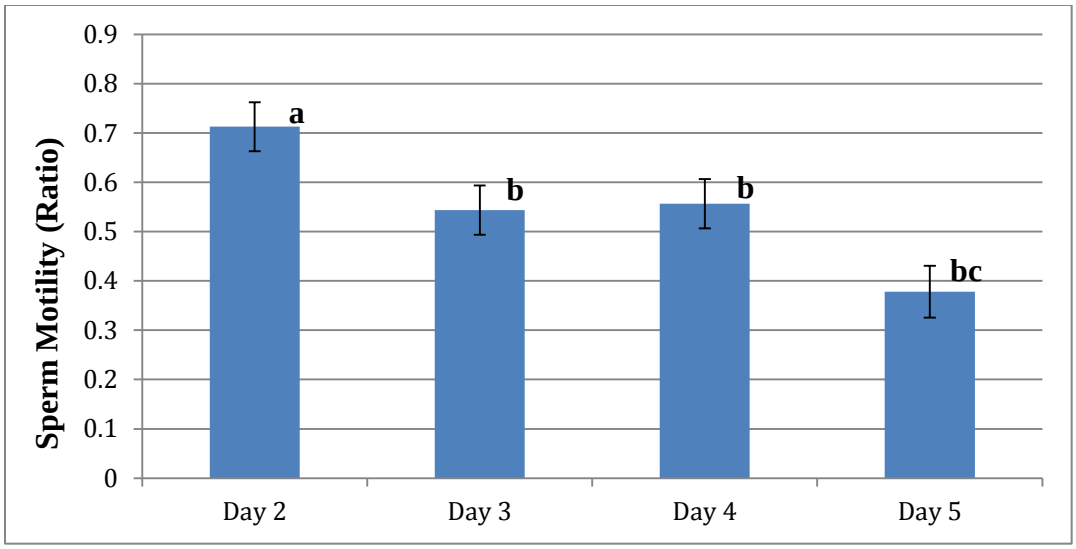

Figure 3. Effect of incubation time at 4C on sperm motility. Bars with different letters indicate statistical differences $p<0.05$. The values in this graph represent mean +- standard error.

There were also differences between treatments with differing concentrations of glycerol Sperm incubated with 0% glycerol were significantly less motile than those exposed to 3% and 6% glycerol (Figure 4).

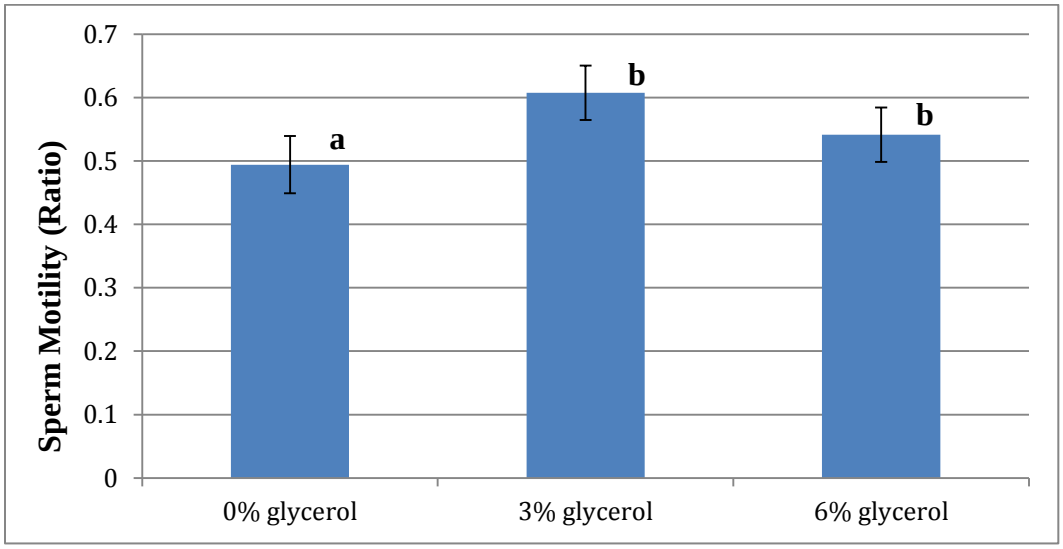

Figure 4. Effect of percent glycerol on motility at 4C. Bars with different letters indicate statistical differences $p<0.05$. The values in this graph represent mean +- standard error.

From the CASA analysis, there appeared to be a linear decrease in progressive sperm movement over time. Data from assessments on day 2 and day 5 are statistically different from day 3 and day 4 as well as each other. This difference is presented in Figure 5.

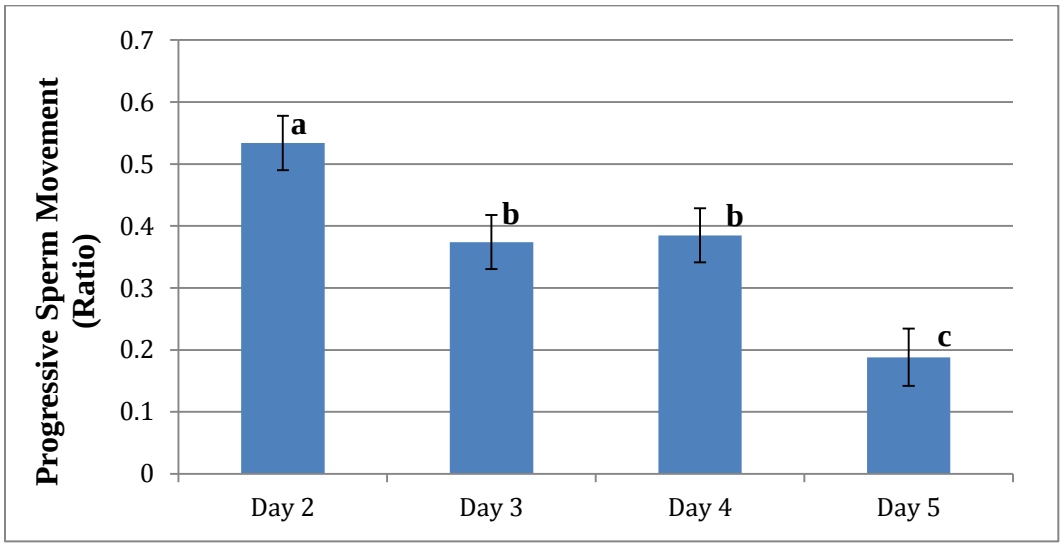

Figure 5. Effect of incubation time at 4C on progressive sperm movement. Bars with different letters indicate statistical differences $p<0.05$. The values in this graph represent mean +- standard error.

While the ratio of progressively moving sperm decrease over days, progressive sperm cell movement was increased when cells were incubated with increased percentages of glycerol (see Figure 6).

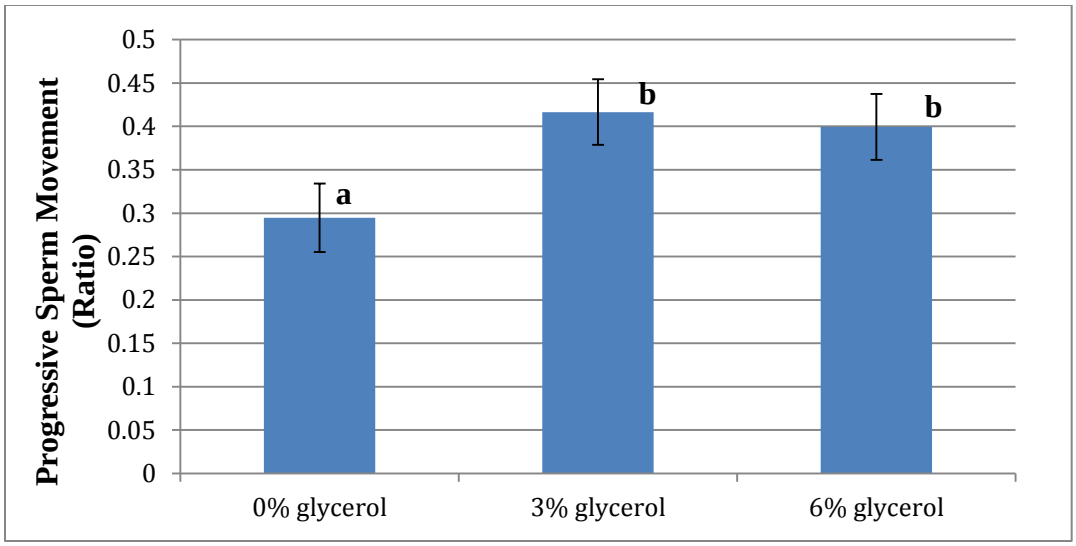

Figure 6. Effect of percent glycerol on progressive sperm movement at 4C. Bars with different letters indicate statistical differences $p<0.05$. The values in this graph represent mean +- standard error.

Rapid movement or velocity of the sperm cells was also objectively measured by CASA. This measurement follows the same trends as the previous measurements by decreasing over time. Velocity of cells decreased over time and was least active on day 5 of the experiment. These results are depicted in Figure 7 below.

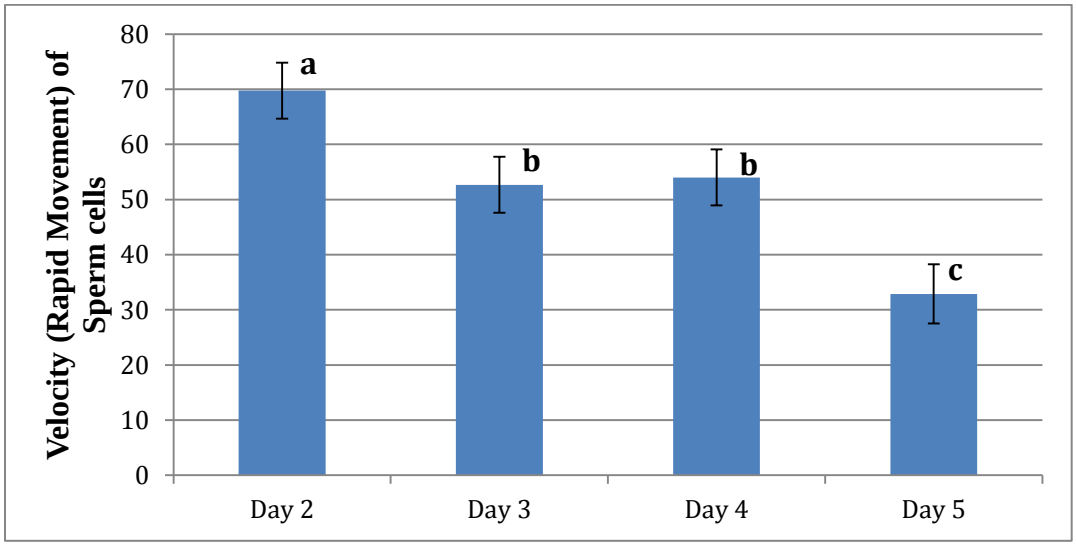

Figure 7. Effect of incubation time at 4C on velocity (rapid movement) of sperm cells. Bars with different letters indicate statistical differences $p<0.05$. The values in this graph represent mean +- standard error.

Rapid movement velocity of the sperm cells was additionally measured across glycerol treatments. There was no statistical difference ($p<0.05$) between any of the treatments (Figure 8).

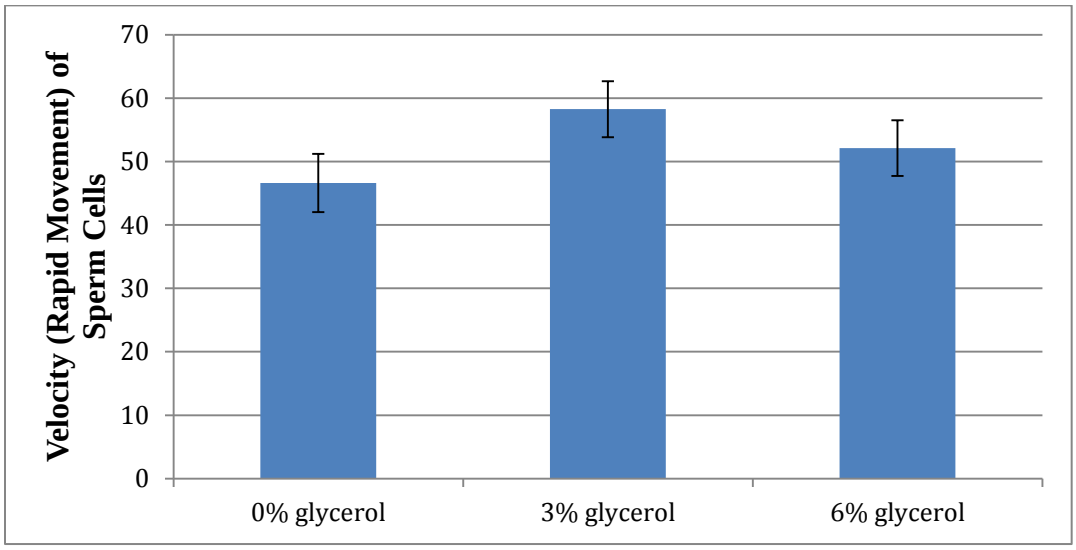

Figure 8. Effect of percent glycerol on velocity (rapid movement) of sperm cells at 4C. The values in this graph represent mean +- standard error. This graph shows no significant difference.

Differences in straightness of sperm cells were also identified between day 2 and day 5 of the study (Figure 9), which followed the trend observed previously for other CASA measurements.

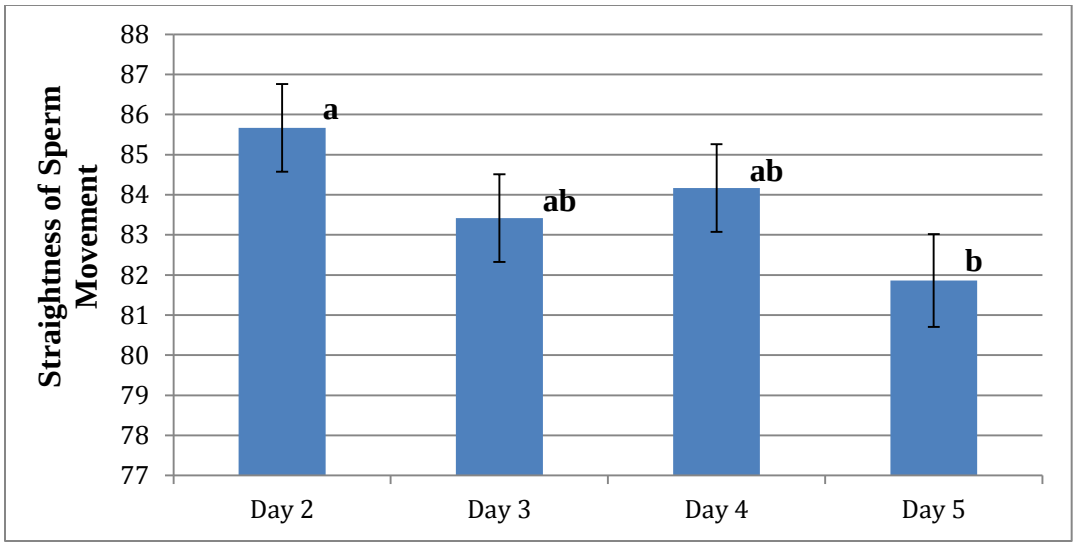

Figure 9. Effect of incubation time at 4C on straightness of sperm cell movement. Bars with different letters indicate statistical differences $p<0.05$. The values in this graph represent mean +- standard error.

The straightness of sperm movement was significantly different across treatments. Results indicate that increasing concentrations of glycerol provided a greater straightness of movement compared with 0%glycerol (Figure 10).

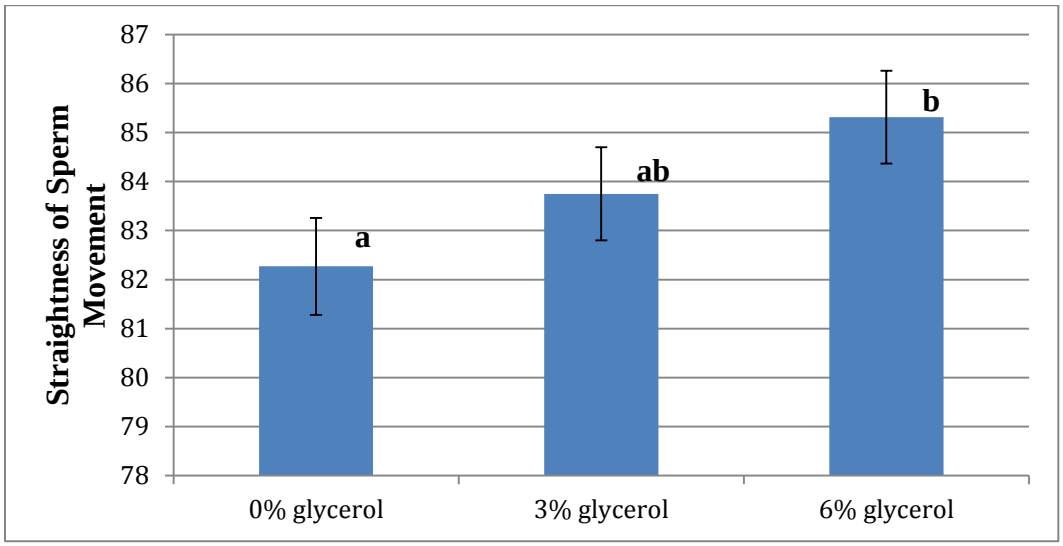

Figure 10. Effect of percent glycerol on straightness of sperm cells movement at 4C. Bars with different letters indicates statistical differences $p<0.05$. The values in this graph represent mean +- standard error.

The linearity of sperm cells followed the same trends as most of the previous assessments, although there were differences between day 3 and day 4 (Figure 11). Linearity of sperm cells movement across treatments, however, were not different (Figures 12).

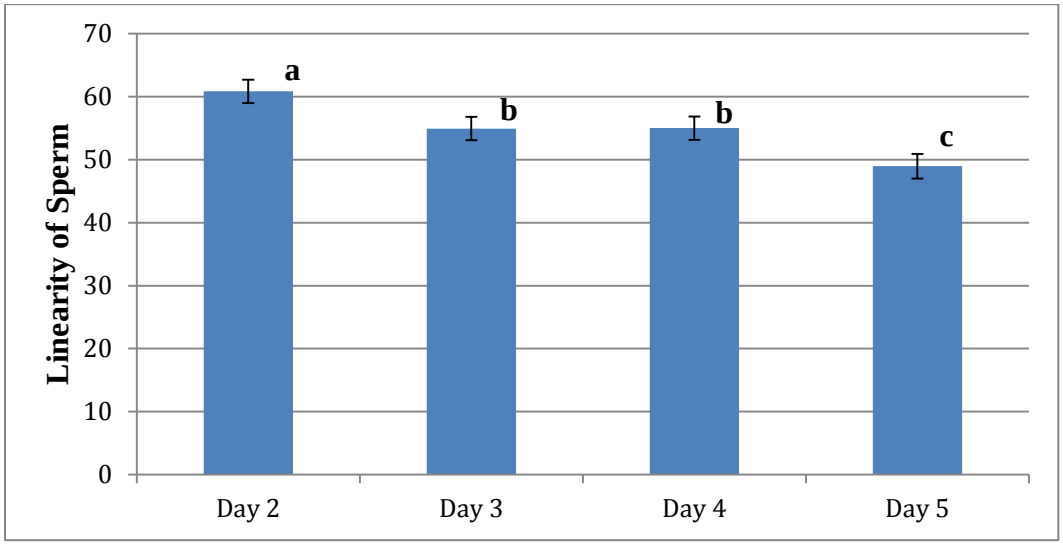

Figure 11. Effect of incubation time at 4C on linearity of sperm cell movement. Bars with different letters indicate statistical differences $p<0.05$. The values in this graph represent mean +- standard error.

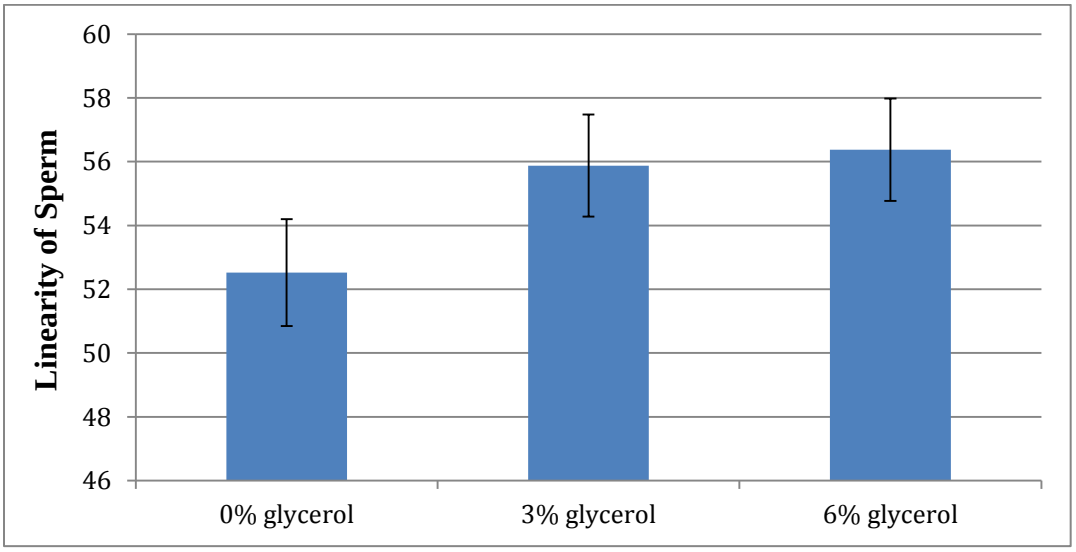

Figure 12. Effect of percent glycerol on linearity of sperm cells movement at 4C. The values in this graph represent mean +- standard error. This graph shows no significant difference.

The results of experiment I indicate that sperm viability and sperm movement were negative affected over time; however, the decline did not appear to be correlated either positively or negatively to concentration of glycerol used in the incubation medium.

Experiment II

Evaluation of the statistical model indicated significant effects of concentration of glycerol and days post thaw; however, there were no significant interactions between concentration of glycerol and days post thaw. Because the objective of experiment II was to determine if concentration of glycerol during incubation would affect sperm survival and movement, and because there was no treatment by day/times interaction, statistical comparisons were only made within treatment.

In the live/dead assessment, there was no difference between treatments (concentration of glycerol) at 0 hr or 1 hr post thaw. However, at 3 hr post thaw, there was a reduction in percent live sperm at 6% glycerol when compared to both 0% and 3% glycerol (Figure 13).

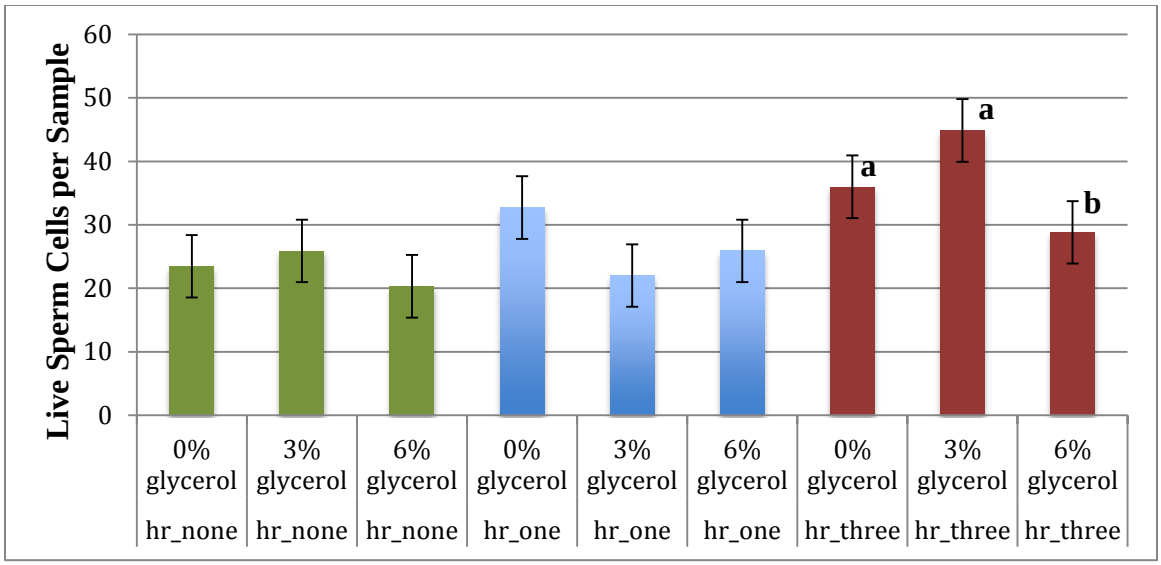

Figure 13. Effect of thaw time and glycerol concentration on sperm mortality. Bars with different letters indicate statistical differences $p<0.05$. The values in this graph represent mean +- standard error.

Other indicators of fertility measured in experiment II included sperm motility ratio, progressive sperm cell percentage, and velocity (rapid movement) of sperm cells. There was no statistical difference for these assessments between assessment times or between treatment groups (Figures 14, 15, 16).

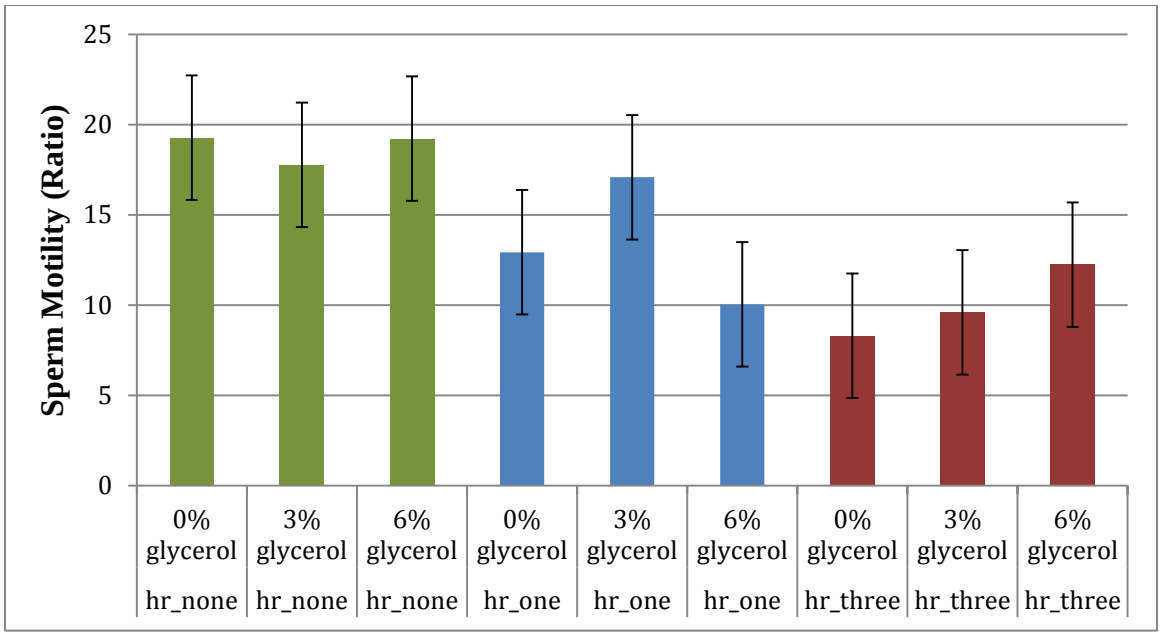

Figure 14. Effect of thaw time and glycerol concentration on sperm motility. The values in this graph represent mean +- standard error. This graph shows no significant difference.

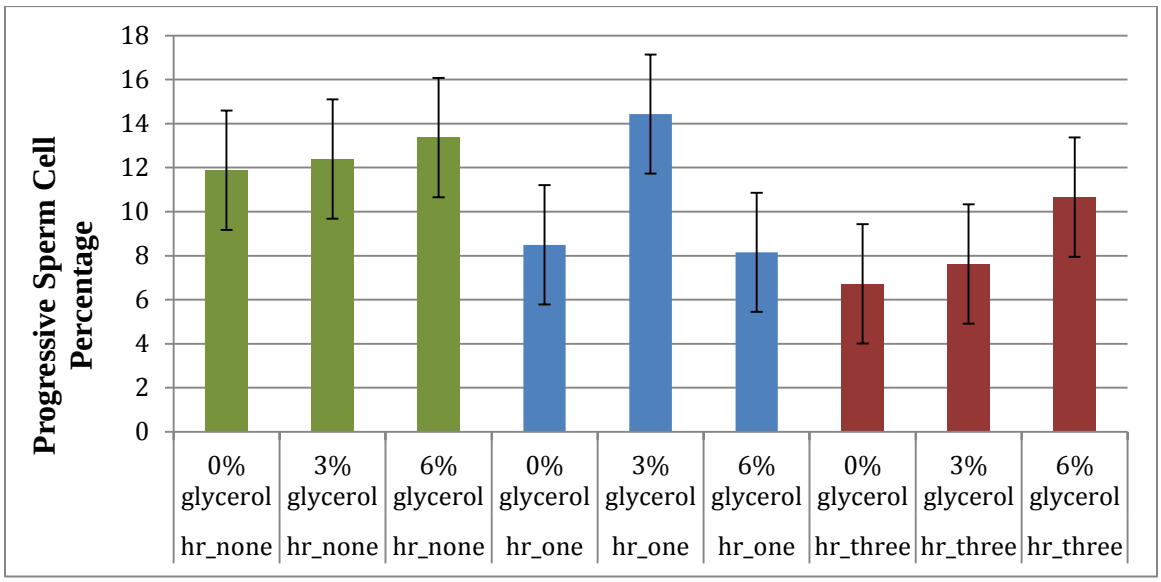

Figure 15. Effect of thaw time and glycerol concentration on progressive movement in sperm cells. The values in this graph represent mean +- standard error. This graph shows no significant difference.

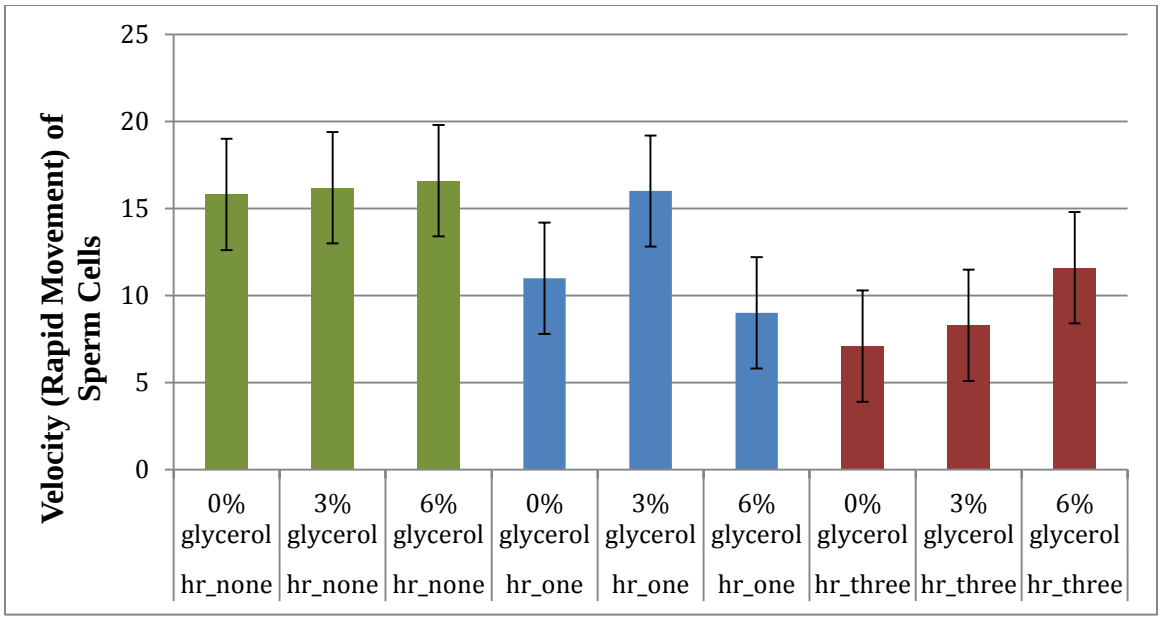

Figure 16. Effect of thaw time and glycerol concentration on velocity (rapid movement) of sperm cells. The values in this graph represent mean +- standard error. This graph shows no significant difference.

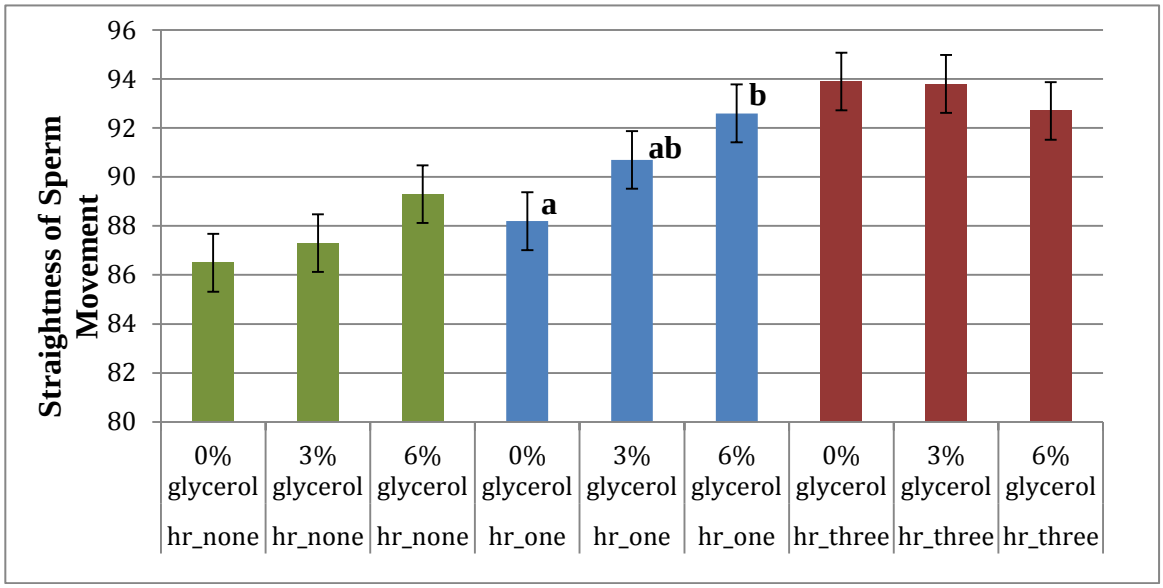

Figure 17. Effect of thaw time and glycerol concentration on straightness of sperm cell movement. Bars with different letters indicate statistical differences $p<0.05$. The values in this graph represent mean +- standard error.

Linearity of sperm cells appeared to follow the same trend as straightness of sperm cells. There was no statistical difference between treatments at 0 or 3 hr. There

was a difference between 0% glycerol and 6% glycerol at 1 hr post thaw. Moreover, cells incubated in 0% glycerol had decreased linearity of sperm cells movement compared with 6% glycerol.

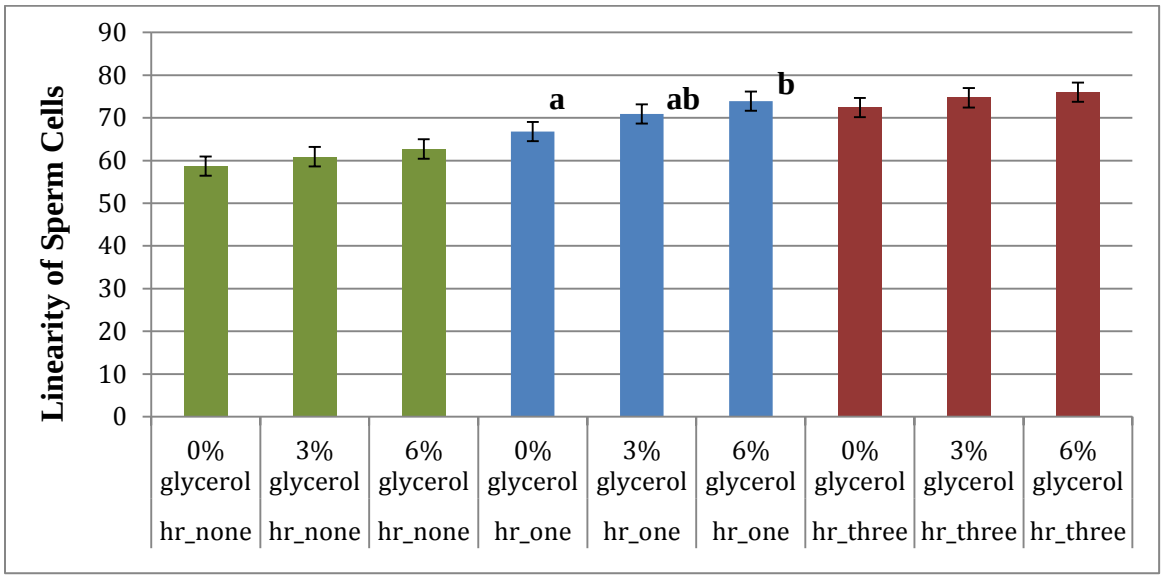

Figure 18. Effect of thaw time and glycerol concentration on linearity of sperm cell movement. Bars with different letters indicate statistical differences $p<0.05$. The values in this graph represent mean +- standard error.

Live sperm cell assessment stood alone in its differences during the 3-hr post thaw assessment. In addition to percent live sperm cells, there were statistical differences within the one-hour post thaw assessment of felid sperm cells. Straightness and linearity of sperm cells in 0% and 6% treatments were different at 1 hr.

The results of experiment II indicate that sperm viability and sperm movement were largely unaffected by concentration of glycerol. With the exception of the live/dead assessment, in those instances where differences in percent glycerol were observed, sperm appeared to move with greater velocity in 6% glycerol.

CHAPTER V

DISCUSSION

Overview

Although several studies in the domestic cat have demonstrated that semen can be frozen in glycerol, the optimum concentrations of the cryoprotectant and rate of freezing have not been established (Luvoni, 2006). Most studies have reported that cat semen can be effectively frozen using about 5% glycerol, with glycerol being the most ideal cryoprotectant for felids (Luvoni, 2006). However, few studies have been designed to determine if greater successes in post-thaw recovery could be obtain if the percent glycerol used during the cooling phase of freezing was varied. Results from this study were consistent with previous studies in cats, which showed that glycerol can be used effectively to freeze sperm. To access quality of semen in this study, sperm samples were analyzed with live/dead staining procedures and with CASA instrumentation. CASA provides precise and accurate information on different sperm motion characteristics (Kathiravan, Kalatharan, Karthikeya, Rengarajan, & Kadirvel, 2011).

The CASA parameters were previously modeled and refined mathematically to best describe the motion of a spermatozoon as it travels through a microscopic field (Cancel, Lobdell, Mendola, & Perreault, 2000). The fertility indicators measured in

these experiments are some of the most useful identifiers described by the CASA manufacturer (Kathiravan, Kalatharan, Karthikeya, Rengarajan, & Kadirvel, 2011). CASA was used in studies described herein because of it is more accurate in discriminating motility percentage increments compared with estimates made by laboratory technicians. The main advantage of CASA is that it allows an objective analysis of sperm motility in samples, and that identical parameters can be used in serial sample analysis (Broekhuijse, Sostaric, Feitsma, & Gadella, 2011).

Live-dead staining has been used to assess semen quality in human sperm (Dougherty, Emilson, Cockett, & Urry, 1975), as well as many mammalian species (Fillers et al., 2008). The Live/Dead viability kit measures the ability of the stains to pass through the membrane, and provides an easy one step process for live/dead measurement. Live dead staining was used in this study to determine if different percentages of glycerol added to sperm prior to freezing would alter sperm viability.

Previous studies have demonstrated that addition of glycerol to cells at either 37 C or 4C affected survival of sperm due to the large volume changes that are induced by the cryoprotectant (Fiser & Fairfull, 1989). While glycerol is known to be toxic to spermatozoa, and must be added when samples have been slowly cooled before freezing, addition of the cryoprotectant at high concentrations before cooling, as done in this research, resulted in higher sperm motility post-thaw. These findings are in agreement of those of Fahy, 1986. Moreover, addition of glycerol at 30C resulted in a highly significant decrease in the mean spermatozoa motility immediately post-thawing compared with addition of the cryprotectant at 4C (Colas, 1975). These findings are in agreement with those reported by Fahy, 1986, who demonstrated that sperm membrane

integrity, including intactness and stability, were superior when glycerol was added to sperm at 5C compared to 15C. The ability of glycerol to maintain membrane integrity better at 5C rather than 15C could be explained by the reported effect of glycerol as a promoter of capacitation-like changes, or by its toxic effect on sperm metabolism (Fahy, 1986).

Experiment I

Results from experiment I indicate that survival of feline sperm was affected over time when exposed to extended refrigeration time (4C). Using live/dead ratios, data from experiment I indicated that sperm survival consistently decreased over time. These results are consistent with those in red tailed deer in which sperm motility was significantly ($P<0.05$) lower from sperm harvested from the epididymis and incubated in a defined medium for 2, 3, and 4 days, when compared to controls (Soler, Perez-Guzman, & Garde, 2003). In another study conducted in rhesus monkeys, post thaw motility decreased significantly after 48 hr of refrigerated storage at 4C (Dong, Rodenburg, Huang, & Vandevoor, 2008). In dogs, motility decreased significantly within the first 5 hr of refrigeration ($P<0.05$), but then declined more gradually thereafter (Yu & Leibo, 2002). However, in experiment 1, semen incubated with either 3 or 6% glycerol displayed greater motility scores over time, compared to 0% glycerol. Glycerol addition was in agreement with Zambelli et al. who added glycerol at room temperature to allow increased penetration into spermatozoa during the refrigeration time prior to freezing in straws resulting in increased motility (Zambellie et al., 2002).

As the concentration of glycerol increased, other indicators of motility, including progressive sperm movement ratio, increased. These results indicate that the presence of

glycerol increases not only sperm motility ratio, but also the progressive sperm movement ratio, whereas velocity at which these cells moved, as measured by rapid movement, was not affected by the varying concentrations of glycerol. While the apparent velocity of cells movement favored treatments with the highest glycerol concentration, motility and progressively motility measurements were similar across treatments. Interestingly, there were no differences between linearity of the sperm movement, but straightness of sperm movement was increased in the presence of 6% glycerol. These data are consistent with those reported by Mantovani et al., 2002, who said the presence of glycerol rather than ethylene glycol was more effective at increasing straightness in equine (Mantovani, Rota, Falomo, Bailoni, & Vincenti, 2002).

In summary, sperm motility ratio, progressive ratio and straightness of sperm cells were positively affected by the increase in glycerol during an extended refrigeration incubation period. The variation between treatments had no apparent effect, however, on live sperm cells, velocity (rapid cell) movement, and linearity of sperm movement. As reported in this and other studies in other species (Vera-Munoz, et al., 2011), indicators of fertility over time were decreased, regardless of glycerol concentration. Because no significant detrimental effects to the felid sperm cells containing glycerol during refrigeration were observed in this study, a subsequent experiment was conducted to determine the effect of glycerol concentrations during refrigeration on post thaw sperm characteristics.

Experiment II

Results from the present experiment demonstrate that incubation of sperm at 0% or 3% glycerol during refrigeration is optimum for sperm survival 3 hr post thaw. These

results are in agreement with most studies in felids, suggesting that semen can be successfully frozen in glycerol at concentrations between 3 and 6 percent (Luvoni, 2006). In rams, exposure time to glycerol had no significant effect on survival of spermatozoa after thawing and incubation, but fertility was significantly higher with 4% than with 2% glycerol (Colas, 1975). Previous studies in felids did not include glycerol in medium during refrigeration prior to cryopreservation. The importance of cooling sperm in a glycerol medium is always clear until after freezing and subsequent thawing (Hammerstedt, 1990). A study conducted in Rhesus Monkeys showed consistently high post thaw motility with 3% glycerol after refrigerated storage for 24 or 48 hours. However, the glycerol was not added during refrigeration (Dong, Rodenburg, Huang, & Vandevoor, 2008). Although the present study indicated no differences between 0% and 3% glycerol at any of the three post-thaw sperm movement assessments, consistently higher post thaw motility was observed with 3% glycerol after refrigerated storage for 24 or 48 hours in Rhesus Monkeys (Dong, Rodenburg, Huang, & Vandevoor, 2008). It appears that the addition of small concentrations of glycerol prior to refrigeration does not adversely affect sperm.

Sperm motility, progressive sperm cell percentage, and velocity (rapid movement) of sperm cells was not affected by the varying concentrations of glycerol during any of the three assessment times. These results are in contrast to those suggesting that glycerol is toxic to cells when at ambient temperature in humans (McGonagle, Goldstein, Feldschuh, & Foote, 2002). Sperm motility and acrosomal morphology were greatly affected by the freeze-thaw procedures (Pope, Yong, & Dresser, 1991). In a similar study,

frozen ejaculated cat semen resulted in approximately 30% motility after thawing, similar motility was observed in this study (Tsutsui, Wada, Anzai, & Hori, 2003).

As previously reported in experiment I, straightness of sperm cells was positively affected by increased glycerol percent during refrigeration. Sperm straightness was significantly reduced in treatments with 0% glycerol compared to 6% glycerol added prior to refrigeration. This held true for linearity of sperm cells as well. These results are in contrast to the idea of glycerol toxicity (McGonagle, Goldstein, Feldschuh, & Foote, 2002).

It is not clear why glycerol affected these specific fertility identifiers differently. More extensive research must be done to better understand the intricate chemical role of glycerol in preserving felid sperm cells. Results of the present experiments indicate that when felid sperm cells were treated with glycerol and then refrigerated for 4 hr, positive effects in the presence glycerol were observed. These results indicate that glycerol does not have a toxic effect on sperm, when present in media for 4 hr prior to freezing,

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