

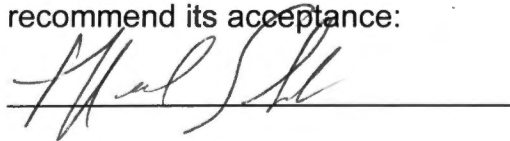
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I am submitting herewith a thesis written by Melody Nakisha Malone entitled "Evaluation of stressors as potential indicators of male fertility ." I have examined the final paper copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.



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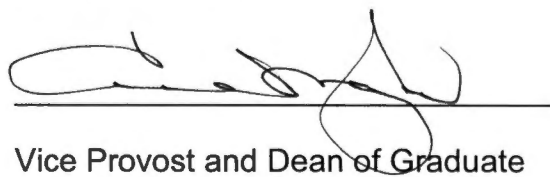


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**EVALUATION OF STRESSORS AS POTENTIAL INDICATORS OF MALE
FERTILITY**

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Melody Nakisha Malone

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DEDICATION

This thesis is dedicated to my extraordinary parents Reverend Sam Malone and Dorothy Malone for teaching me to believe in the impossible, to my grandfather Curtis Foreman who taught me to never lose heart, to my grandmother Dorothy Foreman for lessons on love, patience, and self-respect, to my grandmother Mae Rachel Malone for teaching me to pray, and to my Heavenly Father who completes me.

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ABSTRACT

Overall objective of this thesis was to determine if the ability of individual bulls' sperm within a breed to withstand stress could be correlated with fertility.

Specific objectives included 1) examine variation among individual bulls' sperm within breed to withstand stress of aging for extended time periods; 2) investigate the effects of a moderate (41.0°C) or more severe (45.0°C) elevated temperature on sperm motility over time; 3) examine variation of individual bulls' sperm within a breed to withstand stress of elevated temperature of 43.0°C for 3 h post-thaw; 4) and determine whether ability of individual bulls' sperm within the Holstein breed to withstand elevated temperature is correlated with fertility. For sperm motility assessments, sperm were diluted 1:20 in Sperm-TALP containing 6.0 mg/ml BSA, 1.0 mM sodium pyruvate, 50 U/ml of penicillin, and 50 µg/ml of streptomycin. For in vitro fertilization, sperm were exposed to elevated temperature before fertilization of oocytes. On d 3 and 8 (d 0 = day of fertilization), ability of putative zygotes (PZ) to cleave and develop to blastocyst, respectively, was determined. Results demonstrated variation among individual bulls' sperm within breed to withstand stress of aging or elevated temperature for extended time periods. However, sperm from high fertility bulls resulted in similar embryo development as sperm from bulls of lower fertility. Surprisingly, motility of frozen-thawed bovine sperm within an AI straw can persist for as long as 15 h post-thaw, which is a greater duration than previously proposed.

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NOMENCLATURE

°C	Celsius
d	days
h	hour(s)
min	minutes
ml	milliliter
mm	millimeter
mM	millimolar
P	probability
rpm	revolutions per minute
μl	microliter
U	unit

Abbreviations

NRR	nonreturn rates
AI	artificial insemination
COC	cumulus oocyte complexes
PZ	putative zygotes
IVF	in vitro fertilization
HEPES	N-2-hydroxyethyl piperazine-N'-ethanesulfonic acid
KSOM	potassium simplex optimized medium
OMM	oocyte maturation medium
SEM	standard error of the means
TALP	Tyrodé's Albumin Lactate Pyruvate

CHAPTER I

INTRODUCTION

Although numerous studies have sought to identify key factors that would be predictive of male fertility, no single laboratory test can assess a male's total fertility (Evenson et al., 1999). Accurate prediction of fertility would be of benefit especially in bovine, where variations in conception rates up to 25% are seen in a population of bulls used commercially for artificial insemination despite having previously passed routine exams (Larson and Miller, 2000). To date, nonreturn rates remain the most reliable indicator of a sire's fertility. However, obtaining data from nonreturn rates is time consuming, labor intensive, and expensive. The established procedures and criteria of breeding soundness evaluations provide a quick and economical way to identify those bulls that may be infertile based on a gross examination of sperm, scrotal circumference, and body composition (Hopkins and Spitzer, 1997). However, two of the parameters examined in breeding soundness evaluations, sperm motility and morphology, have not been consistent indicators of fertility (Mayenco-Aguirre and Cortes, 1998). A sperm sample may lose fertility before motility (Guylas, 1968). Specifically, bovine sperm within the oviduct may remain fertile for up to 48 h and motile up to 96 h (as reviewed by Austin, 1975). Despite promising alternatives for predicting male fertility such as sperm chromatin structure assay, (Evenson et al., 1999); sperm ubiquitin tag immunoassay, (Sutovsky et al., 2001a); hypoosmotic swelling test, (Rota et al., 2000); and sperm protein P25b, (Larson

and Miller, 2000) a demand still remains for the development of more reliable testing for prediction of fertility in males. An observation made by Miller and Edwards (unpublished data, 1999) suggested that the ability of sperm to withstand stress may be indicative of fertility. Specifically, changes in sperm motility differed for two bulls diluted with the same extender and within the same breed. Sperm motility for Bull A, a bull of known high fertility, averaged 68% immediately post-thaw and maintained motility 15 h later at 71% in a water bath at 34.4°C. Conversely, sperm motility for Bull B, a bull of unknown fertility, averaged 68% immediately post-thaw and 17% after aging for 15 h. Physiological responses of sperm from individual bulls to environmental or induced stressors in correlation to fertility still remain unclear. This lack of knowledge slows the development of testing that could identify factors indicative of fertility.

The *long-term goals* of this project are to provide information that could be ultimately used in determining an alternative for predicting male fertility. The *overall objective* of the described research is to determine if the ability of individual bulls' sperm within a breed to withstand stress can be correlated with fertility. The *central hypothesis* of the research is sperm from high fertility bulls withstand stress better than sperm obtained from bulls of lower fertility. The basis for our hypothesis is based on preliminary findings made by Miller and Edwards (unpublished data, 1999).

Testing of the central hypothesis and pursuit of the overall objective will be conducted by addressing the following *research objectives*:

1. To examine variation among individual bulls' sperm within breed to withstand stress of aging for extended time periods. Based upon previous work conducted by Miller and Edwards (unpublished data, 1999), the working hypothesis for this objective is that sperm from individual males may vary in ability to withstand stress of aging.

2. To investigate the effects of a moderate (41.0°C) or more severe (45.0°C) elevated temperature on sperm motility over time. The working hypothesis for this aim is sperm motility would be reduced by a moderate elevated temperature (41.0°C) and those reductions in sperm motility would be more pronounced when a more severe elevated temperature (45.0°C) was incorporated.

3. To examine variation of individual bulls' sperm within a breed to withstand stress of elevated temperature of 43.0°C for 3 h post-thaw. The working hypothesis for this objective is that sperm from individual bulls' within breed may vary in their ability to withstand elevated temperature of 43.0°C for 3 h.

4. To determine whether ability of bull sperm within the Holstein breed to withstand elevated temperature is correlated with fertility. The working hypothesis is that sperm from high fertility bulls will better withstand elevated temperature than bulls of lower fertility. The basis for this hypothesis is based on preliminary findings made by Miller and Edwards (unpublished data, 1999), which suggests that the ability of individual bulls' sperm to withstand stress could be indicative of fertility.

Efforts of the proposed research will investigate whether the ability of individual bulls' sperm to withstand stress is indicative of fertility. Stressors of aging and elevated temperature on sperm within an AI straw will be incorporated to test the hypothesis. Experimental endpoints will include sperm motility, sperm morphology, and embryo development as assessed by proportion of cleaved embryos and blastocyst yield. This approach is both simplistic and informative for investigating responses of individual bulls' sperm to induced stressors and their correlation with fertility. The research is expected to yield the following outcomes: 1) characterize variation among individual bulls' sperm to withstand environmental stressors, 2) further characterize effects of elevated temperature post-thaw on bull sperm by means of sperm motility and sperm morphology assessments, 3) determine if ability of sperm from individual bulls to withstand environmental stressors is indicative of fertility status, and 4) evaluate developmental competency of bovine embryos to cleave and develop to blastocysts after fertilization of oocytes with sperm exposed to elevated temperature sperm.

CHAPTER II

REVIEW OF THE LITERATURE

The growing incidence of male infertility has resulted in a concerted effort to develop testing that can both accurately predict fertility status and be applicable for a variety of male species. Today, no single test can completely predict fertility status in males (Evenson et al., 1999). The following is a review of literature, which describes male infertility, current methodologies used for prediction of male fertility, alternative approaches for predicting fertility in males, process of spermatogenesis, process of fertilization and the fertile lifespan of sperm, effects of aging on sperm, and effects of elevated temperature on sperm.

Male Infertility

Roughly 20% of bulls are considered of unsatisfactory fertility according to breeding soundness evaluations (Spitzer et al., 1988; Hopkins and Spitzer, 1997). Reasons for infertility in these animals may be due to disease, poor body confirmation, or disturbances during spermatogenesis (Spitzer and Hopkins, 1997; Larson and Miller, 2000). Another factor that contributes to bull infertility is fertilization failure (i.e. the inability of sperm to fertilize an ovum). Fertilization failure averages approximately 15% in bulls (Hawk, 1983) and contributes to overall reproductive inefficiency, which costs the US Dairy Industry in excess of \$300 million dollars annually (as reviewed by Shannon, 1978; Dalton et al., 2001). Whereas the remaining 80% of bulls are given a rating of satisfactory fertility according to breeding soundness evaluations (Spitzer and Hopkins,

1997), variations ranging from 20-25% in conception rates may be reported (Kidder et al., 1954; Bearden et al., 1956; Linford et al., 1976; Larson and Miller, 2000).

Accurate testing for the prediction of male fertility is imperative given that 15-20% of human couples have fertility problems of which the male factor accounts for 40-50% of these infertility cases worldwide (Glover et al., 1999; Sutovsky et al., 2001b). Environmental factors such as estrogen mimicking compounds (including pesticides and phthalates) and endocrine modulators are implicated in both lowering sperm counts and increasing incidence of testicular cancer (as reviewed by Turner and Sharpe, 1997; Cheek and McLachlan, 1998; Veeramachaneni, 2000; Olivia et al., 2001).

Current Methodologies Used For Prediction of Male Fertility

There are several methods used to evaluate potential fertility in males. Methods include nonreturn rates, breeding soundness evaluations, sperm motility, and sperm morphology.

Nonreturn rates

To date, nonreturn rates (NRR) remain the most reliable and informative indicator of a sire's fertility (Zhang et al., 1999; Larsson and Rodríguez-Martínez, 2000). Nonreturn rates examine the proportion of cows not requiring re-insemination, which is generally determined 28-90 d post-insemination (Salisbury and VenDemark, 1961; Hunter and Greve, 1997; Larson and Miller, 2000; Larsson and Rodríguez-Martínez, 2000). Despite the reliability of NRR as an indicator of fertility in bulls, generating data from several hundred (Kelly and

Hurst, 1962) or thousands of inseminations (Salisbury, 1968; Foote and Kaproth, 1997) required to obtain the most accurate NRR is time consuming, labor intensive, and expensive (Larsson and Rodríguez-Martínez, 2000). Furthermore, NRR considers both conception rates and calving rates; failure of one or both leads to changes in the NRR, ultimately changing fertility ranking of a bull (Koops et al., 1995; Grossman et al., 1995). In an effort to correct for outside influences such as the integrity of inseminates as well as maternal or paternal influences on subsequent embryonic growth that can ultimately alter NRR, investigators have developed models to compute the estimated conception rate for bulls (ERCR; Grossman et al., 1995). Furthermore, extensive testing of the semen samples of commercially used AI bulls is conducted to account for variations that may exist among ejaculates prior to insemination (Otoi et al., 1993; Zhang et al., 1997).

Breeding Soundness Evaluations: Sperm Motility and Morphology

Given the effort and time when assessing fertility of bulls via NRR, evaluation of the semen quality as an indicator of fertility has been investigated. Maddox and co-workers in 1959 are credited with the earliest report of 'fertility testing' based on gross examination of the semen sample (as cited by Spitzer and Hopkins, 1997). From the first documented 'fertility testing', scientists of the Society of Theriogenology over the past 40 years have established procedures and criteria for breeding soundness evaluations (BSE; Hopkins and Spitzer, 1997). Use of BSE provides both a quick and economical means to identify infertile bulls based upon body composition, evaluation of scrotal circumference,

and gross examination of sperm motility and morphology (Hopkins and Spitzer, 1997).

Sperm Motility

Sperm motility assessments within BSE evaluate the proportion of progressively motile sperm moving in a forward direction with average speeds ranging from 6-7mm/min (Salisbury and VanDemark, 1961). Sperm motility assessments are inexpensive, easily done, and requires little time (Hopkins and Spitzer, 1997). However, sperm motility has been an inconsistent indicator of fertility. Some previous studies have demonstrated positive correlations of progressive motility to field fertility of bulls as expressed by NRR (Hirao, 1975; Linford et al., 1976; Correa et al., 1997; Januskauskas et al., 2001a); whereas others have reported no significant correlation (Salisbury et al., 1978; Zhang et al., 1999). In fact, a previous study demonstrated that although no drastic changes in sperm motility were observed after rabbit sperm had been aged under conditions *in vitro* for up to 6 d at room temperature, fertility, as measured by proportion of ova fertilized, had decline markedly (Guylas, 1968). Additionally, subjective measurements of sperm motility carry potential for variations among individual observers (as reviewed by Amann, 1989; as reviewed by Braundmeier and Miller, 2001). More objective assessments can be determined however, using Computer Automated Semen Analyzers (CASA; Larsen et al., 2000; Mortimer, 2000), which uses video framing to evaluate the trajectory of motile sperm.

Sperm Morphology

Morphology of sperm, that is a sperm's composition of a normally shaped head and tail, is another parameter examined in BSE (Hopkins and Spitzer, 1997). Semen samples containing 20-25% abnormal sperm are related to reductions in fertility (Salisbury et al., 1978). However, when used alone, sperm morphology is not always an indicator of fertility, as no single classification system exists to identify all abnormalities in sperm morphology (Braundmeier and Miller, 2001). Typically, abnormalities are grouped generally as either primary or secondary abnormalities (Johnson, 1997). Specifically, primary sperm abnormalities such as pyriform heads and nuclear vacuoles arise during spermatogenesis, whereas secondary abnormalities, including knobbed acrosomes and distal midpiece reflexes originate during spermiogenesis (Barth and Oke, 1989; Johnson, 1997). Sperm abnormalities may also be classified as either *major* (proximal cytoplasmic droplets and pyriform heads) or *minor* (distal cytoplasmic droplet and detached heads), which is dependent upon the abnormalities effect upon fertility in males (Barth and Oke, 1989; Johnson, 1997).

Alternative Approaches for Predicting Fertility in Males

Intensive efforts are currently underway to develop alternative methods for predicting male fertility and are generally classified as either molecular or functional based assays (Braundmeier and Miller, 2001). Molecular based assays consider specific proteins or chromatin structure in the seminal sample as markers of fertility or those that indicate any perturbations that have occurred during spermatogenesis or epididymal transit (Braundmeier and Miller, 2001),

while functional based assays examine the ability of spermatozoa to function normally during steps of fertilization. Molecular based assays currently under investigation include sperm chromatin structure assay, sperm ubiquitin tag immunoassay, and several proteins assays such as spermatozoal β -1,4 galactosyltransferase (Larson and Miller, 2000), and sperm protein P25b (Parent et al., 1999). Examples of functional based assays include hypoosmotic swelling test (Rota et al., 2000), and in vitro fertilization testing, which considers sperm ability to penetrate the zona pellucida (Zhang et al., 1998) or zona-free oocyte (Henault and Killian, 1995).

Sperm Chromatin Structure Assay

The sperm chromatin structure assay (SCSA) evaluates the susceptibility of sperm chromatin to denaturing conditions (Evenson et al., 1999; Bochenek et al., 2001; Januskauskas et al., 2001b). Chromatin from sperm of males of low fertility may be more susceptible to denaturation after sperm are subjected to low pH or heat compared to chromatin of highly fertile bulls and humans (Evenson et al., 1999). Evaluation of chromatin structure is done by staining sperm with acridine orange. Semen samples with sperm that are deemed morphologically normal may contain aberrant chromatin, which can potentially penetrate oocytes (Bochenek et al., 2001; Evenson et al., 2002). Therefore, detection of sperm with damaged chromatin is conducted with flow cytometry versus visually with a microscope.

A previous study demonstrated SCSA was capable of distinguishing a low fertility bull from a bull of high fertility (1.2 to 3.0%; Bochenek et al., 2001). The

bull of low fertility had proportion of disturbed chromatin ranging from 2.1 to 23.8%, whereas the high fertility bull had lower percentage of chromatin abnormalities (1.2 to 3%). The presence of defective chromatin within sperm of the low fertility bull (23%) is consistent with findings by Evenson et al. (1999), where a "threshold value" of 27% of abnormal chromatin was established as negatively affecting fertility in human males. Other recent efforts have used SCSA in bulls, stallions, and men (Love and Kenney, 1998; Evenson, 1999; Evenson et al., 1999; Bochenek et al., 2001; Januskauskas et al., 2001b).

Sperm Ubiquitin Tag Immunoassay

The sperm ubiquitin tag immunoassay (SUTI) evaluates the cross-reactivity of damaged sperm to anti-ubiquitin antibodies (Sutovsky et al., 2001a, b). The basis of this assay is dependent upon the process of ubiquitination, where proteins targeted for destruction are "tagged" by ubiquitin, a small 8.5 kDa 76-amino acid polypeptide (Sutovsky et al., 2001b). Defective sperm proteins are subjected to ubiquitination, which occurs during epididymal transit of sperm (Sutovsky et al., 2001a). So upon ejaculation, defective sperm are tagged with ubiquitin, which is detectable upon analysis with immunofluorescence (Sutovsky et al., 2001a, b). Increased activity of ubiquitination has been associated with low fertility in human males. Furthermore, use of SUTI positively identified the male factor as the reason for infertility in five cases where previously reasons for infertility were unknown (Sutovsky et al., 2001b).

Spermatozoal galactosyltransferase activity

The ability of sperm to bind to the zona pellucida during fertilization may be related to fertility (Larson and Miller, 2000). Previous efforts using mice have demonstrated that content of β -1,4 galactosyltransferase (GalTase) on sperm surface may facilitate binding to zona pellucida, specifically to the ZP3 glycoprotein (Miller et al., 1992; Larson and Miller, 2000) in mice sperm. This enzyme is also present in bull sperm, but may not be a marker of fertility (Larson and Miller, 2000), as GalTase activity in sperm from 24 bulls did not correlate to NRR (Larson and Miller, 2000).

Use of epididymal proteins in assay for prediction of male fertility

Proteins of the epididymis participate in the maturation of sperm, which ultimately yield their fertilizing potential (Parent et al., 1999). In hamster sperm, levels of an epididymal protein, sperm protein 26 (P26h) have been correlated with fertility (Robitaille et al., 1991). Levels of P26h in hamster sperm have been associated with sperm binding to the zona pellucida (Robitaille et al., 1991; Parent et al., 1999). Moreover, levels of sperm protein 22 (sp22) within caudal epididymal sperm in rats were shown to be indicative of fertility (Welch et al., 1998). More recent investigations suggest that levels of P25b in bull sperm are correlated with fertility. Bulls of high fertility, as previously determined by nonreturn rates, showed higher levels of P25b as determined by Western blot analysis in comparison to lower fertility bulls (Parent et al., 1999).

Hypoosmotic swelling test

The hypoosmotic swelling test (HOS) of sperm was originally designed to evaluate biochemical activity of the sperm membrane (Correa et al., 1997; Rota et al., 2000). Normal sperm respond to hypoosmotic conditions by expansion and coiling of the tail (Correa et al., 1997; Rota et al., 2000). This assay may be used to distinguish between semen samples of low versus high fertility. Use of this assay for predicting fertility has been evaluated in the bulls, rams, stallions, boars, and dogs (Rota et al., 2000). When HOS is incorporated into routine semen analysis with other parameters such as sperm motility and morphology, correlations for proportion of swollen sperm, in addition to proportion of motile and morphologically normal sperm are correlated with *in vitro* fertility in bulls (Correa et al., 1997). However, Rota et al. (2000) reported no correlation of proportion of swollen sperm for five Piedmontese bulls to fertility *in vitro* after using HOS. Although the procedure is simple to use and is inexpensive, preliminary efforts suggest that using HOS alone cannot accurately differentiate intermediate fertility semen samples from high or low fertility bull sperm samples (Rota et al., 2000).

In vitro fertilization testing: zona binding and oocyte penetration tests

In vitro fertilization testing is yet another alternative that has been investigated. Testing has evaluated the ability of sperm to bind to the zona pellucida as well as ability of sperm to penetrate oocytes.

Zona-binding test

Ability of sperm to bind to zona pellucida of the oocyte has been positively correlated with fertility in livestock species, particularly bulls (Zhang et al., 1997; Zhang et al., 1998). In bulls, the ability of sperm to bind to zona pellucidae was evaluated in 22 Swedish Red and White, used commercially for AI to determine if there was a correlation with 56-day NRR (Zhang et al., 1998). Investigators demonstrated that in combination with other parameters such as sperm motility, absolute zona-binding assays are correlated with NRR in bulls. In a comparable study, Fazeli et al. (1997) reported ability of bull sperm to bind to zona pellucida using the hemizona assay was related to field fertility.

Oocyte Binding test

Ability of sperm to bind to zona-free oocytes may be correlated to fertility. Henault and Killian (1995) evaluated effects of sperm preparation through heparin-capacitation on ability of bull sperm to penetrate zona-free oocytes. Results from that study demonstrated that sperm treated with heparin penetrated oocytes more readily than sperm incubated without heparin. Also, in a separate experiment evaluating two bulls of either low or high *in vivo* fertility, sperm from a bull of high fertility penetrated more oocytes versus low fertility bull as assessed by fluorescein isothiocyanate (FITC; Henault and Killian, 1995). Currently, conflicting data have been reported in determining if ability of sperm to bind to zona-free oocytes is correlated with fertility (as reviewed by Braundmeier and Miller, 2001) since gametes analyzed are from different animals (Henault and Killian, 1995).

Despite current efforts, no single test can accurately predict fertility in males. Development of a test is dependent upon a better understanding of the molecular and functional aspects of sperm. Therefore, the following section is a brief overview of spermatogenesis.

Spermatogenesis

Spermatogenesis is a process involving the formation of functional haploid spermatozoa from diploid spermatogonia, which occurs within the testes. Since extensive reviews of spermatogenesis by several authors exist (Johnson, 1995; Kerr, 1995; and Johnson et al., 1997); this topic will be described in brief. Spermatogenesis occurs within the seminiferous tubules and is comprised of three distinct phases: spermatocytogenesis, meiosis, and spermiogenesis.

Spermatocytogenesis, the initial phase of spermatogenesis, involves mitotic divisions of spermatogonia in the maintenance of a continuous pool of germ cells that eventually give rise to additional stem cells or further differentiate to give rise to spermatozoa (as reviewed by Johnson, 1995). Spermatogonia are classified as Type A, Type I (intermediate), and Type B, and named according to the length of the chromosome within each type. Spermatogonia are connected by intracellular bridges, which allows for either synchronous development or degeneration of a stem cell line. Prior to the meiotic phase of spermatogenesis, spermatocytes undergo both DNA replication and protein synthesis (reviewed by Erickson et al., 1981).

Meiosis, as reviewed by Johnson (1995), is the second phase of spermatogenesis that involves the formation of four haploid cells after reduction

of a diploid cell. Genetic information is not only duplicated, but also exchanged as primary spermatocytes enter the first prophase, which is the longest period of meiosis. Both RNA and DNA synthesis occur in spermatocytes as well as gene transcription (as reviewed by Kerr, 1995). Secondary spermatocytes undergo a brief interphase, but no major DNA synthesis. Through duplication and two subsequent divisions spherical haploid spermatids are prepared for morphological alteration into elongated spermatozoa (as reviewed by Johnson, 1995).

The final phase of spermatogenesis, spermiogenesis, involves the morphogenetic changes of spermatids to functional spermatozoa (as reviewed by Johnson, 1995). Spherical spermatids, composed of round nuclei, are elongated and nuclei material is condensed. There is minimal DNA synthesis, yet the activity of disulfide bonds increases after elongation of spermatozoa. Additional changes involve the Golgi apparatus that gives rise to the acrosome, an enclosed enzymatic cap that is positioned at the apex of the sperm head. Enzymes, including acrosin, hyaluronidase, and zona lysin are implicated in assisting sperm to make entry pass the glycoprotein barrier of the ovum. Sertoli cells are responsible for removal of excess cytoplasmic material as spherical spermatids evolve into an elongated spermatozoa after enfolding of the plasma membrane has produced the flagellar canal.

Still linked by intracellular bridges of incomplete cytokinesis, the spermatids are nurtured by Sertoli cells. The spermatogenetic cycle of the bull is composed

of 21 d mitosis, 23 d meiosis, and 17 d spermiogenesis. This 61-day cycle overlaps itself with a new cycle beginning approximately every 13.5 d.

Fertilization

Fertilization may be defined as the process of fusion of an oocyte with a spermatozoon. The resulting conceptus, after undergoing cell divisions and differentiation, may result as a live offspring at the end of gestation. Extensive reviews of fertilization have been conducted (Ball et al., 1984; Sutovsky et al., 1996; Van Soom and de Kruif, 1996; Sutovsky and Schatten, 1998; Wassarman 1999 a, b, and c; Töpfer-Petersen, 1999; Töpfer-Petersen et al., 2000; Scott, 2000). The following is a brief overview of the process of fertilization and the fertile lifespan of sperm when diluted and stored at ambient temperatures, while stored in the frozen state, and after thaw.

Prior to sperm and oocyte binding within the oviduct, sperm undergo several modifications. In the female reproductive tract of cows, oviductal cells secrete factors that promote motility of sperm, which functions under the control of hormones (Lapointe et al., 1995). Using bovine oviductal cell monolayer, results demonstrated that motility promoting factors are released via estrogen and progesterone in the isthmus and ampulla regions of the oviduct. Sperm initially adhere to mucosal epithelial of the caudal isthmus in the cow to acquire the capability to fertilize (as reviewed by Van Soom and Kruif, 1996; as reviewed by Töpfer-Petersen, 1999). Once released from oviductal epithelial, capacitated sperm proceed toward the oocyte exhibiting hyperactive motion until they meet at the junction of the ampulla and isthmus (as reviewed by Hawk, 1987; as

reviewed by Töpfer-Petersen, 1999). Sperm then bind to the zona pellucida, a glycoprotein matrix composed of three functionally distinct proteins: zona pellucida 1 (ZP1), zona pellucida 2 (ZP2), and zona pellucida 3 (ZP3; as reviewed by Dean, 1992; Brewis and Wong, 1999; as reviewed by Wassarman, 1999 a, c; as reviewed by Töpfer-Petersen et al., 2000). While, ZP1 and ZP2 have primarily a structural role, ZP3 serves as a receptor for sperm in initial binding (as reviewed by Dean, 1992; Brewis and Wong, 1999). Binding of the sperm with the zona pellucida induces the acrosome reaction releasing enzymatic proteins including acrosin (Brewis and Wong, 1999). In bulls, acrosin is highly correlated with the ability of sperm to penetrate the oocyte (De los Reyes and Barros, 2000); however, the presence of acrosin may not be necessary in mice (Wassarman, 1999 a, b). After entry into the perivitelline space, acrosome-reacted sperm bind to the oolemma (Wassarman, 1999 a; as reviewed by Scott, 2000). Fusion of the sperm with the oocyte causes release of cortical granules, which induces the zona block preventing the incidence of polyspermy (reviewed by Burks and Saling, 1992; reviewed by Dean, 1992; Sutovsky and Schatten, 1998). Within the ooplasm, second meiotic division is completed following release of the second polar body (Ball et al., 1984). Decondensation of the sperm chromatin leading to formation of the male pronucleus is followed by syngamy of both pronuclei yielding a genetically distinguishable zygote (Ball et al., 1984; Sutovsky et al., 1996; Sutovsky and Schatten, 1998; Tanghe et al., 2002). At this point, the 1-cell zygote may begin development to term after necessary differentiation and cell divisions.

The process of in vitro fertilization (IVF) differs from that of in vivo fertilization as sperm that have been confirmed as motile after purification process are deposited directly into a pool of oocytes (Ball et al., 1984; Trounson, 1992). Parrish et al. (1988) reported a greater yield of oocytes fertilized after incubation with sperm at 39.0°C (53%) than when incubated at either 35.0°C or 37.0°C (10%). In vitro fertilization is further facilitated by glycosaminoglycans (GAG) including heparin, which has been implicated in inducing the acrosome reaction in sperm and increasing the number of oocytes fertilized versus sperm not incubated with heparin (Parrish et al., 1985; Parrish et al., 1988). Additionally, energy substrates including pyruvate, glucose, lactate, NaHCO_3 , Ca^{2+} , Low K^+ , and Na^+ are commonly used in fertilization media to mimic the electrolyte composition of oviductal fluid (Visconti et al., 2002). Although specified temperatures and incorporation of GAG and energy substrates have enhanced the process of in vitro fertilization, thousands of sperm are still required to incubate with oocytes to promote likelihood of fertilization (as reviewed by Van Soom and DeKruif, 1996).

Fertile lifespan of diluted sperm stored at ambient temperatures

Storage of sperm in the liquid state requires that constituents of the diluting media are capable of maintaining fertile lifespan of sperm (as reviewed by Vishwanath and Shannon, 2000). To ensure that the lifespan of sperm stored in the liquid state had a minimum shelf life between 2-4 d, metabolic activity had to be minimized, particularly if semen required shipping great distances (as reviewed by Vishwanath and Shannon, 2000). Earlier investigations

demonstrated that sperm maintained at 5.0°C reduced metabolic activity, but not without increases in the influx of sodium (Na^+), which lead to irreversible sperm damage (Makler et al., 1981). Later investigations demonstrated that ambient temperatures generally ranging from 18.0-24.0°C with egg yolk as a preservative were deemed optimal for artificial insemination with fresh semen versus 5.0°C (as reviewed by Vishwanath and Shannon, 2000).

Furthermore, the type of diluent further influences the fertile lifespan of fresh semen. For example, use of CAPROGEN as a diluent maintained fertility for up to 5 d and slowed reduction of sperm motility considerably over a 4-week period (Vishwanath and Shannon, 1997). In another study, Salamon et al. (1979) demonstrated that although diluted spermatozoa stored up to 10 d at an ambient temperature could penetrate oocytes, lambing rates were reduced with the same diluted sperm aged for only 3 d.

Fertile lifespan of frozen sperm

Whether sperm stored in the frozen state (-196.0°C in liquid nitrogen or at -76.0°C under dry ice) can maintain fertilizing capabilities indefinitely is highly argumentative (as reviewed by Salisbury and Hart, 1970; as reviewed by Vishwanath and Shannon, 2000). Leibo et al. (1994) demonstrated that sperm frozen for 37 years at -78.0°C exhibited normal post-thaw motility and was successful in subsequent embryo development. However, others have suggested that the lifespan of sperm while frozen is much shorter, ranging from 4 to 6 years (Lee et al., 1977; Lundgren, 1980). Moreover, previous studies reported an initial improvement in fertility after bovine sperm has been stored

frozen (as reviewed by Salisbury and Hart, 1970; Lee et al., 1977; Lundgren, 1980). Furthermore, effect of season of collecting sperm is implicated in long-term storage of bovine sperm (as reviewed by Salisbury and Hart, 1970; Lundgren, 1980).

Following nearly 50,000 inseminations, minute declines in fertility for five bulls were reported after long-term storage of bovine sperm for 4 to 6 years at -196.0°C (Lundgren, 1980). Lee et al. (1977) had previously reported no drastic decrease in fertility, determined by NRR, when bovine sperm was stored in the frozen state at -196.0°C for 4 to 5 years. Ironically, there was an initial improvement in fertility between 4 to 12 months of storage (as reviewed by Salisbury and Hart, 1970; Lundgren, 1980). This theory proposed by several investigators suggest that upon ejaculation, both normal sperm and sperm with any abnormalities compete equally during fertilization (as reviewed by Salisbury and Hart, 1975). However upon storage of sperm in the frozen state, sperm with disturbances in membrane structure or having aberrant chromatin, are less likely upon thaw to compete with normal sperm; therefore, ova are more likely to be fertilized with normal sperm resulting in improved fertility (as reviewed by Salisbury and Hart, 1970; as reviewed by Salisbury and Hart, 1975; Lundgren, 1980). An effect of season, based upon the time semen was initially collected, has been implicated on affecting the long-term storage of bovine semen (Salisbury, 1967, 1968). When evaluating semen after being frozen at -196.0°C for 6 months, semen collected during warm months (May to October) decreased

in fertility more readily than for semen collected during cooler months (Salisbury, 1967, 1968).

Cryopreservation

It has been documented as early as the 18th century by Spallanzani that spermatozoa could be immobilized when exposed to a freezing temperature and made motile again when warmed (Herman, 1981). Yet, a larger market for cryopreserved bull sperm for artificial insemination did not occur until Polge et al. (1949) incorporated glycerol as a protective agent. Of the 200 million doses of semen used for artificial insemination, approximately 95% are made with cryopreserved semen (as reviewed by Vishwanath and Shannon, 2000). Superior genetic bulls can provide in excess of 60,000 doses of semen annually ultimately increasing genetic merit (as reviewed by Curry, 2000). Benefits of cryopreservation included increasing genetics within herds and subsequent decrease in the generation interval (as reviewed by Vishwanath and Shannon, 2000). Also, conception rates with cryopreserved sperm when artificially inseminated have been comparable with natural mating. Furthermore, a bull is more likely to yield more progeny through AI than by natural mating. However, successes seen in bull sperm have not been reproducible for other domestic livestock, including boars, rams, and stallions (Polge et al., 1970; as reviewed by Holt, 2000).

Despite the use of a variety of cryoprotectants, nearly 40-50% of sperm are irreversibly damaged due to compromises in the sperm membrane integrity (as reviewed by Salamon and Maxwell, 1995; as reviewed by Curry, 2000; as

reviewed by Vishwanath and Shannon, 2000; as reviewed by Watson, 2000). To account for the loss of sperm due to cryopreservation, up to 10X frozen-thawed sperm may be inseminated in comparison to inseminations with fresh sperm. For example, values ranging between 10-25 million sperm/ml of frozen-thawed sperm are inseminated versus 1 million sperm/ml fresh sperm (Shannon, 1978; as reviewed by Holt, 2000; as reviewed by Watson 2000). Losses are attributed to reactive oxygen species, which compromises the integrity of sperm (as reviewed by Holt, 2000; as reviewed by Watson, 2000; Chatterjee and Gagnon, 2001). Cryoinjury including ice crystallization, osmotic stress, changes in pH affect sperm, and a "pre-capacitation" state of sperm are also implicated in shortened lifespan of frozen sperm versus fresh sperm (as reviewed by Holt, 2000; as reviewed by Watson, 2000).

Fertile lifespan of sperm post-thaw

Generally, it is recommended that upon thaw sperm be inseminated within 15 min to avoid significant drops in post-thaw temperature, which may compromise fertility (Barth and Bowman, 1988). However, an earlier study reported no changes in fertility of bulls, as determined by NRR, after sperm were thawed and maintained on ice at 1.0°C up to 4 h post-thaw before insemination (NRR with sperm aged 4 h: 69% after 338 inseminations vs. NRR with sperm immediately post-thaw: 68% after 471 inseminations, respectively; Kelly and Hurst, 1962). However when sperm were maintained at 1.0°C for 6 h post-thaw versus inseminations made immediately post-thaw from the same study, a slight decline in fertility was reported in six Holstein-Friesian bulls (NRR with sperm aged 6 h:

60% after 309 inseminations vs. NRR with sperm immediately post-thaw: 68% after 471 inseminations, respectively).

It has been recommended to thaw sperm at temperatures of at least 35.0°C to 37.0°C for a minimum of 30 seconds versus thawing using the pocket method (approximately 9 min to thaw) or temperatures of 5.0 and 20.0°C (Barth and Bowman, 1988). Kaproth et al. (2002) reported no significant changes in post-thaw motility at 5 or 20 min, when thawed at 35.0°C (48.5 vs. 48.7%, respectively). DeJarnette et al. (2000) also demonstrated that initial thawing of bovine semen in straws from each of five Holstein bull using an AI gun or at 5.0 or 20.0°C before placement in a 37.0°C water bath were not comparable to control (sperm thawed and maintained at 37.0°C for 3 h) in maintaining viability of sperm, as determined by proportion of motile sperm. Specifically, sperm motility after thawing straws of semen at various temperatures were 76, 80, 75, and 77% for the 37.0°C water bath, 20.0°C water bath, 5.0°C water bath, and within an AI gun, respectively (DeJarnette et al., 2000). DeJarnette et al. (2000) further proposed that bulls might differ in their ability to withstand thermal insult post-thaw after one bull of the five Holstein bulls evaluated was "resistant" to post-thaw thermal insults of thawing within an AI gun, or at 5.0 or 20.0°C.

Frozen-thawed sperm generally has a shorter lifespan than diluted sperm (as reviewed by Hawk, 1983; Shannon and Vishwanath, 1995; as reviewed by Watson, 2000). Specifically, mean survival of frozen-thawed sperm rediluted in CAPROGEN was less than freshly collected sperm stored at ambient temperatures ranging from 18.0 to 24.0°C, as determined by at least 10% of

sperm being motile (75.8 vs. 124 h survival, respectively; Shannon and Vishwanath, 1995).

Effects of aging on sperm, sperm functions, and effects of aged sperm on subsequent embryo development

Ongoing research efforts are investigating the detrimental effects of aging sperm *in vivo* within the epididymis, *in utero* (i.e. within the female reproductive tract), or under an environment *in vitro*, which ultimately leads to impairment of sperm function. Additional research has further focused on the effects of aging sperm on embryo development (as reviewed by Tarín et al., 2000).

Sperm aged in vivo within the male tract

Mature sperm are stored within the caudal epididymis where fertility can persist for a finite period (Young, 1929a; Shaver and Martin-DeLeon, 1975). The effects of aging sperm within the male tract after surgical bilateral ligation of the corpus epididymis has been documented in several species including the guinea pig with fertility up to 35 d (Young, 1929a), but motility for 55 d upon collection and evaluation; and in the rabbit with fertility persisting for 38 (Chang and Pincus, 1964), 40 (Hammond and Asdell, 1926), and 49 d (Tesh and Glover, 1969). Tesh and Glover (1969) also reported that rabbit sperm maintained motility up to 60 d.

Aging sperm in a ligated epididymis increased incidence of anomalies in fetuses, embryonic death, chromosomal abnormalities, and reduced litter sizes (Shaver and Martin-DeLeon, 1975). Tesh and Glover (1969) reported anomalies such as fetuses lacking gall bladders and having deformed skull sutures. Also,

embryonic death increased from 3.8 to 20% when sperm of guinea pig was retained within the ligated epididymis beyond 20 d and upon removal inseminated into females (Young, 1929a). Moreover, chromosomal abnormalities, notably triploidy, have been documented when aged sperm derived from ligated epididymides of ICR Swiss rabbits were used for fertilization (Martin-DeLeon and Boice, 1982). From previous research efforts, it has been suggested that both fertility and subsequent embryo development are severely impaired when evaluating the effects of aging sperm within the male tract (Salisbury and Hart, 1970).

Sperm aged within the female tract

The effects of aging sperm *in utero* involves aging of sperm within the female tract after delaying the onset of ovulation, which then allows sperm to age before fertilization of the ovum (as reviewed by Salisbury and Hart, 1970; Blandau, 1969). This form of aging and its effects on fertile lifespan and subsequent impairment on embryo development, which varies among species, has been documented in bulls (Laing, 1945; Vandeplassche and Paredis, 1948), more intensively in rabbits (Hammond and Asdell, 1926; Chang and Pincus, 1964; Tesh, 1969; Martin and Shaver, 1972), and bats (Racey, 1973). Generally, sperm aged *in utero* lose ability to fertilize before motility (Blandau, 1969) as previously documented in bulls with fertility maintained for 24 to 48 h (Laing, 1945; Blandau, 1969), yet motile for 96 h (as reviewed by Austin, 1975); in rats with fertility for 14 d, but motility persisting as long as 17 d (Blandau, 1969); and

in bat sperm, which retained both fertility and motility following 198 d of storage within the oviduct throughout hibernation (Racey, 1973).

Sperm aged *in utero* resulted in smaller litter sizes, increased incidence of chromosomal abnormalities, and impaired pre-implantation of embryos (as reviewed by Salisbury and Hart, 1970). Through insemination of rabbit sperm into females before injection of LH to induce ovulation, sperm were 'aged' within the oviduct from 10 to 32 h before ovulation occurred. As a result, smaller litter sizes were reported when rabbit sperm were aged in this manner at 22 h post-insemination and no litters produced by 30 h of aging sperm (Hammond and Asdell, 1926). A comparable study reported decreases in litter sizes from 7.2 to 2.2% when rabbit sperm were aged beyond 29 h (Tesh, 1969). It has been suggested that decreased in litter sizes are influenced mainly by higher incidence of chromosomal anomalies, which leads to early embryonic death (Martin and Shaver, 1972). Chromosomal abnormalities up to 9.7% in 6-day old rabbit blastocysts were noted compared to 1% in controls, which was reportedly due to improper cytokinesis in addition to formation of blastocysts that were mixploidy (Martin and Shaver, 1972). As sperm age increased, both impairment of pre-implantation and post-implantation increased in rabbit embryos (Tesh, 1969).

Effects of aging on fertile lifespan of sperm post-thaw

Aging sperm *in vitro* results in compromised motility and fertility, as measured by NRR or proportion live offspring and has been documented in cattle (Salisbury and Flerchinger, 1967; Salisbury, 1968; Todorovic et al., 1969; Krzyzosiak et al., 2000); rabbits (Guylas, 1968); mice (Benitz and Graves, 1972; Smith and Lodge,

1987; Sato et al., 2001); and pigs (First et al., 1963). Todorovic et al. (1969) demonstrated that proportion of motile sperm declined following storage of diluted sperm for 21 d at 5.0°C (70 to 30%). In a comparable study, proportion of motile diluted sperm decreased from 80% at d 0 (day of collection and dilution) to 15% on d 8 after bovine sperm were incubated under conditions *in vitro* at ambient temperature (18.0 to 20.0°C; Krzyzosiak et al., 2000). Salisbury (1968) evaluated the effects of aging diluted sperm *in vitro* when held at 4.0°C for up to 5 d. Following an initial rise in fertility when comparing 2-day aged bovine sperm to sperm aged for one day, as determined by NRR, fertility then decreased steadily as embryonic mortality increased (Salisbury, 1968). A study conducted by Guylas (1968) demonstrated that motility of diluted rabbit sperm was not affected when stored at room temperatures for up to 6 days. However, ability of sperm to fertilize oocytes decreased steadily (95, 51, and 8%) at day 0, 3, and 6, respectively. These findings further assert that motility may not be indicative of fertility. In mice, sperm derived from vasa deferentia aged *in vitro* had a significant effect on pronuclear formation in mice (Smith and Lodge, 1987); however, a small proportion of sperm aged *in vitro* for 3 d at 22.0°C were capable of fertilizing ova (Sato et al., 2001). In a comparable study examining the effects of rabbit sperm aged *in vitro*, proportion of fertilized ova had decreased markedly when diluted sperm aged 24 h *in vitro* at room temperature versus 4 h storage (46 vs. 95%, respectively; Cooper and Orgebin-Crist, 1977). Similar findings were reported for undiluted sperm aged in the same fashion.

Sperm aged *in vitro* result in higher incidence of chromosomal abnormalities, early embryonic death, and small litter sizes due to alterations incurred by the sperm membrane and nuclear material (Mann and Lutwak-Mann, 1975; as reviewed by Tarín et al., 2000). Diluted boar sperm aged for 54 h and maintained at 6.0 to 8.0°C resulted in lowered fertilization rate, reduced accessory sperm/ovum, and resulted in fewer 25-day embryos when compared to boar sperm aged for 6 h and maintained at 6.0 to 8.0°C before insemination of sows (First et al., 1963). An explanation for the increased embryonic losses may be due to improper incorporation of the male pronuclei due to damaged DNA (Martin-DeLeon and Boice, 1982). Although aging of sperm under conditions *in vitro* may result in perturbations of sperm membrane, it remains unclear if aging results in loss of DNA content (Anand et al., 1967; Anand and First, 1968; Salisbury and Hart, 1970, 1975). Earlier studies suggested aging of bull or boar sperm *in vitro* resulted in losses of DNA, which ultimately compromises development of embryos (First et al., 1963; Segina and Norman, 1964; Anand et al., 1967; and Tesh and Glover, 1969). Losses of DNA up to 61% were reported when bovine sperm diluted in egg-yolk was aged 21 d (Segina and Norman, 1964). Mean losses of DNA of 15.4% ($P < 0.01$) were reported in aged boar spermatozoa (Anand et al., 1967). At 0 h, mean DNA content for each sperm cell was 3.6×10^{-9} mg but decreased to 3.1×10^{-9} mg 24 h later. However, follow-up studies suggested that losses were from dead sperm only and aging does not lead to loss of DNA content (Anand and First, 1968; Salisbury and Hart, 1970). Aging of sperm *in vitro* induces intracellular changes including an

increase in oxidative damage by free radicals such as superoxide anion and peroxide, increase in proportion of sperm spontaneously undergoing acrosome reaction, and release of metabolic by products (as reviewed by Vishwanath and Shannon, 1997). Another adverse effect of aging sperm *in vitro* in mice sperm resulted in the release of metabolic by-products leading to increases in pH and increasing the proportion of immobilized and dead sperm (Sato et al., 2001). Such was the case when sperm were aged *in vitro* for 3 d at 22.0°C. With each successive day of aging, pH increased beyond 7.5, which is implicated in sperm death; by day 3 of aging all sperm were dead (Sato et al., 2001).

Effects of elevated temperature on sperm and male reproductive functions

Effects of elevated temperature are being investigated on cellular and functional aspects of sperm after thermal insult to the testes or while maintained in an *in vitro* environment. Additional research efforts are investigating the effects of elevated temperature on frozen-thawed sperm and on development of resultant embryos.

Effects of elevated temperature on male testis and sperm function

Application of elevated temperature on the testes of bulls through scrotal insulation reduced proportion of motile sperm, increased incidence of sperm abnormalities, and increased susceptibility of chromatin to denature (Vogler et al., 1991; Vogler et al., 1993; Karabinus et al., 1997). After a mild thermal insult (mean temperature=34.8°C) administered for 48 h in which the scrotum of bulls (n=6) were insulated, reductions in sperm motility up to 20% were most

pronounced 15 d later (Vogler et al., 1991). Vogler et al. (1993) conducted a similar study to examine incidence of sperm abnormalities after scrotal insulation in bulls using differential interference contrast microscopy. Incidence of sperm abnormalities increased 12 d post-insult and peaked at d 18 with more than 80% abnormal sperm from the ejaculate. Recovery of normal sperm in the ejaculate did not begin until 39 d after scrotal insulation (Vogler et al., 1993). Moreover, when Karabinus et al. (1997) used sperm chromatin structure assay (SCSA) to evaluate the stability of chromatin in frozen-thawed sperm, structural changes in bull sperm chromatin peaked 12 d after scrotal insulation for 48 h. Taken together, impairment of sperm function of reduced sperm motility, increased proportion of abnormal sperm, and increased susceptibility of sperm to denature were all reported beginning at d 12 post-insult (Vogler et al., 1991; Vogler et al., 1993; Karabinus et al., 1997).

Impaired sperm function including reduced proportion of motile sperm, disturbances in spermatogenesis, alteration of sperm chromatin, and reduction in testes weight, after exposure of testes to elevated temperature through insulation or local application of heat has been documented in other species including rams (Mieusset et al., 1992), stallions (Love and Kenney, 1999), guinea pigs (Young, 1929b), mice (Jannes et al., 1998; Setchell et al., 1998; Rockett et al., 2001), and rat (Bowler, 1972). In contrast, other studies have shown resistance of sperm and sperm nuclei when briefly exposed to elevated temperatures by normal pronuclear formation and subsequent embryo development (Yanagida et al., 1991; Hoshi et al., 1992; Cozzi et al., 2001). In rams, scrotal insulation for up to

21 d (16 h/day) resulted in marked decrease in sperm motility (79.1% 49 d before insult to 17.0% post-insult) and total number of sperm (3.6×10^9 , 49 d before insult to 2.6×10^9 post-insult; Mieusset et al., 1992). After scrotal insulation in pony stallions (n=4) for 48 h, analysis using SCSA showed spermatocytes were most sensitive to denaturation in sperm chromatin (Love and Kenney, 1999). Incidence of altered sperm chromatin peaked at d 24 after sperm were collected via ejaculation and analyzed using SCSA, which corresponded spermatocyte cell stage of spermatogenesis in horses (Love and Kenney, 1999). In guinea pigs, nearly 100% of epididymal sperm were non-motile after exposure of the excised epididymides to 47.0°C for 30 min (Young, 1929b). In mice, local heating of the testes resulted in perturbations in spermatogenesis, with spermatocytes reported to be most sensitive to elevated temperature (Rockett et al., 2001). In response, expression of hsp70-1 and hsp70-3 increased and was dominant in spermatocytes during the local application of heat (43.0°C for 20 min) to testes in adult male mice (Rockett et al., 2001). Expression of heat shock proteins in response to thermal insult has also been evaluated in embryos (Edwards and Hansen, 1996, 1997; Edwards et al., 1997). Furthermore, when testes of C57BL/6 mice were submerged in 43.0°C water baths for 20 min, apoptosis was evident within 8 h post-shock (Rockett et al., 2001) with increased presence of vacuoles of tubules and pyknotic nuclei of germ cells after TUNEL analysis. Additionally, testes weights were lowest 15 d after submersion of testis in a 43.0°C water bath and were not comparable to relative testis weights in the controls 68 d later (Rockett et al., 2001). In a comparable study, relative testis

weights of mice after local thermal insult to the testis for 20 min at 42.0°C were significantly lower than controls when examined 28 d post-insult (323 vs. 384.7 mg/100 g, respectively; Jannes et al., 1998). Also in rats, Bowler (1972) recorded heat-shocked testis weights 40% of the controls up to 24 weeks post-stress after rat testes were exposed to 43.5°C for 20 min 7 times at 6-week intervals.

Oocytes fertilized with sperm exposed to elevated temperature result in compromised embryo development (rams: Mieusset et al., 1992; rabbits: Burfening and Ulberg, 1968; guinea pigs: Young, 1929b; and mice: Jannes et al., 1998; Rockett et al., 2001). Conception rates after ewes were inseminated with sperm originating from testes subjected to thermal insulation were not changed (Mieusset et al., 1992). However, marked embryonic losses were reported in rams as early as d 4 post-insult when frozen-thawed ram sperm previously retrieved after scrotal insulation for 16 h over 21 d were used to artificially inseminate ewes (Mieusset et al., 1992). After excised epididymides of male guinea pigs were placed in a water bath up to 30 min at 45.0 and 46.0°C, retrieved sperm used for insemination of females resulted in lower pregnancy rates (Young, 1929b). However, pregnancy rates were not affected when oocytes were fertilized with epididymal sperm retrieved from epididymides previously exposed to elevated temperatures of 40.0, 42.0, and 44.0°C. In mice, single exposure of adult male mice testes to elevated temperature of 32.0°C for 24 h reduced rate of fertilization and decreased embryonic survival rates (Burfening et al., 1970). Similar findings were reported with high pre-implantation

losses and early embryonic death in rabbits and mice after insemination with sperm exposed to elevated temperature (Howarth et al., 1965; Burfening and Ulberg, 1968; Burfening et al., 1970). In contrast, previous studies have demonstrated thermostability of mammalian sperm nuclei and sperm after exposure to harsh elevated temperatures with normal pronuclear development and viable offspring (Yanagida et al., 1991). Ability to withstand acute elevated temperature was demonstrated when sperm nuclei derived from hamster, mouse, humans, and other vertebrates (rooster and fish) were isolated and then subjected to elevated temperatures of 60.0 to 125.0°C for 20-120 min prior microinjection into hamster oocytes (Yanagida et al., 1991). After microinjection of sperm nuclei into hamster oocytes, oocytes were incubated for 5 h at 37.0°C before evaluation for presence of pronuclei formation (Yanagida et al., 1991). Approximately 70% of sperm nuclei were capable of decondensing and forming normal pronuclei after exposure to 90.0°C for 30 min. Moreover, it has been documented that microinjection of oocytes with sperm previously subjected to severe elevated temperature resulted in live offspring due to thermostability of male genome (Cozzi et al., 2001). After selecting motile mice epididymal sperm using swim-up, sperm were heated to 60.0°C for 30 min in a water bath prior to microinjection into oocytes. Resultant embryos were then transferred until assessment of embryo development. Although both sperm motility and sperm membrane integrity were compromised for most sperm (98%), there were two resulting live offspring, which were reported to produce young of their own after reaching puberty (Cozzi et al., 2001).

Effects of elevated temperature on frozen-thawed spermatozoa and resultant embryos

More recent efforts have investigated the effects of elevated temperature on function of frozen-thawed sperm in addition to effects on oocytes fertilized with sperm previously exposed to elevated temperature (Monterroso et al., 1995; Chandolia et al., 1999). Incubation of sperm post-thaw at elevated temperature for up to 3 h at 41.0°C (Monterroso et al., 1995) or 4 h at 41.0°C and 42.0°C (Chandolia et al., 1999) reduced motility in bovine sperm upon evaluation with a Hamilton Thorn Motility Analyzer. Chandolia et al. (1995) also reported no breed by temperature interaction for reductions in sperm motility of Brahman-derived bulls and European-derived bulls (Angus and Holstein), although reduction in sperm motility for Holstein was more gradual compared to other breeds (Chandolia et al., 1999). Also, viability was reduced at 42.0°C and 43.0°C in frozen-thawed bull sperm when incubated for up to 3 h post-thaw after evaluation with Trypan blue exclusion (Monterroso et al., 1995). Additionally, sperm velocity was reduced at 41.0°C for 3 h, while the proportion of sperm undergoing swim-up was reduced at 43.0°C. In contrast, neither DNA nor acrosome membrane integrity was affected after sperm were exposed to elevated temperatures of 39.0, 41.0, or 42.0°C under 5% CO₂ for up to 3 h upon evaluation using acridine orange stain (Monterroso et al., 1995). However, there was a gradual increase in proportion of sperm with altered acrosomes. Supplementation of glutathione in Sperm-TALP at concentrations of 0, 1 µM, or 1 mM was beneficial at 39.0°C in sperm undergoing swim-up, but not at 43.0°C. The investigators further reported

no significant effect of incubating sperm at 39.0, 41.0, or 42.0°C for 3 h on ability of oocytes to cleave or develop to 4-cell stage.

Rationale and Significance

Rationale: The proposed research will serve as a preliminary step in establishing if variation of sperm from individual bulls to withstand environmental stressors may be used as an indicator of fertility.

Significance of the proposed research: Although numerous studies have sought key factors that would be predictive of male fertility, no single laboratory test can assess a male's total fertility (Evenson et al., 1999). Accurate prediction of male fertility would be of benefit especially in bovine, where variations in conception rates up to 25% are seen in a population of bulls used commercially for artificial insemination despite having previously passed a breeding soundness evaluation (BSE Larson and Miller, 2000). To date, NRR remain the most reliable indicator of a sire's fertility, yet obtaining data from NRR is time consuming, labor intensive, and expensive. The established procedures and criteria of BSE provide a quick and economical way to identify those bulls which may be infertile based on a gross examination of sperm, scrotal circumference, and body composition (Hopkins and Spitzer, 1997). Unfortunately, two of the parameters examined in breeding soundness evaluations, sperm motility and morphology, have not been consistent indicators of fertility (Mayenco-Aguirre and Cortes, 1998). Despite promising alternatives for predicting male infertility (sperm chromatin structure assay, Evenson et al., 1999; sperm ubiquitin tag immunoassay, Sutovsky et al., 2001a,b; hypoosmotic swelling test, Rota et al.,

2000; and sperm protein P25h, Larson and Miller, 2000), a demand remains for the development of a more reliable test to predict fertility in males. *The proposed research is significant because information gained could ultimately lead to the development of a stress test for predicting fertility in males.* Development of a test predictive of fertility in males may characterize infertile males within livestock species and in humans where the male factor accounts for up to 40% of all infertility cases (Costabile and Spevak, 2001).

Summary of Research Objectives

Taken together, we hypothesize that sperm from highly fertile bulls may withstand stress better than sperm obtained from bulls of lower fertility. The overall objective of the current research is to determine if the ability of individual bulls' sperm within a breed to withstand stress can be correlated with fertility. As a preliminary step toward obtaining our overall goal, objectives outlined in this thesis describe efforts to examine variation among individual bulls' sperm within breed to withstand effects of aging within an AI straw post-thaw, examine effects of elevated temperature on motility of sperm after incubation within an AI straw, characterize variation among individual bulls' sperm post-thaw to withstand effects of elevated temperature, and determine if ability of sperm from individual bulls to withstand elevated temperature after thaw is indicative of fertility status.

CHAPTER III

MATERIALS AND METHODS

Materials

All reagents and chemicals used for in vitro production of bovine embryos were purchased from Sigma (St. Louis, MO) unless otherwise noted. Eosin-nigrosin stain used for assessing morphology of sperm was generously provided by Dr. Fred Hopkins (Department of Large Animal Clinical Sciences, College of Veterinary Medicine; The University of Tennessee). Dr. F. Neal Schrick (Department of Animal Science; The University of Tennessee) generously provided frozen semen and use of Cito Thaw baths originally purchased from American Breeders Service (DeForest, WI). Frozen semen from high and low fertility bulls were generously provided by Dr. J. Mel DeJarnette (Select Sires, Inc. Plain City, OH). Bovine ovaries used for the collection of cumulus oocyte complexes (COC) were purchased from Brown's Packing Company (Gaffney, SC). Medium-199 (M-199), gentamicin, penicillin-streptomycin, and non-essential amino acids (NEAA) were purchased from Specialty Media, Inc. (Phillipsburg, NJ). Fetal bovine serum (FBS) was obtained from BioWhittaker (Walkersville, MD). Folltropin®-V was provided by Dr. F. Neal Schrick (Department of Animal Science; The University of Tennessee) and luteinizing hormone (LH) was provided by USDA (Beltsville, MD). "NARCO" 220A water bath was purchased from National Appliance Company (Portland, Oregon). Light immersion oil, glass microscope slides, stage warmer, and lens cleaner were

purchased from Fisher (Pittsburgh, PA). Modified Tyrode's media (HEPES-TALP, IVF-TALP, and Sperm-TALP; Parrish et al., 1988) and KSOM (Biggers et al., 2000 with modifications provided by Dr. John Hasler) were prepared in the laboratory or purchased from Specialty Media, Inc. (Phillipsburg, NJ).

Assessment of Sperm Motility

Procedures as described by Sorensen (1975) along with a modified protocol provided by Dr. Fred Hopkins (Department of Large Animal Clinical Sciences, College of Veterinary Medicine; The University of Tennessee) were used to conduct sperm motility assessments. Immediately post-thaw, 5 μ l of sperm obtained from a 0.5 ml straw was placed into 95 μ l of Sperm-TALP pre-warmed to 38.5°C (1:20 dilution). Sperm-TALP was prepared according to Parrish and co-workers (1988) and supplemented with 6-mg/ml bovine serum albumin (BSA), 1.0 mM sodium pyruvate, and 50 U/ml of penicillin and 50 μ g/ml of streptomycin. The 1:20 dilution of sperm was gently mixed and maintained at 34.4°C until assessment. The number of progressively motile sperm (10 random sperm live/dead for each field) was assessed in five different microscopic fields in a 20- μ l droplet placed on a pre-warmed petri dish. A separate, 20- μ l droplet of diluted sperm was also evaluated to yield 10 total microscopic fields. Assessments of sperm motility were made at 400 magnification using an inverted light microscope. The mean value (in multiples of ten) of one or two evaluator(s)' assessment was calculated and recorded. See Appendix A for specific procedural details.

Assessment of Sperm Morphology

Procedures for assessment of sperm morphology as described by Barth and Oko (1989) were used to conduct assessment of sperm morphology along with a modified protocol provided by Dr. Fred Hopkins (Department of Large Animal Clinical Sciences, College of Veterinary Medicine; The University of Tennessee). Immediately post-thaw, 25 µl of sperm was placed on a glass slide pre-warmed to 38.5°C adjacent to 4-5 mm drop of pre-warmed eosin-nigrosin stain. The eosin-nigrosin stain and sperm were mixed using the blunt end of an additional slide. A smear of sperm/stain mixture was made using a second slide. The smear was allowed to dry on a slide warmer for 3 min at 38.5°C. Using approximately 2-3 mm drop of immersion oil, 100 sperm cells were evaluated at 1000 magnification. Refer to Appendix B for specific procedural details.

In Vitro Production of Bovine Embryos

Modifications of procedures for in vitro production of bovine embryos described by Edwards and Hansen (1996) were used. Briefly, COC were harvested from follicles ranging 2-8 mm in diameter. After evaluation, only COC having an evenly darkened and granulated ooplasm and compact cumulus were cultured in M-199 containing Earle's salts, 10% FBS, 50.0 µg/ml gentamicin, 5.0 µg/ml FSH, 0.3 µg/ml LH, 0.2 mM Na Pyruvate, and 2.0 mM L-glutamine (OMM) for 22-24 h under maximum humidity, 5.5% CO₂ at 38.5°C. For in vitro fertilization, OMM was aspirated from COC housed in four-well dishes and replaced with 500 µl of IVF-TALP fertilization medium containing penicillamine, hypotaurine, and epinephrine (PHE).

Straws of frozen sperm were thawed and layered on top of a discontinuous Percoll gradient (2 ml 45% Percoll on top of 2 ml 90% Percoll). Sperm were centrifuged at 2200 rpm for 15-20 min. After Percoll centrifugation, sperm were washed in Sperm-TALP and centrifuged at 1100 rpm for 8 to 10 min. The sperm pellet was resuspended with approximately 500 μ l of IVF-TALP. Oocytes were fertilized with 200,000 motile sperm/0.5 ml and then incubated in 5.5% CO₂ at 38.5°C in a humid environment. Approximately 18 h after addition of sperm to COC, putative zygotes (PZ) were denuded of sperm and cumulus by vortex mixing for 4 min and then washed in HEPES-TALP. Putative zygotes were cultured in KSOM at 5.5% CO₂, 7.0% O₂, and 87.5% N₂ at 38.5°C for up to 8 days post-fertilization. On d 3 and 8 (day 0 = day of fertilization), ability of PZ to cleave and develop to blastocyst, respectively, was determined.

Objective 1: Motility of frozen-thawed bovine sperm after aging for extended time periods

The aim of objective 1 was to examine variation among individual bulls' sperm within a breed to withstand stress of aging for extended time periods. Sperm motility was evaluated after aging frozen-thawed sperm within an AI straw for up to 15 h in nine different bulls representing Charolais (n=3; 49CH9845, 137CH221, and 54CH530), Holstein (n=3; 11HO3348, 11HO4245, and 11HO4385), and Senepol (n=3; 140SE5, 140SE14, and 140SE56) breeds. Seven straws of frozen sperm from a given bull were thawed and incubated in a Cito Thaw water bath at 34.4°C. Sperm motility was assessed at 0, 5, 7, 9, 11, 13, and 15 h post-thaw. Two evaluators independently assessed proportion of

progressively motile sperm. The mean value (in multiples of ten) of evaluator(s)' score was calculated and recorded. The experiment was replicated three times. Statistical models were fit to include effects of individual bulls and time as a regression variable.

Objective 2: Motility of frozen-thawed bovine sperm following exposure to a moderate (41.0°C) or severe (45.0°C) elevated temperature

Aging sperm for 15 h is not only detrimental, but also time consuming and inconvenient to incorporate into a potential fertility test. Therefore, we sought an alternative stressor that was shorter in duration, but sufficient to maximize potential differences in individual bulls' sperm. Our selection of elevated temperature as the alternative stressor was influenced by previous work that examined effects of elevated temperature on function of frozen-thawed bull sperm (Monterroso et al., 1995; Chandolia et al., 1999). For this objective, we utilized a preliminary approach to investigate the effects of a moderate (41.0°C) or more severe (45.0°C) elevated temperature on sperm motility after incubation within an AI straw over time.

Elevated temperature of 41.0°C

Sperm motility was evaluated after subjecting frozen-thawed sperm to a moderate elevated temperature of 41.0°C for up to 6 h within an AI straw in four different bulls representing Holstein (n=3; 11HO4245, 11HO4385, and 11HO3348), and Senepol breeds (n=1; 140SE36). Specifically, straws of frozen sperm (n=8 for each bull) from each bull were thawed by placing in a Cito Thaw

water bath at 34.4°C for 45 seconds. Four straws of sperm from each bull were maintained in the 34.4°C Cito Thaw water bath, while the other four straws were transferred to a 41.0°C water bath (0 h post-thaw motility assessments for bull sperm at 41.0°C treatment group was conducted 5 min after transfer). To maintain sperm at 41.0°C, 400 or 600 ml beakers were filled with water and encased in aluminum foil before placement inside of the "NARCO" 220A water bath for at least 2 h before sperm were thawed. Periodic temperature readings were taken to ensure the required temperature had been reached. Sperm motility was assessed at 0, 2, 4, and 6 h post-thaw using a single straw of sperm from each bull at each time point. Experiment was replicated two times. Data were analyzed by least-squares analysis using mixed procedures of SAS (version 8.2, 2001). Sources of variation in the model included effects of time (0, 2, 4, and 6 h post-thaw), temperature (34.4°C or 41.0°C), and interaction of time*temperature.

Elevated temperature of 45.0°C

Sperm motility was evaluated after subjecting frozen-thawed sperm to an elevated temperature of 45.0°C for up to 8 h within an AI straw in four different bulls representing Holstein (n=3; 11HO4245, 11HO4385, and 11HO3348), and Senepol (n=1; 140SE36) breeds. Specifically, straws of frozen sperm from each bull (n=10) were thawed by placing in a Cito Thaw water bath at 34.4°C for 45 seconds. Five straws of sperm from each bull were maintained in the 34.4°C Cito Thaw water bath, while the other five straws were transferred to a 45.0°C water bath (0 h post-thaw motility assessments for bull sperm at 45.0°C

treatment group was conducted 5 min after transfer). To maintain sperm at 45.0°C, 400 or 600 ml beakers were filled with water and encased in aluminum foil before placement inside of the "NARCO" 220A water bath at least 2 h before sperm were thawed. Periodic temperature readings were taken to ensure the required temperature had been reached. Sperm motility was assessed at 0, 2, 4, 6, and 8 h post-thaw using a single straw of sperm from each bull at each time point. Experiment was replicated two times. Data were arranged in a randomized block design and analyzed using mixed procedures of SAS (version 8.2, 2001). Sources of variation included in the model considered the effects of time (0, 2, 4, 6 and 8 h post-thaw), temperature (34.4°C or 45.0°C), and interaction of time*temperature.

Objective 3: Effects of elevated temperature (43.0°C) on frozen-thawed bovine sperm for 3 h post-thaw

Given that exposure of sperm to 45.0°C decreased sperm motility within 2 h, we hypothesized that elevated temperature of 43.0°C on sperm would be sufficient to establish possible variation among individual bulls' sperm in their ability to withstand this stressor of elevated temperature. The aim of objective 3 was to examine variation of individual bulls' sperm within a breed to withstand a stressor of 43.0°C for 3 h post-thaw. Sperm obtained from bulls representing Charolais (n=2; 36CH9257 and 137CH295), Holstein (n=2; 11HO4245 and 11HO4385), Senepol (n=4; 158SE3, 158SE5, 158SE17, and 158SE55), and Simmental (n=2; 41SM1893 and 30SM002) breeds were evaluated. Sperm motility was assessed after maintaining sperm within an AI straw at 34.4°C or

43.0°C for 0, 1, 2, or 3 h post-thaw. On a given day, straws of frozen sperm (n=8 for each bull) from two bulls within the same breed were thawed by placing in a Cito Thaw at 34.4°C for 45 seconds. Four straws from each bull were maintained at 34.4°C in a water bath, while the four remaining were transferred to a 43.0°C water bath (0 h post-thaw motility assessments for bull sperm at 43.0°C treatment group was conducted 5 min after transfer). To maintain sperm at 43.0°C, 400 or 600 ml beakers were filled with water and encased in aluminum foil before placement inside of the "NARCO" 220A water bath. Periodic temperature readings were taken to ensure the required temperature had been reached. Motility assessments were made at 0, 1, 2 and 3 h post-thaw. Data were arranged in a randomized block design with 2*4 factorial treatment arrangements (SAS: version 8.2, 2001). The experiment was replicated three times for each bull. Statistical models included effects of time (0, 1, 2, or 3 h post-thaw), temperature (34.4°C or 43.0°C), bull-id, time*temp, time*bull-id, temp*bull-id, and time*temperature*bull-id.

Objective 4: Differences in individual Holstein bull sperm to withstand elevated temperature as influenced by fertility status

The aim of objective 4 was to evaluate whether ability individual bulls' sperm within the Holstein breed to withstand elevated temperature is correlated with fertility status. The basis for this hypothesis was derived from preliminary observations made by Miller and Edwards (unpublished, 1999). Frozen-thawed sperm were obtained from bulls of high or low fertility and maintained within an AI straw at 34.4 or 43.0°C for 3 h (treatment arrangements: Table 1).

Table 1: Treatment arrangements (2X2 factorial)

Fertility Status	Temperature (°C)	
	34.4	43.0
High (n=1)	H-34.4	H-43.0
Low (n=1)	L-34.4	L-43.0
Lab Control (n=2; pooled)	Thawed 0 h post-thaw at 34.4°C	

Sperm motility and sperm morphology were assessed at 3 h post-thaw.

Additional effects of fertility and elevated temperature on sperm contained within an AI straw were evaluated by examining embryo development after fertilization of oocytes.

Effects of temperature and time were examined on motility and morphology of sperm over 10 replicates. As a control, sperm motility and morphology from two bulls (140SE5 and 140SE55) were assessed along with high and low fertility bull over 10 replicates. Additionally, a lab control was included in this experiment to assess quality of in vitro system for producing embryos using bulls (n=2 pooled; 140SE5 and 140SE55) previously shown to yield high proportion embryos that develop to blastocyst. Replicates for which development of oocytes into blastocyst was greater than 20% were included in analysis (n=5). Data were arranged in a randomized block design with 2X2 factorial arrangement of treatments and analyzed using mixed models of SAS (version 8.2, 2001). The Shapiro-Wilk test was used to check for normality of data; data were transformed when normality values were less than 0.90.

Frozen semen from bulls of high or low fertility (n=6; 20 straws of semen/bull) were utilized to test our hypothesis. Data used to determine fertility status of

those bulls were generated from NRR for which a relative breeding efficiency was determined by technicians at Select Sires (personal communication with Dr. Mel DeJarnette). Using the relative breeding efficiency scale with 5.0=high fertility and 3.5=average fertility, "Hunter" (7HO3847), "Image" (7HO4146), and "Roadmark" (7HO4364) were considered to be of high fertility status each having a score of 4.0. However, "Nike" (7HO4687) and "Bryan" (7HO4610) were rated average fertility with a score each of 3.0. "High" (7HO4888) was rated as a low fertility bull with a score of 2.0. However, immediately prior to starting this project (approximately one year after semen had been provided by Dr. DeJarnette), the fertility status of the bulls had changed based upon estimated relative conception rates (ERCR; personal communication with Dr. DeJarnette). Using ERCR scores (score of 0=average fertility bull when compared to herd mates), the following bulls could only be defined as high fertility and low fertility respectively: "Nike": 7HO4687 having a +2 on ERCR and "High": 7HO4888 having a -4 on ERCR. The same medium was used to extend semen for the high fertility bull versus bull of low fertility with the exception of one constituent that Dr. DeJarnette would not reveal (personal communication with Dr. DeJarnette).

CHAPTER IV

RESULTS

Motility of frozen-thawed bovine sperm after aging

In this experiment, sperm motility was evaluated after aging frozen-thawed sperm for up to 15 h at 34.4°C within an AI straw in nine different bulls representing Charolais, Holstein, and Senepol breeds (n=3 bulls/breed). Regressing over time, the rate of decline in motility of aged sperm was very similar for three Senepol bulls examined (Figure 1; SEM=0.4; $P>0.7$; slope = -1.7, -1.3 and -1.2% per hour). In contrast, large amounts of variation among individual bulls were noted in Holsteins (Figure 2; SEM=0.4; $P<0.0001$; slope = -4.2 and -3.0% vs. -0.6% per hour) and Charolais (Figure 3; SEM=0.4; $P<0.02$; slope = -3.8% vs. -2.6 and -2.1% per hour).

Motility of frozen-thawed bovine sperm after exposure to a moderate (41.0°C) or more severe (45.0°C) elevated temperature

The aim of this objective was to investigate the effects of a moderate (41.0°C) or more severe (45.0°C) elevated temperature on sperm motility after incubation of sperm within an AI straw over time. Sperm motility decreased throughout experimental time period (Figures 4 and 5; $P<0.0001$). However, incubation of sperm within an AI straw at 41.0°C did not reduce sperm motility (Figure 4; pooled SEM=5.9; $P=0.9693$). In contrast, incubation of sperm within an AI straw at 45.0°C greatly reduced sperm motility as early as 2 h post-thaw (Figure 5; pooled SEM=5.2; $P=0.0027$).

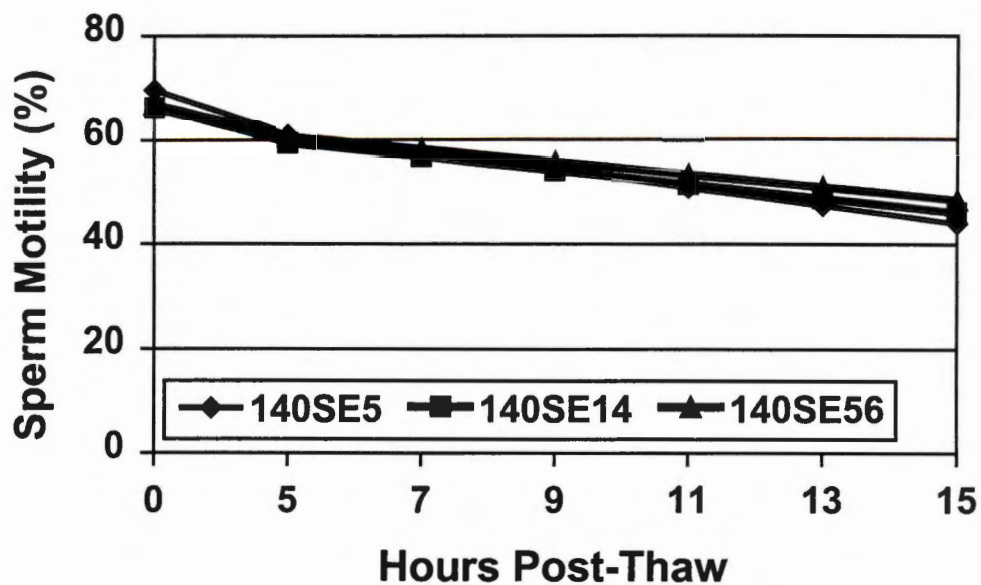


Figure 1: Sperm motility of Senepol bulls up to 15 h post-thaw within an AI straw (SEM=0.4; $P>0.7$; slope = -1.7, -1.3 and -1.2% per hour).

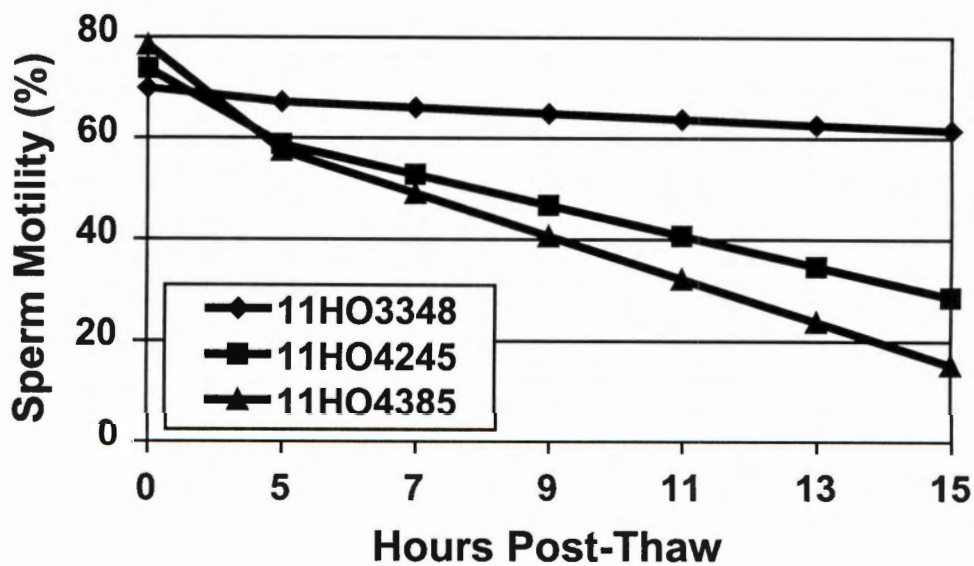


Figure 2: Sperm motility of Holstein bulls up to 15 h post-thaw within an AI straw (SEM=0.4; $P<0.0001$; slope = -4.2 and -3.0% vs. -0.6% per hour).

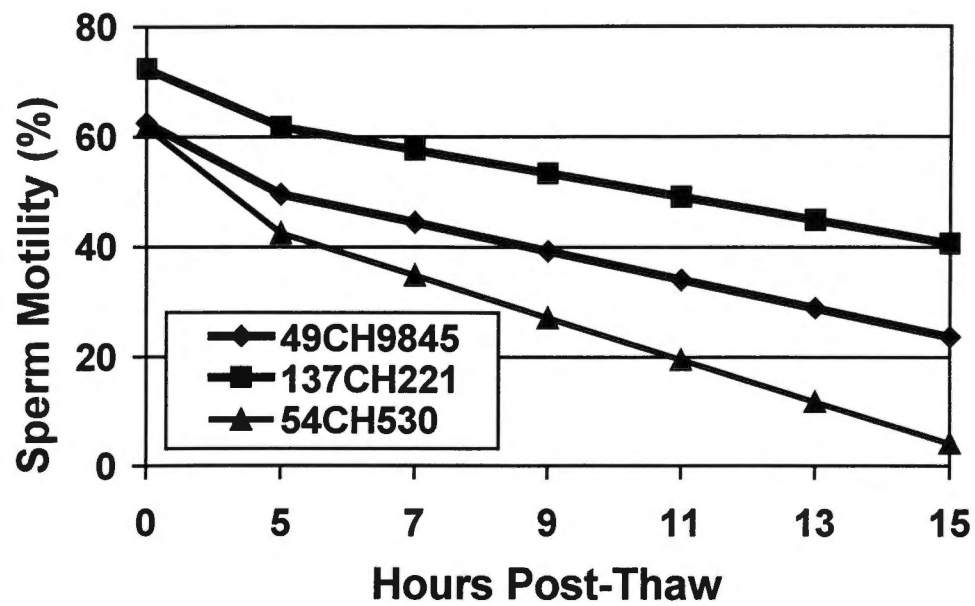


Figure 3: Sperm motility of Charolais bulls up to 15 h post-thaw within an AI straw (SEM=0.4; $P<0.02$; -3.8% vs. -2.6 and -2.1% per hour).

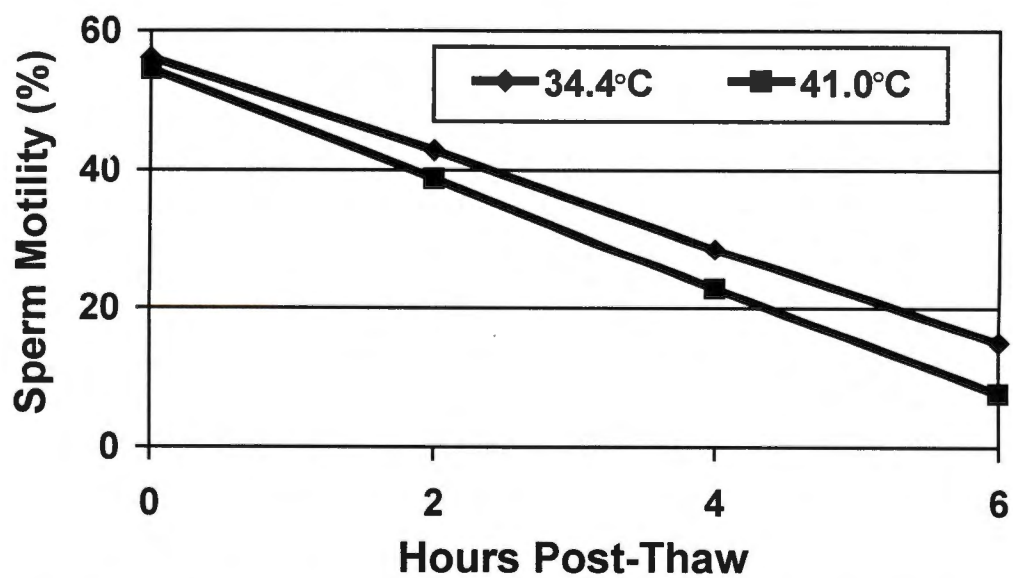


Figure 4: Motility of bovine sperm after incubation at 34.4°C or 41.0°C for 0, 2, 4, and 6 h within an AI straw (temp*time: pooled SEM=5.9; $P=0.9693$).

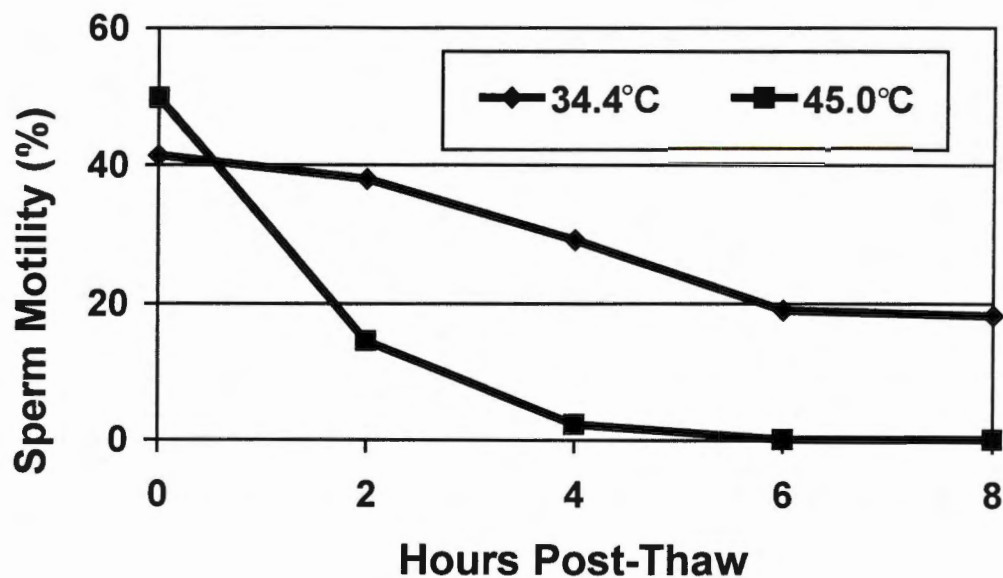


Figure 5: Motility of bovine sperm after incubation at 34.4°C or 45.0°C for 0, 2, 4, 6, and 8 h within an AI straw (temp*time: pooled SEM=5.2; P=0.0027).

Effects of elevated temperature on frozen-thawed bovine sperm for 3 h post-thaw

Variation of individual bull sperm within breed (Charolais, n=2; Holstein, n=2; Senepol, n=4; and Simmental, n=2) to withstand a stressor of 43.0°C for 3 h post-thaw within an AI straw was examined in this experiment. Sperm motility decreased throughout experimental time period (Figure 6; SEM=1.4; P<0.0001). Moreover, incubating sperm within an AI straw at 43.0°C for 3 h decreased proportion of motile sperm over time (Figure 7; time*temp; SEM=1.8; P=0.0220). Variation among individual bulls within Holstein and Simmental breeds to withstand stress of 43.0°C after incubation within an AI straw was noted (Figures 8 and 9). In contrast, no variations were found among individual Charolais and Senepol bulls in their ability to withstand elevated temperature of 43.0°C for 3 h

post-thaw (Figures 10 and 11). Variation among individual bull sperm of Holstein and Senepol bulls to withstand aging within an AI straw was noted (Figures 12 and 13).

Differences in individual Holstein bull sperm to withstand elevated temperature as influenced by fertility status

The aim of objective 4 was to evaluate whether ability of sperm from a highly fertile bull versus a low fertility bull within the Holstein breed to withstand elevated temperature for 3 h post-thaw is correlated with fertility status. Sperm motility was greater for the high fertility bull versus the low fertility bull when sperm were incubated within an AI straw (Table 2). Incubating sperm at an elevated temperature of 43.0°C reduced proportion motile sperm (Table 3). Similar findings were noted after Percoll purification (Tables 2 and 3). There was no temperature*time interaction.

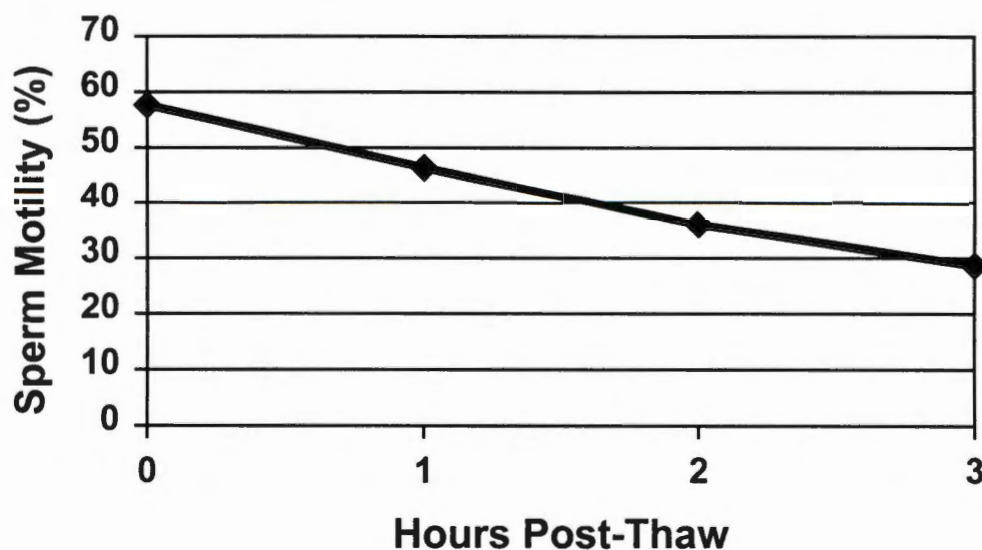


Figure 6: Motility of bovine sperm after incubation for 0, 1, 2, and 3 h in an AI straw (SEM=1.4; $P<0.0001$).

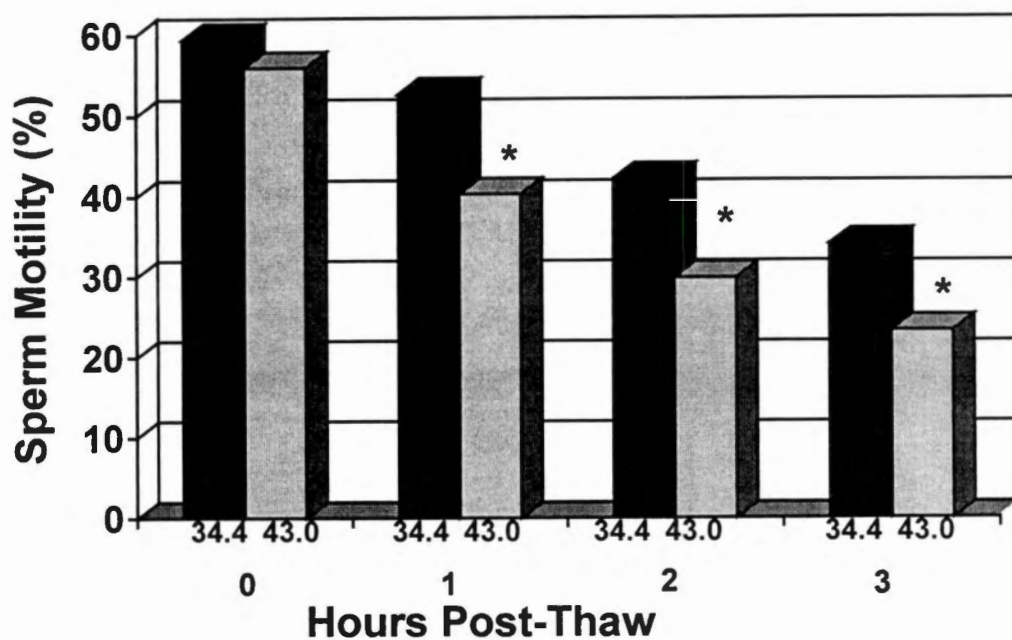


Figure 7: Effects of elevated temperature of 43.0°C on sperm motility up to 3 h post-thaw (time*temp; SEM=1.8; P=0.0220).

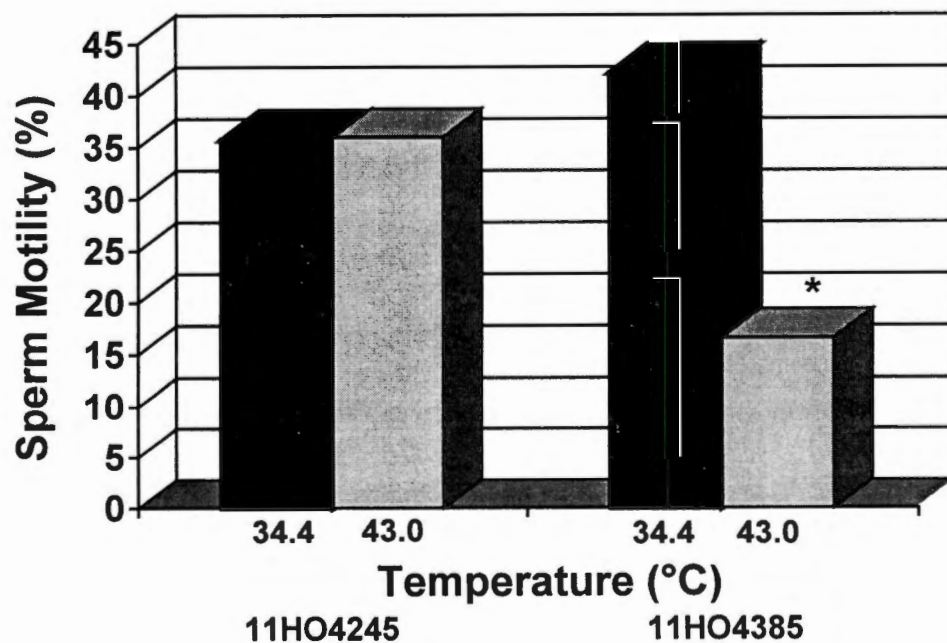


Figure 8: Variations in sperm motility of individual Holstein bulls to withstand elevated temperature of 43.0°C (temp*bull-id; SEM=2.3; P<0.0001).

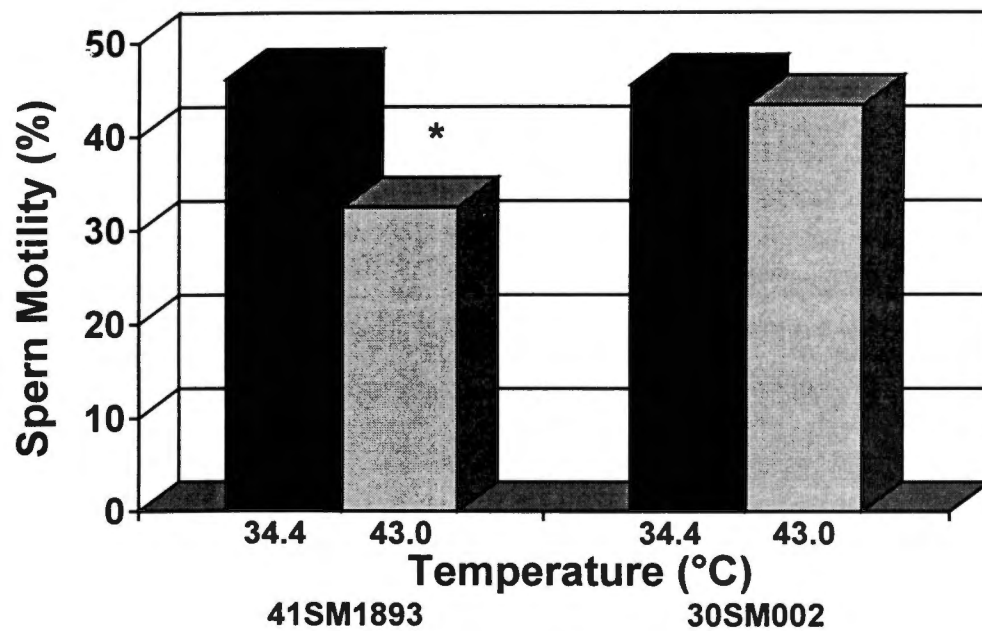


Figure 9: Variations in sperm motility of individual Simmental bulls to withstand elevated temperature of 43.0°C within an AI straw (temp*bull-id; SEM=2.8; P=0.05).

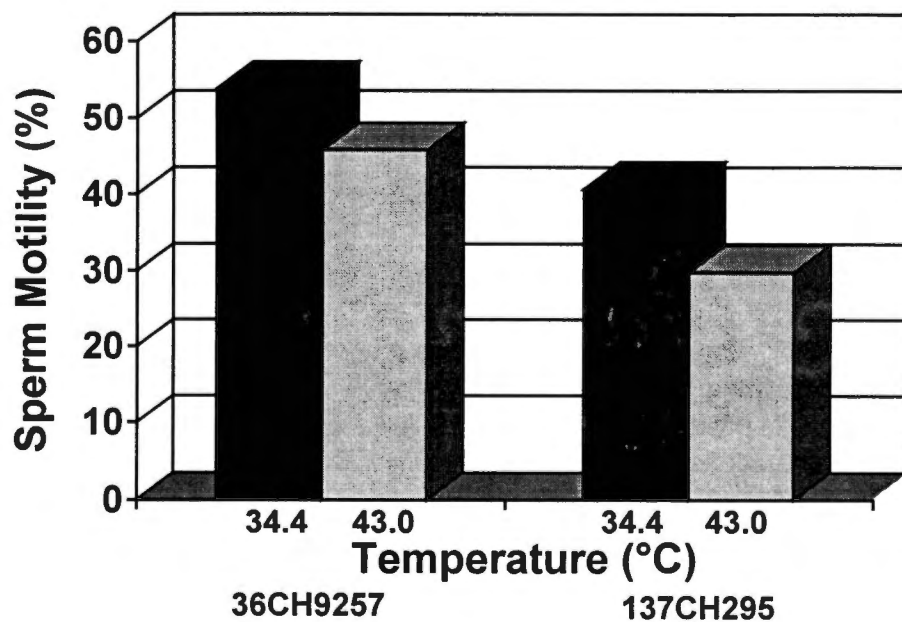


Figure 10: Sperm motility of individual Charolais bulls to withstand elevated temperature of 43.0°C within an AI straw (temp*bull-id; SEM=2.5; P=0.4).

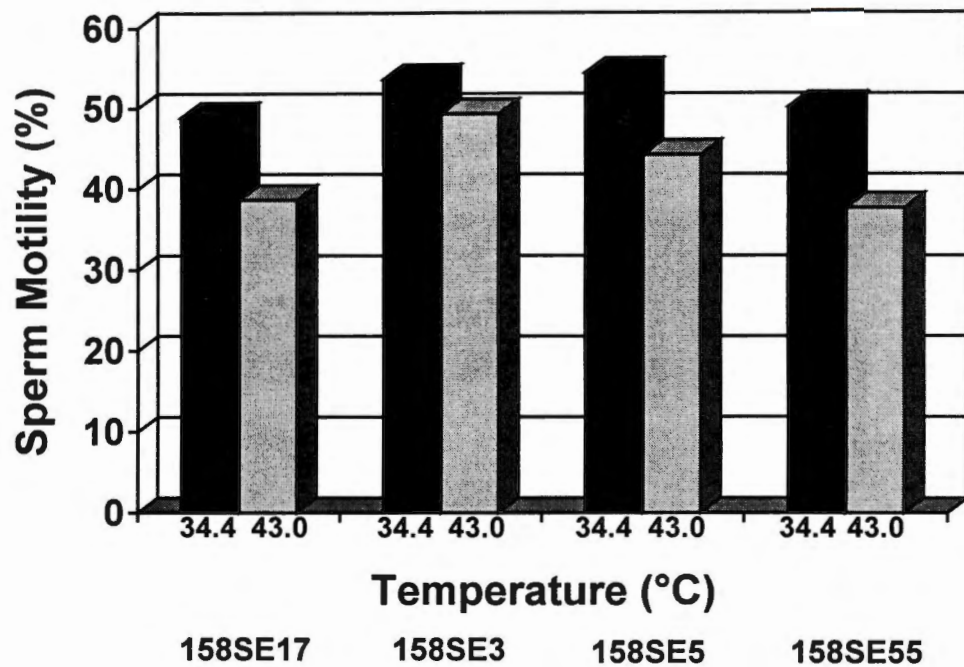


Figure 11: Sperm motility of individual Senepol bulls maintained at 34.4°C or 43.0°C within an AI straw (temp*bull-id; pooled SEM=2.9; P=0.3001).

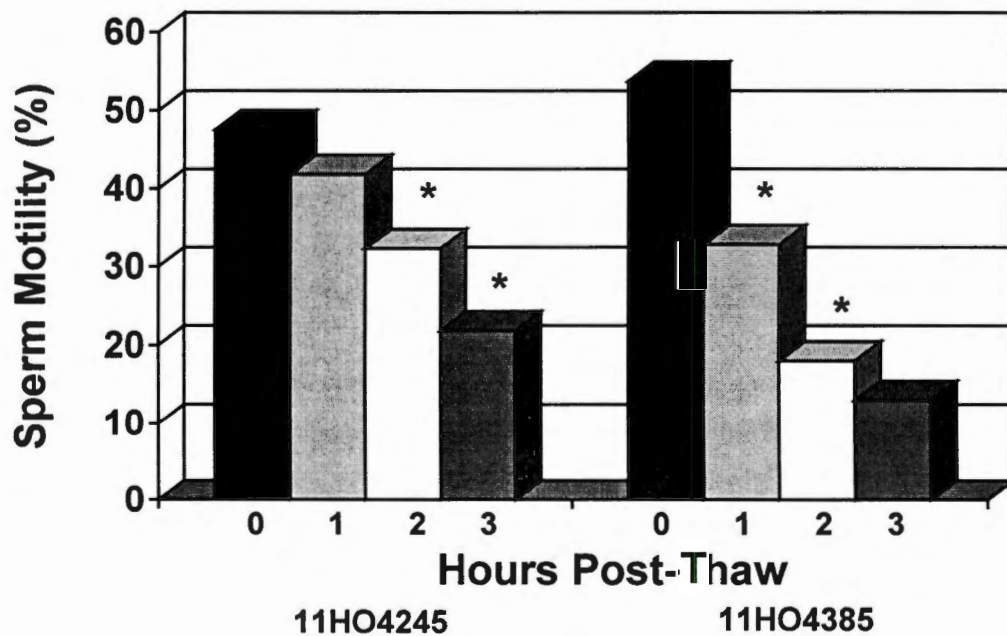


Figure 12: Variations in sperm motility of individual Holstein bulls to withstand aging within an AI straw (time*bull-id; SEM=3.2; P=0.0196).

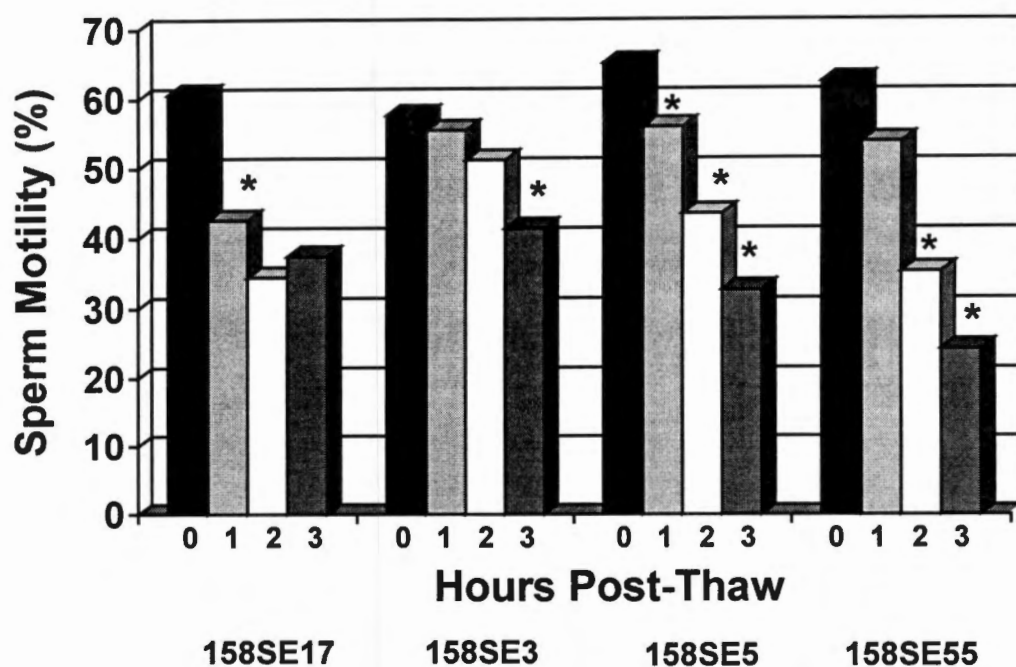


Figure 13: Variations in sperm motility of individual Senepol bulls to withstand aging (time*bull-id; pooled SEM=3.7; P=0.0010).

Table 2: Effects of maintaining sperm within an AI straw for 3 h on motility of high and low fertility bulls.

Fertility Status	Motility 3 h post-thaw	Motility After Percoll Purification
High	58.2 ^a	57.2 ^a
Low	53.1 ^b	46.3 ^b
P-value	P=0.0510	P=0.0164
SEM	2.8	3.5
Lab Control	78.5	78.7

Experiment was replicated 10 times; ^{a, b}-Values within same column differ

* Lab Control motility assessed immediately post-thaw (0 h)

Table 3: Effects of maintaining sperm within an AI straw at 34.4 or 43.0°C for 3 h.

Temperature (°C)	Motility 3 h post-thaw	Motility After Percoll Purification
34.4	72.6 ^a	73.5 ^a
43.0	38.7 ^b	29.9 ^b
P-value	P<0.0001	P<0.0001
SEM	2.8	3.5

Experiment was replicated 10 times; ^{a, b}-Values within same column differ

Low fertility bull had a lower proportion of normal sperm than high fertility bull (Table 4). In particular, a greater incidence of detached heads was noted (Table 4). Incubation of sperm within an AI straw at 43.0°C for 3 h post-thaw decreased proportion of normal sperm and increased proportion having knobbed acrosomes (Table 5). There was fertility *temperature interaction for proportion of distal droplets (Figure 14).

Ability of oocytes to cleave or develop to blastocyst after fertilization with sperm from a high fertility versus low fertility bull was similar (Table 6). In contrast, fertilization of oocytes with sperm incubated within an AI straw at 43.0°C for 3 h reduced proportion of oocytes cleaving, but did not affect the ability of those that did cleave to develop to 8-16 cell stage or to blastocyst (Table 7).

Table 4: Proportion of sperm abnormalities in bulls of high or low fertility.

Fertility Status	% Nrm	% Abnormal								
		pd	ka	pyr	dmr	dh	dd	bt	mh	mac
High	90.5 ^a	0.5	3.2	0.5	1.8	2.5 ^b	0.1	0.4	0.6	0.1
Low	81.8 ^b	0.9	1.8	1.2	1.4	11.7 ^a	0.2	0.2	0.8	0.1
P-value	0.0001	0.07	0.12	0.09	0.57	0.0001	0.15	0.2	0.5	1.0
SEM	0.8	0.2	0.7	0.3	0.4	0.7	0.1	0.1	0.2	0.1
Lab Control	86.7	0.7	2.0	1.2	2.3	6.1	0.1	0.1	0.4	0.2

Experiment was replicated 10 times; ^{a, b}-Values within same column differ
 % Nrm = proportion of normal sperm; pd = proximal droplets; ka = knobbed acrosomes; pyr = pyriform; dmr = distal midpiece reflex; dh = detached head; bt = bent principal piece; mh = micro head; mac = macro head

Table 5: Effects of elevated temperature on proportion of sperm abnormalities.

Temp (°C)	% Nrm	% Abnormal								
		pd	ka	pyr	dmr	dh	dd	bt	mh	mac
34.4	87.6 ^a	0.6	1.5 ^b	0.9	1.7	6.6	0.2	0.4	0.6	0.1
43.0	84.8 ^b	0.8	3.5 ^a	0.8	1.5	7.7	0.1	0.2	0.7	0.1
P-value	0.03	0.23	0.03	0.83	0.69	0.22	0.15	0.13	0.59	1.0
SEM	0.8	0.2	0.7	0.3	0.4	0.7	0.1	0.1	0.2	0.1

Experiment was replicated 10 times; ^{a, b}-Values within same column differ;
 % Nrm = proportion of normal sperm; pd = proximal droplets; ka = knobbed acrosomes; pyr = pyriform; dmr = distal midpiece reflex; dh = detached head; bt = bent principal piece; mh = micro head; mac = macro head

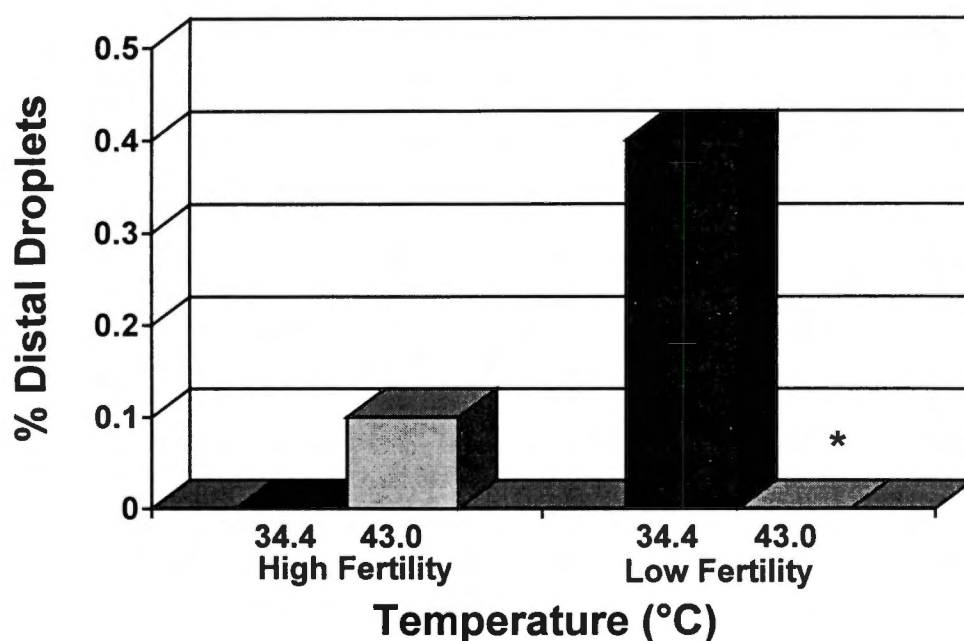


Figure 14. Proportion of distal droplets on sperm as related to fertility status and thaw temperature (temp*bull-id; SEM=0.1; P=0.0152).

Table 6: Developmental competency of bovine embryos derived from oocytes fertilized with sperm from high and low fertility bulls incubated 3 h post-thaw.

Fertility Status	No. Oocytes	Rec. (%)	Cleave (%)	8-16/cleave (%)	Blastocysts of cleaved (%)
High	455	390(85.5)	211(54.5)	109(46.1)	44(22.5)
Low	467	394(84.5)	216(53.0)	133(64.1)	28(16.5)
P-value	---	P=0.7358	P=0.8171	P=0.1	P=0.35
SEM	---	2.1	6.3	7.2	4.9
Lab Control	220	181(81.2)	141 (76.5)	101 (70.6)	50 (36.9)

Experiment was replicated 5 times when lab control >20%; No. oocytes = number of oocytes in maturation medium; % Rec. = number and proportion of PZ recovered after denuding of excess cumulus and sperm; % cleave = number and proportion of PZ that cleaved; % 8-16/cleave = number and proportion of cleaved embryos that developed to 8-16 cell stage; Blastocysts of cleaved (%) = number and proportion of cleaved embryos that developed to blastocyst stage.

Table 7: Developmental competency of bovine embryos derived from oocytes fertilized with sperm incubated at 34.4°C or 43.0°C for 3 h post-thaw.

Temp (°C)	No. Oocytes	Rec. (%)	Cleave (%)	8-16/cleave (%)	Blastocysts of cleaved (%)
34.4	477	403(84.4)	251(62.1) ^a	163(62.6)	47(21.2)
43.0	445	381(85.5)	176(45.4) ^b	79(47.6)	25(17.8)
P-value	---	P=0.7230	P=0.0231	P=0.17	P=0.59
SEM	---	2.1	6.3	7.2	4.9

^{a, b} - least square means values within same column differ

Experiment was replicated 5 times when lab control >20%; No. oocytes = number of oocytes in maturation medium; % Rec. = number and proportion of PZ recovered after denuding excess cumulus and sperm; % cleave = number and proportion of PZ that cleaved; % 8-16/cleave = number and proportion of cleaved embryos that developed to 8-16 cell stage; Blastocysts of cleaved (%) = number and proportion of cleaved embryos that developed to blastocyst stage.

CHAPTER V

DISCUSSION

Overall objective of the present study was to determine if ability of individual bull sperm to withstand stress could be correlated with fertility. Results demonstrated variation among individual bulls' sperm within breed to withstand stress of aging or elevated temperature for extended time periods. However, sperm from high fertility bull resulted in similar embryo development as sperm from low fertility bull.

Variation among individual bulls' sperm within breed to withstand stressors of aging and elevated temperature

Results demonstrated variation among individual bulls' sperm within breed to withstand stress of aging or elevated temperature for extended time periods. In similar findings reported by DeJarnette et al. (2000), one of five Holstein bulls was "resistant" to post-thaw insults of thawing within an AI gun, or at 5.0 or 20.0°C. Reasons for differences among individual bulls' sperm within breed to withstand environmental stressors may include differences among AI studs (methods for semen collection and processing may differ), specific lot(s) of semen evaluated, and differences in types of extender and/or constituents within the same semen extender.

Sperm from individual bulls within the Senepol and Holstein breeds were derived from a single AI stud; however frozen-thawed sperm of Charolais and Simmental bulls originated from different AI studs (objectives 1 and 3). Although

semen from individual bulls within Charolais breed were extended similarly, with exception of one (36CH9257 egg-yolk-TRIS, personal communication with Jean Schofield of North American Breeders, Berryville, VA vs. whole milk extenders: 49CH9845, personal communication with Steve Trantham of Genex, Inc. Strafford, MO; 54CH530, personal communication with Richard Almquist of Hawkeye Breeders Service, Adel, IA; and 137CH221 and 137CH295, Mark Nichols of Nichols Cryo-Genetics, Ankeny, IA), subtle differences contributed by individual AI studs during processing, collection, and freezing of semen may have contributed to variation observed among individual bulls (Pickett and Berndtson, 1974; Ennen et al., 1976). However, variations were noted in sperm from individual Holstein bulls despite originating from a single AI stud (objectives 1 and 3), as well as in Senepol bulls (objective 3). Perhaps differences among semen straws within a single lot contributed to the variation reported in these bulls (Otoi et al., 1993). Specifically, results demonstrated variation among different straws of semen derived from a single ejaculate, as assessed by cleavage and development of bovine embryos. It remains unclear, however, why variation was demonstrated in Senepol bulls derived from a single lot in objective 3, but not in objective 1. Perhaps the different stressors (aging in objective 1 versus elevated temperature in objective 3) that were utilized to establish variation among individual bull's sperm within breed could explain the disparity. Moreover, caution should be used in interpreting data as the sample set of individual Senepol bull sperm may have been too small to establish variation.

Another reason for variation among individual bulls' sperm may be due to extender type and/or constituents within the same extender. Specifically, straws of semen for Senepol, Simmental and Holstein contained egg-yolk, while Charolais semen was extended in whole milk. Earlier studies indicated fertility of bovine sperm could be maintained regardless of extender. However, egg yolk extenders are usually preferred due to ease of viewing sperm when making assessments of motility (as reviewed by Vishwanath and Shannon, 2000). Furthermore, Foote et al. (2002) suggested that egg yolk extenders are superior to milk extenders for maintaining viability of sperm following addition of antioxidants.

Effects of stressors on sperm motility, morphology, and function

Results from the present study further characterized the effects of aging and elevated temperature on sperm for extended time periods within an AI straw post-thaw. Specifically, proportion of motile sperm was reduced markedly when sperm were aged within an AI straw at 34.4°C for up to 15 h post-thaw or subjected to elevated temperature of 43.0°C for up to 3 h post-thaw. Additionally, exposure of sperm within an AI straw to elevated temperature post-thaw altered sperm morphology and compromised ability of oocytes to cleave.

Unique to the studies described in this thesis, sperm were aged within an AI straw and maintained in a water bath at 34.4°C for up to 15 h post-thaw. The majority of previous studies describe negative effects of non-cryopreserved sperm following collection and dilution (Todorovic et al., 1969; Krzyzosiak et al., 2000) or that, which was evaluated after undergoing swim-up or Percoll

purification when removed from AI straws (Monterroso et al., 1995; Chandolia et al., 1999).

Effects of aging on motility of frozen-thawed bovine sperm

Following a literature search, a single study was found that specifically aged bovine sperm within an AI straw. Specifically, DeJarnette et al. (2000) aged straws of bovine sperm within an AI straw; however, sperm were aged for a shorter duration of 3 h post-thaw at 37.0°C. In another study by Kelly and Hurst (1962) evaluating the effects of aging on sperm post-thaw, fertility, measured by nonreturn rates decreased gradually when aged for up to 6 h at 1.0°C. However, sperm were aged within 1 ml glass ampules and incubated at a lower temperature.

Aging of sperm within an AI straw reduced proportion of motile sperm. Results are not surprising given previous studies examining the negative effects of aging on sperm immediately after collection, dilution, or following sperm purification. For example, Todovoric et al. (1969) aged diluted sperm that had been previously collected from an ejaculate of a Holstein-Friesian bull for 21 d at 5.0°C. A gradual decline in proportion of motile sperm was demonstrated (70% motility on day of collection to 30% on d 21). In another study, diluted sperm were aged within 100 ml sterile Schott bottles wrapped in aluminum, which were aerated daily and stored at ambient temperatures (18.0 to 20.0°C) for up to 8 d (Krzyszosiak et al., 2000). Again, decline in sperm motility was gradual over the 8-day period (80% to 15% on d 8).

Reductions in proportion of motile sperm are more pronounced when sperm are aged post-thaw. DeJarnette et al. (2000) reported great reduction in sperm motility after thaw when sperm were aged within an AI straw at 37.0°C for up to 3 h (76% at 0 h to 27% 3 h post-thaw). Reductions in sperm motility in the study conducted by DeJarnette et al. (2000) were greater than for the present study. Differences in temperature (37.0°C versus 34.4°C) may explain disparity in rate of decline of motility. Although sperm were aged post-thaw in the study conducted by Kelly and Hurst (1962), decline in fertility, as determined by NRR, was gradual over the 6-h time period. Furthermore, differences in surface to volume ratios for 1 ml glass ampules (Senger, 1980) versus 0.5 ml Cassou polyvinyl straws used within the present study (2492 vs. 1537, respectively). Previous findings demonstrated that conception or survival rates were similar regardless of container in which sperm are aged (Pickett and Berndtson, 1974). However, straws of semen have been shown to be more sensitive to thermal insults post-thaw in comparison to glass ampules (Senger, 1980).

Although proportion of motile sperm within an AI straw was reduced in the present study, motility was still observed 15 h later. This suggests that fertile lifespan of bovine sperm post-thaw may be much longer than originally proposed. Generally, it is recommended that sperm be inseminated within 15 min post-thaw to avoid drastic decreases in motility or loss of fertility (Barth and Bowman, 1988).

***Effects of elevated temperature on motility, morphology, and function
frozen-thawed bovine sperm***

Results demonstrated that incubation within an AI straw at 43.0°C reduced sperm motility, increased incidence of knobbed acrosomes, and compromised fertility as measured by decrease in number of oocytes that cleaved when fertilized with treated sperm. This is consistent with previous findings that documented impairment of motility and alternations to the sperm membrane and acrosomes following exposure of mice sperm to an elevated temperature of 56.0°C for 0.5 h (Cozzi et al., 2001); however, this is inconsistent with findings by Monterroso et al. (1995).

Exposure of sperm to elevated temperature reduced proportion of motile sperm, alters membrane integrity, and increases incidence of sperm abnormalities. Specifically, incidence of knobbed acrosomes increased following exposure of sperm to elevated temperature of 43.0°C. However, since this is considered a primary abnormality that arises during spermatogenesis, what were incorrectly labeled as "knobbed acrosomes" during morphology assessments were actually acrosomal perturbations that developed due to incubation of sperm at 43.0°C for 3 h. This is consistent with findings by Cozzi et al. (2001) that demonstrated severe alterations to sperm membrane and acrosome in addition to reduction in sperm motility in 98% of epididymal mouse sperm following exposure to 56.0°C for 0.5 h. In contrast, elevated temperature of 39.0, 41.0 or 42.0°C did not affect proportion of frozen-thawed bovine sperm having intact membranes (Monterroso et al., 1995). Also, other studies have demonstrated

thermostability of sperm and sperm nuclei when exposed to severe elevated temperatures. Specifically, nuclei of mammalian species and other species could withstand thermal insults ranging from 60.0 to 125.0°C for up to 2 h and still form pronuclei (Yanagida et al., 1991; Hoshi et al., 1992).

Ability of oocytes to cleaved after fertilization with sperm previously exposed to elevated temperature (43.0°C) was compromised; however, of those embryos that cleave, ability to develop to 8-16 cell stage or to blastocyst was not affected. Cozzi et al. (2001) also reported compromised fertility associated with impairment of embryos to develop to blastocysts. Despite the negative effects of elevated temperature, viable offspring were still obtained (Cozzi et al., 2001). In contrast, findings by Monterroso et al. (1995) documented no significant effect of ability of oocytes to cleave or develop to 4 to 8-cell stage when fertilized with sperm incubated at 39.0, 41.0, or 42.0°C for 3 h.

Following exposure of sperm to elevated temperature post-thaw, Monterroso et al. (1995) reported no alterations to sperm acrosomes nor compromised fertility. Disparity between the present study and with the study by Monterroso et al. (1995) may be influenced by differences in experimental design. Although 200,000 motile sperm/0.5 ml were added to oocytes during IVF following exposure of sperm to elevated temperature, fertility was still compromised. Therefore, a limitation of the present study may be using ability of oocytes to cleave as an indicator of fertility since oocytes can cleave without the presence of sperm.

Sperm motility, morphology and function of sperm of known high and low fertility bulls

In the present study, results demonstrated that the bull of high fertility had greater proportion of motile sperm and less incidence of sperm abnormalities than the low fertility bull following incubation within an AI straw for extended time periods. However, ability to withstand stress was not indicative of fertility status, refuting the hypothesis that sperm from highly fertile bulls would withstand stress better than sperm from bulls of lower fertility. However, data should be interpreted with caution since only two bulls were available for use within this objective.

Results demonstrated that sperm motility was greater for the high fertility bull versus the low fertility bull when sperm was incubated within an AI straw for 3 h (58.2 vs. 53.1%, respectively). Similarly, Correa et al. (1997) using frozen-thawed bovine sperm demonstrated higher proportion of motile sperm for higher fertility Holstein bulls than lower fertility Holstein bulls immediately post-thaw (72.5 vs. 67.6%, respectively). Regarding sperm morphology assessments conducted in the present study, the low fertility bull had fewer morphologically normal sperm than the high fertility bull (90.5 vs. 81.8%, respectively). Correa et al. (1997) also reported fewer morphologically normal sperm in low fertility Holstein bulls after thaw than in higher fertility Holstein bulls (75.9 vs. 82.2%, respectively). In contrast, Whitfield and Parkinson (1992) reported no differences in proportion of abnormal sperm among bulls of differing fertility as determined by 90-d NRR.

Results further demonstrated that ability to withstand stress was not indicative of fertility, which verifies that sperm motility and morphology are not consistent indicators of fertility. Specifically, ability of oocytes to cleave or develop to blastocyst after fertilization with sperm from a high fertility bull versus a low fertility bull was similar, which is inconsistent with other studies (Shi et al., 1990; Eid et al., 1994; Zhang et al., 1997; Comizzoli et al., 2000; Ward et al., 2001). Possible reasons to explain disparity among studies include integrity of the sperm sample, sperm dosage for IVF, effects of capacitating agents, and variation among straws of semen used.

Although progressively motile sperm were purified through Percoll gradient in the present study, only immotile or dead sperm are trapped within the interface of Percoll gradient (Parrish et al., 1995). Therefore, it is possible for motile, "normal appearing" sperm, having subtle alteration to the chromatin or possessing abnormalities such as nuclear vacuoles or craters, to potentially compete with normal sperm during fertilization *in vitro* (Saacke et al., 1998). These traits are considered uncompensable and changes to sperm dosage (current study used 200,000 motile sperm/0.5 ml) to account for abnormalities would be ineffective (Saacke et al., 1998, 2000). Further, despite utilizing standard staining techniques and observing sperm at 1000X, assessments perhaps were not sensitive in detecting sperm with aberrant chromatin or having nuclear vacuoles (Barth and Oko, 1989; Thundathil et al., 1998). In a previous study for example, a bull rated satisfactory according to routine exams, yet the majority of sperm were later found to contain nuclear vacuoles (Barth and Oko, 1989). Therefore, it

is possible that the bulls within the present study had abnormal sperm, which could not be detected using eosin-nigrosin staining. Feulgen staining, scanning electron microscopy, or differential interference microscopy (DIC) are preferred to identify incidence of nuclear vacuoles (Barth and Oko, 1989). Additionally, the present study revealed a higher proportion of detached heads in bull of lower fertility than high fertility (2.5 vs. 11.7%, respectively). Detached heads are considered secondary abnormalities that usually arise during spermiogenesis (Barth and Oko, 1989). Sperm having detached heads are generally considered compensable defects because sperm dosage can be increased to account for the abnormality. Unlike sperm having nuclear vacuoles, sperm having detached heads are unable to compete during fertilization.

In the current study, a dosage of 200,000 motile sperm/0.5 ml was used to fertilize oocytes. However, despite adding the same amount of motile sperm to oocytes for both bulls of high or low fertility, the presence of uncompensable sperm traits within the sperm sample may have affected subsequent development. Also, sires generally require different amounts of sperm to reach their maximum fertility potential (Saacke et al., 2000). Shannon and Vishwanath (1995) proposed that if sperm numbers were adjusted in such a way that concentrations was suboptimal, perhaps differences between sires and various treatments could be detected. However, a recent study conducted by Ward et al. (2002) demonstrated that concentration of sperm of $0.25 \times 10^6/\text{ml}$ and $0.5 \times 10^6/\text{ml}$ were adequate for embryos to cleave and develop to blastocysts.

In the current study, bulls of high and low fertility performed poorly in the IVF system compared to the lab control, even with incorporation of the capacitating agent, heparin. Previous efforts demonstrated that bulls might vary in their responses to glycoaminoglycans such as heparin or chondroitin-sulfate in an *in vitro* system (Lenz et al., 1983). Moreover, poor performance of the high and low fertility bulls was not related to technical differences or quality of oocytes, as the lab control bull performed satisfactory (having greater than 20% blastocysts development). Therefore, this provides a good example that some bulls, regardless of fertility, do not perform well within an IVF system.

When considering variation within ejaculate or from straw to straw, Otoi et al. (1993) indicated differences among straws of semen from a single lot. Leibfried-Rutledge et al. (1989) further demonstrated ejaculates for bulls differ as measured by penetration and fertilization rates of embryos. A likely source of variation was inclusion of an extra constituent within the extender of one of the bulls used within this study. However, the identity of the constituent could not be revealed (personal communication with Dr. JM DeJarnette, Select Sires, Inc- Plain City, OH).

Conclusions/Implications

Results demonstrated variation among individual bulls' sperm within breed to withstand stress of aging or elevated temperature for extended time periods. However, sperm from high fertility bulls resulted in similar embryo development as sperm from bulls of lower fertility. Surprisingly, motility of frozen-thawed

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APPENDICES

Appendix A
Assessment of Sperm Motility

Melody N. Malone
Dr. Fred Hopkins, Large Animal Clinical Sciences, CVM
Summer 2002

Reference: Sorensen AM, Jr. 1975. Semen evaluation. In: Repro Lab. A laboratory manual for animal reproduction. Boston: American Press 3rd edition. p 47-58.

Materials and equipment needed:

Microcentrifuge tubes (0.5 ml)
Cito-Thaw (American Breeders Service; generously provided by Dr. FN Schrick-Department of Animal Science, The University of Tennessee)
Geslab GP-500 water bath (set to 38.5°C)
Slide Warmer (Fisher)
60 X 15 mm Petri dish (Corning 25060-60)
Serological pipettes (sizes: 1 ml, 5 ml, 10 ml, 25 ml and 50 ml)
Gilson Pipettor; P 100-1000 µl (Fisher 21-102-51E)
Gilson Pipettor; P 10-100 µl (Fisher 21-102-51C)
Gilson Pipettor; P 0.5-10 µl (Fisher 21-102-51A)
Pipette tips (for P-10, P-100, P-1000)
Gauze (Medline)
Homemade plunger (for expelling sperm from AI straw)
Scissors
Nikon Eclipse TE300 inverted microscope
Floating, round tube rack (Fisher 14-792-14)
Floating, square tube rack (Fisher 14-792-19)* discontinued item
0.5 ml microcentrifuge tube rack (Fisher 05-408-16)
Ethanol and Roccal

Reagents for Sperm-TALP (50 ml)

50 ml of Sperm-TL stock (Specialty Media OR that which is prepared in the laboratory)
BSA (Sigma A-3311)
Sodium Pyruvate (Stock 9; Sigma P-5280)
Penicillin/Streptomycin (TMS-AB2-C; Specialty Media, Inc.)

Preparing Sperm-TALP (refer to medium sheet for specific details)

Prepare medium in laminar flow hood
-put 50 ml of Sperm-TL in 100 ml glass beaker
-add 300 mg of BSA and allow time for it to dissolve
-add 2.63 ml of Na Pyruvate
-add 250 µl of Pen/Strep
-sterile filter using a 0.2 µm syringe tip filter and store at 2-8°C for up to one week

Motility Assessment of Sperm

1. Prepare motility tubes prior to assessment. You will need 0.5 ml microcentrifuge tubes, P-100 pipette and respective tips, microcentrifuge tube holder, and supplemented Sperm-TALP. Sperm-TALP is a nutrient rich medium used to maintain the optimum level of sperm viability. It is prepared according to Parrish and co-workers (1988) and supplemented with 6-mg/ml bovine serum albumin (BSA), 1.0 mM sodium pyruvate, and 50 U/ml of penicillin and 50 µg/ml of streptomycin.
2. Place 95 µl of Sperm-TALP into 0.5 ml microcentrifuge tubes and place tubes inside a floating tube rack prior to placing in large water bath (in General lab equilibrated to 38.5°C). The floating tube racks (square or round) are located in the box adjacent to the water bath. Ensure that microcentrifuge tubes containing Sperm-TALP are securely shut. Water kills sperm; so avoid any likelihood of water entering tubes.
3. Warm motility tubes for a minimum of 20 min prior to assessment.
Note: Cold medium alters motility of sperm (severe coiling of the tail).
4. Thaw a 0.5 ml straw of frozen/thawed sperm in a Cito-Thaw bath (34.4°C; 94.0°F) for 45 seconds. Keep the lid of the Cito-Thaw closed being aware that exposure to light, air, and cold has adverse effects on sperm. The key is **speed**. The faster one can process sperm prior to sperm motility assessment, the better.
5. Wipe the straw with ethanol and dry with sterile gauze. Gently shake the straw by holding at the sealed end to remove air bubbles. Using a straw cutter or scissors remove the sealed end of the tube.
6. Dispense sperm into an empty 0.5 ml microcentrifuge tube using the homemade plunger.
7. Gently tap to evenly distribute semen contents within the microcentrifuge tube.
8. Remove 5 µl of sperm from microcentrifuge tube using a Gilson, P-10 pipette (white top) and pipette tips (clear tips; 1-10 µl).
9. Place 5 µl of sperm into 95 µl of Sperm-TALP making a 1:20 dilution. This dilution is recommended for assessing motility of frozen-thawed sperm (Sorensen, 1975).
10. Gently tap tube to ensure sperm are mixed properly. Do not tap roughly as you may knock the heads off of the sperm.

11. Using a Gilson, P-100 or P-20 pipette and respective tips (yellow), place 20- μ l droplet of sperm dilution onto a pre-warmed ($\sim 38.5^{\circ}\text{C}$ using slide warmer) 60X15 petri dish bottom. Place remaining contents of tube in a Cito-Thaw bath for a second assessment.
12. Using an inverted light microscope at 400 magnification is recommended for assessing sperm motility (Sorensen, 1975).
13. Evaluate the number of progressively motile sperm (10 random sperm live/dead for each field) in 5 different microscopic fields. **Progressively motile sperm** = proportion of sperm that move in a forward direction. Train your eye to select 10 random sperm (live/dead). Differentiate between those that are progressively motile from those with poor motility.
14. **NOTE:** The eye will be drawn to sperm that are moving. Therefore, this subjective evaluation of sperm motility holds more potential for error. So it is recommended that at least two evaluators independently assess sperm motility.
15. Repeat this process for 5 more fields using an additional 20- μ l droplet of sperm dilution. This will yield a total of 10 total microscopic fields.
16. The proportion of progressively motile sperm from a given bull with the example below is estimated by adding the number of motile sperm in each of 10 fields (i.e. 46 of 100 sperm are progressively motile)

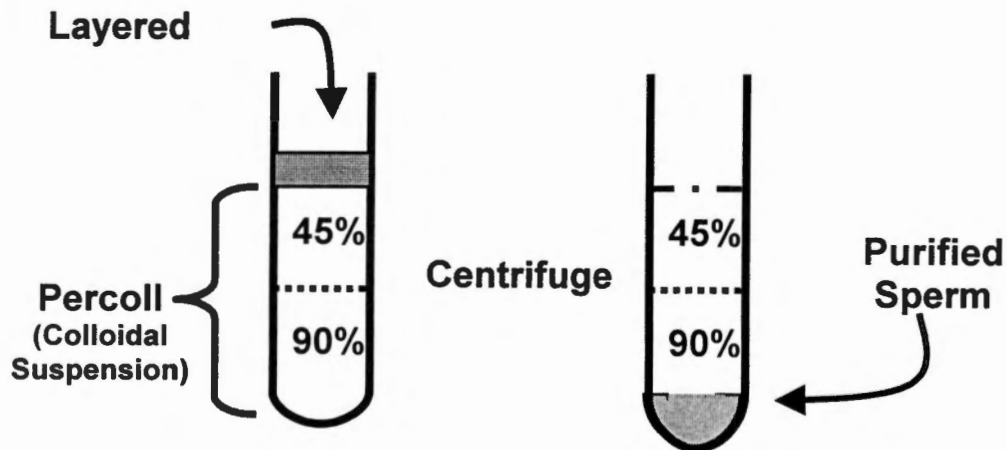
	Drop 1					Drop 2					
# motile	3	6	4	5	4	4	6	6	4	4	= 46
# sperm	10	10	10	10	10	10	10	10	10	10	= 100

Note: Assessing sperm motility prior and after Percoll purification may be conducted using the following steps:

1. Dispense contents of semen straw on top of a discontinuous Percoll gradient (2 ml 45% Percoll/2 ml 90% Percoll) that is housed within a 15 ml conical tube.
2. Being careful not to disturb sperm fraction, remove 5 μ l of the undiluted sperm and place in a pre-warmed motility tube containing 95 μ l of Sperm-TALP yielding a 1:20 dilution (figure 1). Place top back on to conical tube and proceed with spin. Follow steps 11-16.

3. To conduct motility assessment after Percoll purification, remove 5 μ l sperm after it has been re-suspended in (~500 μ l) IVF-TALP. Place sperm in a pre-warmed motility tube containing 95 μ l of Sperm-TALP. Make sure to place remaining sperm + IVF-TALP inside 38.5°C incubator with lid of conical tube slightly loosened. Follow steps 11-16.

Diagram 1: (Edwards' lab schematic)



Appendix B

Morphological Assessment of Sperm

Melody N. Malone-2002

Dr. Fred Hopkins, Large Animal Clinical Sciences, CVM

Reference: Barth AD and Oko RJ. 1989. Abnormal Morphology of Bovine Sperm. Iowa State Press. Ames, Iowa p 8-12.

Modified Eosin-Nigrosin stain formula: Dr. Fred Hopkins generously prepared stain.

Materials:

Eosin Y (Sigma E-4009)

Nigrosin (Sigma N-4754)

Sodium citrate (Sigma S-4641)

Distilled water

Cito-Thaw (American Breeders Service; generously provided by Dr. FN Schrick-Department of Animal Science, The University of Tennessee)

Light immersion oil-Type A (Cargille Lab. 16482)

3' X 1' glass microscope slides (Fisher 12-550-12)

Optical lens cleaner (Fisher 22-143974)

Lens Paper (Fisher 11-996)

Slide Warmer (Fisher)

Slide Holder (Fisher 14-127-50A)

Gilson Pipettor (P 10-100 µl)

Pipette tips

Gauze (Medline)

Phase-Contrast Microscope (adapted for oil immersion analysis)

Counter (hand counter with 2 counting units-Fisher 02-670-12)

Preparing Modified Eosin Nigrosin stain formula:

-Eosin Y-3.3 g

-Nigrosin-20 g

-Sodium citrate-1.5 g

-qs distilled water to 300 ml ** "qs" is **quantity sufficient** (i.e. the quantity sufficient to make a total of 300 ml)

-dissolve by heat and stirring

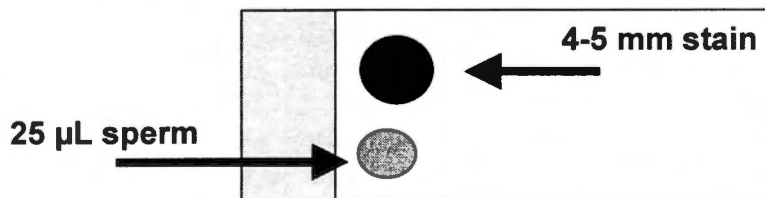
-adjust pH to 6.7 to 6.8

-allow to stand for a few days and filter

Morphological Assessment of Sperm (see Barth and Oko, 1989; Hopkins and Spitzer, 1997)

1. Turn on slide warmer (38.5°C) prior to sperm morphology assessments.
Note: It is important to AVOID cold shocking sperm. Sperm that has been cold shocked will have coiled tails.
2. Place Eosin-nigrosin stain, light immersion oil, and microscopic slides on the slide warmer pre-warmed to 38.5°C.
3. Empty contents of a frozen-thawed straw of sperm into a 0.5 ml microcentrifuge tube using the homemade plunger. Gently tap to evenly distribute undiluted sperm contents within the microcentrifuge tube. Keep tube warm. I suggest placing it into the Cito-Thaw. Use the round, floating tube racks.
4. To prepare smear (i.e. sperm + stain), place 25 µl of undiluted sperm on a pre-warmed slide adjacent to a 4-5 mm drop of warmed stain. Do so near the frosted region of the slide (Diagram 2A).

Diagram 2A:



5. Briefly mix the stain and sperm using the blunt end of another slide (Diagram 2B).

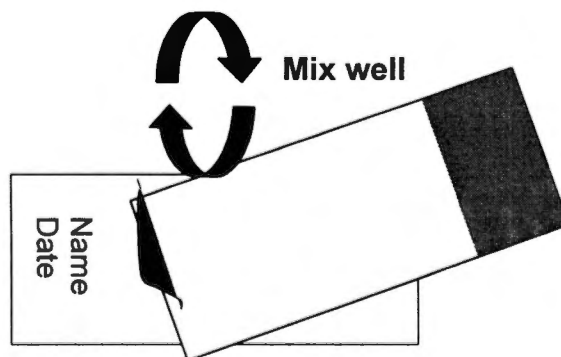
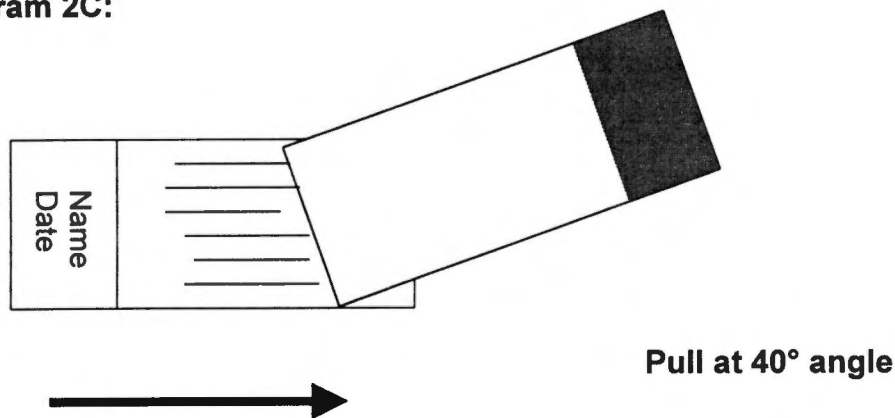


Diagram 2B:

6. To make a smear, pull the accessory slide along a 40° angle from the edge of the mixture of sperm and eosin-nigrosin stain (Diagram 2C).

Diagram 2C:



7. Allow the smear to dry on a slide warmer (38.5°C) for at least 3 min. A wet slide that hasn't been properly dried will have a shiny, glossy blue appearance. A properly dried slide will look a dull blue appearance. Avoid over-drying (i.e. longer than 5 min), as this will promote cracks in the smear usually resulting in sperm with detached heads!
8. Place dried slide on a slide holder until assessment.
9. Using a phase-contrast microscope allowing for oil immersion, examine sperm at 1000 magnification). **NOTE:** Currently, Dr. Edwards does not have a phase-contrast microscope so Dr. Fred Hopkins generously provided a microscope for analysis of sperm.
10. To view sperm, place a drop (~2-3 mm) of immersion oil on top of dried smear and evaluate 100 sperm cells to determine proportion of abnormal sperm. Use the counter with 2 counting units for ease in counting 100 sperm. Why only 100 sperm? Well, Salisbury and Mercier (1945) found that counting more didn't increase precision.
11. It is recommended to evaluate sperm morphology from the middle 1/3 of the slide (Barth and Oko, 1989; Hopkins and Spitzer, 1997) because it usually has the best background. If you think about it, the edge of the smear will be thicker and is most prone to drying and caking.
12. Record data on data sheet provided with protocol.
13. Discard slides after use in glass receptacle. Slides cannot be preserved.
14. Clean microscope after use with lens paper and lens cleaner (Fisher). Gently blot the lens to avoid scratches.

NOTE: Eosin/nigrosin stain housed within a dropper bottle (ours is stored this way) will withhold staining properties for up to 6 months if stored at room temperature. However, these stains are more prone to acquiring fungi and bacteria (Barth and Oko, 1989).

Classification of common abnormalities used in theriogenology: (according to Johnson, 1997)

Primary abnormalities = arise during spermatogenesis.

Secondary abnormalities = arise during spermiogenesis (i.e. sperm maturation)

Primary:

- Pyriform heads
- Proximal cytoplasmic droplet
- Distal midpiece reflex
- Maldeveloped
- Craters

Secondary:

- Detached heads
- Knobbed acrosome
- Simple bent principal pieces
- Distal cytoplasmic droplet
- Microcephalic head
- Macrocephalic head
- Coiled principal pieces

MORPHOLOGY ASSESSMENTS
(adopted protocol from Dr. Fred Hopkins) Standard 100 sperm cell count

Bull-id _____

1	2	3	4	5	6	7	8	9	10
11	12	13	14	15	16	17	18	19	20
21	22	23	24	25	26	27	28	29	30
31	32	33	34	35	36	37	38	39	40
41	42	43	44	45	46	47	48	49	50
51	52	53	54	55	56	57	58	59	60
61	62	63	64	65	66	67	68	69	70
71	72	73	74	75	76	77	78	79	80
81	82	83	84	85	86	87	88	89	90
91	92	93	94	95	96	97	98	99	100

No. Abnormal/ 100 = % Abnormal _____

VITA

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