

**CRYOPRESERVATION OF WHITE-TAIL DEER EPIDIDYMAL SPERM FOR
ARTIFICIAL INSEMINATION**

A Thesis

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ABSTRACT

The ability to cryopreserve epididymal sperm from mature postmortem bucks has long been of interest to both wildlife conservationists and deer ranchers. Increased understanding of the cryobiology of epididymal sperm from a nondomestic species, such as White-tail deer, could aid in development of future protocols to assist in the preservation of endangered species. In Experiment I, results showed that after cooling postmortem bull testes for 22 hours, no significant difference was noted between sperm parameters of epididymal sperm collected at room temperature or at a cool environment. In Experiment II, it was shown that White-tail deer sperm could be successfully cryopreserved using a bovine freezing protocol. Also, if immediate processing of epididymal sperm is not an option, testes can be held within the scrotum at 10°C to 15°C for up to 24 hours prior to processing the sperm for freezing. In Experiment III, post-thaw normal sperm morphology results show that glycerol should be considered over DMSO when freezing White-tail deer epididymal sperm. In Experiment IV, it was shown that White-tail deer epididymal sperm can be held in the presence of glycerol for up to 12 hours and still result in post-thaw motility values >30%. Also, when comparing exposure time of White-tail deer epididymal sperm to glycerol there was relatively no change in post-thaw membrane integrity from 0 hours to 24 hours. In the final experiment, the fawning rates of three different estrous synchronization protocols using timed artificial insemination were compared over two consecutive breeding seasons. The most preferred synchronization protocol was a 14 day CIDR with an eCG injection at the time of CIDR removal. A preliminary experiment was then conducted using the 14 day CIDR with eCG to synchronize and artificially inseminate six does with frozen-thawed epididymal sperm, resulting in five healthy fawns from three pregnancies.

CHAPTER I

INTRODUCTION

With an increased interest in managing exotic species by zoos, sanctuaries and/or private animal refuges, there is a growing concern regarding an increase of the inbreeding coefficient in animal populations. There is also concern of the decreasing numbers of endangered and threatened species remaining in the wild state. To address the problem of inbreeding, reproductive physiologists are now attempting to develop the use of new assisted reproductive technologies (ART) to replenish the genetics of wild and captive animal populations.

The objective of this research project was to develop an effective and plausible method to harvest testes and retrieve epididymal sperm from postmortem White-tailed deer (*Odocoileus virginianus*) that could be successfully cryopreserved for subsequent use in artificially insemination of does.

The cauda epididymidis has been shown to be a source of viable mammalian sperm that is capable of fertilization and has resulted in normal births. A recent example of the practical use of epididymal sperm for the purpose of endangered species conservation is the birth of a single Spanish ibex offspring (*Capra pyrenaica hispanica*) by artificial intrauterine insemination (Santiago-Moreno et al., 2006a).

In this study White-tail deer were used for two reasons. First, they would be an excellent conservation model for endangered Cervidae species. According to International Union for Conservation of Nature and Natural Resources (IUCN) there are currently 11 primary species of Cervidae, with additional subspecies, that are considered anywhere from vulnerable to almost extinct (www.iucnredlist.org). The White-tail deer is proposed for a conservation model because during the hunting season these animals are reasonably accessible. Knowledge gained could later be applied to related species that are currently threatened by inbreeding and/or extinction.

Second, there is an increased commercial interest in the ability to improve desirable animal genetics, such as body weight and antler size, in the deer hunting industry. In recent years trophy deer hunting has generated increasing revenue for ranchers, merchants and state governments. It is estimated that 94.3% of land in Texas is privately owned (http://www.texasep.org/html/Ind/Ind_5pub.html), with many of these land owners being cattle ranchers, who are finding it is more profitable to raise trophy males for hunting, rather than raising cattle. The International Association of Fish and

Wildlife agencies reported that Texas generated \$989,041,737 in retail sales in 2001 from deer hunters alone. Retail sales are dollars that are spent not only on items necessary for hunting but also money spent on food, supplies, lodging and travel. From these retail sales, the federal government collected a total of \$80,132,855 in taxes (IAFWA 2002).

Although these numbers are substantial, they do not include the money that hunters pay directly to the rancher for an opportunity to kill a trophy buck. In some instances, a hunter will pay \$10,000 dollars to harvest a large trophy male. The problem with the deer hunting industry is that bucks with trophy characteristics are the ones that are sought after and killed, thus removing these males from the breeding population. It is hoped that new assisted reproductive technologies will be applied in animal reproductive management, so that males with desirable genetics can continue to maintain their genes in animal populations long after their death. It has been reported that different antler characteristics have different degrees of heritability. For example, basal circumference and antler weight of a 3.5 year old buck is 0.80 and 0.28 heritable, respectively (Harmel et al., 1989)

Hopefully, using research developed in laboratory and farm animals, such as freezing epididymal sperm and artificial insemination, an attempt will be made to develop the White-tail deer into an effective conservation model that could be used for endangered species. Also, since the White-tail deer has in recent years become an increased source of income for the agricultural industry, new approaches to assist in reproductive management will help advancement of animal fertility and productivity.

CHAPTER II

LITERATURE REVIEW

SPERMATOGENESIS

Spermatogenesis is a process in which testicular stem cells develop into mature spermatozoa. There are three different subcategories of spermatogenesis, which include (A) spermatocytogenesis (mitosis), (B) meiosis and (C) spermiogenesis. These developmental events occur in the seminiferous tubules of the testes. (A) Spermatocytogenesis is a series of mitotic divisions that starts with diploid ($2n$) spermatogonia (A_1) cells, which are the most immature male germ cells. Spermatogonia are located at the periphery of the seminiferous tubules, and as they mature, move towards the lumen of the seminiferous tubules. The end result of spermatocytogenesis are primary spermatocytes, which remain as $2n$ cells. The purpose of spermatocytogenesis and the series of mitotic divisions is to provide base cells that will ultimately become spermatozoa (sperm). The spermatogonia serve as a pool of stem cells that allows the process of spermatogenesis to continue indefinitely (Johnson, 1994).

The production of gametes in the male only occurs by meiosis in the testis. The final step of meiosis separates homologous chromosomes to form two $1n$ diploid cells. During the process of meiosis DNA is synthesized, duplicated and divided. The purpose of meiosis is to ensure that each $1n$ cell contains a different combination of the same DNA, which ultimately insures genetic diversity between individuals of the same species (Amann et al., 1983).

The final step of the original spermatogonia is the metamorphic transformation to a $1n$ sperm cell. The sole purpose of sperm is to transport the male's DNA to the site of fertilization in the female. To accomplish this goal round spermatids need to physically change in shape so that their new shape will allow them to be progressively motile and move in a forward motion. This final step of metamorphism is the final testicular product, which is the tadpole-shaped sperm that society is familiar with, head, neck and tail. When the process of spermatogenesis is complete sperm will leave the testes and enter the male reproductive tract where they will complete the process of maturation (Senger, 1997).

The process of spermatogenesis is a very productive and efficient means of producing a large number of sperm capable of fertilization. With millions of sperm

produced per year, sperm morphological abnormalities do occur. In nine animal species that Gomes (1977) studied, the occurrence of abnormal sperm ranged between 10% and 40% (see Harcourt, 1991). Cohen (1973) has proposed that the incidence of abnormal sperm is simply an error in the process of spermatogenesis. In contrast, Barker et al. (1988, 1989) suggest that abnormal sperm were purposefully created and were never meant to partake in the act of fertilizing an ovum, but rather, they were constructed for the purpose of sperm competition and to hinder other male's sperm from achieving fertilization in instances when a female mates with multiple males during estrus.

ANATOMY AND FUNCTIONS OF THE MALE REPRODUCTIVE TRACT

The male reproductive tract is often divided into three categories. The first category is the "primary sex organs", which includes the male gonads usually referred to as testes or testicles. The second category, the accessory sex glands and ducts, include the seminal vesicles, prostate gland and the bulbourethral glands; while the ductile parts are the epididymis, vas deferens and urethra. The final category is the external genitalia frequently termed the copulatory organ. The only component of this group is the penis (Blom, 1968).

Mammalian males generally have a pair of testes, which depending on the species, can either be external, (e.g., bull, sheep and deer) or internal, for example, in beavers (Doboszynska et al., 1981) and elephants (Hildebrandt et al., 1998). Stewardson et al. (1998) reported that only during prime breeding season (November through December) the Cape Fur seals displayed a fully pendulous scrotum. During nonbreeding season the testes of the male seal are drawn into the groin but located external to the abdominal wall (Stewardson et al., 1998). In species with external gonads, the testicles are housed in a protective pouch (the scrotum) made from the skin and fascia of the abdominal wall. Testes serve two purposes: production and secretion of both sperm and sex hormones.

Generally, mammals have three accessory sex glands; however, the absence of one or more of these glands is not uncommon. All farm animals including stallion, boar and ruminants have all three of these accessory glands, however in the domestic cat the vesicular glands are absent, and in the dog the prostate is the only sex gland present, (Purswell et al., 1993).

Vidyadaran et al. (1999) reported that when dissecting six Lesser Mouse deer (*Tragulus javanicus*) the vesicular glands and the bulbourethral glands were identified

and noted that although the prostate was present it was not well defined. Goeritz et al. (2003) reported the presence of both a prostate and seminal vesicles, when ultrasounding five male Roe deer for morphology of testis and accessory glands, however, there were no bulbourethral glands identified. Also using ultrasound, Hafez et al. (2001) reported similar results in the male Dromedary camel with the presence of the bulbourethral and prostate glands and the complete absence of the seminal vesicles.

When present, the accessory glands are located in the pelvic girdle of each animal. Each gland is separate from the actual reproductive ducts and opens up into the urethra. Upon ejaculation, each gland deposits its secretions into the lumen of the pelvic urethra before, during and after the passing of the sperm (Senger, 1997).

The production of sperm is accomplished in an assembly-line type sequence. As the spermatogonia move from the outer edges of the seminiferous tubules to the center, they go through morphological transformations. In domestic animals, the process of spermatogenesis can take anywhere from 61 days in the bull (Johnson, 1994) to 39 days in the boar (Amann, 1986), 49 days in the ram (Amann, 1986), 57 days in the stallion (Amann, 1986) and 60 days in the dog (Amann, 1986)

Although testes weights between animals vary, Amman (1983) reported that sperm production was positively correlated with scrotal circumference in bulls. Carter et al. (1980) stated that scrotal circumference was not an acceptable means of predicting sperm production in Hereford and British Friesian AI bulls. In sheep, Land et al. (1975) measured the diameter of the testes from Black Face, Finn and Merino rams and found that the breed with largest diameter testes also had females with higher ovulation rates. Bailey et al. (1996) more recently reported a positive relation between testicular shape and sperm production in Holstein bulls. Testicles that are ovoid in shape produce more sperm per day than those testicles that are longer in shape, and more rounded testicles were shown to produce the least amount of sperm per day (Bailey et al., 1996).

Production and secretion of the testicular steroids are accomplished by the Leydig cells. These Leydig cells are found in the interstitial compartment of the testis, and have LH receptors. When these receptors are stimulated by LH, the Leydig cells produce progesterone, which is then converted into testosterone, the primary male sex hormone (Senger, 1997). Leydig cells have been found to produce, in addition to testosterone, progesterone and estrogen (Zirkin et al., 1980). The testes in the boar also have been found to produce and secrete Cholesterol₁₆ unsaturated steroid that is thought to be a male pheromone (Booth, 1982; Signoret, 1970).

The Sertoli cells of the testes are often referred to as the nurse cells because they provide the microenvironment necessary for spermatogenesis (Amann et al., 1983). The changes undertaken by the spermatogonia to spermatids are induced by FSH binding to the Sertoli cells, which then allows these cells to provide the environment necessary for spermatogenesis.

In seasonally reproductive male farm animals, such as rams (Lincoln et al., 1980) and stallions (Johnson et al., 1983) there is a reduction in sperm production, but not complete arrest, during the nonbreeding seasons. In the Roe deer, sperm production goes from no spermatogenesis activity and very low testosterone levels in the spring and summer (nonbreeding season) to a highly active state and high circulating concentrations of testosterone in fall and winter (breeding season) (Short et al., 1966; Blottner et al., 1996). Short et al. (1966) reported that a close relationship exists between sperm production and the increase of LH, FSH and testosterone during the rutting season. Studies reported on Red deer (another seasonally breeding Cervidae) have shown no sperm (Lincoln et al., 1971) or reduced sperm (Martinez-Pastor et al., 2005b) production during the nonbreeding/rutting season. In contrast to Red deer and Roe deer, Willard et al. (2002b) reported in Axis deer that although there were some seasonal changes in testes sperm production and morphology, the Axis bucks maintained testicular function throughout the year. Also Garcia-Macias et al. (2006) reported that there was less sperm chromatin condensation in Red deer and Brown bear during the breeding season when compared with the nonbreeding season.

After all the accessory glandular fluid is mixed it is referred to as seminal fluid, and once the seminal fluid is mixed with the sperm the mixture is referred to as semen. Research has shown that seminal fluid is not necessary for fertilization when using assisted reproductive techniques (ART), such as artificial insemination (AI), in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI). However, Poiani (2006) has reported that in natural mating the seminal fluid is very beneficial and serves multiple functions including: helping with sperm capacitation, contributing to sperm competition in the way of mucous plugs, pheromones and assisting in fertilization by maintaining pH, provides sperm nutrients, aiding in regulating acrosome reactions, as well as playing a role in sperm motility and female tract contractions.

In polyandrous species (species where more than one male mates with a fertile female) seminal fluid plays a role in sperm competition (Poiani, 2006). One method of sperm competition is the deposit of a mucous plug into the female tract by the male. A

mucous plug is a mass of hardening semen, which hinders the transport of sperm from other males through that female's tract (Harcourt, 1991). Dixson et al. (2002) evaluated 40 primate species and reported that in those species that were polyandrous, male seminal fluid coagulation was higher than those species that were monoandrous. Ramm et al. (2005) found similar results in rodents and reported a relationship between the testis size and the size of accessory sex glands. In the boar and the stallion, the last part of the ejaculate contains a viscous gel that has been proposed to plug the cervix to stop sperm backflow (Senger, 1997).

Anderson (1980) proposed that the first fraction of an ejaculate from the dog was from the prostate gland (see England, 1990). Since the urethra is responsible for the transport of sperm and urine, and since urine is harmful to sperm, the purpose of the prostatic fluid is to clean the urethra of residual urine. Also it has been suggested that in the farm animals the prostatic fluid along with the bulbourethral fluid helps maintain a pH of ~7.0, which is important for maintaining viable sperm (Blom, 1968).

Once released from the Sertoli cells, testicular sperm are transported from the rete testis into the efferent ducts, where the sperm leave the testis and enters the caput epididymidis. Contractions of the testicular capsule (Davis et al., 1970) and the seminiferous tubules (Roosen-Runge, 1951), along with the continual secretion of fluid from the rete testis and absorption of the fluid in the caput epididymidis (Macmillan, 1953) and cilia action of the efferent ducts (Davis et al., 1970) have all been listed as possible mechanisms in which sperm are transported from the rete testis into caput epididymidis. However, Leeson (1962) using electron microscopy, concluded that cilia movement alone would be of insufficient means to transport enough sperm from the testes to the epididymis.

Upon exiting the testicle the sperm enter into the head (caput) of the epididymis, and then travel to the body (corpus) and eventually the tail (cauda) of the epididymis. The tail of the epididymis is used as a storage compartment where the sperm are held until ejaculation. The time required for sperm to travel from the head of the epididymis to the tail varies between species. Amann et al. (1983) referenced in a review article that it takes sperm about 6.4 days in the bull, 9.8 days in the stallion, 6.4 days in the boar and 13.4 days in the ram to travel from the head of the epididymis to the tail. Movement of the sperm is achieved by continuous peristaltic contractions of smooth muscle surrounding the epididymis. It has been reported in the boar (Swierstra et al. 1971) and

in the bull (Amann et al., 1962) that frequent ejaculations does not affect the rate at which sperm travel through the caput and corpus epididymidis.

The epididymis is a completely separate organ from the testis that runs along the outside surface of each testicle. At the time of ejaculation the sperm will leave the epididymis by way of the vas deferens. The vas deferens then connects to the urethra. At ejaculation sperm will travel through the urethra and out the orifice of the penis. There is substantial amount of early literature describing, sexual arousal or false mounting of bulls (Hafs et al., 1959, 1962; Almquist, 1973), boar (Hemsworth et al., 1979; Arkins et al., 1988) and ram (McGrath et al., 1979) to increase total sperm concentration in an ejaculate. Collins et al. (1951) was one of the first to report a quantitative study addressing sperm concentration and sexual excitement.

Hafs et al. (1974) reported in bulls and rabbits that without sexual stimulation, an injection of prostaglandin $F_2\alpha$ did increase the amount of sperm collected. It should be noted that in the rabbit a significant increase in sperm collection due to prostaglandin $F_2\alpha$ was only found in the first of a series of four (once weekly) injections/collections. Also, in the bull even though prostaglandin $F_2\alpha$ caused a significant increase between injected and noninjected bulls, the greatest amount of sperm collected due to prostaglandin $F_2\alpha$ was still significantly less than bulls that were sexually stimulated.

SPERM ANATOMY AND FUNCTION

Sperm are made up of three distinct segments and their sub-segments. For this review these segments will be referred to as follows: the head, mid-piece and tail. The head consists of the acrosomal cap and the post-acrosomal region. The tail is made up of the principal piece and the end or terminal piece. The sperm head contains the dense and compact DNA. The shape of the head in most mammals is flat and oval shape. However, the rat, Golden hamster (Blandau, 1951; Leblond et al., 1952), Volcano mouse (Villalpando et al., 2000) and approximately 11 other subfamilies (~151 species) of rodents studied by Breed et al. (2005) have a sperm head that folds back onto itself producing a “hook” like shape, referred to as the apical hook. Rooster sperm, unlike the rat or other farm animals, have a very elongated head that comes to a point and is shaped much like a “spear”.

An important structure surrounding the sperm head is the acrosome. The acrosome is a thin double layered membranous sac that covers the anterior two thirds of the sperm head like a crescent cap. The acrosome is applied to the sperm head in the last stages of spermiogenesis. This acrosomal sac contains hydrolytic enzymes, which

include: acrosin (Muller-Esterl et al., 1981), hyaluronidase (Rowlands, 1944; Hunnicutt et al., 1996a; 1996b), esterases (Bradford et al., 1976) and acid hydrolases (NagDas et al., 1996). These enzymes are essential for fertilization. With the activation of these enzymes at the right time they allow the sperm head to fuse with the oocyte and assist in penetration of the oocyte to result in fertilization.

These enzymes are held in the acrosome until the appropriate time of the acrosome reaction or more commonly known as capacitation. Austin (1951) and Chang (1951) reported that before fertilization could occur in vitro the sperm must first reside in the female reproductive tract. This mechanism was termed capacitation, which was further described by Brackett et al. (1975), Aonuma et al. (1973) and Oliphant et al. (1973) as the series of enzymatic reactions, which releases the acrosomal enzymes to allow for fertilization, and naturally occurs in the female reproductive tract.

Aonuma et al. (1973) and Oliphant et al. (1973) reported that capacitation was the removal or change of seminal plasma components located on the sperm surface. Heparin and heparin-binding proteins found in seminal fluid have been shown to stimulate the capacitation of sperm, by allowing heparin-like glycosaminoglycans to induce capacitation in the female tracts of cattle and rats (Nass et al., 1990). If capacitation was to occur too soon or too late, it renders the sperm nonfunctional.

The next segment of the sperm cell is the mid-piece, which in the rat is made up of nine pairs of microtubules that arrange themselves radially around two core filaments (Nagano, 1963). Nagano (1963) compared cross sections of sperm tails from normal and cryptorchid rats. In the cryptorchid males there were 11 microtubules, however they were disarranged and not in a symmetric fashion as they are in sperm from normal males. The arrangement of these 11 tubules is called the axoneme. The axoneme is then again surrounded by more dense longitudinal fibers. The axoneme and dense fibers are then surrounded by mitochondria that twist longitudinally in a helical pattern. The tail is composed of the same components as the mid-piece, minus the mitochondrial helix. The axoneme along with the additional fibers and the mitochondrial sheath allow for flexibility of the mid-piece during the lateral movements of the tail during locomotion. Martinez-Pastor et al. (2004) have reported in ram frozen-thawed ejaculated sperm that mitochondrial activity and mitochondrial membrane potential were positively correlated with sperm motility and sperm velocity.

The final and longest segment of the sperm cell is the tail. As stated above, its make up is identical to that of the mid-piece except for the mitochondrial helix. The

mitochondrial helix is replaced by a fibrous sheath. The origin of movement is generated by the axoneme, which runs the full length of the tail. When microtubule pairs make a sliding movement in opposite direction from its adjacent pair motility is accomplished. These alternating movements from opposite sides of the mid-piece produce the bending waves of the tail, which projects the sperm forward (Satir, 1979; Gibbons, 1981)

The length of sperm and their individual segments can vary among species. The sperm from farm animals are similar in shape, however, can vary in size (Blom, 1968). From this author's observations goat sperm can measure a total length of 58.2 μm with the head, mid-piece and tail measuring 8.2, 12.8 and 37.2 μm , respectively, and a head width of 3.8 μm . Also, White-tail deer sperm measures 34.9 μm in total length, and 5.4, 6.8 and 23.4 μm for the head, mid-piece and tail length, respectively. The width of the sperm head in White-tail deer was measured at 2.6 μm . In a study that reviewed 168 species of rodents, Breed (2005) reported sperm mid-pieces that ranged from 7.5 μm to 63.3 \pm 35.9 μm and total lengths ranging from 55.5 \pm 3.5 μm to 175.5 \pm 46.5 μm . Saravia et al. (2007) reported sperm head measurements in five breeds of pigs. The smallest head length and width measured 8.8 \pm 0.29 μm and 4.5 \pm 0.15 μm , respectively, for the Yorker (hybrid) and 9.1 \pm 0.34 and 4.6 \pm 0.19, respectively, for the largest breed (Duroc). The longest known sperm cell is produced by a type of fruit fly known as *Drosophila bifurca*, with the sperm measuring 58 mm long (Gilbert, 2006).

Esteso et al. (2006) reported in Red deer that when epididymal sperm heads have less surface area, they survive cryopreservation at a better rate than sperm heads that have more surface area. Furthermore, sperm heads that were more elongated and narrow also froze significantly better than those sperm that had more rounded heads. Based on these results, it is likely that Red deer epididymal sperm head and shape can influence sperm freezability.

The purpose and function of the sperm remains consistent between all known mammals. This purpose is to safely transport DNA to the oocyte so the two haploid cells (sperm and ovum) can join to create a new individual to continue the propagation of the species.

FUNCTIONS OF THE EPIDIDYMIS

The epididymis performs various major functions necessary for the reproduction of most mammalian species. First, the epididymis serves as a passage way for sperm to travel into the vas deferens. The epididymis also concentrates the sperm, provides the environment and fluids necessary for sperm maturation and acts as a storage unit for

immature sperm that are ready for ejaculation. As sperm leaves the testicle it is accompanied by rete testis fluid, which is a diluent that makes ductile transport of the sperm easier. Upon reaching the epididymis, epithelial cells absorb rete testis fluid and secrete epididymal fluid, which concentrates the sperm and allows for more storage space (Brooks, 1983). While the sperm travel from the caput to the cauda epididymidis they continue to go through maturation. This maturation involves the migration of the cytoplasmic droplet, located on the mid-piece to travel from the proximal position to the distal position. Also while moving through the epididymal duct, rat sperm acquire the ability of increased progressive motility (Cosentino et al., 1986). Maturation of sperm predominantly takes place while sperm move from caput to the corpus sections of the epididymis (Brooks, 1983).

It has been reported that sperm from all regions of the epididymis have different levels of motility, with the percentage of motile cells and the progressive swimming pattern of these cells being higher in samples closer to the cauda epididymidis rather than the caput epididymidis (Kann et al., 1980; Cosentino et al., 1986; Perez-Sanchez et al., 1996). There is evidence that upon leaving the corpus, sperm are bound with "forward motility protein" (FMP), which allows the caudal sperm to move progressively rather than in circles or thrashing, which is observed in sperm from the caput region (Acott et al., 1981). FMP is attached to epididymal sperm just prior to entering into the proximal epididymis (Acott et al., 1981). It is believed that FMP is activated with elevated levels of cyclic AMP to produce progressive motile sperm. The mechanism by which FMP and cyclic AMP act is not yet understood (Acott et al., 1981).

It is believed that the process by which sperm gains its ability to fertilize is due to the addition and/or subtraction, or altering of surface proteins, until the correct receptors are on the surface of the sperm. These sperm receptors permit same-species recognition between the sperm cell and the oocyte (Brooks, 1983). The process of sperm maturation is very dependent on the presence of androgens, mainly dihydrotestosterone (DHT). The importance of DHT to induce the ability of proximal epididymal sperm to fertilize oocytes was illustrated when Orgebin-Crist et al. (1976) used an organ culture system to mature rabbit sperm in vitro from isolated epididymal tubules of the corpus epididymidis by the addition of DHT to the culture medium. DHT, a metabolite of testosterone, is synthesized in the epididymis, predominantly in the proximal sections of the epididymis when testosterone is acted on by the enzyme 5 α -reductase (see Brooks, 1983).

Since testosterone is needed for the synthesis of DHT and testosterone is produced and secreted primarily by the Leydig cells in the testicle, testosterone is transported to the epididymis primarily by way of rete testis fluid bound to a specific transport protein called androgen binding protein (ABP).

Androgen binding protein is produced in the Sertoli cells, located within the seminiferous tubules, and the release of ABP is regulated by FSH. The hormonal mechanism that starts in the hypothalamus and ends in the testes is a classical hormonal feedback loop. Gonadotropin releasing hormone (GnRH) causes the release of FSH and LH from the pituitary. FSH then acts on the Sertoli cells to release ABP. LH, meanwhile, stimulates the Leydig cells to release testosterone. Testosterone then feeds back on the Sertoli cells to make them more sensitive to FSH stimulation, Testosterone also binds with ABP and is transported to the epididymis (Hansson et al., 1976).

There is some evidence that the epididymis of such species as the rat, ram and rabbit can synthesize some androgen from acetate, a testosterone precursor (Hamilton et al., 1969, 1970, 1972). However, if the epididymis could produce sufficient amounts of androgen there would be no need for ABP to transport the androgen. Hansson et al. (1976) and later Brooks (1983) both report that the concentration of testosterone and testicular ABP in rete testis fluid is similar. This similarity in concentration makes a strong case for the idea that ABP is the transport mechanism by which testosterone reaches the cauda epididymidis.

It is unclear whether the androgens present in the epididymis enhance sperm maturation, by acting directly on the sperm or by acting on the epididymis to provide an environment for sperm maturation (Setty, 1979). Although the mechanism by which androgens cause sperm maturation is unclear, endogenous androgens are necessary for not only maturation but motility and survivability of sperm in the epididymis (Setty, 1979).

The epididymis stores sperm, which occurs primarily in the cauda region. Cosentino et al. (1986) reported that man holds two thirds of extra-testicular sperm in the cauda epididymidis, while the rabbit stores slightly less at 60% (Blom, 1968). The boar is capable of accumulating 3 weeks of its extra-testicular sperm into an enlarged cauda epididymidis (Blom, 1968). The cauda epididymidis not only stores the sperm but provides the proper conditions for holding sperm in a quiescent state of metabolism. The exact mechanisms which maintains potentially motile mature sperm in a quiescent state

are not completely understood. However the duration that the epididymis can maintain viable sperm is variable between species.

In most mammals, including farm animals, viable sperm can be retained in the epididymis for 2 to 3 weeks (see Setchell et al., 1993). It has been recorded in the smaller rodents (e.g., rats, rabbits and guinea pigs) that fertilization of cauda epididymal sperm decreases after being stored in the epididymis for 21 days (see Setchell et al., 1993). However, in these same species, if epididymal sperm is trapped in the epididymis (by ligation of the vas deferens) for up to 50 days, it will still express motility when finally released from the cauda epididymidis (see Setchell et al., 1993). Sperm of Japanese quail sperm loses its ability to be motile after only 3 days in the epididymis (Clulow et al., 1982). In reptiles (e.g., some snakes) epididymal storage lasts for several months, during hibernation. The longest known viable epididymal sperm storage for mammals is found in hibernating bats (Gustafson, 1979). Certain hibernating species of bats hold sperm for 10 months before mating upon emerging from hibernation (Racey, 1974, 1979). Despite the variability in the duration of sperm storage, in most cases, storage of sperm is most often achieved by the cauda epididymidis (Setchell et al., 1993).

Most researchers agree that the purpose for sperm being maintained in an immotile quiescent state is to conserve energy stores and postpone cellular membrane reactions, which will be required when the sperm travel through the upper female reproductive tract. However, there is debate over the mechanism in which sperm are maintained in their quiescent state. Some evidence suggests that sperm are held in quiescence by a change in osmotic pressure, since sodium and chloride ions are in much lesser concentrations in the epididymal fluid than they are in circulating blood (Setchell et al., 1993).

Also it has been speculated that amino acids could play a role in maintaining epididymal sperm immotile. This speculation is based on reports that in the caudal epididymal plasma, the amino acids glutamate, glutamine and asparagine are found at a higher concentration in the ram (Setchell et al., 1967; Jones 1978) and glutamine in the bull (Sexton et al., 1971). Higher concentrations of the amino acids hypotaurine, taurine and glutamate were found in the epididymal fluids of the boar (van der Horst et al., 1966; Johnson et al., 1972; Jones 1978) and taurine, hypotaurine in the rabbit (Jones 1978). In the rat, much higher concentration of glutamate was found in the luminal fluid of the corpus epididymidis (Hinton 1990). All these amino acids are found in much higher

concentrations in the epididymal regions and/or fluids than in the blood (Setchell et al., 1993).

Hoskins et al. (1978) have reported that in the bull an increase in cyclic AMP was required for the initiation of sperm motility. Acott and Hoskins (1981) reported that sperm motility in the bull occurs not only by an increase in cyclic AMP but also by the binding of forward motility protein.

In contrast to what Hoskins et al. (1978) earlier reported, Acott et al. (1984) showed that by inducing bull caudal epididymal sperm motility the cyclic AMP remained relatively constant. However, Acott et al. (1984) did agree with Hoskins et al. (1978) by stating cyclic AMP was a regulator of sperm motility. The latter group also theorizes that during maturation of sperm cyclic AMP steadily increases, so by the time sperm reaches the cauda epididymidis and are capable of motility, cyclic AMP no longer needs to increase for motility activation.

Usselman and Cone (1983) described a high molecular weight protein (>200 kd), located in the caudal epididymal fluid of the rat epididymis. Since the presence of this protein had not been reported previously, they named this protein, "immobilin". It was proposed that due to the high molecular weight of this protein, it made the caudal fluid more viscous and physically inhibited sperm motility.

In contrast, it has been recorded and confirmed on several accounts that pH levels in the caudal epididymal fluid are significantly lower than the rest of the body (6.6, rat; 5.8, bull) (Levine et al., 1978; Acott et al., 1984). Thus, it has been hypothesized that this lower pH suppresses sperm motility. Carr et al. (1984) has shown that when bull cauda epididymal sperm were held in vitro in caudal epididymal fluid at a pH of 5.5, sperm are immotile, however, when held in the same fluid except with a pH of 7.6, sperm become motile. It was concluded that caudal epididymal fluid contains an undefined factor that inhibits cauda epididymal sperm but is only active at a lower pH value, which is present in the cauda epididymidis. If there is a sperm motility inhibitor that is only active at a lower pH, it is completely inactivated once sperm enter the female tract. Lewis et al. (1984) reports that the pH level in the vagina of a dairy cow is 7.32 during estrus. This pH level is just three tenths of a point lower than the pH value reported to induce bull sperm motility by Carr et al. (1984).

Turner et al. (1985) compared the cauda epididymal fluid of five different species (rat, hamster, guinea pig, rabbit and human) to determine how epididymal sperm responded to their native cauda fluid, and how the cauda sperm from one species

responded to the cauda fluid of another species. Along with testing the interaction of cauda fluid and cauda sperm, they also tested the viscosity of each species' caudal fluid. It was concluded that the species with the highest to lowest viscosity was the rat, hamster, guinea pig, rabbit and human, respectively. In contrast to the rat, hamster, guinea pig and human, Turner et al. (1985) also found that the rabbit was the only species whose sperm were not immotile in its native fluids. When comparing how the sperm of one species responded to the caudal fluid of the other species, it was found that the rabbit epididymal sperm were heavily suppressed by all caudal fluids, except its own. Rat epididymal sperm were completely immotile in its own caudal fluid, and strongly suppressed in the guinea pig and hamster fluids, while the rabbit fluid had relatively no effect on it. The guinea pig epididymal sperm were completely hindered in its own and rabbit fluid, and showed little but not much motility in rabbit and hamster fluid. Hamster sperm were completely suppressed by its own and rat fluid, while guinea pig and rabbit fluids strongly inhibited it but did not leave completely immotile.

It has become evident that there is some sort of inhibitory factor and more likely a combination of factors in which sperm are held in a quiescent state. However, it is likely that the mechanism of epididymal quiescence is slightly different for each species.

COMPARISON OF EPIDIDYMAL SPERM AND EJACULATED SPERM

The ultimate goal in reproduction is to produce pregnancies and the method that will produce the best results is always going to be natural mating or at least the use of ejaculated semen. However, in many cases natural mating is not an option and ejaculated semen is unavailable, due to difficulty of handling the animal, death prior to collection or obstructive azoospermia preventing ejaculation (Drouineaud et al., 2003). In these cases, the best alternative source of viable, reproductively capable sperm are those stored in the cauda epididymidis. Research as shown that cauda epididymal sperm, when used with AI can produce offspring in a multitude of species, for example, the eland antelope (Bartels et al., 2001), goat (Blash et al., 2000), dog (Hori et al., 2005), gaur (Hopkins et al., 1988) and Spanish ibex (Santiago-Moreno et al., 2006a). When used with ICSI epididymal sperm has produced offspring in a few species including cattle (Goto et al., 1990) and rats (Hirabayashi et al., 2002). Kolbe et al. (1999) reported that using ICSI with epididymal sperm on in vivo or in vitro matured pig oocytes can result in cleavage, however, using fresh ejaculated sperm produced significantly higher cleavage rates than did epididymal sperm. More recently, Probst et al. (2003) reported

the birth of piglets using ICSI and epididymal sperm. For a more detailed review of success using epididymal sperm see Table 2.1

Despite its obvious reproductive potential, epididymal sperm does have some characteristics that make it noticeably different from ejaculated sperm. The most noticeable difference between epididymal and ejaculated sperm is the cytoplasmic droplet. This droplet can be located anywhere along the mid-piece of the sperm. However, maturity is estimated by the location of the droplet, the more distal the droplet is from the head the more mature the sperm. The cytoplasmic droplet was first described by Retzius in 1909 (see Cooper, 2005). The cytoplasmic droplet is a remnant of the cytoplasm from when the maturing cell was a spermatid. The shedding of the cytoplasmic droplet occurs when the sperm are introduced to seminal fluid (Harayama et al., 1996; Cooper 2005).

Other than the physical differences, there are also metabolic differences between epididymal and ejaculated sperm. Lardy and Ghosh (1952) reported that epididymal sperm respire at a much slower rate than ejaculated sperm, and from this observation they concluded that epididymal sperm is more efficient when it comes to the oxidative generation of utilizable energy. It has been reported in Red deer, that although post-thaw motility of epididymal sperm is equal, 8% glycerol is recommended over 4% glycerol due to the better post-thaw acrosomal protection (Martinez-Pastor et al., 2006b).

Epididymal and ejaculate sperm have been reported to respond differently to caffeine when used as a sperm motility stimulant (Weston et al., 2005). When equine epididymal sperm were incubated in a medium with caffeine, the sperm motility improved over time, however, when ejaculated sperm were incubated in the same medium their motility decreased over time. It was hypothesized that since the epididymal sperm were never coated with seminal plasma components, their susceptibility to caffeine was increased. It was also proposed that caffeine might inhibit cyclic nucleotide phosphodiesterase more efficiently in epididymal sperm, which would increase the levels of cyclic AMP of the epididymal sperm (Weston et al., 2005). Early research indicated that epididymal sperm are less susceptible to cold shock (Lasley et al., 1944; Bialy et al., 1959). In addition, Barrios et al. (2000) showed that proteins in seminal plasma reversed cold shock damage on ram sperm.

The region from which sperm are retrieved is crucial to its fertilizing potential. In the ram, it has been shown that sperm harvested from the corpus or caput epididymidis were unable to penetrate hamster oocytes in vitro. While sperm from the cauda

Table 2.1. Species and assessment of fertility in studies using epididymal sperm for assisted reproductive techniques

Species	Fresh/Frozen	Method of Fertilization	Assesment of Fertiltiy	Reference
Cat	frozen	IUI	offspring	Tsutsui et al. (2003)
Cattle	frozen	AI	pregnancy	Barker (1954)
	frozen	ICSI	offspring	Goto et al. (1990)
Dog	frozen	ICSI	offspring	Guerrero et al. (In press)
	frozen	IUI	offspring	Hori et al. (2005)
Eland	frozen	AI	offspring	Bartels et al. (2001)
Gaur	frozen	AI	third trimester ultrasound	Hopkins et al. (1988)
Goat	frozen	AI	offspring	Blash et al. (2000)
Horse	frozen	AI	offspring	Barker & Gandier (1957)
	fresh/frozen	AI/UTJ	pregnancies	Morris et al. (2002)
Human	fresh	AI	pregnancy	Hanley, (1956)
	fresh	IVF	pregnancy	Temple et al. (1985)
	fresh/frozen	ICSI	offspring	Cayan et al. (2001)
	fresh	ICSI	offspring	Drouineaud et al. (2003)
	fresh	ICSI	offspring	Rhouma et al. (2003)
Marmoset	fresh	AI	offspring	Morrell et al. (1997)
	frozen	AI	offspring	Morrell et al. (1998)
Mouflon sheep	fresh	IUI	offspring	Garde et al. (1995)
Mouse	frozen	IVF	fresh/frozen embryo transfer to produce offspring	Songsasen et al. (1997)
	fresh	IVF	embryo transfer to produce offspring	Songsasen et al. (1998)
	frozen in testes	IVF	embryo transfer to produce offspring	An et al. (1999)
	fresh	IVF	embryo transfer fetal count after recipient death	Kishikawa et al. (1999)
	frozen in testes	Microinjected	offspring	Ogonuki et al. (2007)
Pig	frozen	ICSI	embryo transfer to produce offspring	Probst et al. (2003)
Rat	frozen	IUI	offspring	Nakatsukasa et al. (2001)
	fresh	ICSI	offspring	Hirabayashi et al. (2002)
Red deer	frozen	IUI	Recovered day-7 embryos & transferred to produce fawn	Zomborszky et al. (1999)
	frozen	IUI	offspring	Soler et al. (2003c)
Sheep	frozen	IUI	offspring	Ehling et al. (2006)
Spanish Ibex	frozen	AI	offspring	Santiago-Moreno et al. (2006a)
White-tail deer	frozen	AI	offspring	Jacobson et al. (1989)

AI = artificial insemination; IUI = intrauterine insemination; ICSI = intracytoplasmic sperm injection; UTJ = utero-tubual insemination;

epididymis penetrated hamster oocytes at a rate that was not significantly different from that of ejaculated sperm (Williams et al., 1991). Furthermore, Nishikawa and Waide (1952) artificially inseminated a total of 19 rabbits with sperm from the testis (n = 6), caput epididymidis (n = 6) and the vas deferens (n = 7) and pregnancy rates were: 0, 16 and 57%, respectively (see Orgebin-Crist et al., 1969). Blandau and Rumery (1964) reported similar results in the rat when only 8% of mature oocytes were fertilized with caput epididymal sperm, while 93% of mature oocytes were fertilized with cauda epididymal sperm.

These subtle but obvious differences can all be attributed to the environment or fluid in which the sperm are located (e.g., seminal plasma for ejaculated, epididymal fluid for epididymal sperm). Epididymal plasma has many factors that maintain epididymal sperm through mechanisms that are not yet understood. There are a multitude of components in seminal fluid including: citric acid, ergothioneine, fructose, glycerylphosphorylcholine, sorbitol, ascorbic acid, amino acids, peptides, proteins, lipids, fatty acids and numerous enzymes (Garner et al., 2000).

These chemical elements found in seminal fluid differ between species due to size and absence of different accessory glands. Even though there is a wide array of research on mammalian seminal fluid, there are conflicting theories about whether seminal fluid helps or hinders sperm. For example, Hori et al. (2005) reported that when epididymal sperm was collected into prostatic fluid of the dog there were less sperm possessing a pre-freeze cytoplasmic droplet. Also, the dog epididymal sperm had higher post-thaw motility and viability values in those sperm that were exposed to prostatic fluid compared to those that were not exposed to prostatic fluid. Further more, the dog epididymal sperm in the prostatic fluid treatment showed a higher motility value after 6 hours at 20°C. Similar to the dog, a study in Red deer showed that allowing epididymal sperm to incubate and then freeze with seminal plasma improved post-thaw viability and motility values of the epididymal sperm (Martinez-Pastor et al., 2006a). Also, in a recent PhD dissertation, Guerrero (2006) showed that when seminal plasma is introduced to epididymal bovine sperm, and then removed, the occurrence of cytoplasmic droplets was reduced. The reduction of the droplets was thought to improve morphology of the epididymal sperm post-thaw since it was believed that the cytoplasmic droplets had a negative effect on the sperm morphology when subjected to cryopreservation. Harayama (1996) showed that the presence of fructose encourages the release of the cytoplasmic droplet in boar sperm. This supports Guerrero's findings, since fructose is

found in boar seminal plasma. Dott et al. (1979) showed that 1 hour of incubation of bull and ram sperm increased motility, however, at 3 hours of incubation, sperm motility had declined. Also in this same experiment, they showed that seminal fluid affected rabbit sperm negatively and in a much sooner time frame. Moreover, it was shown that when African buffalo epididymal sperm were frozen with bovine seminal plasma the results were significantly less than when sperm was not exposed to seminal plasma (Herold et al., 2004a).

Whether or not seminal plasma is beneficial to sperm, it does apparently serve a physiological purpose. It has been demonstrated in the bull that seminal plasma indirectly assists in the initiation of sperm capacitation, which is necessary to complete fertilization (Therien et al., 1999). Arangasamy et al. (2005) reported that exposure of Murrah buffalo epididymal sperm to isolated seminal plasma proteins (heparin and gelatin binding) assisted the sperm in mucous penetration and protected sperm membranes in vitro. Furthermore the concentrations 20, 30 and 40 mg of heparin and 30 and 40 mg of gelatin binding proteins showed better membrane protection than 10, 50 and 60 mg. All protein treated epididymal sperm showed a significantly higher membrane protection than did the control.

Another issue which needs to be considered is that when an ejaculate is collected artificially, whether by electro-ejaculation or by an artificial vagina, the sperm and the accessory secretions are mixed together. Since the accessory glands release their stock in a timely sequence during natural mating there is no evidence that all the accessory gland secretions and sperm are uniformly mixed (Dott et al., 1979). Therefore, in vivo sperm may not directly interact with those components of the seminal fluid that would cause a detrimental effect or diminish its capability for fertilization.

LIMITATIONS IN THE USE OF EPIDIDYMAL SPERM

Even with some success in the field of assisted reproduction, epididymal sperm has limitations that include: methods for harvesting sperm from the epididymis, techniques for freezing epididymal sperm, the cytoplasmic droplet and in most cases, collection of epididymal sperm is from a postmortem animal.

There are three main methods being used to collect epididymal sperm. However depending on the laboratory there are subtle variations in these methods. With each method the epididymides along with the vas deferens are dissected away from the testicle. The first method is mincing or dicing the epididymis up while it is in a sperm medium. In the medium the sperm will then swim away from the tissue and be collected

by pipette or filtration (Songsasen et al., 1998; An et al., 1999; Hewitt et al., 2001). This method is often used in smaller species where the epididymides are difficult to manipulate due to their small size (Martinez-Pastor et al., 2005a).

The second method is called the slicing, or puncture, method. In both the first and second method, much of the connective tissue and superficial blood vessels are dissected away from the epididymis. The epididymal ducts are either cut with a scalpel blade or punctured in several places with a needle. After the incisions the epididymis is often milked, to extract as much sperm as possible. Again, the epididymides are placed into a medium and allowed to incubate so the epididymal sperm can swim up into the medium and away from the epididymal tissue. The sperm rich medium is collected by either filtration or pipette (Bissett et al., 2005; Harshan et al., 2005). A disadvantage of these first two methods is the centrifugation of the sperm following collection. The purpose of the centrifugation is to remove the blood and tissue cells that sometimes contaminate the sample (Harshan et al., 2005; Suzuki et al., 2003). Centrifugation can cause structural damage to the acrosome (Mack et al., 1987), reduce motility (Alvarez et al., 1993) and induce enzymatic activity of the sperm (Benau et al., 1987), all of which reduce fertilizability.

The third method of epididymal sperm collection is the retrograde flush method, which has been reported in the boar (Lasley et al., 1944), stallion (Bruemmer et al., 2002), African buffalo (Herold et al., 2004a,b) and Red deer (Martinez-Pastor et al., 2006c). The idea of the retrograde flush is to move the epididymal sperm in a direction opposite of its normal transport. For this method, the vas deferens is threaded with either a needle or small tube, both of which are attached to a syringe, and then, either medium or air is used to push the sperm out of single small incision that was made at a distal location in the cauda epididymidis.

It has been shown in Red deer that the flushing method is preferred over the slicing method, because the flushing method produced less contamination and higher quality sperm (Martinez-Pastor et al., 2005a). Even though this particular project showed no difference in concentration, the authors believed that if executed correctly, flushing could produce higher sperm numbers than the slicing method. Gerber et al. (2001) also stated that the flushing method was an acceptable method to use out in the field when collecting epididymal sperm from African buffalo. In contrast, Cary et al. (2004) showed no difference in sperm concentration, pre-freeze total and progressive motility or morphology between the flushing method and a modified slicing method known as

flotation method, when collecting epididymal sperm in the stallion. Due to the lack of difference between the two methods, they preferred the flotation method because it is easy to prepare (Cary et al., 2004).

Whether it is the species being collected, ease of collection, contamination, concentration, quality of sample or simply capability, each method used to recover epididymal sperm has its advantages and disadvantages. Determining which method to use is just one concern when preparing epididymal sperm. Another problem would be to determine what protocol should be used to freeze epididymal sperm.

CHAPTER III

EVALUATION OF THE EFFECT OF PERFORMING EPIDIDYMAL SPERM EXTRACTIONS AT DIFFERENT TEMPERATURES FOLLOWING A 22 HOUR INTERVAL AT 4°C

INTRODUCTION

In the past, the inability to ejaculate or the unexpected death of a genetically valuable male would have simply been an unfortunate loss of desired genetics. However, advances in technology and understanding of the male reproductive anatomy have allowed researchers to harvest, cryopreserve and utilize these genetics in the form of immature sperm. The epididymides structure was first described in 1668 by de Graaf, however, it was not until 1913 when Tournade reported the first live epididymal sperm experiment (see Orgebin-Crist, 1998). Tournade showed in several different species that sperm found in the proximal region of the epididymis were immotile, while sperm recovered in the distal region were completely motile.

There have been studies on epididymal sperm from many different mammalian species. In many of these studies the epididymal samples came from laboratory or domestic animals with the collection of the testes from animals subjected to laboratory euthanasia (Hamilton et al., 1970; Perez-Sanchez et al., 1996), slaughter (Kundu et al., 2000; Kaabi et al., 2003), culling of wild herds (Herold et al., 2004a,b) or nonlethal castration (Hewitt et al., 2001; Cary et al., 2004). In a few instances, the epididymal sperm were collected by cannulation or fistulation of the vas deferens while the testes remained intact in the bull (Amann et al., 1963; Bennett et al., 1967; Deutscher et al., 1974; Holtz et al., 1974). However, no matter the method used for epididymal sperm collection, the availability of a near by laboratory was utilized to process the testes, thus eliminating the need to transport testes long distances for processing.

As informative as some of the earlier epididymal experiments have been, the access of a trained technician to a functional laboratory for the purpose of epididymal sperm extraction at the time of an animal's unpredicted death is often unavailable. Also, because of remote locations or unexpected death, the testes need to be removed and held for 24 hours or longer before sperm processing (Santiago-Moreno et al., 2006b; Saragusty et al., 2006). Others have held postmortem animal testes for the purpose of epididymal sperm research (Kikuchi et al., 1998; Songsasen et al., 1998; Stillely et al., 2000; Bruemmer et al., 2002; James et al., 2002; Yu et al., 2002a). The basic question remains, 'How does epididymal sperm survive and respond following prolonged

postmortem storage?' Spanish ibex (Santiago-Moreno et al., 2006a) and Desert and Mountain gazelle (Sargusty et al., 2006) testicles were removed and held at environmental temperature for up to 24 hours before harvesting epididymal sperm. In contrast, boar (Kikuchi et al., 1998), stallion (Bruemmer et al., 2002) and dog (Yu et al., 2002a) testes were cooled to 4°C or 5°C after removal from the body but in these studies epididymal sperm extraction was performed at room temperature. Songsasen et al. (1998) euthanized mice, and held their intact bodies at room temperature for up to 24 hours before removing the testicles and extracting the sperm from the epididymis. In the latter study, epididymal sperm were successfully used to produce live offspring using the assisted reproductive technique of embryo transfer of in-vitro produced embryos.

In instances where epididymal sperm are not immediately processed, cooling the testes prior to epididymal sperm extraction likely helps reduce tissue and cell death and thus, maintaining subsequent viability of the sperm (Soler et al., 2003a; 2005). Despite the benefits of cooling, testes are often rewarmed to room temperature when processed. Fluctuations in testicular temperature may present sperm viability problems, since it has long been known that mammalian sperm are very sensitive to temperature fluctuations. To minimize the damage due to temperature fluctuations, Martinez-Pastor et al. (2005a) and Soler et al. (2005) have both reported cooling of Red deer epididymides prior to extraction and then performed the extractions in a cooled environment, Santiago-Moreno et al. (2006b) also retrieved epididymal sperm at 5°C after epididymides were stored at 5°C for greater than 24 hours.

At present, no study has been conducted to evaluate epididymal sperm of bulls that were processed at room temperature versus a cooled temperature following a 24 hour storage period. The objective of this study was to evaluate parameters of bull sperm that were processed at either room temperature or a cooled temperature after being held at 4°C for 22 hours.

MATERIALS AND METHODS

Experimental Design

Experiment 3.1

This experiment was conducted to determine if there was a difference in pre-freeze and post-thaw sperm parameters of bull epididymal sperm that were held at 4°C for 22 hours postmortem, then removed from the epididymides and processed at either room temperature or a cool temperature. During the summer months of 2005, 11 pairs of testes were obtained at a local abattoir from reproductively mature bulls of mixed breeds

and various body weights. Testes within the scrotum were collected, placed in a pre-cooled Styrofoam ice chest and transported to the Embryo Biotechnology Laboratory (EBL).

The ice chest was cooled by frozen gel packs, prior to transport. Upon arrival at the laboratory, testes were placed in a 4°C refrigerator where they were maintained for up to 22 hours. Following the 22-hour storage period, one testicle from the pair was separated from the scrotum and randomly assigned to one of two treatments. The remaining testis was processed immediately following the processing of the first testis. Testes were dissected and epididymides were flushed at either room temperature (~19.2°C) (Treatment A) or cool temperature (Treatment B) at 12°C.

Once collected, sperm from each testis (n = 22) was kept separate and assessed using an inverted light microscope to determine total sperm motility. Also, fluorescent DNA staining (syber 14 and propidium iodide) was used to determine sperm membrane integrity of each sample. Sperm were then frozen in liquid nitrogen vapor and stored. Post-thaw samples were prepared and evaluated in the same manner used to evaluate the pre-freeze sperm samples. Three straws were thawed for each sample and the average of the three straws was the sample value.

Experimental Procedure

Testes Collection

Bovine testes with scrotum were collected postmortem from mature bulls (n = 11) at a local abattoir in Robert, Louisiana. Shortly following death, the scrotum containing both testes was pulled away from the body and a cut removing the testicles made close to the body, in such a way that as much of the spermatic cord as possible remained with the scrotum. Time of animal death and the time the testes were placed into the ice chest were noted. Testes were placed in plastic Ziploc bags and set into a Styrofoam ice chest (31.5 x 26 x 23 cm) that was pre-cooled using frozen gel packs. A piece of cardboard was placed between the testes and the gel packs to reduce rapid cooling of the testes. Before transporting, the temperature of the ice chest ranged from 3.1°C to 16.5°C, with a median temperature of 11.0°C. However, once testes were placed in the ice chest the temperature rose to as high as 28°C before cooling down during transport. The Styrofoam ice chest containing the testes was then transported by motor vehicle ~65 miles to the Embryo Biotechnology Laboratory in St. Gabriel, Louisiana. Upon arrival at the EBL testes in the plastic bags were transferred from the ice chest to a 4°C refrigerator where they were held for 22 hours.

Sperm Collection

After 22 hours at 4°C (24 hours after death), testes were separated from the scrotum, the tunica vaginalis surrounding the testis was dissected and the testis and epididymis were exposed. Before removing the tunica vaginalis, the testicular temperature was taken by placing a temperature probe through the previously made incision until it rested alongside the cauda epididymidis. Once testicular temperature was recorded, the cauda epididymidis and vas deferens were dissected away from the testicle and the cauda epididymidis was cut away from the still attached corpus epididymidis. The testicle was further processed to remove the remaining corpus and caput epididymidis, leaving just the testicle. The weight of the separated testicle and cauda epididymidis were then recorded. As the cauda epididymal tubules regress from the vas deferens to the corpus of the epididymis, a distinct anatomical difference in the diameter of the cauda tubules occurs at the medial portion of the cauda epididymidis, where an incision was made. The superficial blood vessels and thin membrane were dissected away, exposing the epididymal tubules. Using a scalpel blade (no. 10), a single tubule was bisected to allow the retrograde flow of epididymal sperm from the vas deferens side of the cauda epididymidis.

Epididymal flushing was performed using a flushing medium consisting of an egg yolk-based extender Part A (EY-TFC), composed of 3.075 g of Tris (Sigma-Aldrich Inc., St. Louis, MO), 1.261 g of fructose (Sigma-Aldrich, Inc.), 1.639 g of citric acid monohydrate (Sigma-Aldrich, Inc.) and 20 ml of egg yolk (fresh chicken eggs) in 80 ml of distilled water (EY-TFC extender). Before processing the testes, fresh EY-TFC extender was prepared and filtered through a 0.45 µm Nalgene syringe filter (Nalgene Company, Rochester, NY) to remove large egg yolk components. The medium was used in the retrograde flush of the cauda epididymal tubules.

The syringe used for flushing was made by heating the tip of the 12 ml Monoject syringe and then slowly stretching the melted tip until a small enough diameter was produced to fit inside the lumen of the vas deferens. Flushing was performed by threading the vas deferens with this plastic tube attached to the 12 ml syringe containing the EY-TFC extender. Once inserted, the plastic tube was held in the vas deferens using a Desmarres Chalazion forceps (78.74 mm). Cauda epididymal sperm were flushed using retrograde flow into a 15 ml plastic centrifuge tube that contained ~3 ml of EY-TFC extender. The extender prevented the concentrated sperm from sticking to the walls of the 15 ml centrifuge tube.

A 200- μ l volume of epididymal sperm-extender mixture was removed from the sperm sample and placed in a warmed 1.7 ml plastic microcentrifuge tube and diluted 1:5 with 37°C phosphate-buffered saline (PBS). For samples collected at room temperature (Treatment A) the 15 ml centrifuge tube with the remaining epididymal sperm-extender mixture was placed in a 250 ml glass beaker containing 200 ml of room temperature (23°C) water for 30 minutes, to allow the sperm-extender mixture to slowly equilibrate. Those samples collected in the cool temperature (Treatment B) remained in the cool temperature (12°C) until time for further processing. However, 200 μ l of the 'cooled collected' sample (Treatment B) was removed, handled and evaluated in the same procedure as the room temperature samples.

Pre-Freeze Sperm Analysis

The sperm sample analyses were completed on the 1:5 diluted sperm that had been allowed to warm in a 37°C water bath. The first sperm parameter recorded was total sperm motility using an inverted light microscope with a heated stage (Nikon Diaphot, Tokyo, Japan). Total motility of epididymal sperm was assessed after 5 minutes of incubation at 37°C. A 10 μ l droplet of 1:5 diluted sperm was placed on a warmed pre-cleaned glass microscope slide, covered with a warmed 18 x 18 mm glass cover slip and placed on the heated microscope stage (37°C). The motility of each sample (n = 22) was determined by calculating the average total sperm motility of three separate views of the same slide.

Also, membrane integrity was evaluated using fluorescent staining with a inverted light microscope (40X) equipped with ultraviolet fluorescence and a fluorescein isothiocyanate filter. Staining of the sperm to evaluate membrane integrity was accomplished by using the LIVE/DEAD viability kit (Molecular Probes, Inc., Eugene, OR). The kit included SYBR 14 (1 mM solution in DMSO), which stained the DNA of membrane intact sperm green (viable cells), propidium iodide (PI) (2.4 mM solution in water), which stained the DNA of membrane ruptured sperm red (nonviable cells). A 200 μ l volume of the 1:5 sperm dilution was pipetted into a microcentrifuge tube (0.65 ml) and gently mixed with 400 nM of SYBR 14 and 24 μ M of PI. Samples were protected from light (in a dark room) and maintained at 37°C for 10 minutes before being evaluated.

Determining the percentage of membrane intact (viable) sperm was accomplished by randomly counting 200 stained sperm using the fluorescent

luminescence of the Nikon inverted microscope (40X). The number of green fluorescent sperm (viable) was divided by 200 to calculate the percent live cells.

Epididymal sperm concentrations were determined using a hemacytometer and adjusted to a final concentration of 50 to 125 million sperm per ml by the addition of EY-TFC.

Sperm Cooling and Addition of Cryoprotectant

A 250 ml glass beaker containing 200 ml of water and the 15 ml centrifuge tubes were placed in a 4°C refrigerator for 4 to 6 hours to allow the epididymal sperm-extender mixture to cool to 4°C. After reaching 4°C, the second part (Part B) of the EY-TFC extender was slowly added to the mixture. The Part B extender was made of EY-TFC + glycerol at a 12% concentration. The Part B extender was added in a stepwise process until the ratio of Part A extender to Part B extender was equal (1:1). The final sperm mixture contained epididymal sperm, 20% egg yolk and 6% glycerol (freezing medium).

Cryopreservation and Thawing of Epididymal Sperm

The freezing medium was loaded into 0.5 ml semen straws (#005569, Cassou straw, IMV Technologies, Minneapolis, MN) and sealed using a heat sealer, resulting in ~10 straws (units) for each sperm sample (n = 22), or 220 units total. Once loaded, the straws were placed on a custom made Styrofoam boat, so that straws were 2 cm above the liquid nitrogen (Yu et al., 2002b). The boat was a 2 cm high square with the center portion removed, similar to a floating doughnut. On the top surface of the boat there was a thin plastic screen clam netting (1/4 inch squares) where the plastic semen straws laid. The hollowed out center allowed the liquid nitrogen vapor to come up underneath the straws to cool and freeze the sperm. Straws were maintained in liquid nitrogen vapor for 10 minutes and then plunged directly into the liquid nitrogen.

Straws remained in liquid nitrogen for 1 to 5 weeks before evaluating the post-thaw parameters. At the time of thawing, a single straw was removed from liquid nitrogen and immediately placed in a 37°C water bath and allowed to thaw for 30 seconds. Then the straw was removed from the water, dried and the cotton plug cut free. The open end of the 0.5 ml straw was then held upside down over a 1.7 ml microcentrifuge tube that was sitting in 37°C water bath. Using sharp pointed scissors a small cut is made on the top part (sealed side) of the straw. Cutting the straw a second time removes the vacuum of the straw and any fluid below the cut flows into the centrifuge tube. The whole thawing process did not take longer than 90 seconds. Post-thawed samples were prepared and evaluated in the same manner used to evaluate the

pre-freeze sperm samples. Three straws were thawed for each sample and the average of the three straws was the sample value.

Statistical Analysis

The testis morphological measurements, total sperm motility, sperm morphology and sperm membrane integrity statistically were analyzed using a Student t-test to compare pre-freeze and post-thaw values within and between treatments. Measured sperm parameters are reported as mean \pm SEM per treatment group and a P value ≤ 0.05 was used to determine significance differences for this study. Sigma Stat Version 3.0 was used to analyze each data set.

RESULTS

Experiment 3.1

Testes and Epididymides Weights

Testicular and epididymides weights prior to sperm extraction are shown in Table 3.1. The testes ranged from 184 g to 457 g and epididymides weights ranged from 7 g to 20 g. The mean for the left and right testes weights were 271.7 g and 312 g and the mean left and right epididymides weights were 12.3 g and 11.4 g, respectively. Variation in testes and epididymides weights were noted within males. The greatest variation between testes within animal was 153 g, while the least variation was 3 g. The greatest variation between the epididymides within bulls was 4 g, while both epididymides of one animal were of identical weights. Also, the greatest variation in testes and in epididymides were found in the same animal (no. 1). The same was true for the male with the least variation (no. 9). No significant difference ($P \geq 0.05$) was detected between mean values of the left and right testes or left and right epididymides.

Testicular Temperatures

The temperature of testes just prior to being dissected free of the epididymides are shown in Table 3.1. The temperatures at the junction of the cauda epididymidis and the tunica albuginea ranged from 2.4°C to 10.2°C with the mean temperature for the left and right testes being 6.7°C and 5.4°C, respectively. No significant difference was detected between the temperatures of the left and right testes.

Total Sperm Motility

The mean pre-freeze total motility for epididymal sperm was 54.1 \pm 3.7% at room temperature (Treatment A) and 51.4 \pm 3.9% for the cool temperature processing (Treatment B) (Figure 3.1). The mean post-thaw values for epididymal sperm recovered at room temperature was 30.1 \pm 4.4% (Treatment A) and 36.6 \pm 4.9% at the cool

Table 3.1 Testis and cauda epididymides weights and testis temperatures from individual postmortem bulls. Weights and temperature measurements were made at the time of epididymal sperm collection.

Bull number	<u>Testes wt (g)</u>		<u>Epididymides wt (g)</u>		<u>Testis temp (°C)*</u>	
	Left	Right	Left	Right	Left	Right
1	274	427	20	16	5.5	3.6
2	226	206	13	12	6.0	4.0
3	292	407	15	12	5.5	2.4
4	265	250	9	8	4.8	5.8
5	202	196	11	11	8.1	4.7
6	300	265	10	11	7.0	5.5
7	405	457	13	10	4.5	6.0
8	242	216	7	8	7.3	6.0
9	187	184	9	9	7.1	7.3
10	356	346	15	17	7.3	7.5
11	340	378	13	11	10.2	6.8
Mean \pm SEM	271.7 \pm 19.6	312 \pm 30.9	12.3 \pm 1.1	11.4 \pm 0.9	6.7 \pm 0.5	5.4 \pm 0.5

No significant difference was found between epididymal and testes weights or testis temperatures for right or left testes ($P \geq 0.05$).

*Temperatures were taken using a digital thermometer after the 22 hour storage period at 4°C.

temperature (Temperature B). There was a significant ($P \leq 0.05$) decrease from pre-freeze motility to post-thaw motility within Treatment A and within Treatment B, but no significant difference was detected between treatments for either pre-freeze sperm motility or post-thaw sperm motility.

Total sperm motility for both treatments prior to and after freezing for individual bulls is shown in Figure 3.2. There was a variation in sperm motility among males noted both before and after sperm freezing. For example, animals no. 8 and no. 9 achieved their highest post-thaw value of 20% and 45% in Treatment A, while animals no. 7 and no. 10 had sperm motility almost identically with 22% and 45%, except this occurred in Treatment B. Neither treatment consistently produced higher motility values across all bulls. The range of total motility for the room temperature treatment (Treatment A) was 30% to 70% for pre-freeze and 9.5% to 57.5% post-thaw motility. For the cool temperature treatment (Treatment B) the pre-freeze motilities ranged from 20% to 65% and post-thaw motility ranged from 2.5% to 62.5% motile. Bull numbers 1, 2 and 4 had post-thaw values in total motility that were higher than the pre-freeze values for the cooled temperature treatment (Treatment B).

Membrane Integrity

The mean percent pre-freeze membrane integrity for epididymal sperm harvested at room temperature (Treatment A) was $84.1 \pm 4.6\%$ and $81.1 \pm 3.4\%$ in the cool temperature treatment (Treatment B) (Figure 3.3). The mean post-thaw values for epididymal sperm collected at room temperature was $41.1 \pm 5.4\%$ compared with $51.0 \pm 4.3\%$ for the cool temperature treatment. There was a significant decrease ($P \leq 0.05$) in membrane integrity from the pre-freeze to post-thaw values within Treatments, however, there was no significant difference between treatments for either the pre-freeze or post-thaw membrane integrity values.

The percent membrane integrity values for both treatments, prior to and after freezing for individual males, are presented in Figure 3.4. The difference between the pre-freeze values for the two treatments was $\leq 6\%$ for the animals, except bull no. 1, 3, 4 and 6. There was no one treatment in which the epididymal sperm from all animals performed any better in, over the other treatment. For the room temperature treatment, pre-freeze membrane integrity was at least 80% for all animals, with the exception of male no. 3 at 40%. For the cool temperature treatment, animals except no. 1, 4 and 6 had a pre-freeze

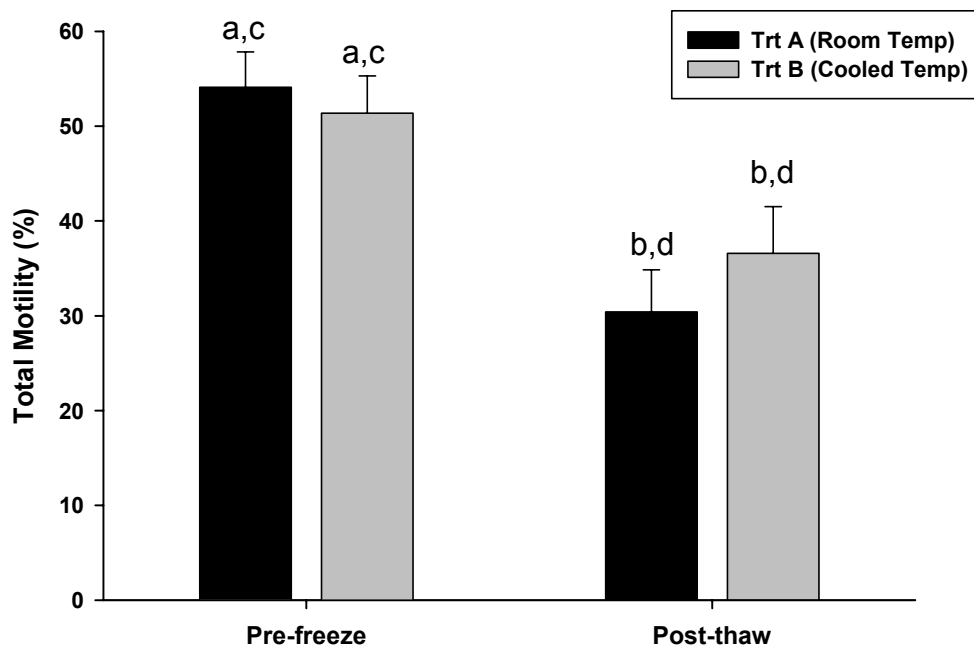


Figure 3.1. Percent total motility (mean±SEM) of pre-freeze and post-thaw bull epididymal sperm that were processed at room temperature (Treatment A) or at a cool environment (Treatment B). ^{a,b}Mean values within treatments with different superscripts are significantly different ($P \leq 0.05$). ^{c,d}Mean values between treatments with different superscripts are significantly different ($P \leq 0.05$).

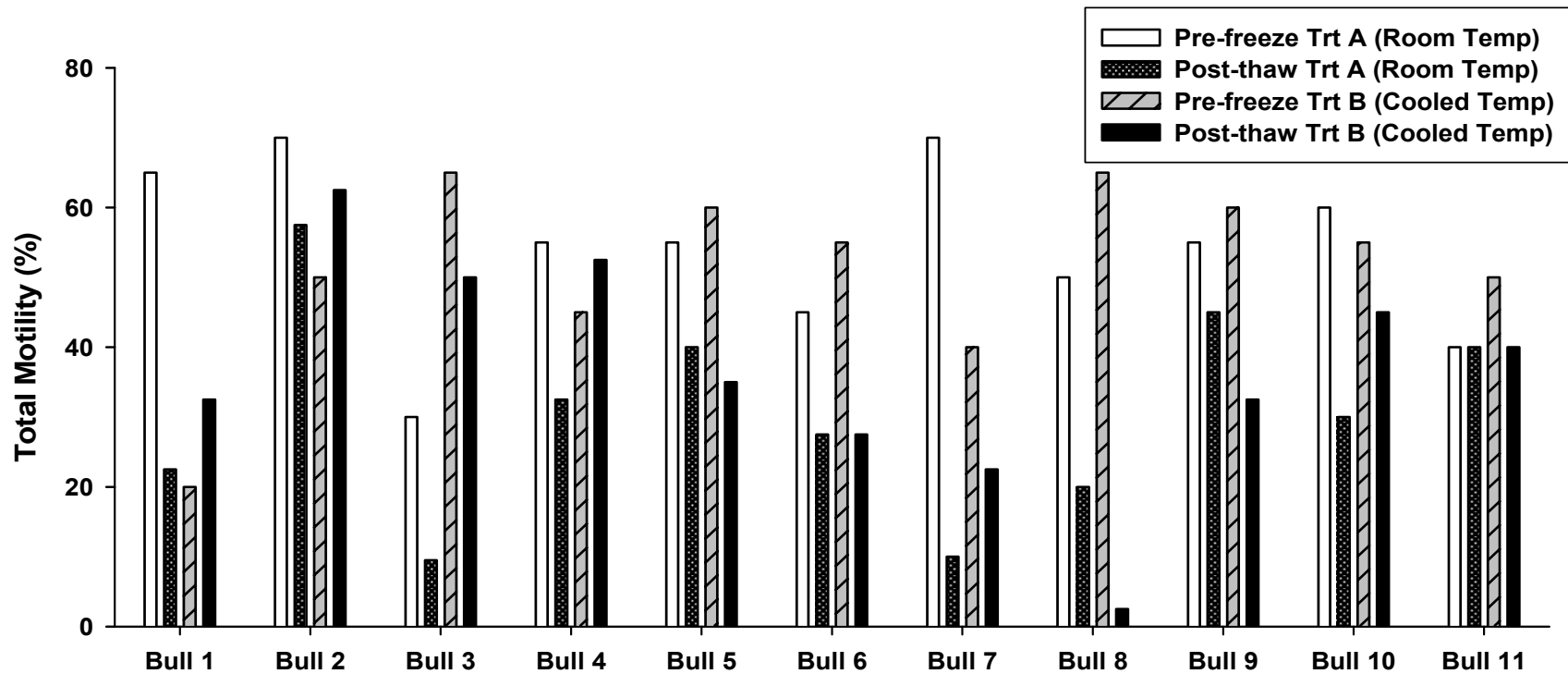


Figure 3.2. Pre-freeze and post-thaw total motility of epididymal sperm from individual bulls whose testes were processed at either at room temperature or at a cool environment.

value of at least 82%. The percent range of membrane integrity for the room temperature treatment (Treatment A) was 40% to 95% for the pre-freeze and 10.5% to 67% the post-thaw sperm. Sperm for the cool temperature treatment (Treatment B) the pre-freeze sperm ranged from 63% to 95% and post-thaw ranged from 22.5% to 69%.

DISCUSSION

Epididymal sperm studies have been conducted in a range of different species from the domestic cat (Niwa et al., 1985; Axner et al., 1998; Pushett et al., 2000) to horses, zebras, rhinoceros (Lubbe et al., 2000; James et al., 2002) and more recently the Spanish ibex (Santiago-Moreno et al., 2006a,b). However in these experiments the temperatures in which the testes were stored and processed have not been consistent. Some epididymal experiments cooled the testes down to 4 or 5°C and then processed at room temperature (James et al., 2002), while others cooled the testes down to 4 or 5°C and then processed the testes at 4 or 5°C (Santiago-Moreno et al., 2006b). While others do not cool the testes at all before processing, depending on the postmortem time that epididymal sperm was harvested (Axner et al., 1998).

To reduce cell degradation during extended holding periods testes are often cooled if processing of epididymal sperm cannot occur in a reasonable amount of time postmortem. However, due to the lack of an available 4°C processing room, many studies process the previously cooled testes at room temperature. In our current study an effort was made to evaluate if extracting epididymal sperm in a cooled environment produced different results than extracting sperm at room temperature. The objective of this study was to cool testes down to 4°C over a 22-hour interval, then remove the epididymal sperm at either room temperature (19 to 23°C) or in a cooled environment of 4°C. Pre-freeze and post-thaw sperm parameters were recorded and statistically evaluated.

When mature bull testes were evaluated, no significant difference was detected between the mean weights of the left and right testis or the left and right epididymides, across males. Correspondingly, it has been reported that no significant difference was found for any of the measured parameters (length, width, circumference and volume) when comparing left and right testes in Awassi rams (Salhab et al., 2001). Although weight of individual testes was not recorded in these intact rams in the latter study, the parameters that were measured correlated with testicular size.

In contrast to the study on Awassi rams, Dorper rams were placed into two groups that received different diets (intensively managed versus nonintensively managed)

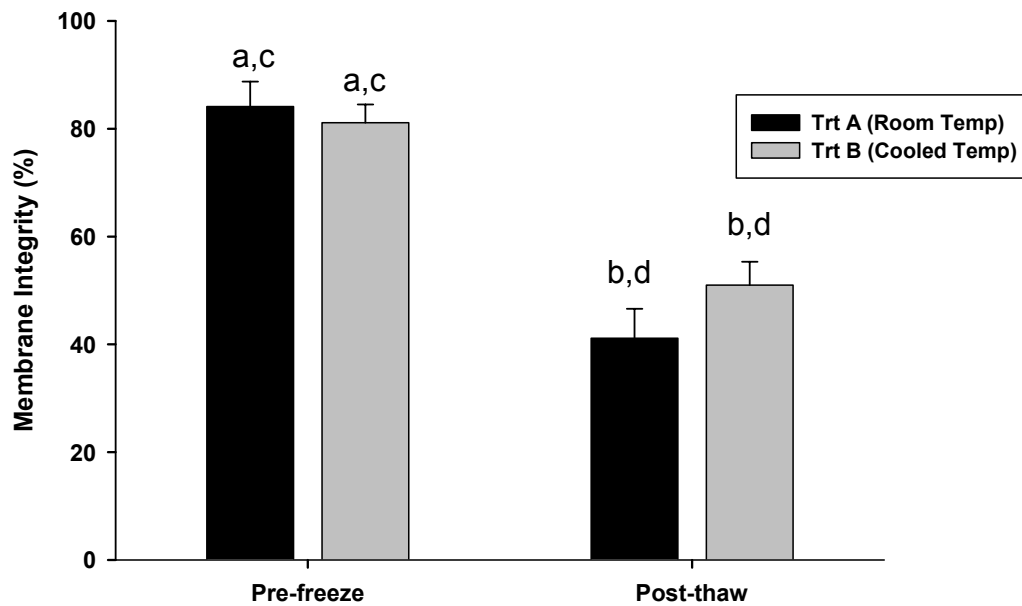


Figure 3.3. Percent membrane integrity (mean±SEM) of pre-freeze and post-thaw bull epididymal sperm that were processed at room temperature (Treatment A) or at a cool environment (Treatment B). ^{a,b}Mean values within treatments, with different superscripts are found to be significantly different ($P \leq 0.05$). ^{c,d}Mean values between treatments with different superscripts are found to be significantly different ($P \leq 0.05$).

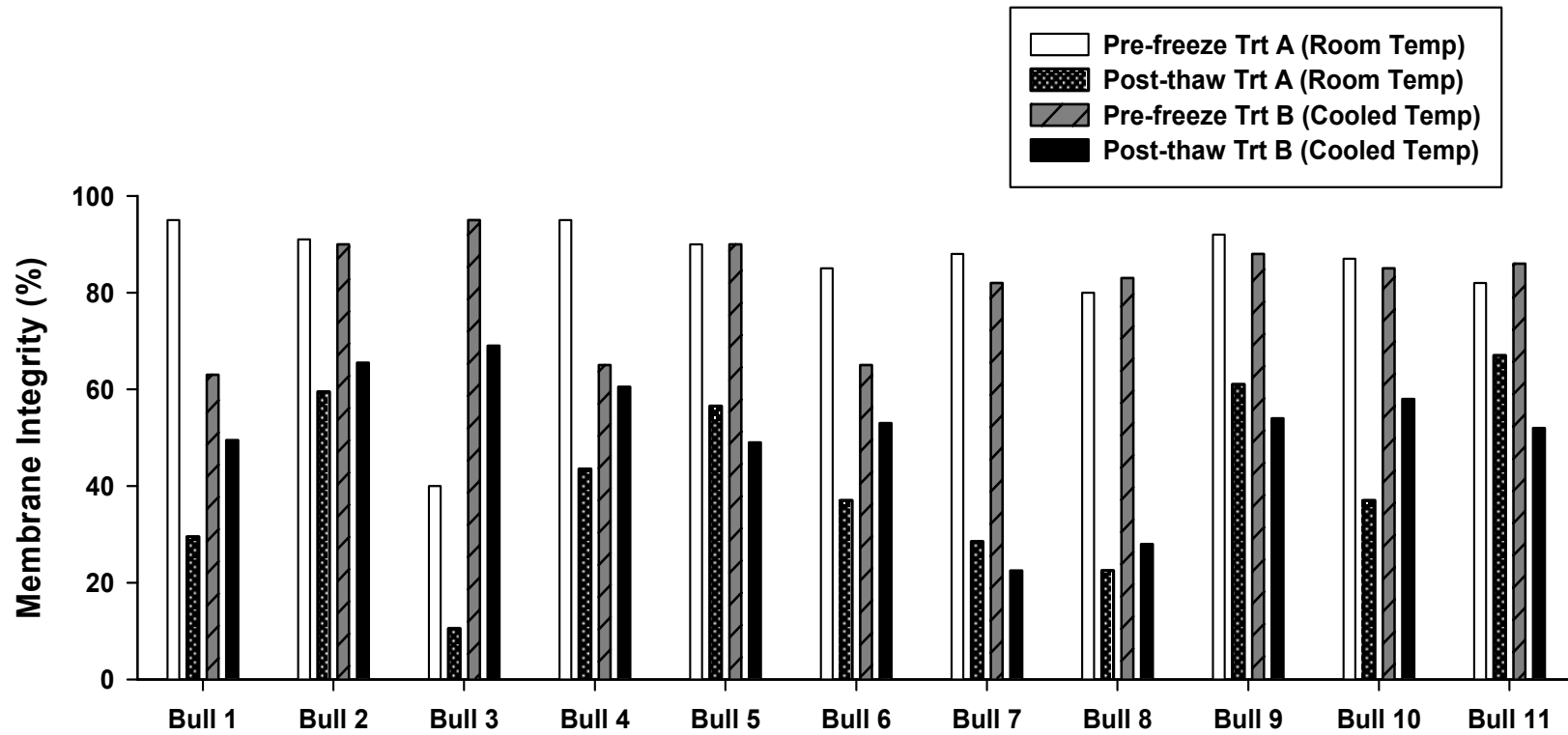


Figure 3.4. Pre-freeze and post-thaw membrane integrity of epididymal sperm from individual bulls whose testes were processed at either at room temperature or at a cool environment.

(Fourie et al., 2004). In this study, the intensively managed rams had a significantly greater scrotal circumference, fat deposit in the scrotal neck, total testes weight and testes volume. These differences, however were attributed to better body condition and more subcutaneous scrotal fat from the diet of the more intensively managed rams. Epididymides weights and volumes were also recorded for these Dorper rams but no significant differences were detected for epididymis parameters between the two groups.

In the present study, pre-freeze total mean motility (54.1%) and membrane integrity (84.1%) at 24 hours postmortem for sperm collected at room temperature was not significantly different from the total motility (51.4%) and the membrane integrity (81.1%) of the sperm collected at a cool temperature (4°C to 10°C). It has been proposed that cooling testes of such animals as the mouse (Kishikawa et al., 1999), dog (Yu et al., 2002a) and Red deer (Soler et al., 2003a; Martinez-Pastor et al., 2005a) during transport helps maintain epididymal sperm and protects them from membrane decomposition. Nonetheless, we found no significant differences for total motility or membrane integrity; further studies will need to be conducted to confirm these observations.

In the present study, it was noted that the post-thaw total motility for bulls no. 1, 2 and 4 was higher than that of the pre-freeze sperm motility for these bulls. This did not occur in bulls no. 3 and 5 through 11. Usually sperm post-thaw parameters are lower than pre-freeze parameters for ejaculated semen. A possible explanation for our results is that pre-freeze sperm were not allowed to completely warm up before evaluation.

An observation seen in all samples both before and after sperm freezing was a tight circular pattern in which sperm were swimming. When first observed, this circular swimming pattern was believed to be a result of cold shock (Leibo et al., 2002). But after continued observation of a sample the tight circular patterns tended to open up into bigger circular patterns, such that sperm would swim in and out of the microscopic view. It has been reported that sperm (Perez-Sanchez et al., 1996) collected from the corpus epididymidis had more progressive motility than did sperm collected from the testes and caput epididymidis. Also sperm collected from the cauda epididymidis and vas deferens had a more linear swimming pattern than did the sperm harvested from the corpus of the epididymides. In addition, Perez-Sanchez et al. (1996) also noted, sperm that were incubated tended to gain linear swimming patterns, as observed in our study with bull epididymal sperm. Acott et al. (1981) describes what is known as 'forward motility protein' (FMP) that could help further explain the circular swimming patterns in the bull.

The FMP is bound to developing sperm as they transcend through the epididymis but the protein does not become activated until the increase of internal cAMP. In goat epididymal sperm, forward motility was artificially induced using epididymal plasma, which was attributed to the presence of FMP in the epididymal plasma (Jaiswal et al., 1998). This resulted in epididymal plasma causing an increase in intrasperm cAMP.

In conclusion, if bovine testes are cooled before epididymal sperm is extracted, it is not necessary for epididymal sperm collection to be performed in a cool environment (4°C to 12°C).

CHAPTER IV

COMPARING TESTICLE HOLDING METHODS PRIOR TO EPIDIDYMAL SPERM EXTRACTION AND FREEZING OF EPIDIDYMAL SPERM FROM WHITE-TAIL DEER

INTRODUCTION

With 11 species of the world's deer being endangered or threatened, the White-tail deer would be an excellent conservation model because they are accessible and fertile. In animal agriculture, pen raising White-tail deer has become a ranching practice in many of the states where White-tail deer are known to be native. Recently deer ranching has been enhanced by producers realizing that trophy deer hunters are willing to pay up to \$10,000 dollars for the opportunity to kill a large antlered, trophy buck. Harmel et al. (1989) reported that antler characteristics and body weight are both heritable characters in White-tail deer. Body weight and number of antler points of a 3.5 year old buck was reported to have a heritability of 0.48 and 0.75, respectively (Harmel et al., 1989).

Most researchers agree that natural mating compared with artificial insemination produces the highest pregnancy rates. However, in some situations natural mating is not physically possible or economical. With the accidental death of a breeding male, epididymal sperm would likely be the best chance to save the genetics from a rare or valuable breeding male. With an increase in habitat destruction and a decrease in animal breeding populations, endangered species are at more risk of producing a homologous gene pool (Gilmore et al., 1998, Thongtip et al., 2004, Saragusty et al., 2006), which in turn makes these animals more susceptible to infection and/or disease. With endangered animals, the genetics of any individual is important to keeping the diversity in the species. Thus conservation of gametes would insure the ability of that individual's genes to remain in the population.

With the interest and increasing profits from raising trophy animals, many cattlemen are turning their cattle ranches into high-fenced hunting ranches. As in the cattle operations, these new deer ranchers will have the need to improve the genetics of their deer herds. But unlike the cattle industry, many states now have issued a moratorium on the movement of any deer species across state lines. This moratorium is due to the recent outbreak of a neurological disease, known as Chronic Wasting Disease (CWD). Fortunately, this moratorium does not apply to gametes, embryos or reproductive tissues, which allows the potential for the movement of genetics from one

state to another without transporting the live animal. In this case, collecting ejaculated semen from desired males would be the ideal situation. However, collecting ejaculated sperm from untamed males that have rarely or never been handled is usually more difficult than economical. Since a deer producer can not sell a genetically valuable male or his progeny across state lines, it would be more logical to hunter harvest the male by a paying hunter, and harvest the buck's epididymal sperm and sell the cryopreserved sperm. For White-tail deer epididymal sperm to be used successfully in commercial animal agriculture, research is essential to modify existing methods, previously developed for collecting and cryopreserving ejaculated sperm. Ejaculated and epididymal sperm are similar in many ways, but despite these similarities there are some physiological differences. With the help of further epididymal sperm research these differences can be addressed and accommodated for.

With the White-tail deer harvest locations usually being in remote areas, proper laboratory equipment and experienced technicians are seldom available for tissue processing. In these instances, testes would need to be shipped overnight or driven to facilities for sperm recovery and cryopreservation. Often the transportation of testes will take longer than 24 hours, so it has been recommended that testes should be cooled to 4°C or 5°C to help preserve the sperm during transit (Kishikawa et al., 1999; Yu et al., 2002a; Soler et al., 2003a; Martinez-Pastor et al., 2005a). These studies have reported that cooling the epididymides often helps maintain epididymal sperm parameters and helps prevent cell damage during transit and/or storage. Although studies have been published on cooling the testes for transport, to our knowledge none have addressed the condition in which the testes should be cooled. For example, should the testes be removed from the scrotum or remain within the scrotum, or should they be placed in a cold water bath to induce a faster cooling rate?

A decrease of total sperm motility, progressive motility and membrane integrity are generally expected when comparing pre-freeze values with post-freeze values. In fact if the data does not show a decrease in these parameters it is assumed that some sort of error was made by the technician. Since epididymal sperm has been reported to have different physiological properties from ejaculated sperm (see Fernandez-Santos et al., 2006b). When developing freezing protocols for epididymal sperm, these need to be considered.

The objectives of this study were to compare two different methods for holding White-tail deer testes during cool storage (24 hours). Also, to successfully freeze White-

tail deer epididymal sperm using the bovine freezing protocol from the previous experiment.

MATERIALS AND METHODS

Experimental Design

Experiment 4.1

Paired testis from 14 mature White-tail deer were collected postmortem from hunter harvested bucks during the 5 weeks prior to the rutting season, during the 2005-2006 Louisiana White-tail deer hunting season. After removal from the body, testes were randomly split into treatments, so that one testis remained within the scrotum (Treatment A), while the other testis of the pair was removed from the scrotum (Treatment B). Both testes were placed in a single Ziploc plastic bag placed into a pre-cooled Styrofoam ice chest containing frozen gel packs, and transported to the Embryo Biotechnology Laboratory (EBL). Testes were held in the ice chest for a 24-hour storage period before epididymal sperm were flushed from the cauda epididymidis. After collection, sperm was extended and allowed to cool slowly to 4°C and then frozen using liquid nitrogen vapor.

Pre-freeze and post-thaw motility was assessed by a single technician using an inverted microscope equipped with a heated stage. The motility of each sample (n = 28) was determined by taking the average total motility of three separate views of the same slide. Also for each straw thawed, motility was assessed at three different time periods after thawing (10 minutes, 30 minutes and 60 minutes). Both pre-freeze and post-thaw morphology was determined using a mixture of the sperm samples (n = 28) with eosin-nigrosin stain on a microscope slide, under a light microscope. Pre-freeze membrane integrity was evaluated visually using fluorescent microscopy and the post-thaw membrane integrity was assessed using multicolor flow cytometry.

Experimental Procedure

Testes Collection

During the 2005-2006 Louisiana White-tail deer hunting season managed hunts were conducted by Louisiana Department of Wildlife and Fisheries (LDWF) on different wildlife refuges. During these hunts, area sportsmen harvested White-tail deer and by LDWF regulations were required to check the animal in at mandatory check stations located on the refuge. At the time of check in, animals were aged, weighed and antler measurements were recorded. Also at this time, paired testes samples were removed from the animal in such a way, that as much of the spermatic cord as possible remained with the scrotum. Testes were then randomly placed in either Treatment A (testis held in

the scrotum) or Treatment B (testis not held in the scrotum) and both were placed in the same Ziploc plastic bag and into a cooled Styrofoam ice chest (31.5 x 26 x 23 cm), containing 4 to 6 frozen gel packs. From previous trials the pre-cooled ice chest temperature was expected to be 10°C to 15°C. A piece of single layered cardboard was placed on top of the gel packs to prevent direct contact and reduce rapid cooling rate of the testes. The Ziploc bags were placed on top of the cardboard with the lid firmly sealing the ice chest, prior to transporting the testes back to the EBL. For each animal (n = 14) the time of death, and the time of testicle removal, was recorded at the same time as the age, body weights and antler measurements. The time from animal death to the time of testicle removal varied from 0.66 hours to 4.09 hours. Testicles were transported to EBL and remained stored in the same cooled ice chest for ~24 hours before epididymal sperm were extracted.

Sperm Collection

Following the 24-hour storage interval one testicle from the pair was removed from the ice chest and processed with a note being made of its treatment (in or out of the scrotum). Processing started by making an incision in the tunica vaginalis surrounding the testis. Before removing the tunica vaginalis testicular temperature was taken by placing a temperature probe (DiGi-Sense[®] Dual Input JTEK Themocouple Thermometer) through the previously made incision until it rested alongside the cauda epididymidis. Once testicular temperature was recorded the cauda epididymidis and vas deferens were dissected away from the testicle and the cauda epididymidis was cut away from the attached corpus epididymidis. The testicle was further processed to remove the remaining corpus and caput epididymidis, leaving just the remaining testicle. The weights of the separated testicle and cauda epididymidis were then determined. An incision was made where the epididymal tubules regress from the vas deferens to the corpus of the epididymides (a distinct anatomical difference in the diameter of the cauda tubules that occurs at the medial portion of the cauda epididymidis). The superficial blood vessels and thin membrane were dissected away exposing the epididymal tubules. Using a scalpel blade (no. 10), a single tubule was bisected to allow the retrograde flow of epididymal sperm from the vas deferens side of the cauda epididymidis.

On the day of testicle processing, Triladyl, a commercial one step extender (Minitube of America, Inc., Verona, WI) was prepared with 20% egg yolk (fresh chicken eggs), and filtered through a 0.45 µm Nalgene syringe filter (Nalgene Company,

Rochester, NY). Before retrieving the epididymal sperm, ~3 ml Triladyl extender was added to a 15 ml centrifuge tube to prevent sperm from sticking to the sides of the collection tube. Epididymal sperm were then flushed from the epididymal tubules with the Triladyl extender, using a retrograde flow.

The syringe used for flushing, was made by heating the tip of the 12 ml Monoject syringe and then slowly stretching the melted tip until a small enough diameter was made, to fit inside the lumen of the vas deferens. Flushing was performed by threading the vas deferens with the plastic tube attached to the modified 12 ml syringe, which contained the Triladyl extender. Once inserted, the plastic tube was held in the vas deferens using a Desmarres Chalazion forceps (78.74 mm). Cauda epididymal sperm was then flushed into the 15 ml centrifuge tube containing the extender.

A 300 μ l volume of epididymal sperm-extender mixture was placed in a warmed 1.7 ml plastic microcentrifuge tube and diluted 1:5 with 37°C phosphate-buffered saline (PBS). The 15 ml plastic centrifuge tube with remaining epididymal sperm-extender mixture were placed in a 250 ml glass beaker containing 200 ml of room temperature water for 30 minutes to allow a slow equilibration of the sperm-extender mixture. After 30 minutes, the beaker with the 15 ml tubes was placed in a 4°C refrigerator and allowed to reach 4°C before being further processed.

Pre-Freeze Sperm Analysis

All sperm parameter measurements were made from the 1:5 diluted sperm that had been allowed to warm in a 37°C water bath. The first sperm parameter recorded was total sperm motility using an inverted light microscope with a heated stage (Nikon Diaphot, Tokyo, Japan) and Hoffman optics (40X objective). Total motility of epididymal sperm was assessed after 5 minutes of incubation at 37°C. A 10 μ l droplet of 1:5 diluted sperm was placed on a warmed pre-cleaned glass microscope slide covered with a warmed 18 x 18 mm glass cover slip and placed on the heated microscope stage (37°C). The motility of each sample (n = 22) was determined by calculating the average total sperm motility of three separate views of the same slide.

Sperm morphology was also evaluated using a single drop of eosin-nigrosin stain (Lane Manufacturing, Inc, Denver, CO), placed on a pre-cleaned glass microscope slide and mixed with ~2 μ l of sperm sample. Using another slide this mixture was then dispersed all along the slide in such a manner that the concentration of the stain decreased from left to right. After the stain dried, morphology for each sample was determined by placing the slide under a 100X oil immersion objective of an upright light

Nikon microscope. A total of 200 sperm were counted at random and placed into one of the six categories as follows: normal, bent tails, broken mid-pieces, proximal droplets, abnormal heads or detached heads. The percentage of sperm in each category was then calculated.

Finally, membrane integrity was evaluated using fluorescent staining with a inverted light microscope (40X objective) equipped with ultraviolet fluorescence and a fluorescein isothiocyanate filter. Staining of the sperm for the purpose of membrane integrity was accomplished by using the LIVE/DEAD viability kit (Molecular Probes, Inc., Eugene, OR). The contents of the kit included SYBR 14 (1 mM solution in DMSO), which stained the DNA of membrane intact sperm green (viable cells), propidium iodide (PI) (2.4 mM solution in water), which stained the DNA of membrane ruptured sperm red (nonviable cells). A 200 μ l volume of the 1:5 sperm dilution was pipetted into a microcentrifuge tube (0.65 ml) and gently mixed with 400 nM of SYBR 14 and 24 μ M of PI. Samples were protected from light (in a dark room) and maintained at 37°C for 10 minutes before being evaluated.

To determine the percentage of membrane intact sperm (viable), 200 sperm were randomly counted using the fluorescent luminescence of the Nikon microscope. The number of green sperm (viable) was divided by 200 to determine the percent of cells with intact membranes.

Epididymal sperm concentrations were determined using a hemacytometer and adjusted to a final concentration of 39 to 120 million sperm per ml by the addition of Triladyl.

Cooling, Cryopreservation and Thawing of Epididymal Sperm

As mentioned above, the 15 ml centrifuge tube with the sperm sample was placed in a 250 ml beaker with 200 ml of room temperature water and then placed in a 4°C refrigerator to cool the sperm sample to 4°C. Once cooled, the sperm samples ($n = 28$) were loaded into 0.5 ml plastic semen straws (005569, Cassou straw, IMV Technologies, Minneapolis, MN), sealed using a heat sealer and prepared for freezing.

Freezing, was accomplished by placing loaded semen straws. Straws from each treatment were placed on a custom made 2 cm Styrofoam floating boat so that straws were always 2 cm above the liquid nitrogen (Yu et al., 2002b). There were 10 straws frozen for each treatment for a total of 20 straws per animal. The freezing boat was square in shape with the center removed, much like a floating doughnut. On top of the square there was a thin plastic screen clam netting (1/4 inch squares) where the plastic

straws laid. The hollowed out center allowed the liquid nitrogen vapor to come up underneath the straws to freeze the sperm. Straws frozen at 2 cm were allowed to sit in liquid nitrogen vapor for 10 minutes and then plunged directly into the liquid nitrogen (LN₂).

Straws remained in liquid nitrogen up to 10 months before post-thaw evaluation. At the time of thawing, a single straw was removed from liquid nitrogen and immediately placed in a 37°C water bath and allowed to thaw for 30 seconds. Then the straw was removed from the water bath, dried and the cotton plug was cut free. The open end of the 0.5 ml straw was then held upside down over a 1.7 ml microcentrifuge tube that was sitting in 37°C water bath. Using sharp-tipped scissors, a small cut was made on the top part (sealed side) of the straw. Cutting the straw a second time removed the vacuum of the straw and any fluid below the cut flowed into the centrifuge tube. The whole thawing process did not take longer than 90 seconds.

Samples used to evaluate post-thaw total motility and morphology were prepared and evaluated in the same manner, using the same equipment used to evaluate the pre-freeze values of these samples. Post-thawed values for total motility were based from the average of thawing three straws from each treatment. Also for each straw thawed, motility was assessed at three different time periods after thawing (10 minutes, 30 minutes and 60 minutes). The purpose of the three time periods was to monitor any increase in motility after allowing the sample to warm up in the 37°C water bath. The motility value reported for each straw was the best motility of the three motility values, regardless of the time the observation was made. The reported post-thaw morphology value was based on counting 200 sperm from a single straw for each treatment.

The post-thaw membrane integrity was determined by multicolor flow cytometry, using a protocol for samples with egg yolk components present (Nagy et al., 2003), with minor modifications. The staining protocol consisted of SYBR 14 and PI. A final concentration of 100 nM SYBR 14 solution and 12 µM PI solutions were added to 250 µl of diluted sperm sample. The samples were thoroughly mixed and incubated at 37°C in the dark for 10 to 15 minutes and then remixed before subjected to flow cytometric analysis. Measurements were completed on a FACSCalibur flow cytometer (Beckton Dickinson Immunocytometry Systems, San Jose, CA).

Forward and side scatter values were recorded on a linear scale, while fluorescent values were recorded on a logarithmic scale. The two fluorochromes were excited at 488 nm with a 20 mW argon laser. Plasma membrane ruptured cells were PI

positive, and their red fluorescent signal was detected on the FL3-channel using a 610 nm long pass filter. Membrane intact sperm were SYBR 14-positive, and its green fluorescent signal was detected on the FL1-channel using a 530/30 nm band pass filter. Dot plots drawn for data analysis were generated by WinMDI 2.8 (software by J. Trotter, available for downloading (<http://www.facs.scripps.edu/software.html>)). On SYBR 14 (FL1) and PI (FL3) dot plots, specific regions were identified to determine the percentage of viable and nonviable sperm.

Flow cytometry was performed by an experienced technician. The procedure written in this study was modified from that described by Guerrero (2006).

Statistical Analysis

The testis morphological measurements, total sperm motility, sperm morphology and sperm membrane integrity were statistically analyzed using a Student t-test to compare pre-freeze and post-thaw values within and between treatments. Measured sperm parameters are reported as mean \pm SEM per treatment group and a P value of ≤ 0.05 was used to determine significance differences for this study. Sigma Stat Version 3.0 were used to analyze each data set.

RESULTS

Experiment 4.1

Testes and Epididymides Weights

The weights of both the testes and epididymides for each animal just prior to sperm extraction are presented in Table 4.1. Weights for testes ranged from 25 g to 53 g, while the range for the epididymides was 2 g to 8 g. The mean for the left and right testes and epididymides were 38.8 g and 38.3 g, and 3.6 g and 3.8 g, respectively. Variation between testes weights within males was minimal, with the greatest difference being 9 g, which only pertained to one animal. Animal no. 8, 9 and 11 had identical testicular weights, while animal no. 2, 4 and 7 only had 1 g difference between the testes pair. The largest testis were coupled to the largest epididymis. No significant difference ($P \geq 0.05$) was found between left and right testes or left and right epididymides.

Testicular Temperatures and Epididymal Sperm Concentrations

Also displayed in Table 4.1 are the temperatures of each testes right before being dissected to remove the epididymides. Temperature ranged from 5.2°C to 14.6°C with the mean temperature for the left and right testes being 8.3°C and 8.0°C, respectively. No significant difference ($P \geq 0.05$) was detected between the temperatures of the left and right testes.

The average for the total number of cells collected from the left epididymides was 2.8×10^9 sperm cells with a range from 390×10^6 to 8.75×10^9 . The average for the total number of cells collected from the right epididymides was 2.6×10^9 sperm cells with a range from 910×10^6 to 6.9×10^9 .

Total Sperm Motility

The mean pre-freeze total motility for epididymal sperm collected from testis held in the scrotum (Treatment A) and not held in the scrotum (Treatment B) was $74.6 \pm 1.9\%$ and $67.5 \pm 2.2\%$, respectively. Treatment A had a significantly ($P \leq 0.05$) higher pre-freeze total motility than Treatment B. Both treatments had a significant decrease in total motility from the pre-freeze to post-thaw values. The post-thaw mean motility for Treatment A and Treatment B was 37.2 ± 5.0 and $26.6 \pm 3.9\%$, respectively (Figure 4.2). No significant difference ($P \geq 0.05$) was detected for post-thaw motility, between Treatment A and Treatment B.

Total motility for both treatments prior to and after freezing, for individual animals is illustrated in Figure 4.3. Pre-freeze motility for Treatment A was consistently higher, or equal to that of Treatment B for all animals, except animal number 2 where Treatment A had a total motility value of only 5% less than that of Treatment B. The range of total pre-freeze and post-thaw motility for Treatment A was 60% to 85% and 3% to 63%, respectively. Range for Treatment B pre-freeze was 50% to 80% and 5% to 53% for the post-thaw range. Animal number 8 produced the minimum post-thaw motility value, for both treatments.

Total Motility In Relation To Time

After thawing, sperm were allowed to incubate in a 37°C water bath to reach their full motility potential. Total motility readings were recorded at 10, 30 and 60 minutes. The final motility value reported for each straw was the best motility of the three motility values, regardless of the time the observation was made. See Table 4.2 (Treatment A) and Table 4.3 (Treatment B). The highest motility value recorded was consistently observed after epididymal sperm sample were sitting in the water bath for either 30 minutes or 60 minutes, for both Treatments A and B. Rarely was the highest total motility value observed at the 10 minute mark.

Sperm Morphology

Sperm harvested from epididymides that were held in the scrotum (Treatment A) had a mean of 74.1% normal pre-freeze morphology. While sperm from epididymides held out of the scrotum (Treatment B) produced a mean of 71.3% normal pre-freeze

Table 4.1 Testis and cauda epididymides weights and temperatures from individual postmortem White-tail bucks. Weights and temperature measurements were taken at time of epididymal sperm collection.

Deer number	<u>Testes wt (g)</u>		<u>Epididymides wt (g)</u>		<u>Testis temp (°C)</u>	
	Left	Right	Left	Right	Left	Right
1	32	28	2	2	-	6.0
2	45	46	3	3	6.0	5.2
3	27	29	3	3	9.2	8.8
4	25	26	2	3	6.1	6.5
5	30	32	2	2	10.2	8.5
6	34	30	4	3	8.4	8.1
7	28	29	2	3	9.8	9.3
8	44	44	6	5	10.8	7.6
9	39	39	3	4	7.8	9.8
10	47	41	3	3	6.5	7.6
11	42	42	5	5	5.7	7.5
12	52	49	5	4	5.5	7.3
13	54	48	4	5	6.8	7.5
14	44	53	6	8	14.6	11.9
Mean±SEM	38.8±2.53	38.3±2.43	3.6±0.39	3.8±0.42	8.3±0.73	8.0±0.45

No significant difference was found between epididymal and testes weights or testis temperatures for right or left testes ($P \geq 0.05$).

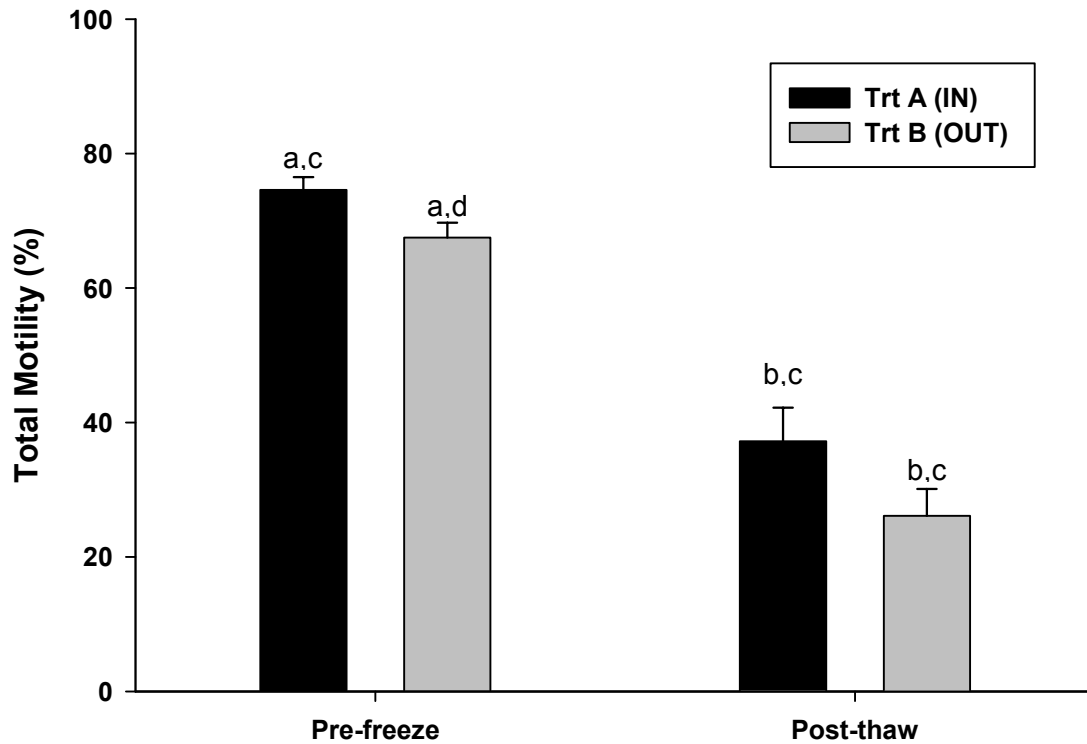


Figure 4.1. Percent total motility (mean \pm SEM) of pre-freeze and post-thaw White-tail deer epididymal sperm from testes that were either held in or out of the scrotum during a 24 hour interval. ^{a,b}Mean values within treatments with different superscripts are significantly different ($P\leq 0.05$). ^{c,d}Mean values between treatments with different superscripts are significantly different ($P\leq 0.05$).

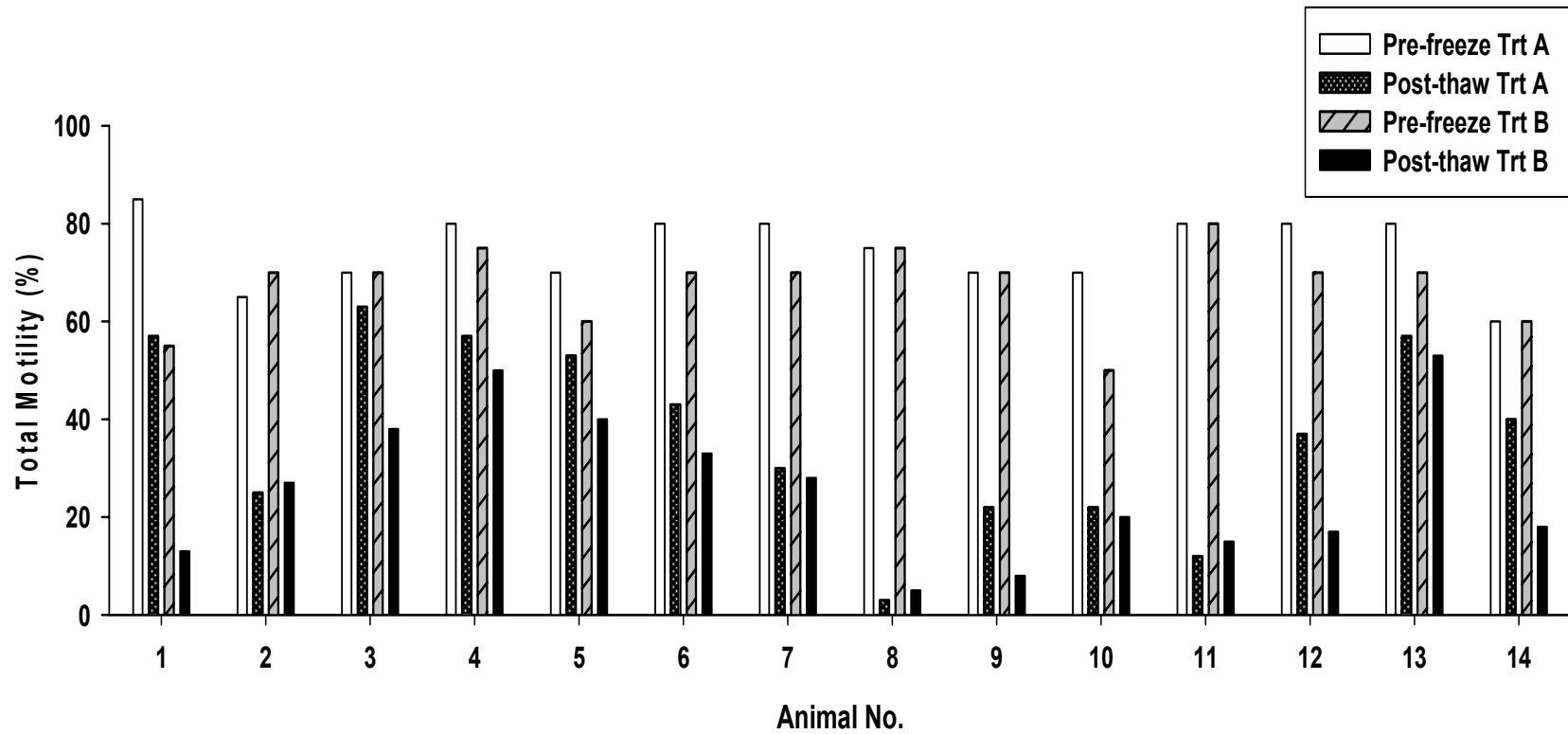


Figure 4.2. Pre-freeze and post-thaw total motility of epididymal sperm from individual White-tail bucks whose testes were held either in or out of the scrotum for a 24 hour interval.

sperm. After freezing, Treatment A recorded 66.9% normal sperm and Treatment B had a normal morphology percentage of 57.8% (Table 4.4). Although there was a decrease in normal sperm due to cryopreservation for both treatments, no significant difference ($P \geq 0.05$) was detected between pre-freeze and post-thaw or between treatments for normal sperm morphology. However, there was a significant decrease ($P \leq 0.05$) when comparing abnormal heads between pre-freeze and post-thaw, within Treatment A.

Membrane Integrity

The mean pre-freeze membrane integrity for epididymal sperm collected for Treatment A was $86.5 \pm 1.5\%$ and $86.3 \pm 1.4\%$ for Treatment B. No significant difference was detected between Treatment A and Treatment B for pre-freeze membrane integrity. However, a significant difference ($P \leq 0.05$) was determined between post-thaw membrane integrity for Treatment A ($54.2 \pm 3.7\%$) and Treatment B ($41.7 \pm 2.7\%$). Also, subjecting the epididymal sperm to cryopreservation did cause an expected significant decrease of the mean membrane integrity, when comparing pre-freeze to post-thaw values for both treatments (Figure 4.4).

Pre-freeze membrane integrity for both treatments prior to and subsequent to freezing for individual animals is illustrated in Figure 4.5. Variation among animals for pre-freeze membrane integrity was minimal for both treatments; the difference between minimum and maximum values for Treatment A and Treatment B was less than 20%. While there was more variation in the post-thaw values, Treatment A produced higher membrane integrity values for 12 of the 14 animals used in the study. Treatment A pre-freeze membrane integrity values ranged from 76% to 95%, and for Treatment B the range was 76% to 92%.

DISCUSSION

The ability to use epididymal sperm from a postmortem White-tail buck is a practice that has both conservational and agricultural implications. If epididymal sperm is going to be successful with assisted reproductive technologies, basic techniques for holding, collecting and cryopreservation must be determined.

No significant difference was found between the weights of the left and right testis or epididymides from mature White-tail bucks in this study. At the time of processing there was no significant difference in the temperatures for testis held in the scrotum and testis held out of the scrotum.

The method in which the testes are cooled may appear to be not that important, but something as simple as cooling or not cooling has repeatedly produced significant differences

Table 4.2. Values represent total motility of epididymal sperm collected from testes that were held in the scrotum (Treatment A). Motility was recorded at respective times post-thaw and priority ranked 1 to 3 (1 = top and 3 = lowest ranking)

Buck number	10 minutes	30 minutes	60 minutes
1	33 ³	57 ¹	50 ²
2*	25 ¹	22 ²	25 ¹
3	32 ³	63 ¹	53 ²
4	60 ¹	52 ³	57 ²
5	20 ³	53 ¹	47 ²
6	17 ³	37 ²	43 ¹
7	7 ³	20 ²	30 ¹
8*	3 ¹	3 ¹	2 ²
9	15 ³	22 ¹	20 ²
10	18 ²	22 ¹	17 ³
11	2 ³	10 ²	12 ¹
12	5 ³	37 ¹	20 ²
13	40 ³	50 ²	57 ¹
14	3 ³	37 ²	40 ¹
Occurrence of top ranking/period	3	7	6
Occurrence of low ranking/period	10	2	2

*The occurrence of top ranking motility occurred at more than one time period.

Table 4.3. Values represent total motility of epididymal sperm collected from testes that were held out of the scrotum (Treatment B). Motility was recorded at respective times post-thaw and priority ranked 1 to 3 (1 = top and 3 = lowest ranking)

Buck number	10 minutes	30 minutes	60 minutes
1	13 ²	12 ³	20 ¹
2	15 ³	23 ²	27 ¹
3	23 ³	38 ¹	35 ²
4	8 ³	50 ¹	40 ²
5	23 ³	40 ¹	33 ²
6	12 ³	33 ¹	30 ²
7*	7 ³	28 ¹	28 ¹
8*	3 ²	3 ²	5 ¹
9*	2 ³	8 ¹	7 ²
10	20 ¹	17 ²	13 ³
11	3 ³	8 ²	15 ¹
12	2 ³	15 ²	17 ¹
13	45 ²	40 ³	53 ¹
14	2 ³	18 ¹	17 ²
Occurrence of top ranking/period	1	7	7
Occurrence of low ranking/period	11	3	1

*The occurrence of top or low ranking motility occurred at more than one time period.

Table 4.4. Sperm morphology parameters (% means \pm SEM) of White-tail deer epididymal sperm prior to freezing and post-thaw. Calculated from 200 sperm counted and categorized across several fields.

Treatments	Normal	Bent tails	Broken Mid-pieces	Proximal droplets	Abnormal heads	Detached heads
Treatment A (IN)						
Pre-freeze	74.1 \pm 4.4	13.7 \pm 3.0	6.7 \pm 2.2	1.2 \pm 0.3	1.5 \pm 0.3 ^a	2.0 \pm 0.5
Post-thaw	66.9 \pm 6.0	21.9 \pm 4.2	7.9 \pm 2.4	1.5 \pm 0.5	0.5 \pm 0.2 ^b	1.3 \pm 0.3
Treatment B (OUT)						
Pre-freeze	71.3 \pm 4.6	15.0 \pm 2.3	7.6 \pm 3.4	2.8 \pm 0.9	1.1 \pm 0.3	2.3 \pm 0.7
Post-thaw	57.8 \pm 6.3	21.8 \pm 3.9	13.3 \pm 4.5	5.3 \pm 2.0	0.5 \pm 0.2	1.4 \pm 0.4

^{a,b}Means with different superscripts within columns are significantly different ($P \leq 0.05$).

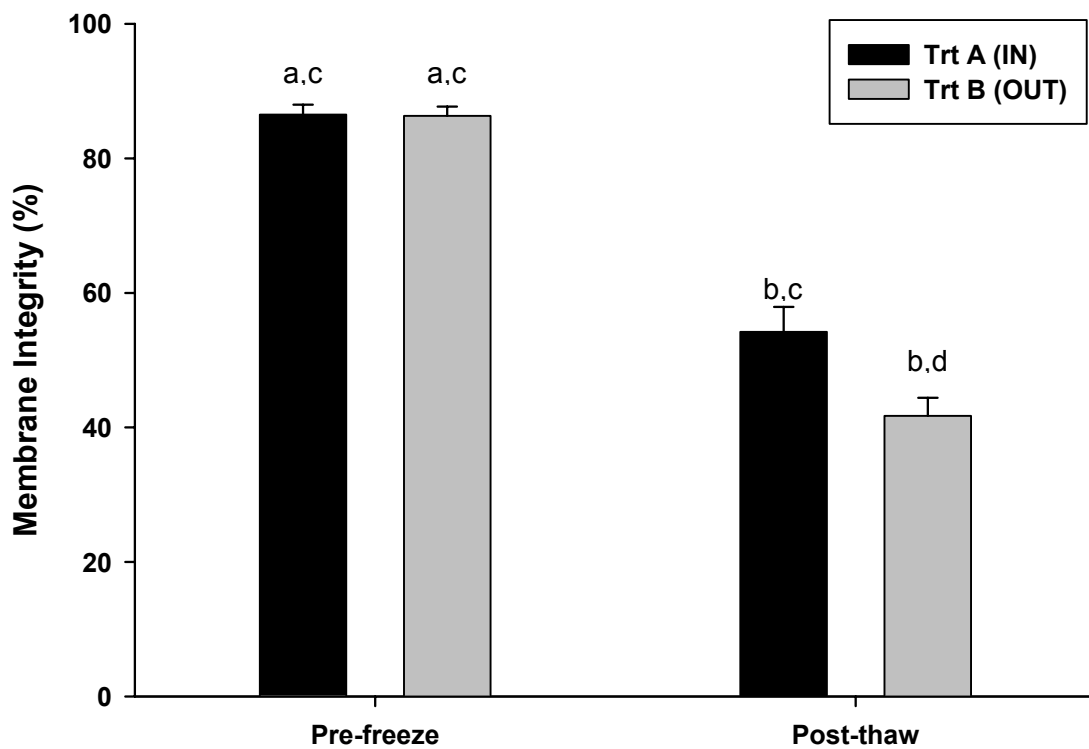


Figure 4.3. Percent membrane integrity (mean±SEM) of pre-freeze and post-thaw White-tail deer epididymal sperm from testes that were either held in or out of the scrotum during a 24 hour interval. ^{a,b}Mean values within treatments with different superscripts are significantly different ($P \leq 0.05$). ^{c,d}Mean values between treatments with different superscripts are significantly different ($P \leq 0.05$).

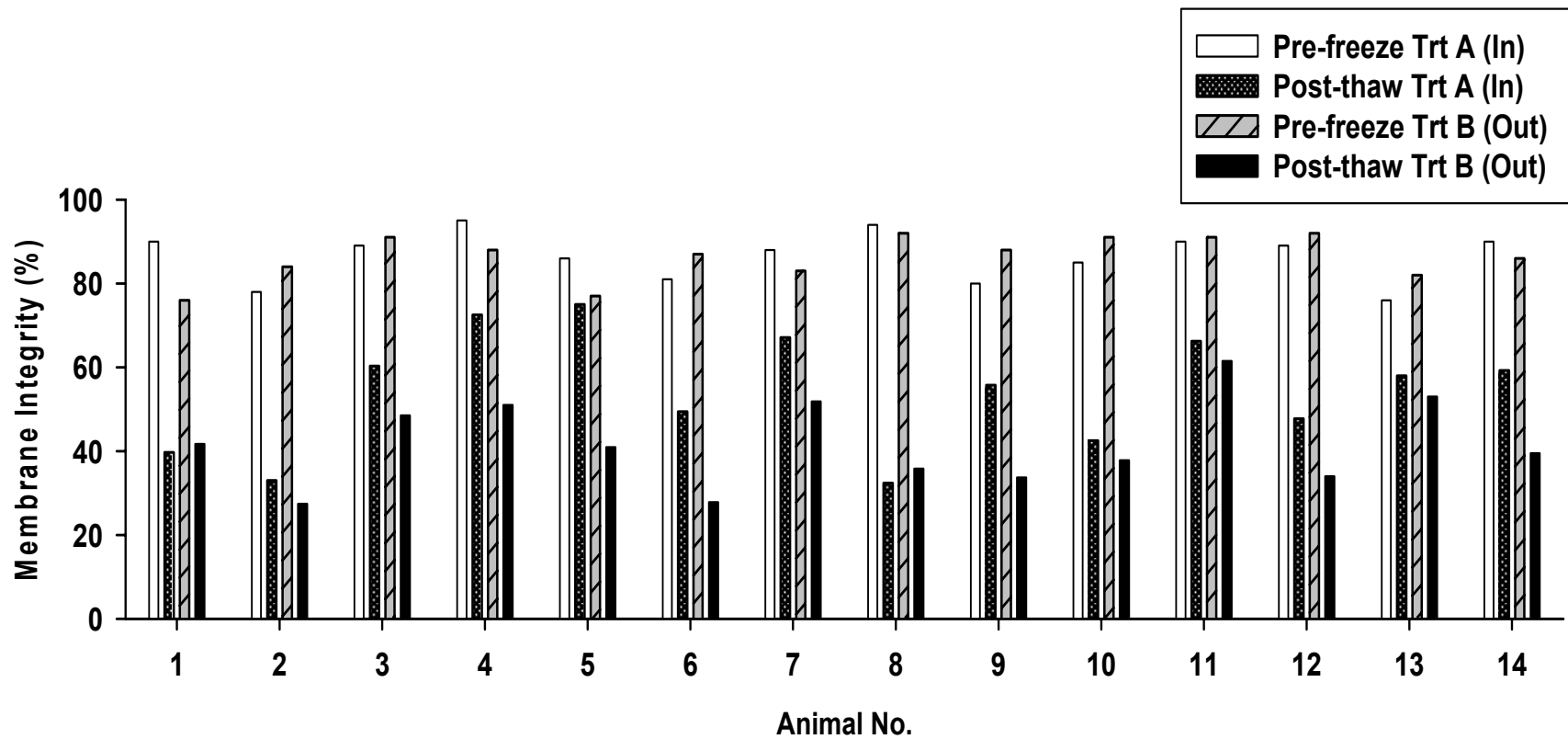


Figure 4.4. Pre-freeze and post-thaw membrane integrity of epididymal sperm from individual White-tail bucks whose testes were held either in or out of the scrotum for 24 hours.

in subsequent post-thaw sperm viability. In the present study, holding testis in the scrotum produced a significantly greater mean pre-freeze total motility (74.6%) than did holding the testis out of the scrotum (67.5%). This difference in motility is possibly due to cold shock, caused by the rapid cooling of testes that were not housed in the scrotum and thus lacked the additional insulation of the scrotum. Zeng et al. (2001) have reported that cold shock causes an irreversible loss of sperm motility in the boar. However, others have reported that epididymal sperm from the ram (Hammerstedt et al., 1982), the boar (Lasley et al., 1944) and the bull (Baily et al., 1959) were more resistant to cold shock than the corresponding ejaculated sperm from these males. In the present study, pre-freeze did not have an effect on sperm morphology between treatments. This coincides with a report that storing testes with attached epididymides had no effect on the percentage of normal morphology of refrigerated Red deer epididymal sperm, on day 3 of storage (Soler et al., 2003a). Also, there was no difference between treatments for pre-freeze membrane integrity.

After holding the testes for cooling in either treatment (holding the testis in the scrotum or holding testis out of the scrotum) sperm was frozen and thawed. The post-thaw motility for White-tail deer sperm for this study seldom reached its full motile potential within 10 minutes of being thawed. Generally, sperm incubated in a 37°C water bath had higher motility values at 30 or 60 minutes when compared with motility at 10 minutes. Unlike our results, when frozen-thawed ejaculated sperm from Formosan Sika deer (Cheng et al., 2004), Formosan Sambar deer (Cheng et al., 2004) and dog (Nothling et al., 2005; Rota et al., 2005) were allowed to incubate in a 37°C to 38°C water bath, motility proceeded to decrease over time. However, Cheng et al. (2004) and Rota et al. (2005) reported this decrease when sperm were checked at 1-hour intervals rather than 20 minute or 30 minute intervals after thawing, as in our study.

We reported there was a significant decrease of abnormal heads within Treatment A when comparing pre-freeze to post-thaw. However, abnormal heads is a primary morphology defect, which is believed to have occurred during spermatogenesis and not from the cooling treatments. Consistent with our results, in the Mexican Gray wolf (*Canis lupus*) and generic Gray wolf (*Canis lupus baileyi*) cooling rate did not result in a significant difference in pre-freeze or post-thaw ejaculated sperm morphology (Zindl et al., 2006). Also it has been reported in the coyote (*Canis latrans*) that freezing rate did have a significant difference in ejaculated post-thaw sperm morphology (Minter et al., 2005).

Post-thaw membrane integrity of White-tail deer epididymal sperm was determined by staining sperm with SYBR 14 and propidium iodide and analyzed by flow cytometry. SYBR 14 and propidium iodide have been proven to accurately label live and dead ejaculated sperm from bulls, boars, rams and rabbits, as well as epididymal sperm from rats (Garner et al., 1994, 1995). Percent of live ejaculated sperm that was evaluated by flow cytometry correlates ($r = 0.68$) with stallion fertility. This correlation demonstrates that determination of the percentage of live sperm by flow cytometry can be a good representation of sperm fertility (Wilhelm et al., 1996). For this study membrane integrity of epididymal sperm from testes held out of the scrotum (41.7%) was significantly lower than sperm frozen from testes held in the scrotum (54.2%) but only for the sperm collected from testes held out of the scrotum. Similarly, Kumar et al. (2003) reported that when ram and boar ejaculated semen was frozen at different freezing rates there was a significant difference in sperm viability.

In conclusion, collection and cryopreservation of White-tail deer epididymal sperm 24 hours postmortem was accomplished. However, there was $\leq 50\%$ post-thaw motility and large variability found among males. It is evident improvements to the present procedures are necessary. Recommendations for collecting epididymal sperm from White-tail deer would be that if immediate processing is not an option, testes can be held within the scrotum at 10°C to 15°C for up to 24 hours prior to processing the sperm for freezing.

CHAPTER V

COMPARISON OF POST-THAW VALUES FROM EPIDIDYMAL SPERM OF WHITE-TAIL DEER THAT WERE FROZEN WITH EITHER GLYCEROL OR DMSO AS THE CRYOPROTECTANT

INTRODUCTION

Cryoprotectants are compounds that specifically act to protect and maintain the viability of cells when being subjected to very cold conditions. These agents include two basic categories, permeating and nonpermeating. Glycerol, a permeating agent, was first reported as a cryoprotectant in the use of freezing plant cells and tissues by Maximov in 1908 (see Salamon and Maxwell, 1995). Also, a Russian paper written by Bernstein and Petropavlovsky in 1937 stated that glycerol was used at a concentration of 9% to protect sperm of the rabbit, guinea pig, bull, ram, boar, stallion and duck when cooled down to -21°C (see Leibo, 2004). The most recognized and cited study indicating the discovery of glycerol as a cryoprotectant was that of Polge (1949). It is said that Polge's use of glycerol was an accident; believing that he was using a fructose solution in the hopes of freezing rooster semen, when in reality it was a mislabeled bottle of glycerol mixed with some protein (see Leibo, 2004). Today glycerol is still one of the most commonly used cryoagents for freezing mammalian sperm (Medeiros et al., 2002).

Even with the wide use of glycerol across many species, other cryoprotective agents have been successfully used for freezing sperm. One such agent is dimethyl sulfoxide (DMSO). DMSO was first reported as a cryoprotectant when freezing red blood cells by Lovelock and Bishop (1959). Once its potential as a cryoagent was realized, DMSO was further studied in many different cells and tissues (see Gilmore et al., 1997). When compared with glycerol, DMSO was shown to produce higher post-thaw sperm motility when freezing epididymal sperm from the Springbok (Loskutoff et al., 1996). Also, other agents in addition to glycerol have produced better post-thaw sperm viability results in similar African antelopes (Loskutoff et al., 1996). In the mouse, DMSO was unable to produce the best post-thaw results, but did perform better than glycerol (Sztejn et al., 2001). In the African elephant, DMSO has produced the best results for both ejaculated and epididymal sperm. DMSO also performed better than glycerol when freezing epididymal sperm from the impala (Gilmore et al., 1998).

Protocols for freezing epididymal sperm are often only slightly modified from those used to freeze ejaculated sperm. It is often assumed, since glycerol produces the best post-thaw results for ejaculated sperm from a specific species, it must be the best for freezing epididymal sperm from the same species (Platz et al., 1982). This assumption has often been proven to be right, for example, in the bull (Guerrero 2006) and the Red deer (Fernandez-Santos et al., 2005).

In contrast, studies from different species have shown repeatedly, that when comparing epididymal and ejaculated sperm, there are distinct differences between the two sperm types. Often these differences are attributed to the sperm's interaction with seminal plasma, which epididymal sperm has never experienced (Lasley et al., 1944; Yu et al., 2002a).

Freezing of White-tail deer sperm has been accomplished and proven to be viable post-thaw, by using artificial insemination (Jacobson et al., 1989; Magyar et al., 1989). Possibly due to this initial success no desire to test alternative freezing protocols has occurred. However, the question still remains, is glycerol an effective cryoprotectant for freezing White-tail deer epididymal sperm? To date there are studies comparing different cryoprotectants for freezing epididymal sperm of other Cervidae, such as Iberian Red deer (Fernandez-Santos et al., 2006a) and other wild ungulates such as the impala, blesbok and springbok (Loskutoff et al., 1996). However to our knowledge, there have been no studies comparing DMSO with glycerol as a possible cryoprotectant in freezing epididymal sperm from White-tail deer.

The objective of this study was to compare the post-thaw survival of White-tail deer epididymal sperm that was frozen using either glycerol or DMSO as the cryoprotective agent.

MATERIALS AND METHODS

Experimental Design

Experiment 5.1

Paired testes from 7 mature White-tail deer were removed postmortem from hunter harvested bucks collected during 3 weeks of the peak rutting season for the 2005-2006 Louisiana White-tail deer hunting season. Testes were collected on 4 individual days over a period of 16 days. Testicles within the scrotum, once removed from the body of a postmortem buck, were placed in a plastic Ziploc bag and placed in a Styrofoam ice chest (at ambient temperature) and transported to the Embryo Biotechnology Laboratory (EBL). Upon arrival at the EBL, testes were processed and

epididymal sperm were flushed from the cauda epididymidis. Epididymal sperm were flushed from both epididymides using an extender as the flushing medium. No cryoprotectant was present in the extender at this time. Sperm from both epididymides were pooled and allowed a slow cool to 4°C. Upon reaching 4°C the pooled epididymal sperm were equally divided and either glycerol or DMSO was added to the divided sperm samples. After the addition of the cryoprotectant sperm were loaded into 0.5 ml straws and cryopreserved using liquid nitrogen vapor. Pre-freeze (n = 7) and post-thaw (n = 14) motility were assessed by a single technician using an inverted microscope equipped with a heated stage. The motility of each sample was determined by calculating the average total sperm motility of three separate areas of the same slide. Also for each straw thawed, motility was assessed at three different time periods after thawing (10 minutes, 30 minutes and 60 minutes). Both pre-freeze (n = 7) and post-thaw (n = 14) morphology were determined using a mixture of sperm samples with eosin-nigrosin stain on a pre-cleaned glass microscope slide under a light microscope with a 100X oil immersion objective. Pre-freeze (n = 7) membrane integrity was evaluated using fluorescent microscopy, while post-thaw (n = 14) membrane integrity was evaluated by multicolor flow cytometry.

Experimental Procedure

Testes Collection

During the 2005-2006 White-tail deer hunting season managed hunts were conducted by Louisiana Department of Wildlife and Fisheries (LDWF) on different wildlife refuges in Louisiana. During these hunts, area sportsmen harvested White-tail deer, and by LDWF regulations were required to check the animal in at mandatory check stations located on the refuge. At the time of check in, the animal was aged, weighed and antler measurements were recorded. Also at this time, paired testes with scrotum were removed from the animal in such a way, that as much of the spermatic cord as possible remained with the scrotum. Testes were then placed in a Ziploc plastic bag and into an ambient temperature Styrofoam ice chest (31.5 x 26 x 23 cm). The time from animal death to the time of testicle removal varied from 1.67 hours to 5.25 hours. Testicles were transported to the EBL for processing.

Extender Preparation

On the day the testes were processed, a two part goat semen extender was prepared containing 3.634 g of Tris (Sigma-Aldrich, Inc., St. Louis, MO), 0.5 g of fructose (Sigma-Aldrich, Inc.) 1.99 g of citric acid (Sigma-Aldrich, Inc.) and 20 ml of egg yolk

(fresh chicken eggs) in 80 ml of double distilled water to reach a final volume of 100 ml of extender. The extender was filtered through a 0.45 µm Nalgene syringe filter (Nalge Company, Rochester, NY). After filtering, the extender was divided into three types: one type Part "A" extender (flushing medium) and two types of Part "B" extender (glycerol or DMSO treatments). Once divided, either glycerol or DMSO was added to each one of the Part "B" extenders. The concentration of both cryoprotectants was 12%. Both Part "B" extenders were then placed into a 4°C refrigerator to cool. Depending on the number of samples processed, a different volume of the extender was prepared. However, concentration of all the extender components was constant no matter what volume of the extender was added.

Sperm Collection

Following the arrival at the EBL, testicles were removed from the scrotum and processed (≤ 12 hours postmortem). Processing started by making an incision in the tunica vaginalis surrounding the testis. Before removing the tunica vaginalis, testicular temperature was taken by placing a temperature probe (DiGi-Sense[®] Dual Input JTEK Thermocouple Thermometer) through the previously made incision until it rested alongside the cauda epididymidis. Once testicular temperature was recorded, the cauda epididymidis and vas deferens were dissected away from the testicle and the cauda epididymidis was excised away from the attached corpus epididymidis. The testicle was further processed to remove the remaining corpus and caput epididymidis, leaving just the remaining testicle.

The weight of the separated testicle and cauda epididymidis were then determined. An incision was made where the cauda epididymal tubules regress from the vas deferens to the corpus of the epididymides (a distinct anatomical difference in the diameter of the cauda tubules occurs at the medial portion of the cauda epididymidis). At this medial portion, the superficial blood vessels and thin membrane were dissected away exposing the epididymal tubules. Using a scalpel blade (no. 10), a single tubule was bisected to allow the retrograde flow of epididymal sperm from the vas deferens side of the cauda epididymidis.

Before retrieving the epididymal sperm, ~3 ml of Part "A" extender was added to a 15 ml centrifuge tube to prevent sperm from adhering to the sides of the collection tube. Epididymal sperm were then flushed from the epididymal tubules using Part "A" extender, using retrograde flow.

The syringe used for flushing was made by heating the tip of the 12 ml Monoject syringe and then slowly stretching the melted tip until producing a small enough tube diameter to fit inside the lumen of the vas deferens. Flushing was performed by threading the vas deferens with the plastic tube attached to the modified 12 ml syringe that contained flushing medium. Once inserted, the plastic tube was held in the vas deferens using a Desmarres Chalazion forceps (78.74 mm). Cauda epididymal sperm were flushed into the 15 ml centrifuge tube containing the extender.

A 300 μ l volume of epididymal sperm-extender mixture was removed from the sperm sample and placed in a warmed 1.7 ml plastic microcentrifuge tube and diluted 1:5 with 37°C phosphate-buffered saline (PBS). The 15 ml centrifuge tube with remaining epididymal sperm-extender mixture was placed in a 250 ml glass beaker containing 200 ml of water at room temperature and allowed to stand for 30 minutes. After the 30 minutes, the beaker with 15 ml tubes was placed in 4°C refrigerator and cooled to 4°C before further processing.

Pre-Freeze Sperm Analysis

All sperm parameter measurements were made from the 1:5 diluted sperm that had been allowed to warm in a 37°C water bath. The first sperm parameter assessed was total sperm motility using an inverted light microscope with a heated stage (Nikon Diaphot, Tokyo, Japan) and Hoffman optics (40X objective). Total motility of epididymal sperm was assessed after 5 minutes of incubation at 37°C. A 10 μ l droplet of 1:5 diluted sperm was placed on a warmed pre-cleaned glass microscope slide, covered with a warmed 18 x 18 mm glass cover slip and placed on the heated microscope stage (37°C). The motility of each sample (n = 7) was determined by calculating the average total sperm motility of three separate views of the same slide.

Sperm morphology was also evaluated using a single drop of eosin-nigrosin stain (Lane Manufacturing, Inc, Denver, CO), placed on a pre-cleaned glass microscope slide and mixed with ~2 μ l of sperm sample. Using another slide this mixture was then dispersed all along the slide in such a manner that the concentration of the stain decreased from left to right. After the stain dried, morphology for each sample was determined by placing the slide under a 100X oil immersion objective of an upright light Nikon microscope. A total of 200 sperm were counted at random and placed into one of the six categories as follows: normal, bent tails, broken mid-pieces, proximal droplets, abnormal heads or detached heads. The percentage of sperm in each category was then calculated.

Finally, membrane integrity was evaluated using fluorescent staining with an inverted light microscope (40X objective) equipped with ultraviolet fluorescence and a fluorescein isothiocyanate filter. Staining epididymal sperm for the purpose of determining membrane integrity was accomplished by using the LIVE/DEAD viability kit (Molecular Probes, Inc., Eugene, OR). The contents of the kit included SYBR 14 (1 mM solution in DMSO), which stained the DNA of membrane intact sperm green (viable cells), propidium iodide (PI) (2.4 mM solution in water), which stained the DNA of membrane ruptured sperm red (nonviable cells). A 200 μ l volume of the 1:5 sperm dilution was pipetted into a microcentrifuge tube (0.65 ml) and gently mixed with 400 nM of SYBR 14 and 24 μ M of PI. Samples were protected from light (in a dark room) and maintained at 37°C for 10 minutes before being evaluated.

To determine the percentage of membrane intact sperm (viable), 200 individual sperm were randomly counted using the fluorescent luminescence of the Nikon microscope (40X objective). The number of green sperm (viable) was divided by 200 to calculate the percent of cells with intact membranes.

Epididymal sperm concentrations were determined using a hemacytometer and adjusted to a final concentration of 200 to 212 million sperm per ml (twice as much as the final concentration), by the addition of more Part "A" extender.

Addition of Cryoprotectant

Each animal unit ($n = 7$) had two 15 ml centrifuge tubes containing the same concentration of Part "A" extender and epididymal sperm ($n = 14$). This volume was half of the final volume. Once cooled to 4°C Part "B" extender (Part "A" extender + cryoprotectant) containing either glycerol or DMSO was added to its respective sample. Adding an equal amount of the "sperm free" Part "B" extender to the sample, doubled the volume and decreased the final sperm concentration to 100 to 106 million cells per ml. Also before the addition of Part "B" extender, the concentration of the cryoprotectant in Part "B" was 12% and after the addition the concentration decreased to the desired concentration of 6%. The Part "B" extender was added to the sample in a stepwise process to prevent shocking of the sperm. Upon completion of the addition of Part "B", samples were loaded into 0.5 ml straws (#005569, Cassou straw, IMV Technologies, Minneapolis, MN), sealed using a heat sealer and prepared for cryopreservation.

Cryopreservation and Thawing of Epididymal Sperm

Ten straws were frozen for each treatment for a total of 20 straws per animal ($n = 7$). Straws from each treatment were placed on a custom made Styrofoam floating

boat, so that straws were 2 cm above the liquid nitrogen (Yu et al., 2002b). The freezing boat was 2 cm tall, square with the center portion removed, similar to a floating doughnut. On the top surface of the boat there was a thin plastic clam netting screen (1/4 inch squares) where the plastic straws laid. The hollowed out center allowed the liquid nitrogen vapor to come up underneath the straws freezing the sperm. Straws were subjected to liquid nitrogen vapor for 10 minutes and then plunged directly into the liquid nitrogen.

Straws remained in liquid nitrogen up to 9 months before being thawed for post-thaw evaluation. At the time of thawing, a single straw was removed from liquid nitrogen and immediately placed in a 37°C water bath and allowed to thaw for 30 seconds. Then the straw was removed from the water bath, dried and the cotton plug cut free. The open end of the 0.5 ml straw was then held upside down over a 1.7 ml microcentrifuge tube that was sitting in 37°C water bath. Using sharp-tipped scissors, a small cut was made on the top part (sealed side) of the straw. Cutting the straw a second time removed the vacuum of the straw and any fluid below the cut flows into the centrifuge tube. This whole process did not take longer than 90 seconds.

Samples used to evaluate post-thaw total motility and morphology were prepared and evaluated in the same manner, using the same equipment used to evaluate the pre-freeze values of these samples. Post-thaw values for total motility were based from the average of thawing 3 straws from each treatment. Also for each straw thawed, motility was assessed at three different time periods after thawing (10 minutes, 30 minutes and 60 minutes). The purpose of the three time periods was to monitor any increase in motility after allowing the sample to warm up in the 37°C water bath. The motility value reported for each straw was the best motility of the three motility values, regardless of the time the observation was made. The reported post-thaw morphology value was based on counting 200 sperm cells from a single straw for each treatment.

The post-thaw membrane integrity was calculated by multicolor flow cytometry. Assessment of plasma membrane was conducted using a protocol for samples with egg yolk components present (Nagy et al., 2003), with minor modifications. The staining protocol consisted of SYBR 14 and a propidium iodide (PI). A final concentration of 100 nM SYBR 14 solution, and a 12 µM PI solution were added to 250 µl of diluted sperm sample. The samples were thoroughly mixed and incubated at 37°C in the dark for 10 to 15 minutes and then remixed before being subjected to flow cytometric analysis.

Measurements were completed on a FACSCalibur flow cytometer (Beckton Dickinson Immunocytometry Systems, San Jose, CA).

Forward and side scatter values were recorded on a linear scale, while fluorescent values were recorded on a logarithmic scale. The two fluorochromes were excited at 488 nm with a 20 mW argon laser. Plasma membrane ruptured cells were PI positive, and their red fluorescent signal was detected on the FL3-channel using a 610 nm long pass filter. Membrane intact sperm were SYBR 14-positive, and its green fluorescent signal was detected on the FL1-channel using a 530/30 nm band pass filter. Dot plots drawn for data analysis were generated by WinMDI 2.8 (software by J. Trotter, available for downloading at (<http://www.facs.scripps.edu/software.html>)). For SYBR 14 (FL1) and PI (FL3) dot plots, specific regions were identified to determine the percentage of viable and nonviable sperm in each sample.

Flow cytometry was performed by an experienced technician. The procedure written in this study was modified from that described by Guerrero (2006).

Statistical Analysis

The testis morphological measurements, total sperm motility, sperm morphology and sperm membrane integrity were statistically analyzed using a Student t-test to compare pre-freeze and post-thaw values within and between treatments. Measured sperm parameters are reported as mean \pm SEM per treatment group and a P value ≤ 0.05 was used to determine significance differences for this study. Sigma Stat Version 3.0 were used to analyze each data set.

RESULTS

Experiment 5.1

Testes and Epididymides Weights

Prior to flushing all testes and epididymides were weighed (Table 5.1). The testes and epididymides weights for this project ranged from 32 g to 44 g and 3 g to 6 g, respectively. The mean for the left and right testes weights were 38.0 g and 38.1 g, respectively. The mean weights for the epididymides were 4.4 g for the left and 4.6 g for the right. No significant difference ($P \geq 0.05$) was found between left and right epididymides or testes weights.

Testicular Temperatures and Epididymal Sperm Concentrations

The temperatures of each testis just prior to being dissected from the epididymides are shown in Table 5.1. Temperature ranged from 18.9°C to 26.5°C, the mean temperature for the left and right testes was 22.1°C and 22.3°C, respectively. The variation of temperature

Table 5.1 Testis and cauda epididymides weights and testis temperatures from individual postmortem White-tail bucks. Weights and temperature measurements were made, at time of epididymal sperm collection (≤ 12 hours postmortem).

Deer number	<u>Testes wt (g)</u>		<u>Epididymides wt (g)</u>		<u>Testis temp (°C)</u>	
	Left	Right	Left	Right	Left	Right
1	42	43	4	4	26.0	26.5
2	44	44	6	5	24.3	24.6
3	42	42	4	4	25.1	26.2
4	36	38	5	6	18.9	18.9
5	35	34	5	5	20.0	20.1
6	32	32	4	3	20.6	20.6
7	35	34	3	3	19.6	19.5
Mean \pm SEM	38.0 \pm 1.73	38.1 \pm 1.86	4.4 \pm 0.37	4.6 \pm 0.37	22.1 \pm 1.11	22.3 \pm 1.25

No significant difference was detected between epididymal and testes weights or testis temperatures for right or left testes ($P \leq 0.05$).

between testes of the same animal was minimal, with the greatest difference within animal was 1.1°C. No significant difference ($P \geq 0.05$) was detected between the temperatures of the left and right testes among the bucks.

The average for the total number of cells collected from both epididymides was 3.8×10^9 with a range from 1.2×10^9 to 7.2×10^9 .

Total Motility

The mean (\pm SEM) pre-freeze total motility for epididymal sperm before being divided into treatments was $69.3 \pm 3.5\%$. The mean post-thaw values for epididymal sperm frozen using glycerol as the cryoprotectant was $44.7 \pm 7.9\%$ and $31.4 \pm 8.4\%$ for sperm frozen with dimethyl sulfoxide (DMSO). No significant difference ($P \geq 0.05$), was detected between post-thaw values for the glycerol and DMSO treatments. There was a significant decrease for both treatments from the initial pre-freeze value (Figure 5.1).

Total motility for both treatments prior to and after freezing, for individual animals is illustrated in Figure 5.2. The variation among animals before freezing was moderate with a difference of 20% between the minimum and maximum values. The variation post-thaw among animals was much greater, with the glycerol treatment ranging from 15% to 80% and 5% to 67% for the DMSO-treated sperm.

Total Motility In Relation To Time

After thawing, sperm were maintained in a 37°C water bath to reach their peak motility. Total motility readings were recorded at 10, 30 and 60 minutes and the reported motility value for each straw was the best motility of the three motility values regardless of the time the observation was made; see Table 5.2 (glycerol treatment) and Table 5.3 (DMSO treatment). Total sperm motility value tended to be higher when checked at 30 minutes and then dropped down by 60 minutes of incubation. The best motility was recorded at 60 minutes only once in both treatments, and most often the least total motility value was observed at the 60 minute time period.

Morphology

Prior to freezing, $47.7 \pm 7.7\%$ of epididymal sperm had normal morphology. Following the freeze, $30.6 \pm 6.4\%$ of sperm treated with glycerol showed normal morphology, and sperm subjected to DMSO had $25.7 \pm 5.4\%$ normal cells (Table 5.4). Although there was a decrease in normal sperm due to cryopreservation for the glycerol treatment, no significant difference was detected. However, a statistical difference ($P \leq 0.05$) was found between the pre-freeze and the post-thaw DMSO treatment for sperm with normal morphology.

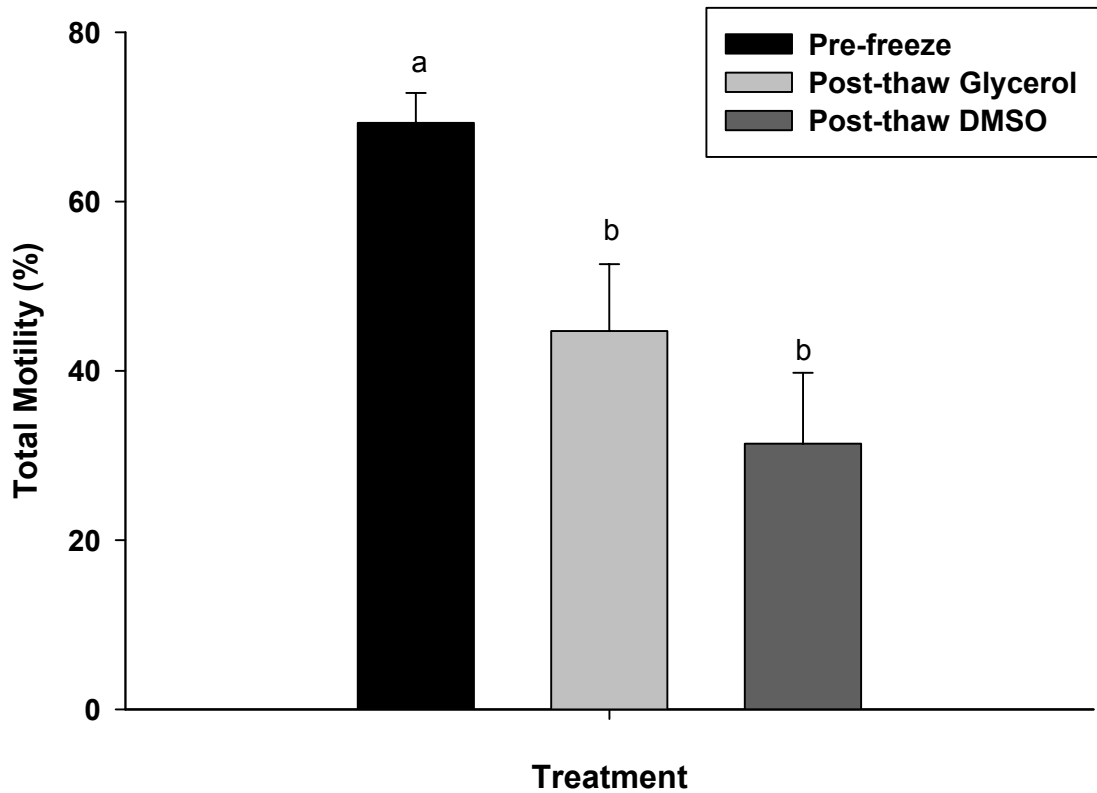


Figure 5.1. Percent total motility (mean±SEM) comparing pre freeze and post-thaw values of epididymal sperm from White-tail deer that were frozen using either glycerol or DMSO as the cryoprotectant. ^{a,b}Mean values between pre-freeze and treatments with different superscripts are significantly different ($P \leq 0.05$).

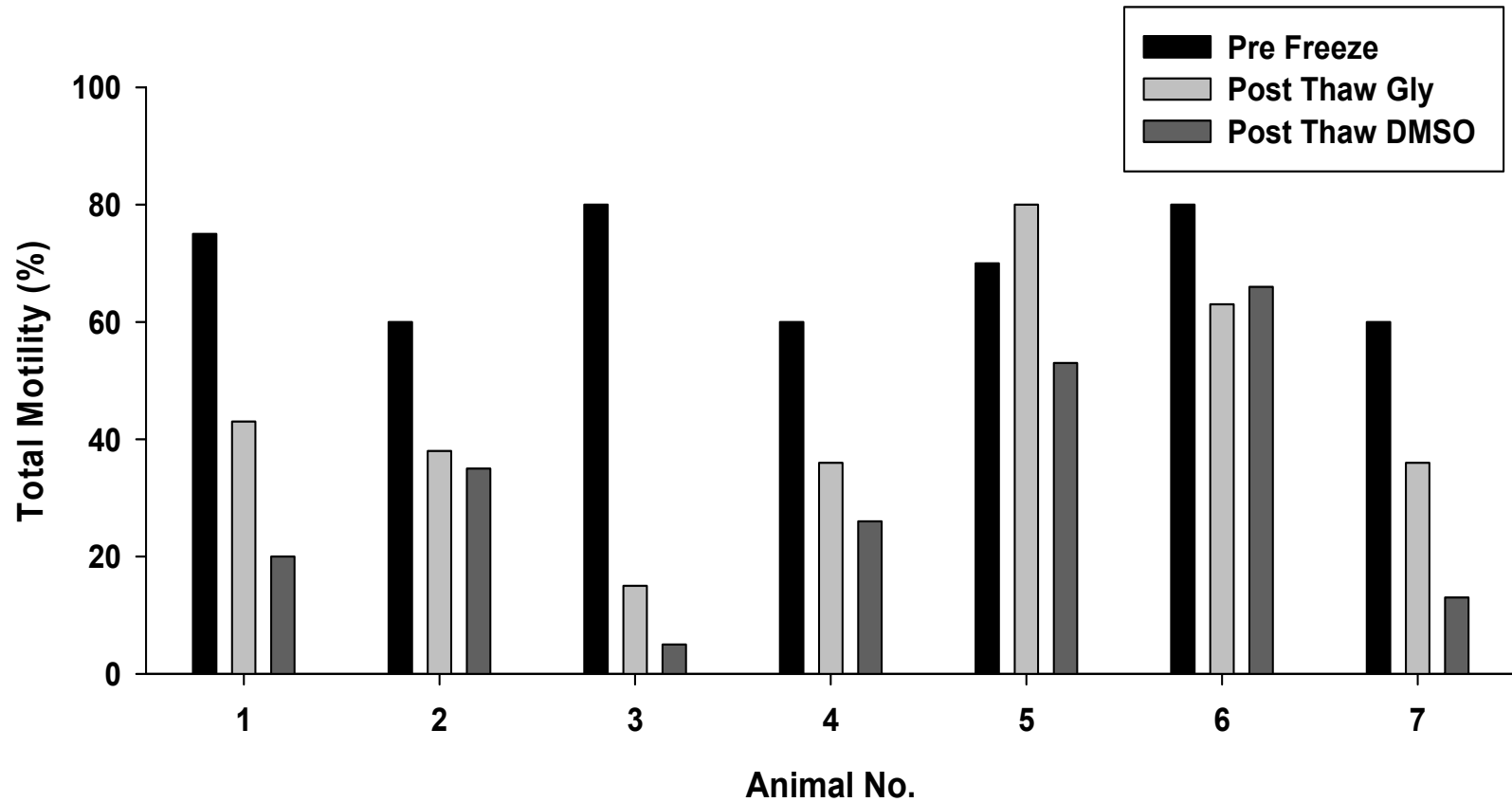


Figure 5.2. Pre-freeze and post-thaw total motility of epididymal sperm from individual White-tail bucks, when sperm was frozen using either glycerol or DMSO as the cryoprotectant.

Table 5.2. Values represent total motility of epididymal sperm that was frozen using glycerol as the cryoprotectant. Motility was recorded at specific times post-thaw and ranked priority 1 to 3 (1 = top and 3 = lowest ranking).

Buck number	10 minutes	30 minutes	60 minutes
1*	27 ²	43 ¹	27 ²
2	23 ³	32 ²	38 ¹
3*	15 ¹	7 ²	7 ²
4	33 ²	37 ¹	20 ³
5	77 ²	80 ¹	60 ³
6	63 ¹	55 ²	48 ³
7	28 ²	37 ¹	12 ³
Occurrence of top ranking/period	2	4	1
Occurrence of low ranking/period	2	1	6

*The occurrence of low ranking motility occurred at more than one time period.

Table 5.3. Values represent total motility of epididymal sperm that was frozen using DMSO as the cryoprotectant. Motility was recorded at specific times post-thaw and ranked priority 1 to 3 (1 = top and 3 = lowest ranking).

Buck number	10 minutes	30 minutes	60 minutes
1	13 ²	20 ¹	12 ³
2	23 ³	35 ¹	25 ²
3*	5 ¹	3 ²	3 ²
4	23 ²	27 ¹	15 ³
5	53 ¹	47 ²	37 ³
6	65 ²	67 ¹	53 ³
7	13 ¹	12 ²	5 ³
Occurrence of top ranking/period	3	4	0
Occurrence of low ranking/period	1	1	6

*The occurrence of low ranking motility occurred at more than one time period.

Table 5.4. Sperm morphology parameters (% means \pm SEM) of White-tail deer epididymal sperm prior to freezing and post-thaw. Calculated from 200 sperm counted and categorized across several fields.

Treatments	Group	Normal	Bent tails	Broken mid-pieces	Proximal droplets	Abnormal Heads	Detached heads
	Pre-freeze	47.7 \pm 7.7 ^a	16.8 \pm 4.4	15.9 \pm 9.3	0.7 \pm 0.2	14.7 \pm 7.8	4.3 \pm 2.6
Glycerol	Post-thaw	30.6 \pm 6.4	28.7 \pm 6.1	16.7 \pm 7.0	0.2 \pm 0.1	15.2 \pm 9.1	8.7 \pm 7.1
DMSO	Post-thaw	25.7 \pm 5.4 ^b	34.8 \pm 6.9	14.1 \pm 4.5	0.4 \pm 0.2	17.0 \pm 9.2	8.2 \pm 5.5

No significant difference was found between pre-freeze or treatments post-thaw for the six different categories of sperm morphology ($P \geq 0.05$).

Membrane Integrity

The mean pre-freeze membrane integrity for epididymal sperm in this study was $89.9 \pm 2.8\%$. The post-thaw percent of membrane intact sperm that were treated with glycerol was $59.5 \pm 5.8\%$ while DMSO had $41.6 \pm 7.2\%$ of sperm with intact membranes. No significant difference ($P \leq 0.05$) was determined for post-thaw membrane integrity between the glycerol and DMSO treatments. Subjecting the epididymal sperm to cryopreservation did cause a significant decrease ($P \leq 0.05$) in the mean membrane integrity when comparing pre-freeze to post-thaw values for both treatments (Figure 5.3).

The pre-freeze and post-thaw values for both treatments are shown in Figure 5.4. Membrane integrity before freezing was fairly consistent between animals, ranging from 75% to 96%. All the animals except animal number 4 had higher values in the glycerol treatment. The glycerol post-thaw values ranged from 42% to 79%, and the DMSO treatment had a range of 9.3 % to 57.8% (Figure 5.4).

DISCUSSION

Successful cryopreservation of sperm is made possible by the presence of cryoprotectants (CPA) that allow sperm to survive extremely low temperatures. Membrane penetrating CPAs achieve this by lowering the temperature at which the sperm are susceptible to intracellular salts, which are detrimental (Muldrew et al., 2004). However, even though glycerol is the most common CPA for freezing mammalian sperm it does not always produce the best post-thaw results.

In our study, no significant differences were found between the weights of the left and right testes or epididymides from mature White-tail bucks. Also mean testicle temperatures taken at the time of processing were not significantly different among males. Although there was variation between animals for their body weights (28.8 to 84.9 lbs.), there was minimal variation between weights of the left and right testes and epididymides weights within each animal. The greatest variation between left and right testes for the same male was 2 g and 1 g for the epididymides.

There was no significant difference when measuring the total motility of White-tail deer epididymal sperm that was frozen using either glycerol or DMSO. These findings are similar to reports in other deer species, such as Fallow deer and Red deer when comparing glycerol with DMSO and propylene glycol (see Veldhuizen 1994 in Asher et al., 2000). Glycerol has outperformed DMSO for the cryopreservation of epididymal sperm in other exotic species including impala and blesbok (Loskutoff et al., 1996; Winger et al., 1997).

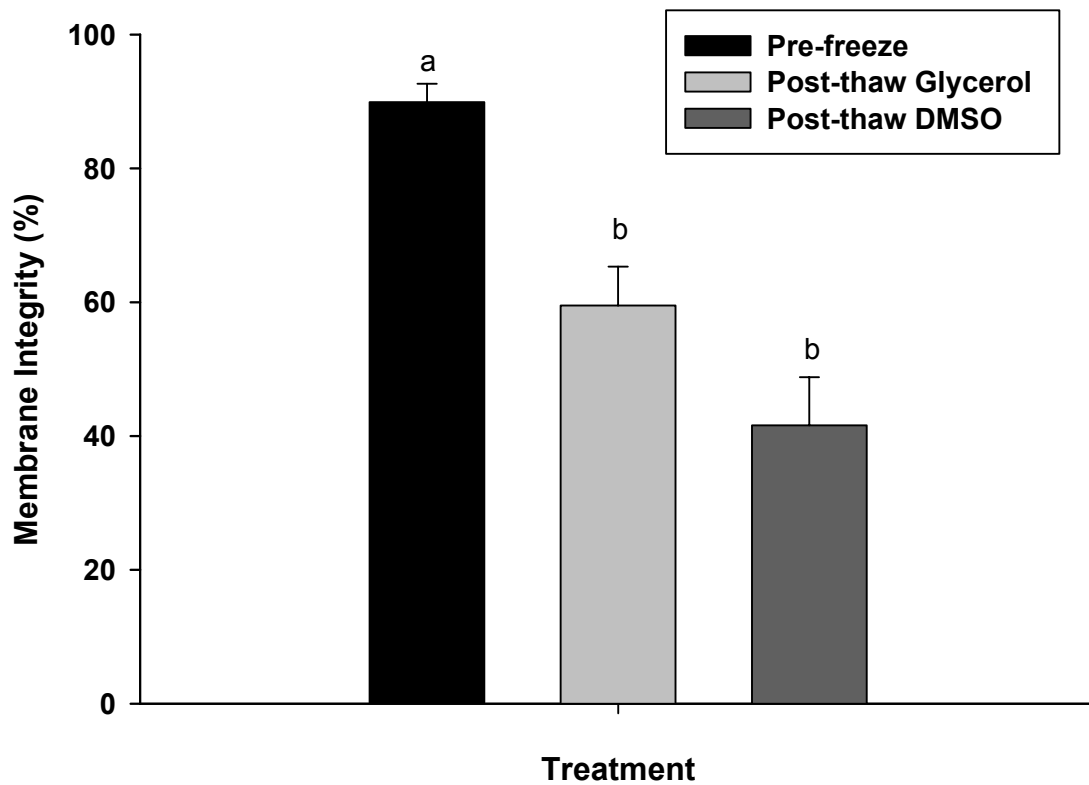


Figure 5.3. Percent membrane integrity (mean \pm SEM) comparing pre-freeze and post-thaw values of epididymal sperm from White-tail deer that were frozen using glycerol or DMSO as the cryoprotectant. ^{a,b}Mean values between pre-freeze and treatments with different superscripts are found to be significantly different ($P\leq 0.05$).

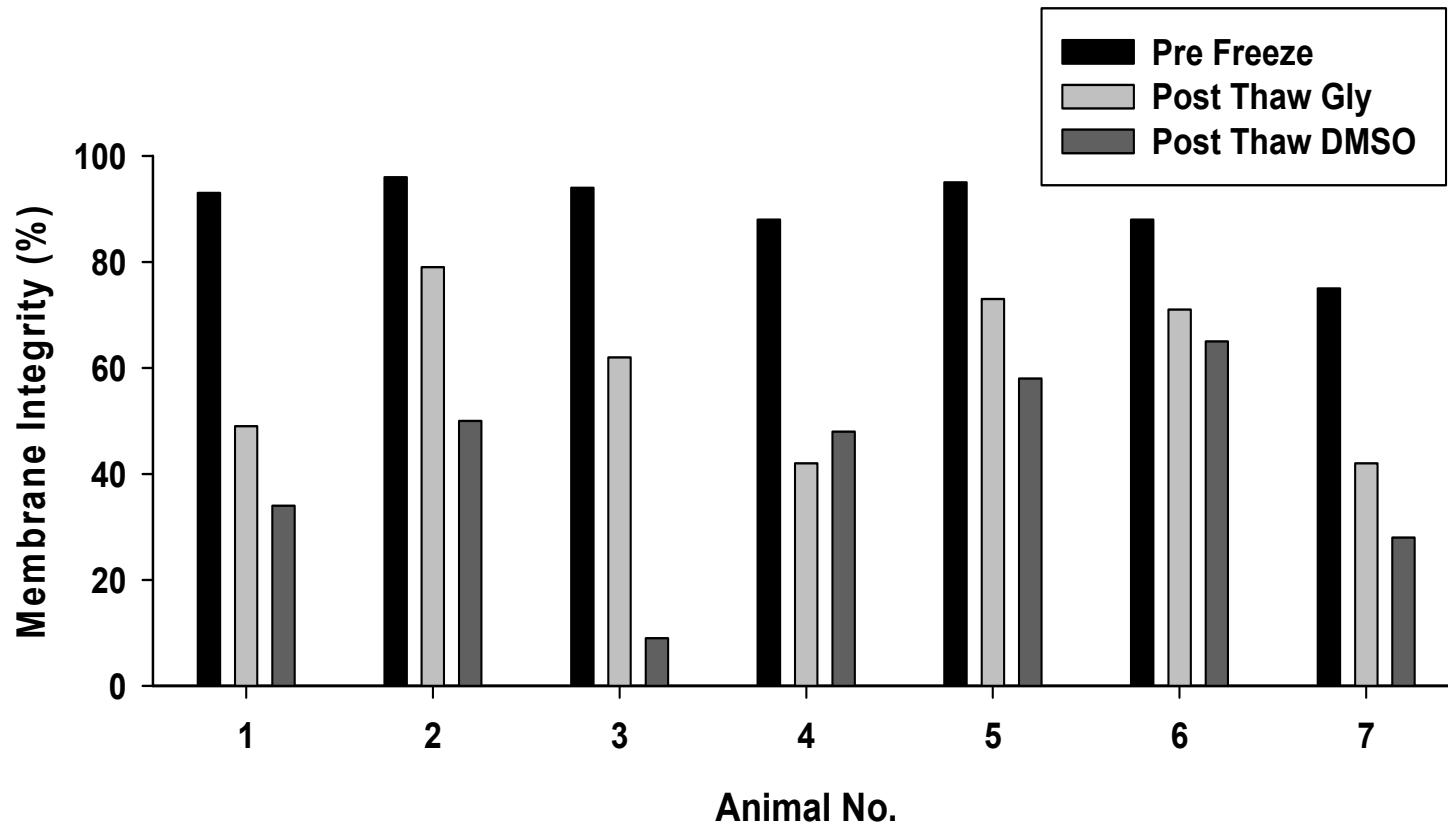


Figure 5.4. Pre-freeze and post-thaw membrane integrity of epididymal sperm from individual White-tail bucks, when sperm was frozen using either glycerol or DMSO as the cryoprotectant.

There are few studies comparing CPAs for freezing sperm of other wild Cervidae. However, Loskutoff et al. (1996) did report that springbok epididymal sperm had better post-thaw motility when frozen with DMSO rather than with glycerol, ethylene glycol or propylene glycol. One report indicated that both ejaculated and epididymal sperm of the wart hog preferred ethylene glycol over that of glycerol and DMSO (Gilmore et al., 1998). This same report showed that ejaculated and epididymal impala sperm, when frozen with DMSO, resulted in better post-thaw motility than when sperm were frozen with glycerol, but less motility than ethylene glycol when used for the ejaculated sperm. Also, DMSO outperformed both glycerol and ethylene glycol when measuring post-thaw motility of ejaculated and epididymal sperm in the African elephant (Gilmore et al., 1998). When freezing ejaculated sperm from the Japanese White rabbit DMSO outperformed glycerol, however, it was less affective than lactamide and acetamide when comparing progressive motility (Kashiwazaki et al., 2006).

In the present study, frozen-thawed sperm samples from White-tail deer were allowed to stand in a 37°C water bath and motility was measured after 10, 30 and 60 minute incubation intervals. When evaluating motility over time, the highest recorded motility value was observed at 60 minutes for only one animal in the glycerol treatment and none at 60 minutes for the DMSO treatment. Our results show that epididymal sperm from bucks in these treatments reach their peak sperm motility at prior to the 60 minute observation time, and decreased over time. These results are similar to those reported for ejaculated Formosan Sika deer and Formosan Sambar deer that reached their peak motility at time zero and then decreased every hour for 4 hours (Cheng et al., 2004). Also when blesbok epididymal sperm was allowed to incubate in 37°C water bath motility was recorded at 2 hours and then decreased by 6 hours of incubation (Winger et al., 1997). However, the results for White-tail deer epididymal sperm in our previous study (previous chapter) are contrary to the results in this current study. The White-tail deer epididymal sperm reached peak motility most often prior to the end of the 60 minute incubation period. This suggests that the procedure used for White-tail deer epididymal sperm has an effect on when sperm will reach peak post-thaw motility.

Assessment of epididymal sperm morphology from mature White-tail deer that were cryopreserved using either glycerol or DMSO only showed a significant difference when comparing percentage of pre-freeze normal sperm to post-thaw normal sperm, which had been frozen in DMSO. This difference can be attributed primarily to the percentage of bent tails before and after freezing. This pattern is similar to results

reported for goats when, sperm acrosome, head and tail morphology of ejaculated sperm were compared between pre-freeze and post-thaw (Singh et al., 1995). There was no significant difference found between sperm head abnormalities but there was a difference in tail abnormalities for goat ejaculated sperm. However, a significant increase in acrosomal and tail abnormalities was noted when freezing ovine sperm with DMSO in place of glycerol (Singh et al., 1995).

When evaluating membrane integrity of epididymal sperm from White-tail deer that were frozen with glycerol or DMSO there was no significant difference for the pre-freeze or post-thaw parameters. Recently, Fernandez-Santos et al. (2005) reported no significant difference in the membrane integrity values of epididymal sperm that were frozen using glycerol over DMSO, propylene glycol and ethylene glycol in Red deer. In the Asian elephant, there was a significant increase in the membrane integrity of ejaculated sperm that was frozen in glycerol compared to DMSO (Thongtip et al., 2004).

DMSO has produced significantly better membrane integrity results over glycerol when used with ejaculated sperm from the Japanese White rabbit (Kashiwazaki et al., 2006). When epididymal sperm from one inbred and one hybrid mouse colonies were frozen in different CPAs and then tested for membrane integrity, one colony had better results with DMSO, while the other colony had better results with glycerol (Sztejn et al., 2001).

Our results showed no significant difference between glycerol and DMSO for total motility or membrane integrity. However, there was a significant decrease in normal sperm morphology for epididymal sperm frozen using DMSO as the cryoprotectant when compared with the pre-freeze value. In contrast to the DMSO treatment, there was no significant difference for normal sperm morphology when comparing the glycerol treatment to the pre-freeze value. In addition, when performing post-thaw motility evaluation on White-tail deer epididymal sperm, it is recommended that the sample be allowed to stand in 37°C water bath for up to 30 minutes before recording sperm motility.

CHAPTER VI

POST-THAW EVALUATION OF EPIDIDYMAL SPERM FROM WHITE-TAIL DEER AFTER A 4°C STORAGE WITH GLYCEROL FOR 0, 2, 4, 12, AND 24 HOURS

INTRODUCTION

Epididymal sperm is a viable source of male gametes that has potential use in different assisted reproductive techniques (ART). Epididymal sperm from such species as the African elephant, impala and wart hog (Gilmore et al., 1998) have been cryopreserved for the purpose of gene banking. Studies in other exotic species including the Red hartebeest, eland and Burchell's zebra (Bezuidenhout et al., 1995) have evaluated cool storage of epididymides at 4°C. Bezuidenhout et al. (1995) reported in the African buffalo that live sperm were collected from epididymides 5 days postmortem when the epididymides were stored at 4°C. Also, in the Red hartebeest and eland live sperm was collected up to 4 days postmortem. However, Bezuidenhout et al. (1995) reported epididymal sperm from the Zebra to be sensitive to postmortem time, when no live sperm were detected in the epididymides only after day 2 postmortem. Lubbe et al. (1999) reported post-thaw results from the White rhinoceros showing acceptable (48%) post-thaw rates. In addition, Lubbe et al. (1999) showed a marked difference in pre-freeze and post-thaw sperm motility between one breeding and one nonbreeding male rhinoceros.

Although breeding female numbers were low, Bartels et al. (2001) showed in a small experiment that frozen-thawed epididymal sperm in the eland is viable enough to produce a calf. Winger et al. (1997) have reported an 88.2% fertilizing rate for blesbok oocytes that reached meiosis II when fertilized in vitro with blesbok epididymal sperm. Due to lack of availability of most exotic species, reliable assisted reproductive procedures have not been developed using epididymal sperm. However, in the domestic species epididymal sperm has been used successfully for ART. For example, in the horse, epididymal sperm has been used in artificial insemination and resulted in pregnancies (Barker et al., 1957). In the domestic dairy goat cleavage (in vitro) and a pregnancy were achieved using frozen-thawed epididymal sperm (Blash et al., 2000), and recently, in our own laboratory, frozen-thawed bovine epididymal sperm produced a live calf using intracytoplasmic sperm injection (ICSI) (Guerrero, 2006).

Due to the unpredictability of death and often the remoteness of exotic males at the time of death, harvesting and freezing of epididymal sperm in a timely manner is

often not feasible. In other instances, epididymal sperm from valuable males has been stored in the epididymis, with or without the testes, before the sperm were processed (Kikuchi et al., 1998; Bissett et al., 2005; Soler et al., 2005). Red deer (Soler et al., 2003b, and 2005) and eland (Bissett et al., 2005) are examples of two species where viable sperm have been recovered from epididymides that were stored with the testes at 5°C and without the testes at 4°C, respectively, for up to 3 days postmortem in the eland and 4 days postmortem in the Red deer. In these studies, the sperm maintained in the epididymides over time, resulted in a steady decline in sperm parameters. It is generally accepted that after death, animal tissue and cells deteriorate over time producing a toxic environment for epididymal sperm. The possibility of maintaining epididymal sperm in various extenders rather than in a deteriorating epididymis has been investigated. Promising results were reported when epididymal sperm from the African buffalo was stored in extender at 4°C for up to 9 hours postmortem (Herold et al., 2006). Furthermore, it was reported that Taurine bull sperm was required to equilibrate with glycerol for at least 2 hours before resulting in optimal freezability and fertility rates (Dhami et al., 1993). A short pre-freeze equilibration time of 30 minutes has also been shown to be an important factor in the cryopreservation of human sperm (Ali et al., 1993). In contrast, equilibration of boar semen up to 75 minutes had neither a positive or negative effect on post-thaw viability (Almlid et al., 1988).

It has long been known that prolonged storage of sperm with glycerol is ultimately detrimental to the sperm. It has been reported that sperm metabolically converts glycerol into a toxic metabolite known as “methyglyoxal” (Riddle et al., 1973).

The objective of this study was to store epididymal sperm with glycerol for 0, 2, 4, 12 and 24 hours before freezing to determine if and when glycerol became toxic to White-tail deer epididymal sperm.

MATERIALS AND METHODS

Experimental Design

Experiment 6.1

Paired testes from 7 mature White-tail deer were removed postmortem from hunter harvested bucks collected during the post rutting season of the 2005-2006 Louisiana White-tail deer hunting season. All seven pairs of testes for Experiment 6.1 were collected and processed in one day. Testicles with scrotum, once removed from the body of a postmortem buck, were placed in a Ziploc plastic bag and placed in a Styrofoam ice chest. Testes were held at ambient temperature, and transported to the

Embryo Biotechnology Laboratory (EBL). Upon arrival at the EBL, testes were processed and epididymal sperm was flushed from the cauda epididymidis. Epididymal sperm was flushed from both epididymides using an extender as the flushing medium. There was no glycerol present in the extender at this time. Sperm from both epididymides were pooled and allowed a slow cool to 4°C. Upon reaching 4°C, glycerol was added to the sample. Following the addition of the glycerol, the sperm samples were loaded into 0.5 ml plastic straws. Each animal (n = 7) had at least two straws cryopreserved at each of the following time periods: 0, 2, 4, 12 and 24 hours after the addition of glycerol. All straws were frozen by liquid nitrogen vapor and then subsequently thawed for post-thaw evaluation. Pre-freeze (n = 7) and post-thaw (n = 35) motility were assessed by a single technician using an inverted microscope equipped with a heated stage. Also for each straw thawed, motility was assessed at three different time periods after thawing (10 minutes, 30 minutes and 60 minutes). Both pre-freeze (n = 7) and post-thaw (n = 35) morphology was determined using a mixture of sperm sample with eosin-nigrosin stain on a pre-cleaned microscope slide under a light microscope and 100X oil immersion objective. Pre-freeze (n = 7) membrane integrity was evaluated using fluorescent microscopy. While post-thaw (n = 35) membrane integrity was calculated by multicolor flow cytometry.

Experimental Procedure

Testes Collection

During the 2005-2006 Louisiana White-tail deer hunting season managed hunts were conducted by the Louisiana Department of Wildlife and Fisheries (LDWF) on different wildlife refuges in the state. During these hunts local sportsmen harvested White-tail deer, and by LDWF regulations were required to check the animal in at mandatory check stations located on the refuge. At time of check-in the animal was aged, weighed and antler measurements were recorded. Also at this time, paired testes with scrotum were removed from the animal in such a way, that as much of the spermatic cord as possible remained with the scrotum. Testes were then placed in a Ziploc plastic bag and into an ambient temperature Styrofoam ice chest (31.5 x 26 x 23 cm). For each animal (n = 7) time of death, and time of testicle removal was recorded at the same time as the age, body weights and antler measurements. The time from animal death to the time of testicle removal varied from 2 hours to 7.3 hours. Testicles were transported to the EBL to be processed.

Extender Preparation

On the day the testes were processed, a two part goat semen extender was prepared using 3.634 g of Tris (Sigma-Aldrich, Inc., St. Louis, MO), 0.5 g of fructose (Sigma-Aldrich, Inc.), 1.99 g of citric acid (Sigma-Aldrich, Inc.) and 20 ml of egg yolk (fresh chicken eggs), in 80 ml of double distilled water to reach a final volume of 100 ml of extender. The extender was filtered through a 0.45 µm Nalgene syringe filter (Nalge Company, Rochester, NY). After filtering extender was divided into two parts: Part "A" extender (flushing medium) and Part "B" extenders (glycerol). Once divided, glycerol was added to the Part "B" extender, the result was a 12% glycerol concentration. Part "B" extender was then placed into a 4°C refrigerator to cool. Depending on the number of samples processed, a different volume of the extender was prepared. However, concentration of extender components was constant no matter the volume of the extender added.

Sperm Collection

Following arrival at the EBL, testicles were removed from the scrotum and processed (≤ 14 hours postmortem). Processing started by dissecting the tunica vaginalis surrounding the testis. Before removing the tunica vaginalis testicular temperature was taken by placing a temperature probe (DiGi-Sense[®] Dual Input JTEK Thermocouple Thermometer) through the previously made slit until it rested alongside the cauda epididymidis. Once testicular temperature was recorded the cauda epididymidis and vas deferens were dissected away from the testicle and the cauda epididymidis was cut away from the attached corpus epididymidis. The testicle was further processed to remove the remaining corpus and caput epididymidis, leaving just the testicle. The weights of the separated testicle and cauda epididymidis were then determined. An incision was made where the epididymal tubules regress from the vas deferens to the corpus of the epididymis (a distinct anatomical difference in the diameter of the cauda tubules occurs at the medial portion of the cauda epididymidis). The superficial blood vessels and thin membrane were dissected away exposing the epididymal tubules. Using a scalpel blade (no. 10), a single tubule was dissected to allow the retrograde flow of epididymal sperm from the vas deferens side of the cauda epididymidis.

Before retrieving the epididymal sperm ~3 ml of Part "A" extender was added to a 15 ml centrifuge tube to prevent sperm from sticking to the sides of the collection tube. Epididymal sperm was then flushed from the epididymal tubules using Part "A" extender, using a retrograde flow method.

The syringe used for flushing was made by heating the tip of the 12 ml Monoject syringe and then slowly stretching the melted tip until making a small enough diameter tube to fit inside the lumen of the vas deferens. The flushing was performed by threading the vas deferens with the plastic tube attached to the modified 12 ml syringe which contained flushing medium. Once inserted, the plastic tube was held in the vas deferens using a Desmarres Chalazion forceps (78.74 mm). Cauda epididymal sperm was then flushed into the 15 ml centrifuge tube containing the extender.

A 300 μ l volume of epididymal sperm-extender mixture was placed in a warmed 1.7 ml plastic microcentrifuge tube and diluted 1:5 with 37°C phosphate-buffered saline (PBS). The 15 ml centrifuge tube with remaining epididymal sperm-extender mixture was placed in a 250 ml glass beaker containing 200 ml of water (at room temperature) and allowed to stand for 30 minutes. After the 30 minutes, the beaker with 15 ml tube was placed in a refrigerator and allowed to reach 4°C before further processing.

Pre-Freeze Sperm Analysis

All sperm parameter measurements were made from the 1:5 diluted sperm that had been allowed to warm in a 37°C water bath. The first sperm parameter assessed was total sperm motility using an inverted light microscope with a heated stage (Nikon Diaphot, Tokyo, Japan) and Hoffman optics (40X objective). Total motility of epididymal sperm was assessed after 5 minutes of incubation at 37°C. A 10 μ l droplet of 1:5 diluted sperm was placed on a warmed pre-cleaned glass microscope slide, covered with a warmed 18 x 18 mm glass cover slip and placed on the heated microscope stage (37°C). The motility of each sample (n = 7) was determined by calculating the average total sperm motility of three separate views of the same slide.

Sperm morphology was evaluated using a single drop of eosin-nigrosin stain (Lane Manufacturing, Inc., Denver, CO), placed on a pre-cleaned glass microscope slide and mixed with ~2 μ l of sperm sample. Using another slide this mixture was then dispersed all along the slide in such a manner that the concentration of the stain decreased from left to right. After the stain dried, morphology for each sample was determined by placing the slide under a 100X oil immersion objective of an upright light Nikon microscope. A total of 200 sperm were counted at random and placed into one of the six categories as follows: normal, bent tails, broken mid-pieces, proximal droplets, abnormal heads or detached heads. The percentage of sperm in each category was then calculated.

Membrane integrity was evaluated using fluorescent staining with an inverted light microscope (40X objective) equipped with ultraviolet fluorescence and a fluorescence isothiocyanate filter. Staining epididymal sperm for the purpose of determining membrane integrity was accomplished by using the LIVE/DEAD viability kit (Molecular Probes, Inc., Eugene, OR). The contents of the kit included SYBR 14 (1 mM solution in DMSO), which stained the DNA of membrane intact sperm green (viable cells), propidium iodide (PI) (2.4 mM solution in water), which stained the DNA of membrane ruptured sperm red (nonviable cells). A 200 μ l volume of the 1:5 sperm dilution was pipetted into a microcentrifuge tube (0.65 ml) and gently mixed with 400 nM of SYBR 14 and 24 μ M of PI. Samples were protected from light (in a dark room) and maintained at 37°C for 10 minutes before being evaluated.

To determine the percentage of membrane intact sperm (viable), 200 sperm were randomly counted using the fluorescent luminescence of the Nikon microscope mentioned above. The number of green sperm (viable) was divided by 200 to conclude the percent of cells with intact membranes.

Epididymal sperm concentrations were determined using a hemacytometer and adjusted to a final concentration of 60 to 250 million cells per ml (twice as much as the final concentration); by the addition of more Part "A" extender.

Addition of Cryoprotectant and Cryopreservation

As previously mentioned, epididymal sperm from each animal was allowed to cool down to 4°C. At this point the volume of the sperm sample was half that of the final volume. Once cooled to 4°C Part "B" extender (Part "A" extender + glycerol), was added to the sample. Addition of an equal amount of the "sperm free" Part "B" extender doubles the volume and decreases the final sperm concentration to 30 to 125 million cells per ml. Also, before and after the addition of Part "B" extender, the concentration of glycerol was 12% and 6%, respectively. Part "B" extender was added to the sample in a stepwise process to prevent shocking of the sperm. When the glycerol addition was finished, samples were loaded into 0.5 ml straws (#005569, Cassou straw, IMV Technologies, Minneapolis, MN), sealed using a heat sealer and prepared for cryopreservation. Then 2 to 5 straws for each animal (n = 7) were frozen at each of the following time periods: 0, 2, 4, 12, and 24 hours after the addition of glycerol.

Straws for all five treatments were placed on a custom made Styrofoam boat (Yu et al., 2002b), so that straws were 7 cm above the liquid nitrogen. The freezing boat was a 7 cm tall, square with the center removed, much like a floating doughnut. However, on

the top surface of the square there was a thin plastic clam netting screen (1/4 inch squares) where the straws laid. The hollowed out center allowed the liquid nitrogen vapor to come up underneath and freeze the straws. Straws rested in liquid nitrogen vapor for 15 minutes, and then were plunged directly into the liquid nitrogen.

Thawing of Epididymal Sperm

Straws remained in liquid nitrogen up to 8 months before being thawed to evaluate the post-thaw values. At the time of thawing, a single straw was removed from liquid nitrogen and immediately placed in a 37°C water bath and allowed to thaw for 30 seconds. Straw was removed from the water bath, dried and the cotton plug cut free. The open end of the 0.5 ml straw was then placed over a 1.7 ml microcentrifuge tube that was placed in 37°C water bath. Using sharp-pointed scissors a small cut was made on the top part (sealed side) of the straw. Cutting the straw a second time removed the vacuum of the straw and any fluid below the cut flows into the centrifuge tube. This whole thawing process did not take longer than 90 seconds.

Samples used to evaluate post-thaw total motility and morphology were prepared and evaluated in the same manner, and using the same equipment used to evaluate the pre-freeze values of these samples. Post-thawed values for total motility were based from the average of thawing 3 straws from each treatment. Also for each straw thawed, motility was assessed at three different time periods after thawing (10 minutes, 30 minutes and 60 minutes). The purpose of the three time periods was to monitor any increase in motility after allowing the sample to warm up in the 37°C water bath. The motility value reported for each straw was the best motility of the three motility values, regardless of the time the observation was made. The reported post-thaw morphology value was based on counting 200 sperm cells from a single straw for each treatment.

The post-thaw membrane integrity was calculated by multicolor flow cytometry. Assessment of plasma membrane was conducted using a protocol for samples with egg yolk components present as described by Nagy et al. (2003), with only minor modifications. The staining protocol consisted of both SYBR 14 and PI. A final concentration of 100 nM SYBR 14 solution and 12 µM PI solution were added to 250 µl of diluted sperm sample. The samples were thoroughly mixed and incubated at 37°C in the dark for 10 to 15 minutes and then remixed before subjected to flow cytometric analysis. Measurements were completed on a FACSCalibur flow cytometer (Beckton Dickinson Immunocytometry Systems, San Jose, CA).

Forward and side scatter values were recorded on a linear scale, while fluorescent values were recorded on a logarithmic scale. The two fluorochromes were excited at 488 nm with a 20 mW argon laser. Plasma membrane ruptured cells were PI positive, and their red fluorescent signal was detected on the FL3-channel using a 610 nm long pass filter. Membrane intact cells were SYBR 14-positive, and its green fluorescent signal was detected on the FL1-channel using a 530/30 nm band pass filter. Dot plots drawn for data analysis were generated by WinMDI 2.8 (free software by J. Trotter, available for downloading at (<http://www.facs.scripps.edu/software.html>)). On SYBR 14 (FL1) and PI (FL3) dot plots, specific regions were drawn to determine the percentage of viable and nonviable sperm in each sample.

Flow cytometry was performed by an experienced technician. The description of the procedure used in this study was modified from that described by Guerrero (2006) for this laboratory.

Statistical Analysis

Total sperm motility, sperm morphology and sperm membrane integrity were analyzed using ANOVA, with the Holm-Sidak method used for multiple time comparison. Testicular measurements were analyzed using a Student t-test to compare left and right testes. Measured sperm parameters are reported as mean \pm SEM per treatment group. A $P\leq 0.05$ was used to determine significance for this study. Sigma Stat Version 3.0 was used to analyze data for this experiment.

RESULTS

Experiment 6.1

Testes and Epididymides Weights

Testes and epididymides weights from each male used in this study are presented in Table 6.1. The testes weights for this study ranged from 19 g to 45 g, while the epididymides ranged from 2 g to 5 g. The mean for the left and right testes weights were 34.3 g and 33.1 g, respectively. The mean weights for the epididymides were 4.0 g for the left and 3.6 g for the right testes. No significant difference ($P\geq 0.05$) was detected between left and right epididymides or testes weights.

Testicular Temperatures and Epididymal Sperm Concentrations

Just before the epididymides were dissected away from the testes, the temperature of each testicle was recorded and is shown in Table 6.1. Testicular temperature ranged from 9.2°C to 13.2°C, with the mean temperature for the left and right testes being 11.5°C and 11.3°C, respectively. The temperature variation between

Table 6.1 Testis and cauda epididymides weights and temperatures from postmortem White-tail bucks. All measurements were taken at time of epididymal sperm collection (≤ 12 hours postmortem).

Deer Number	<u>Testes wt (g)</u>		<u>Epididymides wt (g)</u>		<u>Testis temp ($^{\circ}$C)</u>	
	Left	Right	Left	Right	Left	Right
1	35	35	5	5	11.2	11.2
2	28	28	4	3	10.6	11.0
3	45	45	4	5	12.2	11.4
4	40	40	5	3	10.1	9.2
5	35	34	4	3	11.6	11.8
6	32	31	2	3	12.5	11.1
7	25	19	4	3	12.5	13.2
Mean \pm SEM	34.3 \pm 2.56	33.1 \pm 3.17	4.0 \pm 0.38	3.6 \pm 0.37	11.5 \pm 3.6	11.3 \pm 4.5

No significant difference was found between epididymal and testes weights or testis temperatures for right or left testes ($P > 0.05$).

testes within animals was minimal. The greatest difference within an individual animal was 1.4°C. No significant difference ($P \geq 0.05$) was detected between the temperatures of the left and right testes.

The average for the total number of sperm cells collected from both epididymides was 2.0×10^9 with a range from 390×10^6 to 5.25×10^9 .

Total Motility

The mean pre-freeze total motility for epididymal sperm before being divided into treatments was $78.6 \pm 1.8\%$. After pre-freeze evaluation, sperm were divided into treatments and frozen, the mean post-thaw values for epididymal sperm frozen after being exposed to glycerol for 0, 2, 4, 12 and 24 hours was $52.1 \pm 9.9\%$, $51.4 \pm 9.0\%$, $48.7 \pm 7.6\%$, $39.7 \pm 8.2\%$ and $28.0 \pm 6.8\%$, respectively. As shown in Figure 6.1, post-thaw total motility decreased the longer epididymal sperm was stored with glycerol before being frozen. However, other than the statistical decrease between the pre-freeze and all post-thaw values, no significant difference among the post-thaw treatments was detected. Pre-freeze motility and each animal's sperm motility for all five treatments after freezing are shown in Figure 6.2. There is high variation among animals for each treatment. The pre-freeze for each animal was consistent, with a difference of only 15%, and a range from 70% to 85%. The more varied post-thaw values ranged from 25% to 80%, 5% to 80%, 23% to 78%, 18% to 75% and 10% to 55% for 0, 2, 4, 12 and 24 hour exposure to glycerol, respectively.

Total Motility In Relation To Time

After thawing, sperm samples from each animal and treatment were allowed to sit in a 37°C water bath to reach their full motility potential. Total motility readings were recorded at 10, 30 and 60 minutes. The highest recorded value was the reading used in the final report for each animal. See Tables 6.2 through 6.6 for results from 0, 2, 4, 12 and 24 hour storage with glycerol, respectively. For the 0, 2, 4 and 12 hour treatments, the highest motility reading occurred more often at either 10 and/or 30 minutes, never did the highest motility occur more than once for each treatment at 60 minutes of incubation mark. When pooling and evaluating all the treatments together, the lowest motility occurred at 60 minutes 22 out of 26 times.

Morphology

Prior to freezing and dividing into treatments, epididymal sperm cells had a $58.2 \pm 8.6\%$ normal morphology. Following the freeze, cells that were stored with glycerol for 0, 2, 4, 12 and 24 hours showed a $32.3 \pm 2.6\%$, $37.7 \pm 3.5\%$, $35.1 \pm 4.5\%$, $28.1 \pm 3.8\%$

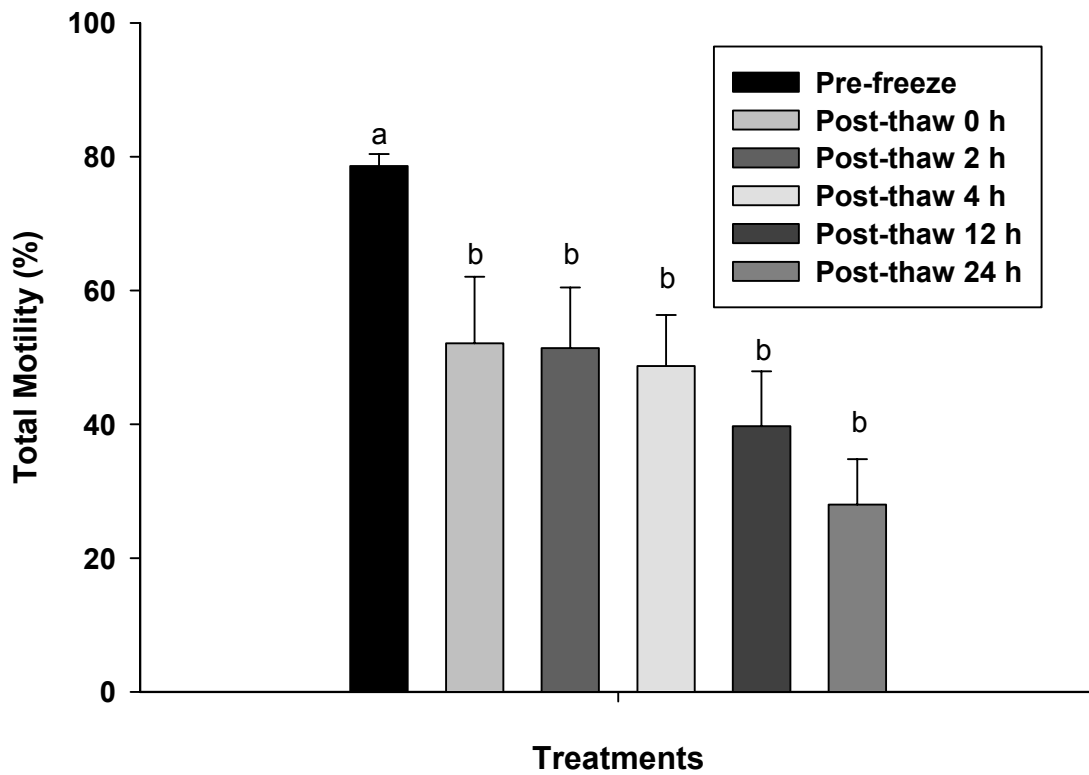


Figure 6.1. Percent total motility (mean±SEM) comparing post-thaw values of White-tail deer epididymal sperm that was subjected to glycerol for different lengths of time before freezing. ^{a,b}Mean values between treatments with different letters are significantly different ($P \leq 0.05$).

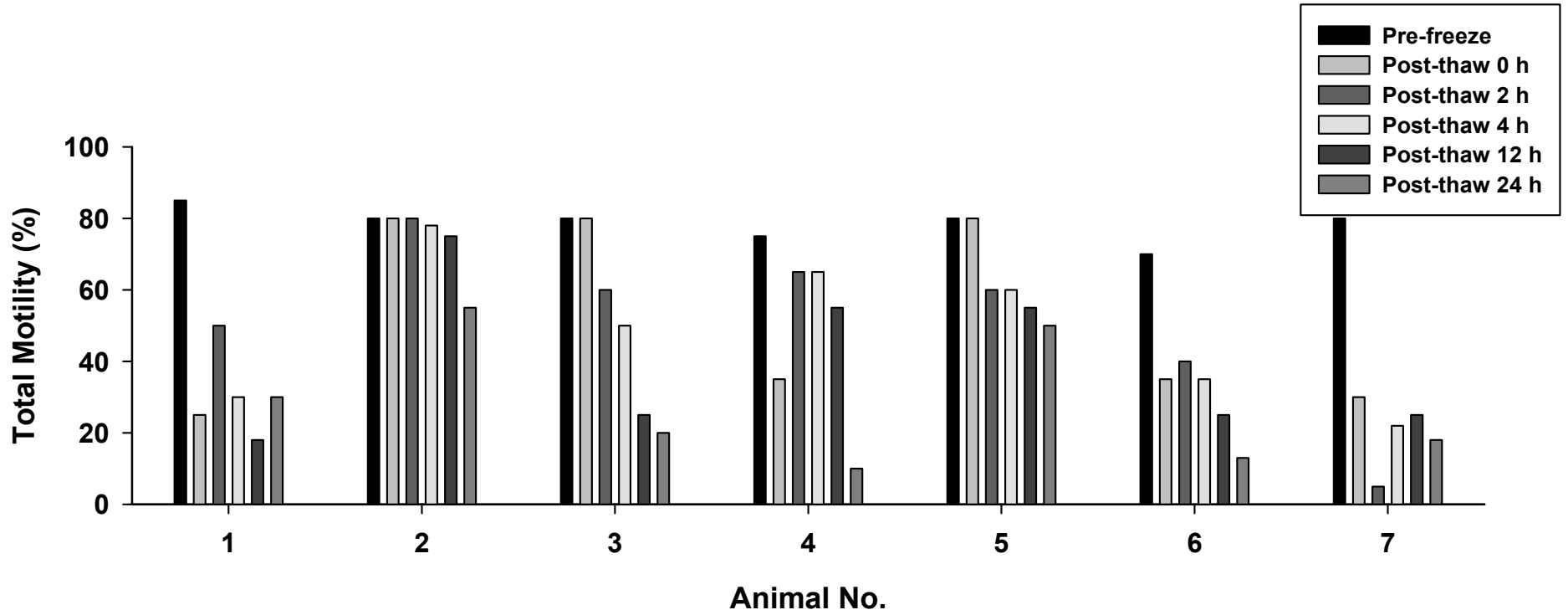


Figure 6.2. Pre-freeze and post-thaw total motility of epididymal sperm from individual White-tail bucks, when sperm was subjected to the cryoprotectant, glycerol, for different lengths of time before being frozen.

Table 6.2. Values represent total motility of epididymal sperm that was frozen 0 hours after the addition of glycerol. Motility was recorded at specific times post-thaw and ranked priority 1 to 3 (1 = top and 3 = lowest ranking).

Buck number	10 minutes	30 minutes	60 minutes
1	25 ¹	23 ²	18 ³
2	80 ¹	50 ²	10 ³
3	70 ²	80 ¹	30 ³
4*	25 ²	35 ¹	35 ¹
5	80 ¹	75 ²	40 ³
6	35 ¹	30 ²	5 ³
7	30 ¹	18 ²	8 ³
Occurrence of top ranking/period	5	2	1
Occurrence of low ranking/period	1	0	6

*The occurrence of top ranking motility occurred at more than one time period.

Table 6.3. Values represent total motility of epididymal sperm that was frozen 2 hours after the addition of glycerol. Motility was recorded at specific times post-thaw and ranked priority 1 to 3 (1 = top and 3 = lowest ranking).

Buck number	10 minutes	30 minutes	60 minutes
1	0 ³	45 ²	50 ¹
2*	80 ¹	80 ¹	70 ²
3*	60 ¹	60 ¹	50 ²
4	65 ¹	53 ²	10 ³
5	60 ¹	50 ²	40 ³
6	28 ²	40 ¹	10 ³
7	5 ⁻	5 ⁻	5 ⁻
Occurrence of top ranking/period	4	3	1
Occurrence of low ranking/period	1	0	5

*The occurrence of top ranking motility occurred at more than one time period.

Table 6.4. Values represent total motility of epididymal sperm that was frozen 4 hours after the addition of glycerol. Motility was recorded at specific times post-thaw and ranked priority 1 to 3 (1 = top and 3 = lowest ranking).

Buck number	10 minutes	30 minutes	60 minutes
1	30 ¹	25 ²	15 ³
2	78 ¹	73 ²	30 ³
3*	20 ²	50 ¹	50 ¹
4	65 ¹	28 ²	8 ³
5	60 ¹	45 ²	35 ³
6	35 ¹	25 ²	8 ³
7	23 ¹	18 ²	8 ³
Occurrence of top ranking/period	6	1	1
Occurrence of low ranking/period	1	0	6

*The occurrence of top ranking motility occurred at more than one time period.

Table 6.5. Values represent total motility of epididymal sperm that was frozen 12 hours after the addition of glycerol. Motility was recorded at specific times post-thaw and ranked priority 1 to 3 (1 = top and 3 = lowest ranking).

Buck number	10 minutes	30 minutes	60 minutes
1	18 ²	13 ³	20 ¹
2	75 ¹	50 ²	23 ³
3	5 ³	25 ¹	20 ²
4*	55 ¹	55 ¹	15 ²
5*	55 ¹	55 ¹	30 ²
6	25 ¹	15 ²	10 ³
7	25 ¹	13 ²	5 ³
Occurrence of top ranking/period	5	3	1
Occurrence of low ranking/period	1	1	5

*The occurrence of top ranking motility occurred at more than one time period.

Table 6.6. Values represent total motility of epididymal sperm that was frozen 24 hours after the addition of glycerol. Motility was recorded at specific times post-thaw and ranked priority 1 to 3 (1 = top and 3 = lowest ranking).

Buck number	10 minutes	30 minutes	60 minutes
1	5 ³	13 ²	30 ¹
2	55 ¹	50 ²	13 ³
3	20 ³	20 ³	20 ³
4	0 ³	5 ²	10 ¹
5	50 ¹	45 ²	25 ³
6	8 ³	5 ²	13 ¹
7*	18 ¹	13 ²	13 ²
Occurrence of top ranking/period	3	0	3
Occurrence of low ranking/period	3	1	3

*The occurrence of top ranking motility occurred at more than one time period.

and 31.2±2.6% normal morphology, respectively (Table 6.7). No significant difference was detected in the post-thaw morphology among any of the treatments.

Membrane Integrity

The mean pre-freeze for membrane integrity of epididymal sperm in this study was 91.0±1.5%. The post-thaw percent of membrane intact sperm that were stored with glycerol for 0, 2, 4, 12 and 24 hours was 66.1±3.7%, 72.1±3.7%, 70.1±3.1%, 69.7±5.1% and 65.2±2.3%, respectively. No significant difference ($P \geq 0.05$) was found for post-thaw membrane integrity values among any of the treatments. Subjecting the epididymal sperm to cryopreservation did cause an expected significant decrease ($P \leq 0.05$) in the mean membrane integrity values when comparing pre-freeze to post-thaw values for all treatments. However the mean post-thaw values remained high, all treatments stayed above 65% even after freezing and thawing. Also, the mean post-thaw membrane integrity between treatments was relatively constant, with no clear pattern of membrane integrity loss over time (Figure 6.3). The integrity of sperm cell membranes prior to and post-thaw for individual males are displayed in Figure 6.4. Membrane integrity before freezing was fairly consistent with a range of only 12% between the minimum value of 85% and the maximum value of 97%.

DISCUSSION

When collecting sperm from postmortem males, the epididymal sperm is often held for extended lengths of time in the epididymis which is still attached to the testis. Holding and transporting the testes can be inconvenient and the decomposition of the tissue can become toxic to the sperm over time (Soler et al., 2003b, 2005; Bissett et al., 2005). Therefore, it would seem to be more logical to extract epididymal sperm and hold or transport the sperm while in an extender. Motility of ejaculated dog sperm held for 4 days at 4°C was higher at days 1 through 4 when sperm were held in an egg yolk-tris based extender instead of being held in autologous seminal plasma, egg yolk-milk or egg yolk-cream extender (Rota et al., 1995). In contrast, epididymal sperm in stallions had higher motility at 0, 24 and 48 hours when sperm were held in seminal plasma instead of a skim milk-glucose extender (Braun et al., 1994). Under field conditions, it is often more practical to use a one-step extender, rather than the usual two-step extender. The problem with a one-step extender is that sperm are exposed to the cryoprotectant (CPA) for a longer period of time than when using a two-step extender. Since CPAs have been proven to be toxic to sperm (Fahy, 1990), the increased exposure time may

Table 6.7. Sperm morphology parameters (% means \pm SEM) of White-tail deer epididymal sperm prior to freezing and post-thaw. Calculated from 200 sperm counted and categorized across several fields.

Treatments	Group	Normal	Bent tails	Broken mid-pieces	Proximal droplets	Abnormal heads	Detached heads
	Pre-freeze	58.2 \pm 8.6 ^a	16.5 \pm 3.7	20.2 \pm 11.5	3.9 \pm 2.7	0.6 \pm 0.2	0.7 \pm 0.6
0 hours	Post-thaw	32.3 \pm 2.6 ^b	22.6 \pm 5.4	39.0 \pm 5.5	5.1 \pm 3.2	0.6 \pm 0.3	0.5 \pm 0.2
2 hours	Post-thaw	37.7 \pm 3.5 ^b	20.5 \pm 6.2	37.8 \pm 5.2	5.3 \pm 3.4	0.2 \pm 0.1	0.9 \pm 0.2
4 hours	Post-thaw	35.1 \pm 4.5 ^b	21.7 \pm 6.8	36.5 \pm 6.5	5.5 \pm 42.8	0.4 \pm 0.2	1.0 \pm 0.2
12 hours	Post-thaw	28.1 \pm 3.8 ^b	26.4 \pm 5.6	38.9 \pm 6.0	4.9 \pm 3.1	0.5 \pm 0.2	1.4 \pm 0.4
24 hours	Post-thaw	31.2 \pm 2.6 ^b	10.2 \pm 2.6	50.8 \pm 6.3	7.2 \pm 4.6	0.9 \pm 0.3	0.5 \pm 0.2

^{a,b}Means with different superscripts within columns are significantly different ($P \leq 0.05$).

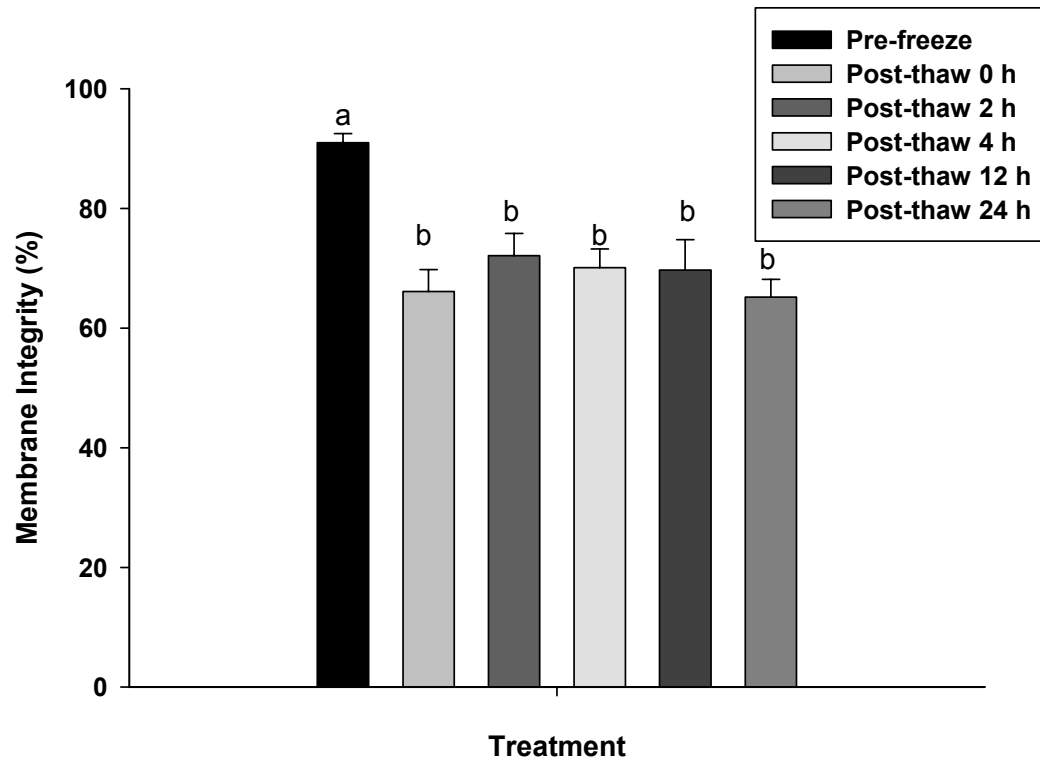


Figure 6.3. Percent membrane integrity (mean±SEM) comparing post-thaw values of White-tail deer epididymal sperm that was subjected to glycerol for different lengths of time before freezing. ^{a,b}Mean values between treatments with different letters are significantly different ($P \leq 0.05$).

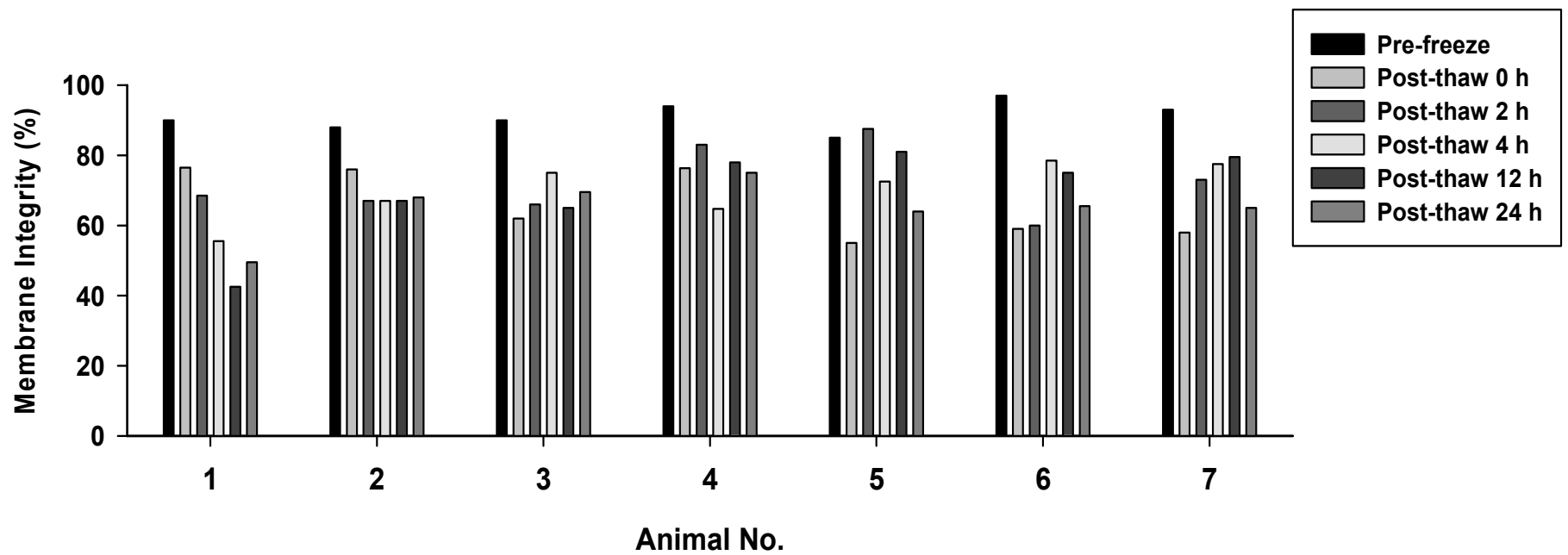


Figure 6.4. Pre-freeze and post-thaw membrane integrity of epididymal sperm from individual White-tail bucks, when sperm was subjected to the cryoprotectant, glycerol, for different lengths of time before being frozen.

cause unacceptable post-thaw results.

The testes and epididymides weights of mature White-tail deer used in this study had no significant difference when comparing the left to the right testes. Also, at the time of processing, the temperatures between the left and right testes were similar with a 0.2°C variation between the temperature means of the left and right testes. However, there was more variation between animals for the testes weights. This variation was likely due to factors such as age, body weight and/or genetics.

When measuring pre-freeze and post-thaw motility of epididymal sperm frozen at 0, 2, 4, 12, and 24 hours of incubation with glycerol, there was an expected significant decrease in sperm motility from pre-freeze to post-thaw within animals. However, no significant difference was detected for post-thaw motility among the five incubation time intervals. Mouse epididymal sperm has also been found to have decreased motility the longer the sperm were exposed to glycerol (Katkov et al., 1998).

Luskutoff et al. (1996) reported that when blesbok, impala and springbok epididymal sperm were incubated with glycerol for extended lengths of time at 30°C to 32°C, sperm motility decreased as the time exposed to glycerol increased. Despite the apparent decrease in sperm motility, no significant difference was detected in the results reported by Luskutoff et al. (1996). The same pattern was observed for both ejaculated and epididymal sperm from the wart hog, impala and African elephant sperm that was incubated with glycerol up to 60 minutes (Gilmore et al., 1998). Ejaculated sperm from the Namibian cheetah showed a decrease from pre-freeze to post-thaw motility but no significant difference when sperm were incubated with glycerol at 5°C for up to 60 minutes (Crosier et al., 2006).

Herold et al. (2006) reported no decrease in sperm motility when epididymal sperm from the African buffalo (*Syncerus caffer*) were stored in one-step extenders (Triladyl and Andromed) for up to 9 hours at 4°C. However, previously Herold et al. (2004) reported a study in the African buffalo that showed when epididymal sperm was allowed to incubate with glycerol for 3 to 9 hours it had significantly higher post-thaw motility than when sperm were only incubated with glycerol for ≤ 2 hours.

Post-thaw total motility was measured at different time periods to determine when White-tail deer epididymal sperm, that were subjected to the respective treatments, reached their peak motility. For sperm that were held with glycerol for 0 to 12 hours, the highest recorded motility was observed within 30 minutes post-thaw, except for one instance at 2 hours and one instance at 12 hours. These results are similar to

those reported for the ram when ejaculated sperm parameters, including motility, decreased from 0 to 2 hours post-thaw incubation at 38°C with glycerol (Gil et al., 2000).

In this study, the only significant difference for morphology was noted when comparing pre-freeze and post-thaw for percent normal sperm morphology. Due to the freezing process, this difference would normally be expected (Medeiros et al., 2002). It appears that the decrease in normal morphology is due to an increase in bent tails and broken mid-pieces. These tail and mid-piece abnormalities can be due to cold shock, which the sperm could have been exposed too during the cooling or freezing process, which has been previously reported for bulls (Barth and Oko, 1989). Our study showed no significant difference in normal sperm morphology when compared among treatments. Okano et al. (2004) reported results from ejaculated dog sperm that showed no significant decrease in the percent of abnormal sperm that were stored with glycerol for up to 16 hours (Okano et al., 2004). Similarly a study on the Japanese Black bear showed no difference in percent of abnormal sperm when comparing frozen-thawed ejaculated sperm that were incubated with glycerol for 1 hour or 3 hours (Okano et al., 2006).

Although motility of White-tail deer epididymal sperm declined over time when exposed to glycerol, the percentage of sperm that maintained membrane integrity remained constant for 0, 2, 4, 12 and 24 hours of glycerol exposure. Purdy (2006) reported that when ejaculated sperm from the ram was equilibrated in the presence of glycerol, no significant decrease was detected when measuring post-thaw membrane integrity. In a study evaluating ejaculated sperm from cynomolgus monkey, Li et al. (2005) reported that the highest percent of sperm with membrane intact cells was recorded at 30 minutes of glycerol equilibration instead of a 10, 60 or 90 minute exposure.

From the results of our study one can conclude that White-tail deer epididymal sperm can be stored in the presence of glycerol for up to 12 hours and result in a post-thaw motility value >30%. Also, storing White-tail deer epididymal sperm in the presence of glycerol for 24 hours before freezing, does not cause a significant decrease in sperm post-thaw membrane integrity. Post-thaw epididymal sperm from our study would be acceptable for intracytoplasmic sperm injection, in vitro fertilization or possibly artificial insemination.

CHAPTER VII

COMPARISON OF DIFFERENT ESTROUS SYNCHRONIZATION PROTOCOLS FOR FIXED-TIMED ARTIFICIAL INSEMINATION OF WHITE-TAIL DEER WITH A PRELIMINARY USE OF WHITE-TAIL DEER EPIDIDYMAL SPERM

INTRODUCTION

Artificial insemination (AI) is a tool that producers can use to maximize the number of progeny from a single ejaculate of an individual male. Also, AI can be used when natural mating from a specific male is not feasible due to location. Estrous synchronization of females is another technique often used to reduce the time interval of the breeding and birthing season of a group of females. Often estrous synchronization and AI are used in conjunction to maximize the number of females that can be mated in the shortest time interval. The combined use of these two assisted reproductive technologies allows producers to turn a 3 month natural breeding season into an efficient 30-day breeding season.

Artificial insemination dates back to Arabians artificially breeding their mares. The first recorded case of AI was conducted by Spallanzani (1784). Centuries later a Russian team headed by Ivanoff reported successful AI in the cow, sheep and two exotic equine species (zebras and Przewalskii horse) (Ivanoff, 1922). By the 1900s reports of successful AI in dogs, guinea pigs, horses, rabbits and humans had occurred (see Foote, 1999).

Today estrous synchronization is most widely used for AI and best understood in cattle. For this reason, most other synchronization protocols for other species are derived from those protocols used in cattle. Depending on the species, there are several different synchronization protocols currently in practice; and most of these use the hormones progesterone, prostaglandin or a combination of these two agents (Twagiramungu et al., 1995).

Synchronizing estrous for the purpose of AI has been successfully reported in several Cervidae species, some of the more common species include the Red deer (Asher et al., 1988; Haigh et al., 1991; Willard et al., 2002a; Bowers et al., 2004) Fallow deer (Asher et al., 1992; Willard et al., 2002a), Sika deer (Willard et al., 1996) and White-tail deer (Haigh 1984; Jacobson et al., 1989; Magyar et al., 1989; Willard et al., 2002a). Asher et al. (2000) have reported that greater than 95% of artificial inseminations in deer are performed on Red deer and Fallow deer, and most occur in New Zealand, Australia and Europe. AI has become popular in these countries because

farming Red deer and Fallow deer has developed into a commercial livestock industry (Soler et al., 2003a). In the United States the White-tail deer industry continues to grow, however, it is not nearly as developed as the Red deer and Fallow deer industries. In the past, White-tail deer has been used more as a conservation model, however, with the development of trophy deer hunting ranches there is a new source of income. With this new source of income, producers are now making efforts to increase the use of estrous synchronization and AI to improve deer herd genetics.

Even with the White-tail deer having a niche in the agriculture industry, they can still play a vital role as a model in conservation. Artificial insemination with ejaculated sperm and estrous synchronization have become common tools in domestic species, for the purpose of herd improvement. However, when these procedures are used in conjunction with epididymal sperm, it is most often for the purpose of conservation. Epididymal sperm can be used in domestic species, but this is usually when ejaculated sperm is unavailable. While in exotic and/or wild species epididymal sperm is frequently the only source of the male gametes. Development of an estrous synchronization and AI program that can be efficiently used with epididymal sperm in White-tail deer can then be further used to develop methodologies for endangered species.

The main objective of this study was to compare three estrous synchronization protocols combined with AI using frozen-thawed ejaculated sperm over two consecutive breeding seasons. At the end of the second breeding season, the most promising estrous synchronization protocol was used for AI using epididymal sperm of subsequent studies.

MATERIAL AND METHODS

Experimental Design

Experiment 7.1

The goal of this experiment was to determine which of two estrous synchronization protocols would produce the highest birth rates when used with AI and ejaculated frozen-thawed White-tail deer sperm. Thirty eight pen-raised White-tail does, born and raised at Idlewild Research Station in Clinton, Louisiana, were randomly assigned to two estrous synchronization treatments (Treatment A and Treatment B) (See Figures 7.1 and 7.2). The does ranged in weight and age from 46 kg to 74 kg and 1.5 years to 5.5 years, respectively. All does were on a timed AI sequence and inseminated at either 60 hours (Treatment A) or at 52 hours (Treatment B) post-synchronization. At the time of insemination, all does received 0.1 ml intravenous

sedative (iv) and all does were checked for pregnancy by transrectal ultrasound at 35 days post-insemination.

Experiment 7.2

The objective of this experiment (Experiment 7.2) was to use the synchronization protocol that produced the highest pregnancy rates from Experiment 7.1 ('Treatment A' now becomes 'Treatment A-I') and compare this protocol with a new estrous synchronization protocol (Treatment B-I). Thirty six pen-raised White-tail does, born and raised at Idlewild Research Station in Clinton, Louisiana, were randomly assigned to either Treatment A-I or Treatment B-I. The only difference between Treatment A-I and Treatment B-I was that females in Treatment B-I received an injection (im) of 200 units of equine Chorionic Gonadotropin (eCG) at the end of the synchronization protocol (See Figures 7.1 and 7.3). The does ranged in weight from 43.5 kg to 67.5 kg, and ranged in age from 2.5 years to 6.5 years. All does were time inseminated at 60 hours post-synchronization. All does were given 0.1 ml of detomidine hydrochloride (Domosadan) iv, and pregnancies were determined by transrectal ultrasound at 48 days post-insemination.

Epididymal Sperm - Preliminary Experiment

This was a preliminary experiment conducted during the same breeding season as Experiment 7.2. The purpose of the experiment was to implement the use of frozen-thawed epididymal sperm into an estrous synchronization protocol which would result in a live fawn. Six 1.5-year-old does ranging in weight from 47.5 kg to 58.0 kg were subjected to estrous synchronization using a 14 day CIDR-G (Controlled Intravaginal Drug Release device – goat) and a 200 IU injection (im) of eCG at the time of CIDR removal (Treatment B-I) (Figure 7.3). All does were artificially inseminated 60 hours after the removal of the CIDR. Does were inseminated using frozen-thawed epididymal sperm collected from a postmortem hunter-harvested male. Sperm were frozen using Triladyl and remained in liquid nitrogen for >1 year before being thawed for insemination. Epididymal sperm recorded a post-thaw motility of 60%. Does were pregnancy checked by transrectal ultrasound at day 48 post-insemination.

Experimental Procedure

Experimental Animals

White-tail does used in all three experiments were born and pen-raised at Idlewild Research Station located ~4.5 miles southeast of Clinton, Louisiana. Does ranged in body weights from 43.5 kg to 74 kg and age from 1.5 years to 6.5 years. The

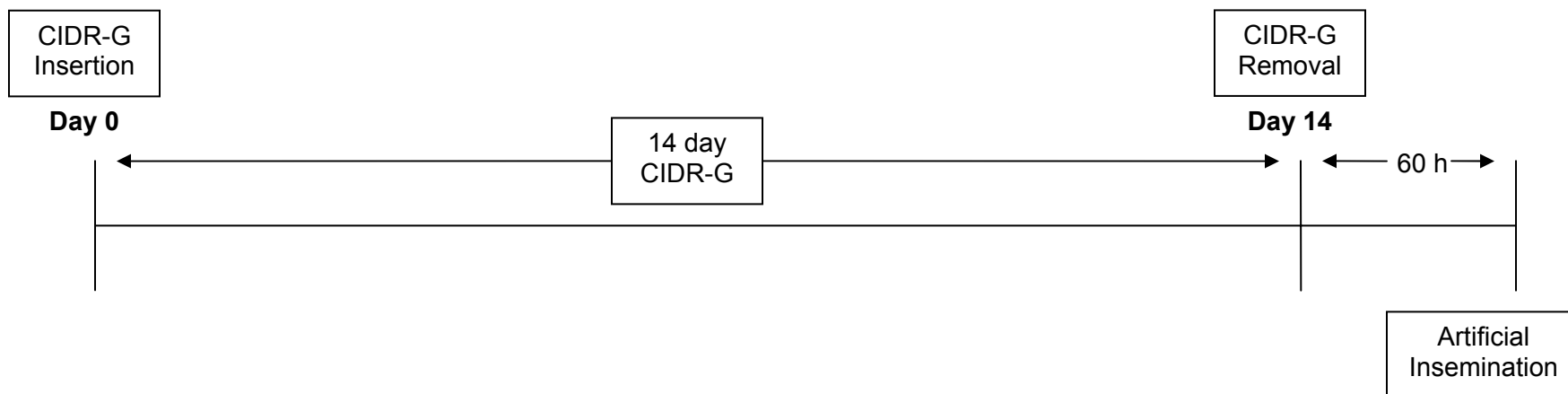


Figure 7.1. Time line for estrous synchronization used in Experiment 7.1 (Treatment A), and again in Experiment 7.2 (Treatment A-I). A 14-day CIDR-G protocol with artificial insemination at 60 hours after CIDR removal.

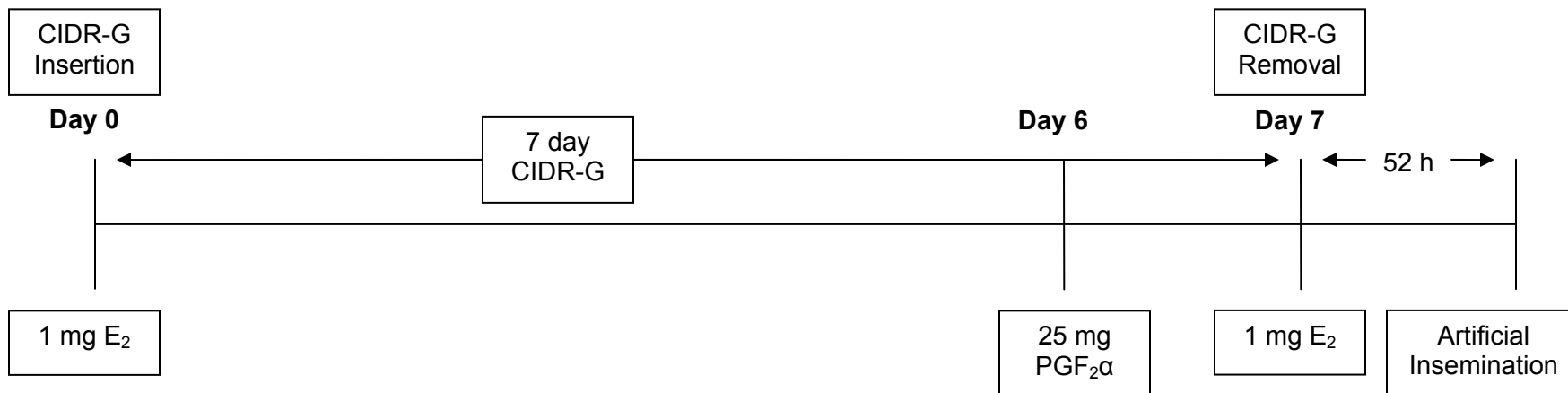


Figure 7.2. Time line for estrous synchronization used in Experiment 7.1 (Treatment B). A 7-day CIDR-G protocol with a 1 mg injection of estradiol benzoate at the time of CIDR insertion. At day 6 of treatment, a 25 mg injection of prostaglandin F₂α was administered. CIDRs were removed on day 7 of treatment and does were given another injection of 1 mg estradiol benzoate. Females were artificially inseminated 52 hours following CIDR removal.

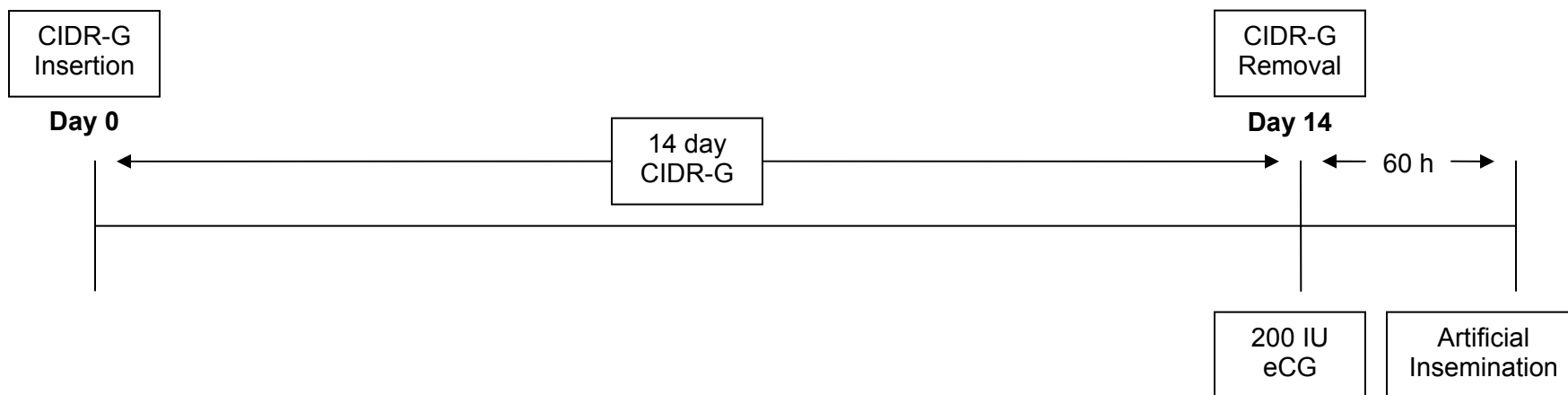


Figure 7.3. Time line of estrous synchronization used for Experiment 7.2 (Treatment B-I). A 14-day CIDR-G with an injection of 200 IU of eCG at the time of CIDR removal. Artificial insemination of the does at 60 hours after CIDR removal.

average weight and age for does used in Experiment 7.1 and Experiment 7.2 was 57.5 kg and 3.1 years, respectively. Females were maintained in a cleared 1.01 hectares grass enclosure surrounded by a 2.44 meter high chain length fence. Females had constant access to shade barns, fresh water and a complete deer pellet ration. The ration was made and purchased from Cargill Foods located in LeCompte, Louisiana. Feed was composed of the necessary ratio of crude protein, crude fiber and fat.

Epididymal sperm used in the preliminary experiment was collected postmortem from a White-tail buck that was harvested during the Louisiana 2004-2005 hunting season. Sperm was harvested from the epididymides within 12 hours postmortem, extended using Trialadyl[®] (Minitube, Verona, WI), a one-step extender, frozen in liquid nitrogen and held for >1 year.

Estrous Synchronization

White-tail does were worked in a custom made deer barn constructed with 3.05 meter tall 6.1 mm thick, solid aluminum walls. The solid walls help calm the animals because they were unable to see what was occurring in the other areas of the barn. Using moveable panels and strategically placed doors, females were herded into a box scale where they were weighed for the start of the project. After being weighed does walked into a drop bottom deer chute (Deer Handler, St. Alberta, Canada) where they were manually restrained. While restrained a CIDR-G (Control Intravaginal Drug Release Device) (Pharmacia Animal Health, Pfizer, Inc.), made for goats, was inserted into each doe.

In Year 1 (Experiment 7.1), does (n = 38) were stratified by age, weight and last fawning date randomly across two estrous synchronization treatments (Treatment A and Treatment B). Does in Treatment A were fitted with a CIDR, released and were not handled again until CIDR removal (14 days later) (Figure 7.1). Does in Treatment B were given an injection (im) of 1 mg of estradiol benzoate at the same time the CIDR was inserted. Then 6 days after CIDR insertion does received a 25 mg injection (im) of prostaglandin F₂α and on day 7 CIDRs were removed and does were given a second injection (im) of estradiol benzoate (Figure 7.2).

In year 2 (Experiment 7.2), does (n = 36) were again stratified randomly across two estrous synchronization protocols. Does in Treatment A-I were subjected to a 14-day CIDR protocol. This was the protocol (Treatment A-I) used in Experiment 7.1. (Figure 7.1). Does in Treatment B-I were also treated with a 14 day CIDR, however, the

females were also treated with an intramuscular injection of 200 IU of eCG at the time of CIDR removal (Figure 7.3).

Artificial Insemination

For females in Treatments A, A-I and Treatment B-I insemination occurred 60 hours after the removal of the CIDR. Females in Treatment B were inseminated 52 hours post CIDR removal. All does regardless of treatment were handled in the same manner. At time of insemination, females were restrained with the drop bottom deer chute and given a 0.1 ml injection (iv) of detomidine hydrochloride (Domosedan) prior to initiating the insemination procedure. After the injection each doe was inseminated with one 0.5 ml straw of chilled or frozen-thawed semen with a concentration of 100 million sperm/straw. Intra-cervical inseminations were attempted on all does, however, penetration of the cervical rings was not always accomplished. In instances where semen was not deposited in the cervix, it was deposited at the cervical os.

To access the cervix, technicians used a clear plastic Jorgenson goat speculum modified with a fiber optic cable that ran the length of the tube and ended just before reaching the cervix. An LED pen light was attached to the opposite end of the fiber optic cable that transferred the emission of the LED light and illuminated the os cervix of the doe. A cattle AI gun and sheath was used to deposit the semen. From the time the doe entered the chute to the time she was released was less than 10 minutes.

The six does used in the preliminary experiment were treated and handled the same way except they were inseminated using frozen-thawed epididymal sperm collected from a postmortem buck. Sperm was frozen in 0.5 ml plastic straws at a concentration of 50 million sperm/straw and had a post-thaw motility of 60%.

At the time of insemination, frozen semen straws were removed from liquid nitrogen and placed in a 37°C water bath for 30 seconds. Straws were then removed from the water bath and placed into a pre-warmed AI cattle gun and held (<3 minutes) until AI technician was ready to deposit the semen.

Pregnancy Confirmation

Females were checked for pregnancy 35 or 48 days post-insemination in both Experiment 7.1 and Experiment 7.2. The six does from the preliminary AI experiment using frozen-thawed epididymal sperm were also checked 48 days post-insemination. Pregnancies were determined by a transrectal ultrasonography using a bovine rectal probe attached to a plastic extension. The extension was necessary because the exterior genitalia of the females was too small to allow rectal palpation. Does were

classified as pregnant only when a defined fetus was detected by the ultrasound. The final pregnancy rate was determined following birth of the fawns.

Statistical Analysis

The fawning rates was analyzed using Chi square analyses to compare between different estrous synchronization protocols. The fecundity rates between synchronization treatments was analyzed using a Student t-test. Fawning results are reported as a percentage (does inseminated/does that fawned). Fecundity rates are reported as a mean of the number of offspring per doe. A $P \leq 0.05$ was used to determine significance for this study. Sigma stat 3.5 was used to run both the Chi square test and Student t-test

RESULTS

Experiment 7. 1

Fawning Rates

Females were allowed to run with a clean-up buck 14 days after insemination. A gestation length of 195 ± 7 days was used to determine whether offspring was a result of artificial insemination or natural breeding.

Does in Treatment A (n = 18) that conceived from AI had gestation lengths that ranged from 183 days to 211 days, with a mean length of 194.7 days. Fawning rate was 55.6% (10/18). Gestation range for females in Treatment B (n = 17) was 193 days to 199 days, with a mean gestation length of 197.5 days. Fawning rates for Treatment B was 23.5% (4/17) compared with the fawning rates produced by the clean-up buck of 75% (6/8) for Treatment A and 100% (14/14) for Treatment B (Figure 7.1). No significant difference ($P \geq 0.05$) was detected between treatments for either the AI rates or the clean-up breeding rates. There was a significant increase ($P \leq 0.001$) in both Treatment A and Treatment B when comparing AI to clean-up pregnancy rates.

Fecundity Rates

Females in Treatment A and Treatment B had an average of 1.4 fawns and 1.0 fawn per doe, respectively. This was significantly lower ($P \leq 0.024$) than the fecundity rates produced by the clean-up buck of 2.0 and 1.8 fawns per doe for Treatment A and Treatment B, respectively.

Experiment 7.2

Fawning Rates

Females in Treatment A-I (n = 18) had gestation lengths that ranged from 192 days to 200 days, with a mean of 196.2 days. Fawning rate for Treatment A-I was 33.3% (6/18). The gestation length range for does in Treatment B-I was 183 days to 202 days,

with a mean gestation length of 191.6 days. The fawning rate was 55.5% (10/18). The clean-up bucks rates were 91.6% (11/12) for Treatment A-I and 50% (4/8) for Treatment B-I (Figure 7.3). No significant difference was detected between treatments, however, there was a significant increase ($P \leq 0.05$) within both Treatment A-I and Treatment B-I when comparing AI rates to clean-up buck rates.

Fecundity Rates

Females in Treatment A-I and Treatment B-I had a mean of 1.3 and 1.7 fawns per doe, respectively. Females exposed to the clean-up resulted in a fecundity rate of 1.5 fawns per doe for both treatments A-I and B-I. There was no difference in fecundity within or between the treatments.

Epididymal Sperm

Preliminary Experiment

Of the 6 does inseminated, three of them were ultrasounded as pregnant at day 48 post-insemination. One of the three was recorded as carrying twins. At day 190 of gestation, the female thought to have a twin pregnancy gave birth to triplets (two females and one male fawn) (Figure 7.4). At day 201 and 203, the two remaining does gave birth to singletons, one of each sex. The overall pregnancy rate was 50% (3/6), with an overall fecundity rate of 1.67 fawns per doe. The five fawns ranged in weight from 1.9 kg to 4.3 kg. Due to lack of size and strength the smallest of the triplets (a female weighing 1.9 kg) died at 1 day of age.

DISCUSSION

Artificial insemination (AI) allows livestock producers to increase the number of progeny sired by a single male. When used in conjunction with epididymal sperm from a postmortem male, AI becomes a method for preventing the loss of a unique male's genetics. Another technique used for reproductive manipulation is estrous synchronization, which is almost always used in conjunction with AI. For estrous synchronization and AI to be used effectively, synchronization protocols often need to be altered from existing protocols to fit the estrous cycle of the species.

The first recorded use of estrous synchronization with AI in White-tail does was accomplished using two injections of a prostaglandin analogue (cloprostenol) 12 days apart on 2 does (Haigh, 1984). Ejaculated semen from an immobilized buck was collected and used to inseminate the does at 73 and 97 hours after the second



Figure 7.4. Set of triplets (two females and one male fawn) born on day 190 of gestation, after the female was synchronized and artificially inseminated using frozen thawed epididymal sperm from a postmortem White-tail buck.

injection of cloprostenol. The result of the inseminations was two sets of live twins at 192 and 198 days of gestation.

Presently, synchronization of most Cervidae is accomplished with the use of a progesterone implant known as a CIDR (Controlled Intravaginal Drug Release Device). The 7 day CIDR protocol is a common synchronization protocol that is used in cattle. The fawning rates for the does that were synchronized with a 14 day CIDR in our study were higher (55.6%) than does synchronized with the 7 day CIDR (23.5%). However, the 12-to 14-day CIDR protocol is a more common protocol used in most of the Cervidae (Asher et al., 1988, 1990, 1993, 2000; Mulley et al., 1988; Haigh et al., 1991; Jabbour et al., 1993; Monfort et al., 1993; Argo et al., 1994; Willard et al., 1996, 1998). Our fawning rates were in the range reported by the latter authors, of 14% to 80%.

More recent studies have used a 10 day CIDR for use in synchronizing Axis deer (Umapathy et al., 2007) and an 11 day CIDR protocol has been used in Red deer (Bowers et al., 2004). These two studies reported pregnancy rates of 30% (Umapathy et al., 2007) and 49% (Bowers et al., 2004), respectively. A study reported on Fallow deer evaluated the number of females exhibiting estrus and pregnancy rates on does that were exposed to a CIDR for 8, 11, 14, 17 or 20 days (Morrow et al., 1995). The 14 day CIDR treatment resulted in the highest percentage of does that exhibited estrus and resulted in a pregnancy, with rates at 100% and 80%, respectively. The 8-day CIDR treatment had the lowest percent of does exhibiting estrus and pregnancy, with pregnancy rates of 50% and 50%, respectively.

The fecundity rate of females from both the 7 day CIDR and 14 day CIDR was significantly lower than the fecundity rates resulting from the clean-up bucks. This decrease in fecundity rate in these does is thought to be from fewer ovulations from individual females, possibly due to the estrous synchronization protocol. The following season the synchronization protocol used to produced the best pregnancy rates from the prior season (14 day CIDR) was used, except an injection of eCG equine chorionic gonadotropin was included at the time of CIDR removal. The eCG likely increased the number of follicles and ovulation rate, since it is known to act like FSH and LH hormones. With the use of eCG and the 14 day CIDR fecundity rates increased from 1.3 to 1.7 fawns per doe, while the fecundity rate for the clean-up buck was 1.5 fawns per doe.

From this study we have verified that White-tail does can be synchronized for the purpose of timed AI. Based on the results from two breeding seasons, we would

recommend using a 14 day CIDR protocol with an injection of 200 units of eCG rather than a 7 day CIDR treatment or a 14 day CIDR treatment alone.

In the epididymal sperm preliminary experiment, we showed that epididymal sperm from a postmortem White-tail buck could be collected, frozen and used to AI synchronized does, resulting in healthy fawns after a normal length gestation (~200 days). Epididymal sperm of Cervidae have been proven to be viable by producing live healthy offspring (Jacobson et al., 1989; Willard et al., 1996; Zomborszky et al., 1999; Asher et al., 2000). The practical use of epididymal sperm from postmortem males may have important implications in situations where exotic animals are threatened by extinction.

CHAPTER VIII

SUMMARY AND CONCLUSIONS

The goal of the experiments was to develop practical and effective methods for the collection and cryopreservation of epididymal sperm from postmortem bucks for use in assisted reproductive techniques (ART). Although there is scientific literature on reproduction of the family Cervidae, little is directed towards the male and even less information is available on the White-tail bucks. Developing protocols for successful collection and cryopreservation of epididymal sperm from a postmortem buck will reduce the loss of superior genetics due to death or injury. Also, procedures developed in White-tail deer could be beneficial in developing new ART protocols in other similar species that are threatened or are in jeopardy of becoming extinct.

When working with White-tail deer, often, collection of the tissue samples occurs in remote areas away from the laboratory. In the first experiment, the bull was used as a model to determine if there was a significant difference in sperm parameters between epididymal sperm that was harvested and processed at room temperature or in a cool environment after the testicle had been stored at 4°C for 22 hours. Since there was no significant difference between the treatments results showed that after cooling, sperm do not need to be processed in a cool environment.

In the second experiment, White-tail deer testicles were held and cooled for 24 hours either in the scrotum or out of the scrotum. It was shown that White-tail deer sperm could be cryopreserved. If testes are to be held for longer than 24 hours it is recommended that they be held in the scrotum and cooled to 10°C.

In the third experiment, post-thaw White-tail deer sperm parameters were evaluated to determine the effectiveness of either glycerol or DMSO when used as a cryoprotective agent. The results for normal sperm morphology indicated that glycerol was the more effective cryoprotecting agent for freezing White-tail deer epididymal sperm. Also, when evaluating post-thaw parameters, epididymal sperm should be held at least 30 minutes in the 37°C water bath before evaluating sperm motility.

In the fourth experiment, sperm were exposed to glycerol for 0 to 24 hours to determine the toxic effects of glycerol on White-tail deer epididymal sperm. Acceptable post-thaw sperm motility values resulted from White-tail deer epididymal sperm that were stored in the presence of glycerol up to 12 hours. While post-thaw mean membrane integrity remained relatively unchanged out to 24 hours incubation.

The final experiment was to determine which of three timed synchronization protocols produced the best fawning rates in White-tail deer. Three synchronization and artificial insemination protocols were used over two consecutive breeding seasons. At the end of the second fawning season, it was determined that the most effective synchronization protocol was the 14 day CIDR-G protocol, with an eCG injection at the time of CIDR removal. Using this protocol, an additional six does were synchronized and inseminated with frozen-thawed epididymal sperm, which resulted in the birth of five live healthy fawns from three pregnancies.

From this series of experiments it has been shown that epididymal sperm from postmortem bucks can be collected, cryopreserved and successfully used for timed artificial insemination on synchronized does. Although progress was made in our study, further research is needed to determine the most effective protocols for use in White-tail deer.

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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 under the supervision of Dr. Robert A. Godke, Boyd Professor. Jesse is currently a candidate for a Master of Science Degree in the field of animal reproduction.