EFFECTS OF ELEVATED TESTICULAR TEMPERATURE ON VIABILITY OF CRYOPRESERVED SEMEN AND MORPHOLOGICAL CHARACTERISTICS OF EJACULATED SPERMATOZOA

by

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(ABSTRACT)

Two successive ejaculates were collected from six mature Holstein bulls at 3 d intervals for 7 wks. Elevated testicular temperature was induced by complete coverage of the scrotum with insulated material for 48 h. Viability (motility and acrosome integrity) and morphological characteristics of sperm before and after thermal insult were examined. For assessment of results, collection days were grouped: days -6, -3, 0 = Period 1 (d 0 = day of testis coverage after semen collection on that day), days 3, 6, 9 = Period 2 , days 12, 15...39 = Period 3. Semen was cryopreserved on each day of collection until morphological abnormalities of sperm increased to >50%. Semen viability before and after freezing was lower in Period 3 than in Period 1 ($P \leq .01$). These differences coincided with increased abnormal morphology. No differences in viability were observed between Period 1 and Period 2 for unfrozen semen. Once frozen, spermatozoa ejaculated during Period 2 were significantly different from Period 1 for both viability measurements, but only after 3 h incubation at 37°C ($P \leq .01$). Mean percent pre-insult abnormal sperm level was 19.6 ± 5.7 and sperm morphology in Period 1 (pre-insult) did not differ from that in Period 2. Morphological change was first noted in Period 3 on d 12 and 15 (47.5 ± 27.4 and 65.0 ± 27.0 % abnormal sperm, respectively). Abnormal sperm peaked on d 21 (83.2 ± 22.8 %). Although bulls varied in degree and time of response post-insult, all bulls exhibited the same sequence of appearance for specific abnormalities. The sequence and peak means for these abnormalities observed over all bulls were as follows: decapitated sperm, d 15 (33.9 ± 28.8 %); diadem defect, d 18 (55.6 ± 25.8 %); pyriform heads and nuclear vacuoles (excluding diadems), d 21 (18.3 ± 17.6 and 20.8 ± 10.5

%, respectively); knobbed acrosomes, d 27 (11.6± 13.6 %). Sperm morphology was followed through d 39, by which time all bulls were producing ≤50% abnormal cells (35.2± 8.0 %).

We concluded that viability of epididymal/rete sperm was adversely affected by elevated testicular temperatures, as noted by lowered viability of cryopreserved semen, and that there is a sequence in appearance of abnormal cell types in repsponse to thermal insult of the testis.

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INTRODUCTION

In the livestock industry, the male is judged not only by the genetic potential he manifests, but also his ability to package genetic material such that it is perpetuated as offspring. The evaluation of male fertility potential has centered on assuring adequate numbers of quality sperm reach the site of fertilization such that oocyte penetration, fertilization, and embryonic development can occur. Semen of fertile bulls is characterized by having more viable sperm and a consistently lower incidence of morphologically abnormal sperm than non-fertile or subfertile bulls (Williams and Savage, 1925; Lagerlof, 1934). Thus, for successful artificial insemination in cattle, the seminal characteristics classically recognized as important to fertility and which must be taken into consideration are sperm viability and sperm morphology. Ambient temperature and efficiency of innate thermoregulatory mechanisms of the testes are two significant factors influencing semen characteristics of the bovine. In one of the earliest works describing the effects of elevated temperature on bulls, Casady et al. (1953) found an impaired efficiency of spermatogenesis as reflected in impaired sperm viability, morphology, and sperm output after exposure of animals to 37°C for 2 wk or 30°C for 5 wk. Although previous reports indicated that epididymal sperm were not affected by elevated environmental as well as elevated testicular temperatures, these studies only considered the neat semen ejaculated by the stressed animals (Skinner and Louw, 1966; Ross and Entwistle, 1979).

Most major artificial breeding organizations routinely monitor viability and morphology of sperm and have established their own minimum standards acceptable for use of semen with respect to these traits. Artificial insemination (AI) requires semen to be collected and extended, packaged, and cryopreserved resulting in inherently lower sperm viability from freeze-thaw associated stress. With respect to the contemporary AI industry, it is important to know if cryopreserved sperm reflect heat stress, particularly sperm that are in the epididymis and rete testis at the time of insult since they would have completed spermatogenesis and would be expected to have normal morphology.

The overall objective of this study was to determine the effects of heat insult to the bull testis on the ability of spermatozoa to be cryopreserved subsequent to the insult. Since the effects of heat on spermatogenesis are well documented, the particular concern of this study was the vulnerability of sperm in the epididymis and rete testis to the effects of heat as expressed by post thaw survival of cryopreserved sperm. Specifically, the objective of this study was to determine the effects of elevated testicular temperature by complete coverage of the testis and pampiniform plexus area (neck of the scrotum) on:

1. viability before and after freezing of sperm collected during and following thermal insult with specific focus on sperm in the epididymis/rete testis at the time of thermal insult.

2. morphological characteristics of sperm resulting from thermal insult.

BACKGROUND LITERATURE

Relationship of Sperm Viability and Morphology to Fertility

A goal of research in laboratory assessment of semen for use in AI has been to predict the fertility achievable with the use of that semen. To establish the relationship of laboratory tests of sperm viability and morphology with fertility, it is evident that the neat semen quality has importance in evaluating the male. Also important is the interaction of that semen with the preservation methods being utilized. This interaction is conveyed to the female in the quality of semen in the AI unit. Thus, important to success in artificial insemination is semen quality represented in the original ejaculate as well as man's interaction with that semen in an effort to preserve it's fertilizability.

Traditionally, traits of semen quality have been classified as viability or morphology related. Sperm morphology is considered to reflect the physiological status of the male for sperm production and storage in the extragonadal ducts and is considered to be best when sperm are uniform in shape and characteristic for the species (Williams and Savage, 1927). Semen viability traits can also reflect the

physiological status of the male but, in addition, become measures of man's interaction with semen as it is collected, preserved, and inseminated (Saacke,1983). Therefore, the viability traits are of extreme importance to semen preservation since the product represents an interaction of the biological entity produced by the male and man's interaction with that product. Linford et al., (1976), Elliott (1978 a,b), and Saacke (1983) reviewed the relationship between sperm viability and fertility. In general, decreased viability is correlated with fertility in that as viability decreases so does fertility. A wide variety of viability traits have been objectively and subjectively measured. A list of viability measurements used in AI and research for bovine are shown in Table 1. Saacke and White (1972) also evaluated the relationship of selected semen quality tests to fertility. They observed a positive correlation ($r = .42$) between percent motility estimates post thaw and percent 90-day non-return (NR) to first service. More highly related to fertility (90-day NR) than motility was acrosomal retention. Furthermore, the results of an acrosome retention determination after 2 h incubation was more closely related to fertility ($r = .60$) than was a similar determination immediately post-thaw ($r = .42$).

The relationship of abnormal cell content to fertility has been extensively documented by many authors (Blom, 1972; Sullivan, 1978; Blom, 1972; and Barth and Oko, 1989). In general, correlations range dramatically depending upon investigation. In the study by Saacke and White (1972), a relationship of abnormal cell content with fertility was found for abnormal heads ($r = -.34$)) and protoplasmic droplets ($r = -.37$), but not for abnormal tails ($r = -.06$). When variation due to differences among bulls was removed from this study, correlations between all semen tests, with the exception of acrosomal integrity after 2h incubation ($r = .20$), became non-significant. Loss of a significant relationship of semen traits to fertility, when measured among ejaculates of the same bull, was thought to be due to a loss of variation in both semen traits and fertility. The major source of variation was from bull to bull rather than from ejaculate to ejaculate within a given bull.

In a review of literature, Sullivan (1978) discussed specific aberrations of sperm cell morphology associated with reduced fertility. He stated that there is no clear experimental evidence of a quantitative relationship between a specific morphological characteristic and fertility. He also suggested

Table 1. VIABILITY TESTS APPLIED TO SEMEN PRESERVATION

Viability Test	Reference
Motility estimate	
Progressive motility-photomotility	Elliott et al. (1973)
Acrosomal integrity (presence of apical ridge)	Saacke and Marshall (1968)
Release of glutamic oxaloacetic transaminase (GOT)	Pace et al. (1970)
Acrosin activity remaining	Schwert and Takenaka (1955) as modified by Johnson et al. (1976) and Pace and Sullivan (1978)
Swelling of spermatoza under hypotonic conditions	Smith and Graham (1976)
Sperm passing through a Sephadex filter	Graham et al. (1976)
Serum-induced head-to-head agglutination	Senger and Saacke (1976)
Lipid loss from spermatozoa	Komarek et al. (1964) & Pickett et al. (1975)
Oxygen uptake by sperm	Coulter and Foote (1974)

that this was probably due to the degree with which abnormal cells exist along with morphologically normal motile cells. Sullivan (1970) lists those sperm aberrations which, if ejaculated in sufficient numbers, have been shown to be related to depressed fertility. They include: acrosomal abnormalities (eg. knobbed acrosome); nuclear vacuoles (eg. diadem defect); decapitated sperm; abnormal midpieces and bent or coiled tails. Knobbed acrosomes in high frequency have been associated with sterility (Hancock, 1953) and subfertility (Saacke et al. 1968). Bulls with normal fertility who develop a corkscrew shaped midpiece become subfertile as the frequency of the corkscrew defect increases (Blom, 1959 and Munroe et al., 1961). The dag defect in which the coiled tail is enclosed in a common cell membrane was observed to be a cause of subfertility in the bovine when 40 to 50% of the cells were affected (Blom, 1966 and Blom and Birch-Andersen, 1966).

In some subfertile Guernsey (Hancock and Rollinson, 1949 and Jones, 1962) and Swedish Red and White bulls (Settergren, 1968), more than 50% of sperm heads were separated from the tails. In contrast to the non-motile tailless sperm associated with testicular degeneration, the sperm tails from bulls with this defect were motile (Settergren, 1968 and Blom and Birch-Andersen, 1970). The separation of head and tail was noted to occur within the caput epididymis (Hancock, 1955). Ultrastructurally, this detachment was observed to occur between the basal plate in the head and the capitulum of the neck. Also, the implantation fossa was characterized as small and irregular (Settergren, 1968 and Blom and Birch-Andersen, 1970).

The diadem defect was investigated in a case study reported by Miller et al., (1982). A Simmental bull fit for show with a history of low fertility was examined. A multiform nuclear lesion (diadem defect with nuclear vacuoles along the apical region of the head) occurred in more than 80% of the sperm produced by this bull and was believed to be the cause of the low fertility. The pregnancy rate obtained after natural service and AI was 5 and 8%, respectively. These values were much lower than the fertilization rate assessed in superovulated heifers (18%) and at an embryo transfer center (41%) when this bull was used. These researchers suggested that a high rate of embryonic

mortality due to deficiencies in the male genome was an explanation for the differences between the fertilization rate and pregnancy rate.

Sperm Motility Analysis

Until recently, the human eye and a microscope were the primary tools for evaluating motility and velocity of a population of sperm cells. This procedure is widely recognized as subjective and prone to human error. Track motility, an objective method for evaluating sperm motility, has been used for bulls (Elliott et al., 1973; Revell and Wood, 1978; Amann and Hammerstedt, 1980) and for human (Makler,1978). This procedure involves human evaluation of the tracks produced by motile sperm on a photomicrographic negative generated from a timed exposure to semen under dark field microscopic illumination (Elliott et al. 1973).

O'Connor et al. (1981) determined the correlations between computer evaluations of spermatozoal track motility and conventional laboratory tests of seminal quality. They determined that the computer evaluation technique chosen, although repeatable and precise, offered no advantage over the less costly and more traditional objective methods of evaluating seminal quality. Aitken et al. (1982a,1982b, 1985) and Jeulin et al. (1986) have discussed the significance of amplitude of lateral head displacement (ALH) in human fertility studies. Aitken et al. (1982b) found that ALH was significantly related to fertilizing capacity of human spermatozoa and indicated that accurate measurement in any motility analysis could be of great importance.

Recent advances in electronic technology have permitted construction of hardware to capture, in real time, a series of video images in digital form. Software has been developed to extract information on sperm location in successive video frames and further process it to provide extensive data on sperm motion. Budworth et al. (1988) have detailed the commercial system Cell Soft (CRYO

Resources, Ltd., New York, NY). Katz and Davis (1987) also detailed a computerized motility analysis system, Expert Vision (EV) automatic motion analysis system (Motion Analysis Corp., Santa Rosa, CA). Another system that has not been documented for use with bovine sperm cells is the Hamilton-Thorn Motility Analyzer (HTM-S), Hamilton-Thorn Research, Inc., Danvers, MA. HTM-S was designed to provide a rapid analysis of motility, velocity, linearity (progressivity), ALH, and beat cross frequency (BCF). The HTM-S is a self contained unit consisting of an stroboscopic optical system, a microprocessor, monitor screen and internal printer. The system can be linked to an external printer for hard copy or to a personal computer for data storage. It is critical to recognize that any of these systems must rely on image size, shape, and/or grey scale characteristics to discern spermatozoa and differentiate them from debris present in the field of view.

Seasonal Variation

Seasonal variations in fertility have been reported in the bovine (Kelly and Hurst, 1963; Monty and Racowsky, 1987) and for bulls, specifically (Johnston and Branton, 1953; Sullivan and Elliott, 1968). Lower semen quality during warmer seasons was characterized by increased morphological abnormalities and decreased sperm motility. Sekoni and Gustafsson (1987) studied the seasonal variations in sperm morphology of 19 dairy bulls used for AI in the northern United States. They observed that semen ejaculated during June, July and August had more total morphologically abnormal cells than ejaculates collected during other months of the year. Their explanation for the increased abnormal cells was the heat stress experienced by the animals.

Sullivan (1970) showed that seasonal increases in abnormal sperm cells were accompanied by decreased sperm motility following cryopreservation. These findings were based on year long semen collections of bulls used for AI. During the months of June through September, total abnormalities, predominantly primary abnormalities increased. It was also during this time that there

was an increased frequency of discarded collections due to inadequate sperm motility. When adjustments in sperm dosage per insemination were made to compensate for the abnormal sperm present, NR rates of cows bred with these samples still decreased as the proportion of abnormals in the inseminate increased. Sullivan (1970) suggested that increasing the number of sperm per dose would be valid for poorer quality semen due to low motility; however poor quality semen due to abnormalities would not be compensated for by lower dilution rates. He further proposed that if there were problems with spermatogenesis resulting in detectable abnormal cells, there would be a strong possibility that morphologically and biochemically abnormal cells undetectable to the evaluator could also be present resulting in decreased fertility.

A primary cause of heat stress damage to the spermatogenic process may be injury to the cell membrane or other cytomembranes at critical stages of sperm development (Blackshaw and Hamilton, 1970). If membranes of the sperm cell are indeed injured, one would expect that spermatozoal ability to be cryopreserved would be reduced due to the known ionic imbalances which accompany cryopreservation. These imbalances are due to the hypertonic solute concentration that results as water is withdrawn from suspension media as ice (Mazur, 1980). Parkinson (1987) observed seasonal trends in cryopreserved semen quality of Holstein bulls collected once weekly. The percentage of sperm surviving freezing decreased in July and September revealing a trend toward lower survival rate with higher ambient temperatures.

Climatic Chamber Studies

Other researchers have placed bulls into climatic chambers at elevated temperatures and have demonstrated an increase in the percentage of sperm abnormalities, especially head abnormalities (primary), to be an outcome of these high temperatures. Johnston et al. (1963) exposed bulls (two each of Holstein, Brown Swiss, and the respective F_1 Red Sindhi crosses with these breeds) to a

maximum temperature of 40°C, 54% relative humidity (RH). This temperature and RH was reached by midday and was maintained for 8 h followed by a decrease to a minimum of 27.7°C, 72% RH, for that day. This cycle was maintained for 7 d. Johnston et al. collected two ejaculates weekly and reported a decrease in initial motility, concentration, and total numbers of sperm per ejaculate in response to the treatment. These decreases were more marked in purebred bulls than in crossbreds. They also reported an increase in abnormal spermatozoa. The increase in sperm abnormalities was greater in purebred than in crossbred bulls. Chronologically, the first type of sperm abnormalities observed were coiled or bent midpieces appearing during the first week after insult followed by pyriform heads appearing 2 wk after the end of heat stress, when total abnormal cell content also peaked. Recovery occurred in all bulls by 9 wk after the end of stress, but was most rapid in crossbred bulls.

Skinner and Louw (1966) exposed bulls ejaculated once each week to either 40°C for one 12 h period, one 24 h period, or 6 d of a 24-h cycle of 40°C daytime temperature (12 h) and 26.6°C nighttime temperature (12 h). The initial motility estimates decreased markedly after exposure to 40°C, declining sharply at the second ejaculate (2 wk) after exposure. Recovery was subsequently evident in the 12 h group (3 wk post exposure), but the 24 h and 6 d groups continued to decline in motility until the fifth and fourth weeks, respectively. Increases in tail abnormalities occurred 15 to 22 d after exposure and were mainly represented by decapitated spermatozoa and bent tails. Bulls also showed a marked increase in head abnormalities for all three treatment groups beginning the third week after the initial exposure to high temperatures. The main feature of this increase was the rise in numbers of pyriform heads. The studies of Johnston et al. (1963) and Skinner and Louw (1966) are in agreement that increased primary abnormalities were particularly characterized by pyriform-shaped heads. However, there was a discrepancy in time of occurrence of this sperm abnormality in relation to the onset of heat stress. The earlier appearance of abnormalities in the Johnston et al. study (1963) was probably due to the ejaculation frequency employed compared to the Skinner and Louw (1966) study (two ejaculates/wk vs one ejaculate/wk, respectively), since increased frequency of ejaculation can hasten epididymal transport (Amann, 1987).

Skinner and Louw (1966) also investigated the effect of elevated temperatures on sperm stored in the epididymis. They evaluated this by exposing bulls to 40°C, as in the prior experiment, followed by slaughter 12 h later. Samples of spermatozoa were taken from three areas of the epididymis, stained, and evaluated for abnormalities. There was little difference in the percentage of tail abnormalities in bulls exposed for 12 h or 24 h. However, there were slightly fewer decapitated spermatozoa in the control group. In the bulls exposed to daily fluctuations in temperature (40 to 26.6°C for 6 d group) there was an increase in the percentage of tail abnormalities at all three epididymal sites, mainly due to the increase in decapitated spermatozoa. In order to determine the site of damage in the testis, bulls were slaughtered 5 d after exposure to 40°C for the previously described periods. Testicular tissue was fixed and microscopically examined for changes in cellular content. In the 24 h and 6 d groups there was only evidence of vacuolation of spermatids. Skinner and Louw concluded that spermatids and spermatozoa in the final stages of development were most susceptible to an ambient temperature of 40°C. They further suggested that vacuolation of the spermatids in stage 2 of the cycle of the seminiferous epithelium may also account for the sharp increase in pyriform heads during the third and fourth week after exposure. Ortavant (1959) stated that in the bull the base of the spermatid nucleus contracts during stage 2 of the cycle of the seminiferous epithelium and that without doubt, pyriform spermatozoa are formed at this stage.

A study by Meyerhoeffer et al. (1985) demonstrated that bulls exposed to elevated temperatures for 8 wk produced semen of a lower quality based on decreased sperm motility and increased percentages of abnormal and aged sperm (deteriorated acrosomes). In their study, 16 yearling bulls were assigned to two groups. Eight bulls were heat stressed by exposure to 35°C. for 8 h followed by 31°C for 16 h. The remaining eight bulls were controls held at 23°C during each 24-h period. Semen was collected by artificial vagina twice weekly. Ejaculate volume was not altered by treatment but, the percentage of motile sperm decreased in stressed bulls by 2 wk after the start of the heat treatment. Motility returned to normal values 16 wk after the onset of heat treatment. The percentage of aged acrosomes (false acrosome reaction) of stressed bulls increased by the second week of treatment and remained greater than controls throughout the stress period. The aged

acrosome was present from the second week of treatment until 15 to 16 wk after onset of heat stress. Total sperm per ejaculate in general was not affected by heat treatment.

Climate Controlled Studies in Other Species

The effect of heat on viability and morphology of spermatozoa in other species has also been documented. Moule and Waites (1963) examined semen collected twice weekly for 9 wk from 12 Merino rams before and after they were exposed during 3 d for two 6-h periods in a climatic chamber at 40.5°C, 8.5 mm Hg vapor pressure and 40.5°C, 31.5 mm Hg, respectively with a 42-h separation period in between exposure periods. Semen of decreased quality was collected from all rams after this treatment. However, the effect varied in severity and duration. The first ejaculate containing abnormal spermatozoa was collected 13-21 d after the treatment. Coiling and loss of tails were the two morphological abnormalities most often observed. The three rams which were most severely affected suffered a seminal degeneration which lasted for 35 to 39 d and was characterized by a sperm concentration decline on d 14 after treatment which remained low between d 21 and 32.

After recovery from the previous experiment the same rams were again divided into three groups. Groups 1 and 2 were exposed to the two 6-h heat treatments previously described. Group 3 was placed in the climatic chamber for 12 h at 21°C and 6.8 mm Hg vapor pressure (control). A subgroup of rams from Groups 2 and 3 were supplied with a cooling mechanism providing water at 17 to 19°C circulating around the scrotum. Semen samples were collected twice weekly for 3 wk before and 12 wk after the experimental treatments. Only the rams exposed to heat without their scrota cooled by circulating water produced semen of inferior quality. Effects of heat treatment observed were decapitated spermatozoa and decreased motility. Moule and Waites concluded that under the conditions of the experiment, the efficiency of the heat dissipating properties of the

scrotum alone affected the magnitude of the seminal response to short periods of elevated air temperature.

Wettemann et al. (1976) investigated the effect of heat stress in males on embryonic mortality. Semen from control (23°C) and heat stressed (34°C for 12 h followed by 30°C for 12 h daily for 90 d) boars was collected twice weekly, extended, and used to inseminate gilts. Evaluation of seminal characteristics revealed no changes in semen volume during the elevated ambient temperature. However, sperm output was reduced in stressed boars during wk 2 through wk 6 of treatment. Also, a decrease in motility and percent normal cells was observed by the second week of treatment. Gilts were bred with semen from control and heat stressed boars to determine the effect of this treatment on the fertility in swine. Results of inseminating gilts with the heat stressed semen revealed a reduction in conception rate and pregnancy. Gilts were slaughtered on d 30 ± 3 of pregnancy and the numbers of corpora lutea and normal embryos (based on embryo length) were recorded. Only 28.6% of gilts bred with semen from heat stressed boars conceived compared to 41.2% of gilts bred with control semen. Embryonic survival at 30 ± 3 d of pregnancy was 71.2 ± 3.7% in gilts bred with semen from control boars and 48.5 ± 5.2% for gilts bred with semen from stressed boars. Since gilts were bred with equal numbers of sperm from heat stressed or control boars, it is apparent that the quality of spermatozoa was important to fertility and embryonic survival.

Scrotal Insulation Studies

Local heating or insulation of the scrotum has resulted in reduced sperm output and semen characterized by reduced viability and normal morphology. Ross and Entwistle (1979) studied the effect of scrotal insulation on semen quality and rate of spermatogenesis. Five beef bulls were selected to undergo scrotal injection with ^{3}H-thymidine and scrotal insulation either for 0, 10, or 20 h. Only

one bull was assigned as a control (0 h). All bulls were collected by electro-ejaculation at 3 or 4 d intervals for 3 wk immediately before the experiment commenced. After injection of ^{3}H-thymidine, ejaculates were collected at the same frequency for a further 18 wk from one bull from the 10-h and one bull from the 20-h groups. In order to determine the rate of spermatogenesis, unilateral castration was performed on a bull from each treatment group. The cycle of the seminiferous epithelium was estimated to be 13.4 d with a range of 12.9 to 13.6 d. Ross and Entwistle determined that length of the seminiferous epithelium cycle was unaltered by the treatment indicating that rate of sperm production did not change in response to scrotal insulation. The ejaculate characteristics of the remaining two bulls were similar after heat insult. The percent live and percent motile sperm ejaculated began to decline between 3-5 wk after insult. In both bulls, a slight recovery was observed followed by an additional decline in live sperm between 6 and 8 wk. By 13 wk, recovery of sperm viability was largely complete in both bulls. The percentage of morphologically abnormal spermatozoa began to increase approximately 3 wk after scrotal heating. High levels of abnormalities (both primary and secondary) were observed in ejaculates between 3 and 6 wk after treatment and again 11 to 13 wk, after which they returned to pre-treatment levels. Abnormal heads and tails (coiled and bent) as well as proximal droplets were observed after heat stress in both bulls. Ross and Entwistle concluded that round and elongating spermatids were susceptible to injury after scrotal temperature elevation and indicated that elongated spermatids and epididymal spermatozoa were not affected by scrotal insulation.

Wildeus and Entwistle (1983) investigated further the effects of scrotal insulation on morphological changes and sperm production rates. Ten bos taurus x bos indicus bulls were assigned randomly to two groups (control and heat treatment). Temperature probes were implanted subcutaneously into the scrotum of each bull and an insulation pouch was placed around the scrotum and glued to the abdominal wall on five bulls (treated). These pouches remained in place for 48 h. The remaining five bulls served as controls. Semen was collected by electro-ejaculation for 23 d after insult at 2-d intervals for the first 14 d and then every third day until slaughter on d 23. The mean scrotal temperature difference between treated and control bulls was 4.05°C. They found no sig-

nificant effect of scrotal insulation on sperm output per ejaculate. However, they reported an increase in the percentage of abnormal spermatozoa. There was an increase in the incidence of decapitated sperm in bulls 6-14 d after scrotal insulation. The percentage of abnormal tails increased at 12 d and those with protoplasmic droplets at 17 d after insulation, the latter remaining high until slaughter at 23 d. The incidence of head abnormalities was not reported. The percentage of spermatozoa with abnormal acrosomes increased significantly 12 d after insulation. Wildeus and Entwistle (1983) noted that response varied among animals despite the fact that they achieved a uniform increase in scrotal temperature among bulls.

Detection of the Interaction of Semen Traits and Environmental Temperature

Previously, research evaluating the effects of high environmental temperatures on semen and spermatogenesis utilized conventional brightfield and phase contrast microscopes with conventional fixation and staining procedures. The use of differential interference contrast microscopy (DIC) has more clearly revealed the presence of abnormalities of the bovine sperm head such as craters and diadem defects, both a form of nuclear vacuoles, (Coulter et al., 1978). Also, development of DIC has made possible another objective measure of sperm viability by virtue of acrosomal integrity evaluations using unfixed wet semen smears (Saacke and Marshall, 1968). Utilizing DIC, Saacke and White (1972) showed that cells surviving freeze-thawing, experience more rapid acrosomal alteration than unfrozen cells during the first 4h of incubation at 37°C and that the rate of this change is associated with fertility. Therefore, the aging of semen apparently conveys additional information of freeze-thaw induced cell injury. Thus, if heat insult to the bull testis changes membranes of the bovine sperm cell, epididymal spermatozoa ejaculated after treatment should show a lower ability to be cryopreserved using these methods. Decreased ability to be

cryopreserved would especially be reflected in the post thaw incubation of cryopreserved semen. On this basis, the DIC optics were utilized in this study to evaluate the viability of fresh and cryopreserved semen as well as sperm morphology in relation to the thermal stress explored.

MATERIALS AND METHODS

Semen Collection

Six Holstein bulls were selected on their ability to produce semen having greater than 70% morphologically normal sperm with 60% or greater estimated progressive motility. Five bulls ranged in age from 14 to 17 mo and one bull was 34 mo of age. Semen was collected via artificial vagina at a frequency of two ejaculates (in succession) every 3 d. Bulls were located at the Bull Research Barn, Virginia Polytechnic Institute and State University, Dairy Cattle Center, Blacksburg, Virginia. The collection of each ejaculate was preceded by a period of sexual preparation consisting of two false mounts separated by 2 min of active restraint.

Thermal Insult to the Testes

The purpose of the thermal insult to the testes was to mimic a mild, naturally occurring environmental interference with testicular thermoregulation. To minimize potential stress from excessive intervention, no effort was made to establish or maintain a uniform elevation in testicular temperature during the insult period. Thermal insult of testes was induced by enclosing the scrotum, including the neck, in a sack constructed of an insulated material. After 30 min, allowing for thermal response of the scrotum to the elevated temperature, the sacks were readjusted to assure complete coverage of the scrotum and scrotal neck. Sacks were fixed in place with Velcro and medical tape. The thermal insult was maintained for a period of 48 h. Bulls were placed on experiment at different times of the year specifically, June, September, October, 1989 and February, 1990. Ambient temperatures and relative humidities were recorded daily, based on 10 min measurements averaged every 2 h, throughout the trial by a CR21 Micrologger, (Campbell Scientific Inc., Logan, Utah). Scrotal surface temperatures were measured when sacks were put on (immediately after readjustment) and again, just before removal using a type T flat thermistor probe attached to a digital thermocouple thermometer (Cole-Parmer Instruments, Chicago, IL). Following final placement of the sack, bulls were partially restrained by halter such that they had the freedom to lie down, stand, eat and drink. This partial restraint was designed to prevent premature removal of the sacks and insure proper bedding, dry conditions, and general comfort.

Experimental Design

Semen was collected at a frequency of two ejaculates in succession every third day beginning one week prior to actual data collection. Data collection began 6 d prior to installation of the scrotal sack and continued until d 39 after installation of the sack. In order to evaluate the vulnerability

to heat stress of spermatozoa in the epididymis and rete testis compared to those in stages of spermatogenic development (seminiferous tubules), three periods of semen collection were defined for statistical analysis.

Period 1 = pre-insult, ie., collections on d -6, -3, and 0, where d 0 represents installation of the scrotal sack after semen collection on that day (control period)

Period 2 = post-insult, ie., collections on d 3, 6, and 9 after installation of the scrotal sack

Period 3 = post-insult, ie., collections on d 12, 15, 18...39 after installation of the scrotal sack

Epididymal transport has been estimated at between 8 and 11 d in bulls (Orgebin-Crist, 1962) and one cycle of the seminiferous epithelium has been documented to be 13.3 to 13.5 d (Amann and Almquist, 1962; Amann, 1970). Therefore, the basis of these three Periods was a separation of collections into a control or baseline period (Period 1), a period when sperm ejaculated would have been present in the epididymis and rete testis at the time of heat insult (Period 2), and a period when ejaculated cells would have been undergoing spermatogenesis at the time of heat insult and thus, where abnormalities in the ejaculated sperm would be expected to become apparent and peak (Period 3).

Neat Semen Evaluation

Immediately after collection, semen was evaluated for volume, estimated in graduated collection tubes; concentration, determined by previously calibrated spectrophotometer; and percent progressive motility, qualitatively estimated to the nearest 10% from wet smears examined under a phase contrast microscope equipped with a heated stage (37°C). Sperm abnormalities were quantified by differential count from wet smears using differential interference contrast microscopy at 1000x

magnification. One hundred microliters of pooled semen from the two ejaculates obtained on each day was fixed in 1 ml of Karnovsky's fixative (Karnovsky, 1965) and placed in a 1.5 ml Eppendorf tube. Evaluation of percent morphologically abnormal sperm was performed on the fixed sample on the day of collection. Counts of 100 sperm cells from each of two smears were averaged for each sample. The percent normal and percent abnormal sperm were derived from this count. Cells with multiple abnormalities were only counted once during the 100 cell count but, each abnormality of a particular cell was recorded. Thus, preemption of one cell abnormality by another was avoided. On this basis, actual percentages of primary, secondary, and tertiary abnormalities were reported and could be collectively greater than the total percent abnormal cells in the sample. Specific cell abnormalities were identified by the classifications of Mitchell et al. (1978) and Barth and Oko (1989).

Freezing Semen

On each collection day, the two ejaculates from each bull were pooled and prepared in a conventional manner for cryopreservation in egg yolk citrate-glycerol extender. Prior to use, extender was clarified by filtering through a glass prefilter connected to a .45 μm Acrodisc filter (Gelman Sciences, Ann Arbor, MI). See Appendix A for extender composition. Semen was extended in the non-glycerol fraction (A) within 15 min and allowed to cool slowly (1.5 to 2.0 h) to 5°C before adding Fraction B in 4 increments at 10 min intervals. Semen was then aspirated into .5 ml French straws at a final concentration of 50 million cells per ml. Straws were frozen 24 $\pm$ 2 h post collection in liquid nitrogen vapor for 10 min followed by plunging into liquid nitrogen. Monitored freeze rates were within acceptable ranges for minimal effect on post-thaw viability (Robbins, et al., 1976). Straws were stored in liquid nitrogen for a minimum of 2 wk before evaluation. Additional clarified extender was frozen in straws to adjust the concentration of thawed semen for computer aided

motility analysis at a later time. Freezing of ejaculates continued until morphologically abnormal sperm exceeding 50% of the ejaculate was evident.

Semen Viability Evaluation

A. Pre-freeze

The contents of three straws from each ejaculate pair (day) were pooled into a 1.5 ml Eppendorf tube and evaluated for viability prior to freezing (approximately 20 to 26 h post semen collection). Evaluation was carried out after warming the semen from 5°C to 37°C in a 37°C dry bath. Evaluation was conducted within 2 min of warming (0 h) and after a 3-h incubation period at 37°C. The percent progressive motility was estimated at 250x under a phase contrast microscope equipped with a heated stage, as previously described. Acrosomal integrity was determined objectively at 0 and 3 h of 37°C incubation, using differential interference contrast microscopy, by determining percent intact acrosomes (PIA) based on the presence of the apical ridge (Saacke and Marshall, 1968). Counts of one hundred sperm cells from each of two smears were averaged.

B. Post Thaw

After at least 2 wk storage at -196°C, three straws from each bull/collection day were thawed in water at 35°C for 45 sec, the contents were pooled and evaluated for viability using the same procedure as described for pre-frozen semen.

Quantitative Motility Analysis:: Straws of frozen semen were also evaluated using the Hamilton-Thorn Motility Analyzer (HTM-S version 7.2n), a computerized quantitative evaluation of track motility and sperm velocity. Since this apparatus had not been validated for use with frozen-thawed bovine spermatozoa, a short validation procedure was used (See Appendix B for validation procedure). The ability of the system to measure percent motile cells above 10μm/sec validated. However, the ability to measure velocity of frozen-thawed sperm did not validate. Thus, only the motility evaluation was used. Three straws of semen were thawed as described above and diluted with clarified extender to a concentration of 15 x 10^6 spermatozoa/ml. Twenty microliters of the diluted semen was placed in a Microcell slide (distributed by Hamilton-Thorn, Danvers, MA) known to have a volume of 10 μl per each of two chambers. Samples from each collection day were analyzed in four replicates within a period of 12 min from thawing for the following parameters:

Percent Motile Cells = the fraction of total cells for which path velocity (the five point average cell velocity in μm/sec) > 10μm/sec.

Percent Progressively Motile Cells = fraction of all cells moving with a path velocity > 10 μm/sec and a threshold straightness (progressive velocity/track speed) of 90%.

Statistical Analysis

All statistical analyses were conducted with programs available through the Statistical Analysis System (SAS, 1985). Data were analyzed by general linear models procedure as well as non-orthagonal contrasts of Period 1 with Period 2 and Period 1 with Period 3 to detect changes in viability measurements. Since semen was frozen only until total sperm abnormalities reached 50%, period 3 consisted of only d 12 for two bulls, d 12 and 15 for two bulls, d 12, 15, and 18 for one

bull, and d 12 through 21 for one bull. For evaluation of morphology, Period 3 consisted of d 12 through d 39 post insulation for all bulls. Morphological counts of total, primary, secondary, and tertiary abnormalities were analyzed by general linear models procedure as well as non-orthagonal contrasts of Period 1 with Period 2, Period 1 with Period 3, and Period 2 with Period 3. The Bonferroni F test for significance levels was used to distinguish significance among specific periods, due to the non-orthagonality of the contrasts. Means and standard deviations were used to detect increases in specific abnormal cellular types by specific day of appearance.

The model used for analysis of variance for the neat semen characteristics of initial motility, morphology, and sperm output was as follows:

$$Y_{ijk} = \mu + B_i + P_j + BP_{ij} + D_{(j)k} + e_{ijk}$$

where

Y_{ijk} = percent initial motility, percent total, primary, secondary, and tertiary abormalities or sperm output per collection day ($x10^9$)

μ = overall mean

B_i = the random effect of the ith bull (i = 1,2,3...6)

P_j = the fixed effect of the jth period (j = 1,2, or 3)

$D_{(j)k}$ = the fixed effect of the kth day within the jth period (k = 1,2,3 or 1,2,3...10)

e_{ijk} = random error

The model used for analysis of variance for the computerized motility evaluation of percent motile sperm and percent progressively motile sperm was as follows:

$$Y_{ijk} = \mu + B_i + P_j + BP_{ij} + H_k + BH_{ik} + PH_{jk} + BPH_{ijk} + e_{ijk}$$

where

Y_{ijk} = percent motile spermatozoa or percent progressively motile spermatozoa

μ = overall mean

B_i = the radom effect of the ith bull (i = 1,2,3...6)

P_j = the fixed effect of the jth period (j = 1,2, or 3)

H_k = the fixed effect of the kth hour (k = 1 or 2) 0h or 3 h

e_{ijk} = random error

The model used for analysis of variance for percent intact acrosomes and percent motility evaulated before and after cryopreservation was as follows:

$$Y_{ijkl} = \mu + B_i + P_j + BP_{ij} + F_k + BF_{ik} + PF_{jk} + BPF_{ijk} + H_l + BHil + PH_{jl} + BPH_{ijl} + FH_{kl} + BFH_{ikl} + PFH_{jkl} + e_{ijkl}$$

where

Y_{ijkl} = percent motile spermatozoa or percent intact acrosomes

μ = overall mean

B_i = the random effect of the ith bull (i = 1,2,3...6)

P_j = the fixed effect of the jth period (j = 1,2, or 3)

F_k = the fixed effect of the kth freeze (k = 1 or 2) frozen or unfrozen

H_l = the fixed effect of the lth hour (l = 1 or 2) 0 h or 3 h

e_{ijkl} = random error

RESULTS

Overall effects of scrotal insulation on neat semen quality (motility and morphology) and quantity (sperm output) are presented in Figure 1. A notable increase in total abnormalities (averaged over all bulls) was evident in Period 3, compared to Period 1 ($P \leq .01$) or Period 2 ($P \leq .01$). Corresponding to this increase in total abnormalities was a lower sperm motility for Period 3 compared to either Period 1 ($P \leq .01$) or Period 2 ($P \leq .01$). Also, for neat semen, sperm motility did not differ between Periods 1 and 2. Sperm output per collection day (2 ejaculates) was depressed only in Period 3 relative to Period 2 ($P \leq .05$). Based upon the relatively small reduction in sperm output and motility, the heat insult was considered mild (ie., no destruction of the germinal epithelium resulting in marked reduction in sperm output). The analyses of variance for sperm output,motility, and morphology based on the neat semen are presented in Tables 1, 2, and 3, Appendix C. The influence of individual bulls on sperm output in response to thermal stress is presented in Table 2. Only bull 9827 showed an apparent decrease in sperm output from Period 1 to 2 and again from 2 to 3. Whereas, other bulls revealed an elevated sperm output in Period 2 over both Periods 1 and 3.

The range in ambient temperature experienced by bulls during thermal insult was marked. The temperature recorded at the weather station during thermal insults ranged from -4.9°C to 33.7°C. Variation from maximum and minimum ambient temperatures, experienced by bulls, during the

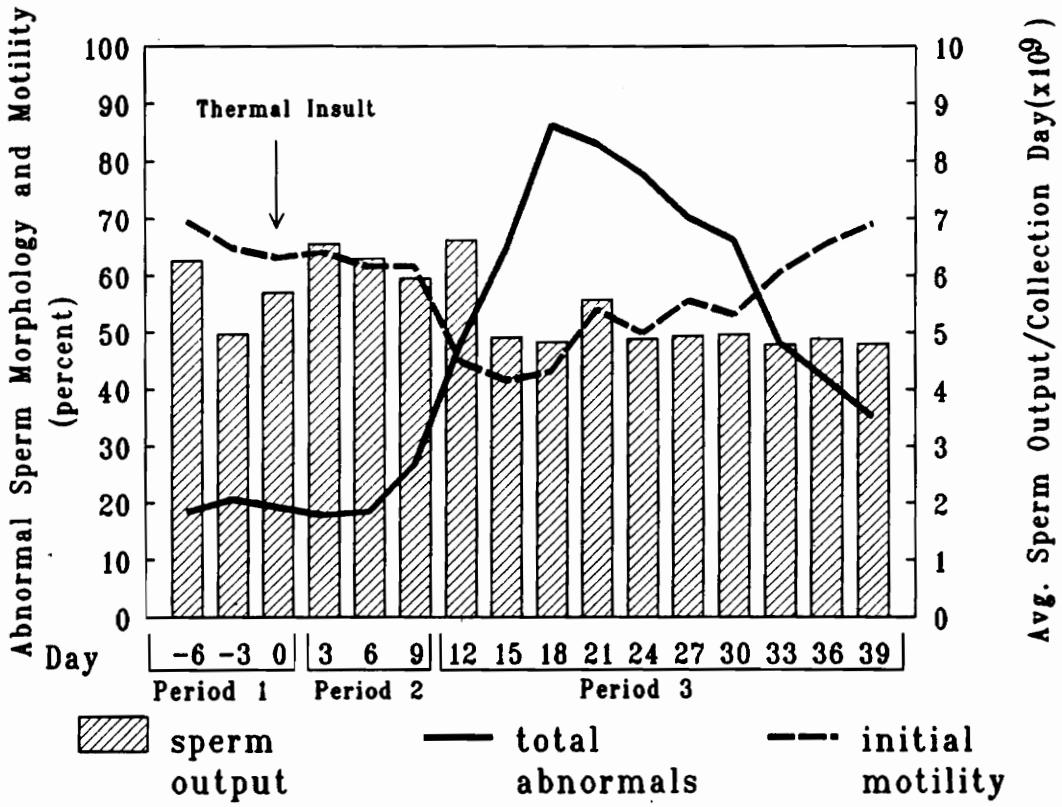

Figure 1. Effects of 48 h scrotal insulation (day 0, Period 1) on morphology and motility of neat semen and mean sperm output (n = 6 bulls).

Table 2. BULL VARIATION IN SPERM OUTPUT PER COLLECTION DAY (x 10^9) BEFORE AND AFTER 48 H SCROTAL INSULATION (DAY 0, PERIOD 1)

Bull	Period 1 [a]	Period 2 [b]	Period 3 [c]
1825	3.87±1.44 [d]	5.04±2.76	3.49±1.73
1914	2.48±1.72	2.80±1.77	2.15±1.64
1921	2.10± .54	2.84±1.57	2.26± .61
1922	3.15±1.86	3.19±1.45	2.92±1.25
2004	2.12±1.44	2.63±1.31	2.44±1.32
9827	3.16±1.03	2.34±1.41	1.64±1.41

a Period 1 = day -6, -3, 0 pre-insult
b Period 2 = day 3, 6, 9 post insult
c Period 3 = day 12, 15...39 post insult
d Mean±SD for days of semen collection within each period

48 h scrotal insulation ranged from 9.6 to 17.0°C. Relative humidities during 48 h thermal insults ranged from a low of 29.7% to a high of 93.9%. Scrotal surface temperatures increased from 33°C directly after scrotal insulation to 36°C (maximum) after removal of the thermal sack.

Viability Characteristics

The effect of heat insult on the viability characteristics of unfrozen and frozen extended semen are presented in Tables 3 and 4, respectively. For unfrozen semen, a decrease in percent of viable sperm was observed between Periods 1 and 3 for both parameters (motility and intact acrosomes) initially (0 h) and after incubation (3 h) at 37°C ($P \leq .01$). Sperm ejaculated during Period 2 were not different from cells ejaculated during Period 1 for these same viability components. As noted in Table 4, frozen-thawed cells obtained during Period 3 were also of lower viability (motility and intact acrosomes) initially (0 h) and after incubation (3 h) compared to Period 1 ($P \leq .01$). However, different from unfrozen semen, relative to Period 1, frozen sperm cells from Period 2 exhibited lower acrosomal integrity and depressed motility after 3 h incubation ($P \leq .01$). Analyses of variance for the fresh and frozen extended semen for motility and acrosomal integrity is presented in Tables 4 and 5, Appendix C.

To aid visualization of viability differences between Period 1 (control) and Period 2, which represented sperm that were in the epididymis or rete testis at the time of thermal insult, the data in Table 5 were assembled. Differences between Period 1 and 2 relative to freezing and post-thaw incubation are apparent. Both freezing and incubation of ejaculated semen independently appeared to improve the ability to detect a thermal effect on sperm in the epididymis or rete testis at the time of the thermal insult. The effects of freezing and incubation were additive as apparent by the greater difference for motility and PIA of freeze-thawed semen after incubation at 37°C for 3 h. Variation among bulls based upon the effect of heat on epididymal/rete testis sperm was to be expected.

Table 3. EFFECT OF 48 H SCROTAL INSULATION (DAY 0, PERIOD 1) ON MEAN VIABILITY OF UNFROZEN EXTENDED SEMEN BEFORE (0 H) AND AFTER (3 H) INCUBATION AT 37°C (N = 6 BULLS)

Period*	% Motility		% Intact Acrosomes	
	0 h	3 h	0 h	3 h
1	66.4 [a]	56.1 [a]	86.0 [a]	79.6 [a]
2	62.5 [a]	47.1 [a]	82.2 [a]	74.6 [a]
3	41.4 [b]	25.7 [b]	62.4 [b]	49.9 [b]

Column values with different letters are significantly different ($P \leq .05$).

* Period 1=day -6, -3, 0 pre-insult
Period 2=day 3, 6, 9 post insult
Period 3=day 12, 15, until morphologically abnormal cells reached >50%

Table 4. EFFECT OF 48 H SCROTAL INSULATION (DAY 0, PERIOD 1) ON MEAN VIABILITY OF FROZEN EXTENDED SEMEN BEFORE (0 H) AND AFTER (3 H) INCUBATION AT 37°C (N = 6 BULLS)

Period *	% Motility		% Intact Acrosomes	
	0 h	3 h	0 h	3 h
1	55.3 [a]	46.4 [a]	79.3 [a]	73.0 [a]
2	48.6 [a]	30.8 [b]	73.8 [a]	62.8 [b]
3	37.8 [b]	12.1 [c]	54.3 [b]	40.7 [c]

Column values with different letters are significantly different ($P \leq .05$).

* Period 1 = day -6, -3, 0 pre-insult
Period 2 = day 3, 6, 9 post insult
Period 3 = day 12, 15, until morpholgically abnormal cells reached >50%

Table 6 reveals the variation in difference among bulls comparing semen produced in Period 1 and Period 2 on the basis of motility and PIA measurements after semen was frozen, thawed, and incubated for 3 h. As may be noted, bull no. 2004 was a low responder to the heat insult, while bull no. 1921 had a high response and others were intermediate in their response.

Computer-aided motility evaluation of frozen-thawed semen utilizing the Hamilton-Thorn motility analyzer (HTM-S) is presented in Table 7. Decreases in percent motile as well as progressively motile cells were observed before and after 3 h incubation at 37°C, distinguishing semen produced in each Period from that produced in another ($P \leq .01$). Analyses of variance for computer aided motility parameters are presented in Tables 6 and 7, Appendix C. Comparisons of the subjective post-thaw motility estimates with the computerized post-thaw motility measurements are presented in Table 8. The HTM-S was able to detect changes in motility of samples collected during Period 2 compared to Period 1 collections at 0 h incubation before these changes would be detected by the human eye aided by phase contrast microscopy. At 3 h incubation differences between Period 2 and Period 1 as well as Period 3 (0 and 3 h incubation) were detected subjectively and through computerized means.

Morphological Characteristics

The effects of scrotal insulation on sperm morphology characteristics (total abnormalities across all bulls) are presented in Figure 1. The total abnormalities in semen obtained during Period 3 was significantly greater than that for Period 1 ($P \leq .01$) or Period 2 ($P \leq .01$). Despite the slight increase in total abnormalities apparent on d 9 (Period 2) there was no significance associated with this increase, i.e., Period 1 did not differ from Period 2 in total abnormal sperm. This apparent increase was due to secondary abnormalities (protoplasmic droplets) on d 9 for two bulls (no. 9827 and 1914).

Table 5. EFFECTS OF 48 H SCROTAL INSULATION ON PERCENT MOTILITY (MOT) AND INTACT ACROSOMES (PIA) BEFORE AND AFTER FREEZING EXPRESSED AS DIFFERENCES BETWEEN PERIOD 1 AND 2

	0 Hour Incubation			
	Unfrozen		Frozen	
	Mot	PIA	Mot	PIA
Period 1 [a]	66.4	86.0	55.3	79.3
Period 2 [b]	62.5	82.2	48.6	73.8
Difference	-3.9	-3.8	-6.7	-5.5
	3 Hour 37°C Incubation			
	Unfrozen		Frozen	
	Mot	PIA	Mot	PIA
Period 1	56.1	79.6	46.4	73.0
Period 2	47.1	74.6	30.8	62.8
Difference	-9.0	-5.0	-15.6	-10.2

a Period 1 = d -6, -3, 0 pre-insult
b Period 2 = d 3, 6, 9 post insult

Table 6. VARIATION IN PERCENT MOTILITY AND INTACT ACROSOMES (PIA) AMONG BULLS: DIFFERENCE BETWEEN PERIOD 1 (CONTROL) AND PERIOD 2 (POST THERMAL INSULT) AFTER INCUBATION (37°C)

Bull#	Motility	PIA
1825	-28.4	- 9.8
1914	-15.0	- 4.1
1921	-43.4	-26.3
1922	1.6	-24.1
2004	5.0	- 0.4
9827	-13.4	3.3
	$\bar{X}$ = -15.6	$\bar{X}$ = -10.2

Table 7. COMPUTER-AIDED ANALYSIS OF FROZEN-THAWED SEMEN PRODUCED BEFORE AND AFTER 48 H SCROTAL INSULATION (DAY 0, PERIOD 1) AND BEFORE AND AFTER INCUBATION AT 37°C FOR 0 OR 3 H

	% Progressive Motility		% Motile	
	0 h	3 h	0 h	3 h
Period 1*	36.5 a	30.2 a	52.3 a	37.5 a
Period 2**	30.1 b	17.6 b	44.1 b	23.8 b
Period 3***	18.7 c	12.0 c	27.5 c	16.3 c

Column values with different letters are significantly different ($P \leq .01$).

* Period 1 = d -6, -3, 0 pre-insult

** Period 2 = d 3, 6, 9 post insult

*** Period 3 = d 12, 15, until morphologically abnormal cells in the ejaculate reach >50%

% progressively motile=the fraction of all cells moving with a path velocity (the five point average cell velocity in μm/sec) >10μm/sec and a threshold straightness of 90%

% motile=the fraction of all cells for which path velocity >10μm/sec

Table 8. COMPARISON OF SUBJECTIVE (PHASE CONTRAST ESTIMATED) AND COMPUTERIZED MOTILITY MEASUREMENTS BEFORE AND AFTER 48 H SCROTAL INSULATION

	Incubation	Period 1	Period 2	Period 3
% Motility (estimated)	0 h	55.3 [a]	48.6 [a]	37.8 [b]
	3 h	46.4 [a]	30.8 [b]	12.1 [c]
% Motility (Computerized)	0 h	52.3 [a]	44.1 [b]	27.5 [c]
	3 h	37.5 [a]	23.8 [b]	16.3 [c]

Row values with different letters are significantly different ($P \leq .05$).

*period 1 = d -6, -3, 0 pre-insult
**period 2 = d 3, 6, 9 post insult
***period 3 = d 12, 15, until morphologically abnormal cells in the ejaculate reach >50%

The effect of thermal insult to the bull testis on the incidence of primary (head), secondary (protoplasmic droplet), and tertiary (tail) abnormalities is presented in Figure 2. It is evident from Figure 2 that the increase in total abnormalities observed in Period 3 (Figure 1), as compared to levels in Period 1, was mainly due to an increase in primary abnormalities ($P \leq .01$). Although secondary abnormalities showed an apparent increase in Period 3, the level of this abnormality was not significantly different from levels produced in Periods 1 or 2. Tertiary abnormality increases observed in Period 3 were significantly different from levels produced in Period 1 ($P < .01$) and in Period 2 ($P < .01$). Analyses of variance for primary, secondary, and tertiary abnormalities are presented in Tables 8, 9, and 10, Appendix C.

The first obvious sperm morphology change observed among bulls was with primary abnormalities first noted on d 12 post initiation of the insult (Figure 2). The percentage of total abnormalities peaked on d 18 post insult and returned to near normal values by the last collection on d 39 post insult (Figures 1 and 2). The morphological changes observed during Period 3 consisted of specific types which were indicative of heat stress. Bulls varied with respect to the degree and time of response for specific abnormalities post insult; however, bulls exhibited the same sequence of appearance for each specific abnormality produced. The sequence and peak means for specific abnormalities (averaged over all bulls) are presented in Figure 3. A peak in decapitated spermatozoa (mean $\pm$ sd = 33.9 ± 28.6) occurred first (d 15) followed by a peak in cells with the diadem defect (mean $\pm$ sd = 55.6 ± 25.8) on d 18. Pyriform heads and nuclear vacuoles (excluding diadems) were observed next on d 21 (means $\pm$ sd = 18.3 ± 17.6 and 24.8 ± 13.8, respectively). Knobbed acrosomes (d 27) and dag defects on d 30 (means $\pm$ sd = 11.8 ± 13.5 and 3.2 ± 2.4, respectively) were the final abnormalities observed before the quality of ejaculates returned to near normal levels. The variance among bulls in sperm morphology response to thermal stress during each period is presented in Tables 9, 10, and 11 for primary, secondary, and tertiary abnormalities, respectively.

The difference among each of the six bulls in degree or depth of response to the heat stress based on the incidence of the six specific abnormal cell types ejaculated in Period 3 can be observed from

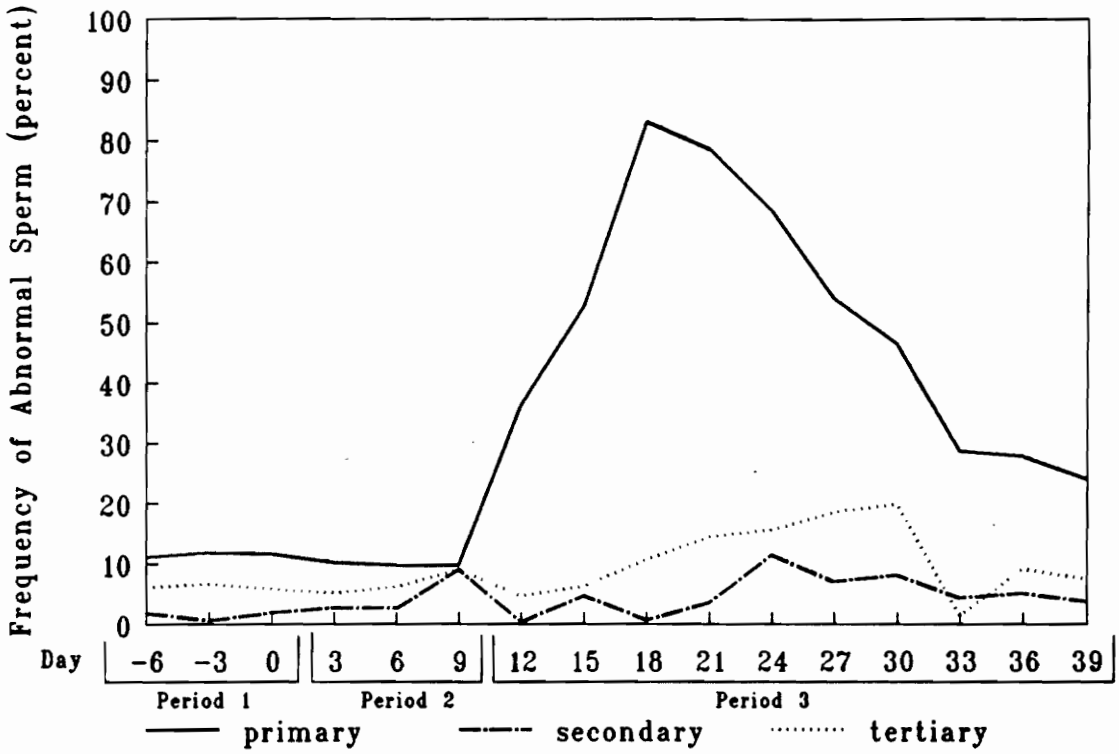

Figure 2. Incidence of primary, secondary, and tertiary abnormalities before and after 48 h scrotal insulation (day 0, Period 1) n = 6 bulls.

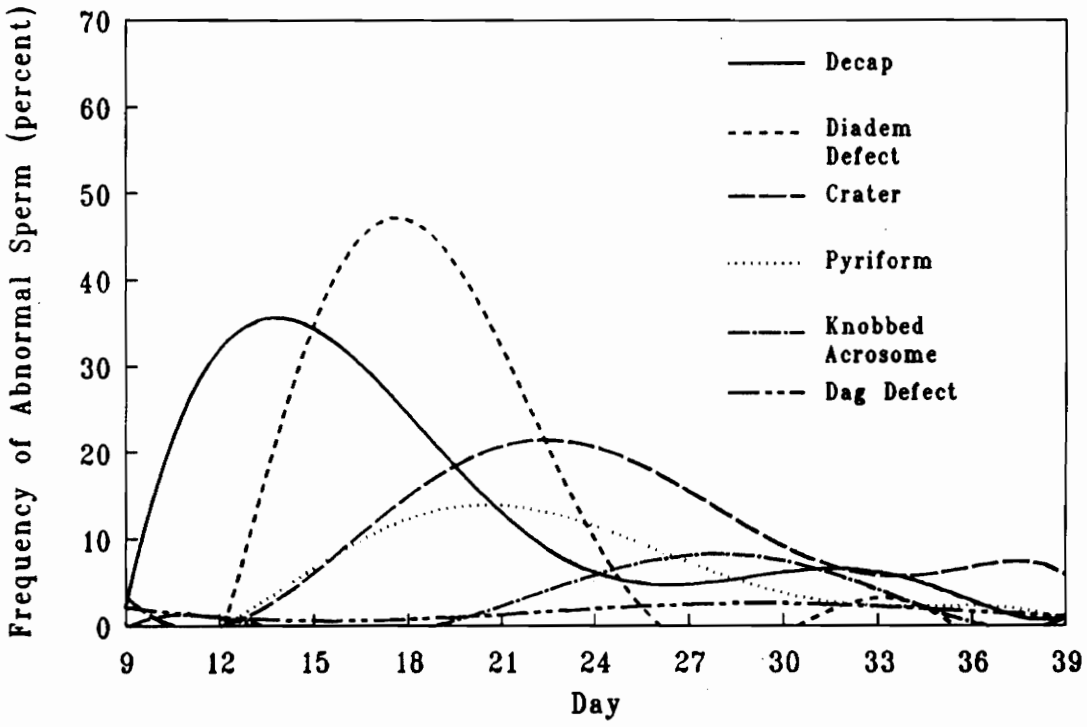

Figure 3. Least square polyomial regression for the incidence of specific abnormal cell types beginning 9 d after 48 h scrotal insulation (n = 6 bulls).

Table 9. BULL VARIATION IN PERCENT PRIMARY ABNORMALITIES (MEAN ± SD) BEFORE AND AFTER 48 H SCROTAL INSULATION (DAY 0, PERIOD 1)

Bull	Period 1 [a]	Period 2 [b]	Period 3 [c]
1825	6.0±3.0	7.0±1.8	48.1±34.5
1914	15.5±1.7	10.8±4.0	64.8±27.3
1921	8.2±0.8	7.8±0.6	47.2±20.0
1922	10.7±0.8	14.2±1.8	51.2±22.2
2004	12.0±1.3	8.0±0.5	23.3± 7.1
9827	17.0±4.3	11.8±1.1	69.2±31.3

a Period 1 = d -6, -3, 0 pre-insult
b Period 2 = d 3, 6, 9 post insult
c Period 3 = d 12, 15...39 post insult

Table 10. BULL VARIATION IN PERCENT SECONDARY ABNORMALITIES (MEAN ± SD) BEFORE AND AFTER 48 H SCROTAL INSULATION (DAY 0, PERIOD 1)

Bull	Period 1[a]	Period 2[b]	Period 3[c]
1825	3.7±1.5	4.2± 1.3	2.0± 2.0
1914	1.3±1.3	10.3±10.1	10.0±10.7
1921	3.3± .8	2.2± .3	7.0± 5.2
1922	2.3±1.3	3.0± 1.3	6.5± 4.6
2004	1.7±2.0	1.7± .8	2.6± 1.6
9827	.7± .7	8.0±10.1	4.6± 5.8

a Period 1 = d -6, -3, 0 pre-insult
b Period 2 = d 3, 6, 9 post insult
c Period 3 = d 12, 15...39 post insult

Table 11. BULL VARIATION IN PERCENT TERTIARY ABNORMALITIES (MEAN ± SD) BEFORE AND AFTER 48 H SCROTAL INSULATION (DAY 0, PERIOD 1)

Bull	Period 1[a]	Period 2[b]	Period 3[c]
1825	1.2±0.8	3.5±3.5	12.0± 7.0
1914	9.0±0.5	6.8±2.3	13.3±10.3
1921	6.3±0.3	9.3±0.8	10.8± 4.4
1922	5.8±2.6	5.5±1.8	8.7± 2.9
2004	6.3±1.5	5.7±3.3	10.2± 2.9
9827	9.2±1.8	10.5±2.2	18.7±13.0

a Period 1= d -6, -3, 0 pre-insult
b Period 2= d 3, 6, 9 post insult
c Period 3= d 12, 15...39 post insult

Figures 4, 5, 6, 7, 8, and 9. The specific cell types are graphed to display the variance among bulls in timing and degree of response for each of these specific cell types. Variance around the incidence of decapitated spermatozoa was observed. Bulls no. 9827, 1914, and 1921 produced ejaculates with a peak increase in number of decapitated spermatozoa on d 12. Day 18 was the peak day for decapitated sperm observed in bulls no. 1922 and 1825. Bull no. 2004 did not respond to thermal stress by exhibiting increases in decapitated sperm. Peak increases in decapitated sperm ranged from 3.5% (bull no. 2004) to 90.5% (bull no. 9827). The diadem defect provided variation in level of response (numbers of abnormals) as well as a slight difference in timing of occurrence for this trait. Bulls no. 9827 and 1914 produced peak increases in diadem defects on d 15 whereas all other bulls had increases on d 18. Peak increases in the diadem defect ranged from a low of 10.5% (bull no. 2004) to a high of 90.0% (bull no. 1825). Although the incidence of the abnormality classified as a crater was elevated for most bulls from d 18 to d 24, peak levels of cratered sperm ranged from 16.0% (bull no. 2004) to 50.0% (bull no. 1825). The occurrence of the pyriform abnormality varied little among bulls. Only bull no. 1922 had a peak in pyriforms on d 24. All other bulls showed a peak of pyriforms on d 21, except for bull no. 2004. The range in peak levels of pyriforms was from a low of 1.5% (bull no. 2004) to a high of 45.5% (bull no.9827). The peak occurrence of knobbed acrosomes varied from d 24 to d 30 and the level of response ranged from basically no response of 1.0% (bull no. 1921) to 35.5% (bull no. 1825). The peak occurrence in the dag defect was relatively small when compared to those of the primary abnormalities. The ranges in peak levels of dag defects was a low of 1.5% (bull no. 1914) to a high of 7.5% (bull no. 1825).

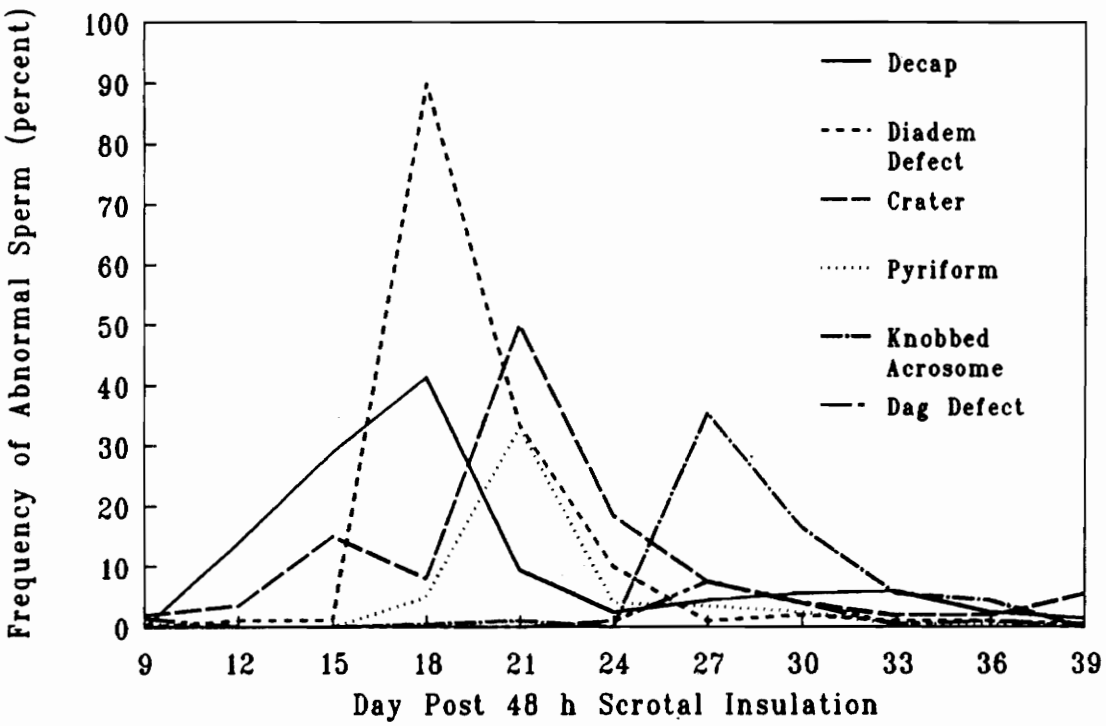

Figure 4. Response of bull no. 1825 to 48 h scrotal insulation based on incidence of specific abnormal cell types in the ejaculate.

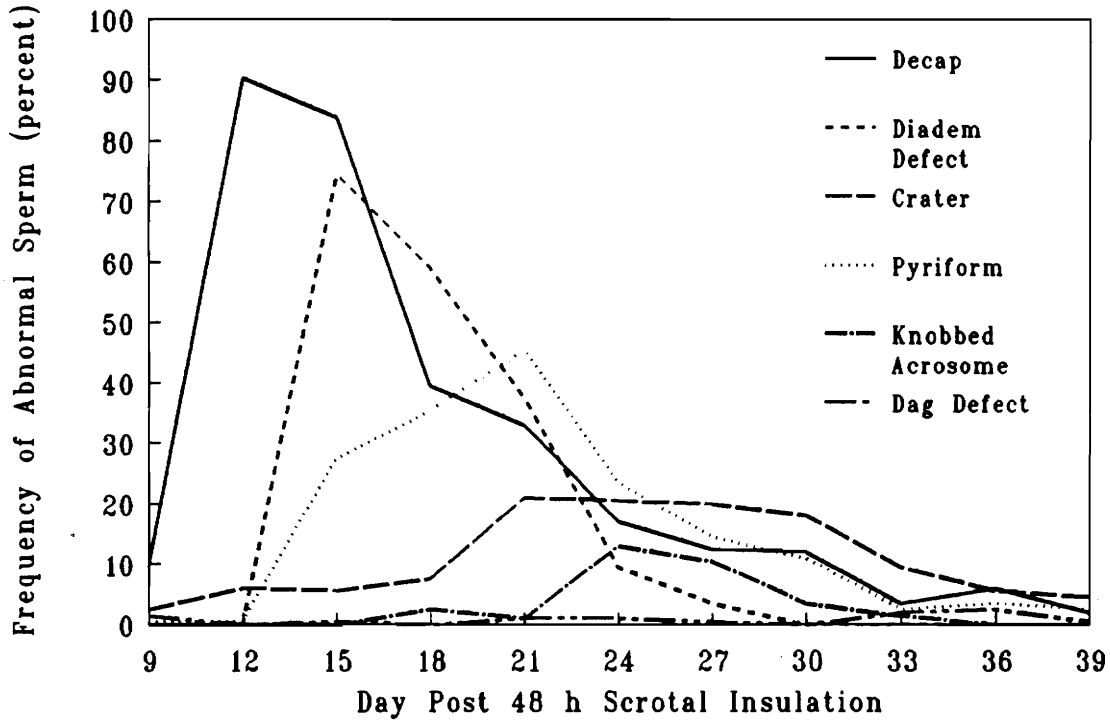

Figure 5. Response of bull no. 9827 to 48 h scrotal insulation based on incidence of specific abnormal cell types in the ejaculate.

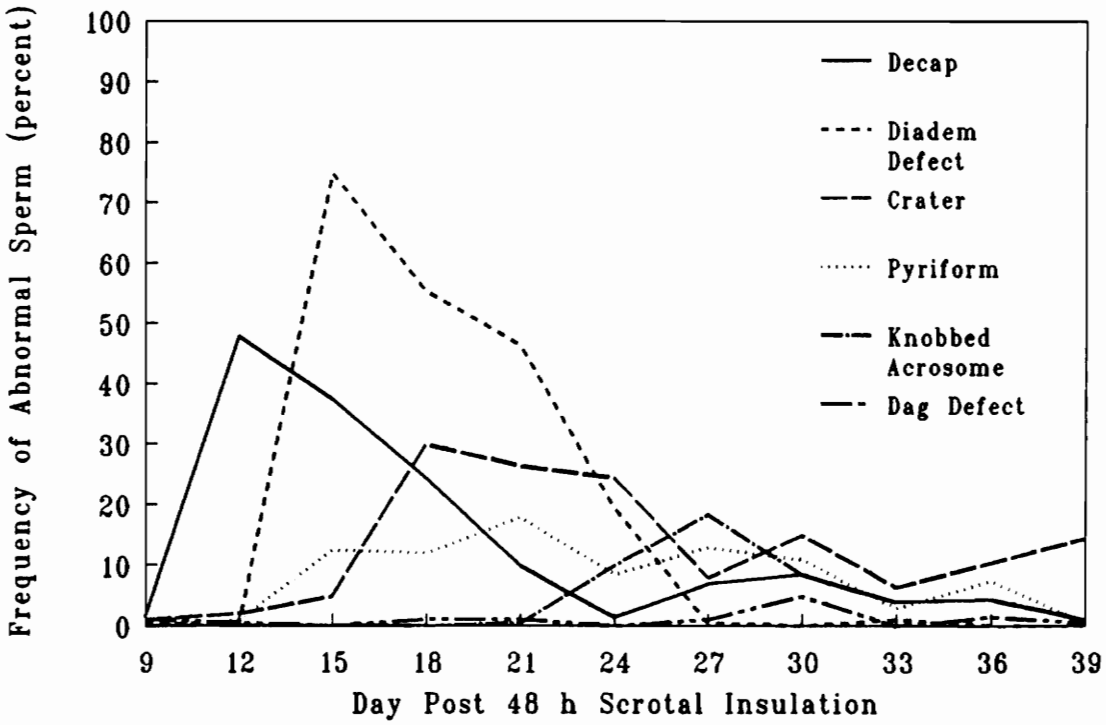

Figure 6. Response of bull no. 1914 to 48 h scrotal insulation based on incidence of specific abnormal cell types in the ejaculate.

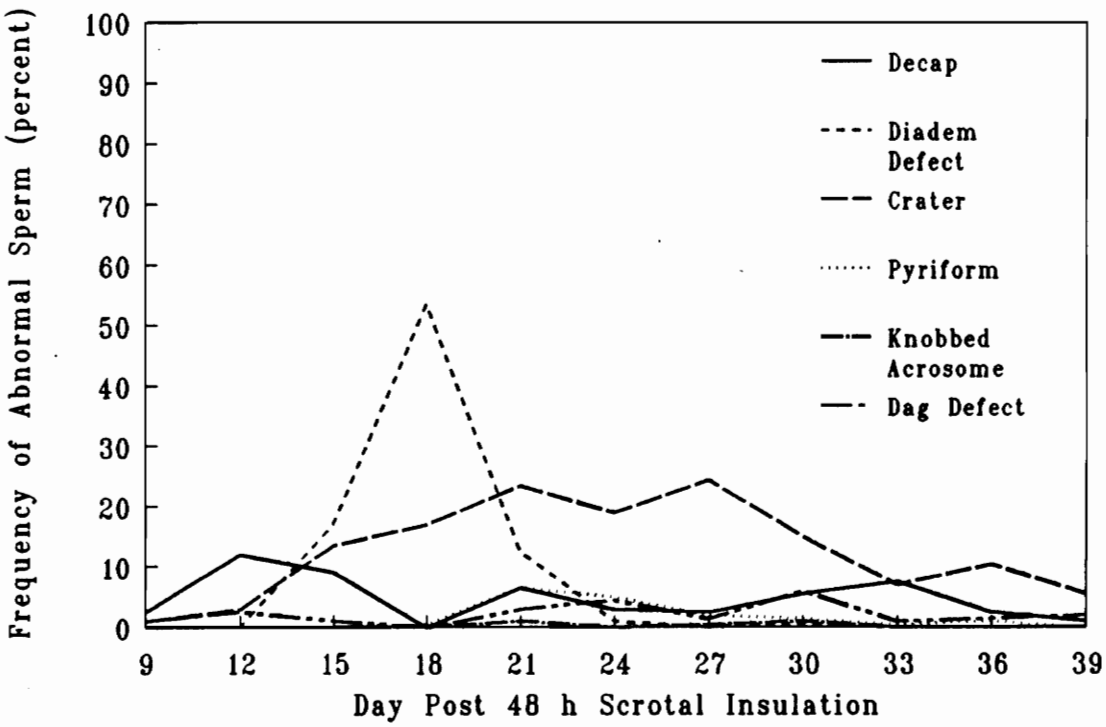

Figure 7. Response of bull no. 1921 to 48 h scrotal insulation based on incidence of specific abnormal cell types in the ejaculate.

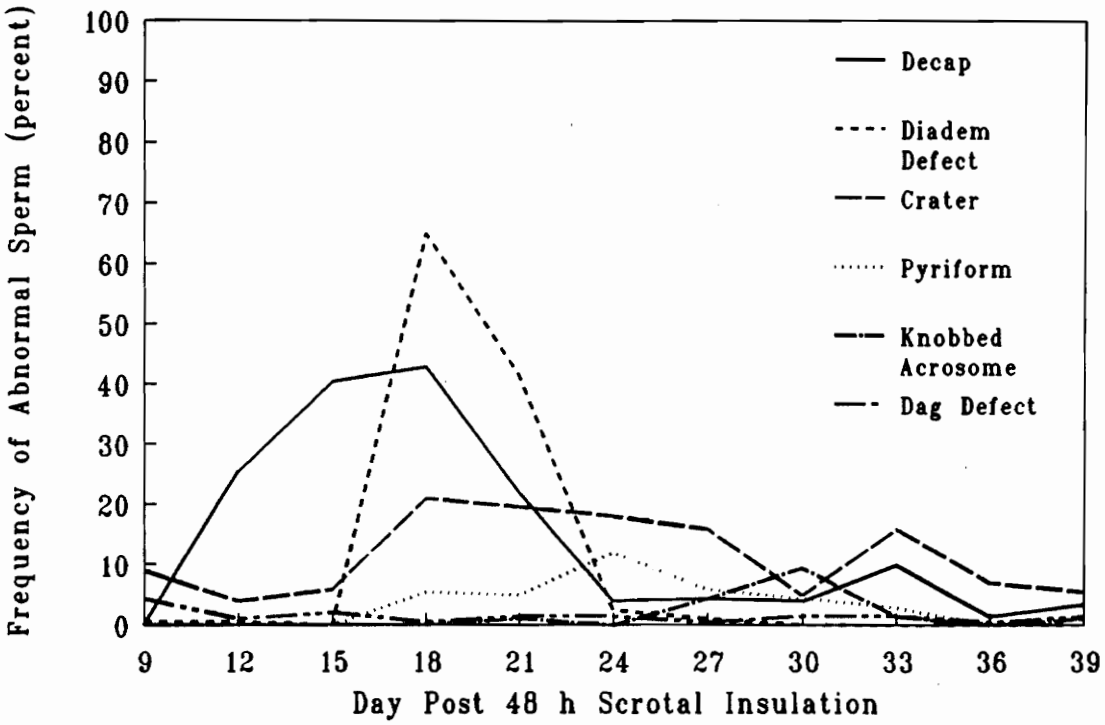

Figure 8. Response of bull no. 1922 to 48 h scrotal insulation based on incidence of specific abnormal cell types in the ejaculate.

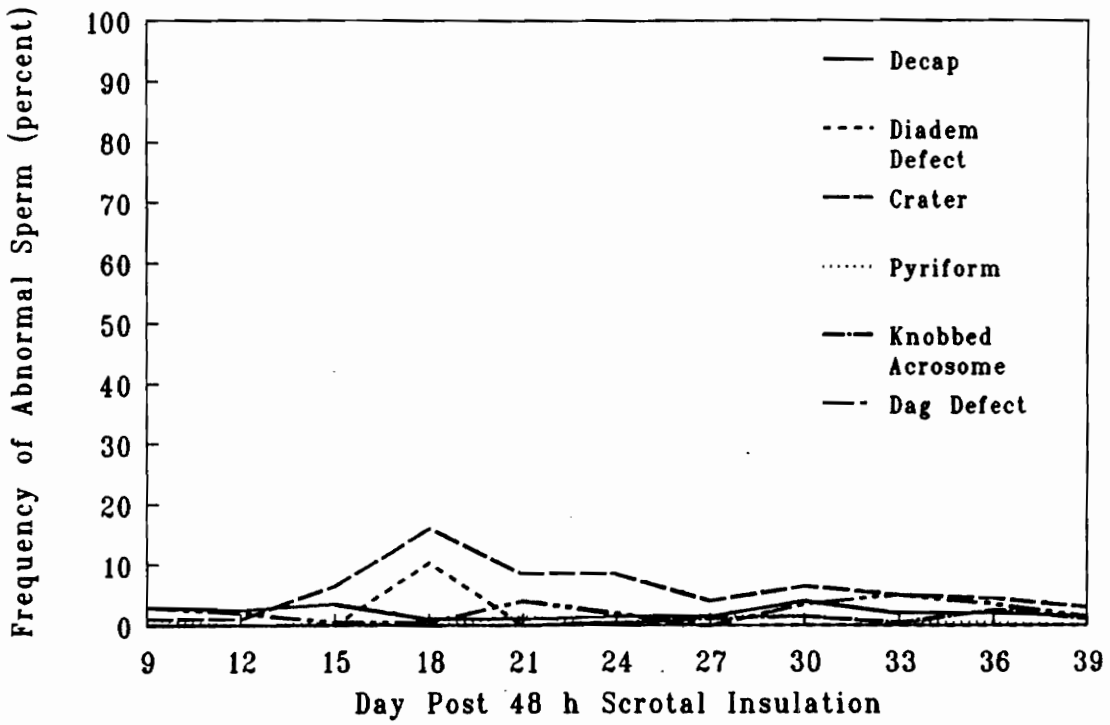

Figure 9. Response of bull no. 2004 to 48 h scrotal insulation based on incidence of specific abnormal cell types in the ejaculate.

DISCUSSION

The response to thermal insult achieved in this experiment was characterized by relatively small changes in sperm output (production) and motility, but a marked change in sperm morphology (Figure 1 on page 26), the latter due to increased primary abnormalitites (Figure 2 on page 37). Thus, thermal insult achieved in this experiment was considered mild and mimicked that observed during the warm months at AI stations in North America for the Holstein breed (Sullivan, 1970). On this basis, post-treatment trends in neat semen characteristics were also similar to those seen in bulls after scrotal insulation (Wildeus and Entwistle, 1983) and high ambient temperatures (Skinner and Louw, 1966; Sekoni and Gustaffson, 1987). Thermal stress resulted in reductions in the percentage of motile sperm in neat semen coincident with increases in abnormal sperm beginnning 12 d after onset of scrotal insulation. Considering that epididymal transport is approximately 8 to 11 d in bulls (Orgebin-Crist, 1962), this indicates that thermal stress interferred with the spermatogenic process rather than sperm maturation in the epididymis, when only neat semen is evaluated.

In the present study, sperm output was not dramatically decreased by thermal insult as in the studies of Casady et al. (1953) and Meyerhoeffer et al. (1985). They reported marked decreases in sperm concentration and total sperm numbers per ejaculate, but in response to higher temperatures (range 35-37°C) in climatic chambers for extended periods rather than increases in scrotal surface temperatures used in this study. Scrotal temperatures measured in the present study increased from

33°C directly after scrotal insulation to 36°C (maximum) after removal of the thermal sack, whereas ambient temperatures during 48 h insult ranged from a low of -4.9°C to a high of 33.7°C. The variation, within bulls, in scrotal surface temperatures was dependent upon placement of the temperature probe. This was to be expected based on a Waites (1970) report of a 4°C difference between body and testicular temperatures which decreased as temperature was taken further away from the body cavity. Even so, scrotal surface temperatures can indicate the internal testicular temperature as evidenced by Coulter et al. (1988). Coulter et al. utilitized infrared thermography in rams and reported that scrotal surface temperatures were correlated with subcutaneous testicular temperatures ($r = .95$).

Viability Characteristics

In contrast with prior studies, the results obtained in this study show that the mild thermal insult achieved also adversely affected the viability of ejaculated spermatozoa housed in the epididymis/rete testis at the time of insult. This observation is based upon the significant differences in viability of cryopreserved and incubated (3 h) sperm obtained in Period 2 compared to that in Period 1. Period 2 consisted of semen collected 3 to 9 d post thermal insult and represented sperm that were assumed to be in the rete testis/epididymis at the time of insult. Previous reports (Skinner and Louw, 1966; Ross and Entwistle, 1979; Meyerhoeffer et al. 1985), indicated that epididymal spermatozoa were not affected by thermal insult.

Since cryopreservation and incubation was not employed in the previous studies, the effect of thermal stress on spermatozoal ability to be cryopreserved could not be identified. In this study, the adverse effects of thermal insult on viability of bovine epididymal/rete testis spermatozoa was seen only after semen cryopreservation and thawing followed by incubation for 3 h at 37°C (Table 5 on page 32). Motility as well as acrosomal integrity decreased significantly after the in-

cubation period. Therefore, stress of incubation after freeze-thawing seems to be imperative to detecting changes in semen viability due to heat stress.

Individual variation in susceptibility to heat stress within bulls was both expected and apparent. This difference could have been due to either an environmental affect at the time of insult and(or) differences in thermoregulatory mechanisms among bulls. The bull with the lowest response, no. 2004, (Figure 9 on page 48), was heat stressed during the coldest month of the study, February, with a temperature range of -4.9 to 12.1°C in relation to the other bulls who were heat stressed during warmer months (range of 7.3 to 33.2°C). On this basis, there could be a relationship between differences in semen viability measurements between Period 1 (control) and Period 2 (post thermal insult) at 3 h post thaw and numbers of morphologically abnormal cells observed during Period 3. This relationship is presented in Table 12. Bull no. 2004 was the only bull not responding to thermal stress by exhibiting dramatic increases in abnormal spermatozoa during Period 3. Likewise, bull no. 2004 revealed the least difference in freezability of sperm collected in Period 2 when compared to that collected in Period 1 (control). Bulls showing differences between Period 1 and 2 in either motility, intact acrosomes or both also had appreciable numbers of abnormal cells during Period 3. Of course, the sample size of this study cannot verify a relationship between viability in Period 2 compared to Period 1 with morphology counts in Period 3. Further studies may be able to confirm this trend, indicating that changes observed in epididymal sperm viability post insult predict morphological changes to come in later ejaculates. The AI industry could benefit from further studies to confirm the relationship of viability measurements of frozen-thawed semen with subsequent appearance of morphologically abnormal cells due to thermal stress. Recognition of an impending problem would permit sounder decisions in culling semen as well as managing the bull toward a more rapid recovery. Detection of viability changes (acrosomal integrity and motility) after freeze-thaw and incubation, but prior to detection of morphological changes due to heat stress, could enable managers to move bulls into less stressful areas thereby minimizing the length of time that bulls are heat stressed.

Table 12. VARIATION IN SEMEN VIABILITY AMONG BULLS: DIFFERENCE BETWEEN PERIOD 1 (CONTROL) AND PERIOD 2 (POST THERMAL INSULT) AT 3 H POST THAW IN RELATION TO ABNORMAL CELLS OBSERVED IN PERIOD 3

Bull#	% Motility	% Intact Acrosomes	% Abnormal Cells during Period 3 (mean±sd)
1825	-28.4	- 9.8	60.2± 29.6
1914	-15.0	- 4.1	74.3± 23.2
1921	-43.4	-26.3	61.2± 17.7
1922	1.6	-24.1	64.0± 17.1
2004	5.0	- 0.4	34.0± 7.5
9827	-13.4	3.3	79.6±26.8
	$\bar{X}$= -15.6	$\bar{X}$= -10.2	$\bar{X}$= 62.2

Computer-aided analysis of motility of frozen-thawed semen revealed differences between thermally stressed epididymal/rete testis spermatozoa (Period 2) and spermatozoa ejaculated during the control period (Period 1). These differences were observed before and after 37°C incubation for 3 h (Table 7 on page 34). It was expected that the use of a computer-aided system would help detect changes in semen viability more precisely than the human eye could detect changes using phase contrast microscopy. Quantitative measurements of the percentage of motile and progressively motile cells evaluated by the HTM-S did reveal differences in heat stressed cells earlier than did subjective estimations of percentage of motile cells using phase contrast microscopy (Table 8 on page 35). It should be noted, however that many of the other parameters of motility were not available because HTM-S velocity measurements could not be validated for frozen-thawed spermatozoa (Appendix B).

Morphological Characteristics

In response to heat stress, the increases in total sperm abnormalities observed in this study (Figure 1 on page 26) were not different than other studies (Johnston et al., 1963; Skinner and Louw, 1966; Ross and Entwistle, 1979). Spermatozoa ejaculated during Period 2 were morphologically normal and increases in abnormalities were not observed except for a slight rise on d 9 (Figure 2 on page 37). This slight increase was due to protoplasmic droplets observed in sperm collected from two bulls. Therefore, spermatozoa located in the epididymis/rete testis, with the possible exception of those exhibiting protoplasmic droplets, were not morphologically affected by heat insult. Sullivan (1978) suggested that large numbers of protoplasmic droplets are the result of abnormal spermiogenesis or epididymal sperm maturation. Failure of sperm epididymal maturation may be a direct result of heat stress effects on the epididymis.

The principle increase in total abnormal cells was seen in Period 3 and was mainly due to primary abnormalities. Tertiary abnormalities observed in Period 3 were different than levels observed in Period 1 or Period 2 and therefore are in agreement with the literature (Johnston et al., 1963 and Wildeus and Entwistle, 1983) in which significant increases in secondary and tertiary abormalities have been reported after thermal insult. Although secondary abnormalities were observed, the changes were not significant. This observation is not in agreement with the literature. The general lack of secondary abnormalities in the present study could be due to the fact that a milder heat insult was achieved.

When specific abnormalities were considered, they were found to peak at different intervals. Previously, cell types indicative of heat stress have been documented only as decapitated spermatozoa (Wildeus and Entwistle, 1983; Parkinson, 1987; Sekoni and Gustaffson, 1987), nuclear vacuoles (Skinner and Louw, 1966), pyriforms (Johnston et al., 1963), droplets (Wildeus and Entwistle, 1983; Parkinson, 1987; Sekoni and Gustaffson, 1987), and abnormal tails (Johnston et al., 1963; Wildeus and Entwistle, 1983; Sekoni and Gustaffson, 1987). The results of this study reveal the presence of a more inclusive list of abnormalities resulting from thermal stress and the order in which they occur. The first abnormality to be observed was decapitated spermatozoa followed by the diadem defect and cratered sperm (nuclear vacuoles). Pyriform cells were observed next, followed last by increases in knobbed acrosomes and the dag defect. Refer to Figure 3 on page 38 for the choronological sequence of abnormal cell types observed during Period 3. The presence of decapitated spermatozoa on d 12 in ejaculates produced by bulls no. 9827 and 1914 had an interesting component in that the lost tails from decapitated sperm were usually motile. This phenomenon has been previously documented in the bovine (Settegren, 1968; Blom and Birch-Andersen, 1970). Hancock (1955) suggested that this incident occurred in the caput epididymis. We assumed that cells ejaculated on d 12 post insult were in the testis at the time of heat insult, but some mixing of spermatozoa during epididymal transport could have occurred.

The semen collection scheme of this study allowed for an evaluation of the relationship between spermatogenic events and abnormal cell types. The incidence of specific abnormal cell types in this

study appeared to be temporally related to stage of development that cells were in at the time of heat stress. Figure 10 describes the temporal relationship based on the eight-stage cycle of the seminiferous epithelium described by Amann (1962a). Cells ejaculated on d 12, 15, and 18 exhibited decapitation, craters, and the diadem defect, but otherwise normal head shape. These cells should have been spermatids in stages 4 through 8 and therefore would have already possessed definite head shape (Amann, 1962a,b and Amann, 1970), but condensation of nuclear material (DNA) would still have been in progress during these stages (Gledhill, 1975). Therefore, normal shaped cells could have nuclear vacuoles if heat stress interferred with condensation. Cells ejaculated on d 18, 21, and 24 had abnormalities classified as diadem, along with continued crater defects, pyriforms and generally misshapen heads. These cells should have represented spermatids in the early stages of the epithelial cycle (1 through 3) and therefore would have been experiencing head morphogenesis (Amann, 1962a and Amann, 1970). These morphological changes seem to be affected by thermal stress in that misshapen heads are prevalent. Cells ejaculated on d 27, 30, and 33 revealed abnormalities such as knobbed acrosomes and the dag defect. The knobbed acrosome is formed during inadequate spreading of the acrosomic granule (Hancock, 1953). The dag defect is a tail abnormality in which the tail is coiled within a continuous cell membrane (Blom, 1966). Therefore, these cells are abnormal and immotile. Both of these types of cells would have chronologically been very young spermatids (round spermatids) in stages 6 through 8 of spermatogenesis at the time of thermal insult. Tail formation and acrosomal spreading occurring during stage 6 through 8 seem to be affected by thermal stress as evidenced by the presence of knobbed acrosomes and the dag defect. Saacke et al. (1968) showed that the knobbed acrosome was due to a defective spreading of the acrosomic granule in the young round spermatids in stages 8 and 1 of the cycle of the seminiferous epithelium.

The degree of bull variation in level of response to heat stress based on the incidence of abnormalities during Period 3 could be due to individual differences in thermoregulation or to ambient conditions at the time of scrotal insulation or the interaction of the two. The variation among bulls observed in timing of the sequence of specific abnormalities could be due to differences among bulls

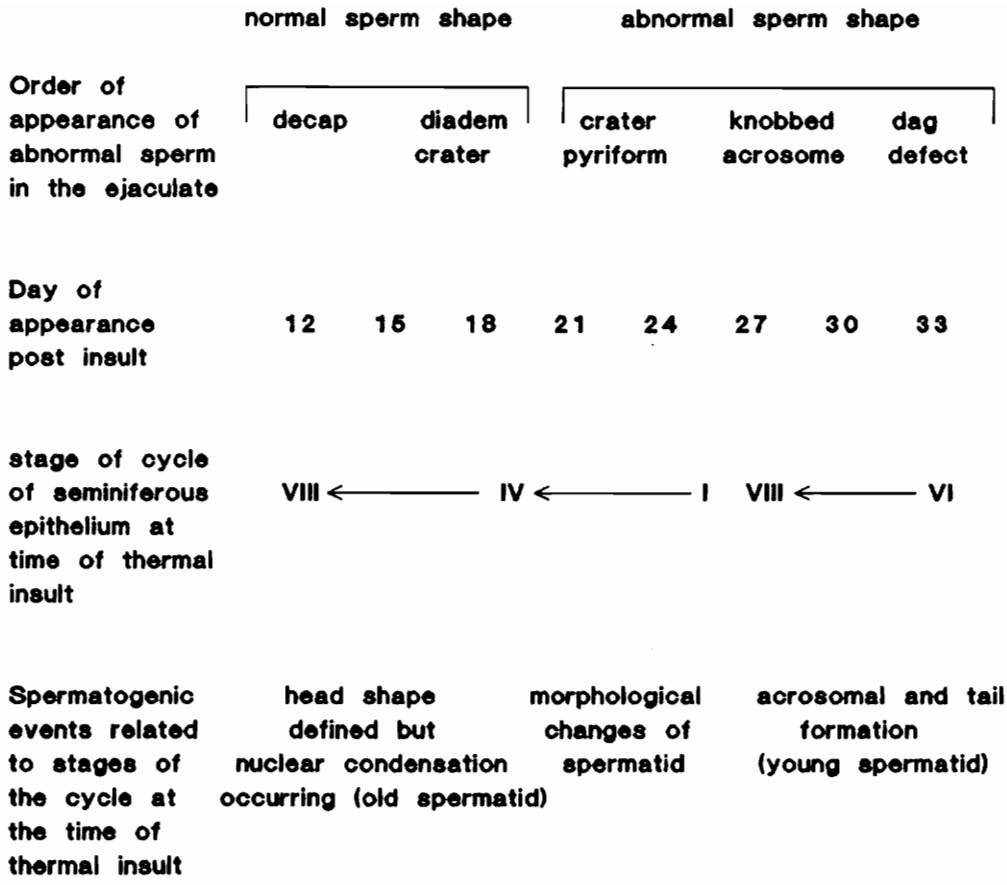

Figure 10. Temporal relationship of morphologically abnormal cell type and events of spermatogenesis that cells encountered during the 48 h thermal insult.

in epididymal transport time. Previous authors (Johnston et al., 1963; Ross and Entwistle, 1979; Meyerhoeffer et al., 1985) related occurrence of abnormalities to the post insult week of collection. However, the variation in semen collection regimen used in different studies, particularly regarding frequency of ejaculation, were difficult to interpret. For the most part, ejaculation frequencies used in other studies were lower than those in the present study. Thus, mixing of sperm in the epididymis was probably greater in earlier studies when compared with the present study. The chronological order of observation of cell types was as follows: decapitated (d 12 to 15), diadems (d 18), cratered (d 18 to 21), pyriform (d 21), knobbed acrosomes (d 27) and dag defect (d 30). Perhaps an even greater chronological separation of abnormal cell types could be achieved if ejaculation frequency was greater than two ejaculates every 3 d.

In order to examine the repeatability within bulls to exhibit responses to thermal stress, a method that could effectively regulate the ambient temperature and(or) uniformity in thermal stress must be used. The utilization of climatic chambers would be essential to study the repeatability of viability and morphological semen characteristics of bulls exposed to specific thermal conditions. Such was not possible utilizing the scrotal sack system employed in the present study.

CONCLUSIONS

These studies showed that a mild heat stress to the testis caused by a 48 h coverage of the scrotum with thermal insulating material reduced viability of ejaculated spermatozoa housed in the epididymis at the time of the insult. The effect of heat stress was observed after semen was cryopreserved when utilizing the aid of a computerized motility analysis system. However, when using conventional subjective phase contrast microscopy, the effect of heat stress was not observed until after semen was cryopreserved and incubated for 3 h at 37°C post thaw. The effect of heat stress on viability measurements was observed within 3 to 9 d post insult and was not accompanied by alterations in sperm morphology.

Spermatogenesis was also adversely affected by the mild heat insult resulting in a depression of sperm motility and increased abnormal sperm morphology in neat semen beginning 12 to 15 d after insult. Morphologically, the sperm head was most vulnerable to heat. Specific abnormal cell types associated with heat stress and order of appearance post insult were: decapitated (d 12 to 15), diadem (d 18), cratered (d 18 to 21), pyriform (d 21), knobbed acrosome (d 27) and dag defect (d 30). The sequence in appearance of abnormal cell types was consistent among bulls and appeared to be temporally related to the stage of spermiogenesis in which cells were engaged at the time of heat insult. Bulls varied in the response to thermal stress in viability of semen, the level of abnormal sperm ejaculated and the predominant type of abnormal sperm produced.

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Appendix A. Egg Yolk Citrate Extender Preparation

The extender was made up the day before collection and the antibiotics Tylosin (100μg/ml), Gentamicin (500 μg/ml), and Linco-spectin (300/600 μg/ml) were added to fraction A on the morning of collection to make up the final 2% of fraction A. Fraction A of extender consisted of (v/v) 20% egg yolk and 78% sodium citrate dihydrate adjusted to pH 6.8 with citric acid monohydrate. Extender was centrifuged and filtered through a glass prefilter then through a 0.45 μm filter. Fraction B consisted of 14% glycerol (v/v) added to a 66% sodium citrate, 20% egg yolk (Fraction A) filtered by the above procedure with 200 μl of antibiotics added on the morning of collection.

EYC extender::

Stock Citric Acid (5% w/v)

5.0 g citric acid monohydrate

deionized water to 100 ml

Stock Sodium Citrate

30.0 g Na Citrate dihydrate

5.6 ml stock 5% citric acid

Deionized water to 1000 ml

Antibiotics (GTLS)

800 μl Gentamicin (100 μg/ml)

600 μl Tylosin (500 μg/ml)

600 μl Linco-spectin (300/600 μg/ml)

Fraction A non-glycerol extender 100 ml

78.0 ml stock Na Citrate

20.0 ml egg yolk

2.0 ml antibiotics

Fraction B glycerol extender 100 ml

65.9 ml stock Na Citrate

20.0 ml egg yolk

14.0 ml glycerol

.1 ml antibiotics

Appendix B. Quantitative Motility Analysis

Validation of the Hamilton-Thorn Motility Analyzer (HTM-S version 7)

In order to evaluate accuracy, five mixtures of live and killed spermatozoa were analyzed at 0 h (1-3 min post thaw). The live cells were incubated for 1 h at 37°C then spiked with the killed cells to make duplicate ratios and analyzed. All semen was from frozen-thawed samples (two pooled ejaculates from one bull) prepared in clarified egg yolk citrate extender. To prepare killed cells, spermatozoa packaged and frozen in .5 ml straws were allowed to thaw in air to room temperature and plunged into liquid nitrogen three times. Four replicates were analyzed in order to minimize potential sources of error. Budworth et al. (1988) and P. Lumas, Hamilton-Thorn Research, (personal communication, 1990) suggested that when using 20 frames per each of nine fields, the concentration of spermatozoa in the sample should be diluted to 15 x 10^6 spermatozoa/ml. A dilution of 200 μl of extended semen in 400 μl of clarified extender was used to decrease the amount of coincident track crossing. Ten microliters of the sample was loaded into each of two chambers in a Microcell slide. The criteria used by the HTM-S to determine motile from immotile sperm

as well as sperm cells from debris were previously set through the analyzer set-up option. The parameters settings were:

Frames at frame rate: 20 at 30/s

Minimum Contrast: 6 units of brightness

Minimum size: 4 pixels

Lo/Hi size gates: 0.5 to 1.8 pixels

Lo/Hi intensity gates: 0.5 to 1.8 pixels

Non motile head size: 6 pixels

Non motile intensity: 55 pixels

Medium VAP (average path velocity) value: 25 μm/s

Low VAP value: 10 μm/s

Threshold straightness: 90 %

The nine fields of view, located at pre-selected sites on the microcell slide were analyzed for the following criteria:

Path Velocity (VAP) = the five point average of cell velocity in μm/sec

Progressive Velocity (VSL) = the velocity measured in a straight line from beginning to end of track, averaged over all cells for which VAP > LVV (low velocity value) in μm/sec

Track Speed (VCL) = average value of the track speed over all cells for which VAP > LVV in μm/sec

Straightness (STR) = ratio VCL/VAP, measures departure of the cell path from a straight line

Linearity (LIN) = ratio VSL/VCL measures departure of the cell track from a straight line

Amplitude of Lateral Head Displacement(ALH) = the maximum lateral head displacement measured in the cell track, averaged over all cells for which the straightness exceeds the threshold straightness and for which VAP > LVV in μm

Beat Cross Frequency (BCF) = frequency with which the sperm track crosses the sperm average path in Hz (cycles/sec)

The HTM-S system was highly repeatable on identical samples (same ratio and hour) for the following criteria.

Measurement	**Repeatability**
Mean ALH	88.9%
Percent motile	93.6%
Percent progressively motile	95.2%

Velocity measurements, did not validate for frozen-thawed bovine spermatozoa as can be noted by the correlations between the ratio of live:killed cells and velocity measurements presented below.

Correlation Coefficients	**Significance Level**
Mean VAP -.53	P < .01
Mean VSL -.53	P < .01
Mean VCL -.45	P < .01
Mean LIN -.01	NS
Mean STR -.01	NS

As expected, velocity decreased with aging but ratio of live:killed cells had a negative effect on the interaction between velocity and aging. As the number of killed cells increased, the velocities decreased. Since there was an interaction seen with use of frozen thawed cells, velocity measurements

did not validate and therefore were not used in further study analysis. Linearity and straightness validated but were not used in further analysis since they were functions of velocity.

Correlations between mean ALH and BCF and the live:killed cell ratio are presented below.

Correlation Coefficients	Significance Level
Mean ALH -.25	NS
Mean BCF -.05	NS

Although beat cross frequency validated for frozen-thawed semen, these measurements were not used for the study since the relationship to fertility is unknown. As can be seen below, Mean ALH was positively correlated with velocity measurements and therefore, was not used to evaluate further data due to its high correlation to velocity measurements.

Correlation Coefficients	Significance Level
Mean VAP .51	$P < .01$
Mean VSL .39	NS
Mean VCL .65	$P < .01$

Correlations between the ratio of live:killed cells and motility measurements are presented below.

Correlation Coefficients	Significance Level
% Motile -.95	$P < .01$
% Prog. Motile -.96	$P < .01$

As was expected, motility measurements decreased proportionately as the number of killed cells increased. The system was used to evaluate only motility measurements (percent motile and percent progressively motile).

Appendix C. Statistical Analysis of Semen Characteristics Before and After 48 h Thermal Stress

Table 1. Analysis Of Variance Of Estimated Initial Motility

Source	df	Mean Squares	F value	PR>F
Bull	5	241.79	3.26	.0109
Period	2	2516.11	11.12	.0029
Day(Period)	13	352.01	4.75	.0001
Bull*Period	10	226.36	3.06	.0031
Residual	65	74.09		

Contrast	df	Mean Squares	F value	PR>F
Period 1 vs 2	1	44.44	.20	NS
Period 1 vs 3	1	3544.62	15.66	<.01
Period 2 vs 3	1	2628.38	11.61	<.01

Table 2. Analysis Of Variance Of Total Abnormalities

Source	df	Mean Squares	F value	PR>F
Bull	5	756.84	5.54	.0003
Period	2	19378.42	63.87	.0001
Day(Period)	13	1406.42	10.29	.0001
Bull*Period	10	303.43	2.22	.0271
Residual	65	136.63		

Contrast	df	Mean Squares	F value	PR>F
Period 1 vs 2	1	21.78	0.07	NS
Period 1 vs 3	1	24739.01	81.53	<.01
Period 2 vs 3	1	22951.67	75.64	<.01

Table 3. Analysis Of Variance Of Sperm Output Per Collection Day

Source	df	Mean Squares	F value	PR>F
Bull	5	2354785.4	9.72	.0001
Period	2	13020372.0	4.49	.0407
Day(Period)	13	3420557.9	1.41	.1784
Bull*Period	10	2902711.8	1.20	.3091
Residual	65	2307692.3		

Contrast	df	Mean Squares	F value	PR>F
Period 1 vs 2	1	4405521.14	1.52	NS
Period 1 vs 3	1	5509019.03	1.90	NS
Period 2 vs 3	1	24507810.10	8.44	<.01

Table 4. Analysis Of Variance Of The Viability Measurement Estimated Motility

Source	df	Mean Squares	F value	PR>F
Bull	5	222.65	4.31	.0045
Period	2	4509.28	7.60	.0099
Bull*Period	10	593.70	11.50	.0001
Freeze	1	2308.22	17.32	.0088
Hour	1	4360.74	38.85	.0016
Freeze*Hour	1	63.59	4.01	.1015
Bull*Freeze	5	133.24	2.58	.0468
Bull*Hour	5	112.25	2.17	.0836
Bull*Freeze*Hour	5	15.85	.31	.9049
Period*Freeze	2	68.72	1.33	.2795
Period*Hour	2	184.01	3.56	.0409
Period*Freeze*Hour	2	53.49	1.04	.3673
Residual	30	51.64		

Contrast	df	Mean Squares	F value	PR>F
Period 1 vs 2 unfrozen 0 h	1	133.33	2.58	NS
Period 1 vs 2 unfrozen 3 h	1	725.93	14.06	NS
Period 1 vs 2 frozen 0 h	1	45.37	.88	NS
Period 1 vs 2 frozen 3 h	1	244.50	4.73	<.01
Period 1 vs 3 unfrozen 0 h	1	921.67	17.85	<.01
Period 1 vs 3 unfrozen 3 h	1	3530.61	68.37	<.01
Period 1 vs 3 frozen 0 h	1	1921.11	37.20	<.01
Period 1 vs 3 frozen 3 h	1	2770.45	53.65	<.01

Table 5. Analysis Of Variance Of The Viability Measurement Percent Intact Acrosomes

Source	df	Mean Squares	F value	PR>F
Bull	5	618.71	39.34	.0001
Period	2	5052.47	5.39	.0259
Bull*Period	10	938.10	59.65	.0001
Freeze	1	1290.32	28.48	.0031
Hour	1	1639.26	93.46	.0002
Freeze*Hour	1	9.90	.96	.3727
Bull*Freeze	5	45.30	2.88	.0306
Bull*Hour	5	17.54	1.12	.3733
Bull*Freeze*Hour	5	10.34	.66	.6583
Period*Freeze	2	18.06	1.15	.3307
Period*Hour	2	68.83	4.38	.0215
Period*Freeze*Hour	2	4.70	.30	.7439
Residual	30	15.73		

Contrasts	df	Mean Squares	F value	PR>F
Period 1 vs 2 unfrozen 0 h	1	91.67	5.38	NS
Period 1 vs 2 unfrozen 3 h	1	315.19	20.04	NS
Period 1 vs 2 frozen 0 h	1	43.76	2.78	NS
Period 1 vs 2 frozen 3 h	1	76.47	4.86	<.01
Period 1 vs 3 unfrozen 0 h	1	1867.72	118.77	<.01
Period 1 vs 3 unfrozen 3 h	1	3136.33	199.44	<.01
Period 1 vs 3 frozen 0 h	1	1666.36	105.96	<.01
Period 1 vs 3 frozen 3 h	1	2646.52	168.29	<.01

Table 6. Analysis Of Variance Of The Computerized Viability Measurement Percent Progressively Motile Sperm

Source	df	Mean Squares	F value	PR>F
Bull	5	34.59	.37	.8677
Period	2	2198.76	3.94	.0547
Bull*Period	10	557.82	5.96	.0001
Hour	1	1544.65	3.44	.1226
Bull*Hour	5	448.56	4.79	.0009
Period*Hour	2	100.27	4.46	.0413
Bull*Period*Hour	10	22.50	.24	.9908
Residual	64	3.61		

Contrasts	df	Mean Squares	F value	PR>F
Period 1 vs 2 frozen 0 h	1	367.52	16.33	<.01
Period 1 vs 2 frozen 3 h	1	1417.52	62.99	<.01
Period 1 vs 3 frozen 0 h	1	2061.92	91.63	<.01
Period 1 vs 3 frozen 3 h	1	2141.66	95.17	<.01

Table 7. Analysis Of Variance Of The Computerized Viability Measurement Percent Motile Sperm

Source	df	Mean Squares	F value	PR>F
Bull	5	24.71	.18	.9694
Period	2	3535.34	3.93	.0551
Bull*Period	10	900.19	6.54	.0001
Hour	1	5109.47	12.94	.0156
Bull*Hour	5	394.83	2.87	.0213
Period*Hour	2	148.21	10.0	.0041
Bull*Period*Hour	10	14.82	.11	.9997
Residual	64	137.74		

Contrasts	df	Mean Squares	F value	PR>F
Period 1 vs 2 frozen 0 h	1	617.32	41.66	<.01
Period 1 vs 2 frozen 3 h	1	1699.16	114.66	<.01
Period 1 vs 3 frozen 0 h	1	4027.95	271.80	<.01
Period 1 vs 3 frozen 3 h	1	2928.63	197.62	<.01

Table 8. Analysis Of Variance Of The Morphological Characteristic Primary Abnormalities

Source	df	Mean Squares	F value	PR>F
Bull	5	547.31	3.17	.0126
Period	2	17866.18	43.70	.0001
Day(Period)	13	1822.39	10.57	.0001
Bull*Period	10	408.88	2.37	.0184
Residual	65	172.39		

Contrast	df	Mean Squares	F value	PR>F
Period 1 vs 2	1	21.78	.05	NS
Period 1 vs 3	1	21126.05	51.67	<.01
Period 2 vs 3	1	22842.19	55.86	<.01

Table 9. Analysis Of Variance Of The Morphological Characteristic Secondary Abnormalities

Source	df	Mean Squares	F value	PR>F
Bull	5	35.45	1.42	.2279
Period	2	74.20	2.84	.1058
Day(Period)	13	49.05	1.97	.0380
Bull*Period	10	26.17	1.05	.4130
Residual	65	24.92		

Contrast	df	Mean Squares	F value	PR>F
Period 1 vs 2	1	66.69	2.55	NS
Period 1 vs 3	1	147.75	5.65	NS
Period 2 vs 3	1	4.10	.16	NS

Table 10. Analysis Of Variance Of The Morphological Characteristic Tertiary Abnormalities

Source	df	Mean Squares	F value	PR>F
Bull	5	111.40	.65	.6642
Period	2	641.87	17.96	.0005
Day(Period)	13	302.28	1.76	.0696
Bull*Period	10	35.74	.21	.9949
Residual	65	172.01		

Contrast	df	Mean Squares	F value	PR>F
Period 1 vs 2	1	3.06	.09	NS
Period 1 vs 3	1	850.22	23.79	<.01
Period 2 vs 3	1	728.35	20.38	<.01

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