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Cryopreservation of Domestic Cat Epididymal Spermatozoa

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CRYOPRESERVATION OF DOMESTIC CAT EPIDIDYMAL SPERMATOZOA

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ABSTRACT

The primary lines of defense in preventing any exotic species from becoming endangered or extinct should be the protection of their habitat and from poaching. For wildlife conservationists, these primary measures are often not feasible, and thus cannot be the only means to prevent extinction. Additional steps to prevent species from becoming extinct are the use of assisted reproductive techniques (ART). The objective of this research was to identify a semen extender that contained little to no egg yolk and could still effectively freeze domestic cat epididymal spermatozoa, for the purpose of sex sorting, using flow cytometry. In the first experiment we showed no significant difference among any of the treatments when measuring membrane integrity or acrosomal status. There was significant difference when comparing the pre-cool motility value between the control and the human sperm preservation medium extender (HSPM), but this difference was not detected in either of the post-thaw evaluations. In the second experiment, it was determined that even at the lowest concentration, 2% egg yolk was not significantly different from the 10% or the 5% when comparing motility, membrane integrity and acrosomal status. In the third series of experiments it was determined that there was no difference in post-thaw sperm motility, membrane integrity or acrosomal status when the epididymal sperm were exposed to the pentoxifylline both before and after freezing. When spermatozoa were exposed to pentoxifylline during post-thaw only, the 1mM treatment, sperm had a significantly greater motility at the 3 hour post-thaw assessment time than the control treatment. In the last series of experiments again, the inability to detect a difference between the extenders gives further evidence that non-egg yolk extenders such as BioXCell® maybe an effective replacement for the commonly used egg yolk-based extenders. Finally there was no difference between the two TesT extenders frozen after cooling to 4°C for any of the parameters measured. The TesT without egg yolk, frozen from room temperature, consistently had lower sperm motility and membrane integrity then sperm frozen in one or both of the extenders frozen after cooling to 4°C.

CHAPTER I
INTRODUCTION

The International Union for Conservation of Nature (IUCN) classifies plants and animals into one of seven categories to designate their threat of extinction, which include: least concerned, near threatened, vulnerable, endangered, critically endangered, extinct in wild and finally, extinct. Of the 5,500 species of mammals in the world, 13% are listed as endangered, critically endangered, extinct in the wild or extinct (IUCN 2012). A recent taxonomy publication lists as many as 40 felid species (Wozencraft, 2005), while the IUCN lists 36, seven of which are listed as either endangered or critically endangered (Sunquist and Sunquist, 2002; IUCN 2012).

A potential option of enhancing the propagation of endangered species in the future is by the application of assisted reproductive technologies (ART). Artificial insemination (AI), the least invasive and oldest of the ART methods, was first reported in the dog by Lazzaro Spallanzani in 1780 (see Senger, 1997). In vitro fertilization (IVF) and the birth of offspring after embryo transfer was first reported in the rabbit (Chang, 1959). This occurred after the importance of spermatozoa capacitation had been discovered earlier in the decade by Chang (1951) and Austin (1951). Embryo transfer (ET) was first accomplished in the domestic rabbit in England by Walter Heape (1891). Successful repeatable cryopreservation of spermatozoa was first reported in 1949, with the finding that the addition of glycerol to the extender solution allowed rooster spermatozoa to survive the freezing process (Polge et al., 1949).

The ART methods listed above have been used across various exotic species. For example, Pope et al. (1984) reported the birth of a live baboon following the transfer of an embryo that had been cryopreserved and subsequently thawed. More than a decade later, Pope et al. (1997) reported the birth of a Western Lowland gorilla resulting from the transfer of an embryo that had been produced using IVF. Artificial insemination has also been successfully used to produce offspring in various exotic species. Examples of some of the more difficult species where pregnancies and parturition were finally achieved after decades of effort include the elephant (see review Hermes et al., 2007) and more recently the rhinoceros (Hildebrandt et al., 2007).

There are also examples of artificial insemination being used in exotic canine and feline species. Thomassen and Farstad (2009) reported the birth of live pups when AI was used to breed three Mexican Gray wolves in 2005. One year later, Asa et al., (2006) reported the birth of live pups after artificially inseminating a single Gray wolf. Swanson et al. (1996) reported the

birth of a single male ocelot kitten after a female ocelot was laparoscopically inseminated with frozen thawed semen. |

ART methods have also been used, with some success, in exotic ungulate species. Dresser et al. (1985) and Pope et al., (1991), showed that in vivo-produced embryos could be nonsurgically flushed and nonsurgically transferred, in the bongo antelope and the Scimitar-Horned oryx , respectively, resulting in live healthy calves. Furthermore, Dresser et al. (1985) also showed that in vivo-produced bongo antelope embryos could be transferred into a eland antelope recipient (interspecies embryo transfer) resulting in a live healthy bongo calf, which was then raised by the eland recipient.

Over time, new methodologies have been added to the list of assisted reproductive techniques. Promising examples include intracytoplasmic sperm injection (ICSI) and sex sorted spermatozoa. As before, the new techniques were first developed in the domestic species and then were eventually attempted in exotic species. When performing ICSI, a single spermatozoon is physically microinjected into the cytoplasm of an oocyte to achive fertilization. This technique was first described in the hamster, where the outcome was the formation of both male and female pronuclei (Uehara and Yanagimachi, 1976). Ten years later, Mann (1988) produced the first offspring using ICSI in the mouse. To our knowledge, no mammalian exotic species have been born from the ICSI procedure. To date, there have been only a few reports of ICSI-derived embryos produced from exotic species. Pope et al. (1998) reported the transfer of 10 ICSI-produced Jaguarundi embryos into a domestic cat recipient but no kittens resulted from the transfer. Damiani et al. (2004) also reported the use of ICSI to produce lion embryos using spermatozoa aspirated from the epididymis of a vasectomized male but again, no offspring were born following transfer of the embryos to a recipient female after they had been frozen/thawed.

Also, predetermined gender offspring have now been produced using flow cytometry to separate X-bearing spermatozoa from the Y-bearing spermatozoa prior to insemination in both domestic and exotic species (O'Brian et al., 2009, Garner 2006). The first offspring produced from sex sorted spermatozoa occurred >20 years ago after sorted sperm were surgically inseminated in to the uterus of a rabbit (Johnson et al., 1989). To date, offspring from other domestic species have been born including cattle (Cran et al., 1993), pigs (Johnson 1991), sheep (Catt et al., 1996), horses (Buchanan et al., 2000), dogs (Meyers et al., 2008) and cats (Pope et al., 2009).

The goal in the present research experiment was to determine a suitable nonegg yolk, semi-transparent semen extender that would effectively cryopreserve domestic cat epididymal

spermatozoa. The hope is to eventually use flow cytometry to sort the X and Y spermatozoa in previously frozen cat sperm samples. Due to the opaque coloring and slight viscosity of egg yolk, semen samples extended and/or frozen in an egg yolk-based extender must be washed and centrifuged to clarify the sample so that spermatozoa can be sorted more effectively by flow cytometry.

Accordingly the primary objective was to use a non-egg yolk-based extender to freeze feline sperm for later use in the sex sorting procedure. The long term objective of our experiments was to develop a procedure for cryo-storage of feline sperm that would allow for efficient gender sorting and subsequent production of offspring of a predetermined gender.

CHAPTER II
LITERATURE REVIEW

Basic Principles of Cooling and Freezing Cells

The ability to preserve or suspend cells in a perpetual frozen state and later thaw those cells unharmed has long been of interest to scientists. Mazur et al. (1972) hypothesized that two factors affect the cryosurvival of cells during the cooling, freezing and thawing process: (1) intracellular and extracellular exposure to precipitated and concentrated solutes, pH changes and dehydration all which occur during conversion of water to ice and, (2) formation of intracellular ice during freezing and recrystallization of the ice at thawing. Both of these factors are believed to be dependent on cooling rate, and may interact with each other to influence the survival of cells during freezing. This hypothesis generally referred to as the "two factor hypothesis (freezing injury), is now widely recognized and accepted. The formation of intracellular ice, which causes a substantial amount of cell damage, is the primary problem (Asahina, 1965; Leibo and Mazur, 1971; Meryman, 1974; Griffiths et al., 1979). Intracellular ice formation is caused by a fast cooling rate that does not allow intracellular water to exit the cell before freezing occurs. Furthermore, optimal cooling rates differ among cell types. The optimal cooling rate of large spherical cells, which have a low surface area to volume ratio, such as hamster oocytes (Griffiths et al., 1979) and marrow stem cell suspension (Leibo et al., 1970) is ~1°C/minute. In contrast, small flat cells, such as erythrocytes or spermatozoa that have a high, surface area to volume ratio, a cooling rate of ≥100°C/minute is more effective and less likely to allow the formation of intracellular ice than the slower rates of ~1°C/minute. (Leibo et al., 1970; Miller and Mazur 1976).

More recently, it has been suggested that the formation of intracellular ice during rapid cooling is not the primary cause of sperm cell damage, but rather it is the osmotic imbalance created during thawing (Morris et al., 2007). Mazur and Koshimoto (2002) reported that the actual formation of intracellular ice in mouse spermatozoa did not follow the models predicting that intracellular ice should form only at cooling rates of ≥1000°C/minute. They indicated that intracellular ice occurred at cooling rates of 130 to 261°C/minute.

Cell injury and/or death during cryopreservation is also due to "solution effect injury" that causes damage either by dehydration and shrinkage or by exposure to high concentrations of solutes (Lovelock, 1953a). During slow cooling, intracellular and extracellular water is removed in the form of ice. While ice is being formed, water remaining becomes increasingly concentrated with solutes, usually electrolytes. Although the exact mechanism is not

understood, it is widely accepted that exposure to elevated concentrations of salts is lethal to cells (Lovelock, 1953a).

Cell shrinkage and dehydration also have been hypothesized to cause damage during slow cooling (Muldrew et al., 2004). It has been reported that cell damage during freezing is not caused by shrinkage but by high intracellular concentrations of electrolytes that can result in complete dehydration. Then, presumably, at thawing, rehydration occurs so rapidly that the cell membrane is lysed from over expansion (Lovelock, 1953a; Zade-Oppen, 1968; Farrant and Woolgar, 1972).

Cryodamage caused by intracellular ice formation and slow cooling injury is reduced when glycerol, a cryoprotective agent (CPA), is present in both the intra- and extracellular enviorment (Lovelock, 1953b). A cryoprotectant, as defined by Karow (1969), is a class of "drugs" that acts specifically to protect and maintain the viability of frozen animal cells. Cryoprotectants are divided into two groups (a) permeating and (b) nonpermeating that protect cells during freezing by two different mechanisms, although both rely on membrane diffusion and osmosis. Permeating CPAs are characteristically low molecular weight, nonionic compounds that are highly soluble in water at low temperatures and have low cellular toxicity (Muldrew et al., 2004). An intracellular CPA protects the cell in two ways (1) both intra- and extracellular presence eventually reduces the amount of intracellular water and thus, reduces the amount of intracellular ice being formed at a specific temperature (Leibo and Mazur, 1971) and, (2) by acting as a secondary solvent for intracellular salts, and exposure to lethal levels of electrolytes is reduced (Pegg 1984).

Usually, nonpermeating CPAs have higher molecular weights than their permeating counterparts, and are long-chained polymers (sucrose being an exception). Also, their osmotic coefficients are considered to be large, which allows them to increase the osmolality much higher than their molar concentration (Muldrew et al., 2004). Since nonpermeating CPAs do not pass through the cell membrane, intracellular water is transported out of the cell so as to yield equal intracellular and extracellular osmolality. The loss of water dehydrates the cell, thereby reducing or preventing intracellular ice formation.

Historical Perspective on Methods for Preserving Spermatozoa

There are three major methods used to perverse spermatozoa (1) cryopreservation in liquid nitrogen (-196°C), (2) short-term storage 4°C to 15°C for and (3) freeze drying. Since Polge et al. (1949) reported that spermatozoa could be cryopreserved successfully at sub-zero temperatures by the addition of glycerol, cryopreservation has become the most widely used method of mammalian sperm storage (see reviews by Karow, 1969; Foote, 1982; Watson,

1995; Holt, 2000). Also, storage in the liquid state between 4° to 10°C is used predominantly for short-term maintenance of mammalian spermatozoa (Pope et al., 1989; Goodrowe et al., 1993; Axner, 2012). Freeze drying sperm is another approach that has long been of interest and the subject of numerous studies. Recently, the technology of freeze drying spermatozoa has been revisited (Ward et al., 2003; Kawase et al., 2005; Kaneko and Nakagata 2006) and live mouse offspring have been born following the transfer of ICSI derived embryos (Ward et al., 2003; Kaneko and Nakagata 2006)

Early Attempts at the Cryopreservation of Spermatozoa

Glycerol was first used as a cryoprotectant for freezing plant cells and tissues by Maximov in 1908. Later, Bernstein and Petropavlovsky (1937) had minimal success using glycerol (9.2%) as a cryoprotectant for sperm of the rabbit, guinea pig, bull, ram, boar, stallion and duck, but only for cooling spermatozoa to -21°C. It was also reported that 18% glycerol was toxic to spermatozoa (see review Salamon and Maxwell, 1995).

However, the first successful results after freezing spermatozoa was performed in Cambridge England by Polge et al. (1949). The post-thaw success with avian spermatozoa was the result of mislabeling glycerol as a “sugar”. The first offspring born using glycerol for freezing semen were chickens, only three live chicks were produced from 600 eggs (Smith and Polge, 1950). The first pregnancies in cattle with frozen-thawed semen resulted from the use of artificial insemination (AI) and were reported by Stewart (1951; one bull calf born) and Polge and Rawson (1952; 30 out of 38 cows were pregnant six weeks after insemination).

Subsequently, spermatozoa from both domestic and some nondomestic species have been frozen using glycerol, each with variable rates of success. Smith and Polge (1950) attempted to freeze equine spermatozoa, but the first foal from AI using semen frozen with glycerol was not reported until 7 years later (Barker, 1957). Emmens and Blackshaw (1955) were among the first investigators to use ram semen that had been frozen to -79°C to produce pregnancies from AI in sheep. Graham et al. (1971) reported the birth of five piglets from a single litter after inseminating eight sows with semen that was frozen in pellet form. The first canine pups born using frozen-thawed semen was reported by Saeger (1969). Platz et al. (1978) reported the first kittens born using frozen-thawed semen. The first gorilla produced using frozen-thawed semen and AI was reported by Douglass and Gould (1981). Pope et al. (1997) used frozen semen to produce IVF-derived embryos that resulted in the first gorilla born from embryo transfer. However, due to lack of accessibility and inconsistent quality of thawed semen from exotic species, routine freezing of semen is done only in a few species.

Since the first calf, using frozen-thawed semen was produced in the early 1950s, frozen semen has become the mainstream in the North American and European dairy cattle industries. Thibier and Wagner (2002) reported, that >252 million doses of bovine semen, from 648 semen collection centers were frozen worldwide in 1998. Over 43 million doses were produced in North America from 69 semen collection centers. In comparison, 136 million doses originated in Europe from 285 semen collection centers. More recently, similar results have been reported by the Food and Agriculture Organization (FAO) (Rodriguez-Martinez, 2012).

The number of nondomestic mammalian species where offspring have been produced using AI with cryopreserved spermatozoa has also continued over the decades (see examples in Table 2.1). A more comprehensive table listing >55 mammalian species where various ART technigues were successful resulting in births of offspring has been compiled by Wirtu (2004).

Cryoprotectants

Cryoprotectants are divided into two categories, based on their ability to pass through the sperm membrane (permeating) or those that do not pass through the sperm membrane (nonpermeating). Both types of cryoprotectants involve osmosis and depend on the ability of water to follow the concentration gradient and move freely across the cell membrane. Since the discovery of glycerol as an effective permeating cryoprotective agent, other chemically similar (low molecular weight, extremely low freezing temperature) compounds have been evaluated in attempts to further improve spermatozoa cryo-survival. Other widely used permeating cryoprotectants include dimethyl sulfoxide (DMSO) (Lovelock and Bishop, 1959), ethylene glycol, propylene glycol and other “neutral solutes” (Lovelock, 1954).

DMSO and is possibly the most versatile cryoprotectant with its ability to successfully preserve a variety of cell types (Karow 1969). One example is DMSO’s effectiveness in cryopreserving mouse bone marrow (Ashwood, 1961), as well as DMSO’s ability to produce better post-thaw sperm motility values over other cryoprotectants such as glycerol, formamide, propanediol and adonitol when freezing mouse semen (Sztein et al., 2001).

For example, Loskutoff et al. (1996) showed that Springbok epididymal spermatozoa frozen in DMSO had greater post-thaw motility than did spermatozoa frozen in glycerol, ethylene glycol or propylene glycol. Ejaculated spermatozoa from the African elephant exhibited significantly greater motility when incubated for 60 minutes with 0.5M DMSO or 0.5M ethylene glycol compared with incubation with 0.5M glycerol (Gilmore et al., 1998). Correspondingly, Gilmore et al. (1998) and Loskutoff et al. (1996) (30-32°C) both reported that DMSO was less toxic to impala epididymal sperm during holding periods of 24 to 60 hours respectively.

Table 2.1. A list of some exotic mammalian species in which pregnancies and/live offspring have been produced after the use of artificial insemination procedures with cryopreserved spermatozoa

Species	Method of fertilization	Assesment of fertiltiy	Reference
Addax	AI at the os cervix	Live offspring	Densmore et al. 1987
Alpaca	AI	Live offspring	Bravo et al. 2013
American bison	AI	Live offspring	Dorn 1995
Bactrian camel	AI	Live offspring	Zhao 1994
Banteng	AI	Live offspring	Johnston et al. 2002
Blackbuck	Transcervial IUI	Live offspring	Holt et al. 1988
Black-footed ferret	Laproscopic IUI	Live offspring	Howard et al. 1991
Bottlenose dolphin	Transcervial IUI	Live offspring	Robeck et al. 2005
Cheetah	Laproscopic IUI	Live offspring	see Howard et al. 2009
Chimpanzee	Transcervial IUI	Live offspring	Gould 1989
Common marmoset	AI intracervically	Live offspring	Morrell et al. 1998
Common eland	Transcervial IUI /intracervical	Live offspring	Bartels et al. 2001
Eld's deer	Laproscopic IUI	Live offspring	Monfort et al. 1993
Fallow deer	Laproscopic IUI	Live offspring	Mulley et al.1988
Gaur	AI	Live offspring	Junior et al. 1990
Giant panda	Intracervical	Live offspring	Moore et al. 1984
Leopard cat	Laproscopic IUI	Pregnancy	see Howard et al. 2009
Mohor gazelle	Laproscopic IUI	Live offspring	see Roldan et al. 2006
Ocelot	Laproscopic IUI	Live offspring	Swanson et al. 1996
Pacific white-sided dolphin	Transcervial IUI	Live offspring	Robeck et al. 2009
Red deer	AI and Laproscopic IUI	Live offspring	Asher et al. 1993
Rhesus monkey	Transcervial IUI	Live offspring	Sanchez-Partida et al. 2000
Scimitar Horned oryx	Transcervial IUI	Live offspring	Morrow et al. 2000
Spanish ibex	Laproscopic IUI	Life offspring	Santiago-Moreno et al. 2006a
Suni antelope	Transcervial IUI	Pregnancy	Raphael et al. 1989
White rhinoceros	Transcervial IUI	Live offspring	Hermes et al. 2009
White-tailed deer	AI at the os cervix	Live offspring	Haigh 1984

AI = artificially inseminated nonsurgically with no description of the location where sperm were deposited. IUI = intrauterine insemination;

Ethylene glycol has been used as a cryoprotectant for many cell types including rat liver cells (Magalhaes et al., 2012), bovine fetal and adult cartilage cells (Cetinkaya and Arat, 2011), freezing and vitrification of mouse and rat embryos (Miyamoto and Ishibashi 1977; Mochida et al., 2011) and for freezing bovine embryos (Voelkel and Hu, 1992; Massip, 2001). Due to its low molecular weight, cells are more permeable to ethylene glycol than larger compounds such as glycerol, and thus, osmotic shock is reduced. For this reason, ethylene glycol has been evaluated as a cryoprotectant for preservation of spermatozoa.

Epididymal spermatozoa from blesbok and impala antelope had equal post-thaw motility values when frozen with ethylene glycol or glycerol (Loskutoff et al., 1996). Equine semen used for AI after freezing in 6% ethylene glycol resulted in pregnancies of 3 of 5 mares (Squires et al., 2004). Alvarenga et al. (2000) reported that acrosomal status and motility were similar after cryopreservation of stallion semen in either ethylene glycol or glycerol.

In a theoretical simulation ethylene glycol was compared with glycerol, DMSO and propylene glycol, to determine memebrane permeability and volume excursions induced during cryoprotectant addition and removal (Gilmore et al., 1997). From the results of the computer-generated simulation, it was hypothesized that ethylene glycol was the most permeable, and was therefore, experimentally compared with glycerol. After treatment with ethylene glycol (1M), human spermatozoa exhibited a higher percentage of motility than spermatozoa treated with 1M glycerol when the cryoprotectants were added and removed at 22°C. Similarly when ethylene glycol or glycerol were added to sperm samples at 22°C and the samples cooled to -80°C (programmable freezer), plunged in liquid nitrogen and later thawed at 22°C (bench top method), motility was higher in ethylene glycol treated samples than I was in glycerol treated samples.

Another permeating cryoprotectant that has been used with some success for freezing spermatozoa is propylene glycol. Varisli et al. (2009) showed that motility and Acrosomal status of ejaculated and epididymal ram spermatozoa, following a one-step addition and removal, were similar in all four of the 1M cryoprotectants evaluated (propylene glycol, glycerol, DMSO and ethylene glycol).

The osmotic tolerance and membrane permeability of rhesus monkey and chimpanzee spermatozoa have been measured (Agca et al., 2005a, 2005b). The results showed that chimpanzee and rhesus monkey spermatozoa are more permeable to propylene glycol and ethylene glycol than they are to glycerol or DMSO, with ethylene glycol being the most permeable and DMSO the least permeable. In contrast, when red deer (*Cervus elaphus hispanicus)* epididymal spermatozoa were frozen in glycerol, ethylene glycol and propylene

glycol, motility index, and acrosome and membrane integrity were lower in propylene glycol samples as compared with those frozen in glycerol and ethylene glycol (Fernandez-Santos et al., 2006).

Cool Storage of Spermatozoa

Extended storage of spermatozoa is most often accomplished by some form of cryopreservation. Effective cryoprotective extenders allow spermatozoa to survive at sub-zero temperatures. Storage in liquid nitrogen is the method used universally for long-term storage (-196°C), which has allowed bovine spermatozoa to retain viability for at least 47 years and possibly indefinitely (Carwell, 2008). However, due to the risk of reduced motility and possible acrosome damage in some mammals (Lengwinat and Blottner, 1994), cryopreservation is not always the best method for storing spermatozoa. When spermatozoa are used within days following collection, temporary storage at supra-zero temperatures (4° to 15°C) has been another option to freezing.

Cool Storage of Bovine Spermatozoa

The use of egg yolk as a cryoprotectant in semen extenders was first reported by Phillips (1939) and Phillips and Lardy (1940). It was reported that after 150 hours of storage, spermatozoa extended in an egg yolk extender (10°C) exhibited higher motility than spermatozoa in the control which was not exposed to an egg yolk extender. Willett et al. (1940) showed that bull semen stored in a phosphate-egg yolk diluent at 5°C, could be held for 4 days and still result in a pregnancy rate (after AI) equal to that of fresh semen. Dairy cows inseminated with semen that was extended in a citrate-egg yolk diluent before cooling had a significantly lower 60-to-90 day non-return rate than cows inseminated with semen extended after cooling (Foote and Bratton, 1949).

De Pauw et al. (2003), compared, cooling and/or holding bull versus freezing bull semen. Ejaculates held at room temperature had a higher percentage of spermatozoa with intact membranes and mitochondrial function at pH 6, than at lower (4 or 5) or higher (7 or 8) pH. In addition, the percentage of spermatozoa with intact membranes was significantly higher in isotonic medium (300 mOsm/kg) as compared with those held in either hypotonic or hypertonic solution.

Also, De Paw et al. (2003) showed that the timing of adding of the egg yolk extenders influenced the optimal sperm concentration for holding samples. Membrane integrity was improved in spermatozoa stored at concentrations between 100 X 10^6 and 100 x 10^7/mL after extension in Triladyl®-egg yolk-glycerol and removal by washing after 5 minutes. When

spermatozoa were not exposed to the Triladyl® extender, membrane integrity was greater when spermatozoa were stored at lower concentrations (100 X 10^5 and 500 X 10^6/mL).

The consistently high post-thaw motility values achieved from frozen bull semen, has allowed the cattle industry to depend heavily on cryopreserved spermatozoa. Furthermore, offspring from numerous different mammalian species have been born using AI and cryopreserved spermatozoa (see Wirtu, 2004). However, Watson (2000) emphisized that post-thaw viability of cryopreserved spermatozoa in the majority of mammalian species has been inconsistent and the development of species-specific cryotechniques is necessary if cryopreserved spermatozoa are to be used routinely in nondomestic species.

Cool Storage of Equine Spermatozoa

In a study evaluating the functional viability of frozen equine semen, Cochran et al. (1983) found a 26% decrease in pregnancy rate following AI of mares with frozen-thawed compared with fresh semen. Also, Cochran et al. (1983) reported that only 62% of frozen ejaculates resulted in a post-thaw progressive motility value of ≥35%. Similarly, Loomis et al. (1983) reported that only 31% (17/54) of frozen-thawed stallion ejaculates had ≥ 50% progressive motility and the pregnancy rates after AI was lower than that obtained using fresh semen.

Loomis (2001) reported that cooled stallion semen used for AI during the first estrus of the season resulted in a significantly greater pregnancy rate than did frozen semen (59 % and 51 %, respectively). More recently, Backman et al. (2004) showed that cooled (5°C) horse semen held for 18 hours, before freezing, could be thawed and used for AI to produce pregnancies (70%). Also, they reported that there was no significant difference in pregnancy rates when spermatozoa were frozen at different concentrations (200 x 10^6 or 400 X 10^6 sperm/mL).

Cooling horse semen for the purpose of AI is a practical alternative to freezing, however, the facility that collected and shipped the semen is another factor that may influence the quality of the semen after shipment. When horse semen was cooled and shipped breeding facilities, Heckenbichler et al. (2011) reported a 67% pregnancy rate (86 mares) for a single breeding season. They noted that processing differences between six different collection centers had significant effects on semen volume, arrival temperature, pH, concentration of sperm, total number of sperm, percentage of sperm with intact membranes, morphological defects, total motility, progressive motility and total number of progressively motile spermatozoa.

When stallion semen was extended in a skim milk–sugar-saline extender, cooled (8 to 12°C), and shipped to be used for AI in 56 mares, the foaling rate was 50%, with some of this

semen being used 24 hours after collection (Rota et al., 2004). In an earlier study, Douglas-Hamilton et al. (1984) reported that 13 of 14 mares established pregnancy after AI with stallion semen extended in skim-milk extender and cooled to 6 to 8°C for 12 to 23 hours.

Recently, it was shown that spermatozoa could be collected from stallion epididymides andheld at 4°C ≤ 72 hours post-castration. Furthermore, the recovered spermatozoa were capable of fertilizing oocytes in vitro (Vieira et al., 2013) after cryopreservation. Braun et al. (1994) reported that adding 25% seminal plasma to equine epididymal spermatozoa resulted in a significant increase in sperm motility at 0 hours: however motility at 24, 48 and 72 hours was not significantly increased.

Cool Storage of Ovine Spermatozoa

Purdy (2006) has found that ram semen could be held for up to 48 hours at 5°C without a significant decrease in total motility, progressive motility, membrane integrity and acrosomal status. Previously, Salamon et al. (1979) reported that there was no difference in early pregnancy rates (day 18 or day 19) when ewes were inseminated with semen that had been cooled to 5°C and held for 0, 2, 4, 6 and 8 days, although pregnancy rates, later in gestation, did significantly decrease after AI with semen held for ≥ 4 days. Later, Salmon et al. (1979) did report the birth of a live lamb using semen stored at 5°C for 9 days.

Cool Storage of Swine Spermatozoa

Boar semen was frozen with some success in the early 1970s (Pursel and Johnson, 1971), although boar spermatozoa are less cryo-tolerant than some other widely studied species. It has been reported that farrowing rates and litter size decreased 20% to 30% and 2 to 3 piglets per liter, respectively when cryopreserved semen was used to AI sows (cited by Bailey et al., 2008). Due to poor post-thaw survival the use of four straws/female, rather than one or two straws was recommended. The necessity of using multiple straws for AI is one disadvantage of using frozen-thawed boar semen (Casas et al., 2012).

Artificial insemination is widely used in swine breeding, and 99% of all inseminations (19 million worldwide) are done with extended liquid semen (Johnson et al. 2000). In some European countries, 90% of pigs are artificially inseminated, as compared with 45 to 50% in the United States (Johnson et al. 2000). Most inseminations are performed ≤24 hours after semen collection but semen can be used for up to 5 days post-collection when held between 8°C and 17°C (Pursel et al., 1973; Waberski et al., 1994a; Waberski et al., 1994b; Althouse et al., 1998; Severo et al., 2011). Funahashi and Sano (2005) showed that boar spermatozoa stored at 10°C for ≤ 29 days could penetrate oocytes in vitro when treated with the antioxidant, cysteine.

Cool Storage of Canine Spermatozoa

Cool storage of liquid dog semen has some advantages over freezing. For example, fresh ejaculates of dog semen are simple and easy to acquire. In addition, the cooled samples can be stored for ≤ 10 days before losing viability (Rijsselaere et al., 2011). Inseminations with dog semen cooled for 24 or 48 hours resulted in pregnancy rates and litter sizes that were similar to those obtained with fresh semen (Pinto et al., 1999). Furthermore, Froman et al. (1984) stated that frozen-thawed dog spermatozoa penetrated the zona pellucida (IVF study) of only 5% (6 of 120) of oocytes, as compared to a 70% (84 of 120) penetration rate by fresh spermatozoa. They found the lack of zona penetration may have been due to the early activation of a vital zona penetrating enzyme (acrosine), or the complete loss of the enzyme due to acrosomal damage.

Rodenas et al. (2014) cooled dog semen extended in 20% egg yolk extender at 2.25, 0.9, 0.45 and 0.2°C/minute from 23°C to 5°C, and found no difference between cooling rates in sperm motility and viability at 0, 24, 48, 72 and 96 hours of storage. In an effort to extend this life of cooled semen Verstegen et al. (2005) reported that cooled canine semen (4°C) extended in 20% egg yolk-based extender could retain motility up to 27 days when extender was replaced with fresh extender on days 11, 21 and 27. Motility significantly increased from 68% to 95.6% at the day 11 extender-exchange and from 19.9% to 73.0% at the day 21 exchange. Extender-exchange on day 27 did not improve motility.

The positive effects provided to sperm by egg yolk during cooling is well established; however, under certain conditions cooled epididymal (and ejaculated) sperm can be maintained without egg yolk in the extender. Yu and Leibo (2002) showed that canine epididymal spermatozoa held at 4°C within epididymides for up to 8 days were capable of binding in vitro to dog zonae pellucidae. Motility decreased significantly after 5 hours at 4°C but there was no significant decrease in their ability to bind with the zona until after 48 hours.

While the benefits of chilling canine semen are evident, it has been shown that dog semen can be extended, cooled to 4°C and held for ≤ 2 days and then frozen with no detrimental effects on post-thaw parameters due to the holding period (Hermansson and Forsberg, 2006).

Cool Storage of Feline Spermatozoa

Studying methods of cryo-storaging cat spermatozoa is a good model for determining its potential for propagating endangered felid species (Pukazhenthi et al., 2006; Cocchia et al., 2010). Pope et al. (1989) showed that domestic cat semen stored in TesT egg yolk buffer for 20±2 hours (2°C) resulted in significantly greater cleavage rate in vitro than did freshly collected

semen. Furthermore, there were two live kittens as a result of transferring embryos that were in vitro fertilized with the chilled domestic cat semen.

Filliers et al. (2008) showed that the percentage of motile, progressively motile and rapid or static moving epididymal cat spermatozoa, was significantly reduced after 1 day of storage at 4°C when compared with fresh epididymal spermatozoa. However, average sperm velocity did not decrease significantly until days 3 and 7 of storage. Similarly, Villaverde et al. (2006) reported that cat epididymal sperm motility and vigor decreased significantly from day 0 to day 3 of storage at 5°C. Also, the percentage of morphologically normal spermatozoa decreased significantly during the first day of cool storage.

Harris et al. (2001) demonstrated the resilience of cooled cat ejaculated and epididymal spermatozoa after it was extended and held at 4°C for up to 23 days. During the 23 day storage period, sperm motility ranged from 66.2 (day 0) to 21.2% (day 23) and 79.3 (day 0) to 31.2 (day 23) for ejaculated and epididymal spermatozoa, respectively. During that same time period, the percentage of spermatozoa with intact membranes, ranged from 91.0 to 65% for ejaculated and 95 to 59% for epididymal spermatozoa. Additionally Siemieniuch and Dubiel (2007) also reported that cat epididymal spermatozoa held at 4°C decreased in progressive motility from 73.7% at 24 hours of cooled storage to, 65.0 and 52.5% at days 2 and 3 of storage, respectively. Even though progressive motility decreased by 20%, due to the ease of cooled storage compared with freeze-thawing, using 3-day cooled spermatozoa (52.5%) was proposed as a practical alternative to using frozen-thawed samples in particular circumstancessubstitute over a frozen-thawed sample.

When feline semen was exposed to repeated rapid cooling cycles (3 cycles) in ice water (0°C), motility significantly decreased from one cycle to the next, but the presence of 20% egg yolk minimized the decrease in motility between cycles when compared with non-egg yolk samples (Glover and Watson, 1985). Furthermore, Tittarelli et al. (2006) reported that feline spermatozoa recovered from epididymides held at 4°C in medium containing egg yolk had significantly greater motility, membrane integrity and sperm velocity than sperm recovered from epididymides held in a isotonic saline solution, for 24, 48 and 72 hours. Martins et al. (2009) showed that epididymal cat sperm could be held at 5°C in an egg yolk-based extender for 24 hours before freezing without any significant difference in total motility, progressive motility, membrane integrity or morphology as compared to sperm cooled for 60 minutes before freezing.

Jimenez et al. (2013) tested three commercially available extenders (Triladyl, Andromed and Gent), for their efficacy in freezing domestic cat epididymal sperm. They fout that samples frozen in Triladyl, an egg yolk-based extender, had greater post-thaw motility, vigor,

morphology, acrosome status, membrane integrity and DNA integrity when compared with Andromed and Gent, neither of which contains egg yolk. Domestic cat epididymal spermatozoa held in the epididymides overnight at 5°C have been shown to be capable of zona binding and sperm head nuclear de-condensation, when incubated with zona free hamster oocytes (Goodrowe and Hay, 1993). Furthermore when compared with freshly collected epididymal sperm, the cooled overnight treatment had a greater percentage of spermatozoa that attached to the zona.

Freeze-Drying of Spermatozoa

Freeze-drying spermatozoa is the least used method for preserving spermatozoa and will only be mentioned here briefly. Freeze-drying (lyophilization) procedures are widely used for storage of food (Sosa et al., 2012) and vaccines (Wang et al., 2011; Okada et al., 2012). The process of freeze-drying differs from conventional dehydration. Dehydration is the removal of water by evaporation, while freeze-drying involves the removal of water from a specimen while it is in a frozen state (Meryman, 1960). The process of freeze-drying depends on the direct transition from a solid (ice) into a gas, which now is known as the “sublimation phenomenon” (Hochi et al., 2011).

Freeze-drying of spermatozoa was first attempted in the mid 20th century (see Hochi et al., 2011). Sherman (1954) unsuccessfully attempted to freeze-dry human spermatozoa. Bialy and Smith (1957) also reported negative results in their efforts to freeze-dry bull spermatozoa in the presence of glycerol. In a more recent study Kusakabe et al. (2001) reported that the pregnancies could be produced in mice after transfer of embryos produced by ISCI of oocytes with reconstituted freeze-dried spermatozoa. Furthermore, Kusakabe et al. (2008) demonstrated that human spermatozoa were capable of undergoing freeze-drying without chromosome damage.

In combination with ICSI, freeze-dried spermatozoa have been shown to be a valid source of male genetics. Keskintepe et al. (2002) reported the formation of bovine blastocysts from embryos generated by ICSI of oocytes with spermatozoa that had been freeze-dried for 3 months. Abdalla et al. (2009) showed that injection of freeze-dried bull sperm-heads into mouse and bovine oocytes induced calcium oscillations and resumption of meiosis, respectively, but at lower rates than in oocytes injected with control sperm (not freeze-dried). Recently, Hara et al. (2011) reported that when freeze dried spermatozoa are used with ICSI, the resulting impaired formation of a microtubule-organizing center and reduced embryo development may be a result of the ICSI process, not a result of using freeze-dried spermatozoa.

The first (confirmed) live offspring (mice) from use of freeze-dried spermatozoa were produced using ICSI to fertilize the oocytes (Wakayama and Yanagimachi 1998). Six years later, rabbits were born after transfer of embryos produced by ICSI with freeze-dried spermatozoa (Liu et al., 2004). Pig embryos produced by injecting freeze-dried spermatozoa into oocytes were capable of developing into blastocysts (Kwon et al., 2004). Furthermore, results were improved when only the sperm heads were injected rather than intact spermatozoa.

Semen Extenders Without Egg Yolk

In the context of sperm preservation, "extender" is defined as solutions/media/diluents containing components that are beneficial sperm viability and extend the sample volume. Most extenders consist of five components (1) a buffer, such as 2-[[1,3-dihydroxy-2-(hydroxymethyl)propan-2-yl]amino]ethanesulfonic acid (Tes) and/or tris(hydroxymethyl)aminomethane (Tris) to prevent pH shifts, (2) an energy source, such as glucose or fructose, (3) a protein source such as egg yolk or milk, to protect spermatozoa from cold shock and provide cryoprotection, (4) antibiotics such as gentamicin, penicillin or streptomycin and (5) a cryoprotectant, such as glycerol (Mitchell and Doak, 2004). The latter component is usually not included in extenders used only for temporarily maintaining sperm samples in a liquid state (>0°C).

Milk and egg yolk have proven to be very effective agents for preventing cold shock (Thacker and Almiquist, 1951; Phillips and Lardy, 1940). However, for the purpose of consistency and prevention of disease transmission, the development of synthetic extenders (without animal protein) has long been an area of high interest (Choong and Wales, 1963). Furthermore, Roy (1957), and more recently Cabrera et al. (2005), reported that the bulbourethral gland of the goat releases an enzyme (phospholipase) and a protein (BUSgp60) (Pellicer-Rubio et al., 1997) into the ejaculate that reacts with egg yolk or skim milk. The result is a conversion of egg yolk phospholipids into lysophospholipids, such as lysolecithins, a class of detergent-like compounds that can be toxic to spermatozoa. The lipase activity of the BUSgp60 protein on skim milk negatively affect goat spermatozoa (Pellicer-Rubio et al., 1997).

<u>Use of Bovine Serum Albumin (BSA) in Semen Extenders</u>

As an alternative to milk and egg yolk-based extenders, bovine serum albumin (BSA) has been used with some success in extending longevity of motility of ejaculated bovine semen (Bredderman and Foote, 1971). For example, Matsuoka et al. (2006) showed that ram spermatozoa had significantly greater post-thaw motility after freezing in an extender with 10% and 15% BSA compared with freezing in extenders containing egg yolk with fructose. Also,

Fukui et al. (2007) reported no differences in day 60 pregnancy rates or lambing rates after AI of 60 ewes with semen frozen either in 15% egg yolk extender or 10% BSA extender.

Goat spermatozoa held at 15°C in two commercially available extenders containing BSA [Zorlesco and Androhep (Minitube of America, Inc., Verona, WI, USA)] had greater percentages of motility at 4 and 6 days than spermatozoa held in two other synthetic extenders that did not contain BSA (Xu et al., 2009). Furthermore, spermatozoa held for 3, 5 and 7 days at 5°C in either of two additional synthetic extenders (mZA and mZAP, containing BSA and polyvinyl alcohol, respectively) maintained greater motility than did sperm in the Androhep extender. Xu et al. (2009) also reported 34% progressive sperm motility in mZA extender on day 9 of storage. In addition, five does delivered kids when inseminated with spermatozoa held for 7 days in mZAP extender.

A commercially available extender, Androhep is widely used in the swine industry for long-term (≤5 days) liquid storage of boar semen (see Johnson et al., 2000). Waberski et al. (1994a) reported boar semen stored for 4 days in Androhep (with BSA and HEPES buffer) produced significantly greater pregnancy rates in comparison with semen stored in Kiev porcine medium (without BSA and HEPES buffer).

Fantinati et al. (2009) compared four commercial extenders, including Androhep, to an extender [swine fertilization medium (SFM)], contianing BSA, ethylene diamine tetra-acetic acid (EDTA) but no HEPES, milk or egg yolk. On days 3, 6 and 9 of storage, spermatozoa held in SFM had significantly greater motility than did spermatozoa in the other extenders. Also, SFM was the only treatment in which progressive motility was ≥60% at day 12 of storage. Therefore, it was concluded that, if boar semen is to be stored for more than 4 days, SFM was more economical than commercial extenders, and produces comparable results when properly prepared.

An optically-clear extender (without milk or egg yolk) has other practical advantages in addition to lowering the risk of disease transmission. The equine industry can benefit greatly from sex-sorting of stallion spermatozoa. However, sex-sorting of spermatozoa requires the use of lasers during flow cytometry to recognize Hoeschst stained spermatozoa. Gibb et al. (2011) showed that the X and Y split ratio improved in efficiency from 0.08 to 0.17 when stallion semen was sorted in an optically clear diluent (1% BSA) when compared with samples extended in skim milk. Also, after 18 hours of storage in the clear extender (containing BSA) sperm motility was greater than that of spermatozoa stored in skim milk (74% vs. 64%, respectively).

Although the incidence of milk or egg yolk transferring harmful microbes to semen is low there is a possibility of microbial contamination and exposure of semen to infectious animal borne diseases. For this reason the development of synthetic extenders, void of animal protein, is a priority. In a pig in vitro fertilization study, Jang et al. (2011) reported that using semen frozen in egg yolk resulted in contamination (*Enterobacter cloacae*) during IVF, however, contamination did not occur in the fresh semen that was not exposed to the egg yolk extender.

Use of Low Density Lipoproteins (LDL) in Semen Extenders

The use of an animal by-product, such as egg yolk or milk in semen extenders, does not allow control of standardization due to the presence of unidentified molecules that may have negative effects on spermatozoa (Hinsch et al., 1997 reviewed by Moustacas et al., 2011). Mayer and Lasley (1945) were the first to identify the low density lipoprotein (LDL), which is the specific fraction of egg yolk that provides the cryoprotective benefits when cooling spermatozoa. The subsequent discovery of the cryoprotective properties of glycerol (Polge et al., 1949) resulted in a delay in further studies on the cryoprotective nature provided by LDL. Subsequently, Pace and Graham (1974) confirmed and extended the earlier study of Mayer and Lasley (1945), to show that even in the absence of glycerol, the low density fraction aids in the survival of spermatozoa during cryopreservation. Shortly thereafter, other reports (Watson and Martin, 1975; Watson, 1981; Graham and Foote, 1987, Garcia and Graham, 1987) provided further information relating to the effects of lipid extracts and fractions on survival of bull and ram spermatozoa during cold storage.

Moussa et al. (2002) considered previous methods of purifying LDL to be inadequate and impractical. Accordingly they developed an improved economical method to extract and purify LDL, and reported that post-thaw motility and sperm movement of bull spermatozoa were significantly greater when spermatozoa were cryopreserved in an extender containing 8% LDL when compared with extenders containing egg yolk. More recently, Vera-Munoz et al. (2009) also showed that replacing egg yolk with 8% LDL in Triladyl® was significantly more effective in maintaining post-thaw bovine sperm motility and membrane integrity when compared with both the soy lecithin-based and egg yolk-based extenders BioXCell® and Triladyl®, respectively.

Similarly, Jiang et al., (2007) showed extender containing 9% LDL was significantly more effective at protecting boar sperm DNA from damage during cryopreservation when compared with that containing 0, 6, 7 and 8% LDL. Goat sperm motility, straight line velocity (VSL), amplitude of lateral head displacement (ALH) and Acrosomal status were greater for spermatozoa frozen with LDL compared with egg yolk (Al Ahmad et al., 2008; Moustacas et al., 2011).

Bencharif et al. (2010) reported that dog semen cryopreserved in a commercially available extender containing 6% LDL (CANIXL freeze, IMV, Aigle, France) had numerically higher sperm motility, average path of velocity (VAP) and straight line velocity (VSL) values, while curvilinear velocity (VCL) and lateral head displacement (ALH) values were significantly greater than sperm cryopreserved in egg yolk (20%) extender (Equex® STAMP, Nova Chemical Sales, Scituate Inc., MA, USA). Semen frozen in extender containing 6% LDL was used to inseminate six bitches, with all of the females becoming pregnant and delivering a total of 16 pups (Bencharif et al., 2010).

Use of Soybean Extract Lecithin in Semen Extenders

Further attempts to remove animal proteins from semen extenders have included investigations of a soy-derived lecithin. Cows inseminated with semen frozen in an extender containing soybean extract (Biociphos-Plus® IMV, France) had significantly lower 56-day nonreturn rates than those inseminated with semen frozen in a 20% egg yolk-based extender (van Wagtendonk-de Leeuw et al., 2000; Thun et al., 2002). In contrast, Forouzanfar et al. (2010) reported that post-thaw sperm motility, viability and cleavage rate after IVF with ram semen frozen in a 1% (w/vol) soybean lecithin-based extender were similar to those of spermatozoa frozen in 20%, but not 15% egg yolk-based extender.

Angora buck semen frozen in another soybean lecithin-based semen extender (BioXCell®, IMV Technologies, L'Aigle, France), exhibited greater motility (CASA), average sperm path velocity (VAP), sperm straight linear velocity (VSL), sperm linearity (LIN = VSL/VCL), fewer acrosomal abnormalities and greater pregnancy rates as compared with a Tris + egg yolk extender (Sariozkan et al., 2010). Likewise, buffalo (*Bubalus bubalis*) semen frozen in BioXCell® showed similar post-thaw characteristics (motility, viability, membrane integrity, normal apical ridge, abnormalities) to those of samples frozen in a Tris-citric acid egg yolk extender (Akhter et al., 2010).

The Use of Pentoxifylline to Stimulate Sperm Motility

Pentoxifylline is a small molecule with a molecular compound (M.W. = 278.31 $C_{13}H_{18}N_4O_3$) belonging to a class of drugs called hemorrheologic agents. Caffeine, a chemically related molecule, is also used to increase and/or improve sperm motility. In the buffalo, caffeine increased post-thaw motility when added to spermatozoa before freezing and it further increased motility when present in the thawing medium (Fattouh and Abdou, 1991). Hicks et al. (1972) stated that one contributing factor involved in sperm motility was the presence of intracellular cAMP. When human spermatozoa were incubated with pentoxifylline sperm intracellular cAMP increased at all time points (Calogero et al., 1998). Also, pentoxifylline

added after thawing, improved motility characteristics (total motility, progressive motility and motility velocity) of ram spermatozoa (Maxwell et al., 1995).

Post-thaw addition of pentoxifylline stimulated total and progressive motility of stallion spermatozoa. Pre-freeze addition produced a post-thaw decrease in total and progressive motility when compared with control samples (Gradil and Ball, 2000). Caffeine was shown to increase motility of equine epididymal, but not ejaculated spermatozoa. Also, caffeine had no effect on the acrosomal status of either epididymal or ejaculated spermatozoa (Weston et al., 2005). In contrast, Ortgies et al., (2012) reported that addition of pentoxifylline to ejaculated stallion spermatozoa produced no significant difference in the motility pattern but did increase the percentage of live-capacitated spermatozoa.

When administered as an oral treatment 400 mg of pentoxifylline significantly improved sperm concentration, sperm motility and spermatozoa with normal morphology from infertile men diagnosed with idiopathic oligoasthenoteratozoospermia (Safarinejad et al., 2011).

Treatment of frozen-thawed canine spermatozoa, with pentoxifylline (2.5, 5 and 7.5 mM, 120 minutes) significantly increased total sperm motility (Milani et al., 2010). In addition, treatment with pentoxifylline at high concentrations (10 mM and 100 mM, 1 hour of incubation) increased the capacitation rate and the percentage of acrosome reacted canine spermatozoa (Mirshokraei et al., 2011).

Effect of pentoxifylline has also been studied in the domestic cat. Post-thaw exposure of cat epididymal and ejaculated spermatozoa, to pentoxifylline has been shown to increase motility parameters (as measured by CASA) without affecting longevity of motility (Stachecki et al., 1994). The effects of different concentrations of pentoxifylline will be investigated in this research study.

Feline Epididymal Spermatozoa in Assisted Reproduction

Collection of Feline Epididymal Spermatozoa

There are at least five methods of collecting epididymal spermatozoa, three of which are *ex situ* collections (flushing, mincing, incisions), either post-mortem or post-castration. The other two methods [percutaneous epididymal sperm aspiration (PESA)], microsurgical epididymal sperm aspiration (MESA)] are *in situ* procedures performed on anesthetized males.

The in vitro fertilizing capability of nonejaculated domestic cat (*Felis catus*) spermatozoa was first reported by Bowen (1977). Using spermatozoa from minced vas deferens, Bowen reported a cleavage rate of 79%, including four embryos that developed into the first in vitro-derived blastocysts. Bowen (1977) pointed out that, unlike ejaculated spermatozoa in the cat (Hamner et al., 1970) and epididymal spermatozoa in the rabbit (Ogawa et al., 1972),

spermatozoa from the vas deferens of the cat did not need to undergo capacitation in vivo to fertilize oocytes in vitro.

Using epididymal sperm collected by the flushing method, Niwa et al. (1985) showed that sperm penetrated the zona pellucida by 30 minutes after insemination and the first observation of male and female pronuclei were noted within 4 to 5 hours of co-incubation (Niwa et al., 1985). Pope et al. (1993), using ejaculated sperm, confirmed the chronology of early fertilization events found by Niwa et al. (1985) with epididymal sperm. In the later report they observed that both male and female pronuclei were completely developed by 6 hours post insemination.

Mincing of the epididymides is a widely used method for obtaining domestic cat spermatozoa (Filliers et al., 2010). Because of tissue and blood contamination of the sperm samples obtained by mincing, centrifugation over a density gradient to remove the blood and tissue cells is necessary. Additionally, centrifugation produces a highly concentrated sperm pellet that can then be diluted to the desired concentration (Lengwinat and Blottner, 1994; Tsutsui et al., 2003; Chatdarong et al., 2010).

Lengwinat and Blottner (1994) demonstrated that fresh and cryopreserved epididymal spermatozoa could be used to fertilize oocytes and produce feline 8-cell embryos in vitro. Tsutsui et al. (2003) reported the first births of domestic kittens produced after AI using epididymal spermatozoa (27.3% pregnancy rate).

Comparison of Feline Ejaculated and Epididymal Spermatozoa

Various studies have been conducted to compare the fertilizing ability freezing and cooling ability, and morphology of ejaculated and epididymal cat spermatozoa (Goodrow, 1992; Axner et al., 1998; Harris et al., 2001; Tebet et al., 2006; Hermansson and Axner, 2007; Filliers et al., 2010). Goodrowe (1992) reported that capacitation times of epididymal spermatozoa and ejaculated spermatozoa were similar. Also, at 3 and 4 hours after in vitro insemination, there were more epididymal spermatozoa attached to the zona pellucida than ejaculated spermatozoa (9.6 vs. 5.2 and 12.0 vs. 5.6, respectively). In addition, Goodrowe (1992) compared collection of epididymal spermatozoa immediately after castration (fresh) compared with epididymides maintained overnight at 5°C. Cooled samples were found to have lower motility and lower proportions of abnormal spermatozoa post-thaw than did fresh samples. Even with reduced motility and an increase in abnormalities, cooled spermatozoa had a higher occurance of zona attachment than did fresh spermatozoa (112.2 vs. 84.5, respectively) but both groups had higher post-thaw zona attachment, than did their pre-freeze counterparts.

Axner et al. (1998) compared epididymal and electro-ejaculated spermatozoa from the same male, pre-castration and post-castration. The comparison was accomplished by performing a unilateral castration before electroejaculation was done and then removing the second testicle. They found no significant difference in total proportions of abnormal spermatozoa between the two types of sperm; however, the incidence of specific tail abnormalities was greater in ejaculated spermatozoa than in epididymal spermatozoa. This could suggest that the abnormalities in ejaculated semen resulted from the seminal fluid or from the process of ejaculation.

Also, Axner et al. (1999) used another method of determining the origin of feline teratospermia. They found that epididymal spermatozoa gain initial motility, where the caput epididymis transitions into the corpus portion, with the highest motility found in the cauda portion or the last one third of the epididymis. Head abnormalities, detached sperm heads, midpiece abnormalities and acrosomal defects/abnormalities decreased significantly when the first and last regions were compared, but there were no decreases between adjoining regions, suggesting a gradual decrease along the length of the epididymis. In contrast, tail abnormalities significantly increased (although still relatively low) between the efferent duct and cauda portion of the epididymis (Axner et al., 1999). Thus, the cat epididymis appears to serve as a filter to eliminate spermatozoa with specific abnormalities during transit but the majority of tail abnormalities seem to occur in the epididymis.

Epididymal and ejaculated cat spermatozoa have been compared for their ability to be stored in the liquid (cool, > 0°C) and frozen (liquid nitrogen -196°C) state. Harris et al. (2001) showed that after 23-day storage at 4°C in TEST yolk buffer, epididymal and ejaculated spermatozoa maintained similar motility and membrane integrity parameters. Hermansson and Axner (2007) showed that neither electro-ejaculated nor epididymal spermatozoa were susceptible to cold shock when cooled slowly (0.5°C/minute) or rapidly (3°C/minute) from room temperature (+20°C) to 4°C. Also, they noted that 20% egg yolk in the extender helped maintain sperm motility and acrosomal status but it had a negative effect on maintenance of membrane integrity. Furthermore, ejaculated and epididymal spermatozoa from the same male cats showed no significant difference in survival parameters, both pre-freeze and post-thaw (Tebet et al., 2006).

Feline Sperm Assessments

Motility is generally considered a good indicator of the ability of sperm to fertilize an oocyte (Linford et al., 1976). Morphology is another important characteristic that is widely used for assessing sperm quality and potential fertilizing ability. A common technique for assessing

cat sperm morphology is by counting 500 spermatozoa on a carbol fuchsine-stained smear using light microscopy at a magnification of 1000X (see Axner et al., 1998; 2004a).

An intact acrosome is essential for a sperm to penetrate and fertilize an oocyte, however, damage or loss of the acrosome can occur prematurely due to physical stress or chemical induction. Pope et al. (1991) developed a simple single step acrosomal assay for feline spermatozoa by using 1% fast green, 1% rose bengal and 40% ethyl alcohol in a 0.1M citric acid and 0.2M disodium phosphate buffer. A common method for evaluating Acrosomal status is the use of fluorescent-conjugated lectins called FITC-PNA (fluorescent isothiocyanate conjugated peanut agglutinin) (Silva and Gadella, 2006). Since the acrosome contains hydrolytic enzymes and carbohydrate moieties of glycoproteins, the fluorescently tagged lectin binds specifically to the glycoproteins.

Determining sperm membrane integrity (the percentage of live vs. dead sperm) is another method to assessing spermatozoa. Garner et al. (1994) developed a competitive binding dual-stain using SYBR 14 and propidium iodide (PI). Although PI has a higher affinity for DNA than does SYBR 14, it is also a larger molecule and in contrast to SYBR 14 cannot pass freely through an intact membrane (Garner and Johnson 1995). So, sperm with a viable or intact membrane show a green fluorescence (SYBR 14), while those with a damaged or ruptured membrane will fluoresce red (PI). The combination of SYBR 14 and PI is commercially available as a kit (Live/Dead Sperm Viability Kit, Molecular Probes, Inc., Eugene, OR, USA), and its effectiveness has been shown with spermatozoa from various mammalian species (Garner and Johnson 1995) including the domestic cat (Filliers et al., 2008).

Recently, the Live/Dead Kit was used to determine plasma membrane integrity of domestic cat cryopreserved epididymal spermatozoa (Kashiwazaki et al., 2005). Siemieniuch and Dubiel (2007) showed that SYBR 14/PI could be used in conjunction with flow cytometry for efficient and objective analysis of cat spermatozoa, however, a major disadvantage to the method was that high concentrations of 1×10^6 feline spermatozoa were required. When eosin-nigrosin staining was used to compare and confirm the SYBR14/PI dual staining in domestic cat sperm, Filliers et al. (2008) noted that the results for the two methods were similar, although SYBR 14/PI consistently resulted in lower values. The authors attributed the consistently lower values, to the higher sensitivity of SYBR 14/PI and its ability to detect minor membrane ruptures with a rapid response thus, making it a more accurate indicator of membrane integrity.

Computers can also be used to accurately and objectively assess sperm samples. Computer assisted sperm analysis (CASA) is currently the most objective and accurate way to determine motility characteristics and patterns. Measurements, such as average curvilinear

velocity (VCL), linearity of the cells swim path (LIN), straight line velocity (VSL) and amplitude of lateral head displacement (ALH) are calculated in a matter of seconds measuring thousands of cells simultaneously (Stachecki et al., 1993; Filliers et al., 2008). Stachecki et al. (1993) were the first to report results on sperm motion parameters derived by CASA in the domestic cat. Initially, they used the CASA parameters set for human spermatozoa and slowly adjusted the settings until the CASA system was more specifically calibrated to more accurately measure cat spermatozoa parameters. Filliers et al. (2008) considered that the CASA parameters they described for domestic cat spermatozoa were a suitable reference point for future feline sperm studies.

Siemieniuch and Potocka (2008) proposed that their CASA program more accurately measured motility of feline spermatozoa than did traditional subjective methods. Their parameters were modified from those used for evaluating canine spermatozoa rather than those using the few feline sperm studies done at the time (Siemieniuch and Potocka, 2008).

Epididymal Cat Spermatozoa in Assisted Reproductive Techniques (ART)

Assisted reproductive techniques (ART) such as artificial insemination (Sojka et al., 1970; Tsutsui, 2006; Chatdarong et al., 2007), in vitro fertilization (Harris et al., 2002), embryo transfer (see Kraemer et al., 1979; Pope et al., 1993; Pope, 2000; Gomez et al., 2004), intracytoplasmic sperm injection (Pope et al., 1998; Gomez et al., 2000) and use of sexed sorted semen (Spinaci et al., 2006; Pope et al., 2009) have been applied to the domestic cat. Most of these techniques have been used with varying success using domestic cat epididymal spermatozoa.

Bowen (1977) demonstrated that spermatozoa collected from the vas deferens were capable of fertilizing feline oocytes in vitro and that resulting embryos could develop to the blastocyst stage in vitro. Niwa et al. (1985) showed that fresh epididymal spermatozoa were capable of penetrating cat oocytes. They reported the presence of an enlarged sperm head with attached tail, appearance of the first polar body and activated chromosomes as soon as 30 minutes after IVF.

Goodrowe and Hay (1993) reported that cat epididymides could be held at 5°C overnight without affecting sperm progressive motility or number of normal spermatozoa when compared with spermatozoa recovered from fresh epididymides. Furthermore, fertilization of zona-free hamster ova and attachment to the zonae of feline oocytes were higher using cooled sperm as compared with fresh spermatozoa. Lengwinat and Blottner (1994) found that the cleavage rate of cat oocytes after IVF with cryopreserved epididymal spermatozoa (25%, 43/170) to be lower than that obtained with fresh spermatozoa.

Cat epididymal spermatozoa have also been used for intracytoplasmic sperm injection (ICSI) (Gomez et al., 2000). Bogliolo et al. (2001) reported that 7 of 129 in vitro matured oocytes developed to the blastocyst stage when the oocytes were injected with cat epididymal spermatozoa. More recently, after ICSI, there was no significant difference in cleavage rates after using spermatozoa held for 72 hours at 4°C in the epididymis or in sperm TALP medium when compared with the control group using fresh spermatozoa (Ringleb et al., 2011).

Since seven of 36 feline species are endangered or critically endangered, the development of optimal protocols for assisted reproductive techniques, such as sperm cryopreservation, is an important priority. Due to the similarity in the genome of all felids, the domestic cat is an excellent model for developing these assisted reproductive techniques. Using a combination of assisted reproductive techniques and gene banking, cryopreserved cat spermatozoa could help insure the long-term survival of all species of cats.

CHAPTER III
GENERAL EXPERIMENTAL PROCEDURES FOR ALL EXPERIMENTS

Tissue Collection and Transport

Testes and ovaries from the domestic cat (*Felis catus*) were collected from routine cat neuters/spays performed at local veterinary clinics in New Orleans, Louisiana. Tissues were collected year round and were used randomly across seven different experiments. Immediately after removal, the reproductive tissue (testes with epididymides and vas deferens and ovaries with oviducts and ovarian bursa) were placed in a 150 mL sterile vial containing ~25 mL of HEPES-buffered saline solution (UltraSaline A Solution, Bio-Whittaker, Walkersville, MD) with 50 µg/mL of Gentamicin,(Sigma #G1272) at ambient temperature (~27°C). Reproductive tissue was transported within 120 minutes to the Audubon Center for Research of Endangered Species (ACRES) laboratory in New Orleans, Louisiana. Tissues (epididymides, vas deferens and ovaries) were trimmed and washed in HEPES-199 medium before gametes were harvested.

Sperm Collection and Designating Treatments

After removal from the testes, epididymides and vas deferens were rinsed, and further processed in HEPES-199 medium (at ~22°C). The vas deferens was dissected away from the epididymis so that it was a straight duct, open at both ends. Each vas deferens was then squeezed and milked in a manner that removed the spermatozoa (sperm) from the lumen. The epididymides were sliced repeatedly and minced and incubated in HEPES-199 medium for 30 minutes to allow sperm to migrate from the epididymal ducts. Before being assigned to a treatment, the sperm suspension (containing HEPES-199 medium, minced tissue and sperm) was filtered through a 40 µm cell strainer (Falcon® 40 µm Cell Strainer, Corning, Inc., Corning, NY, USA) into a 50 mL plastic conical centrifuge tube. The sperm suspension was then layered gently over two-layer (45% upper/90% lower) density gradient column (#99264, Isolate®, Irving Scientific, Santa Ana, CA, USA), that was prepared in a 15 mL conical centrifuge tube, and centrifuged at 650 × g for 20 minutes. After centrifugation, the sperm pellet was removed and placed into a 1.6 mL conical aliquot tube (Thomas Scientific #2591K99). Before treatment, an initial assessment of sperm motility, membrane integrity and acrosomal status was recorded (see below for details).

Following the initial assessment, sperm samples were divided into one of three treatments in each experiment. Each of the three fractions of the sperm pellet was placed into a 1.6 mL aliquot tube and a calculated volume of extender without glycerol, was added to each fraction of the sperm pellet. The desired sperm concentration per straw, determined the total

volume of extender to be added to each sperm fraction. After each tube was extended to the desired concentration, they were placed in a ~200 mL room temperature water bath (22°C) and gradually (~3 h) cooled to 4°C±1°C in a walk-in cooler. After cooling (4°C/post-cooling reading), the three treatments were again assessed for sperm motility, membrane integrity and Acrosomal status. Following the post-cool (4°C) sperm assessment, extenders with glycerol, were added to treatments. Samples were diluted 1:1 with extender containing 12% glycerol (Part B) in four steps: 15%, 20%, 25% and 40% of the total that was added to the mixture (modified from Gao et al. 1995) there was approximately 10 minutes between the four dilution steps.

Sperm Analyses

To assess sperm motility 1 µl of the sperm pellet was diluted in 20 µl of HEPES-199 medium. Then, a 10 µl aliquot was taken and placed on a warm pre-cleaned glass microscope slide, covered with a warm 18 x 18 mm glass cover slip and placed on a heated microscope stage (37°C). Motility was determined by calculating the average total sperm motility in three separate fields on each slide. In Experiments 6.1, 6.2, 7.1, 7.2 and 7.3, sperm motility was determined using 1 µl of raw sperm pellet diluted in 20 µl of HEPES-199 medium. Three micoliters of this 1:20 mixture was then placed on a warmed Leja® 4 chambered slide and placed on the heated microscope stage (37°C) of an Olympus CX41 microscope (Olympus America, Inc. Center Valley, PA, USA) equipped with a Hamilton Thorne CASA (computer assisted sperm analysis) Helos™ system and used with the Ceros software (Hamilton Thorne Research, Beverly, MA, USA). The motility of these samples was determined using the CASA system, in which three fields on each slide were digitally analyze.

To determin membrane integrity a LIVE/DEAD viability kit (Molecular Probes, Inc., Eugene, OR, USA) was used. To view cell staining, a light microscope equipped with ultraviolet florescence and a flourescien isothiocyanate filter was used. The LIVE/DEAD kit incorporated two stains, SYBR 14, which stains the DNA of membrane intact cells, green (viable/live), and Propidium Iodide (PI), which stains the DNA of the membrane ruptured cells, red (nonviable/dead). An aliquot of ≤ 1 µl of sperm was diluted with 4 µl of SYBR 14 and 5 µl of PI. Also 5 µl of formal citrate was added to immobilize sperm without damaging intact membranes (Dott and Foster, 1975).

Samples were protected from light and allowed to incubate with stain for 5 minutes before evaluation. Determining the percentage of membrane intact (viable) sperm was accomplished by randomly counting ≥ 200 sperm using fluorescent luminescence (40X). The

number of sperm with green fluorescence was divided by the total count of ≥ 200 sperm to determine the percentage of live sperm.

Acrosomal status was evaluated using FITC-PNA (see Silva and Gadella, 2006) staining (1 mg of lectin, FITC from peanut in 2 mL of HEPES-199 medium). The stock solution was then aliquoted into 0.6 mL tubes (30 uL/tube) and frozen at -80°C until use. An aliquot of ≤ 1uL sperm sample was diluted in 5 uL of FITC-PNA stain along with 5 µl of formal citrate to immobilize sperm. The same fluorescent microscope to evaluate membrane integrity was again used to determine acrosomal status. A total of ≥ 200 sperm were randomly counted and the percentage of nonreacted sperm was divided by the total number counted to calculate the percentage of intact acrosomes.

Sperm concentration was determined either by using a spectrophotometer designed specifically for measuring sperm concentration (1:5 dilution; SpermaCue, Minitube of America, Verona, WI, USA) or by using a standard hemocytometer (Bright-Line, Hausser Scientific , Horsham, PA, USA; 1:1,000 dilution; Experiments 6.1,6.2, 7.1, 7.2, and 7.3). Samples were adjusted to the desired concentration by adding appropriate amounts of the extenders with and without glycerol.

Cryopreservation and Thawing of Sperm Treatments

After loading, semen straws (0.25 or 0.5 mL) were sealed with an ultrasound sealing unit (UltraSeal 21, Minitube of America, Verona, WI, USA) and placed for 20 minutes on a block of dry ice (-78.5°C) in a small Styrofoam box or in a -80°C freezer. After placing straws on the dry ice block, the lid was placed on the box. After 20 minutes, straws were plunged into a 13 mm goblet/cane that was submerged in liquid nitrogen. The cane with straws was then held in a 35 liter liquid nitrogen storage tank for up to 3 months before thawing.

Straws were removed from liquid nitrogen and exposed to air (~22°C) for 5 (0.25 mL) or 10 (0.5 mL) seconds and then immediately immersed in a 60°C water bath for 5 seconds. Once removed from the water bath, contents of straws were emptied into a pre-warmed (37°C) 1.6 mL aliquot tube. Glycerol was removed from the sperm using a seven step dilution of HEPES-199 medium with pentoxifylline, there was approximately 10 minutes between each step to avoid osmotic shock to the sperm. The volumes of the first four dilution steps of HEPES-199 + pentoxifylline were 15, 20, 25 and 40% of the thawed sample. The volumes of the last three dilution steps were 33, 66, and 200% of the sample after the 40% addition from the fourth step. The seven step solution was then centrifuged at 200 x g for 10 minutes, and the resulting sperm pellet was removed and resuspended in 20 µl of HEPES-199 medium and the 1.6 mL tube containing the sample was placed in a 37°C water bath and incubated at 37°C for 3 hours.

Motility, membrane integrity and acrosomal status were evaluated after 0 and 3 hours incubation, as described previously for the initial analysis. For IVF, sperm straws were thawed as described above; then after centrifugation 3 to 6 µL of sperm pellet was diluted with 35 µl of HEPES Tyrode's balanced salt solution.

In Vitro Production Medium (IVF/IVC)

The three balanced salt solutions (BSS) used to prepare the IVF/IVC media were Tyrode's balanced salt solution, TCM-199 medium (with $NaHCO_3$ but without glutamine) and TCM-199 medium (without glutamine or $NaHCO_3$). Additional supplements were added to each BSS to create in vitro maturation (IVM), in vitro fertilization (IVF), in vitro culture-I (IVC-I), in vitro culture-IA (IVC-IA) and in vitro culture-II (IVC-II) medium. Tyrode's balanced salt solution (Irving Scientific, Santa Ana, CA #9298) was the base solution for IVF and IVC media. Tyrode's BSS was also used to prepare HEPES-buffered medium, used for diluting and maintaining sperm used in IVF. TCM-199 medium with $NaHCO_3$ but without glutamine (Sigma #9120) was the base medium used for IVM of oocytes. TCM-199 medium without $NaHCO_3$ or glutamine (Sigma #3769) was the base for the HEPES-buffered holding medium used for maintaining oocytes and embryos during handling in atmospheric air outside of a CO_2 incubator.

The 100X supplement added to TCM-199 for IVM medium (see Appendix III) was prepared by adding 0.292 g glutamine, 0.200 g cysteine, 0.040 g sodium pyruvate, 0.242 g calcium lactate and 0.050 g gentamicin to 10 mL of Tyrode's BSS. The 100X solution was filter sterilized and aliquoted (100 uL) into sterile 1.6 mL tubes for storage at -80°C until use.

Also, equine chorionic gonadotropin (eCG; Calbiochem, San Diego, CA #36722) and human chorionic gonadotropin (hCG; Pregnyl Organon, Inc., West Orange NJ) were used in the IVM medium.

The gonadotropins arrive, in powder form, were diluted in sterile Tyrode's BSS, aliquoted into sterile 1.6 mL tubes and stored at -80°C until use. They were diluted to a 100X final concentration (hCG = 100 IU/mL; eCG = 50 IU/mL). Also Epidermal Growth Factor (EGF, Sigma #9644) was added to the IVM medium. The EGF, in powder form, was diluted with Tyrode's BSS to 1,000 ng/mL (100X final concentration), filtered into 1.6 mL sterile tubes and stored at -80°C until use. Finally bovine serum albumin (BSA; Fraction-V #82047, Serological Proteins, Inc., Kankakee, IL, USA) was added to the IVM medium (0.3g/100 mL).

The 100X supplement for IVF and IVC media was prepared by adding 0.146 g of glutamine, 0.040 g of sodium pyruvate, 0.242 g of calcium lactate, and 0.050 g of gentamicin to 10 mL of Tyrode's solution. Then, the supplement was filter sterilized, aliquoted into 1.6 mL tubes and stored at -80°C until use. The IVF and IVC-I media contained 0.6 g and 0.3 g/100 mL

BSA (Fraction-V), respectively. The MEM Nonessential amino acids solution (100X; Sigma#M7145) was added to the IVC-I, IVC-IA and IVC-II media to give a final concentration of 1%. Upon arrival the NEAA solution was aliquoted into 1.6 mL tubes and store at -80°C. Finally, the IVC-IA and IVC-II media contained MEM essential amino acids (50X; EAAs) and either BSA (IVC-IA) or fetal bovine serium (IVC-II: FBS #30070; Hyclone, Inc., Logan, UT, USA). Upon arrival the NEAA and the EAAs were aliquoted into 1.6 mL sterile tubes (1 mL/tube) and stored in -80°C freezer. The IVC-II medium contained 10% FBS instead of BSA. Upon arrival FBS was thawed, aliquoted into 15 mL tubes (4.5 mL/tube) and stored at -80°C until use. After thawing, before addition to IVC-II medium, FBS was heat-treated at 56°C for 30 minutes.

Aliquotes of frozen supplements were thawed weekly and added to the Tyrode's BSS, TCM-199 media. Each week IVM/IVC media were adjusted to 285 to 295 mOsm and 7.7 to 7.8, respectively. After sterilization by filtration (0.22 µ) into 15 mL sterile centrifuge tubes (5 mL/tube) each tube was gassed with 5% CO_2 in air (or 5% CO_2, 5% O_2, 90% N_2) for 30-40 sec before sealing. Then, tubes were placed in a tilt rack (~30°) angle and stored at 4°C. until use within 7 days.

Oocyte Collection and Maturation

Upon arrival at the laboratory (see Tissue Collection and Transport), ovaries were trimmed of excess tissue, rinsed once in Phosphate-buffered saline, rinsed three times in TL-HEPES and minced in HEPES-199 medium (He199). Cumulus-oocyte complexes were washed three times in HEPES-199. Cumulus-oocyte complexes (COC) with uniformly dark, finely granulated ooplasm surrounded by several layers of cumulus cells and COC with less darkly pigmented, not uniformly and densely granulated ooplasm surrounded by several layers of cumulus cells were rinsed three times in Hi-199 and placed in fresh He-199 medium at 35°C to 38°C. Oocyte recovery, washing, and grading were performed under a laminar flow hood using a stereomicroscope with a heated stage (38°C).

For in vitro maturation (IVM), COC were cultured for 24 hours, in a 4-well culture dish (Nunclon, #176740 Nunc, Denmark) containing 500 µl of Modified TCM-199 medium (IVM medium, Appendix III). The 4-well dish containing the maturing oocytes along with a 60 x 15 mm Petri dish containing 7 mL of sterile water, were placed on an inverted lid from a micro titer plate. Several small holes were drilled in the lid of 60 mm Petri dish. This arrangement of dishes was then placed inside a 16.5 x 20.3 cm plastic bag (Kapak SealPAK pouch, #402, Kapak Corporation, Minneapolis, MN) and filled with 5% O_2, 5% CO_2 and 90% N_2. During the

filling/sealing process the bag was placed on a heating block (38°C). The inflated bag and its contents were placed in a 38°C incubator (Forma Scientific, Model 3130, Marietta, OH).

In Vitro Fertilization and Culture

After 24 hours IVM culture, COC were rinsed in He199 and placed in 40 µL droplets of IVF medium under 3 mL of pre-equilibrated sterile mineral oil (ART -4008-5; Copper surgical, Turnbull, CT USA) in a 35 mm sterile petri dish (#1008, Falcon). The desired concentration of motile sperm was ~1 million sperm/mL. IVF droplets containing oocytes, sperm and surrounded by mineral oil were then placed into a 38°C incubator with 5% CO_2 in air.

At 4-6 hours post-insemination, oocytes were rinsed four times in He199 and then transferred into 500 µl droplets of modified Tyrode's containing nonessential amino acids and BSA (IVC-I) in a four-well dish. The dish was placed in the closed bag system as described for IVM culture and cultured at 38°C. On Day 2, the number of embryos per well was recorded, uncleaved oocytes removed, and embryos were placed into a fresh IVC-I medium containing 2% essential amino acids (IVC-IA). On Day 5, embryos were placed into fresh IVC-I medium containing 2% essential amino acids in which BSA was replaced with 10% FBS (IVC-II medium). Presumptive zygotes and embryos were cultured in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at at 38°C. On Day 8, embryos were evaluated visually to determine the rate of development to the blastocyst state (% blastocysts/embryos X100).

Statistical Analyses

Sigma Stat Version 3.0 was used to analyze the data set of each parameter. Student t-test was the statistical test used to compare the initial, pre-freeze, and 0 and 3 h post-thaw values for sperm motility, membrane integrity and acrosomal status. The statistical analysis compared treatments within a given time point as well as that of individual treatments across times. These parameters (total sperm motility, membrane integrity and acrosomal status) are reported as mean±SEM/treatment group. A P value <0.05 was used to determine significance differences.

CHAPTER IV
SURVIVAL AND *IN VITRO* FUNCTIONALITY OF DOMESTIC CAT EPIDIDYMAL SPERMATOZOA FOLLOWING CRYOPRESERVATION IN EXTENDERS WITH OR WITHOUT EGG YOLK

Introduction

Currently, animal by-products, such as milk and egg yolk are major components in extenders used to cool store and freeze mammalian semen. These animal by-products have been added to semen extenders since 1856 and their use increased in the early 1900s. Kolliker (1856) reported the use of milk in a bull semen extender, while Milovanov and Selivanova (1932) were the first to report the use of egg yolk lecithin and its beneficial effect on bull spermatozoa (sperm) (see Salisbury and VanDemark 1961). The presence of these animal by-products are potential sources for microbial contamination and transfer of zoonotic agents (Wrathall, 2000). Also, the unknown composition of these animal by-products produces inconsistencies among semen extenders. Furthermore, the presence of egg yolk or milk adds additional challenges when spermatozoa are to be sex sorted with a flow cytometer.

These concerns have prompted researchers to search for synthetic or defined components to replace animal by-products in semen extenders, while maintaining cryoprotective properties. Soy lecithin has been investigated as a potential alternative to egg yolk and milk-based semen extenders (Gil et al., 2003; Aires et al., 2003; Vick et al., 2011). Vick et al. (2012) reported that cat sperm frozen in soy lecithin-based extender had higher percentage of motile sperm at 3, 6 and 24 hours post-thaw, than did sperm frozen in egg yolk-based extender. In addition, soy lecithin treatment had a lower percentage of spermatozoa that had undergone capacitation when compared with the sperm frozen with egg yolk.

Bovine serum albumin (BSA) is another animal protein that can be sterilized, monitored for consistency/quality and purchased commercially. Harrison et al. (1978) found that BSA helped maintain sperm motility in washed boar, rabbit, ram and stallion semen after 1, 3, and 5 hours incubation at 30°C. The primary benefit of BSA supplementation was a reduction in the rate of decline in sperm motility. Also, in a more recent study, Xu et al. (2009) showed that the presence of BSA in goat semen extender was beneficial to sperm motility, membrane integrity, acrosomal status and capacitation in buck semen held at 5°C up to 7 days of storage.

When equine semen was incubated at 38°C in a sucrose medium (with or without BSA) percentages of motile sperm and that of those in forward movement were higher in 1% or 3% BSA as compared with the control (0% BSA) (Klem et al., 1986). Furthermore, Nang et al. (2012) reported that 1 mg/mL BSA significantly improved bull sperm viability during storage ≤ 7 days at 4°C.

Matsuoka et al. (2006) concluded, based on post-thaw sperm motility and viability, that 10% and 15% BSA were suitable substitutes for egg yolk for freezing ram semen. Also, Fukui et al. (2007), from the same laboratory, reported that after AI of synchronized ewes with ram semen frozen in BSA extender, 60-day pregnancy rates and lambing rates were equivalent (not significantly different) to those of semen frozen in an egg yolk extender.

Similarly, Sariozkan et al. (2009) reported that bull semen was frozen in the BioXCell® extender and supplemented with either oxidized glutathione, reduced glutathione or BSA. Pregnancy rate was higher in the BSA group (72%) than that of the other two treatments (68.4 and 53.2%), and the control (BioXCell® with no antioxidants; 54.2%) but was only significantly higher than the oxidized glutathione and control treatments.

There are many studies on evaluation of components to replace milk and/or egg yolk-based semen extenders that produce acceptable sperm motility, membrane integrity and acrosomal status values. However, few have investigated the possible benefits of BSA as a replacement for egg yolk in the freezing of feline epididymal spermatozoa. In the present study, we compared a commercially available egg yolk-based extender, that has been used previously for freezing feline spermatozoa with two BSA-based extenders. We evaluated sperm post-thaw motility, membrane integrity and acrosomal status. In addition we compared the ability of sperm frozen in egg yolk extender vs. BSA extender to fertilize oocytes in vitro as well as subsequent developmental competence in vitro of resultant embryos.

Materials and Methods

Experimental Design

Experiment 4.1

A total of 55 sets of domestic cat (*Felis silvestris catus*) testes were collected on 9 days during February through April. Testes were processed and epididymal spermatozoa (sperm) were analyzed and cryopreserved on the day of harvest. From 3 to 15 sets of testes were processed/day (Table 4.1). Sperm collected each day were pooled and divided across three treatment groups (extenders). Treatment A, a commercially available extender, TesT egg yolk buffer (TesT) (Refrigeration Medium #9972, Irving Scientific, Santa Ana, CA). TesT consists of: Tes (2-[(2-Hydroxy-1,1-bis(hydroxymethyl)ethyl)(amino)ethanesulfonic acid), Tris (Tris(hydroxymethyl)amino methane), dextrose and 20% egg yolk.

Treatment B (Human Sperm Preservation Medium; HSPM) was prepared in the laboratory using the method as described by Mahadevan and Trounson (1983). The reagents were added at 28°C as follows: sodium dihydrogen phosphate, calcium chloride, potassium

chloride, magnesium chloride, sodium hydrogen carbonate, sodium chloride, sodium lactate, HEPES ($C_8H_{18}N_2O_4S$), sucrose, glycine, glucose and bovine serium albumin (BSA).

In Treatment C, Tris citrate BSA (TCBSA), was prepared in the laboratory. This extender consists of Tris, citric acid, fructose, glucose, BSA and trehalose.

After each sample was pooled, washed and centrifuged, a pre-cool initial analysis was done. Then the sperm sample was divided into three 1.6 mL aliquot tubes containing one of the three extenders (Treatments A, B and C). Each sperm aliquot (sample) was then placed in separate 125 mL plastic bottles filled with 22°C water and placed into a 4°C walk-in cooler. After gradual cooling to 4°C±1°C, sperm in each treatment underwent a second analysis (post-cool to 4°C). Then extender with glycerol was added to each tube and the samples were loaded into 0.25 mL plastic straws and frozen on a block of dry ice.

To further asses the viability of the cryopreserved epididymal sperm, additional straws from each of the three treatments were thawed and the sperm were incorporated into an in vitro fertilization IVF protocol to produce of feline blastocysts. Cumulus oocyte complexes (COC) from domestic cats were placed in modified TCM-199 (Irving Scientific, Santa Ana, CA) and cultured for 24 hours in in vitro maturation medium (IVM) (see Appendix III) in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2 at 38°C (IVM). For IVF, COC were co-incubated with sperm frozen in either the TesT or HSPM extenders (1 million sperm/mL), under 5% CO_2 in air at 38°C. After 4-6 hours, oocytes were rinsed and cultured using a 3-step system (Pope et al., 2006). On day 2 uncleaved oocytes were removed and number of embryos was determined. On IVC day 8 the number of embryos developing into blastocysts was assessed.

Experimental Procedures

Basic Procedures

The standard procedures used for Experiment 4.1 were as follows: ovarian and testes tissue collection and transport, sperm collection and treatment designation, sperm analysis, cryopreservation and thawing of sperm, in vitro embryo production medium, oocyte collection and in vitro maturation, in vitro fertilization and culture, statistical analyses. Details of the standard procedures are described in Chapter 3.

Semen Extender Preparation (Appendix I and II)

TesT Yolk Buffer was purchased commercially (Irving Scientific, Santa Ana, CA) and shipped frozen on dry ice and arrived in two boxes each containing 20 x 5 mL vials. One box contained TesT Yolk Buffer vials without glycerol (Refrigeration Medium/Part A). The second box contained vials of TesT Yolk Buffer with 12% glycerol (Freezing Medium/Part B). The

reagents and specific molarities for the two extenders prepared in the laboratory are listed in Appendix I. The suppliers and product numbers for each reagent are listed in Appendix II.

Extenders were prepared using purified, Type I water and checked for desired osmolarity (310 to 330 osmoles) and a pH of 7.0 to 7.5. After adjusting to the desired osmolarity and pH, each extender was split equally into two tubes. One of the halves (Part B) received a specific amount of glycerol to make a final concentration of 12% glycerol. Both Part A and Part B for all extenders (including TesT Yolk Buffer), were aliquoted into 1.6 mL aliquot tubes and frozen at -80°C until use.

Sperm Collection and Designating Treatments

Once removed from the testes epididymides and vas deferens were rinsed, and minced in HEPES-199 medium at laboratory temperature (~22°C) with a number 12 scalpel blade. Before being placed into treatments, the suspension of minced tissue and sperm was filtered through a 40 µm cell strainer (BD Biosciences Discovery Labware, Franklin Lakes, NJ) into a 50 mL conical centrifuge tube. The filtered sperm suspension was then gently layered onto a 45/90 density gradient column (Isolate®, Irving Scientific, Santa Ana, CA), in a 15 mL conical centrifuge tube, and centrifuged at 600 × g for 20 minutes. After centrifugation, the sperm pellet was removed and placed into a 1.6 mL conical aliquot tube. Before dividing the daily sperm pellet into the designated extenders, an initial assessment of sperm motility, membrane integrity and acrosomal status was recorded (see below for specific analyses).

Following the initial assessment, sperm samples were divided into three treatments (extenders) in 1.6 mL aliquot tubes and a calculated volume of the Part A (no glycerol) extender was added. The desired sperm concentration/straw, determined the volume of extender added to the pellet. After adding the volume of extender needed to produce the correct ratio of "sperm/extender" the 3 aliquot tubes were placed in a 125 mL plastic bottle filled with ~22°C water and placed in a 4°C walk-in refrigerator and allowed to cool down to 5°C±1°C over a period of ~3 hours. After cooling to 4°C, motility, membrane integrity and acrosomal status were evaluated for each treatment. Following the pre-freeze (4°C) sperm assessment, Part B (with glycerol) of the extenders was added to their respective treatments in four steps (15%, 20%, 25% and 40% of initial volume) at intervals of 20 to 30 seconds and 10 minutes between steps modified the fixed molarity methods by Gao et al. (1995).

Results

Experiment 4.1

Testes Collection, Epididymal Sperm Concentration and Straws Frozen

Over a period of three months, 3 to 15 sets of testes were processed from 9 different collection days (n = 9) for a total of 52 usable sets of testes. Routinely, 3 to 5 sets were processed in a day; however, there was one day with 9 usable sets and another day with 15 usable sets harvested.

The average total number of pooled sperm collected each day was 113 x 10^6, with a range from 43 x 10^6 to 221 x 10^6. Total daily number of sperm was divided evenly across the three treatments and depending on the total sperm count, a range of 1 to 6 (0.25 mL) straws were frozen/treatment/day. On two days, the total number of sperm collected resulted in the freezing of extra 0.25 mL straws. These extra straws allowed for two thaw replicates for these two days (see table 4.1).

Motility

The mean (±SEM) initial motility for the pooled sperm samples was 67.7±4.2% (Control). The mean post-cool (4°C) values for sperm frozen in HSPM (Treatment A), TCBSA (Treatment B) and TesT (Treatment C) were:, 53.2±4.9%, 54.1±6.2%, and 66.8±4.6%, respectively. Sperm motility at 0 hour post-thaw in HSPM, TCBSA and TesT treatments were: 42.3±4.8%, 40.5±4.5%, and 53.2±7.1%, respectively. After 3 hour post-thaw incubation, motility in HSPM, TCBSA and TesT was 20.4±6.2%, 25.0±6.2% and 31.8±5.0% respectively (Table 4.2).

Motility after cooling was not different from initial motility in TesT, and TCBSA samples, but motility was lower in the HSPM group ($P \leq 0.05$). After thawing, motility at 0 hour was reduced in the non-egg yolk treatment groups (HSPM and TCBSA) ($P \leq 0.05$), but not in the TesT group, as compared to initial motility. At 3 hour after thawing motility in all three groups was lower than initial pre-freeze motility ($P \leq 0.05$) (Table 4.2).

Motility at 0 hour after thawing was not different from that recorded after cooling in any of the treatment groups. In the HSPM and TesT groups, motility at 3 hour after thawing was lower than at post-cooling and at 0 hour post-thawing ($P \leq 0.05$). In the TCBSA group, motility at 3 hour after thawing was lower than after cooling ($P \leq 0.05$), but there was no difference in motility at 0 hour versus 3 hour post-thawing (Figure 4.1).

After cooling, motility in the TesT group was higher than in the HSPM group; but, motility in the TCBSA group was not different from that of the other two groups. After thawing, there were no differences in motility among the three groups either at 0 hour or 3 hour (Figure 4.2).

Table 4.1. Summary of the numbers of sets of testes processed, number of sperm recovered and number of straws frozen on each of nine collection days.

Thaw date	Collection date	Sets processed	Total no.	No. of straws per extender/per day
3/29/2008	3/24/2008	5	46×10^6	1.5
3/30/2008	3-19-08 Rep 2	3	221×10^6	6
4/7/2008	4/2/2008	9	70×10^6	2
5/31/2008	3/31/2008	3	53×10^6	2
6/1/2008	3-19-08 Rep 1	4	221×10^6	6
6/3/2008	3-4-08 Rep1	3	128×10^6	4
6/7/2008	3-4-08 Rep 2	3	128×10^6	4
6/7/2008	2/27/2008	?	43×10^6	1
6/8/2008	4/8/2008	4	92×10^6	2
6/8/2008	4/9/2008	3	143×10^6	4
6/8/2008	4/13/2008	15	103×10^6	3
Daily average (no.)		5.2	113×10^6	3.2
Total no.		52	-	35.5

The last two rows show the mean values for all the collection days and the overall total values for the experiment.

Table 4.2. Effect of egg yolk (TesT) versus BSA-based (HSPM and TCBSA) extenders on cat epididymal sperm motility before (pre- and post-cool) and after (0 h and 3 h) freezing.

	Initial (Control)	Post-cool	0 hour Post-thaw	3 hour Post-thaw
HSPM (Trt A)	67.7 ± 4.3^a	53.2 ± 4.9^b	42.3 ± 4.8^b	20.4 ± 6.2^b
TCBSA (Trt B)	67.7 ± 4.3^a	54.1 ± 6.2^a	40.5 ± 4.5^b	25.0 ± 6.2^b
TesT (Trt C)	67.7 ± 4.3^a	66.8 ± 4.6^a	53.2 ± 7.1^a	31.8 ± 5.0^b

Trt = Treatment; ^{a,b}Superscripts only designate a significant difference between initial motility and the value for each extender at each time period ($P<0.05$).

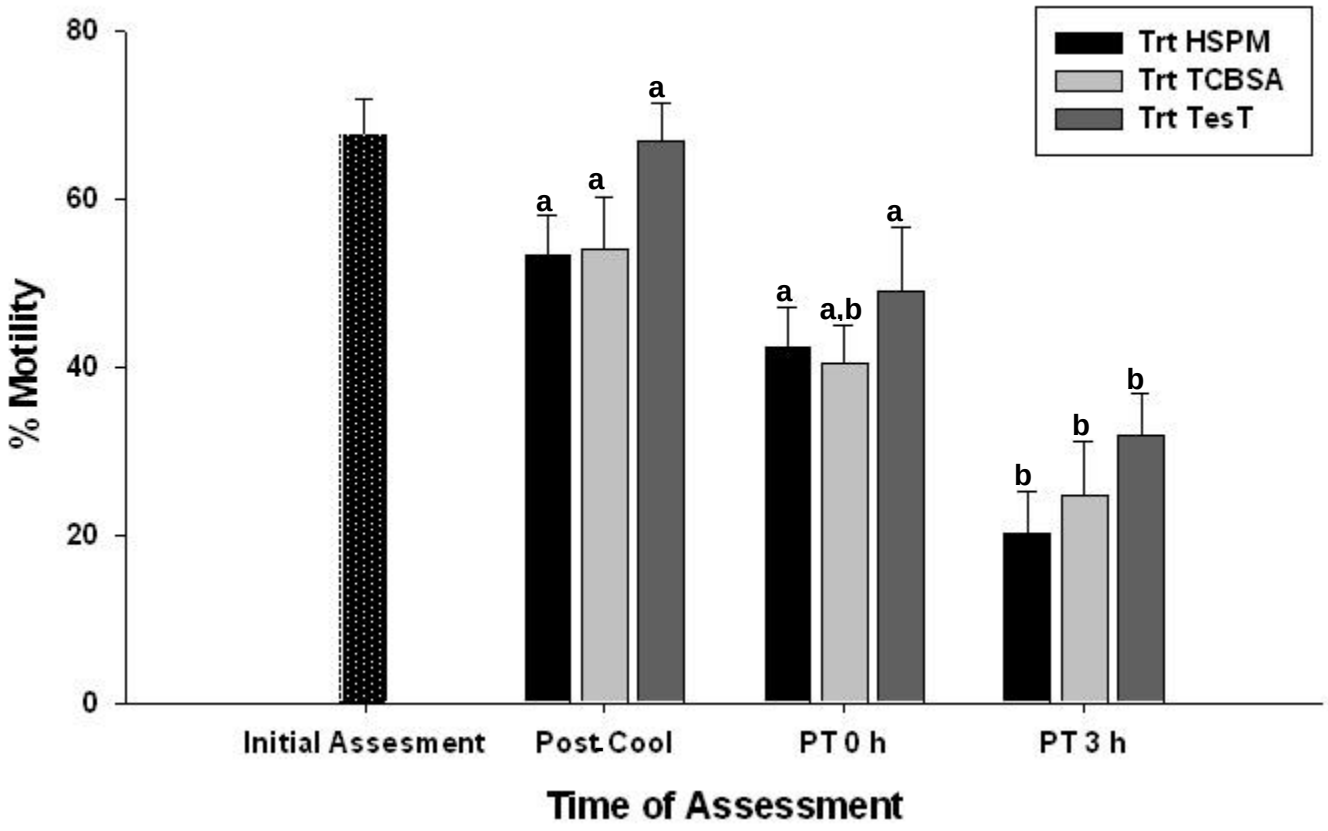

Figure 4.1. Effect of egg yolk (TesT) versus BSA-based (HSPM and TCBSA) extenders on cat epididymal sperm motility before (pre- and post-cool) and after (0 h and 3 h) freezing. [a,b]Mean values within treatments but across time intervals with different superscripts are significantly different (P<0.05).

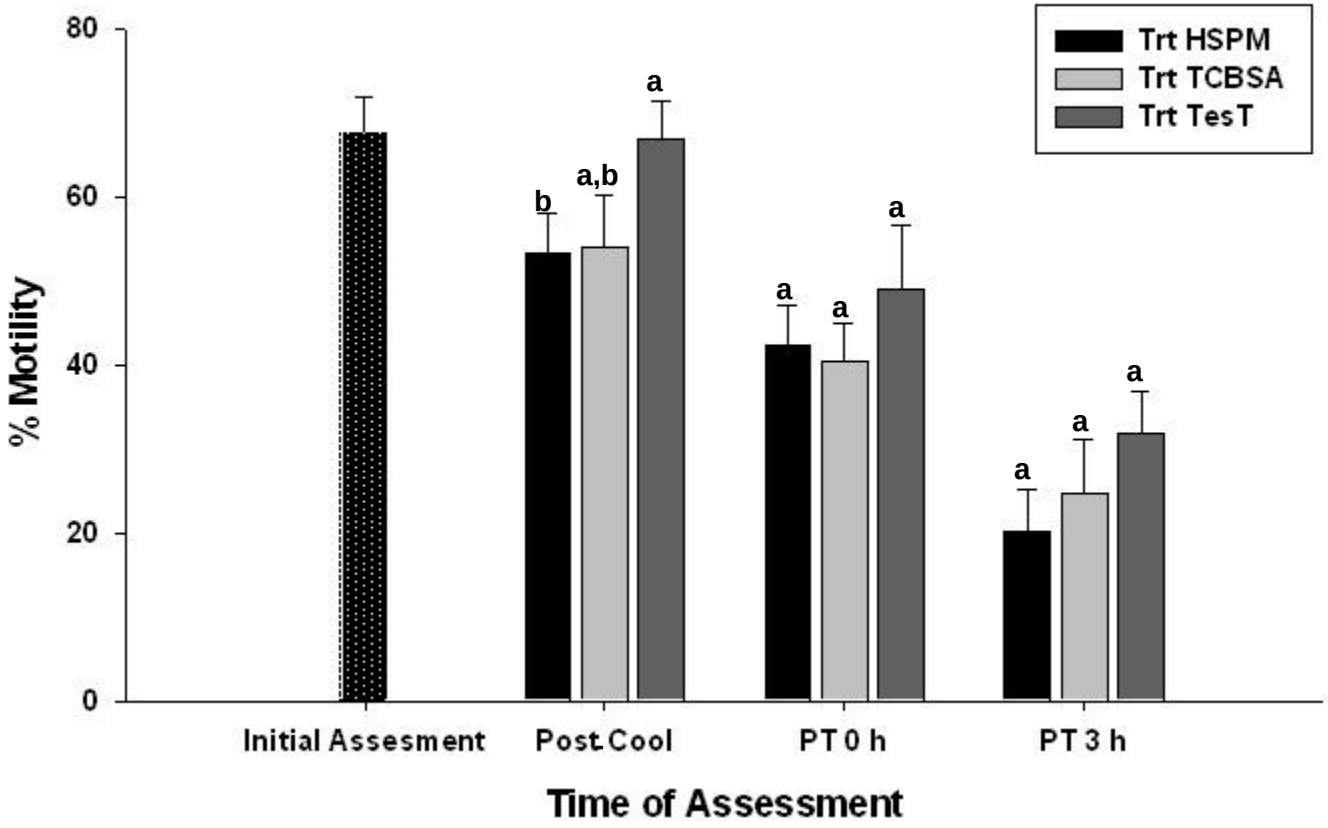

Figure 4.2. Effect of egg yolk (TesT) versus BSA-based (HSPM and TCBSA) extenders on cat epididymal sperm motility before (pre- and post-cool) and after (0 h and 3 h) freezing. [a,b]Mean values across treatments but within time intervals with different superscripts are significantly different (P<0.05).

Membrane Integrity

The mean initial percentage of sperm with intact membranes was 75.3±2.8%. After cooling to 4°C the percentages of sperm with intact membranes were: 72.0±2.7%, 71.4±2.6% and 76.8±3.0% for the HSPM, TCBSA and TesT treatments, respectively. At 0 hour and 3 hour after thawing, membrane integrity percentages were 58.8±5.3% and 52.7±2.7%, 53.6±2.8% and 58.2±3.0%, and 58.4±2.4% and 51.2±2.3% in the HSPM, TCBSA and TesT treatments, respectively. In all three treatments, the initial percentage of sperm with intact membranes was higher than the percentages seen at 0 hour and 3 hour after thawing ($P<0.05$), but not after cooling to 4°C (Table 4.3).

There was no difference between initial membrane integrity and after cooling to 4°C. However, the initial membrane integrity was significantly higher than at 0 hour and 3 hour post-thawing in all treatments.

Within treatments and across time periods, membrane integrity in the non-egg yolk extenders (HSPM and TCBSA) was higher at post-cool than that seen after thawing (0 hour and 3 hour). However, comparing between 0 hour and 3 hour after thawing, sperm membrane integrity was not different in either of the non-egg yolk extenders. In contrast, membrane integrity in the egg yolk extender (TesT), before cooling was higher than after thawing, both at 0 hour and 3 hour. Also, membrane integrity in the TesT treatment was higher at 0 hour after thawing that at 3 hour (Figure 4.3).

Across the three treatments there was no differences in membrane integrity at the three time periods--after cooling and at 0 hour and 3 hour after thawing (Figure 4.4).

Acrosomal Status

Initially 76.6±3.6% of epididymal sperm had intact acrosomes. After cooling the percentage of sperm with intact acrosomes in HSPM, TCBSA and TesT extenders was 74.6±5.2%, 72.0±4.0% and 76.5±2.6%, respectively. At 0 hour after thawing 58.8±2.4%, 63.2±2.7% and 56.2±2.8%, of sperm in HSPM, TCBSA and TesT, respectively had intact acrosomes. At 3 hour after thawing, 55.4±3.6%, 55.0±3.2% and 51.6±2.0% of sperm in the HSPM, TCBSA and TesT, respectively had intact acrosomes. These results are shown in Figure 4.5.

There was no difference between the pre and post-cool percentage of sperm with intact acrosomes in any of the treatments. However, there was a higher percentage of sperm that had intact acrosomes before cooling than after thawing in all treatments (0 h and 3 h after thawing), (Table 4.4).

Table 4.3. Effect of egg yolk (TesT) versus BSA-based (HSPM and TCBSA) extenders on cat epididymal sperm membrane integrity before (pre- and post-cool) and after (0 h and 3 h) freezing.

	Initial (Control)	Post-cool	0 hour post-thaw	3 hour post-thaw
HSPM (Trt A)	75.3±2.8[a]	72.0±2.7[a]	58.8±5.3[b]	52.7±2.6[b]
TCBSA (Trt B)	75.3±2.8[a]	71.4±2.6[a]	53.6±2.8[b]	58.2±3.0[b]
TesT (Trt C)	75.3±2.8[a]	76.8±3.0[a]	58.4±2.4[b]	51.2±2.3[b]

Trt = Treatment; [a,b]Superscripts only designate a significant difference between initial membrane integrity and individual values for each extender at each time period ($P<0.05$).

Table 4.4. Effect of egg yolk (Test) vs. BSA-based (HSPM and TCBSA) extenders on cat epididymal sperm acrosomal status before (pre- and post-cool) and after (0 h and 3 h) freezing

	Initial (Control)	Post-cool	0 hour post-thaw	3 hour post-thaw
HSPM (Trt A)	76.6±3.6[a]	74.6±5.2[a]	58.8±2.4[b]	55.4±3.6[b]
TCBSA (Trt B)	76.6±3.6[a]	72.0±4.0[a]	63.2±2.7[b]	55.0±3.2[b]
TesT (Trt C)	76.6±3.6[a]	76.5±2.6[a]	56.2±2.8[b]	51.6±2.0[b]

Trt = Treatment; [a,b]Superscripts only designate a significant difference between initial membrane integrity and individual values for each extender at each time period ($P<0.05$).

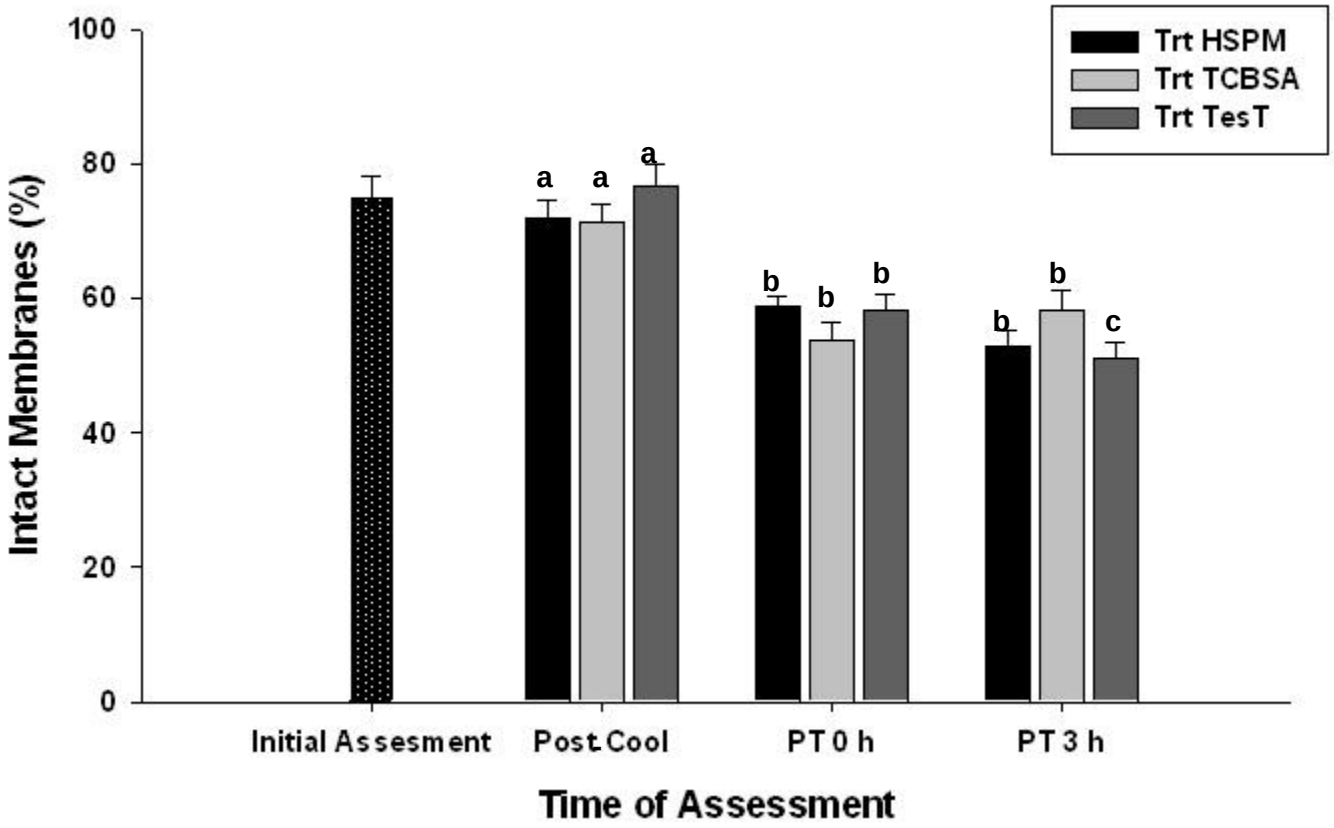

Figure 4.3. Effect of egg yolk (TesT) versus BSA-based (HSPM and TCBSA) extenders on cat epididymal sperm membrane integrity before (pre- and post-cool) and after (0 h and 3 h) freezing. [a,b]Mean values within treatments but across time with different superscripts are significantly different (P<0.05).

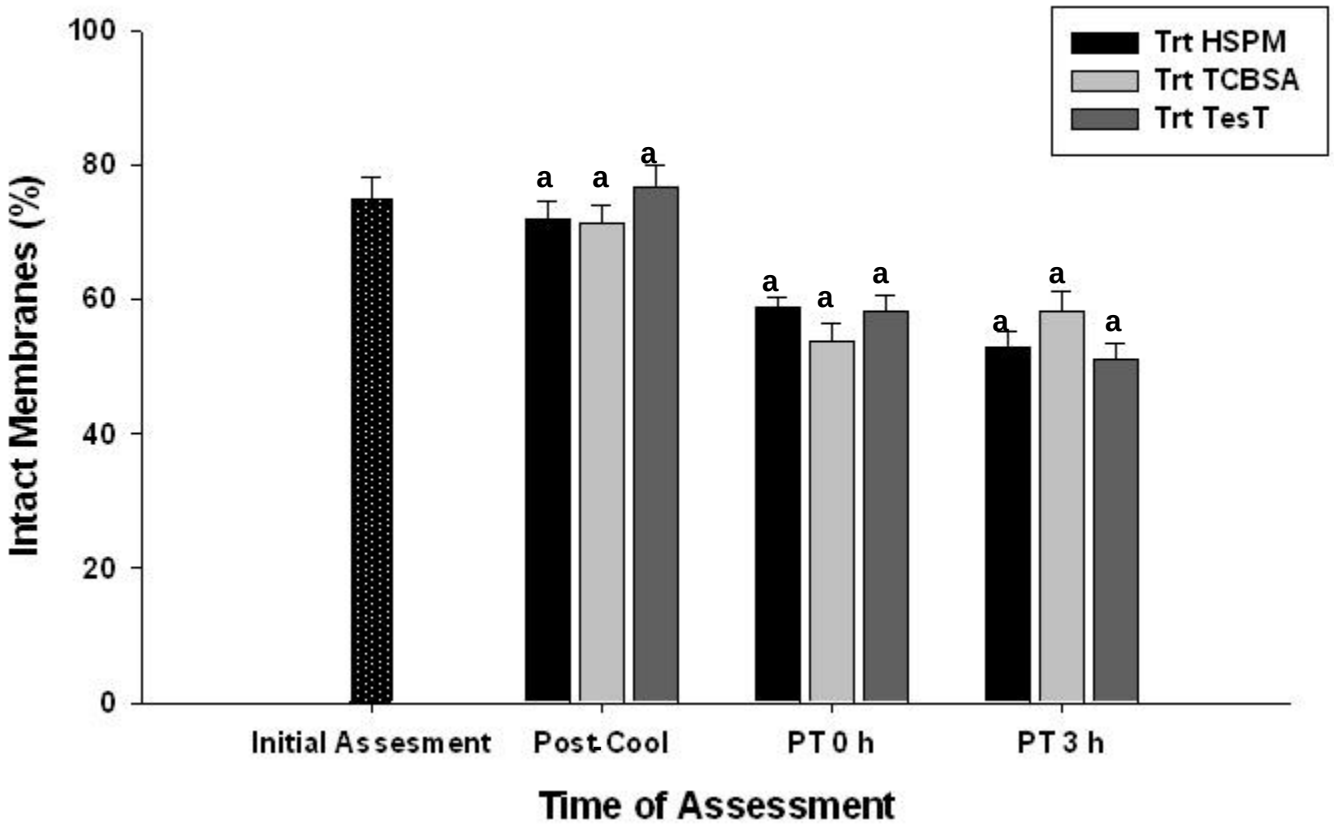

Figure 4.4. Effect of egg yolk (TesT) versus BSA-based (HSPM and TCBSA) extenders on cat epididymal sperm membrane integrity before (pre- and post-cool) and after (0 h and 3 h) freezing. [a,b]Mean values across treatments but within time with different superscripts are significantly different (P<0.05).

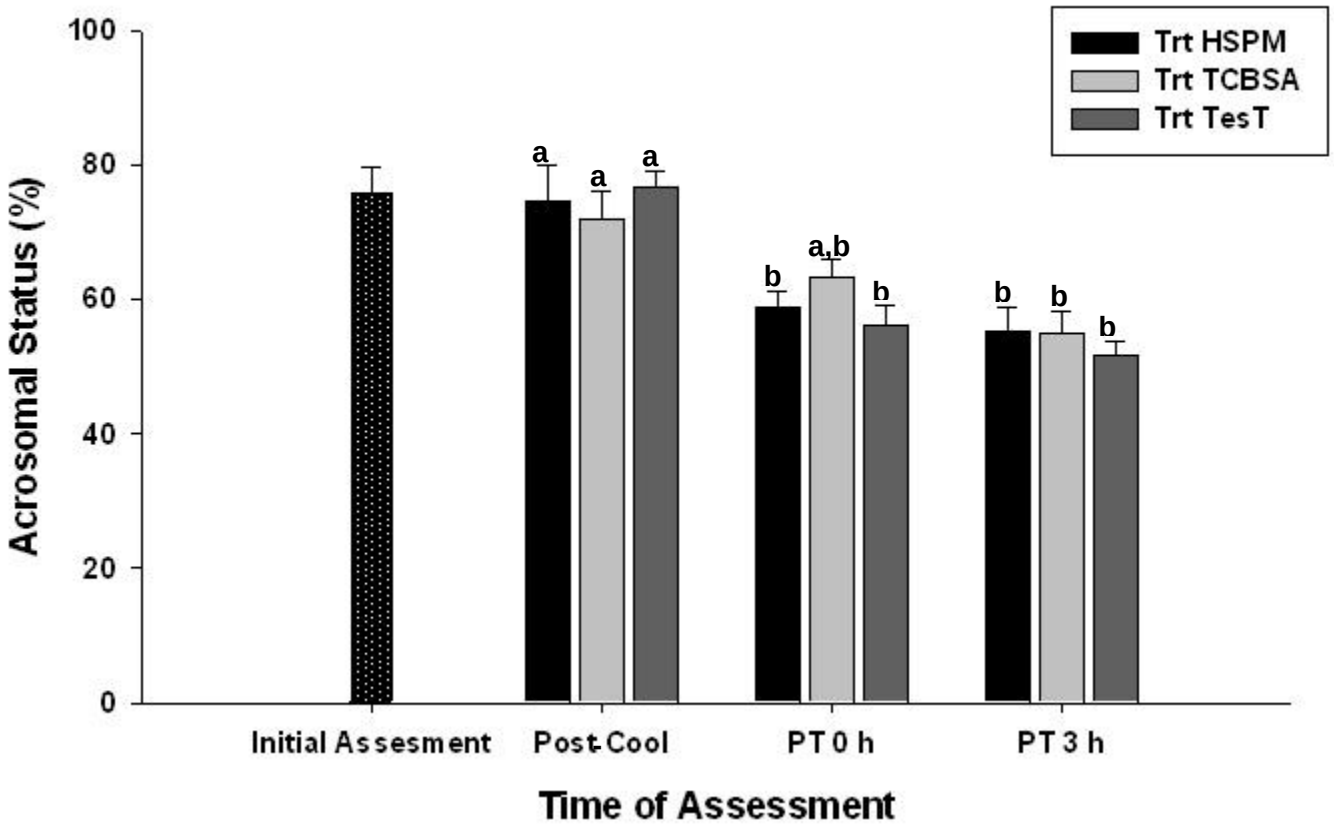

Figure 4.5. Effect of egg yolk (TesT) versus BSA-based (HSPM and TCBSA) extenders on cat epididymal sperm acrosomal status before (pre- and post-cool) and after (0 h and 3 h) freezing. [a,b]Mean values within treatments but across time with different superscripts are significantly different (P<0.05).

The percentage of sperm in HSPM and TesT extenders with intact acrosomes was lower ($P<0.05$) at 0 hour after thawing as compared to that of sperm after cooling. However, there was no significant difference between the post-cool and 0 hour post-thaw values in TCBSA treatment. Also, there was no significant difference between the 0 and 3 hour post-thaw values within the three treatments. The post-cool values for all three treatments were significantly higher than at 3 hour post-thaw (Figure 4.5).

When comparing across treatments but within time periods, there was no significant difference ($P\geq0.05$) among the treatments at the three assessment times (Figure 4.6).

In Vitro Fertilization

Due to oocyte availability we were only able to evaluate sperm frozen in TesT and HSPM extenders. Cleavage frequency of 203 in vitro-matured oocytes was 36% and 33% for the TesT and HSPM treatments, respectively. The rate of in vitro embryo development to the blastocyst stage for TesT and HSPM treatments were 50% and 44%, respectively (Figure 4.7).

Discussion

Knowledge gained from reproductive research in the domestic cat could play a major role in the understanding of exotic feline reproduction for the purpose of conservation. The proper techniques and media used to collect, process, store and cryopreserve feline epididymal sperm has major implications in the propagation of genetics from valuable males that otherwise may be lost. Over the last decade, there have been several studies evaluating domestic cat epididymal sperm. Axner (2004b) compared characteristics of ejaculated and epididymal sperm. Others have analyzed the post-thaw survival of epididymal cat sperm after ex situ storage in the epididymis (Chatdarong et al., 2009; Ganan et al., 2009) or after cool storage in an egg yolk-based extender (Martins et al., 2009) before cryopreservation.

In efforts to find replacements for egg yolk in semen extenders other components, including bovine serum albumin has been used in boars, bulls, buck rabbits, rams and stallions (Harrison et al., 1978). Soy lecithin extract has been tested in several species including the cat (Vick et al., 2012) the dog (Beccaglia et al., 2009; Kasimanickam et al., 2012), the bull (Aires et al., 2003) the buck (goat) (Salmani et al., 2014). Low-density lipoproteins have been evaluated in the bull (Moussa et al., 2002), Red deer (Martinez-Pastor et al., 2009), dog (Bencharif et al., 2010), male goat (da Silva et al., 2014) and the Brown bear (Rodriquez et al., 2012).

The majority of studies on cat epididymal sperm do not report concentrations or total number of sperm recovered. However, Zambelli et al. (2008) did report the concentration of sperm collected by urethral catheterization ($21.0\pm18 \times 10^6$) and electro-ejaculation ($33.6\pm34.5 \times 10^6$) of 11 toms. The daily numbers of sperm recovered in our study were considerably higher

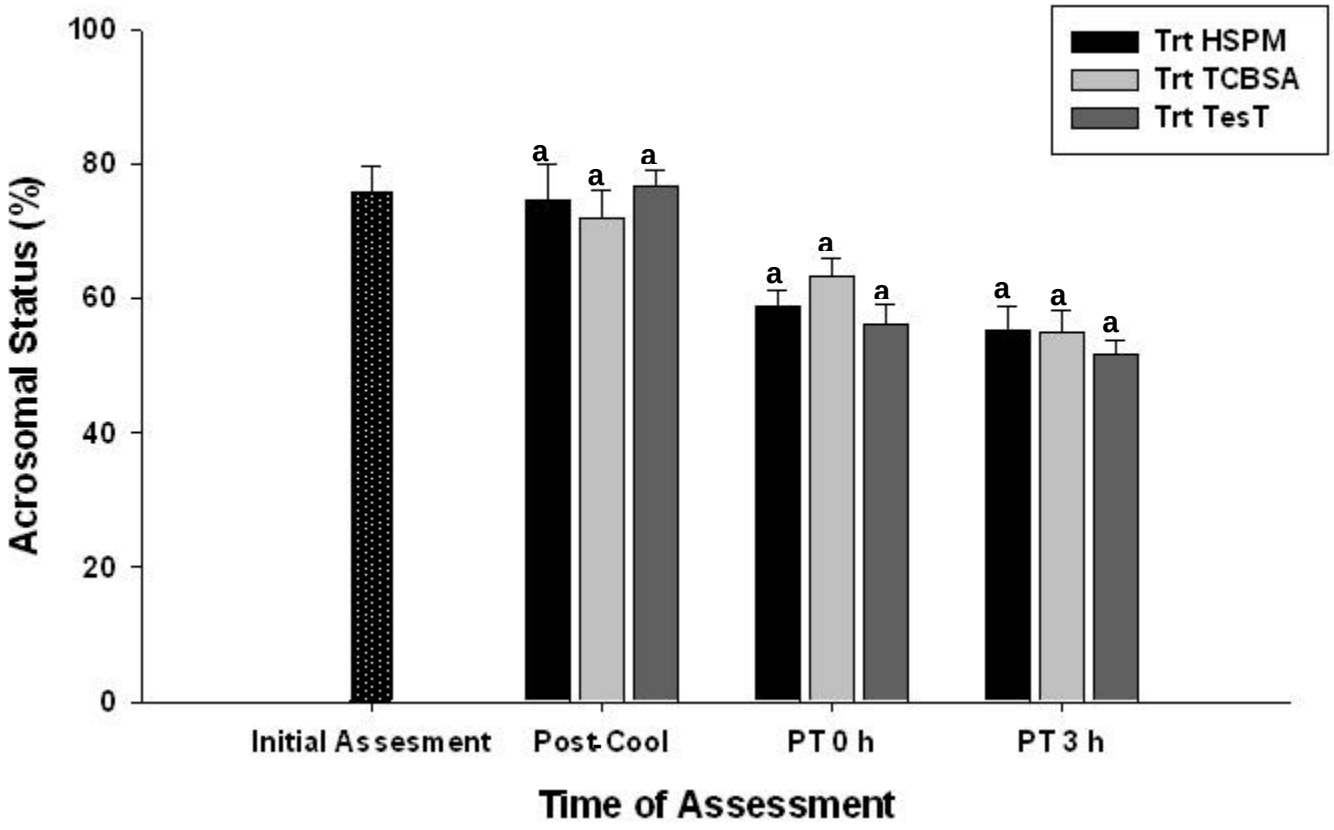

Figure 4.6. Effect of egg yolk (TesT) versus BSA-based (HSPM and TCBSA) extenders on cat epididymal sperm acrosomal status before (pre- and post-cool) and after (0 h and 3 h) freezing. [a,b]Mean values across treatments but within time with different superscripts are significantly different (P<0.05).

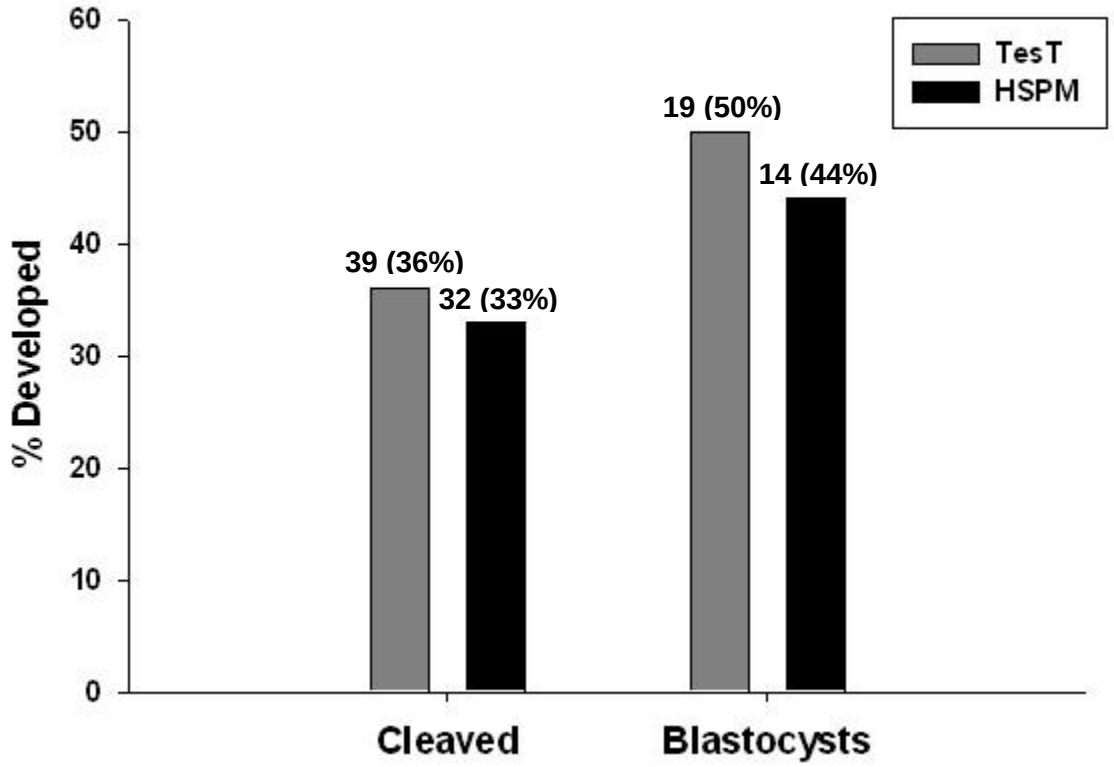

Figure 4.7. IVF percentages when oocytes were fertilized with domestic cat epididymal sperm frozen in HSPM extender(Treatment A) or TesT extender (Treatment C). Blastocysts percentages were derived from the number of embryos produced (Day 2) not from the total number of oocytes placed into IVF (Blastocysts% = no. of blastocysts/no. of embryos X 100).

than those noted by Zambelli et al. (2008) because we pooled testes from multiple individuals. Franca and Godinho (2003) measured daily sperm production per gram of testis. They found that domestic cat testis produce 15.7±1.6 x 10^6 of sperm/gram of testis. Average testis weight of 13 toms was 1.17±0.07 grams. Without knowing the storage capacity of the cat epididymis it is not possible to know if our sperm recovery rates are within the pooled mean. One could make the argument that since are results are based on pooled sperm; our recovery numbers should be significantly greater than an individual male sperm production. Furthermore, the upper range of our sperm recovery number is not so high to be considered inaccurate, and therefore our epididymal sperm recovery values fall within the domestic cat range.

Our results showed no major differences between the egg yolk extender (TesT) and BSA-based extenders (HSPM and TCBSA), and although they did not measure sperm motility Fukui et al. (2007) similarly reported no significant difference between pregnancy and lambing rates when ram semen was frozen in either a 10% bovine serum albumin (BSA) or a 15% egg yolk-based extender.

Cryopreservation causes increased stress and has damaging effects on sperm membranes that result in an expected decrease in post-thaw sperm values of most mammalian species (Parks and Graham 1992; Medeiros et al., 2002). This could help explain the significant decrease we reported between our pre-freeze and post-thaw values.

Swanson et al. (1996) also showed that freezing resulted in a significant decrease in the percentage of intact acrosomes jaguar and cheetah sperm after thawing. In addition, Pukazhenthi et al. (1999) proposed that in the domestic cat, acrosomal damage was caused prior to freezing when ejaculated sperm were rapid cooled (~4°C/minute) and ultra-rapid cooled(~14°C/minute).

Domestic feline epididymal sperm has been shown to be capable of producing embryos in vitro when used for in vitro fertilization (Lengwinat and Blottner, 1994) and offspring when used with artificial insemination (Tsutsui et al., 2003). We report that cat epididymal sperm frozen in an extender containing bovine serum albumin results in post-thaw sperm values that are not significantly different from sperm frozen with a traditional egg yolk-based extender. Furthermore, we showed that sperm frozen in a BSA extender can be used to produce IVF-derived embryos.

CHAPTER V
EFFECT OF EGG YOLK CONCENTRATION IN TEST BUFFERED EXTENDER ON SURVIVAL AND *IN VITRO* FUNCTIONALITY OF DOMESTIC CAT EPIDIDYMAL SPERMATOZOA FOLLOWING CRYOPRESERVATION

Introduction

TesT buffered extender containing 20% egg yolk is a commercially available extender and is commonly used in the cryopreservation of domestic cat spermatozoa (sperm) (Pope et al. 1998; Harris et al., 2001); however, the presence of egg yolk concentrations ≤ 20% presents obstacles that possibly could be avoided by reducing the amount of egg yolk in the extender.

Extenders containing 20% egg yolk are widely used for cryo-storage of sperm in several mammalian species. However, in the 1950s and 1960s egg yolk was commonly used at a concentration of 50% in bull semen extenders (Stewart et al., 1950; Foote et al., 1960). There is some evidence that sperm from some species, including the cat, could survive cryopreservation with an egg yolk concentration ≤ 20% (Glover and Watson, 1987).

The lower concentration of egg yolk in feline extenders is not necessarily used for the purpose of improving post-thaw sperm survival but rather for when sperm are used in IVF or ICSI, where egg yolk needs to be removed by dilution and centrifugation. Furthermore, another reason for examining the effects of lower concentrations of egg yolk on cat sperm functions, is the importance of using a clear extender for sperm samples to be separated into 'X' and 'Y' population by flow cytometry. The presence of egg yolk tends to interfere with the efficiency of the sorting process so the sperm sample needs to be washed prior to sorting. It should be noted that Gibb et al. (2011) has reported that the efficiency of sex sorting stallion sperm was improved when sperm were extended in a clear nonegg yolk extender. Also, Pope et al., (2009) showed that cat ejaculated sperm stored and shipped in a nonegg yolk medium could be sex sorted and result in gender specific kittens when used for IVF at 48 hours after collection.

Santiago-Morano et al. (2006b) showed that Spanish ibex epididymal sperm frozen in a 6% egg yolk extender had a greater fertility rate then a 20% egg yolk extender when sperm were inseminated into domestic goats. Ritar and Salamon (1991) reported that depending on the month, Angora goat ejaculated sperm displayed greater motility after 6 hours incubation in extenders containing 1.5% or 6% egg yolk as compared to 12% egg yolk.

Webb et al. (2011) found that after freezing stallion semen using a "fast freeze" (no cooling or freezing rates) post-thaw motility was greater in 22% or 19% egg yolk as compared with 16% and 13% egg yolk. They also reported egg yolk concentration did not affect post-thaw motility of stallion semen frozen by a "slow freeze" method.

Smith et al. (1979) compared different egg yolk concentrations for freezing beef bull semen. It was reported that when freezing bull sperm in ampules, the low (1% or 2%) and high (16% or 32%) concentrations of egg yolk should be avoided due to reduced motility and acrosome integrity. However, when freezing bull sperm in straws, there were no differences in motility among 4, 8, 16 or 32% egg yolk.

The purpose of the present study was to determine if freezing domestic cat epididymal sperm in an extender containing an egg yolk concentration as low as 2% would alter sperm survival after thawing as compared to that of sperm frozen in an extender with 20% egg yolk. Sperm survival as determined by in vitro viability, sperm motility, membrane integrity and acrosomal status were evaluated at different time points: pre- and post cooling, and at 0 hour and 3 hour after thawing.

Materials and Methods

Experimental Design

Experiment 5.1

The objective of this experiment, was to compare in vitro survivability and functionality of domestic cat (*Felis catus*) epididymal spermatozoa (sperm) that was cryopreserved in TesT buffer (TB) extender containing 2% (Treatment A), 5% (Treatment B) or 10% (Treatment C) egg yolk (EY). For this experiment, domestic cat testes were collected on 10 separate days over a 3 month period (n = 10) and epididymal sperm were recovered, processed and frozen on the same day testes were collected. On the 10 days, a range of 1 to 10 sets of testes were processed/day for a total of 50 sets of testes. For each day, sperm collected from all testes was pooled and equally divided across the three treatments. The three treatments all contained the Tes-Tris buffer (TesT) with the concentration of egg yolk varying from 2, 5 or 10%.

After sperm samples were, washed and centrifuged they underwent the initial analysis (pre-cool, control). Then the sperm samples were divided into three 1.6 mL aliquot tubes containing one of the three egg yolk treatment extenders (Part A). Each tube was placed in its own 125 mL plastic bottle water bath filled with 22°C water, and then placed into a 4°C walk-in cool room and gradually cooled to 4°C±1°C. After cooling, a second sperm evaluation (pre-freeze/4°C) was recorded for each of the treatments. Following the second evaluation, samples were extended 1:1 with TesT yolk buffer (2, 5, or 10%) containing 12% glycerol (Part B) to give a final glycerol concentration of 6%. Then, samples were loaded into 0.25 mL straws and frozen on a block of dry ice for ≥ 20 minutes before plunging inot liquid nitrogen for storage.

To further asses the functionality, cryopreserved epididymal spermatozoa from each treatment were thawed and used for in-vitro fertilization protocol. Cumulus oocyte complexes

(COC) were placed in modified TCM-199 with $NaHCO_3$ (Irving Scientific, Santa Ana, CA) and cultured for 24 hours in 5% O_2, 5% CO_2, and 90% N_2 at 38°C (IVM). For IVF, COC were co-incubated with sperm frozen in either TYB or HSPM (1 million sperm/mL), under 5% CO_2 in air at 38°C. After 18 hours, oocytes were rinsed and cultured using a 3-step system (Pope et al. 2006). Cleavage frequency and blastocyst development were assessed on IVC days 2 and 8, respectively.

Experimental Procedure

Basic Procedures

The basic procedures used for Experiment 5, unless otherwise specified were: tissue collection and transport, sperm collection, sperm analysis, cryopreservation and thawing of sperm treatments, in vitro production medium, oocyte collection and maturation, in vitro fertilization and culture and statistical analyses. Full details for each procedure were provided in Chapter 3.

Sperm Extender Preparation

The base extender, which all three extenders were made from, was the commercially available extender, TesT Yolk Buffer (Refrigeration Medium, Irving Scientific, Santa Ana, CA). This commercially available extender is sold as a 20% egg yolk-based extender that contains Tes (176 mM), Tris (80 mM), dextrose (9 mM), penicillin-G (1,000 units/mL), streptomycin sulfate (1,000 mcg/mL) and egg yolk (20% v/v). An identical extender, but without egg yolk, prepared in the lab. To generate the three treatment extenders with the desired egg yolk concentrations of 2, 5 and 10%, the two base extenders (the commercial and the lab preparations) were mixed in proportions to reduce the 20% egg yolk extender, down to 10, 5, 2% egg yolk. For example, 1 part 20% egg yolk was mixed with 1 part 0% egg yolk to produce the 10% egg yolk extender; and 1 part 10% egg yolk was mixed with 1 part 0% egg yolk to generate the 5% egg yolk extender. For each of the three extenders there were two parts, Part A (without glycerol) and Part B (with 12% glycerol).

Results

Experiment 5.1

Testes Collection, Epididymal Sperm Concentration and Straws frozen

Routinely, three sets were processed each day, except for two separate days when only one set was processed and produced enough sperm to be divided across the three treatments. When the epididymal sperm was pooled, the average total number of cells collected for each day was 187×10^6, with a range from 83×10^6 to 441×10^6.

The total number of epididymal sperm was divided evenly across the three treatments and depending on the sperm concentrations a range of 1 to 4 (0.25 mL) straws were frozen per treatment/day, with a concentration ranging from 19 x 10^6 to 35 x 10^6/straw (Table 5.1).

Motility

The mean (±SEM) initial motility for the daily pooled epididymal sperm, was 67.0±3.7% (Control). Mean motility of sperm in 2% egg yolk after cooling (4°C), and at 0 hour and 3 hour after thawing was 63.0±7.1%, 44.5±4.1% and 31.5±3.3%, respectively. Mean motility of sperm in 5% egg yolk after cooling and at 0 hour and 3 hour after thawing was 75.5±2.0%, 50.0±4.0% and 34.5±3.2%, respectively. The equivalent motility values in 10% egg yolk were 79.0±2.3%, 50.5±3.7% and 31.5±3.0%, respectively (Table 5.2)

There were no significant differences ($P \geq 0.05$) between initial motility and that of sperm in the three treatments after cooling /freezing. However, motility at 0 hour and 3 hour post-thaw motility was lower ($P \leq 0.05$) than the initial Control value (Table 5.2).

Sperm motility after cooling was significantly greater than that of sperm at 0 hour and 3 hour post-thaw. Similarly, motility at 0 hour post-thaw was significantly greater than at 3 hour post-thaw in all three groups (Figure 5.1).

When comparing across treatments for the post-cool assessment point, the after cooling mean motility of sperm in 2% egg yolk was significantly less ($P \leq 0.05$) than that of sperm in 10% egg yolk but motility in 5% egg yolk was not significantly different ($P \geq 0.05$) that of the other two treatments. There was no significant difference in motility among treatments at either the 0 hour or the 3 hour post-thaw (Figure 5.2).

Membrane Integrity

The mean (±SEM) percentage of sperm with an intact membrane at the initial evaluation (pre-cool) was 81.3±2.6% (Control). After cooling to 4°C, membrane integrity percentages were: 79.5±1.7%, 76.9±1.7% and 82.5±2.8% for the 2, 5 and 10% egg yolk treatments, respectively. Mean membrane integrity at 0 hour post-thaw was 53.4±3.1%, 53.1±2.0% and 56.5±1.4% for sperm in 2, 5 and 10% egg yolk, respectively. After 3 hour of post-thaw incubation at 37°C, membrane integrity of sperm frozen in 2%, 5% and 10% egg yolk was 52.6±3.1%, 53.6±1.7% and 53.7±2.3% (Table 5.3).

The percentage of membrane intact sperm at the initial evaluation (pre-cool) was not significantly different ($P \geq 0.05$) from that of cooled sperm in the three treatments. However, it was significantly greater ($P \leq 0.05$) than the 0 and 3 hour post-thaw values in all three treatments (Table 5.3).

Table 5.1. Summary of the numbers of sets of testes processed, number of sperm recovered and number of straws frozen on each of ten collection days.

Thaw date	Collection date	Sets processed	Total Cell Count	No. of straws per extender/day	Concentration per straw
10/7/2008	8/4/2008	1	83×10^6	1	19×10^6
10/8/2008	8/18/2008	3	97.5×10^6	1.5	18×10^6
10/21/2008	10/10/2008	4	108×10^6	1.5	18×10^6
10/22/2008	10/17/2008	7	346×10^6	4	28×10^6
12/4/2008	10/24/2008	8	171×10^6	2	23×10^6
12/4/2008	10/30/2008	4	117×10^6	1	27×10^6
12/5/2008	10/31/2008	8	225×10^6	2	28×10^6
12/9/2008	11/7/2008	10	441×10^6	4	35×10^6
12/10/2008	11/12/2008	4	200×10^6	2	32×10^6
12/12/2008	11/13/2008	1	83×10^6	1	27×10^6
Daily Average (no.)		5	187×10^6	2	25.5×10^6
Total no.		50	-	20	-

The last two rows show the mean values for all the collection days and the overall total values for the experiment.

Table 5.2. Percentage of sperm motility (mean±SEM) before cooling, post-cooling (4°C), 0 hour and 3 hour post-thaw for cat epididymal sperm frozen in an extender containing either 2%, 5% or 10% egg yolk.

	Initial (Control)	Post-cool	0 hour post-thaw	3 hour post-thaw
2% EY	67.0 ± 3.7^a	63.0 ± 7.1^a	44.5 ± 4.1^b	31.5 ± 3.3^b
5% EY	67.0 ± 3.7^a	75.5 ± 2.0^a	50.0 ± 4.0^b	34.5 ± 3.2^b
10%EY	67.0 ± 3.7^a	79.0 ± 2.3^a	50.5 ± 3.7^b	31.5 ± 3.0^b

EY = egg yolk, Trt = (Treatment); ^{a,b}Superscripts designate a significant difference only between initial motility and individual values for each extender at each time period ($P<0.05$).

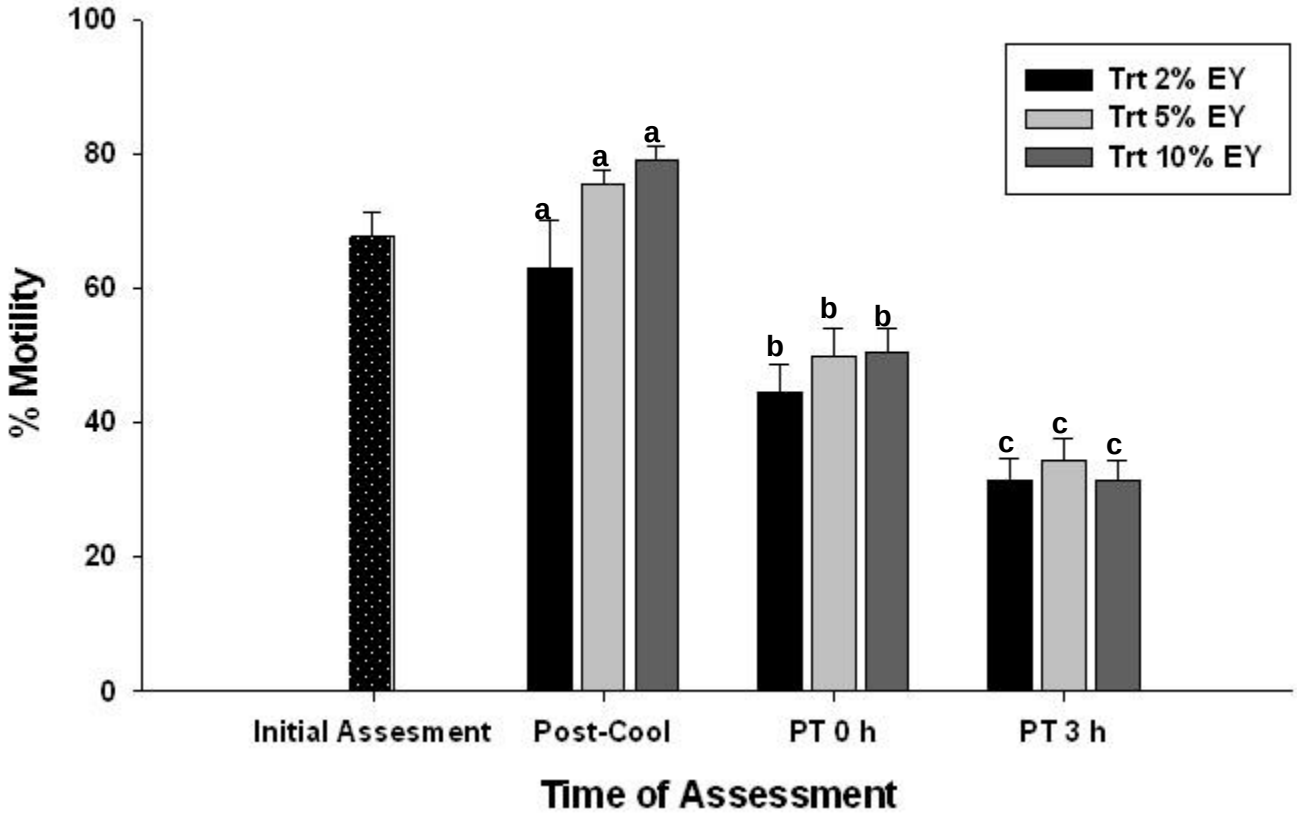

Figure 5.1. Effect of different concentrations of egg yolk extenders (2%, 5% and 10%) on cat epididymal sperm motility before (pre- and post-cool) and after (0 h and 3 h) freezing. [a,b,c]Mean values within treatments but across time intervals with different superscripts are significantly different ($P<0.05$).

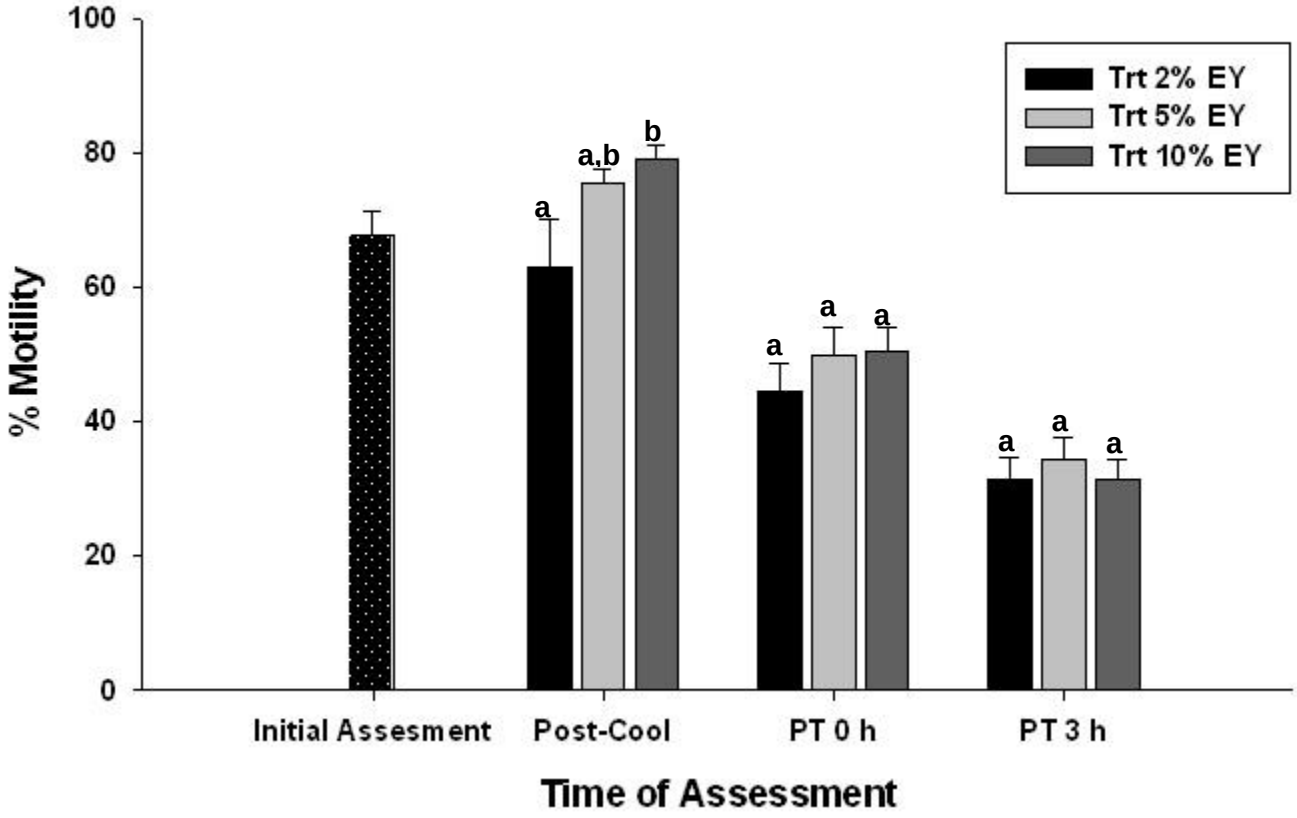

Figure 5.2. Effect of different concentrations of egg yolk extenders (2%, 5% and 10%) on cat epididymal sperm motility before (pre- and post-cool) and after (0 h and 3 h)freezing. [a,b]Mean values across treatments but within time intervals with different superscripts are significantly different ($P<0.05$).

Membrane integrity after cooling was significantly greater than after thawing, both at 0 and 3 hours post-thaw in all three treatments; however, the 0 and 3 hours post-thaw values were not significantly different in any of the three treatments (Figure 5.3).

Lastly, there were no significant differences in sperm membrane integrity among the three treatments for any of the three assessment times (Figure 5.4).

Acrosomal Status

For this experiment, the mean initial value for epididymal sperm with intact acrosomes was 85.6±1.6% (Control). After cooling to 4°C, 85.7±2.2% and 85.9±1.9% of sperm in 2 and 5% egg yolk extender had intact acrosomes, respectively. The 10% egg yolk extender resulted in sperm with a slightly lower mean intact acrosomal value of 82.1±2.2%. At 0 hour post-thaw, the percentage of sperm with intact acrosomes in 2, 5 and 10% egg yolk extender were: 62.5±2.5%, 60.1±3.1% and 59.1±2.4%, respectively. Of the three treatments, the 2% egg yolk extender had the highest mean percentage of sperm with intact acrosomes at 3 hour post-thaw (56.3±2.5%). The 10% egg yolk treatment had the lowest value (52.8±3.0%), while the 5% egg yolk treatment had 56.1±2.3% of sperm with intact acrosomes (Figure 5.5).

The percentage of sperm with intact acrosomes before cooling was not different from that of the three post-cool percentages; however, the pre-cool percentage was significantly different ($P \leq 0.05$) from both the 0 hour and 3 hour post-thaw values in the three treatments (Table 5.4).

After cooling the percentages of sperm with intact acrosome were significantly greater than both the 0 hour and 3 hour post-thaw values within the three treatments. There was no significant difference in acrosomal status between the 0 hour and 3 hour evaluations within the three treatments.

There were no significant differences among the three treatments at any of the assessment times (Figure 5.6).

In Vitro Fertilization

Due to limited availability of oocytes, we examined in vitro functionality of sperm frozen in 2% egg yolk extender as compare to that of a non-egg yolk extender (Human Sperm Preservation Medium, HSPM) evaluation in an earlier experiment (Experiment 5.1). Cleavage frequency of 283 in vitro-matured oocytes was 76% in the TesT Buffer extender with 2% egg yolk, versus 73% in HSPM extender. The percentage of embryos that developed to the blastocysts stage after IVF with sperm frozen in 2% egg yolk extender was 42%, versus 38% in HSPM extender (Figure 5.7).

Table 5.3. Percentage of sperm with intact membranes (membrane integrity) (mean±SEM) before cooling, post-cooling (4°C), 0 hour and 3 hour post-thaw for cat epididymal sperm frozen in an extender containing either 2%, 5% or 10% egg yolk.

	Initial (Control)	Post-cool	0 hour post-thaw	3 hour post-thaw
Trt 2% EY	81.3±2.6[a]	79.5±1.7[a]	53.4±3.1[b]	52.6±3.1[b]
Trt 5% EY	81.3±2.6[a]	76.9±1.7[a]	53.1±2.0[b]	53.6±1.7[b]
Trt 10%EY	81.3±2.6[a]	82.5±2.8[a]	56.5±1.4[b]	53.7±2.3[b]

EY = egg yolk, Trt = Treatment; [a,b]Superscripts designate a significant difference only between initial membrane integrity and individual values for each extender for each time period ($P<0.05$).

Table 5.4. Percentage of cat epididymal sperm with intact acrosomes (acrosomal status) (mean±SEM) before cooling, post-cooling (4°C), 0 hour and 3 hour post-thaw for cat epididymal sperm frozen in an extender containing either 2%, 5% or 10% egg yolk.

	Initial (Control)	Post-cool	0 hour post-thaw	3 hour post-thaw
Trt 2% EY	85.6±1.6[a]	85.7±2.2[a]	62.5±2.5[b]	56.3±2.5[b]
Trt 5% EY	85.6±1.6[a]	85.9±1.9[a]	60.1±3.1[b]	56.1±2.3[b]
Trt 10%EY	85.6±1.6[a]	82.1±2.2[a]	59.1±2.4[b]	52.8±3.0[b]

EY = egg yolk, Trt = Treatment; [a,b]Superscripts designate a significant difference only between initial acrosomal status and individual values for each extender for each time period ($P<0.05$).

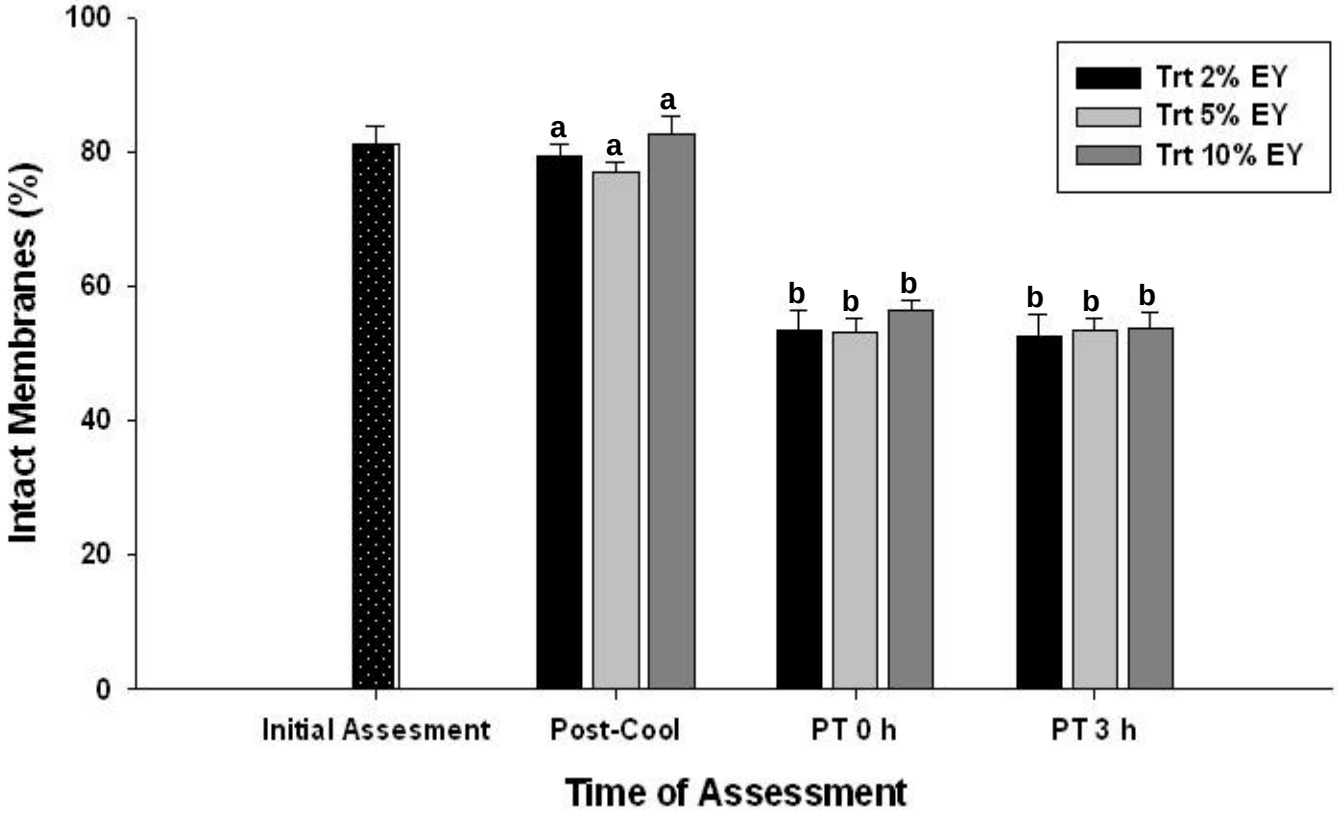

Figure 5.3. Effect of different concentrations of egg yolk extenders (2%, 5% and 10%) on cat epididymal sperm membrane integrity before (pre- and post-cool) and after (0 h and 3 h) freezing. [a,b]Mean values within treatments but across time intervals with different superscripts are significantly different ($P<0.05$).

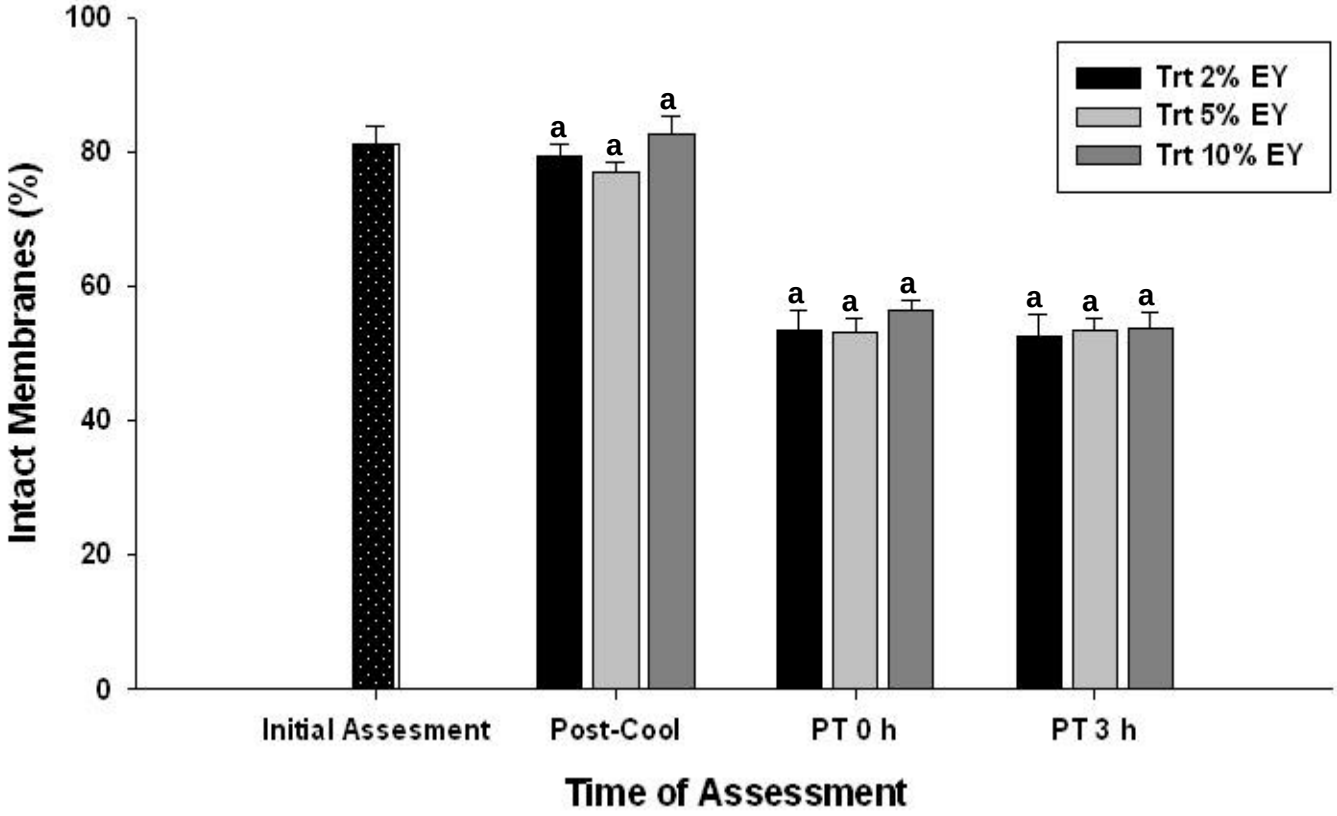

Figure 5.4. Effect of different concentrations of egg yolk extenders (2%, 5% and 10%) on cat epididymal sperm membrane integrity before (pre- and post-cool) and after (0 h and 3 h) freezing. [a,b] Mean values across treatments but within time intervals with different superscripts are significantly different ($P<0.05$).

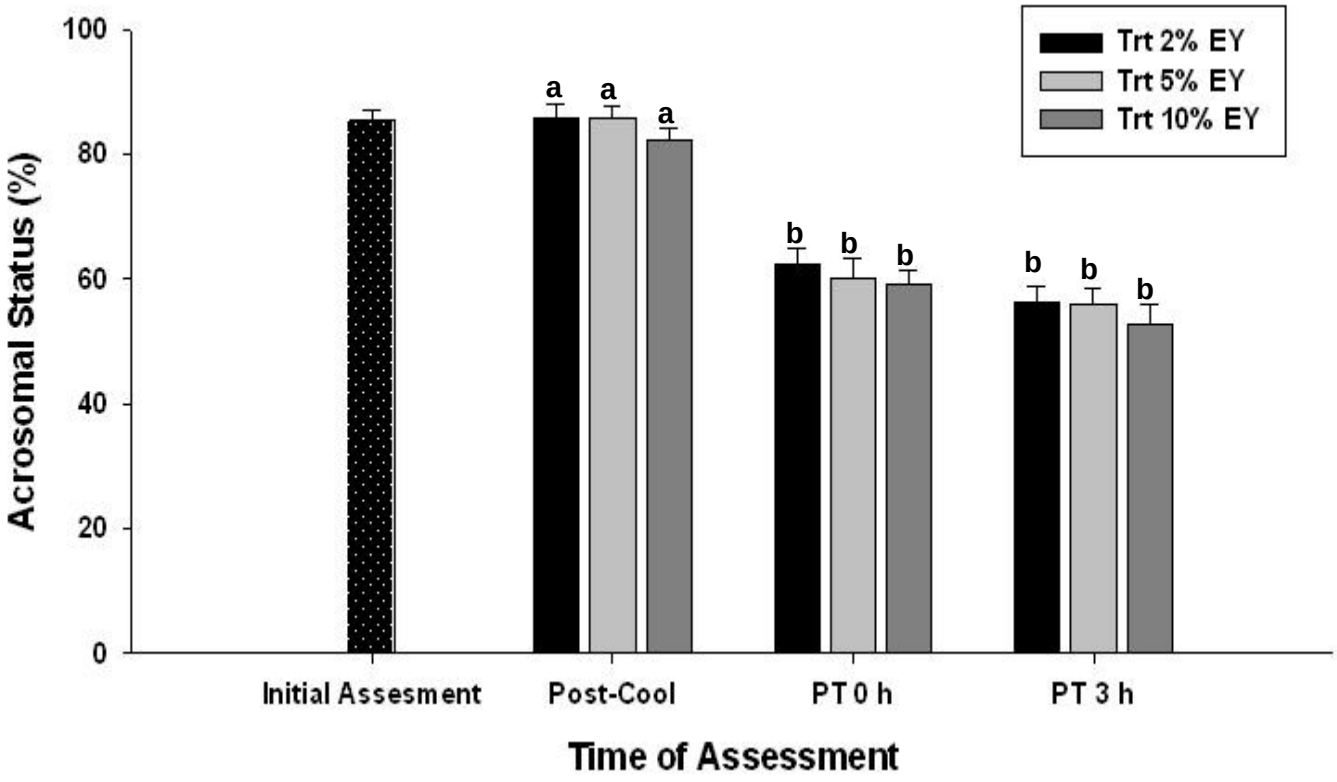

Figure 5.5. Effect of different concentrations of egg yolk extenders (2%, 5% and 10%) on cat epididymal sperm acrosomal status before (pre- and post-cool) and after (0 h and 3 h) freezing. [a,b]Mean values within treatments but across time intervals with different superscripts are significantly different (P<0.05).

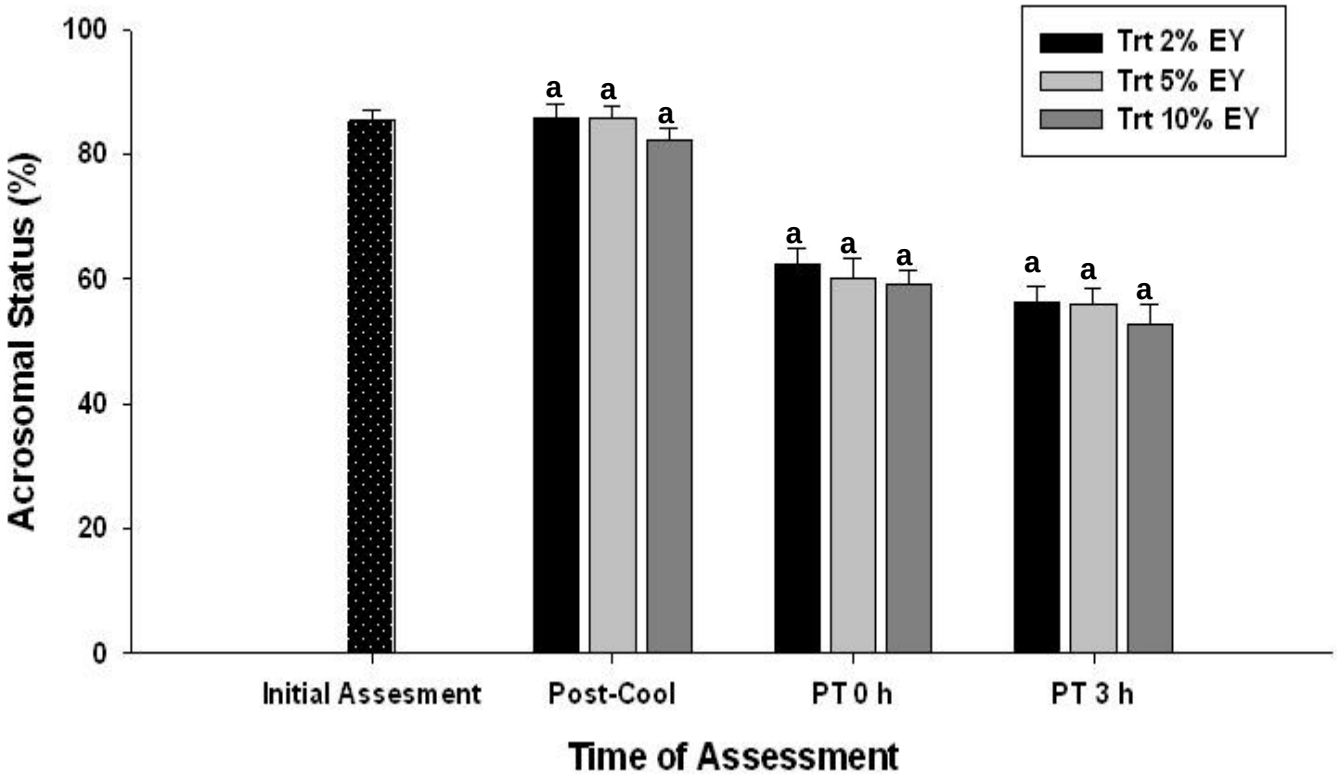

Figure 5.6. Effect of different concentrations of egg yolk extenders (2%, 5% and 10%) on cat epididymal sperm acrosomal status before (pre- and post-cool) and after (0 h and 3 h) freezing. [a,b]Mean values across treatments but within time intervals with different superscripts are significantly different ($P<0.05$).

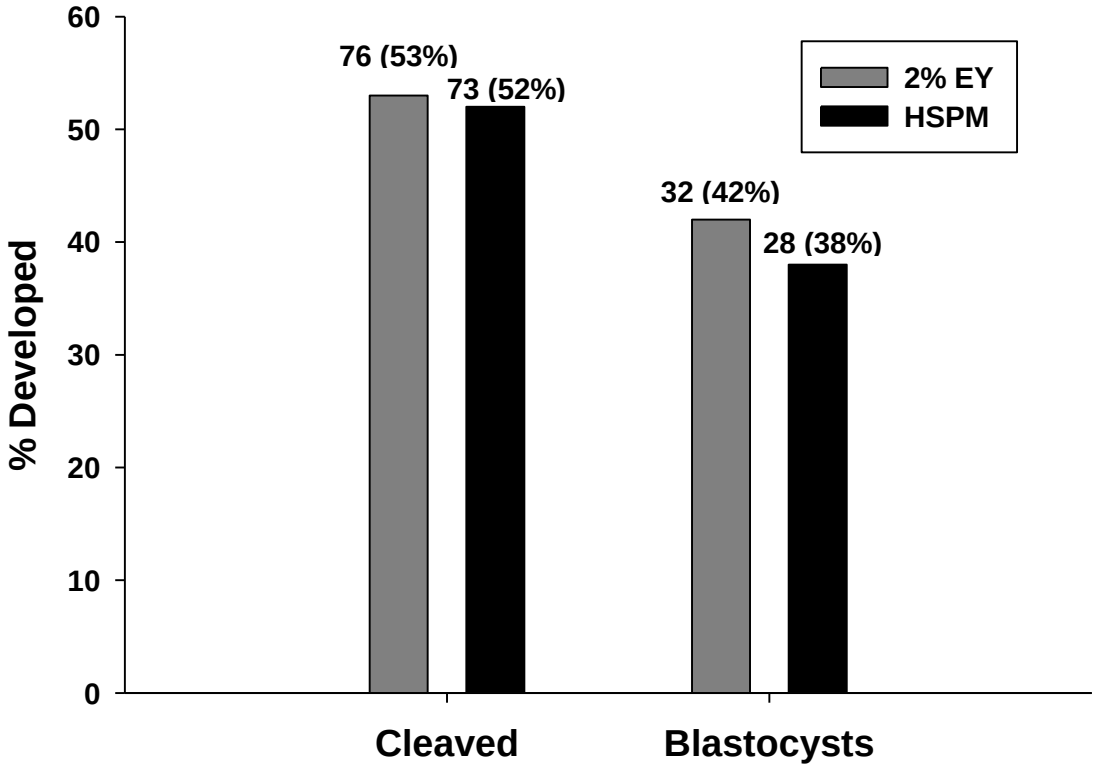

Figure 5.7. IVF percentages when oocytes were fertilized with domestic cat epididymal sperm frozen in TesT buffer extender containing 2% egg yolk or HSPM extender (from Experiment 4.1). Blastocysts percentages were derived from the number of embryos produced (Day 2) not from the total number of oocytes placed into IVF (Blastocysts% = no. of blastocysts/no. of embryos X 100).

Discussion

Currently, with the use of biological stains (Mito tracker green, SYBR 14/PI, FITC/PNA, Hoechst 33342), flow cytometry is used to analyze mitochondrial function, membrane integrity, acrosomal status and for sex sorting sperm (Hossain et al., 2011). However, in the presence of extenders containing egg yolk or other particle matter from animal products (egg yolk or milk) sperm concentration and percentage of non-viable sperm may be over-estimated by flow cytometry (Petrunkina and Harrison, 2010). Reducing the amount of egg yolk in semen extenders would allow for more accurate sperm analysis. Mechanical analysis and categorization of sperm during sorting is improved in a 'clear' extender (Gibb et al., 2011). However, for a reduced egg yolk extender to be practical it needs to protect sperm as effectively as extenders containing animal proteins.

The lack of a statistical difference when comparing across treatments for the post-thaw values reported in our results were not in agreement with those from a recent report on the cryopreservation of boar ejaculates (Blanch et al., 2014). A significant decrease between 0 hour post-thaw motility was noted when boar semen was frozen in a 20% egg yolk extender when compared with a 10% egg yolk extender (Blanch et al., 2014).

Unlike our findings, Cabrera et al. (2005) reported that frozen-thawed Canary buck semen diluted in 12% egg yolk extender resulted in significantly higher sperm motility, membrane integrity and acrosomal status than 6% egg yolk; and likewise the 6% egg yolk had significantly higher values than the 1.5% egg yolk. Ritar and Salamon (1991), also showed a difference between post-thaw survival rates when angora buck semen was frozen in extenders containing different concentrations of egg yolk. However, it was noted that the egg yolk concentration that produced the highest post-thaw values varied by month. When bucks were collected in March sperm in the 12% egg yolk extender had the greatest post-thaw survival. Between the months of April and June, the 6% egg yolk produced the best post-thaw values. In July, 1.5% egg yolk was the superior concentration and by August 0% egg yolk yielded the best post-thaw survival.

No significant difference among the post-thaw sperm values along with the IVF/IVC data showing the viability of sperm that was frozen-thawed in a 2% egg yolk extender to produce in vitro-derived cat embryos, leads us to conclude that a 2% egg yolk extender would be an acceptable replacement for the more commonly used 20% egg yolk extender when freezing domestic cat epididymal sperm. The present results are supported by those of Mulley et al. (1988) who reported the birth of a Fallow deer fawn, after 3 does were artificially inseminated with ejaculated semen, frozen in a 2.25% egg yolk extender.

CHAPTER VI
EFFECT OF PENTOXIFYLLINE CONCENTRATIONS AND TIME OF EXPOSURE ON THE POST-THAW SURVIVAL OF DOMESTIC CAT EPIDIDYMAL SPERM

Introduction

The ability to increase sperm motility may have benefits in assisted reproductive techniques. An increase in motility could mean an increase in the total number of spermatozoa (sperm) that reach the site of fertilization following artificial insemination. Increased sperm motility could also enhance in vitro fertilization rates. For the purpose of this study, stimulating sperm motility could increase the number and quality of sperm recovered in an isolate type of gradient after centrifugation. The increased viable sperm number would likely translate into an increased recovery rate when sperm where sex sorted with a flow cytometer.

Various agents have been used to stimulate sperm motility in different mammalian species. Stachecki et al. (1994; 1995) showed that the stimulating agents pentoxifylline, caffeine and 2'-deoxyadenosine all increased the post-thaw motility of domestic cat ejaculated and epididymal sperm. In an extensive study in the dog, Milani et al. (2010) found that pentoxifylline, caffeine and 2'-deoxyadenosine all had a positive effect on post-thaw sperm motility but at different time points, relating to thawing rates and stimulant concentration. Mirshokraei et al. (2011) reported that when ejaculated dog sperm were incubated for 2 hours with extender plus pentoxifylline, sperm overall motility was increased over the control samples. Also, a significant increase in acrosome reacted and sperm capacitation over that of the controls was reported when dog sperm were exposed to higher concentrations (10 mM and 100 mM) of pentoxifylline.

Recent studies have investigated the effects of pentoxifylline on equine epididymal sperm parameters. In one example, Guasti et al. (2013) reported that using pentoxifylline in flushing medium when collecting equine epididymal sperm, increased motility, progressive motility, straight line velocity and curvilinear velocity. The number of rapid moving sperm were also analyzed immediately after collection and again after 15 minutes of incubation with pentoxifylline. Subsequently, sperm were washed and pentoxifylline removed, difference in the motility characteristics between the pentoxifylline treatment and the control were no longer significantly different at the pre-freeze or post-thaw evaluations. Furthermore, it was reported that when flushing equine epididymal sperm with medium containing pentoxifylline there was no negative post-thaw effects on the sperm membrane integrity during the 0 hour post-thaw evaluations.

Stephens et al. (2013) showed that post-thaw exposure of stallion ejaculated sperm to pentoxifylline resulted in an increase of sperm overall motility, progressive motility, curvilinear velocity, average path velocity and straight-line velocity. Furthermore, the sperm treated with the pentoxifylline treatment had values that were significantly greater than the caffeine-treated or taurine-treated sperm. Also, the pentoxifylline-treated sperm exhibited an increase in in-vitro longevity that was greater than the other two treatments.

In the current study, the effect of three concentrations of pentoxifylline on feline epididymal sperm was evaluated before and after cryopreservation. Sperm motility, membrane integrity and acrosomal status were all measured and these results would help to determine if it would be more beneficial to expose feline epididymal sperm to pentoxifylline before centrifugation and/or during the thawing process.

Materials and Methods

Experimental Design

Experiment 6.1

In an effort to increase the post-thaw motility of domestic cat (*Felis catus*) epididymal sperm, frozen semen straws were thawed and then diluted with HEPES-199 containing different concentrations of pentoxifylline (0mM (Treatment A), 1mM (Treatment B) or 5mM (Treatement C)). This experiment consisted of collecting domestic cat testes over a one month period, from local veterinary clinic in the New Orleans, LA area. A total of 16 sets of domestic cat testes were collected and processed to fill and freeze a total of 16 (0.5 mL) straws. Epididymal sperm were frozen in 2% egg yolk-based extender, and 11 of 16 straws were used in this experiment. Epididymal sperm were recovered and frozen on the same day that the testes were collected. From three to five sets of testes were processed/day on the four collection days and sperm collected from all the testes was pooled and frozen.

Experiment 6.2

The second experiment was designed to increase post-thaw motility of domestic cat (*Felis catus*) epididymal sperm using different concentrations of pentoxifylline. The collecting, processing, freezing, analyzing and thawing of the epididymal sperm for this second experiment was the same as in Experiment 6.1, however, unlike the previous experiment the sperm were not pre-exposed to pentoxifylline for the first centrifugation stage and only exposed to pentoxifylline for the post-thaw treatments. Collection of testes from local veterinary clinics in the New Orleans area occurred daily Monday thru Friday, however, the processing and freezing of the epididymal sperm for this experiment took place on three separate days over a one week

time period. During that week, the processing of 13 set of testes resulted in 12 (0.5 mL) straws and ten (n = 10) of those straws were thawed for use in this experiment.

Experimental Procedure

Basic Procedures

Experiments 6.1 and 6.2 used the following basic procedures, unless otherwise stated: tissue collection and transport, sperm collection, sperm analysis, cryopreservation and statistical analysis. Full details for each basic procedure were provided in Chapter 3.

Preparation of Semen Extender and Pentoxifylline Treatment Solutions

The extender, which all epididymal sperm was frozen, was a 2% egg yolk-based extender which was made by diluting a commercially available 20% egg yolk extender (Irving Scientific, Santa Ana, CA). This commercially available extender is sold as a 20% egg yolk-based extender that contains Tes (176 mM), Tris (80 mM), dextrose (9 mM), penicillin-G (1,000 units/mL), streptomycin sulfate (1,000 mcg/mL) and egg yolk (20% v/v). The formulation of the 2% egg yolk extender was accomplished by making a separate extender containing the same concentrations of Tes, Tris, dextrose, penicillin-G and streptomycin sulfate but no egg yolk (0% egg yolk). The 20% egg yolk extender was then diluted with the 0% egg yolk extender until the extender was down to the desired concentration of 2% egg yolk. For example, 1 part 20% egg yolk mixed with 9 parts 0% egg yolk would generate the 2% extender. There was also a 12% glycerated portion of the extender (Part B) which also had 2% egg yolk and was made in the same manner as above.

Before use in the experiments, two stock solutions of pentoxifylline (PX) were made for each of the 1mM and 5mM treatments. The 1mM and 5mM stock solutions were made by adding 0.13915 and 0.3478 grams of powdered pentoxifylline respectively to 10 mL of Tyrodes buffer. The two stock solutions were then aliquoted, labeled and placed in a -80°C freezer until use. Two thaw solutions were made for each of the pentoxifylline dilution treatments, the make-up and volumes of the pentoxifylline stocks vs. He-199 are given in Table 6.1.

Pre-freeze Exposure, Thawing and Sperm Dilution with Pentoxifylline

After pooled sperm was collected and washed sperm was exposed to a 1mM concentration of pentoxifylline with hopes that the exposure would increase sperm pellet size during centrifugation in a sperm density gradient. This 1mM dose of pentoxifylline applied before the first centrifugation did not occur for experiment 6.2. Following centrifugation, pelleted sperm were evaluated for initial pre-cool values. Straws were removed from liquid nitrogen and exposed to air (22°C) for 10 seconds and then immediately immersed in 60°C water bath for 5 seconds. Once removed from the water bath, straw contents were emptied into pre-warmed

Table 6.1. Post-thaw dilution treatments and the formulas of how the treatments were constructed on each day the straws were thawed.

0mMPentoxifylline	Pentoxifylline Stock 0mM (µl)	HEPES-199 (µl)
Thaw Solution I	0	5000
Thaw Solution II	0	5000
1mMPentoxifylline	Pentoxifylline Stock 1mM (µl)	HEPES-199 (µl)
Thaw Solution I	200	4800
Thaw Solution II	100	4900
5mMPentoxifylline	Pentoxifylline Stock 5mM (µl)	HEPES-199 (µl)
Thaw Solution I	400	4600
Thaw Solution II	200	4800

(37°C) 1.6 mL tubes. The volume of the thawed sperm was measured and divided equally into 3 (1.6 mL) micro centrifuge tubes and diluted with HEPES-199 + 0mM(Treatment A), 1mM(Treatment B) or 5mM(Treatment C) of pentoxifylline in a 7 step fixed molarity method modified from Gao et al. (1995). After dilution with HEPES-199 + pentoxifylline, samples were centrifuged and supernate was removed. The sperm pellet was resuspended with HEPES-199 and post-thaw analysis was recorded, samples were placed in a 37°C incubator for three hours and final post-thaw evaluations were recorded.

Thaw Solution I from Table 6.1 was added to samples in 4 steps, the first addition was 15% of the original thaw volume, second step was 20% of the original thaw volume, this continues for the third and fourth steps with 25 and 40% of the original thaw volume, respectively (~10 minutes between steps). At the completion of step 4 the volume of the samples was 2X that of the initial volume. Steps 5 thru 7 used the Thaw Solution II from Table 6.1 and were added to the samples at 33, 66 and 200% of the volume after step 4. After all the dilutions are added the final volume of each sample is ~8 times that of the initial volume at thawing. The fully diluted samples are then centrifuged at 200 x g for 10 minutes then, supernate was removed and sperm pellets were resuspended in 20 µl of HEPES-199 and placed in a 37°C incubator for 3 hours. At 0 and 3 hours post-thaw incubation motility, membrane integrity and acrosomal status were analyzed as previously described.

Results

Experiment 6.1

Testes Collection, Epididymal Sperm Concentration and Straws frozen

For this experiment, three to five sets of testes were processed per day, for a total of at least 16 sets of testes. Epididymal sperm from the 16 pairs of testes produced a total of 16 0.5 mL straws which were frozen and 11 of those straws were later thawed for use in this experiment (n=11). When the epididymal sperm was pooled, the average total number of cells collected for each day was 190.25 x 10^6, with a range from 108 x 10^6 to 295 x 10^6.

Total number of epididymal sperm determined how many straws would be loaded and frozen for that day, three to five straws were frozen for each of the four processing days. Concentration of sperm per each of the 16 straws ranged from 59 X 10^6 to 44.7 x 10^6 cells per straw (Table 6.2).

Motility

The mean (±SEM) motility for the epididymal sperm at the initial and post-cool (4°C) time periods were 76.3±2.4% and 72.5±1.4%, respectively. Post-thaw epididymal sperm that was diluted with 0mM(Treatment A), 1mM(Treatment B) and 5mM(Treatment C) of pentoxifylline,

resulted in mean 0 hour post-thaw motility values of 55.5±4.1%, 58.2±3.0% and 65.5±3.1%, respectively. After 3 hour of post-thaw incubation at 37°C, total sperm motility for the 0mMtreatment was 36.4±2.4%, while the other two treatments (1mM and 5mM) had identical 3 hour post-thaw motility values of 38.2±1.8 and 38.2±2.6%, respectively. (Table 6.3).

No significant difference was detected ($P \geq 0.05$) when comparing between the initial motility and the motility of the sperm at the post-cool assessment. Predictably, there was a significant decrease ($P \leq 0.05$) between the initial motility value and both the 0 hour and 3 hour post-thaw values of all three thawing treatments (Table 6.3).

There was no significant difference between the post-cool value and the 0 hour post-thaw motility value for the 5mMtreatments. However, there was a significant difference between the post-cool value and the 0 hour post-thaw values for both the 0mMand 1mMtreatments. Also, all the 3 hour post-thaw motility values were significantly less than their 0 hour counterparts for all three treatments (Figure 6.1).

When comparing across the three treatments for the 0 hour post-thaw assessment, no significant difference was detected. Similarly, there was no significant difference when comparing across treatments for the 3 hour post-thaw evaluation time (Figure 6.2).

Membrane Integrity

The mean percentage of epididymal sperm with intact membranes at the initial assessment was 83.8±1.8%, after allowing samples to cool to 4°C (post-cool), the mean percentage was 84.5±2.7%. When evaluating the membrane integrity percentages of the epididymal sperm for the 0 hour post-thaw treatments, the 0mMtreatment had a value of 64.3±1.5% while the 1 and 5mMtreatments had values of 63.4±1.4 and 62.7±1.4%, respectively. At 3 hours post-thaw the 0nM, 1mMand 5mMtreatments had membrane integrity values of 55.7±2.0, 51.8±1.8 and 53.1±1.6, respectively (Table 6.4).

There was no significant difference ($P \geq 0.05$) between the initial and post-cool values when measuring percentage of intact membranes. However, the initial membrane integrity value was significantly greater than the all three of the 0 hour and 3 hour post-thaw treatments (Table 6.4).

The post-cool value was also significantly greater than all the 0 hour and 3 hour post-thaw values. Also, there was a significant decrease when comparing within treatments but across time, between the 0 hour and 3 hour post-thaw values for all treatments (Figure 6.3).

There was no significant difference detected when comparing among the three treatments for neither the 0 hour or 3 hour post-thaw time periods (Figure 6.4).

Table 6.2. Summary of the numbers of sets of testes processed, number of sperm recovered and number of straws frozen on each of four collection days.

Collection date	Sets processed	Total Cell Count	No. frozen per day	Concentration per straw
2/12/2009	4	295×10^6	5	59×10^6
2/19/2009	5	108×10^6	4	54×10^6
3/3/2009	3	134×10^6	3	44.7×10^6
3/4/2009	4	224×10^6	4	55.8×10^6
Daily average values	4	190.25×10^6	4	53.37×10^6
Total no.	16	-	16	-

The last two rows show the mean values for all the collection days and the overall total values for the experiment.

Table 6.3. Percent motility (mean±SEM) of initial, post-cool (4°C), 0 hour post-thaw and 3 hours post-thaw for pooled domestic cat epididymal sperm that were frozen, thawed then diluted with He-199 medium containing either 0, 1, or 5 nanomoles of pentoxifylline.

	Initial (Control)	Post-cool	0 hour post-thaw	3 hourss post-thaw
0mMPX (Trt A)	76.3 ± 2.4^a	72.5 ± 1.4^a	55.5 ± 4.1^b	36.4 ± 2.4^b
1mMPX (Trt B)	76.3 ± 2.4^a	72.5 ± 1.4^a	58.2 ± 3.0^b	38.2 ± 1.8^b
5mMPX (Trt C)	76.3 ± 2.4^a	72.5 ± 1.4^a	65.5 ± 3.1^a	38.2 ± 2.6^b

PX = pentoxifylline, Trt = Treatment; ^{a,b}Superscripts designate a significant difference only between initial motility to the post-cool and individual values for each pentoxifylline thawing treatments at each post-thaw time period ($P<0.05$).

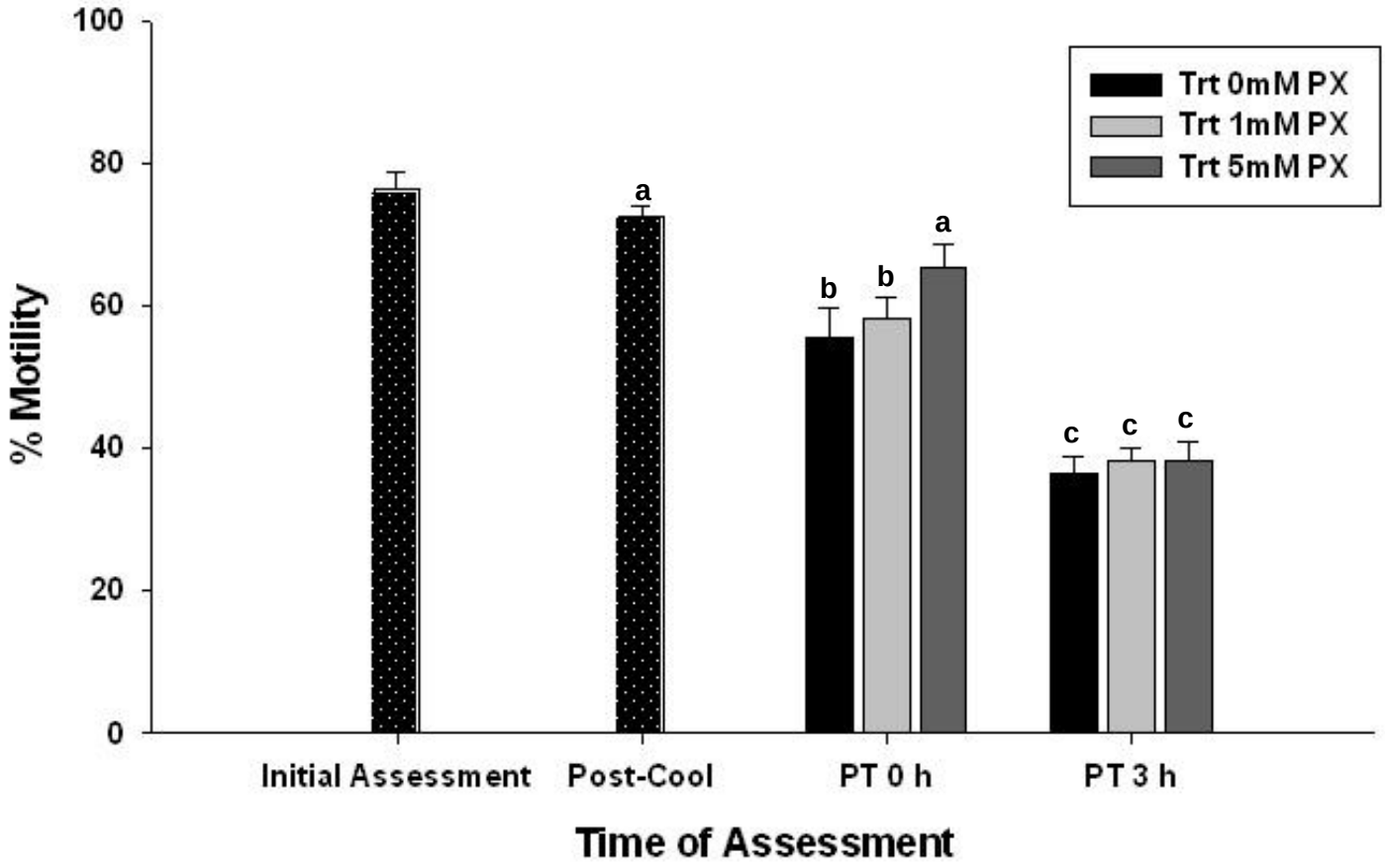

Figure 6.1. Percent motility (mean±SEM) of initial, post-cool (4°C), 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were processed, frozen and later thawed with a 0, 1 or 5 nanomolar pentoxifylline solution. [a,b,c]Mean values within treatments but across time intervals with different superscripts are significantly different ($P<0.05$).

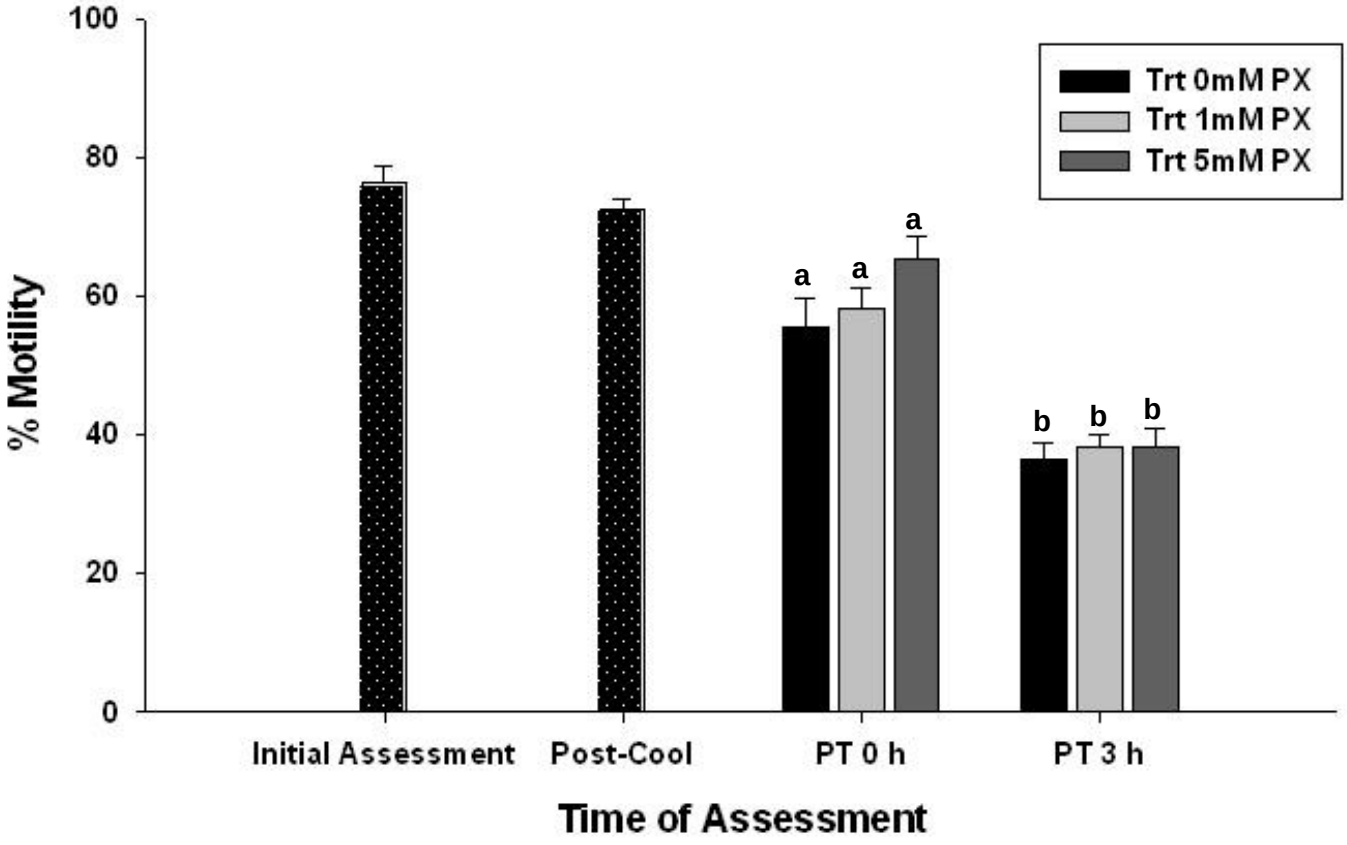

Figure 6.2. Percent motility (mean±SEM) of initial, post-cool (4°C), 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were processed, frozen and later thawed with a 0, 1 or 5 nanomolar pentoxifylline solution. [a,b]Mean values across treatments but within time intervals with different superscripts are significantly different ($P<0.05$).

Acrosomal Status

The initial mean value for feline epididymal sperm with intact acrosomes was 83.0±3.4%.After the sample was extended and cooled to 4°C the percent of intact acrosomes was 89.0±2.8%. The 0 hour post-thaw percentage of intact acrosomes for epididymal sperm that were treated with the 0nM, 1mMand 5mMpentoxifylline treatments were 68.9±1.8, 63.4±2.0 and 64.7±3.1%, respectively. While, the results for the 3 hour post-thaw assessment were 59.1±1.6, 51.6±1.5 and 53.5±1.2% for the 0nM, 1mMand 5mMof pentoxifylline treatments, respectively (Table 6.5).

There was no significant difference ($P \geq 0.05$) when comparing the acrosomal status between the initial and 4°C values There was however, a significant difference ($P \leq 0.05$) between the initial acrosomal status and all of the 0 hour and 3 hour post-thaw values (Table 6.5).

There was a significant decrease between the single post-cool value and all of the 0 hour post-thaw values. Acrosomal status continued to significantly decrease when comparing within the treatments, between the 0 hour and 3 hour post-thaw values for all treatments (Figure 6.5).

There was no significant difference detected when comparing across treatments for neither the 0 hour nor the 3 hour post-thaw values (Figure 6.6).

Experiment 6.2

Testes Collection, Epididymal Sperm Concentration and Straws Frozen

Sperm assessment values for this experiment are from thawing 9 straws (n= 9) frozen on the three separate days over the one week period. During that week, a total of 13 sets of testes were processed with a range of three to seven sets processed per day. The average number of cells collected for each day was 221.0 x 10^6, with a range from 108 x 10^6 to 284 x 10^6. Total number of epididymal sperm determined how many straws would be frozen for that day, 2 to 5 straws were frozen for each of the three processing days. Concentration of sperm per straw ranged from 53 x 10^6 to 55 x 10^6 cells (Table 6.6).

Motility

The mean (±SEM) initial and 4°C (post-cool) motility values of the epididymal sperm for this experiment was 80.0±2.9 and 83.3±3.3, respectively. Motility values for the 0mM (Treatment A), 1mM(Treatment B) and 5mM(Treatment C) treatments, at the 0 hour post-thaw evaluation were 60.0±2.5, 59.5±2.6 and 63.0±2.6, respectively. Total sperm motility after being held in a 37°C incubator for 3 hours post-thaw was 33.0±3.0, 44.0±2.7 and 39.0±2.3% for the 0nM, 1mMand 5mMtreatments, respectively (Table 6.7.).

Table 6.4. Percent membrane integrity (mean±SEM) of initial, post-cool (4°C), 0 hour post-thaw and 3 hours post-thaw for pooled domestic cat epididymal sperm that were frozen, thawed then diluted with He-199 medium containing either 0, 1, or 5 nanomoles of pentoxifylline.

	Initial (Control)	Post-cool	0 hours post-thaw	3 hours post-thaw
0mMPX (Trt A)	83.8±1.8[a]	84.5±2.7[a]	64.3±1.5[b]	55.7±2.0[b]
1mMPX (Trt B)	83.8±1.8[a]	84.5±2.7[a]	63.4±1.4[b]	51.8±1.8[b]
5mMPX (Trt C)	83.8±1.8[a]	84.5±2.7[a]	62.7±1.4[b]	53.1±1.6[b]

PX = pentoxifylline, Trt = Treatment; [a,b]Superscripts designate a significant difference only between initial motility to the post-cool and individual values for each pentoxifylline thawing treatments at each post-thaw time period (P<0.05).

Table 6.5. Percent acrosome integrity (mean±SEM) of initial, post-cool (4°C), 0 hour post-thaw and 3 hours post-thaw for pooled domestic cat epididymal sperm that were frozen, thawed then diluted with He-199 medium containing either 0, 1, or 5 nanomoles of pentoxifylline.

	Initial (Control)	Post-cool	0 hour post-thaw	3 hours post-thaw
0mMPX (Trt A)	83.0±3.4[a]	89.0±2.8[a]	68.9±1.8[b]	59.1±1.6[b]
1mMPX (Trt B)	83.0±3.4[a]	89.0±2.8[a]	63.4±2.0[b]	51.6±1.5[b]
5mMPX (Trt C)	83.0±3.4[a]	89.0±2.8[a]	64.7±3.1[b]	53.5±1.2[b]

PX = pentoxifylline, Trt = Treatment; [a,b]Superscripts designate a significant difference only between initial motility to the post-cool and individual values for each pentoxifylline thawing treatments at each post-thaw time period (P<0.05).

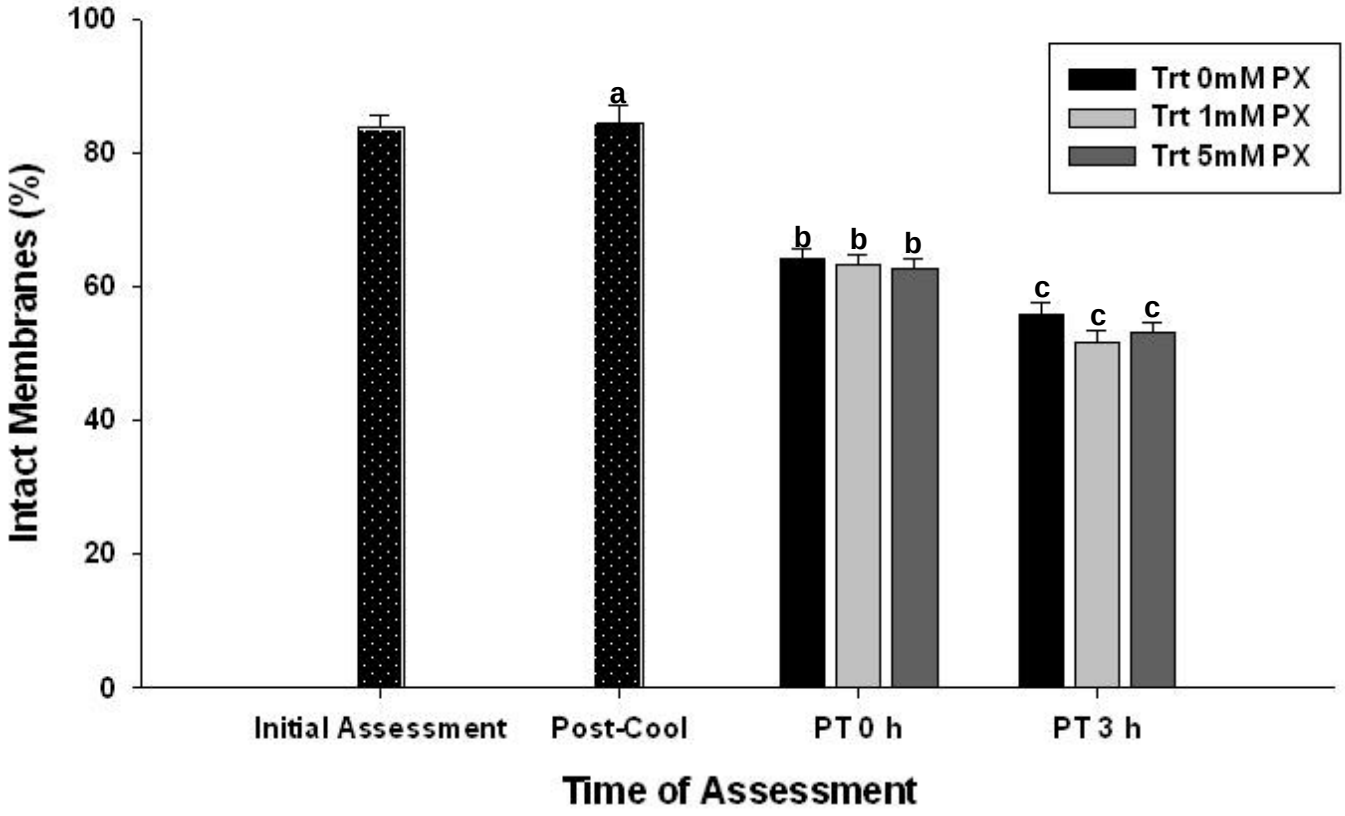

Figure 6.3. Membrane intact percentage (mean±SEM) of initial, post-cool (4°C), 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were processed, frozen and later thawed with a 0, 1 or 5 nanomolar pentoxifylline solution. [a,b,c]Mean values within treatments but across time intervals with different superscripts are significantly different ($P<0.05$).

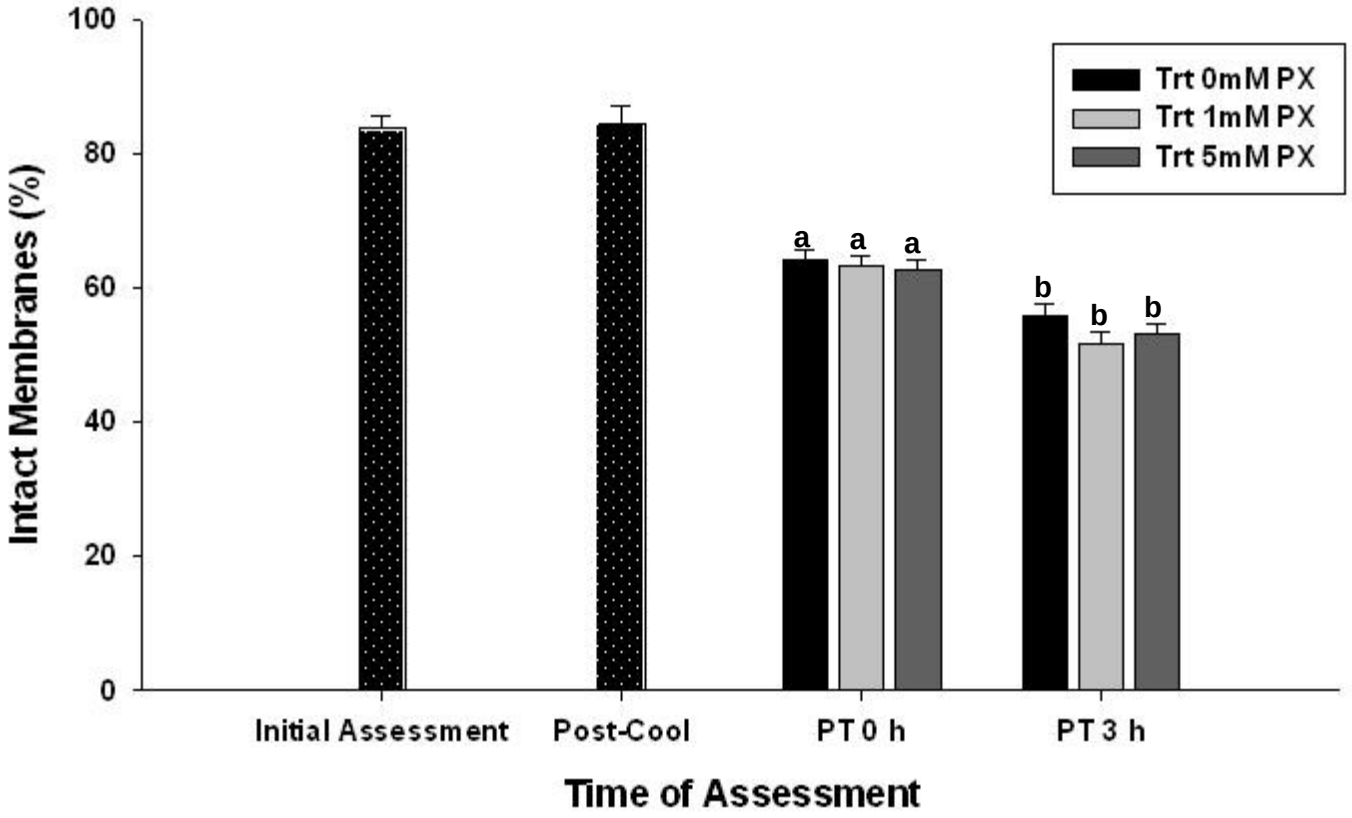

Figure 6.4. Membrane intact percentage (mean±SEM) of initial, post-cool (4°C), 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were processed, frozen and later thawed with a 0, 1 or 5 nanomolar pentoxifylline solution. [a,b]Mean values across treatments but within time intervals with different superscripts are significantly different ($P<0.05$).

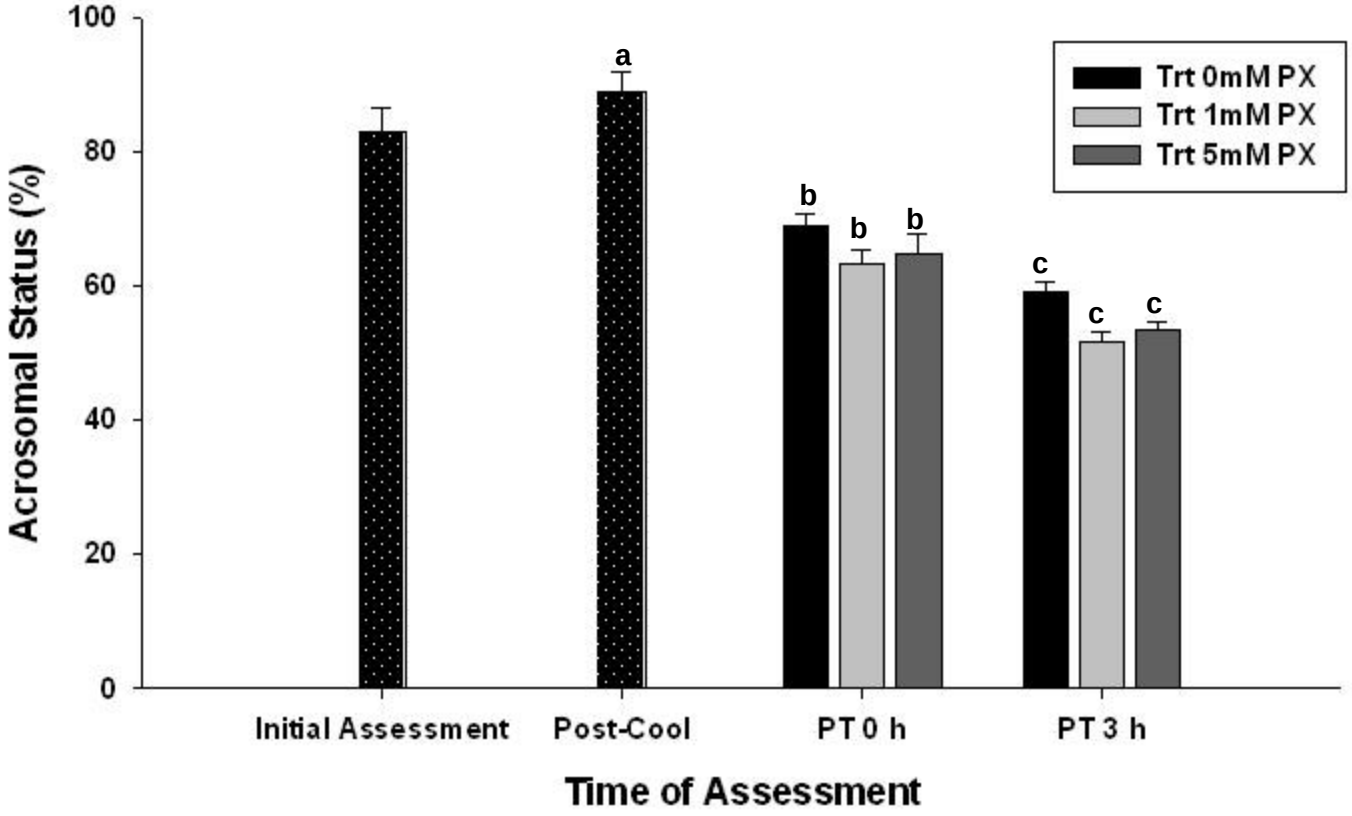

Figure 6.5. Acrosomal status percentage (mean±SEM) of initial, post-cool (4°C), 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were processed, frozen and later thawed with a 0, 1 or 5 nanomolar pentoxifylline solution. [a,b,c]Mean values within treatments but across time intervals with different superscripts are significantly different ($P<0.05$).

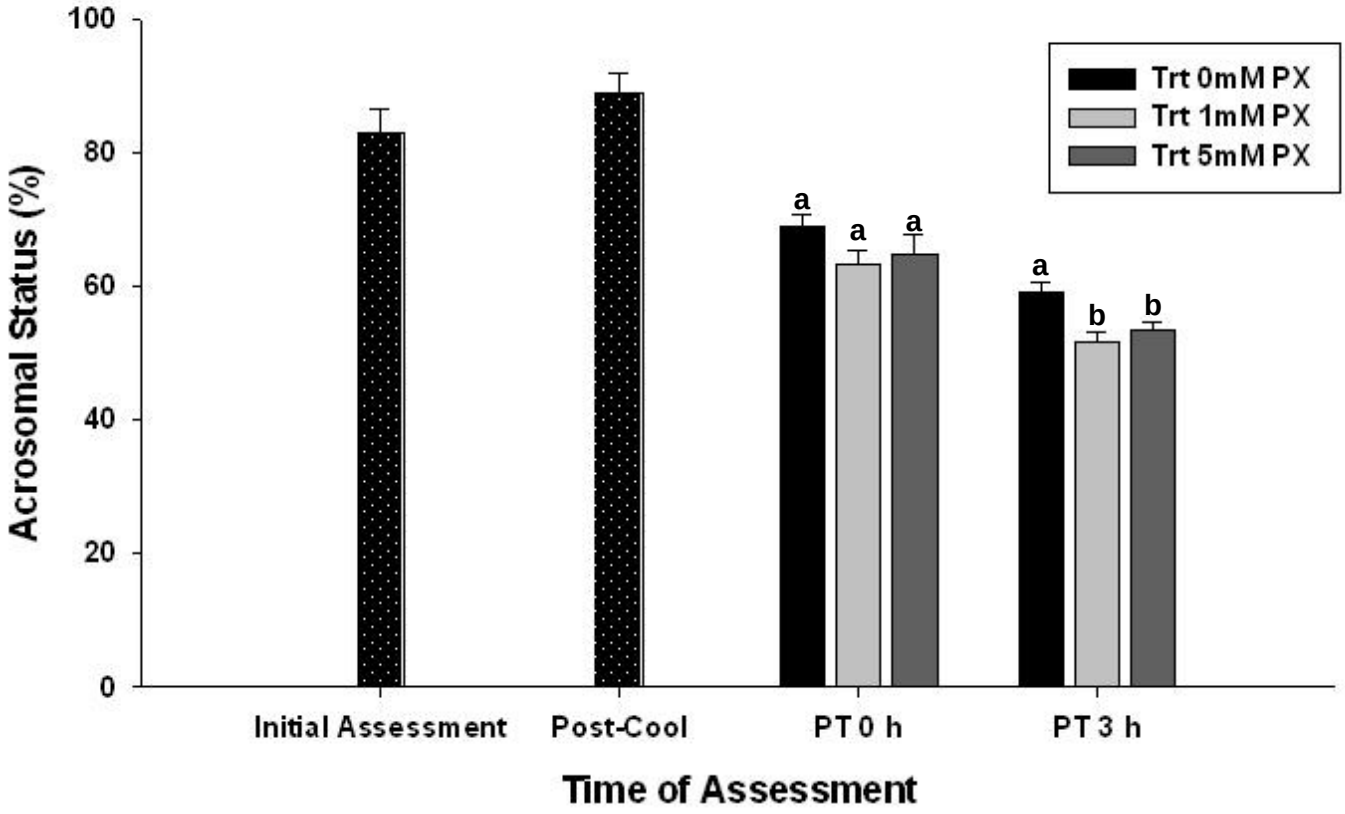

Figure 6.6. Acrosomal status percentage (mean±SEM) of initial, post-cool (4°C), 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were processed, frozen and later thawed with a 0, 1 or 5 nanomolar pentoxifylline solution. [a,b]Mean values across treatments but within time intervals with different superscripts are significantly different ($P<0.05$).

There was no significant difference ($P \geq 0.05$) when comparing between the initial and 4°C assessments. However, there was a significant difference ($P \leq 0.05$) when comparing the initial motility value to the 0 hour and 3 hour post-thaw values for all three treatments (Table 6.7).

The 4°C post-cool value was significantly greater than all three treatments at the 0 hour and 3 hour post-thaw assessments. Also, when comparing within treatments all three 0 hour post-thaw values were significantly different from their 3 hours post-thaw counterparts (Figure 6.7).

When comparing among treatment groups, there was no significant difference at the 0 hour post-thaw assessment time. However, at the 3 hour post-thaw assessment, there was a significant difference between the 0mMand 1mMtreatments, but the 5mMtreatment was not significantly different from either the 0 or 1mMtreatments (Figure 6.8).

Membrane Integrity

The mean (±SEM) value for the initial membrane integrity assessment of the feline epididymal sperm was 83.0±3.8%. After extending the sample and allowing it to cool to 4°C, the post-cool evaluation had a value of 86.7±1.2%. The membrane integrity at the 0 hour post-thaw period for the 0nM, 1mMand 5mMpentoxifylline treatments were 66.6±2.3, 69.7±2.0 and 68.4±1.6%, respectively. Following the 0 hour post-thaw readings, treatments were placed into a 37°C incubator and allowed to sit for 3 hours, at the completion of the three hours the membrane integrity values for the 0nM, 1mMand 5mMtreatments were 60.0±1.8, 56.8±1.8 and 54.9±1.6%, respectively (Table 6.8).

There was no significant difference ($P \geq 0.05$) between the initial and post-cool values. In contrast, all of the 0 hour and 3 hour post-thaw values were significantly lower ($P \leq 0.05$) than the initial membrane integrity value (Table 6.8).

The post-cool value was significantly higher than the 0 hour and the 3 hour post-thaw values for all three treatments. Furthermore, all three of the 0 hour post-thaw values were significantly greater than their 3 hour post-thaw counterparts for all three treatments (Figure 6.9).

Although, there was no significant difference across treatments for the 0 hour post-thaw assessment, at 3 hours post-thaw the percentage of membrane intact cells for the 0mM treatment was significantly different ($P \leq 0.05$) from the 5mM treatment. However, the 1mM treatment was not significantly different from either the 0mM or 5mM treatments (Figure 6.10).

Table 6.6. Summary of the numbers of sets of testes processed, number of sperm recovered and number of straws frozen on each of three collection days.

Collection date	Sets processed	Total Cell Count	No. frozen per day	Concentration per straw
4/1/2009	3	108×10^6	2	54×10^6
4/2/2009	3	284×10^6	2	55×10^6
4/8/2009	7	271×10^6	5	53×10^6
Daily average values	4.3	221×10^6	3	54×10^6
Total no.	13	-	9	-

The last two rows show the mean values for all the collection days and the overall total values for the experiment.

Table 6.7. Percent motility (mean±SEM) of initial, post-cool (4°C), 0 hour post-thaw and 3 hours post-thaw for pooled domestic cat epididymal sperm that were frozen, thawed then diluted with He-199 medium containing either 0, 1, or 5 nanomoles of pentoxifylline.

	Initial (Control)	Post-cool	0 hour post-thaw	3 hours post-thaw
0mMPX (Trt A)	80.0 ± 2.9^a	83.3 ± 3.3^a	60.0 ± 2.5^b	33.0 ± 3.0^b
1mMPX (Trt B)	80.0 ± 2.9^a	83.3 ± 3.3^a	59.5 ± 2.6^b	44.0 ± 2.7^b
5mMPX (Trt C)	80.0 ± 2.9^a	83.3 ± 3.3^a	63.0 ± 2.6^b	39.0 ± 2.3^b

PX = pentoxifylline, Trt = Treatment; ^{a,b}Superscripts designate a significant difference only between initial motility to the post-cool and individual values for each pentoxifylline thawing treatments at each post-thaw time period ($P<0.05$).

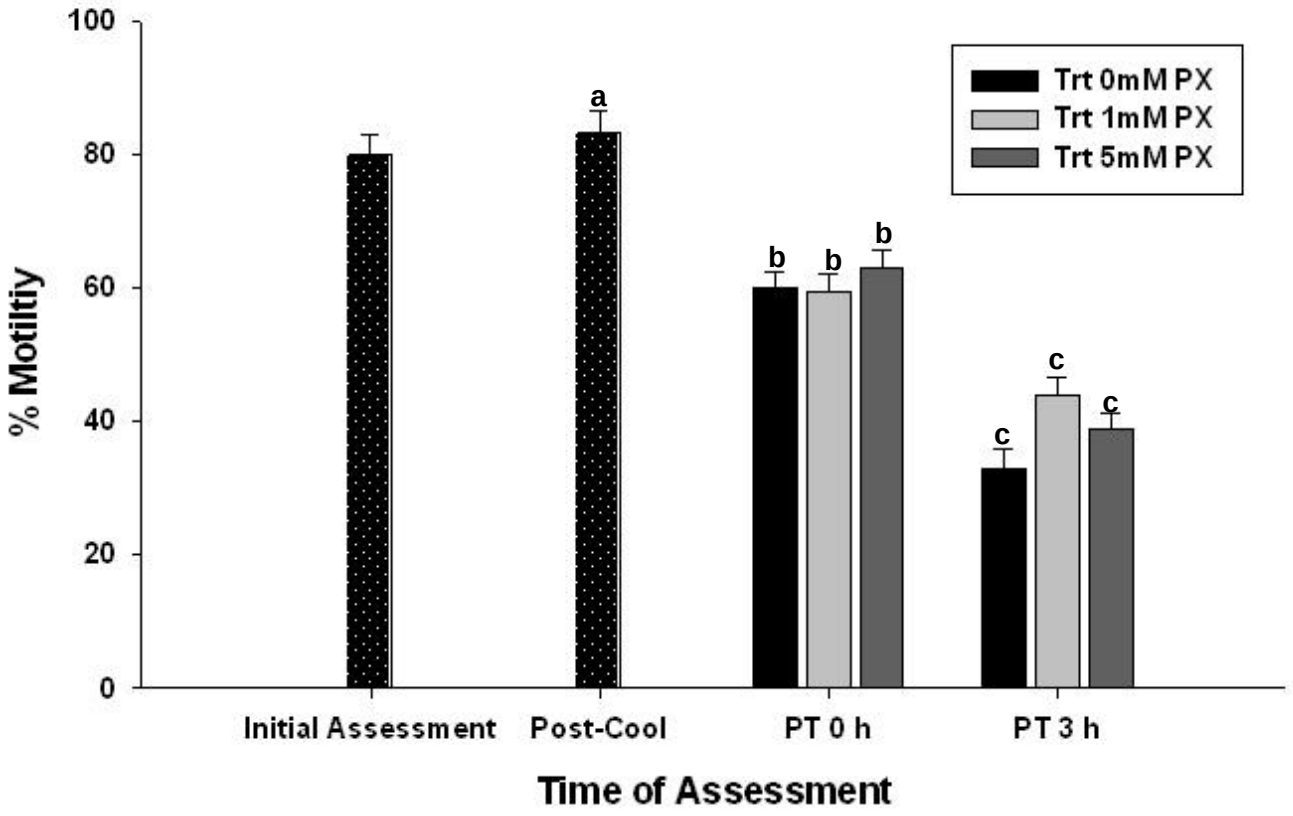

Figure 6.7. Motility percentage (mean±SEM) of initial, post-cool (4°C), 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were processed, frozen and later thawed with a 0, 1 or 5 nanomolar pentoxifylline solution. [a,b,c]Mean values within treatments but across time intervals with different superscripts are significantly different ($P<0.05$).

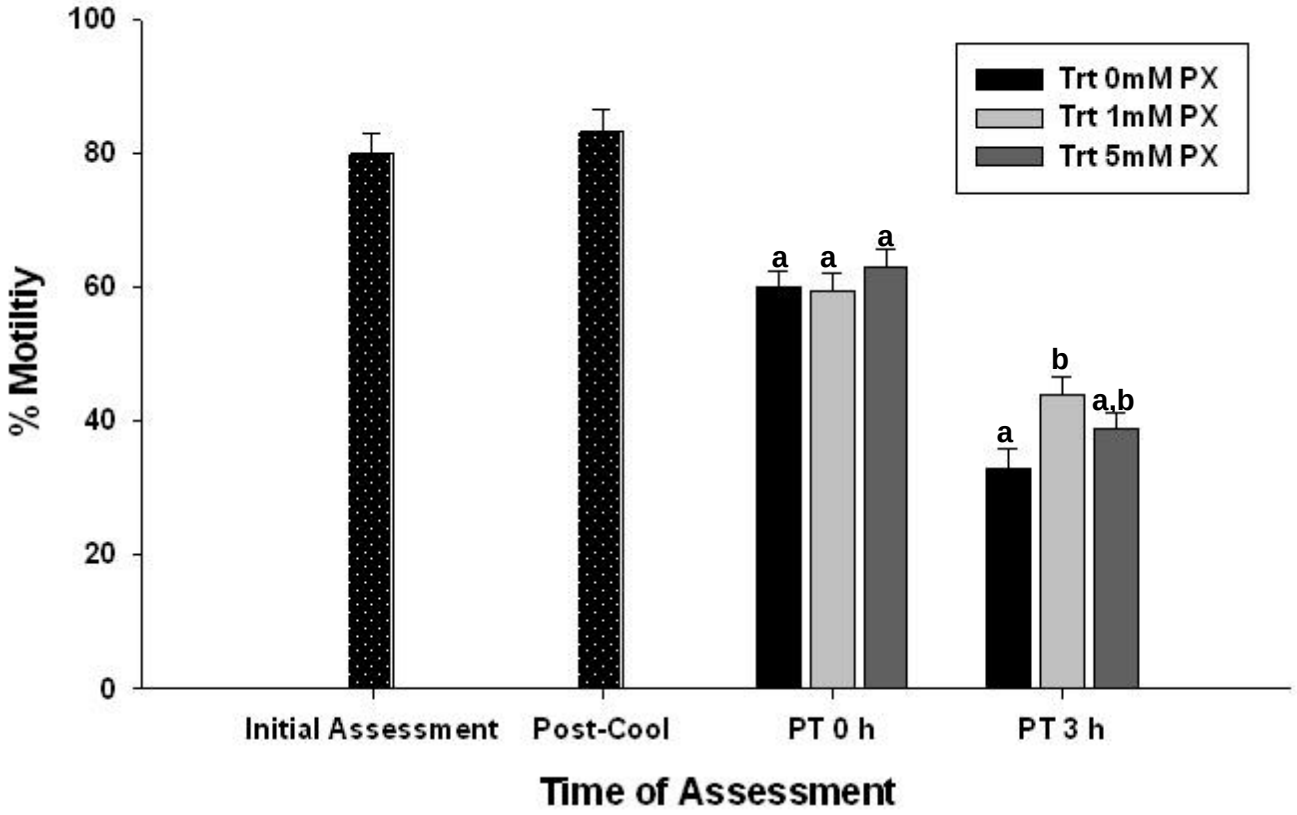

Figure 6.8. Motility percentage (mean±SEM) of initial, post-cool (4°C), 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were processed, frozen and later thawed with a 0, 1 or 5 nanomolar pentoxifylline solution. [a,b]Mean values across treatments but within time intervals with different superscripts are significantly different ($P<0.05$).

Acrosomal Status

The mean Acrosomal status values for the initial and post-cool (4°C) assessments were 93.0±2.1 and 93.7±1.9%, respectively. After thawing and diluting samples with the pentoxifylline treatments the 0 hour post-thaw Acrosomal status values were 73.1±2.1, 71.7±1.9 and 68.3±2.1% for the 0nM, 1mMand 5mMtreatments, respectively. The 3 hour post-thaw assessment values for the 0nM, 1mMand 3mMtreatments were 62.2±2.9, 61.3±2.0 and 58.8±2.4, respectively (Table 6.9).

There was no significant difference ($P \geq 0.05$) between the initial and the post-cool values. However, the initial acrosomal status percentage was significantly greater ($P \leq 0.05$) than all the 0 hour and 3 hours post-thaw values (Table 6.9).

Like the initial value for acrosomal status, the post-cool value was significantly better than the three 0 hour and the three 3 hours post-thaw values. When comparing within the treatments, all the 0 hour post-thaw treatments were significantly different from all the 3 hours post-thaw values (Figure 6.11).

The percentages of intact acrosomes across treatments at 0 hour and 3 hours post-thaw were not significantly different (Figure 6.12).

Discussion

Pentoxifylline is a small molecule used to increase sperm motility and is used most often in human assisted reproductive laboratories (Calogero et al., 1998). Some examples of its use in domestic species are Stachecki et al. (1994), Stachecki et al. (1995) in the domestic cat , Maxwell et al. (1995) in the goat and Milani et al. (2010) in the dog. There are certain time points when it would be beneficial to expose sperm to pentoxifylline, for instance, when collecting epididymal sperm it is often necessary to wash and centrifuge the sperm sample through a density gradient (Filliers et al., 2008). This density gradient separates live motile sperm from blood, tissue cells and immobile sperm (Phillips et al., 2012). Theoretically one could increase the number of sperm recovered the density gradient, by increasing the percentage of motile sperm going into the gradient. Holt et al. (1997) have reported that boar sperm motility (velocity and duration) were good in vitro indicators of boar fertility. Another crucial time to stimulate sperm motility could be during a post-thaw evaluation to record an estimation of a male fertility before artificial insemination or when incubating sperm with oocytes during in vitro fertilization.

For these two experiments the two most notable events for feline sperm motility was in Experiment 6.1, when the mean 0 hour post-thaw motility value for sperm treated with 5mMpentoxifylline did not decrease enough, even with the stress of cryopreservation, to be

Table 6.8. Percent intact membranes (mean±SEM) of initial, post-cool (4°C), 0 hour post-thaw and 3 hour post-thaw for pooled domestic cat epididymal sperm that were frozen, thawed then diluted with He-199 medium containing either 0, 1, or 5 nanomoles of pentoxifylline.

	Initial (Control)	Post-cool	0 hours post-thaw	3 hours post-thaw
Trt 0mMPX	83.0±3.8[a]	86.7±1.2[a]	66.6±2.3[b]	60.0±1.8[b]
Trt 1mMPX	83.0±3.8[a]	86.7±1.2[a]	69.7±2.0[b]	56.8±1.8[b]
Trt 5mMPX	83.0±3.8[a]	86.7±1.2[a]	68.4±1.6[b]	54.9±1.6[b]

PX = pentoxifylline; [a,b]Superscripts designate a significant difference only between initial motility to the post-cool and individual values for each pentoxifylline thawing treatments at each post-thaw time period ($P<0.05$).

Table 6.9. Percent acrosome integrity (mean±SEM) of initial, post-cool (4°C), 0 hour post-thaw and 3 hours post-thaw for pooled domestic cat epididymal sperm that were frozen, thawed then diluted with He-199 medium containing either 0, 1, or 5 nanomoles of pentoxifylline.

	Initial (Control)	Post-cool	0 hours post-thaw	3 hours post-thaw
Trt 0mMPX	93.0±2.1[a]	93.7±1.9[a]	73.1±2.1[b]	62.2±2.9[b]
Trt 1mMPX	93.0±2.1[a]	93.7±1.9[a]	71.7±1.9[b]	61.3±2.0[b]
Trt 5mMPX	93.0±2.1[a]	93.7±1.9[a]	68.3±2.1[b]	58.8±2.4[b]

PX = pentoxifylline; [a,b]Superscripts designate a significant difference only between initial motility to the post-cool and individual values for each pentoxifylline thawing treatments at each post-thaw time period ($P<0.05$).

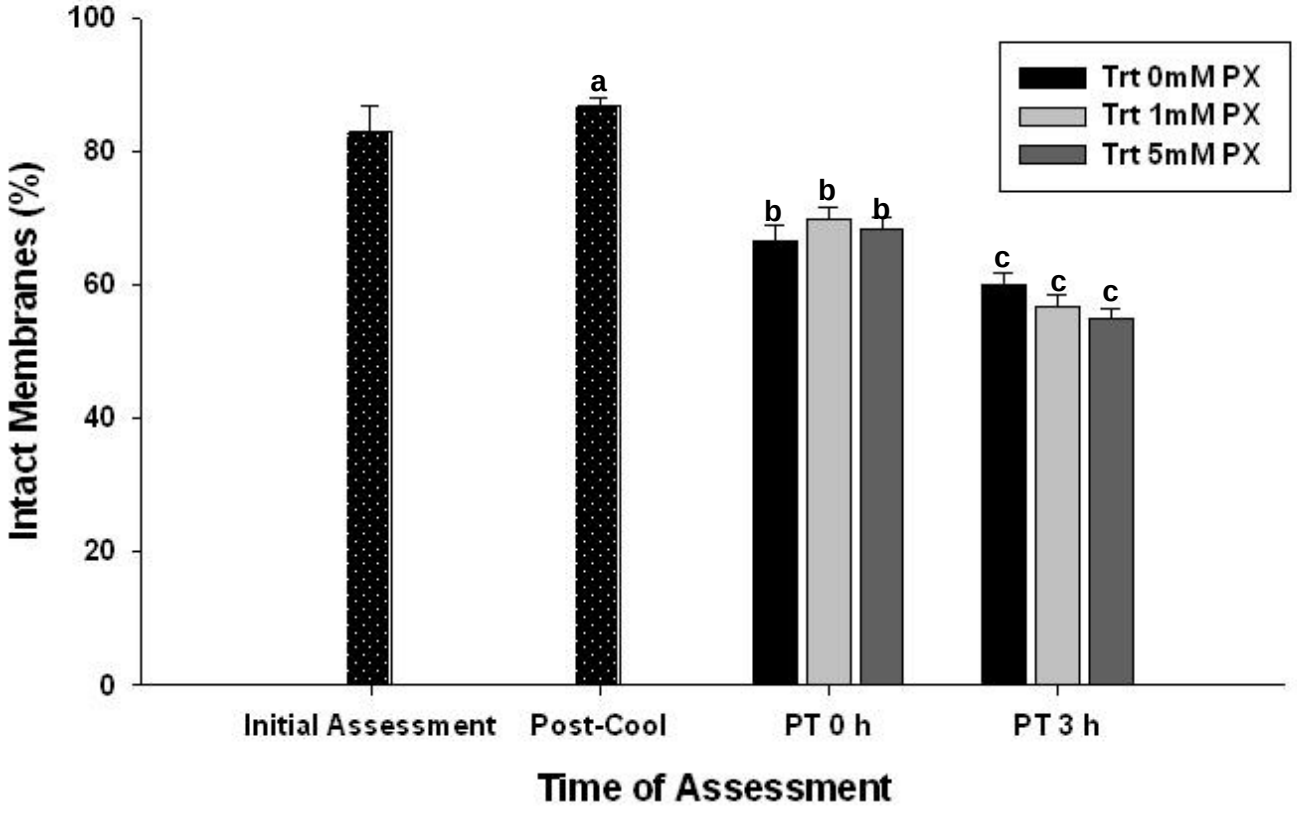

Figure 6.9. Membrane integrity percentage (mean±SEM) of initial, post-cool (4°C), 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were processed, frozen and later thawed with a 0, 1 or 5 nanomolar pentoxifylline solution. [a,b,c]Mean values within treatments but across time with different superscripts are significantly different ($P<0.05$).

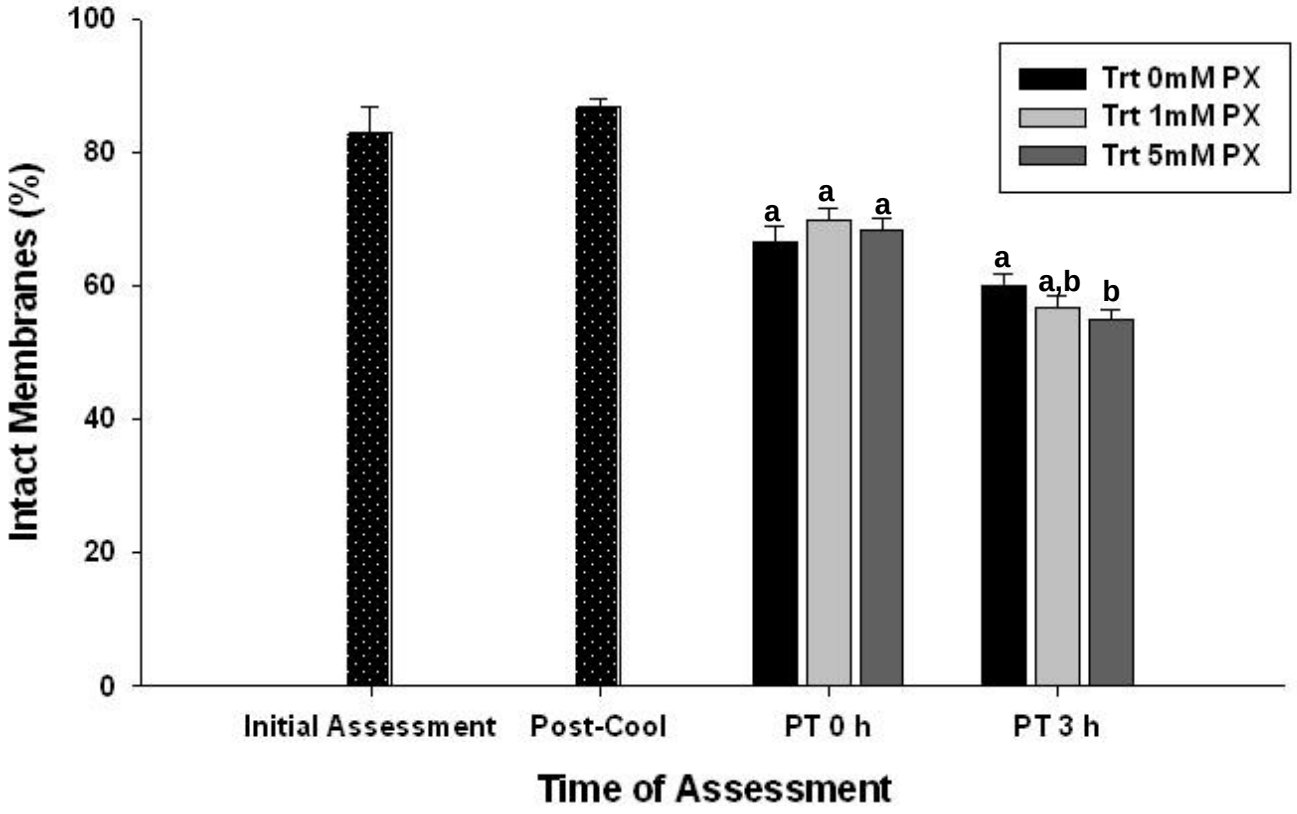

Figure 6.10. Membrane integrity percentage (mean±SEM) of initial, post-cool (4°C), 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were processed, frozen and later thawed with a 0, 1 or 5 nanomolar pentoxifylline solution. [a,b]Mean values across treatments but within time with different superscripts are significantly different ($P<0.05$).

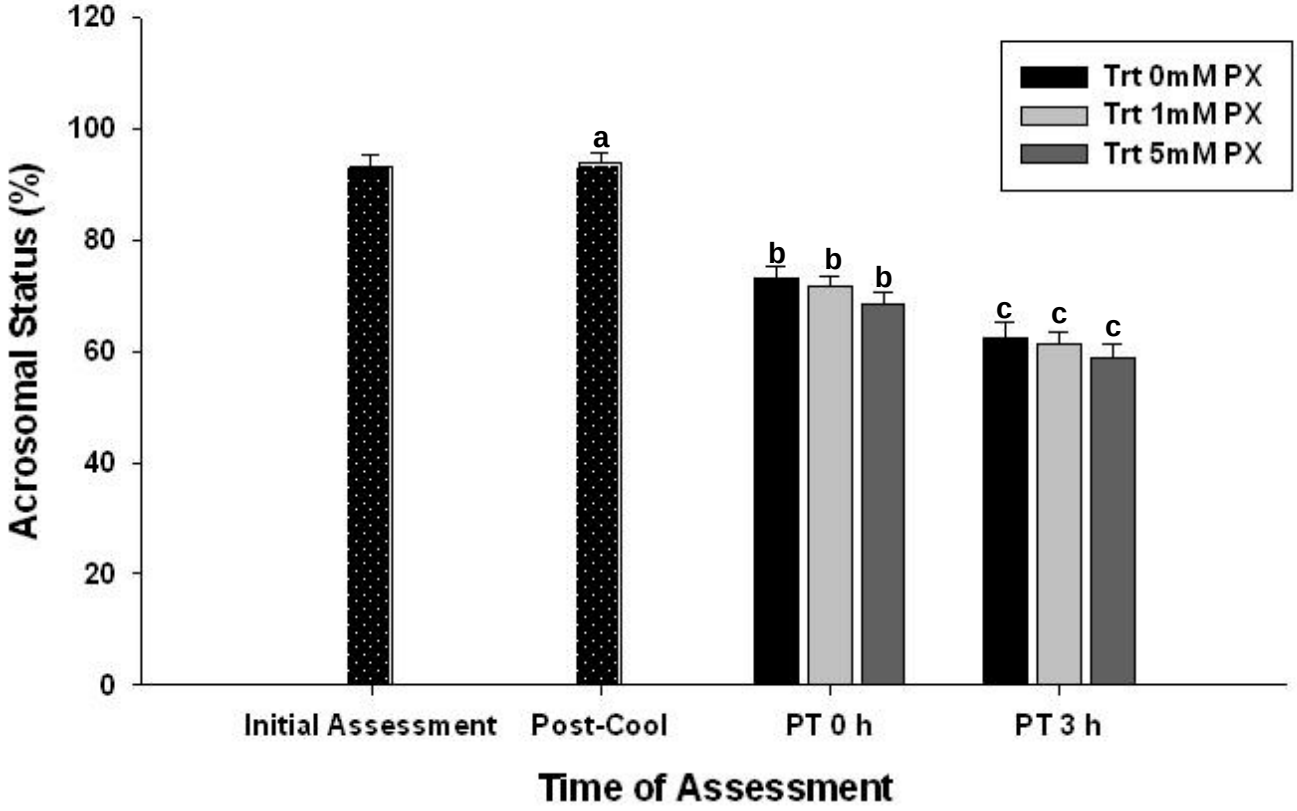

Figure 6.11. Acrosomal status percentage (mean±SEM) of initial, post-cool (4°C), 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were processed, frozen and later thawed with a 0, 1 or 5 nanomolar pentoxifylline solution. [a,b,c]Mean values within treatments but across time with different superscripts are significantly different ($P<0.05$).

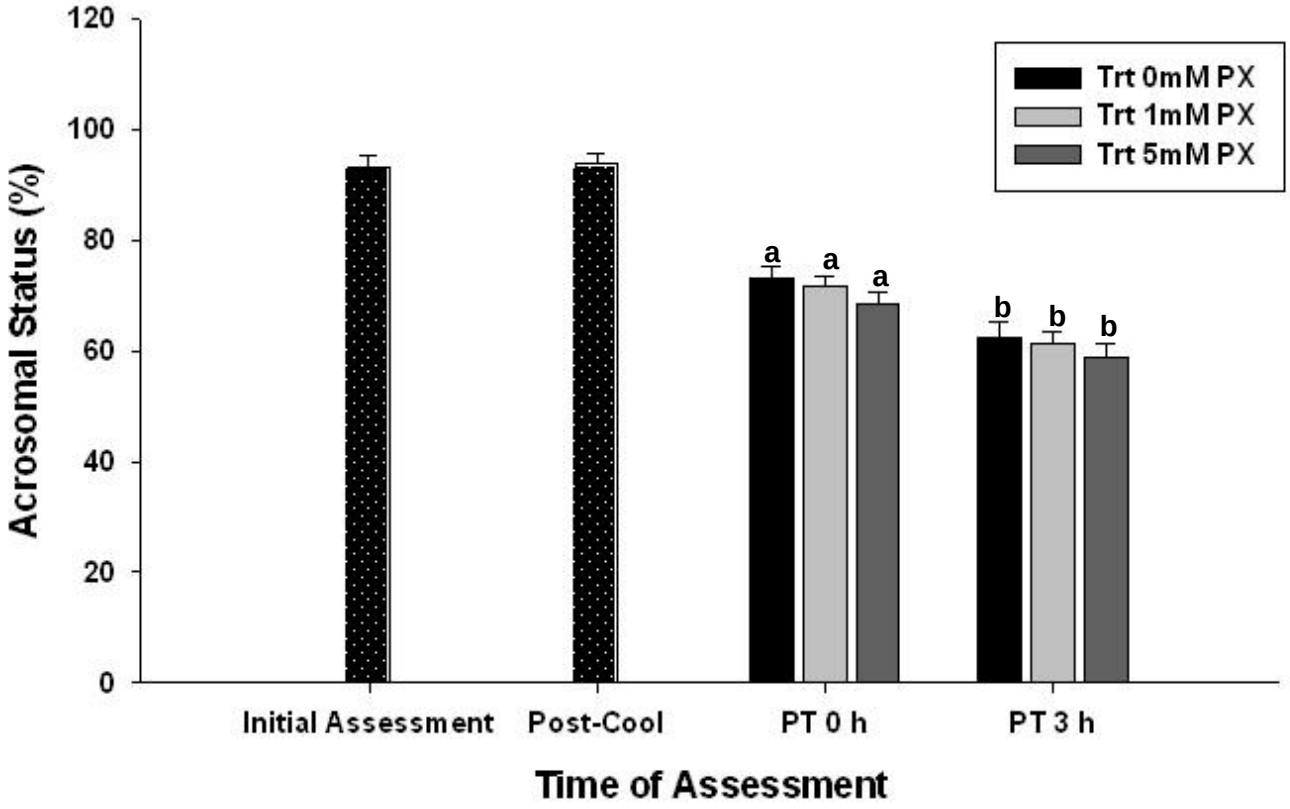

Figure 6.12. Acrosomal status percentage (mean±SEM) of initial, post-cool (4°C), 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were processed, frozen and later thawed with a 0, 1 or 5 nanomolar pentoxifylline solution. [a,b]Mean values across treatments but within time with different superscripts are significantly different ($P<0.05$).

significantly different from the initial or the post-cool motility values. The second event occurred in Experiment 6.2 when the 1mM pentoxifylline treatment had significantly greater sperm motility than the 0mM treatment for the 3 hour post-thaw assessment.

In both experiments, feline sperm samples were not divided into treatments until the 0 hour post-thaw assessment, so as a result there is only one initial and one post-cool motility value. There was no significant difference between the initial and post-cool motility values for either experiment. Also, in both experiments there was no significant difference among treatments for any time point when measuring sperm motility. Silva et al. (2009) also showed that pentoxifylline had no affect on the post-thaw motility of frozen-thawed ejaculated equine sperm. However, our results contradict the findings by Stachecki et al. (1994) who reported that although pentoxifylline had no effect on the motility of freshly collected epididymal cat sperm, there was a significant increase in post-thaw sperm motility parameters when pentoxifylline was used to treat thawed cat epididymal sperm.

When evaluating the sperm membrane integrity results, the only significant difference was noted in Experiment 6.2 at the 3 hour post-thaw assessment when the 0mMpentoxifylline treatment had a membrane integrity value that was significantly greater than the 5mMpentoxifylline treatment. The membrane integrity results of our study correlate with the results reported by Ortgies et al. (2012), who reported that the membrane integrity of equine ejaculated sperm was not affected by pentoxifylline or any of the other sperm stimulants evaluated.

Other than the expected decline in overall acrosomal status resulting from cryopreservation, there was only one significant difference when comparing among treatments (0nm, 1mMand 5nm) in both experiments. Our acrosomal status results in the cat, contradict the findings by Ortgies et al. (2012) who reported that pentoxifylline was the only agent tested that increased the percentage of live-capacitated equine sperm when compared with control sperm.

A possible explanation for the discrepancies between our results and other reports on the effects of pentoxifylline on domestic cat epididymal sperm is the exposure time that we allowed pentoxifylline to be in contact with the sperm. Differences in published results on the same species but from different labs are not uncommon. Stephens et al. (2013) reported that pentoxifylline improved equine post-thaw values for motility, progressive motility, semen curvilinear velocity, average path velocity, straight line velocity, and sperm longevity over the untreated control. Gustai et al. (2013) reported contradicting results, noting that although pentoxifylline increased the pre-freeze equine sperm values for motility, progressive motility,

straight line velocity, curvilinear velocity, and percentage of rapid sperm. The pentoxifylline had no significant effect on any the post-thaw sperm motility parameters.

It may have may have been beneficial if we would have analyzed the post-thaw sperm before introducing pentoxifylline. However, given that our results are similar to what was noted in the stallion, we were initially satisfied with our findings. Nevertheless, due to the difference in sperm motility response compared with other feline ejaculated and epididymal sperm studies further research would be encouraged. Since our results showed no negative effects of pentoxifylline and there is a possible benefit to its use with feline spermatozoa, it is recommended to use pentoxifylline in future experiments.

CHAPTER VII
SURVIVAL OF DOMESTIC CAT EPIDIDYMAL SPERM AFTER TEMPORARY COOL STORAGE OR CRYOPRESERVATION IN DEFINED EXTENDERS

Introduction

The ability to sex sort spermatozoa (sperm) could aid in the reproductive management of genetically valuable exotic species that are in captive breeding programs. Since a portion of sperm are lost during the flow cytometry process (Evans, 2009; Seidel, 2009), the more live motile sperm that enter the sorting procedure, should result in a higher overall sperm recovery rate. Usually sperm sorting is performed on freshly collected or extended and cooled sperm that have not yet been frozen. Pope et al. (2009) collected ejaculated sperm from a domestic tom cat, extended the semen and shipped it overnight to a sperm sorting facility. Subsequently, they reported the first kittens to be born from sex gender sorted sperm (Pope et al., 2009).

In species, such as bovine (Baker et al., 1953), ovine (Lezama et al., 2001) or equine (Pickett et al., 1975) ejaculated sperm concentrations are great enough that sperm loss due to the sorting process can be tolerated. In contrast, when collecting feline epididymal or even ejaculated sperm the total volumes are in the microliters and the sperm concentrations are below 5 million/mL (unpublished data). Therefore, actions must be made to ensure that the highest possible number of live sperm are entering the sorting process. A possible solution for low sperm numbers would be to combine sperm collections from different days. However to accomplish this, sperm collected from earlier days would either need to be cryopreserved or stored at a cooled temperature in an extender that could maintain sperm viability for up to a week.

The benefits egg yolk provides to mammalian sperm during the cooling and freezing process are undeniable. However, finding an effective extender that does not contain egg yolk would eliminate the need to remove the egg yolk particles before sperm sorting. The elimination of this step would also mean a decrease in the stress that centrifugation imposes on the sperm and thereby, allowing more sperm to be available for the sorting process. Furthermore, the survival of sperm and other cell types is not solely dependent on the presence of egg yolk in a freezing medium. This point is demonstrated by the recent successful freezing and thawing of other feline gametes (Galiguis et al., 2014) and embryos (Pope et al., 2012).

The objectives of the following studies were: (1) to evaluate two commercially available, clear extenders that are marketed for the cool storage of semen, on their ability to maintain the viability of domestic cat epididymal sperm when stored at 4°C and then compared with a 2% egg yolk extender, (2) to determine the effectiveness of these cool storage extenders on their

ability to protect domestic cat epididymal sperm during cryopreservation and (3) to determine the effectiveness of a semen extender that contained only salts, buffers and glycerol (no egg yolk), when freezing sperm from room temperature, and after cooling to 4°C and how they compared with sperm frozen in a conventional 2% egg yolk extender that was frozen after cooling to 4°C.

Materials and Methods

Experimental Design

Experiment 7.1

The objective of this experiment was to determine which of three semen extenders (2% Egg Yolk TesT (Treatment A), Biolife® (Treatment B) and BioXCell® (Treatment C)), maintained the highest epididymal sperm survivability when used to store domestic cat sperm at 4°C for 72 h. Testes were collected daily Monday thru Friday from local veterinary clinics in the New Orleans area. Epididymides where then processed and the epididymal sperm that was recovered was extended and cooled all on the same day that they were collected from the epididymides. Over that 4 month period only 5 daily collections resulted in enough epididymal sperm to be spread sufficiently across the three treatments. A total of 24 sets of domestic cat testes were used across the 5 different processing days (n = 5) reported in this expermiment. During those 5 days, a range of 2 to 9 sets of testes were processed per day, and on each of the 5 processing days, all sperm collected was pooled and equally divided across the three different semen extenders mentioned above.

After the pooled epididymal sperm was collected, washed and centrifuged they were evaluated for initial pre-treatment analysis and divided into 3 separate 1.6 mL aliquot tubes and the 3 treatment extenders were added. Each aliquot was placed in its own 22°C water bath, and placed into a 4°C walk-in cooler and allowed to cool for 24 h. Future sperm evaluations were recorded at 24, 48 and 72 hours from when samples were placed in the 4°C.

Experiment 7.2

This experiment was to test the two most effective extenders from the previous experiment on their freezing protection of domestic cat epididymal sperm (2% Egg Yolk TesT (Treatment A), BioXCell® (Treatment B)). Testes were collected daily Monday thru Friday from local veterinary clinics in the New Orleans area, enough daily epididymal sperm was collected to disperse across the treatments on only a fraction of those days. Data for this experiment were derived from processing 36 sets of testes on six different days over a 3 month period. A total of 22 (0.25 cc) and 2 (0.5 cc) straws were frozen with a range of two to eight frozen per day, and from 24 frozen straws 20 (n = 10) were thawed for this experiment (ten straws per treatment).

Sperm were collected from minced epididymides and then washed, centrifuged, analyzed and divided into either TesT extender with 2% egg yolk (Treatment A) or into the commercial extender BioXCell® (Treatment B). Before dividing into treatments an initial motility was taken to confirm sample was good enough to continue with experiment, then sample was split into treatments. Once divided and extended to the desired concentrations, samples were set into separate but equal laboratory temperature (22°C) water baths and placed in a 4°C walkin refrigerator and allowed to reach 4°C. After samples reached 4°C a second motility was recorded as well as the first membrane integrity and acrosomal status reading. Upon completion of the 4°C readings (post-cool), samples were further extended to their final concentrations, loaded into straws and frozen in a -80°C freezer for 20 min before being plunged and held in liquid nitrogen until thawed. Two more assessments were recorded for motility, membrane integrity and acrosomal status at 0 and 3 hours post-thaw.

Table 7.1. Experimental design for experiment 7.2. This table illustrates what readings were recorded and at what time point they were recorded for this experiment.

	Initial	Post-Cool	0 hour PT	3 hours PT
Pre Trt.	Mot	--	--	--
2% EY	--	Mot, MI, AS	Mot, MI, AS	Mot, MI, AS
BioXCell®	--	Mot, MI, AS	Mot, MI, AS	Mot, MI, AS

Mot = Motility, MI = Membrane integrity, AS = Acrosomal status

Experiment 7.3

Realizing that the TesT extender with 2% egg yolk (Treatment A) was the most affective extender from the previous two experiments, but still wanting an extender with no egg yolk we decided to compare the TesT 2% egg yolk extender versus the TesT extender with 0% egg yolk (Treatment B). Also a third treatment was added which involved using the TesT extender with 0% egg yolk but freezing from room temperature (22°C) (Treatment C), instead of cooling sperm to 4°C then freezing. Domestic cat testes were harvested from local veterinary clinics in the New Orleans area for a two month period, however only 10 of those days resulted in enough epididymal sperm to divide across the three treatments. For those 10 processing days a total of 60 sets of testes were collected resulting in a total of 33 (0.25 cc) frozen semen straws. From the 33 total straws, 30 were thawed for this experiment 10 from each treatment (n = 10).

Epididymides were minced and epididymal sperm were collected, washed and centrifuged to acquire a clean and highly concentrated sperm pellet that was divided equally into the three treatments (2%EY (Treatment A), 0%EY (Treatment B), RT 0%EY (Treatment C)). Before dividing the pellet into treatments an initial motility, membrane integrity and acrosomal status were obtained. After the sample was analyzed and extended, the 0 and 2%EY treatments were placed in 22°C water baths and then allowed to cool to 4°C in a walkin cooler. The RT 0%EY treatment was then further extended 1:1 with the second glycerated part of RT 0%EY extender containing 12% glycerol. The fully extended sample was loaded into 0.25 cc straws and frozen on a dry ice block for 20 minutes. After cooling to 4°C the remaining two treatments (0% and 2%EY) were additionally extended 1:1 with their perspective second parts containing 12% glycerol. These samples were also loaded into 0.25 cc straws frozen on a dry ice block, plunged and held in liquid nitrogen until being thawed. One straw from each treatment was thawed on the same day (n=10) and evaluated for motility, membrane integrity and acrosomal status at 0 and 3 hours post-thaw.

The 2%EY treatment was derived in the laboratory from a commercially available TesT buffer extender containing 20% egg yolk (Test Yolk Buffer). The 0%EY treatment was the same TesT buffer extender as the 2% EY treatment except instead of modifying the egg yolk percentage down to 2%, no egg yolk was added at all. The RT 0%EY treatment was the exact same extender, but the difference between the treatments was that the 0%EY and the 2%EY treatments were cooled down to 4°C before being frozen while RT 0%EY was frozen straight from room temperature.

Experimental Procedure

Basic Procedures

The basic procedures used for Experiment 7.1, 7.2 and 7.3, unless otherwise specified were: tissue collection and transport, sperm collection, sperm analysis, cryopreservation and thawing of sperm treatments, in vitro production medium, oocyte collection and maturation, in vitro fertilization and culture, statistical analyses. Full details for each procedure were provided in Chapter 3.

Semen Extender Preparation

The TesT buffered 2% EY extender that was used in all three experiments was modified from the commercially available extender, TesT Yolk Buffer (Irving Scientific, Santa Ana, CA). This commercially available extender is a 20% egg yolk-based extender that contains Tes (176 mM), Tris (80 mM), dextrose (9 mM), penicillin-G (1,000 units/mL), streptomycin sulfate (1,000 mcg/mL) and egg yolk (20% v/v). A second TesT extender was formulated using the recipe

Table 7.2. Experimental design for experiment 7.2. This table illustrates what readings were recorded and at what time point they were recorded for this experiment.

	Initial	Post-Cool	0 hour PT	3 hours PT
Pre Trt.	Mot, MI, AS	--	--	--
4°C-2%EY	--	Mot	Mot, MI, AS	Mot, MI, AS
4°C-0%EY	--	Mot	Mot, MI, AS	Mot, MI, AS
22°C-0% EY	--	--	Mot, MI, AS	Mot, MI, AS

Mot = Motility, MI = Membrane integrity, AS = Acrosomal status

above with the absence of egg yolk. To generate the 2% egg yolk concentration the 20% egg yolk extender was diluted with the identical TesT extender containing 0% egg yolk. For example, 1 part 20% egg yolk mixed with 9 parts 0% egg yolk would generate the 2% extender. For experiments 7.2 and 7.3 when sperm freezing was involved there was also a 12% glycerated portion of the TesT extenders which had the matching concentration of egg yolk depending on which TesT extender it was used with. BioXCell® (IMV Technologies, Maple Grove, MN) and Biolife® (BioLife® Solutions, Bothell, WA) are both commercially available synthetic extenders containing no animal protein. The Biolife® arrives ready to use while the BioXCell® needs to be diluted with ultrapure water. Also BioXCell® is marketed and used for cool storage ($\underline{\geq}$4°C) or for freezing sperm, so when used in Experiment 7.2 it was used as a single step extender and has no second glycerated part to be added.

Method of Applying Treatment Extenders

Following the initial assessment, sperm samples were divided into 1.6 mL conical centrifuge tubes containing one of the specified semen extenders. For Experiment 7.1, 200 µl of each extender was added to its respective treatment sample. In Experiments 7.2 and 7.3 the volume of extender added was calculated depending on the desired sperm concentration per straw. In Experiment 7.2, since BioXCell® was a one step extender, the total volume desired was all added at the same time, before the sample was cooled to 4°C. For Experiment 7.2 and 7.3 the TesT extenders had two parts, Part A (sperm + extender) and Part B (glycerated part). The extender portion of Part A was added before cooling, and Part B was added after cooling. For the RT 0%EY treatment (which was never cooled) both parts were added separately, at room temperature, but one right after the other. Once treatments had the correct ratio of "sperm/extender" samples (except RT 0%EY) were placed in a 22°C water-bath and placed in a

4°C walk-in cooler and allowed ~3 hours to cool to 4°C±1°C. The volumes of Part A and B were identical and each one was exactly half of the final volume. To avoid osmotic injury Part B was added to all treatments in a step wise manner in four steps; 15%, 20%, 25% and 40% of the total Part B to be added (modified from Gao et al. 1995). After Part B (glycerated part) of the extenders was added to their appropriate treatments, samples were loaded into (0.25 cc) straws and frozen on a block of dry ice for 20 minutes before being plunged in liquid nitrogen. All samples were held in liquid nitrogen until thawed, no longer than 3 months after being frozen.

Results

Experiment 7.1

Testes Collection and Epididymal Sperm Concentrations

For this experiment, two to nine sets of testes were processed on five (n = 5) different days for a total of 24 sets of testes. When the epididymal sperm was pooled, the average total number of cells collected for each day was 187 x 10^6 with a range from 83 x 10^6 to 441 x 10^6. Each collection day, the total number of epididymal sperm was divided evenly across the three treatments (Table 7.3).

Motility

The mean (±SEM) initial motility for the pooled epididymal sperm, before being split into treatments was 71.0±3.4%. The mean 24 hours values for epididymal sperm held at 4°C in extenders 2% EY (Treatment A), BioLife® (Treatment B) and BioXCell® (Treatment C) was 69.6±2.9%, 55.0±3.0% and 64.2±1.1%, respectively. At 48 hours of cold storage, the mean motility for the 2% EY, BioLife® and BioXCell® extenders was 67.4±4.8%, 50.4±6.3% and 55.4±4.0% respectively. After cooling in 4°C for 72 hours (3 days) the 2% EY treatment maintained epididymal sperm motility at 66.4±5.2% while the sperm motility in the BioLife® and BioXCell® treatments decreased from their previous values, to 40.0±5.5% and 49.4±4.8% respectively (Table 7.4).

The only treatment that showed a significant decrease ($P \leq 0.05$) in motility between the initial pre-cool and the 24, 48 and 72 hour assessments was the BioLife® treatment. At 48 and 72 hours, the BioXCell® motility values had become significantly less than the initial pre-cool motility value. The 2% EY extender was the only treatment that never differed significantly from the pre-cool value even out to day 3 of the experiment (Table 7.4).

The 2% EY treatment was the only treatment that did not show any significant difference ($P \geq 0.05$) when comparing across the three time points. Likewise, the BioLife® and BioXCell® treatments did not differ significantly between the 24 and 48 hour or between the 48 and 72

hour assessments; but there was a significant difference within both the BioLife® and BioXCell® treatments when comparing the 24 to the 72 hour values (Figure 7.1).

At the 24 hour assessment the mean motility value for sperm held in the BioLife® extender was significantly less than the 2% EY and BioXCell® treatments. There was no significant difference in motility when comparing across treatments at the 48 hours assessment. At 72 hours the 2% EY treatment had a significantly greater motility value than both the BioLife® and the BioXCell® extenders (Figure 7.2).

Membrane Integrity

The initial mean value for epididymal sperm with intact membranes was 75.0±5.2%. After dividing the epididymal sperm into equal parts, extended and allowed to cool to 4°C, the 24 hours membrane integrity assessments were 76.6±1.5%, 80.0±2.8% and 80.2±4.5% of intact membranes for the 2% EY, BioLife® and BioXCell® extenders, respectively. After 48 hours of cold storage at 4°C, sperm diluted in the 2%EY extender had 76.0±5.7% intact membranes, the BioLife® extender produced a membrane integrity value of 69.8±3.7% and the BioXCell® value dropped to 68.6±5.4%. Following the 3 days of cold storage at 4°C, the 2% EY, BioLife® and BioXCell® extenders reported membrane integrity values of 66.2±4.5, 58.6±5.3, and 60.6±3.3%, respectively (Table 7.5).

There was no significant difference ($P \geq 0.05$) when comparing the initial membrane integrity value to the 24 hour or the 48 hour values of all three treatments. The 72 hour values were significantly different ($P \leq 0.05$) from the initial membrane integrity for all three treatments (Table 7.5).

There was no significant difference within any of the treatments when comparing between the day 1 and day 2 values or between the day 2 and day 3 values. However, there was a significant difference when comparing the day 1 to day 3 values within all three treatments (Figure 7.3).

There was no significant difference when comparing across any of the three treatments for any of the three time points measured (Figure 7.4).

Acrosomal Status

The mean value of epididymal sperm with intact acrosomes before being divided into treatments was 86.4±4.3%. After dividing into their respective treatments and allowed to cool in 4°C for 24 hours the treatments maintained acrosomal status values of 87.0±3.2%, 92.6±1.5% and 91.2±3.1% for the2% EY, BioLife® and BioXCell® treatments, respectively. The 2% EY, BioLife® and BioXCell® treatments reported 48 h, mean acrosomal values of 84.4±4.3%, 95.2±1.1% and 80.4±3.8%,respectively. The last assessment recorded for each treatment was

Table 7.3. Summary of the numbers of sets of testes processed, number of sperm recovered and number of straws frozen on each of five collection days.

Collection date	Sets processed	Total Cell Count	No. of sperm per ext.
11/2/2009	9	455×10^6	151×10^6
12/14/2009	6	204×10^6	68×10^6
2/15/2010	2	92.5×10^6	30.8×10^6
2/22/2010	4	176×10^6	87.7×10^6
3/1/2010	3.5	60×10^6	20×10^6
Daily average no.	4.9	197×10^6	71.5×10^6
Total no.	24.5	-	-

The last two rows show the average values for all the collection days, and the total number of processed testes for the experiment.

Table 7.4. Percent motility (mean±SEM) of initial, 24, 48 and 72 hours of cold storage at 4°C for pooled domestic cat epididymal sperm that were extended and held in 2% egg yolk, BioLife® and BioXCell® extenders.

	Initial (Control)	24 hours	48 hours	72 hours
2% EY (Trt A)	71.0 ± 3.4^a	69.6 ± 2.9^a	67.4 ± 4.8^a	66.4 ± 5.2^a
BioLife® (Trt B)	71.0 ± 3.4^a	55.0 ± 3.0^b	50.4 ± 6.3^b	40.0 ± 5.5^b
BioXCell® (Trt C)	71.0 ± 3.4^a	64.2 ± 1.1^a	55.4 ± 4.0^b	49.4 ± 4.8^b

EY = egg yolk, Trt = Treatment; ^{a,b}Superscripts designate a significant difference only between initial motility value and individual values for each extender at each time period ($P<0.05$).

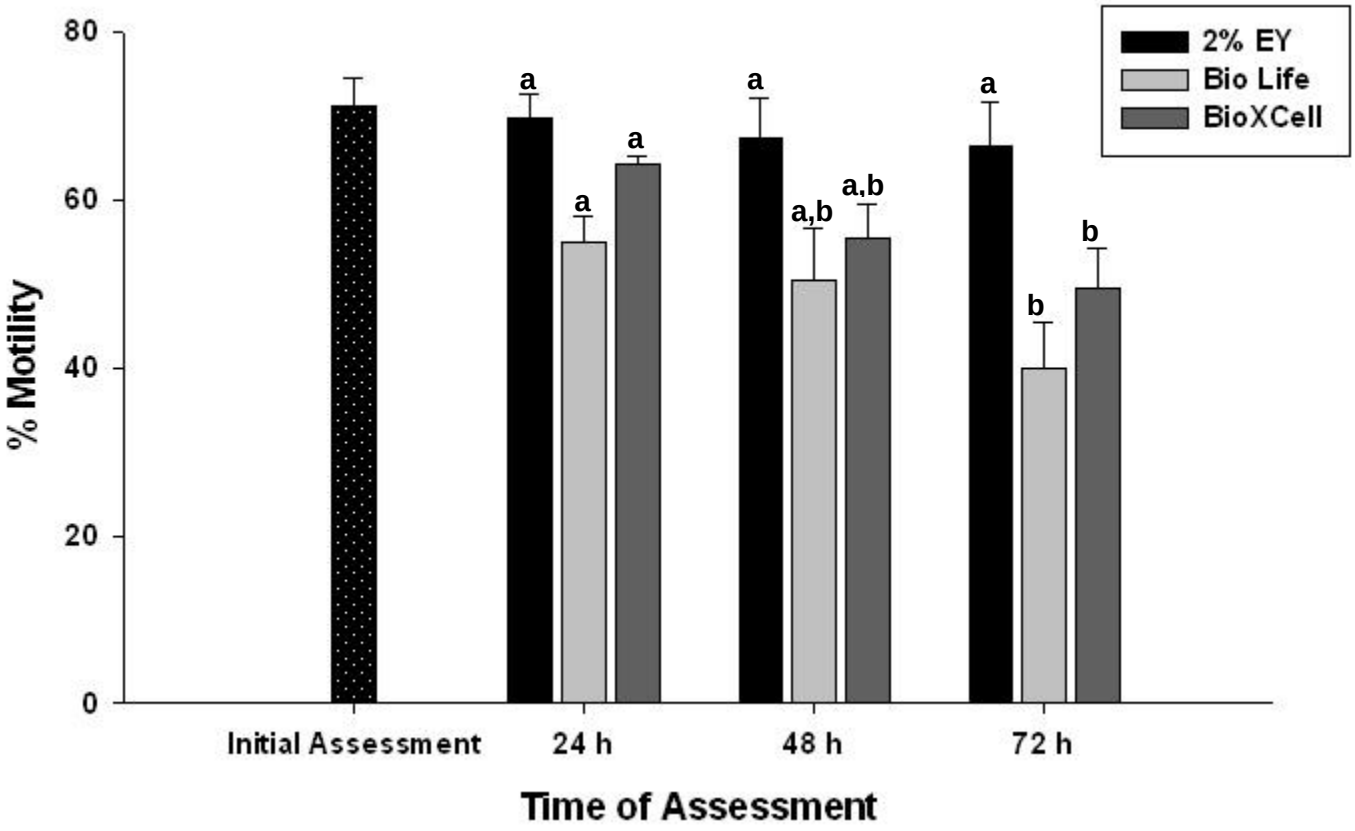

Figure 7.1. Percent motility (mean±SEM) of initial, 24, 48 and 72 hours of cold storage at 4°C for pooled domestic cat epididymal sperm that were extended and held in 2% egg yolk, BioLife® and BioXCell® extenders. [a,b]Mean values within treatments but across time intervals with different superscripts are significantly different (P<0.05).

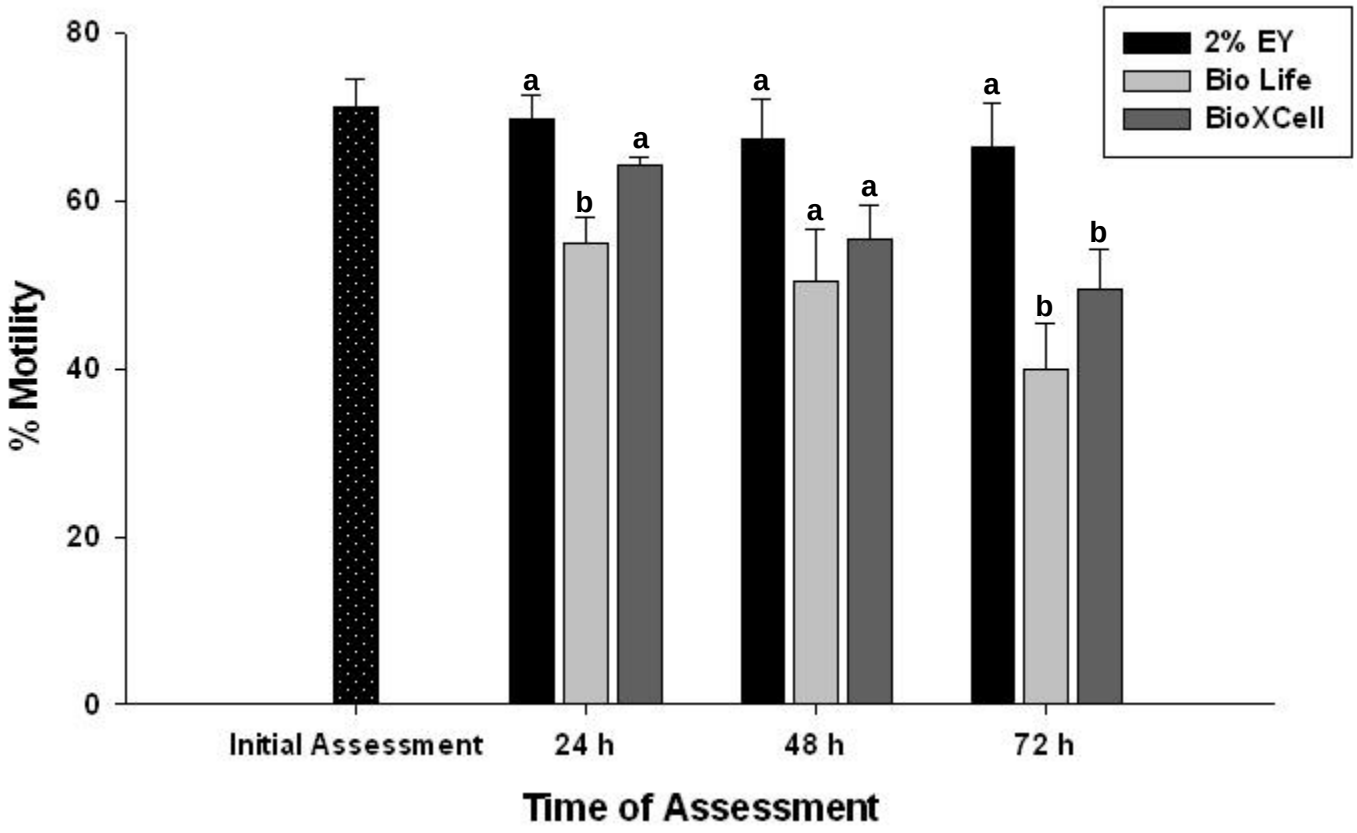

Figure 7.2. Percent motility (mean±SEM) of initial, 24, 48 and 72 hours of cold storage at 4°C for pooled domestic cat epididymal sperm that were extended and held in 2% egg yolk, BioLife® and BioXCell® extenders. [a,b]Mean values across treatments but within time intervals with different superscripts are significantly different (P<0.05).

Table 7.5. Percent membrane integrity (mean±SEM) of initial, 24, 48 and 72 hours of cold storage at 4°C for pooled domestic cat epididymal sperm that were extended and held in 2% egg yolk, BioLife® and BioXCell® extenders.

	Initial (Control)	24 hr	48 hr	72 hr
2% EY (Trt A)	75.0±5.2[a]	76.6±1.5[a]	76.0±5.7[a]	66.2±4.5[b]
BioLife® (Trt B)	75.0±5.2[a]	80.0±2.8[a]	69.8±3.7[a]	58.6±5.3[b]
BioXCell® (Trt C)	75.0±5.2[a]	80.2±4.5[a]	68.6±5.4[a]	60.6±3.3[b]

EY = egg yolk, Trt = Treatment; [a,b]Superscripts designate a significant difference only between initial motility value and individual values for each extender at each time period ($P<0.05$).

Table 7.6. Percent acrosomal status (mean±SEM) of initial, 24, 48 and 72 hours of cold storage at 4°C for pooled domestic cat epididymal sperm that were extended and held in 2% egg yolk, BioLife® and BioXCell® extenders.

	Initial (Control)	24 hr	48 hr	72 hr
2% EY (Trt A)	86.4±4.3[a]	87.0±3.2[a]	84.4±4.3[a]	89.0±3.3[a]
BioLife® (Trt B)	86.4±4.3[a]	92.6±1.5[a]	95.2±1.1[a]	95.8±1.2[a]
BioXCell® (Trt C)	86.4±4.3[a]	91.2±3.1[a]	80.4±3.8[a]	79.0±2.1[b]

EY = egg yolk, Trt = Treatment; [a,b]Superscripts designate a significant difference only between initial motility value and individual values for each extender at each time period ($P<0.05$).

after 72 hours cold storage, the 2% EY extender exhibited a value of 89.0±3.3%, while the BioLife® extender recorded the highest value for this time point of 95.8±1.2%, and the BioXCell® reported the lowest acrosomal value of 79.0±2.1% (Table 7.6).

When comparing the initial acrosomal status value to the 24, 48 and 72 hour values for all three treatments, the only significant difference is between the initial value and the 72 hour value of the BioXCell® treatment (Table 7.6).

When comparing within treatments there was no significant difference when comparing the 24 and 48 hour or the 48 and 72 hour assessments for any of the treatments. BioXCell® was the only treatment that showed a significant decrease within treatment when comparing between day 1 and day 3 values (Figure 7.5).

There was no significant difference across any of the treatments when comparing their acrosomal status for the 24 hours assessment. However, at the 48 hour assessment the BioLife® acrosomal status value was significantly greater than both the 2% EY and the BioXCell® treatments, but the 2% EY and the BioXCell® treatments were not significantly different from each other. Likewise for the 72 hour assessment, BioXCell® had a significantly lower acrosomal status value than both the 2% EY and BioLife® treatments, but the 2% EY and BioLife® treatments were not significantly different from each other (Figure 7.6).

Experiment 7.2

Testes Collection, Epididymal Sperm Concentration and Straws frozen

For this experiment, two to eight sets of testes were processed on six different days (n = 6) for a total of 36 sets of feline testicles. When the epididymal sperm was pooled, the average total number of cells collected for each day was 147.5 x 10^6 with a range from 77 x 10^6 to 336 x 10^6 (Table 7.7).

The total number of daily epididymal sperm was divided evenly across two treatments and depending on the sperm concentrations a range of 1 to 4 (0.25 cc) straws were frozen per treatment, per day. The concentration of the straws ranged from 26 X 10^6 to 67.5 X 10^6 per straw (Table 7.7).

Motility

The initial mean (±SEM) motility for the epididymal sperm before being equally divided into two treatments was 75.8±3.6%. The mean motility value for epididymal sperm post-cool (4°C) in the 2% EY extender was 76.5±3.5%, while the sperm in the BioXCell® extender had a mean motility of 72.7±5.2%. The mean 0 hour post-thaw motility values for the 2% EY and the BioXCell® extenders were 58.9±3.1% and 50.9±2.3%, respectively. After incubating at 37°C for

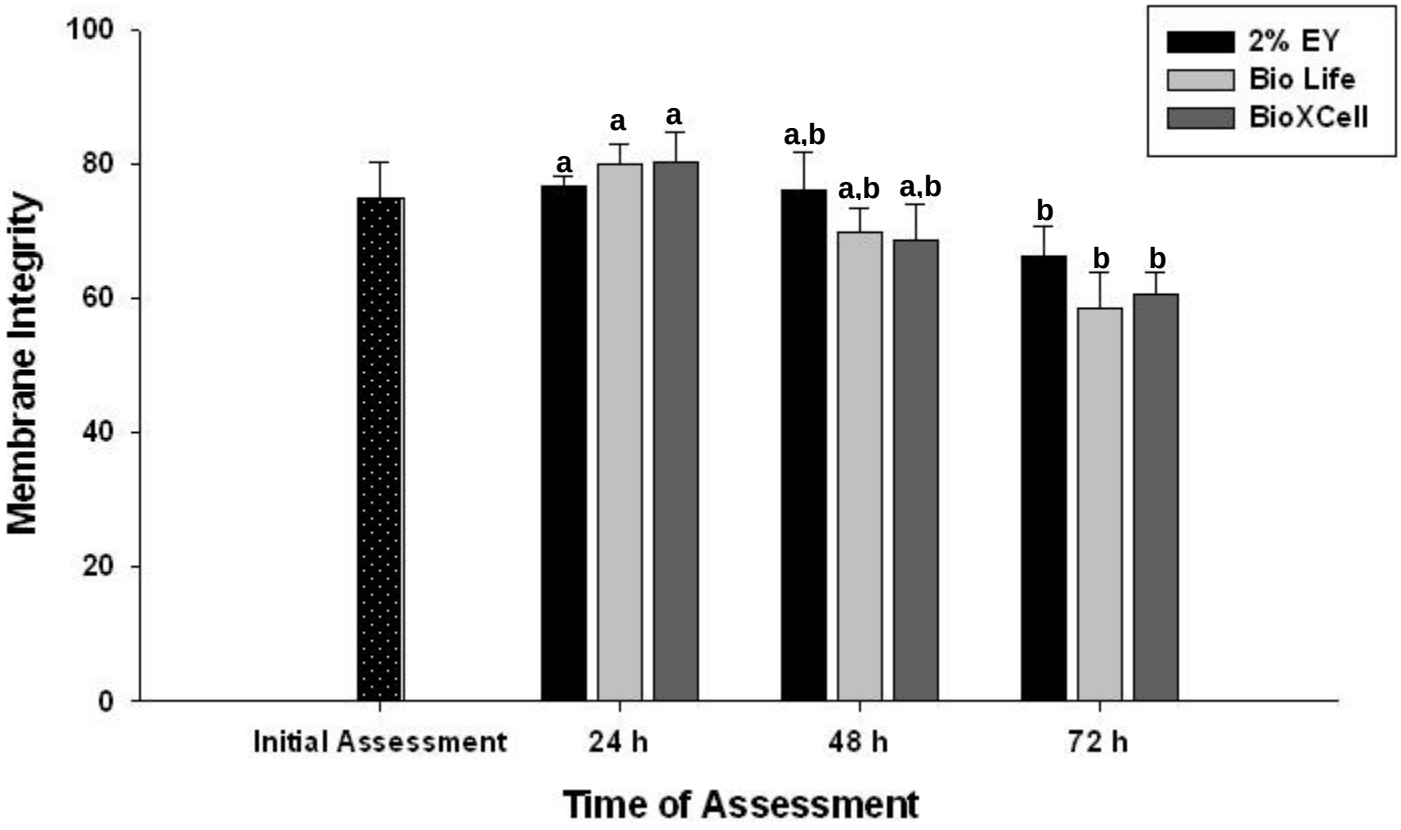

Figure 7.3. Membrane intact percentage (mean±SEM) of initial, 24, 48 and 72 hours of cold storage at 4°C for pooled domestic cat epididymal sperm that were extended and held in 2% egg yolk, BioLife® and BioXCell® extenders. [a,b]Mean values within treatments but across time intervals with different superscripts are significantly different (P<0.05).

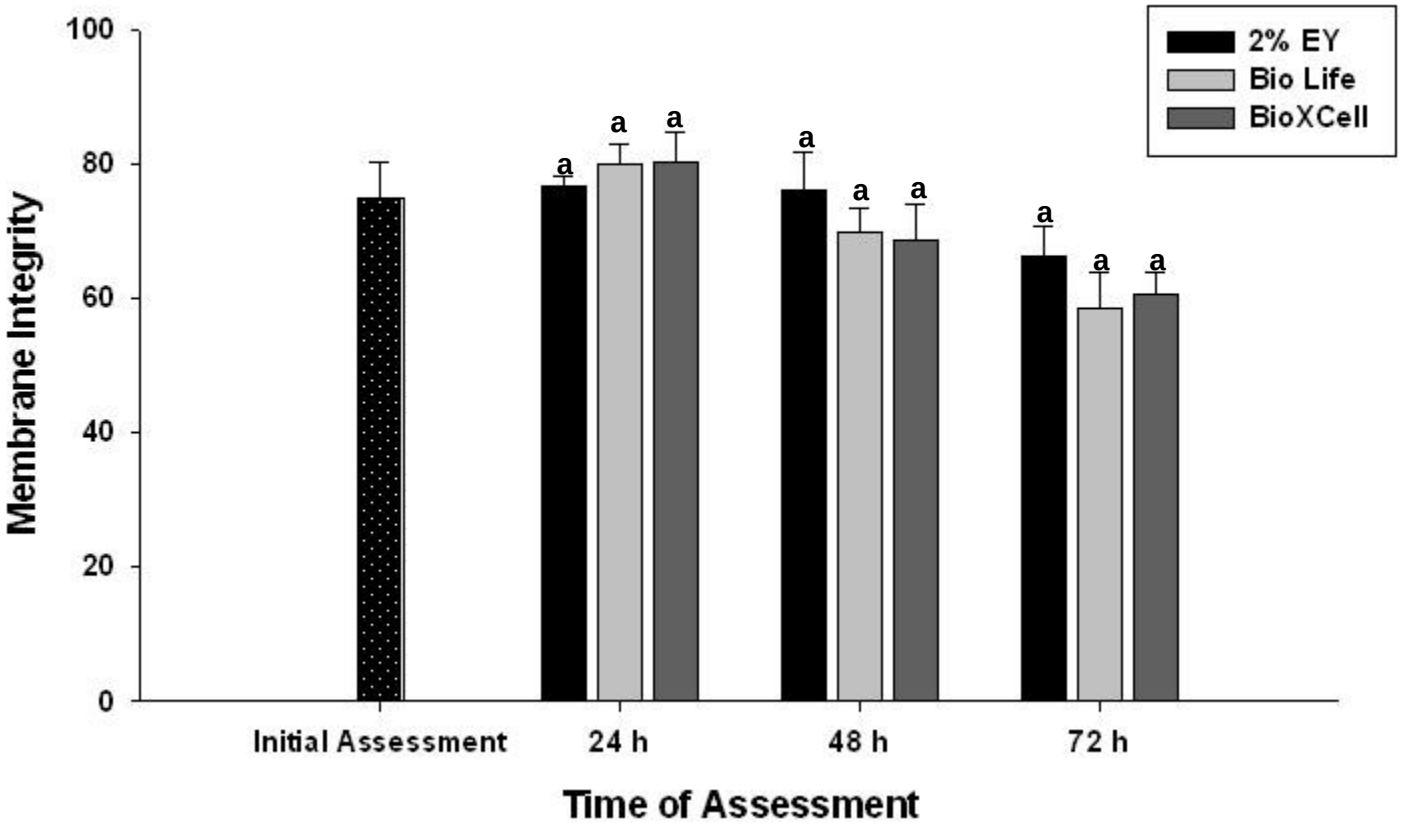

Figure 7.4. Membrane intact percentage (mean±SEM) of initial, 24, 48 and 72 hours of cold storage at 4°C for pooled domestic cat epididymal sperm that were extended and held in 2% egg yolk, BioLife® and BioXCell® extenders. [a,b]Mean values across treatments but within time intervals with different superscripts are significantly different ($P<0.05$).

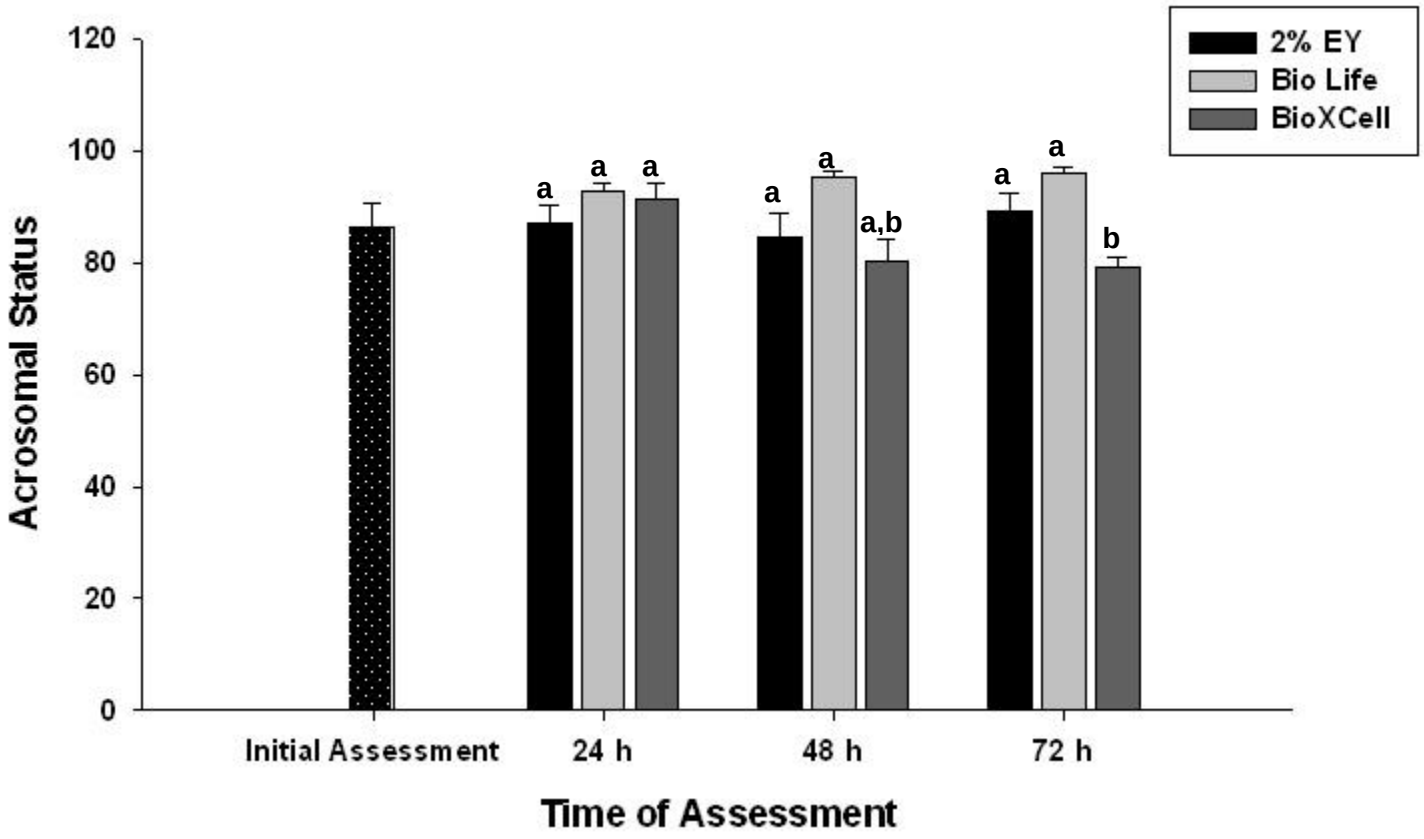

Figure 7.5. Acrosomal status percentage (mean±SEM) of initial, 24, 48 and 72 hours of cold storage at 4°C for pooled domestic cat epididymal sperm that were extended and held in 2% egg yolk, BioLife® and BioXCell® extenders. [a,b] Mean values within treatments but across time intervals with different superscripts are significantly different (P<0.05).

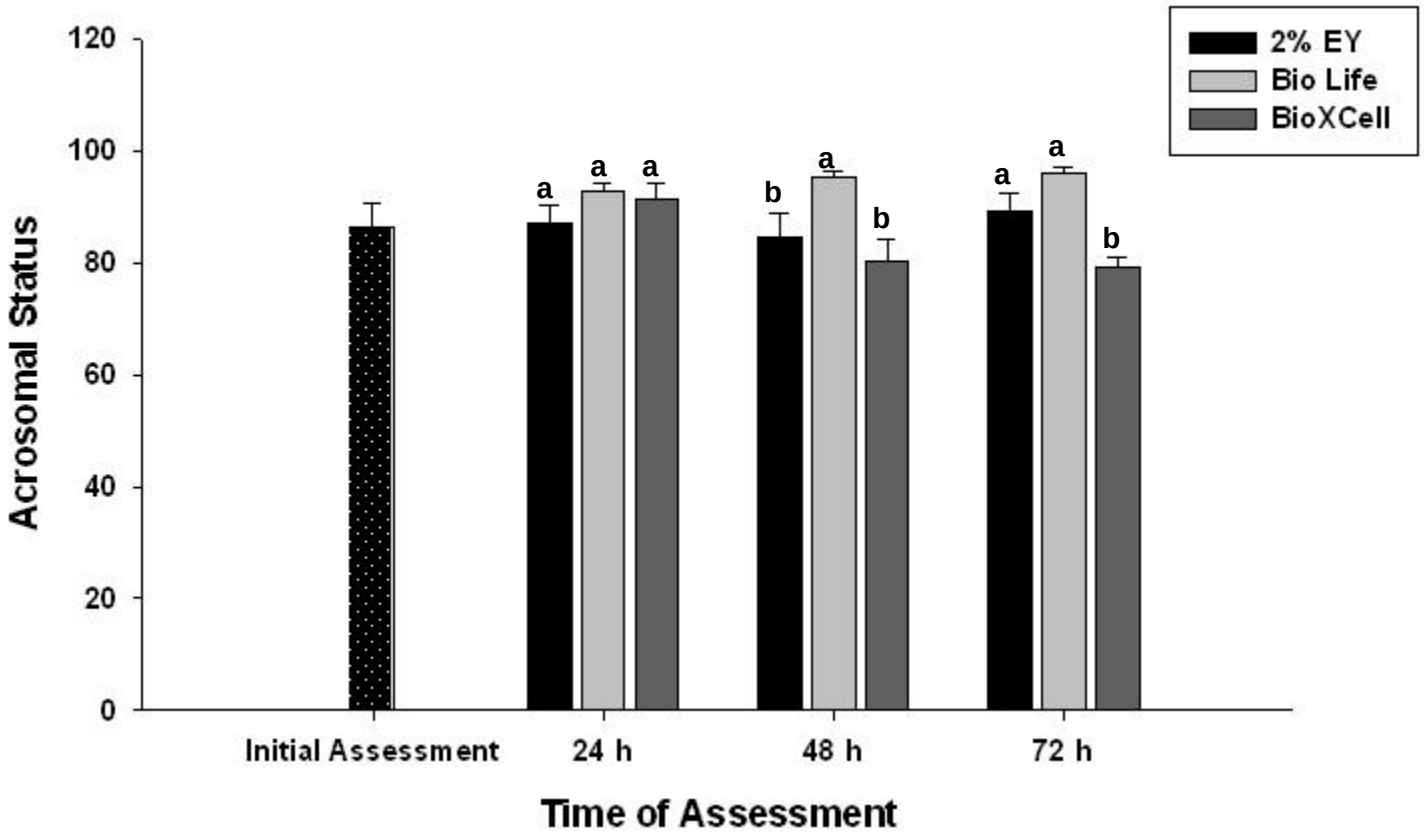

Figure 7.6. Acrosomal status percentage (mean±SEM) of initial, 24, 48 and 72 hours of cold storage at 4°C for pooled domestic cat epididymal sperm that were extended and held in 2% egg yolk, BioLife® and BioXCell® extenders. [a,b]Mean values across treatments but within time intervals with different superscripts are significantly different (P<0.05).

3 hours, the epididymal sperm in the 2% EY extender had a motility value of 36.5±1.5% while the BioXCell® extender produced sperm with a motility of 35.4±2.3% (Table 7.8).

In both treatments there was a significant decrease ($P \leq 0.05$) in motility from the post-cool to the 0 hour post-thaw and again from the 0 hour to the 3 hour post-thaw assessments (Figure 7.7). However, there was no significant difference when comparing between treatments at any of the assessment times (Figure 7.8).

Membrane Integrity

After splitting the sample into equal treatments, the extended sperm was placed in a walk-refrigerator to cool to 4°C, the first membrane integrity value for the 2% EY treatment was 81.8±3.5% while the BioXCell® treatment had 86.0±2.2% intact membranes. After thawing straws from both treatments, the percent of intact membranes at the 0 hour post-thaw time point were 63.2±2.3% and 57.2±2.2% for the 2% EY and BioXCell® treatments, respectively. Following 3 hours incubation in a 37°C incubator, thawed epididymal sperm samples were evaluated for a final assessment. The epididymal sperm frozen in the 2% EY extender had a mean value of 58.6±2.3%, while the BioXCell® treatment recorded a mean value of 52.0±1.9% (Table 7.9).

The post-cool values for both treatments were significantly greater ($P \leq 0.05$) than both the 0 hour and 3 hour assessments. However there was no significant decrease ($P \geq 0.05$) for either treatment when comparing between the 0 hour and 3 hour assessments (Figure 7.9). There was no significant difference when comparing between treatments for the post-thaw or the 0 hour assessments. However, the 2% EY treatment did have a significantly higher percentage of intact membranes than did the BioXCell® treatment for the 3 hour post-thaw assessment time (Figure 7.10).

Acrosomal Status

Like the membrane integrity evaluation the first acrosomal status reading was taken after the sample had been divided into treatments and cooled to 4°C. The first acrosome values were 92.0±2.5% for the 2% EY treatment and 94.7±1.7% for the BioXCell® treatment. The mean 0 hour post-thaw acrosomal status values for the 2% EY and the BioXCell® treatments were 73.5±2.0% and 72.9±3.1%, respectively. The last reading for each straw thawed was after being held in a 37°C incubator for 3 hours post-thaw. The two mean values for the 3 hours post-thaw acrosomal assessment were virtually identical with the 2% EY treatment giving a value of 62.8±1.6% and the BioXCell® reported a 62.3±1.8% (Table 7.10).

Table 7.7. Summary of the numbers of sets of testes processed, number of sperm recovered and number of straws frozen on each of six collection days.

Collection date	Sets processed	Total Cell Count	No. of straws per ext per day	Concentration per straw
3/17/2010	5	135×10^6	1	67.5×10^6
3/24/2010	7	112.5×10^6	2	28×10^6
3/31/2010	8	104×10^6	2	26×10^6
4/1/2010	2	77×10^6	1	38.5×10^6
4/12/2010	8	336×10^6	4	42×10^6
6/16/2010	6	121×10^6	2	30×10^6
Daily average no.	6	147.5×10^6	2	38.6×10^6
Total no.	36	-	12	-

The last two rows show the average values for all the collection days, and the overall total values for the entire experiment.

Table 7.8. Percent motility (mean±SEM) of initial, post-cool, 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were extended and frozen in 2% egg yolk or BioXCell® extenders.

	Initial (Control)	Post-cool	0 hour post-thaw	3 hours post-thaw
2% EY (Trt A)	75.8 ± 3.6^a	76.5 ± 3.5^a	58.9 ± 3.1^b	36.5 ± 1.5^b
BioXCell® (Trt B)	75.8 ± 3.6^a	72.7 ± 5.2^a	50.9 ± 2.3^b	35.4 ± 2.3^b

EY = egg yolk, Trt = Treatment; ^{a,b}Superscripts designate a significant difference only between initial motility value and individual values for each extender for each time period ($P<0.05$).

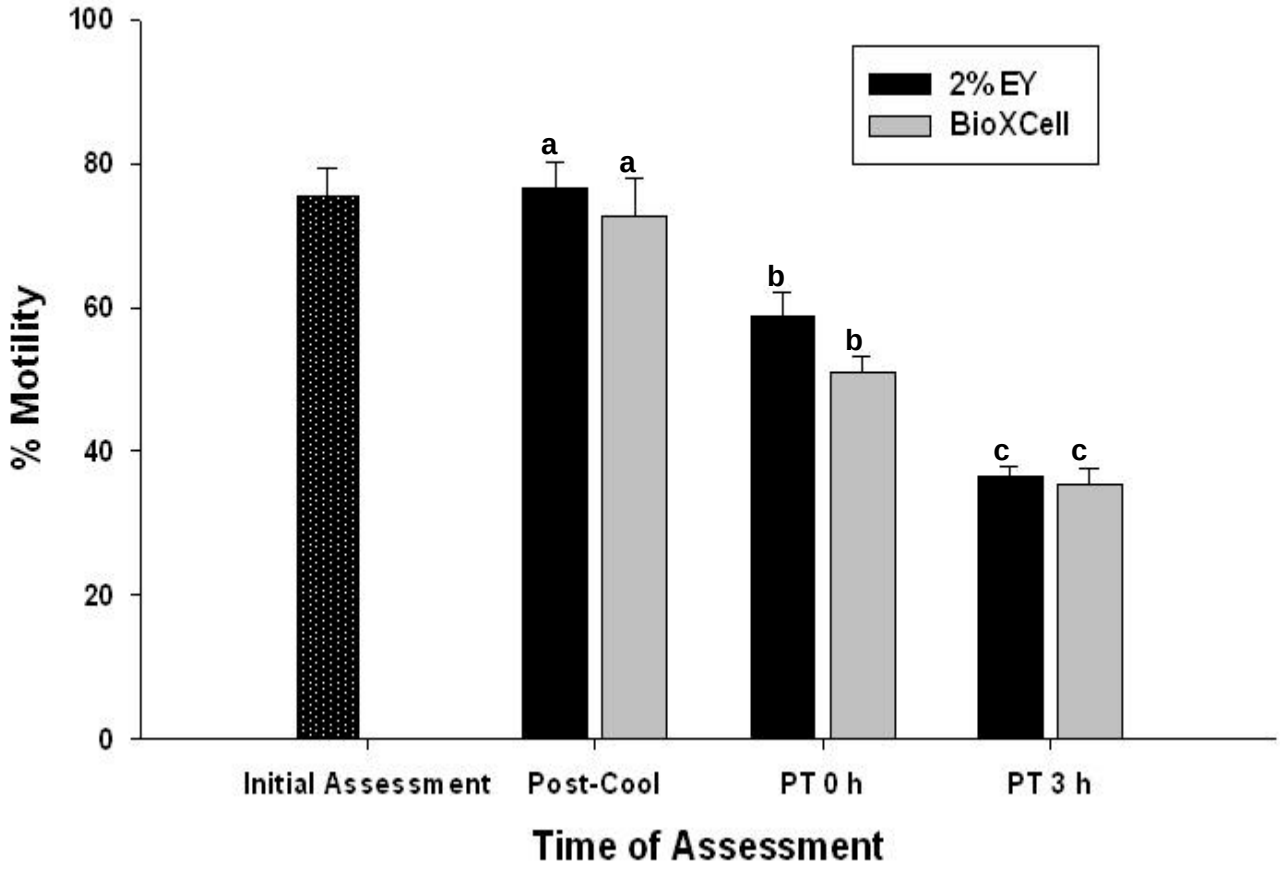

Figure 7.7. Percent motility (mean±SEM) of initial, post-cool, 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were processed and frozen in 2% egg yolk and BioXCell® extenders. [a,b,c]Mean values within treatments but across time intervals with different superscripts are significantly different (P<0.05).

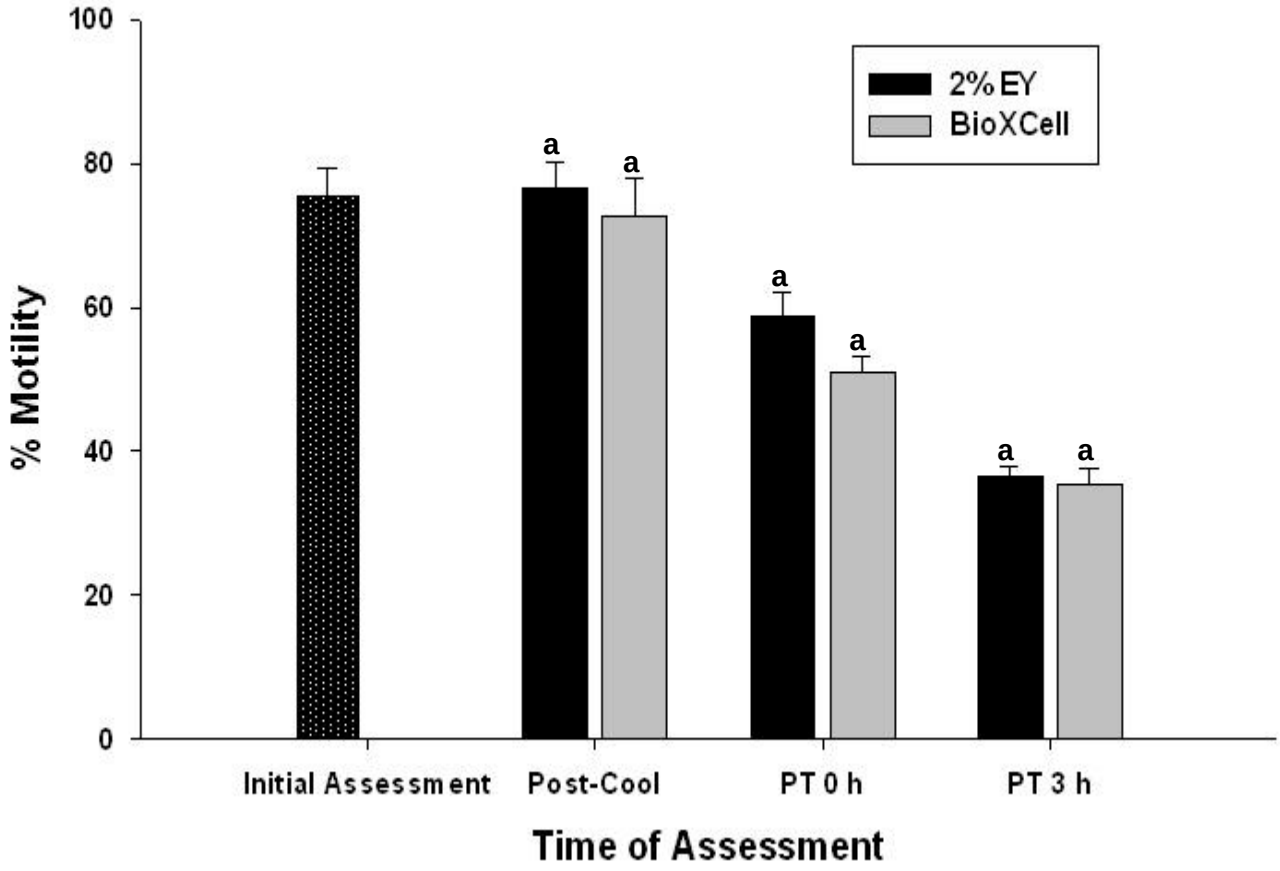

Figure 7.8. Percent motility (mean±SEM) of initial, post-cool, 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were processed and frozen in 2% egg yolk and BioXCell® extenders. [a,b]Mean values across treatments but within time intervals with different superscripts are significantly different (P<0.05).

Table 7.9. Percent membrane integrity (mean±SEM) post-cool, 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were extended and frozen in 2% egg yolk or BioXCell® extenders.

	Post-cool	0 hour post-thaw	3 hours post-thaw
2% EY (Trt A)	81.8±3.5	63.2±2.3	58.6±2.3
BioXCell® (Trt B)	86.0±2.2	57.2±2.2	52.0±1.9

EY = egg yolk, Trt = Treatment;

Table 7.10. Percent acrosomal status (mean±SEM) post-cool, 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were extended and frozen in 2% egg yolk or BioXCell® extenders.

	Post-cool	0 hour post-thaw	3 hours post-thaw
2% EY (Trt A)	92.0±2.5	73.5±2.0	62.8±1.6
BioXCell® (Trt B)	94.7±1.7	72.9±3.1	62.3±1.8

EY = egg yolk, Trt = Treatment;

When comparing within treatments, the acrosomal status values for both the 2% EY and the BioXCell® treatments decreased significantly ($P \leq 0.05$) from the post-cool to the 0 hour and from the 0 hour to the 3 hour assessments (Figure 7.11).

There was no statistical difference ($P \geq 0.05$) when comparing between the 2% EY and BioXCell® treatments for the post-cool, 0 hour post-thaw or the 3 hour post-thaw evaluation times (Figure 7.12).

Experiment 7.3

Testes Collection, Epididymal Sperm Concentration and Straws frozen

Testes were collected over a six week period from veterinary clinics in New Orleans. Testes were processed on the day of collection and sperm were pooled. For the present experiment three to eight sets of testes were processed across 11 days for a total of 55 sets of feline testicles. After pooling an average of 170.3 x 10^6/day, with a range of 94 x 10^6 to 260 x 10^6 (Table 7.11).

The total number of sperm/day was divided evenly across three treatments and depending on concentration, a range of 1 to 2 (0.25 cc) straws were frozen/treatment/day. The concentration of the straws ranged from 31.5 X 10^6 to 54.0 X 10^6/straw (Table 7.11).

Motility

The initial motility for the epididymal sperm before being divided into the three treatment extenders was 66.4±1.0%. The mean motility value for epididymal sperm after cooling to 4°C in the 2% EY (Treatment A) and 0% EY (Treatment B) extenders was 61.4±2.6% and 60.2±3.5%, respectively. The 22°C-0%EY treatment (Treatment C) was never cooled and was frozen from room temperature so there is no 4°C value for that treatment. The mean 0 hour post-thaw motility values for epididymal sperm frozen in the 4°C-2% EY treatment was 31.4±2.2%, the 4°C-0% EY treatment had a post-thaw motility value of 26.0±3.1% and the sperm in the 0% EY extender, frozen from room temperature (22°C-0% EY) had a motility of 22.5±3.0%. After incubating at 37°C for 3 hours post-thaw, the epididymal sperm in the 4°C-2% EY treatment had a motility value of 17.9±2.0% while the 4°C-0% EY treatment resulted in sperm with a motility of 15.3±2.5% and the 22°C-0% EY treatment had the lowest motility value of 9.4±1.2% (Table 7.12).

Neither of the two post-cool values was significantly different from the initial motility reading. At the 0 hour post-thaw assessment all three 0 hour post-thaw values were significantly less the initially motility value. Likewise the 3 hour post-thaw values were significantly less than the initial motility value (Table 7.12).

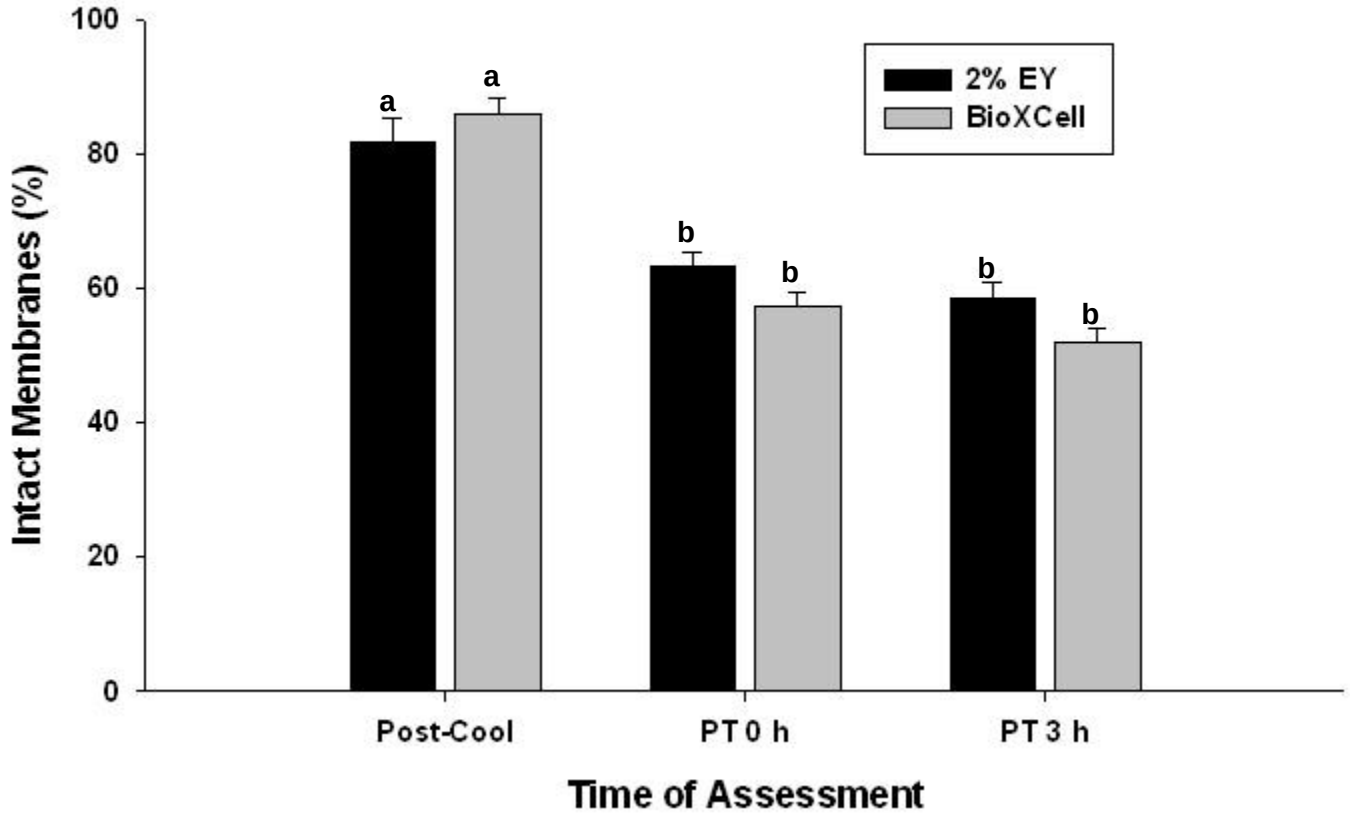

Figure 7.9. Membrane intact percentage (mean±SEM) of initial, post-cool, 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were processed and frozen in 2% egg yolk and BioXCell® extenders. [a,b]Mean values within treatments but across time intervals with different superscripts are significantly different (P<0.05).

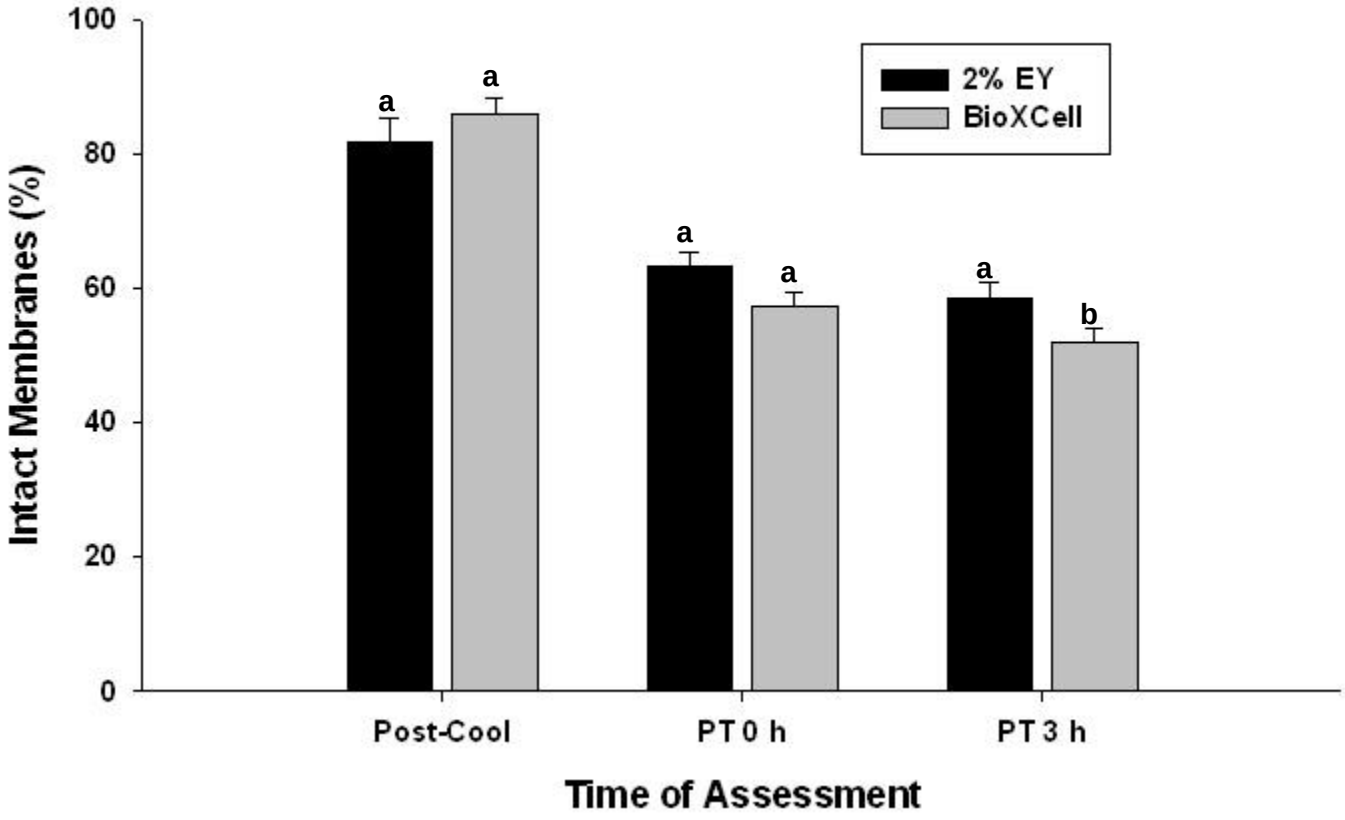

Figure 7.10. Membrane intact percentage (mean±SEM) of initial, post-cool, 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were processed and frozen in 2% egg yolk and BioXCell® extenders. [a,b]Mean values across treatments but within time intervals with different superscripts are significantly different (P<0.05).

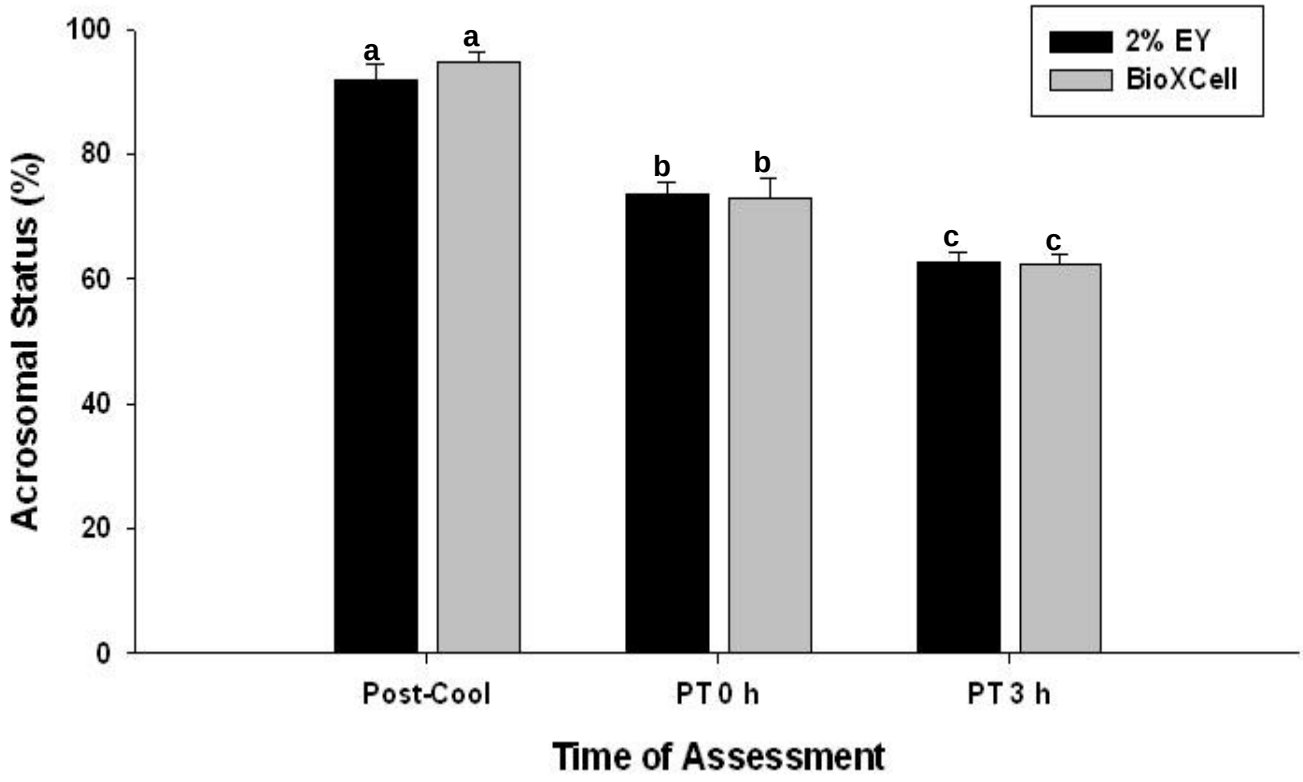

Figure 7.11. Acrosomal status percentage (mean±SEM) of initial, post-cool, 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were processed and frozen in 2% egg yolk and BioXCell® extenders. [a,b,c]Mean values within treatments but across time intervals with different superscripts are significantly different (P<0.05).

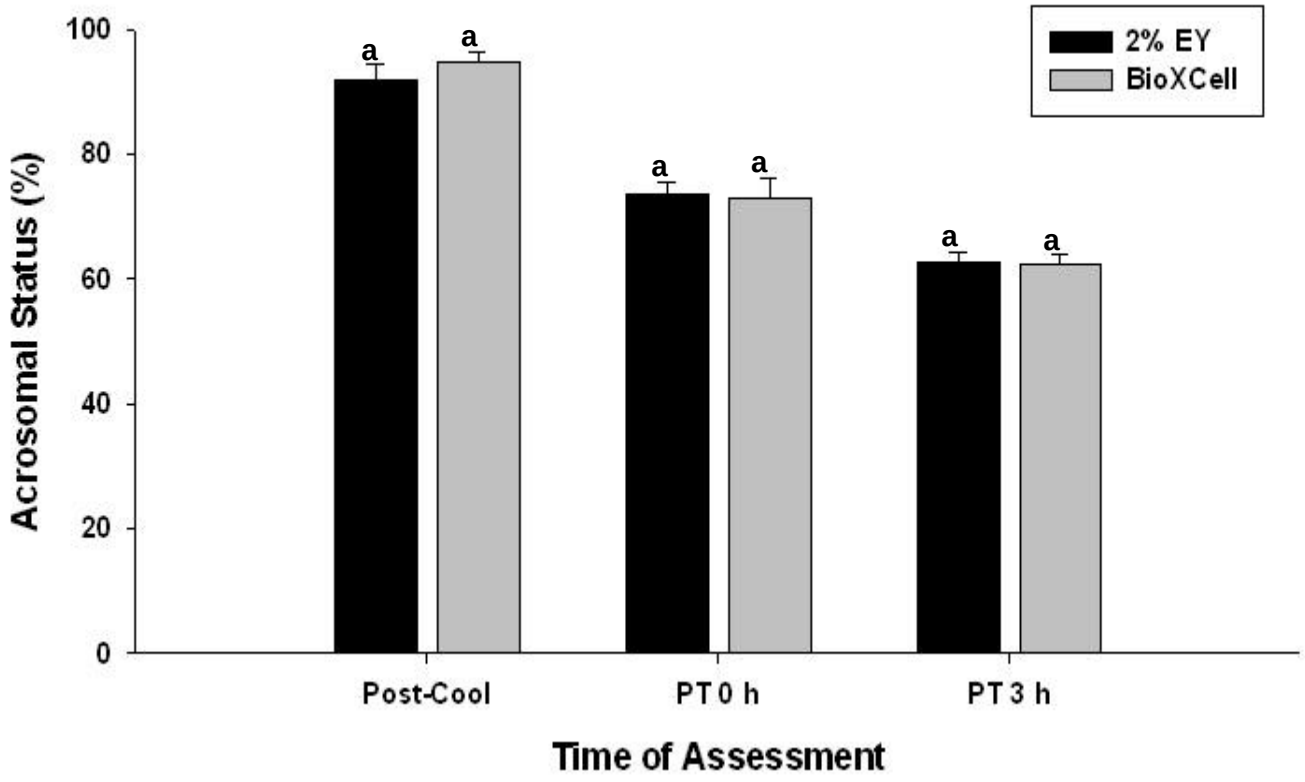

Figure 7.12. Acrosomal status percentage (mean±SEM) of initial, post-cool, 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were processed and frozen in 2% egg yolk and BioXCell® extenders. [a,b]Mean values across treatments but within time intervals with different superscripts are significantly different ($P<0.05$).

Table 7.11. Summary of the numbers of sets of testes processed, number of sperm recovered and number of straws frozen on each of 11 collection days.

Collection date	Sets processed	Total Cell Count	No. of straws per ext per day	Concentration per straw
2/15/2011	6	260×10^6	2	39×10^6
2/16/2011	8	157×10^6	1	47×10^6
2/24/2011	6	121×10^6	1	40.5×10^6
3/3/2011	4	148×10^6	1	48.6×10^6
3/9/2011	3	168×10^6	1	54×10^6
3/15/2011	3	143×10^6	1	49×10^6
3/17/2011	5	140×10^6	1	47×10^6
3/22/2011	4	150×10^6	1	49.5×10^6
3/24/2011	5	127×10^6	1	43×10^6
3/28/2011	6	94×10^6	1	31.5×10^6
3/29/2011	5	195×10^6	2	32.5×10^6
Daily average no.	5	170.3×10^6	1.18	43.8×10^6
Total no.	55	-	12	-

The last two rows show the average values for all the collection days, and the overall total values for the entire experiment.

Table 7.12. Percent motility (mean±SEM) of initial, post-cool, 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were extended and frozen in 0 or 2% egg yolk extender; and frozen from either room temperature or after cooling to 4°C.

	Initial (Control)	Post-cool	0 hour post-thaw	3 hours post-thaw
4°C-2% EY (Trt A)	66.4 ± 1.0^a	61.4 ± 2.6^a	31.4 ± 2.2^b	17.9 ± 2.0^b
4°C-0% EY (Trt B)	66.4 ± 1.0^a	60.2 ± 3.5^a	26.0 ± 3.1^b	15.3 ± 2.5^b
22°C-0% EY (Trt C)	66.4 ± 1.0^a	-	22.5 ± 3.0^b	9.4 ± 1.2^b

EY = egg yolk, Trt = Treatment; ^{a,b}Superscripts designate a significant difference only between initial motility value and individual values for each extender at each time period ($P<0.05$).

When comparing within treatments, both the 4°C assessment readings were significantly greater ($P \leq 0.05$) than their 0 hour post-thaw counterparts. Also, the 3 hour post-thaw values of all three treatments, significantly decreased when compared with their 0 hour post-thaw equivalent (Figure 7.13).

There was no significant difference ($P \geq 0.05$) when comparing the post-cool (4°C) values, between the two treatments. When comparing across the three treatments, for the 0 hour post-thaw motility, the only significant difference ($P \leq 0.05$) was between the 4°C-2% EY and 22°C-0% EY treatments. For the 3 hour post-thaw assessment the 22°C-0% EY treatment was significantly different ($P \leq 0.05$) from both the 4°C-2% EY and 4°C-0% EY treatments (Figure 7.14).

Membrane Integrity

The first evaluation of membrane integrity was recorded prior to dividing the sample into treatments. This first reading showed that 79.9±1.3% of the epididymal sperm had intact membranes. After splitting the sample into equal treatments, the extended sperm was allowed to cool to 4°C in a walkin cooler, and then frozen in the respective extenders. The 0 hour post-thaw values for the 4°C-2% EY, 4°C-0% EY and RT-0% EY treatments were 52.0±2.9%, 50.0±3.9% and 40.7±2.8%, respectively. After thawing straws from the three treatments and allowing the sperm a 3 hours incubation in a 37°C incubator, the membrane integrity values were 46.9±2.8%, 43.0±3.1% and 33.2±3.2% for the 4°C-2% EY, 4°C-0% EY and RT-0% EY treatments, respectively (Table 7.13).

The initial membrane integrity value was significantly greater than the 0 hour and the 3 hour post-thaw values for all three treatments (Table 7.13). There was no significant difference within any of the treatments when comparing their 0 hour to their 3 hours post-thaw readings (Figure 7.15).

At the 0 hour post-thaw assessment, the only cross treatment difference ($P \leq 0.05$) was when comparing the 4°C-2% EY treatment to the 22°C-0% EY treatment. For the 3 hour post-thaw assessment, both of the 4°C treatments were significantly ($P \leq 0.05$) greater than the 22°C treatment but were not significantly different from each other (Figure 7.16).

Acrosomal Status

The first acrosomal assessment was recorded just before dividing the daily sperm pellet into treatments, this initial reading showed that 89.4±1.6% of the epididymal sperm had intact acrosomes. After the samples had been divided into treatments and frozen, they were thawed and sperm were again evaluated. The 0 hour post-thaw acrosomal values were 61.9±3.3%, 58.2±3.3% and 53.1±2.8% intact acrosomes for the 4°C-2% EY, 4°C-0% EY and RT-0% EY

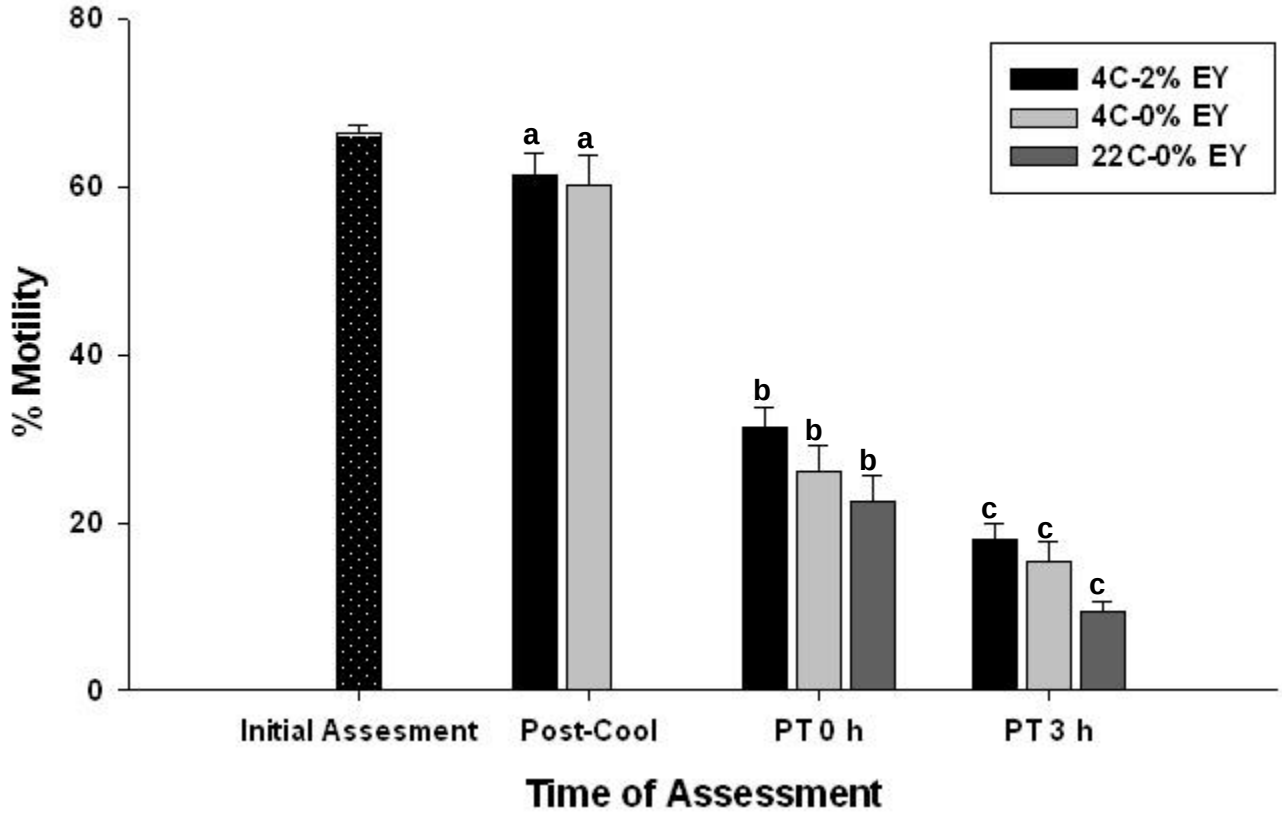

Figure 7.13. Percent motility (mean±SEM) of initial, post-cool, 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were processed in an extender containing 0 or 2% egg yolk; and frozen from either room temperature or after cooling to 4°C. [a,b,c]Mean values within treatments but across time intervals with different superscripts are significantly different (P<0.05).

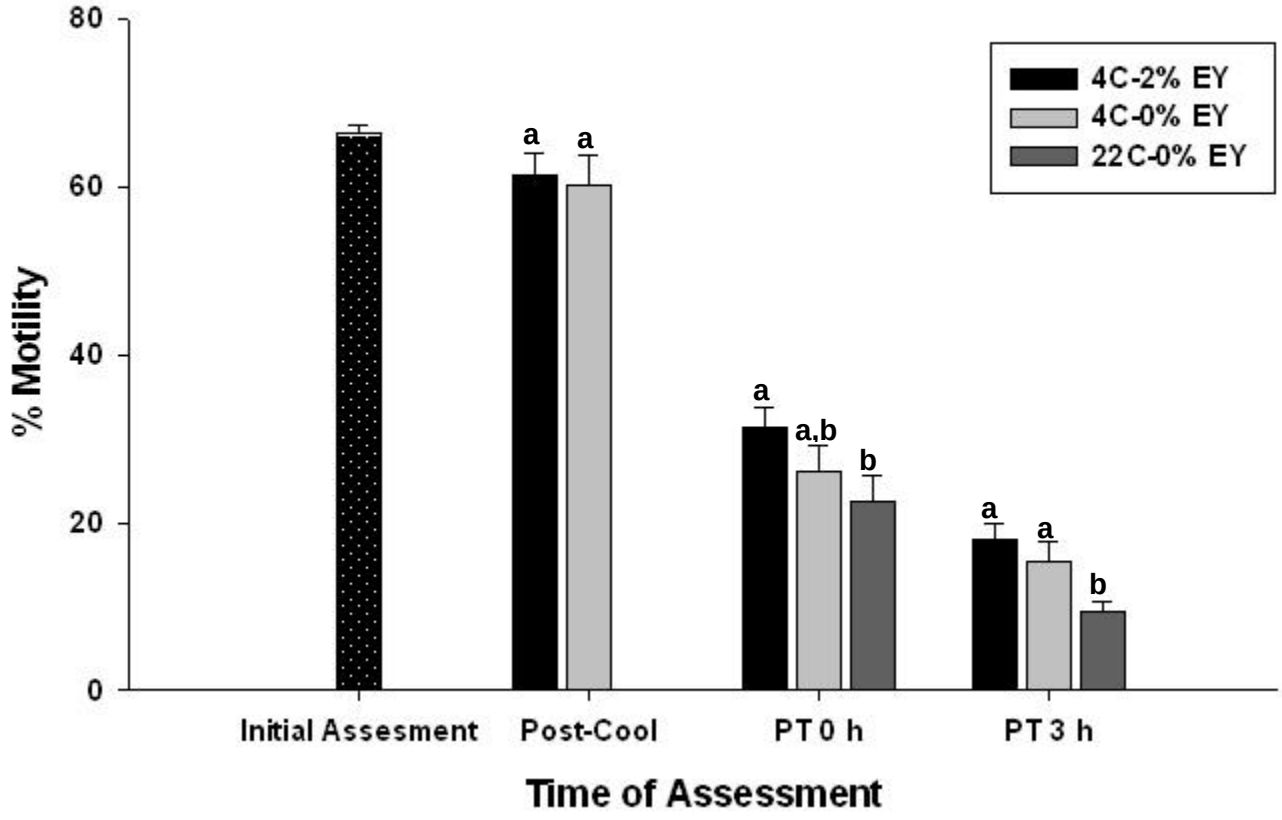

Figure 7.14. Percent motility (mean±SEM) of initial, post-cool, 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were processed in an extender containing 0 or 2% egg yolk; and frozen from either room temperature or after cooling to 4°C. [a,b]Mean values across treatments but within time intervals with different superscripts are significantly different (P<0.05).

treatments, respectively. The final assessment for each straw thawed was the 3 hours post-thaw, which occurred after being held in a 37°C incubator for three hours. The mean value for the 4°C-2% EY and the 4°C-0% EY treatments were 48.8±3.9% and 47.2±3.8%, while the RT-0% EY treatment was almost ten percentage points less at 38.9±4.0% (Table 7.14).

The initial value was significantly greater than all three of the 0 hour and the 3 hours post-thaw readings (Table 7.13). Also when comparing within the treatments all three 0 hour post-thaw values were significantly greater than their 3 hour post-thaw counterparts (Figure 7.17).

There was no significant difference ($P \geq 0.05$) when comparing across the three treatments for the 0 hour post-thaw assessment. Despite the ten points numerical difference there was no significant difference among any of the treatments for the 3 hours post-thaw assessment either (Figure 7.18)

Discussion

Gradual cooling to 5°C is often a precursor to the cryopreservation of mammalian sperm, cat and dog sperm can be held at 5°C for one (Martins et al., 2009) or 2 days (Hermansson and Forsberg, 2006) before freezing or instead of freezing (Riel et al., 2011; Buarpung et al., 2014). Cooling to 5°C is a crucial event for mammalian sperm and can leave them in an irreversible state of cold shock (Watson, 1981; White, 1993). Egg yolk (EY) has long been a common ingredient added to semen extenders to aid in protecting against cold shock (Philips and Lardy, 1940). However, inconsistency between egg yolk sources, the opaque coloring of egg yolk and its tacky nature have prompted my search of extenders that are completely void of egg yolk but still provide adequate protection from cold shock.

The objective of the present experiment was to compare in vitro survival of epididymal cat sperm during storage at 4°C, in two nonegg yolk-based extenders (BioXCell® and BioLife®) with that obtained in a 2% egg yolk-based extender (TesT 2%). Subsequently, I evaluated post-thaw survival of epididymal sperm after cryopreservation in TesT 2% vs. BioXCell®. Lastly I examined the cryoprotective effectiveness of 2% EY versus 0% EY when sperm were frozen before (22°C with 0% EY) or after (4°C with 2% EY, 4°C with0% EY) cooling to 4°C.

When domestic cat sperm were held at 4°C for 72 hours (Experiment 6.1) in 2% egg yolk motility did not significantly decrease. In contrast, sperm motility at 48 and 72 hours of storage in BioXCell® was significantly lower that at 0 hours. Sperm motility in the BioLife® showed a significant decrease at 24, 48 and 72 hours as compared to motility at 0 hours. BioLife® medium was developed for maintenance of organ cells (Mathew et al., 1997) and has never been used previously for maintaining cat sperm during cool storage. Also across

Table 7.13. Percent membrane integrity (mean±SEM) of initial, post-cool, 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were extended and frozen in 0 or 2% egg yolk extender; and frozen from either room temperature or after cooling to 4°C.

	Initial (Control)	0 hour post-thaw	3 hours post-thaw
4°C-2% EY (Trt A)	79.9±1.3[a]	52.0±2.9[b]	46.9±2.8[b]
4°C-0% EY (Trt B)	79.9±1.3[a]	50.0±3.9[b]	43.0±3.1[b]
22°C-0% EY (Trt C)	79.9±1.3[a]	40.7±2.8[b]	33.2±3.2[b]

EY = egg yolk, Trt = Treatment; [a,b]Superscripts designate a significant difference only between initial motility value and individual values for each extender at each time period ($P<0.05$).

Table 7.14. Percent acrosomal status (mean±SEM) of initial, post-cool, 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were extended and frozen in 0 or 2% egg yolk extender; and frozen from either room temperature or after cooling to 4°C.

	Initial (Control)	0 hour post-thaw	3 hours post-thaw
4°C-2% EY (Trt A)	89.4±1.6[a]	61.9±3.3[b]	48.8±3.9[b]
4°C-0% EY (Trt B)	89.4±1.6[a]	58.2±3.3[b]	47.2±3.8[b]
22°C-0% EY (Trt C)	89.4±1.6[a]	53.1±2.8[b]	38.9±4.0[b]

EY = egg yolk, Trt = Treatment; [a,b]Superscripts designate a significant difference only between initial motility value and individual values for each extender at each time period ($P<0.05$).

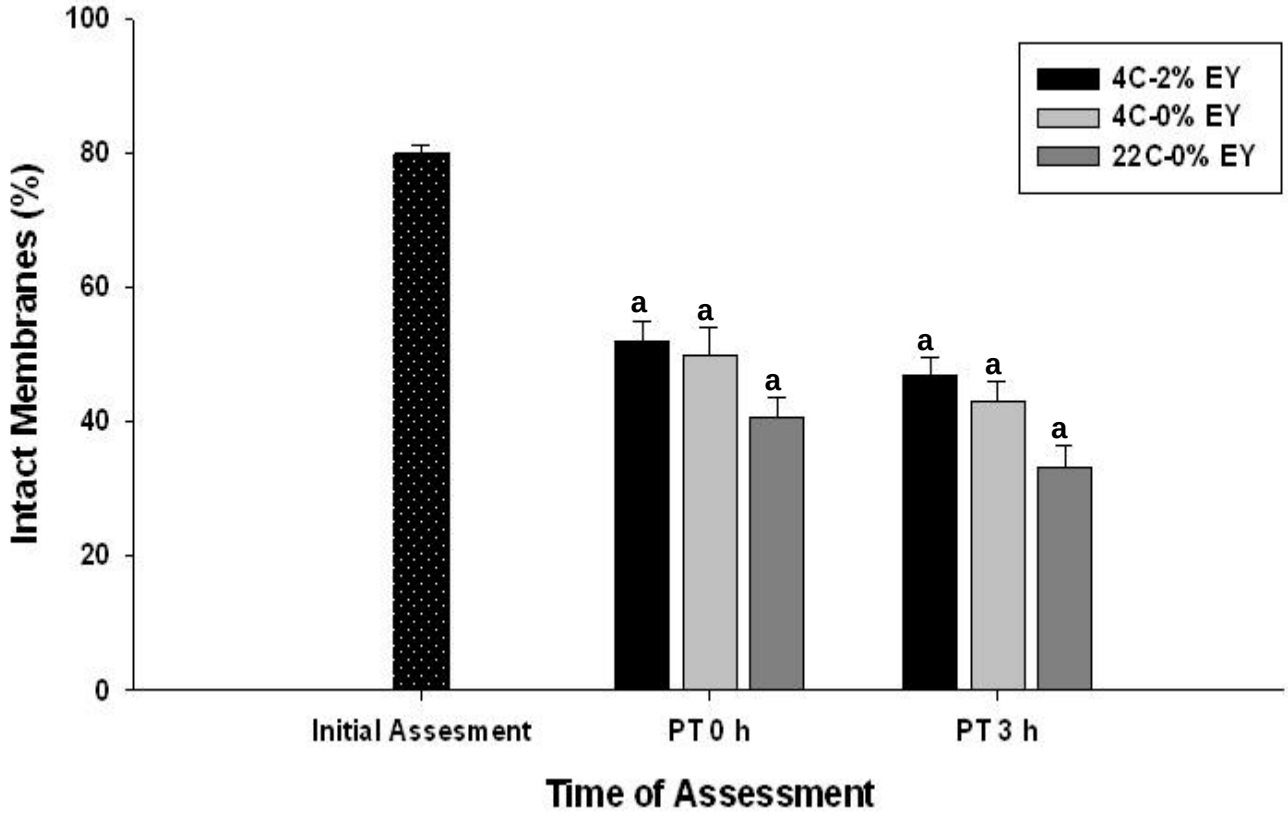

Figure 7.15. Membrane intact percentage (mean±SEM) of initial, post-cool, 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were processed in an extender containing 0 or 2% egg yolk; and frozen from either room temperature or after cooling to 4°C. [a,b]Mean values within treatments but across time intervals with different superscripts are significantly different (P<0.05).

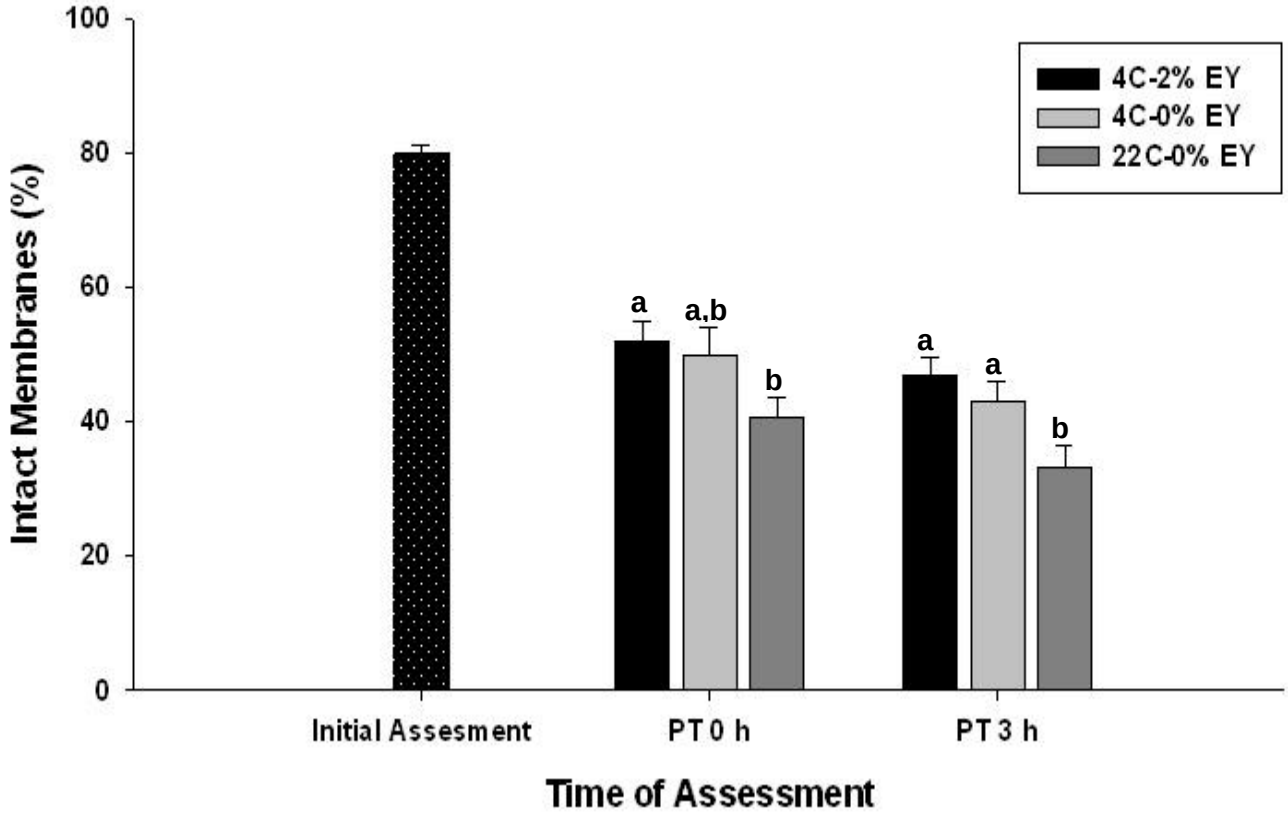

Figure 7.16. Membrane intact percentage (mean±SEM) of initial, post-cool, 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were processed in an extender containing 0 or 2% egg yolk; and frozen from either room temperature or after cooling to 4°C. [a,b]Mean values across treatments but within time intervals with different superscripts are significantly different (P<0.05).

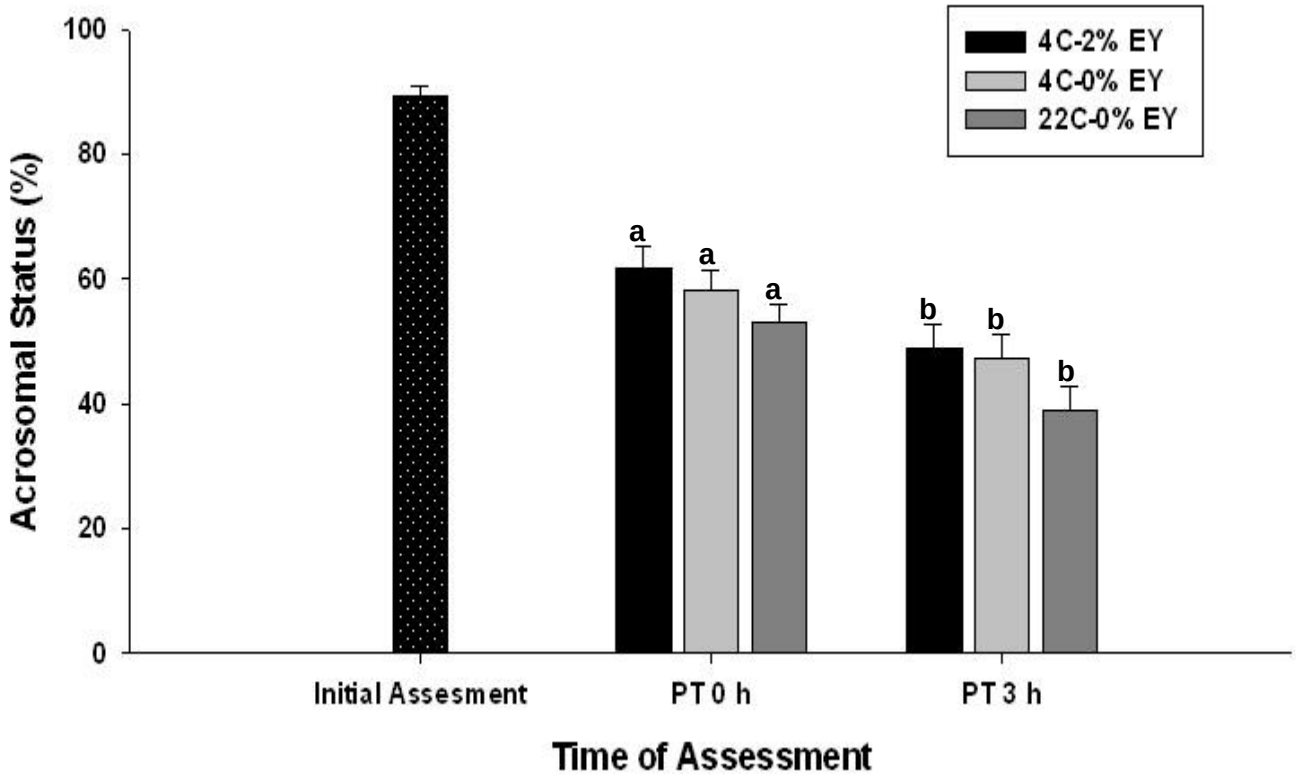

Figure 7.17. Acrosomal status percentage (mean±SEM) of initial, post-cool, 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were processed in an extender containing 0 or 2% egg yolk; and frozen from either room temperature or after cooling to 4°C. [a,b,]Mean values within treatments but across time intervals with different superscripts are significantly different (P<0.05).

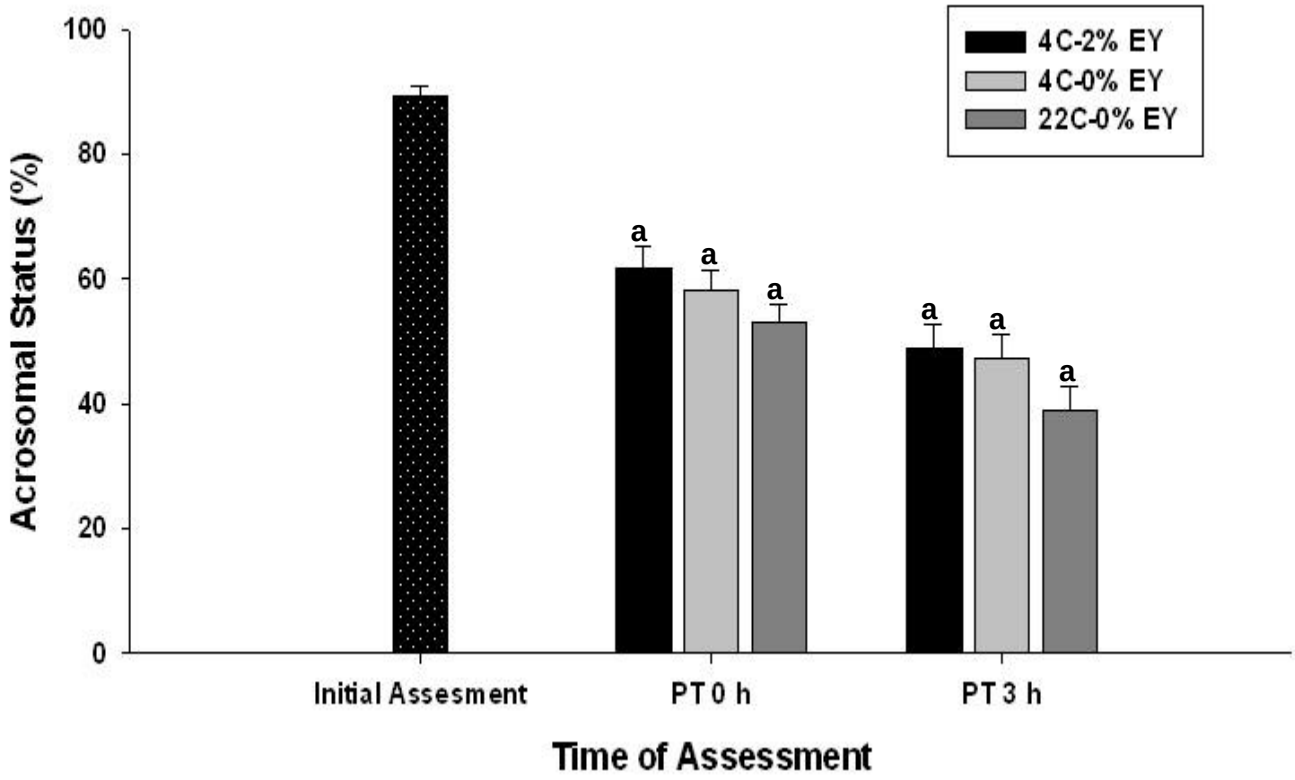

Figure 7.18. Acrosomal status percentage (mean±SEM) of initial, post-cool, 0 hour and 3 hours post-thaw for pooled domestic cat epididymal sperm that were processed in an extender containing 0 or 2% egg yolk; and frozen from either room temperature or after cooling to 4°C. [a,b]Mean values across treatments but within time intervals with different superscripts are significantly different (P<0.05).

extenders, sperm motility in 2% egg yolk and BioXCell® treatments was significantly higher than that of sperm in BioLife® at 24 hours of storage at 4°C. There was no significant difference among treatments at the 48 hour assessment, but at the 72 hours motility in 2% egg yolk was significantly higher than BioXCell® and BioLife® extenders. The lack of significant difference in sperm motility between the 2% egg yolk and the BioXCell® treatments was similar to that reported by Akhter et al. (2011) when they held Nili Ravi buffalo semen at 5°C in egg yolk-based, milk-based and BioXCell® extenders.

The membrane integrity results in the present study were similar to those of Akhter et al. (2011) when Nili Ravi buffalo semen was held at 5°C for 5 days in egg yolk-based extenders or BioXCell® extender.

Within treatments the percentage of sperm with intact acrosomes decreased significantly during 72 hours of storage, only in the BioXCell® treatment. While acrosomal intact sperm in TesT 2% EY and BioLife® did not decrease during storage. However, the percentage of sperm with intact acrosomes at 48 hours of storage in BioLife ® was significantly higher than that of sperm in 2% egg yolk and BioXCell®. For the 72 hour acrosomal assessment, the BioXCell® treatment had a significantly lower percentage of sperm with intact acrosmes than the TesT 2% EY and BioLife® treatments.

BioLife® extender is a commercial solution sold for use in preserving tissue cells and organs at temperatures between 2°C and 8°C (Ostrowska et al., 2009). Thus, we examined the ability of BioLife® extender to support survival (membrane integrity and acrosomal status) of cat sperm during cool storage. However sperm motility is a more complicated cellular process and possible for that reason the BioLife® extender was not able to maintain sperm motility.

In the next experiment (Experiment 6.2), we compared what we believed to be the top two extenders (2% egg yolk and BioXCell® treatments) on their ability to protect cat sperm during cryopreservation. When comparing sperm motility within treatments, both treatments performed similarly with no significant difference between the initial and post-cooled values. There was however an expected decrease in sperm motility when comparing the initial motility to the 0 and 3 hour post-thaw values. Also, motility significantly decreased from one time point to the next for both extenders when comparing within treatments. However, when comparing between treatments there was no significant difference for any of the sperm motility assessments. Contradictory to our results, Kulaksiz et al. (2012) reported that the ram semen frozen in the BioXCell® extender had significantly lower motility and morphologically normal sperm than those frozen in a milk-based extender.

When comparing within treatments for the 2% egg yolk-based and BioXCell® extenders, the post-cooled membrane integrity value was significantly greater than both the 0 and 3 hour post-thaw values but the two post-thaw values were not significantly different from each other. When comparing between treatments the only significant difference detected was at the 3 hour post-thaw assessment and the 2% egg yolk treatment had a significantly greater membrane integrity value than the BioXCell® treatment.

There was no significant difference among treatments at any of the time points across treatments when measuring acrosomal status. However, when comparing within treatments the post-cool assessment was significantly higher than the 0 hour, and the 0 hour assessment was significantly greater than the 3 hour assessment value for both extenders.

Both the 2% egg yolk and BioXCell® treatments exhibited a, within treatment, decline in values for all parameters (overall motility, membrane integrity, acrosomal status) when comparing pre- to post-thaw values and from 0 hour to 3 hours post-thaw. However, the lack of significant difference between the BioXCell® and the egg yolk extenders is similar to what Akhter et al. (2010) reported when they compared the post-thaw results of buffalo sperm motility, viability, plasma membrane integrity and normal apical ridge of sperm that were frozen in either the BioXCell® extender or a 20% egg yolk extender.

In the final experiment (Experiment 6.3), the cryoprotective ability of three extenders containing different concentrations of egg yolk were compared. The only difference among the extenders being the presence or absence of egg yolk and freezing after cooling or without cooling (4°C with 2 % EY, 4°C with 0% EY, 22°C with 0% EY). The initial motility, membrane integrity and acrosomal status values were significantly different from their 0 and 3 hour post-thaw counterparts for all three treatments. Also, when comparing within treatments, overall sperm motility significantly decreased from the post-cool (4°C) to the 0 hour post-thaw and from 0 hour to 3 hour post-thaw assessment times for all treatments. Likewise, when measuring acrosomal status, the 0 hour post-thaw values were significantly different from their 3 hour post-thaw counterparts for all three treatments. The difference between the 0 and 3 hour assessments was not detected for the membrane integrity evaluation. It is believed that the difference within treatments across time is due to the stress of cryopreservation. In a review article Bailey et al. (2000) discusses the damage to mammalian sperm during cryopreservation and the mechanisms of membrane damage and states that the sperm plasma membrane is the primary site of damage during cryopreservation. Holt and North (1994) reported that freezing ram sperm to -30°C damaged sperm head membranes. They also noted that thawing and warming ram sperm up to 30°C damaged to sperm middle piece. The damage caused to the

sperm plasma membrane would explain the decrease in sperm motility, membrane integrity and acrosomal status noted in our results when comparing between pre- and post-thaw assessments.

When comparing across the three treatments both the sperm motility and membrane integrity behaved in the same manner, with the 4°C with 2% egg yolk treatment having a significantly greater overall motility and membrane integrity value than the 22°C with 0% egg yolk treatment for the 0 hour post-thaw assessment. Also, for the 3 hour post-thaw assessment, the 4°C with 2% EY and the 4°C with 0% EY treatments both had significantly greater sperm motility and membrane integrity than the 22°C with 0% EY treatment. There was no significant difference across treatments when analyzing the acrosomal status. From these results we conclude that allowing the sperm to cool down to 4°C is more important than the absence or presence of egg yolk during freezing. This is different from what Aboagla and Terada (2004) reported in the male goat, where they reported that the presence of egg yolk significantly improved sperm motility and progressive motility.

In conclusion, domestic cat epididymal sperm can be stored at 4°C for up to 3 days in egg yolk and nonegg yolk-based extenders. Moreover, sperm can also be frozen in these egg yolk and nonegg yolk-based extenders with no significant differences between the extenders for sperm motility, membrane integrity or acrosomal status. Although, based on our findings it is not recommended, but feline epididymal sperm can be frozen in a Tes-tris-based extender completely void of egg yolk. Finally, allowing cat sperm to cool down to 4°C appears to be more beneficial to post-thaw sperm survival than whether or not the sperm are frozen with or without egg yolk. The results addressed in this discussion highlight possible extenders and/or concepts that may benefit cat sperm that are used in the future for the sex sorting process.

CHAPTER VIII
SUMMARY AND CONCLUSION

The primary lines of defense in preventing any exotic species from becoming endangered or extinct is habitat protection and elimination of poaching. These primary measures are often not feasible, and thus should not be the only means to prevent extinction. An additional approach to prevent species from becoming extinct is the use of assisted reproductive techniques (ART). Efforts should be made to develop new assisted reproductive techniques and improve the effectiveness and reliability of these technologies for in field use. This requires practice and repetition, and more often than not these technologies depend on first developing domestic animal models, such as the use of ART in cattle for the exotic ungulates and domestic cats for non-domestic felines.

The objective of this research was to identify a semen extender containing minimal or no egg yolk that would be effective for freezing domestic cat epididymal spermatozoa. Although egg yolk is effective in protecting spermatozoa of most species during cooling and freezing, its presence interferes with the cell sorting process.

In the first experiment we compared a TesT (Tes + Tris) 20% egg yolk extender (control) with modified human sperm preservation medium (HSPM) and Tris citrate extender that contained bovine serum albumin (TCBSA) to compare their effectiveness for preserving feline epididymal spermatozoa. Results showed no significant difference in membrane integrity and acrosomal status among treatments. There was a significant difference in the pre-cool motility values of the control versus HSPM extender, but not at 0 or 3 hours post-thaw. The absence of a post-thaw difference indicates that non-egg yolk extenders such as HSPM and TCBSA are possible alternatives to egg yolk extenders especially when a ‘clear’ extender is beneficial.

In the second experiment, I examined the effects of lowering the percentage of egg yolk in TesT extender on sperm survival after cryopreservation. Three egg yolk concentrations (10%, 5% and 2%) were evaluated. It was determined that motility, membrane integrity and acrosomal status were not significantly different among the three levels of egg yolk. Thus, 2% egg yolk was further evaluated in subsequent experiments.

In the third series of experiments, I examined the effect of exposure of spermatozoa, to 0mM, 1mM and 5mM of pentoxifylline (a motility stimulant) before and after freezing, on post-thaw survival of sperm frozen in TesT + 2% egg yolk extender. It was determined that there was no significant difference in post-thaw sperm motility, membrane integrity or acrosomal status when epididymal sperm were exposed to pentoxifylline both before and after freezing. When spermatozoa were exposed to pentoxifylline only after thawing sperm in the 1mM

treatment, had a significantly greater motility at 3 hour post-thaw than did sperm in the control treatment. Also at 3 hour post-thaw, membrane integrity for the 5mM treatment was significantly lower than the control treatment.

In the last series of experiments, two commercially available, non-egg yolk extenders, BioXCell® and BioLife®, were compared to TesT 2% egg yolk extender for their ability to maintain spermatozoa during a 72 hour storage period. Although no significant difference was noted, the TesT 2% egg yolk and the BioXCell® extenders were chosen as the two extenders to be tested for cryopreservation. Subsequently, I compared TesT 2% egg yolk extender versus the BioXCell® extender for their ability to protect feline spermatozoa during cryopreservation. There was no significant difference in sperm motility, membrane integrity or acrosomal status of spermatozoa frozen in the TesT 2% egg yolk when compared with sperm frozen in the BioXCell®. Again, the lack of significant difference between the extenders gives further evidence that non-egg yolk extenders such as BioXCell® maybe an effective replacement for the commonly used egg yolk-based extenders.

Finally, sperm were frozen in the TesT extender with and without egg yolk and frozen from room temperature and after cooling to 4°C. The TesT without egg yolk, frozen from room temperature, consistently had lower sperm motility and membrane integrity then sperm frozen in one or both of the extenders frozen after cooling to 4°C. There was no difference between the two TesT extenders frozen after cooling to 4°C for any of the parameters measured.

These experiments have shown that viable epididymal cat sperm can be collected from the epididymides of castrated toms, cryopreserved in extenders that contain little to no egg yolk and thawed, resulting in acceptable post-thaw values sufficient enough for IVF, possibly for AI and most certainly for ICSI. Using the domestic cat as a model Jewgenow et al. (1997) reported the production of in vitro-produced lion and puma 8-cell embryos using in vitro matured oocytes and frozen-thawed epididymal sperm recovered from the reproductive organs of postmortem animals. Furthermore, live offspring have been produced in the ocelot (Swanson et al., 1996) and the cheetah (Howard et al., 1997) when females were artificially inseminated laparoscopically with either fresh or frozen-thawed semen.

Also, with the identification of possible alternatives to the common 20% egg yolk extenders, the probability of future research successfully using flow cytometry to sex sorted previously extended or frozen-thawed cat sperm will likely be used in the future. Pope et al. (2009) reported the production of domestic kittens from predetermined sex and the first exotic species born using gender-sorted spermatozoa was the Elk (Schenk et al., 2003). While the

first aquatic mammal born using gender sorted sperm was the Bottle-nosed dolphin (O'Brien et al., 2006).

The ability to produce offspring of a predetermined gender from sorted feline epididymal spermatozoa (X and Y factions) could be very helpful in an endangered feline population when one gender would be more valuable in a captive breeding program. When working with feline spermatozoa one often deals with low concentrations, and after sex sorting the concentration is even further reduced. With such low concentrations it is likely that the utilization of sex sorted feline spermatozoa will need to be used in conjunction with the technique of intracytoplasmic sperm injection (ICSI). To date ICSI has not been effective in producing any exotic offspring; however, it has been used to produce domestic kittens (Pope et al., 1998) and exotic feline embryos (Pope et al., 1998; Damiani et al., 2004).

In conclusion, there is still further research that needs to be performed in the area of effective feline semen extenders that contain little to no egg yolk, however, the need for such an extender is undeniable. In the future, egg yolk free semen extender will likely improve ART procedures for animals in the future and will most defiantly help in the conservation of exotic species.

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APPENDIX

Appendix I. Components and specific molarities or concentrations of the three semen extenders evaluated in Experiments 4.1 (HSPM, TCBSA, and TesT) and Experiments 4.1, 5.1, 6.1 – 6.2, 7.1 – 7.3 (TesT).

Human Sperm Preservation Medium (HSPM)		Tris Citrate BSA (TCBSA)		TesT Yolk Buffer (TesT)	
Component	Concentration	Components	Specification	Components	Specification
Sodium dihydrogen phosphate	0.321mM	Tris	200mM	Tes	176mM
Calcium chloride	2.721mM	Citric acid	96.3mM	Tris	80mM
Potassium chloride	5.365mM	Fructose	27.8mM	Dextrose	9mM
Magnesium chloride	0.492mM	Glucose	9.0mM	Penicillin-G	1,000 units/mL
Sodium hydrogen carbonate	30.949mM	Bovine serum albumin	100 g/L	Streptomycin sulfate	1,000 mcg/mL
Sodium chloride	100mM	Trehalose	99.9mM	Egg yolk	20% (v/v)
Sodium lactate	12.856mM	Penicillin/ Streptomycin	10 µl/mL	Sterile filtered	Yes
HEPES	20mM	Sterile filtered	Yes		
Sucrose	50mM				
Glycine	133.21mM				
Glucose	5.506mM				
Bovine serium albumin	0.4 g/L				
Penicillin/Streptomycin	10 µl/mL				
Sterile filter	Yes				

HSPM (Mahadevan and Trounson 1983); TCBSA (modified from Fukui et al, 2007); TesT (commercially available).

Appendix II. Individual reagents used to formulate extenders used in the Experiments 4.1 and 5.1

Extender Reagents	Manufacturer	Product No.
Bovine serum albumin	Serologicals Proteins, Inc.	81-068-3
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	Sigma	C-7902
Citric acid ($C_6H_8O_7H_2O$)	Sigma	247529
Fructose ($C_6H_{12}O_6$)	Sigma	F-3510
Glucose ($C_6H_{12}O_6$)	Sigma	G-6152
Glycine ($C_2H_5NO_2$)	Sigma	G-8790
HEPES ($C_8H_{18}N_2O_4S$)	Sigma	H-6147
Magnesium chloride ($MgCl_2 \cdot 6H_2O$)	Sigma	M-2393
Potassium chloride (KCl)	Sigma	P-5405
Sodium chloride (NaCl)	Sigma	S-5886
Sodium dihydrogen phosphate ($NaH_2PO_4 \cdot 2H_2O$)	BioChemika	71502
Sodium hydrogen carbonate ($NaHCO_3$)	Sigma	S-5761
Sodium lactate ($C_3H_5NaO_3$)	Sigma	L-7022
Sucrose ($C_{12}H_{22}O_{11}$)	Sigma	S-1888
Trehalose ($C_{12}H_{22}O_{11} \cdot 2H_2O$)	Sigma	T-0167
Tris base ($C_4H_{11}NO_3$)	Sigma	T-1410

Appendix III. Quantities of base media and supplements used to prepare 100 mL of each type of culture media used for In vitro production of feline embryos

Item	IVM	IVF	IVC-I	IVC-II
Tyrodes Balanced Salt Solution	-	89.5 mL	88.5 mL	76.5 mL
Water, type I, fresh	-	8.0 mL	8.0 mL	8.0 mL
IVF/IVC 100X supplement	-	1.0 mL	1.0 mL	1.0 mL
$NaHCO_3$, 7.5% solution	-	1.5 mL	1.5 mL	1.5 mL
100X MEM NEAAs	-	-	1.0 mL	1.0 mL
50X MEM EAAs	-	-	-	2.0 mL
FBS	-	-	-	10.0 mL
BSA Fraction V	0.3 g	0.6 g	0.3 g	-
TCM 199 w/$NaHCO_3$ and w/o glutamine	9 6.75 mL	-	-	-
IVM 100X supplement	1.00 mL	-	-	-
hCG (1 IU/mL: 100 IU/mL stock)	1.00 mL	-	-	-
eCG (0.5 IU/mL: 200 IU/mL stock)	0.25 mL	-	-	-
EGF (10 ng/mL: 100X stock)	1.00 mL	-	-	-

IVM: in vitro maturation; IVF: in vitro fertilization; IVC-I: in vitro culture I; IVC-II: in vitro culture II (Pope 2006)

VITA

Jesse Ray Saenz was born in 1979 in Alamogordo, New Mexico. Jesse is the only son out of six children born to Martha and Raymond Saenz. Along with his parents, four of Jesse's sisters live in New Mexico, while one sister currently resides in Texas. Jesse grew up in the deserts of New Mexico and was often found running around the Sacramento Mountains where he acquired his passion for wildlife and the outdoors. In the spring of 1999, Jesse graduated from Alamogordo High School. Following graduation Jesse moved to Las Cruces, New Mexico, where he attended and graduated with a bachelor degree in animal science from New Mexico State University in the winter of 2003. In the spring of 2004 Jesse moved to Baton Rouge, Louisiana, where he lived and worked until able to enroll at Louisiana State University in the fall of 2004. In the fall of 2004, Jesse enrolled in the Master of Science degree program in reproductive physiology. With the approval of Dr. Robert A. Godke, Boyd Professor Jesse graduated with his Master of Science Degree in the field of animal reproduction in the fall of 2007. In the spring of 2008 and still a graduate student of Dr. Robert A. Godke, Jesse moved to New Orleans, Louisiana to begin his PhD research at the Audubon Center for Research of Endangered Species (ACRES) under the guidance and expertise of Dr. Charles E. Pope. While still a PhD candidate Jesse married his wife Melissa Pacheco and the two of them had a son, Carson Edward Saenz in December of 2013. Jesse is currently a candidate for a Doctorate of Philosophy degree in the field of animal reproduction with a specialty in collection and cryopreservation of epididymal spermatozoa.