LONG-TERM STORAGE OF LIQUID BOAR SPERMATOZOA

by

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Chapter I. INTRODUCTION

Improving reproductive efficiency is one of the major concerns of swine producers. One such method of reproductive improvement is through the use of an artificial insemination (AI) program. Benefits of an AI program include permitting the producer to maximize the number of inseminations per ejaculate from genetically superior boars, while reducing management and labor costs.

At present, three types of semen can be used in an AI program: fresh liquid semen, stored liquid semen and frozen semen. Fertilization rates for natural service and fresh liquid semen average approximately 85% throughout the swine industry. The fertilization rate for frozen boar semen falls to approximately 60%. Due to the lower fertilization rates reported for frozen semen and until the reliability and popularity of frozen semen increases, its value appears to be more potential than realized.

The use of fresh liquid semen in an AI program offers the advantage of multiple inseminations without sacrificing fertilization rate, but it does not reduce labor and management costs, since it necessitates daily collection of semen. Therefore, the development of an economical extender system that will maximize the long-term storage of liquid semen could be an important management tool in the swine industry.

A major deterrent to the widespread use of liquid semen is the short *in vitro* storage life of boar spermatozoa. The development of an extender system that will increase the storage life of liquid semen and maintain a fertilization rate comparable to that of fresh semen and natural service, will be an economical and practical method of improving reproductive efficiency. Therefore, the objectives of this study were to:

1. Determine the optimum extender system for two extenders that will maintain the highest level of cellular integrity when stored at either 5C or 15C for a minimum of 72 hours.
2. Evaluate the fertilizing capacity of stored spermatozoa using the extender system that maintained the highest level of cellular integrity.
3. Critically analyze enzymatic and morphological changes of boar spermatozoa associated with storage and aging.
4. Characterize properties of boar spermatozoa important to fertilization.

Chapter II. REVIEW OF LITERATURE

Introduction

Historically, the use of artificial insemination (AI) in swine has been highly variable throughout the world. In North America, swine AI has been used very little; whereas, swine AI has been used extensively in Eastern and Western Europe and Japan (Pursel, 1979). Pursel (1979) reported that these differences in acceptance were largely due to economic factors rather than to technical differences.

In 1932, Milovanov reported that the advantage of increasing the number of sows fertilized by a single boar using AI was accomplished by diluting the semen and not by changing the site of semen deposition. Milovanov (1932) prepared an isotonic extender for boar semen, SP-1, and reported that the optimum dilution rate was 1:8 (semen:diluter) with a maximum of 1:30. Rodin and Lipatov (1935) stated that best results were obtained with a dilution of 1:4 (semen to diluter) for an average of 70% pregnancy rate. Pregnancy rate was defined as the percentage of females bred that wee pregnant. Wiggins et al. (1951) reported an average of 91% fertilized ova when gilts were inseminated with 20 ml of freshly collected semen diluted to a final volume of 50 ml with a modified Krebs solution. Ito et al. (1948a) observed that 88.7, 71.4 and 40.0% of the

females were pregnant for semen stored up to 24 hr, 24 to 48 hr and 48 to 75 hr, respectively. Polge (1956a) stated that between 50 and 60% of the females bred should become pregnant at the first insemination with no reduction in litter size, when AI was done with fresh diluted semen, two to six hours old, on the second day of estrus. Polge (1956a) diluted semen with either a yolk-glycine or a yolk-glucose extender, with or without the addition of antibiotics, and reported that there seemed to be no difference between the two extenders for pregnancy rate. He suggested that the addition of antibiotics to the extenders had a beneficial effect on the pregnancy rate but stated that a larger experiment was needed to verify this effect.

Aamdal and Hogset (1957) found that AI with fresh diluted semen, 3 to 8 hr old resulted in a 65.5% pregnancy rate. Semen was diluted with a yolk-citrate extender containing penicillin and streptomycin. Polge (1956a) and Aamdal and Hogset (1957) reported an inverse relationship between semen age and percentage of pregnant females. Dziuk and Henshaw (1958) reported pregnancy rates of 56% for fresh, undiluted semen and 55%, 5% and 42% for diluted semen stored for 1, 2 and 3 days, respectively. The extender used was a yolk-glucose-bicarbonate extender with antibiotics only added to the semen stored for 3 days. They suggested that the higher conception rate for semen stored three days

compared with semen stored two days was due to the addition of antibiotics to the 3-day samples.

Aamdal (1966) reported a 10% decline in the use of swine AI in Norway. The main reason given for this decline was that AI generally resulted in lower pregnancy rates than natural matings. Boender (1966) stated that in the Netherlands, conception rates following AI were about 10 to 25% lower than following natural service. Lindstrom (1966) reported 10 to 15% lower pregnancy rates for swine AI, in Finland, than the 75 to 85% reported for natural matings.

The major problems associated with the use of swine AI during its early development, were the generally lower fertilization rates reported for fresh, diluted semen compared to natural matings and the reduction in fertilizing capacity of stored, liquid semen compared with either natural mating or AI with fresh, diluted semen. A great deal of research has been conducted over the past 20 years on the factors affecting the use of liquid boar semen. Areas of research that have been investigated include the effects of: 1) storage temperature and storage time; 2) incubation time; 3) cooling rate; and 4) extender composition. Additional research has been conducted with frozen boar spermatozoa. However, pregnancy rates obtained from frozen semen have been from 30 to 40% below those obtained from either natural mating or fresh diluted semen (Graham et al., 1971; Pursel and Johnson, 1971, 1972 ; Johnson et al. 1981).

Extenders

History of Extender Usage

One of the early extenders for boar semen was prepared by Milovanov (1932). This extender consisted of an isotonic solution of sodium sulphate with small amounts of potassium sulphate, calcium chloride and peptone. Lasley and Bogart (1944) reported that addition of egg yolk to a phosphate buffer increased the resistance to cold shock of both epididymal and ejaculated sperm. Mayer and Lasley (1945) isolated an active resistance factor from egg yolk. This resistance factor-phosphate buffer mixture proved more effective than the original yolk-buffer mixture in increasing the resistance of bull spermatozoa to cold shock. However, these authors stated that the resistance factor-buffer mixture was not as effective with spermatozoa from the ram or boar as with the bull (Mayer and Lasley, 1945). Noll (1950) observed that pure egg yolk added to boar semen, in a ratio of 1:2, increased the survival of sperm cells during storage at 5C up to 11 days.

Roy (1955) extended boar semen in 3.2% sodium citrate-egg yolk or 3% glycine-egg yolk extenders and stored the extended semen at 4C. After 24 hours, motile sperm cells were found in the glycine-egg yolk extender but none were found in samples extended with the citrate-egg yolk exten-

der. Polge (1956a) compared stored boar semen extended in either yolk-citrate, yolk-phosphate, yolk-glucose or a yolk-glycine. The extenders that maintained the highest sperm cell motility after storage at 5C were the yolk-glycine and yolk-glucose extenders. The yolk-phosphate extender produced slightly higher motility than the yolk-citrate extender (Polge, 1956a). However, Aamdal and Hogset (1957) found that the motility was highest for boar semen that had been extended in a 3% sodium citrate-yolk extender containing penicillin and streptomycin. The citrate-yolk extender was compared with a phosphate extender, a skimmed milk extender and a glycine-yolk extender. In addition, Illinois Variable Temperature extender (IVT), which contains sodium citrate, has been used widely in commercial swine AI (Graham et al., 1978). Graham et al. (1978) quoted reports that in the Swedish AI program in 1973, a 68.6% farrowing rate was obtained with an IVT buffer after first inseminations of 9.860 sows. Farrowing rate was defined as the percentage of females bred that farrowed. Boender (1966) reported an average of 67.5% pregnancy rate for extended semen stored for 72 hours in a modified IVT. This extender was saturated with carbon dioxide and contained 12 grams of fructose per liter of extender.

Graham et al. (1978) stated that possibly the most commonly used extender for "on the farm AI programs" con-

tained skim milk, which was effctive when semen was extended and inseminated on the day of collection. Koh et al. (1976) used a skim milk-glucose extender and reported an overall farrowing rate of 69.8%.

Currently, most swine semen extenders are based on glucose as an energy substrate with the addition of buffering materials and antibiotics. Proteins in the form of egg yolk or milk have sometimes been added but appear to be non-essential (Graham et al. 1978).

Extender Types

Semen extenders can be divided into three major categories: 1) extenders containing egg yolk; 2) extenders containing milk; and 3) extenders containing defined chemical reagents.

Lasley et al. (1942) reported that egg yolk buffer increased the resistance of bull spermatozoa to cold shock by approximately 400% and increased storage time approximately 600% over non-diluted semen. Several investigators have added varying amounts of egg yolk to swine semen extenders to reduce the adverse effect of cold shock on boar spermatozoa. Dziuk (1958) and Pursel et al. (1969, 1970, 1973c) used a glucose-bicarbonate extender containing 30% egg yolk. Benson et al. (1967) used Tris buffer containing 5% yolk. Boar semen was extended in a Tris-tricine-fructose extender containing 20% egg yolk (Bower et al., 1973).

Dziuk (1958) found that the most satisfactory extender for boar semen stored at 7C was the yolk-glucose-bicarbonate extender. Similar results were reported by Pursel et al. (1973c). Pursel et al. (1972b) investigated the effects of adding 0, 5, 10, 20 and 30% egg yolk to a tris-citric acid-lactose extender to determine the resistance of boar sperm cell to cold shock. These authors reported that the addition of 5 to 30% egg yolk to the tris-lactose extender did not protect boar spermatozoa from cold shock. These results were in agreement with Benson et al. (1967) and Pursel et al. (1970).

Thacker and Almquist (1951) described the use of boiled, pasteurized homogenized milk and boiled, pasteurized skim milk as extenders of bull semen. Bull semen diluted in boiled milk resulted in a 79% pregnancy rate based on 30 to 60 day non-returns. Milk was boiled for ten minutes and cooled before dilution. Using the milk extender described by Thacker and Almquist (1951) to extend and store boar semen, Stratman et al. (1958) reported conception rates % 90% and 95% for fresh extended semen and 12 hr stored semen, respectively. Stratman and Self (1961) reported a conception rate of approximately 70% using semen diluted with the milk extender. Conception rate was defined as the percentage of possible ova that were normally developing feti. Koh et al. (1976) found that AI with milk extended semen

yielded a 69.8% overall farrowing rate. First et al. (1963) reported an average fertilization rate of 61%, using fresh semen extended with milk. Fertilization rate was defined as the percentage of possible ova that were normally developing embryos.

Although quantities vary, defined ingredient extenders generally contain the following: an energy substrate, usually a sugar; ionic salts; and antibiotics to retard bacterial growth. Extender antibiotics are discussed in a later section of this review. There are many defined ingredient extenders described in the literature. Only the major components are discussed in this review.

The most common energy substrate added to extenders has been glucose. Several investigators (Waltz et al., 1968; Pursel et al. 1972a,b, 1973b,c; Foley et al., 1967, as modified by Pursel et al. 1974; and Plishko 1965 as quoted by Johnson et al. 1980) have reported using glucose as an energy substrate. Quantities of glucose varied from 3 g per liter (Waltz et al. 1968) to 60 g per liter (Johnson et al. 1980). The following sugars have also been used in swine semen extenders: fructose (Benson et al., 1967; Foley et al., 1967; and Pursel et al., 1972b); lactose (Pursel et al., 1972a,b, 1973a, 1974); sucrose (Pursel et al., 1972b); and raffinose (Pursel et al., 1972b). Pursel et al. (1972b) reported that sperm cells were significantly more

resistant to cold shock in extenders that contained lactose, sucrose or raffinose than in extenders that contained either glucose or fructose.

The combination of Tris (hydroxymethyl) aminomethane and citric acid has been used in semen extenders (Benson et al., 1967; Pursel et al. 1972ab; 1973 a,c, 1974). The Illinois Variable Temperature (IVT) developed by du Mensil du Buisson and Dauzier (1958) contained the combination of sodium citrate, sodium bicarbonate and potassium chloride. Pursel et al. (1973,b) developed the Beltsville L1 extender, sodium citrate, sodium bicarbonate and potassium chloride. Pursel et al. (1974) used a modified Purdue extender (Foley et al., 1967) that contained a combination of the same three ionic salts. The salt composition of Kiev extender consisted of sodium citrate, sodium bicarbonate and disodium ethylenediamine tetraacetate (Plishko, 1965 as quoted by Johnson et al. 1980).

Extender Effects on Acrosome Morphology

Extender pH, sugar composition and percent egg yolk significantly affect the cold shock resistance of boar sperm acrosomes as measured by the percentage of cells having a normal apical ridge acrosome (NAR; Pursel et al., 1972b). Sperm cells were significantly more resistant to cold shock after 7 hr incubation in extenders with pH of 5.9, 6.6 and 7.3 than in extenders with pH of 8.0 and 8.3 (Pursel et al.,

1972b). After 5 hr incubation, boar sperm cells were more resistant to cold shock in extenders containing either lactose, sucrose or raffinose than in extenders containing glucose or fructose (Pursel et al., 1972b). Pursel et al. (1972b) reported that sperm acrosomes were more cold shock resistant after 5 hr incubation in an extender without egg yolk than in an extender containing 30% egg yolk or 20% egg yolk.

Pursel et al. (1973c) have reported that semen extended with egg yolk-glucose-bicarbonate maintained a significantly higher percentage of NAR than semen extended in either of the defined ingredient extenders, Beltsville L1 (BL1) or Beltsville L2 (BL2). Beltsville L2 exteder maintained a significantly higher percentage NAR than Beltsville L1 (Pursel et al., 1973c). After storage for 7 days at 15C, semen extended in BL1, Purdue extender (PE), IVT + CO2 and IVT - CO2 had higher percentages of NAR than semen extended in glucose-bicarbonate (GB) (Pursel et al. 1974). Pursel et al. (1974) reported that the percentage of NAR for the BL1 extender was significantly higher than for the PE extender, following storage at 15C for 7 days. Pursel (1979) stated that the optimum sperm concentration for the maintenance of NAR was $80x10^6$ cells/ml for BL1 extender and $40x10^6$ cells/ml for Kiev extender. Boar sperm acrosomes were more resistant to cold shock when semen was diluted with exten-

ders that: 1) had a pH from 5.9 to 7.3; 2) contained lactose, sucrose or raffinose; and 3) contained no egg yolk.

Extender Effects on Sperm Motility

Roy (1955) observed motile sperm cells after 24-hr storage at 4C when semen was extended in glycine-egg yolk extender but no motile cells were found when semen was extended in citrate-egg yolk extender. Similar results were reported by Polge (1956a). In contrast, Aamdal and Hogset (1957) reported the highest motility in semen extended with a citrate-yolk extender that contained penicillin and streptomycin. Dziuk (1958) found that semen maintained the highest motility when extended with a yolk-glucose-bicarbonate extender. Pursel et al. (1973c) stated that the percentage of motile spermatozoa was significantly higher for semen stored in egg yolk-glucose-bicarbonate extender than in BL2 or BL1 and values were higher for semen stored in BL2 than in BL1. However, Benson et al. (1967) found no difference in sperm motility after cold shock, when semen was extended with a Tris-fructose-citric acid extender containing either 0% or 5% egg yolk. Using a Tris-lactose extender, Pursel et al. (1972b) varied the level of egg yolk from 0 to 30% and found no significant effect on sperm motility after cold shock.

The percentages of motile spermatozoa cold shocked after two hours incubation were significantly higher in

extenders with a pH of 7.3, 8.0 and 8.3 than in extenders with a pH of 5.9 and 6.6 (Pursel et al. 1972b). These authors observed the same trend for percent motile sperm cells after 4.5 hr of incubation as after 2-hr incubation. This trend was not seen after a 7-hr incubation (Pursel et al., 1972b).

When the extenders containing glucose, fructose, lactose, sucrose and raffinose were compared, no significant differences for percent motility after cold shock could be detected among extenders. Sperm motility was higher when semen was diluted with extenders that had a pH from 7.3 to 8.3. Motility was not affected by the sugar composition or the presence of egg yolk.

Extender Effects on Fertility

Wiggins et al. (1951) reported an average of 91% fertilized ova when gilts were inseminated with 20 ml of semen diluted to a final volume of 50 ml. with a modified Krebs solution. Polge (1956a) stated that 50 to 60 percent of the females bred should become pregnant with no reduction in litter size when inseminated with semen extended with either yolk-glucose or a yolk-glycine extender. Aamdal and Hogset (1957) reported that semen diluted with a yolk-citrate extender containing penicillin and streptomycin resulted in a 65.5% pregnancy rate. Pregnancy rates of 55, 5 and 42% for diluted semen stored 1, 2 and 3 days, respectively, were

reported by Dziuk and Henshaw (1958). The extender used was a yolk-glucose-bicarbonate extender with antibiotics added only to semen stored for 3 days (Dziuk and Henshaw, 1958). The mean percent conception rate reported by Stratman and Self (1961) was 73.9% for $2.5x10^9$ sperm in 100 ml of milk extender. In Finland, the average conception rates using a yolk-citrate extender were 74.3, 73.2 and 69.4% for the years 1961, 1962 and 1963, respectively (Lindstrom, 1966). Rutgers (1966) reported that in the Netherlands, 63.9% and 65.3% of females were pregnant after the first insemination, using a skim milk-yolk extender or a glucose-bicarbonate extender, respectively. In a review, Graham et al. (1978) quoted reports that in the Swedish AI program in 1973, a 68.6% farrowing rate was obtained after first inseminations using IVT buffer.

Pursel et al. (1973b) extended semen in Beltsville L1 and held it at a 15C storage temperature. These authors obtained 87.2, 91.4 and 92.6% of normal fertilized ova for semen stored for one, three and five days, respectively. Kabishima et al. (1975) compared semen stored for 96-120 hr with fresh semen and found a decreased pregnancy rate between fresh and stored semen, 94% and 84%, respectively. Johnson et al. (1980) reported significantly more females farrowed when inseminated with semen extended in Kiev extender than in BL1 extender (Johnson et al., 1980). The

defined ingredient extenders appeared to have a more beneficial effect on fertility than the extenders that contained either milk or egg yolk.

Storage Temperature

A wide range of storage temperatures has been used for liquid boar spermatozoa. Boar semen has been stored at temperatures of 4C (Roy, 1955); 5C (Polge, 1956ab; Boender, 1966; Pursel et al. 1973c, 1974); 7C (Dziuk, 1958); 9C (Stratman et al. 1958); 10C (Koh et al., 1976); 10 to 12C (Lasley and Bogart, 1944); 15 to 20C (Ito et al., 1948b; Aamdal and Hogset, 1957; Boender, 1966; Melrose, 1966; Pursel et al. 1973c, 1974); 25C (Boender, 1966; Pursel et al., 1974; and 35C (Boender, 1966).

Effects of Storage Temperature on Acrosome Morphology

Pursel et al. (1973c) stated that an important factor during storage at 5C was the cold damage to boar spermatozoa even though cooling from room temperature (24 to 25C) to 5C was done over a 2-hr period. These authors cited three observations that supported the preceeding statement: 1) The percentage of NAR was higher in samples stored at 15C than in those stored at 5C; 2) There were higher percentages of sperm cells having either a missing apical ridge acrosome (MAR) or a loose acrosomal cap (LAC) in samples

stored at 5C than in samples stored at 15C. Typical acrosomal changes associated with cold shock were MAR and LAC (Pursel et al., 1972a); and 3) Holding undiluted semen at room temperature for 1.25 hr to 7.2 hr maintained higher acrosomal integrity for samples stored at 5C than samples held for .25 hr (Pursel et al. 1973,c). The percentages of MAR and LAC were higher for BL1 and BL2 extended semen than for egg-glucose-bicarbonate extended semen stored at 5C (Pursel et al., 1973c).

Pursel et al. (1974) compared 25C and 15C storage temperatures. Semen was extended with either 2.9% citrate, .9% saline, Tris-lactose-citric acid (Tris) or IVT without carbon dioxide saturation. Sperm acrosomes deteriorated more rapidly when stored at 25C than at 15C in all four extenders. At both 25C and 15C, acrosomes deteriorated at a significantly faster rate when semen was extended in Tris and saline extender than in citrate and IVT without carbon dioxide saturation. In another experiment, semen was extended in either BL1, BL2, IVT+CO2, IVT-CO2, PE or GB and stored at either 25C for three days or 15C for 7 days (Pursel et al., 1974). Semen samples stored at 15C were removed after 7 days and evaluated or incubated for 3 hr at 37C and then evaluated for acrosomal integrity. A significantly higher percentage of NAR was observed after 3 days at 25C for samples in BL1, PE and IVT+CO2 extenders than in GB and

IVT-CO2 extenders. The percentage of NAR was higher in BL1, IVT+CO2 and IVT-CO2 and PE extenders than in GB extender for samples stored at 15C. Following storage at 15C, percent NAR was greater for semen extended in BL1. Similar results were reported for semen stored at 15C and incubated for 3 hr at 37C before acrosomal evaluation. Acrosome morphology was highest at a 15C storage temperature.

Effects of Storage Temperature on Sperm Motility

Ito et al. (1948b) reported that 15 to 20C was the proper storage temperature for optimal sperm motility. Sperm stored at 4C maintained their initial motility values for over 30 hr in samples extended with equal volumes of seminal plasma and 4.5 percent glycine-egg yolk and for over 48 hours in samples extended with the glycine-yolk extender without seminal plasma (Roy, 1955). Polge (1956a) reported 60 to 70% motility in samples stored at 5C for 5 or 6 days. Aamdal and Hogset (1957) diluted semen with a yolk-citrate extender and stored the semen at 15 to 20C for 120 hours. The mean percentages of motile sperm ranged from 79.2 to 1.6 for 0 hr and 120 hr, respectively. Dziuk (1958) stated that motility of sperm stored at 16C was often superior to samples stored at 7C for the first 48 hr, but motility was never superior at 16C after 48 hours. Stratman et al. (1958) stored semen at 9C for 12 hours. The mean percentage of motile spermatozoa for fresh semen was 72.2% and 47.2%

for stored semen. Boender (1966) reported that semen stored at 15C maintained higher sperm motility than semen stored at either 5C, 25C or 35C. Pursel et al. (1974) reported a significantly faster decline in motility of spermatozoa extended in saline and citrate extenders than those extended in Tris and IVT-CO2 extender at both a 15C and 25C storage temperatures. At 25C for 3 days, the percentage of motile spermatozoa was higher for semen extended in BL1, PE and IVT+CO2 than for semen extended in GB and IVT-CO2. For semen stored at 15C the percent motility was higher for IVT+CO2 extended samples than for the other extenders. Semen extended in BL1 and PE had significantly higher percentages of motile spermatozoa than samples in GB and IVT-CO2. When semen stored at 15C was incubated for 3 hr at 37C before evaluation the percentages of motile sperm cells were higher for the BL1, PE and IVT+CO2 extenders than for the GB and IVT-CO2 extenders (Pursel et al. 1974). Sperm motility was highest at a 15C storage temperature.

Effects of Storage Temperature on Fertility

When diluted semen was stored at 15 to 20C, Aamdal and Hogset (1957) reported an overall pregnancy rate of 59.4 percent. This overall pregnancy rate included semen that had been stored at 15 to 20C for 3 to 60 hours. Stratman et al. (1958) reported that the mean percent fertilized ova for fresh diluted semen was 76.9% compared at 9C with 83.6%

for semen stored for 12 hours. However, Kabishima et al. (1975) reported a decreased pregnancy rate for stored semen versus fresh semen. Semen was stored at either a constant 15C or at a range of 8 to 12C for 96 to 120 hr. Pregnancy rates were 94, 83 and 85% for fresh, constant temperature semen and varied temperature semen, respectively. et al., 1975). These authors found no significant difference for pregnancy rate between either of the storage temperatures. Johnson et al. (1980) stored diluted semen at 18C for 3 days. There was no significant difference between storage times of 1 and 2 days at 18C (70.2% versus 65.9%, respectively); however, there was a significant reduction in farrowing rate between one and 3 days (70.2% versus 58.7%, respectively) (Johnson et al. 1980). The storage of semen at 15C appeared to have a more beneficial effect on fertility than did storage at lower temperatures.

Storage Time

Effects of Storage Time on Acrosome Morphology

When extended semen was held for 72 hr at either 15C or 25C Pursel et al. (1974) found that the percentage NAR decreased more rapidly for samples extended with .9% saline and Tris-lactose-citric acid extenders than with 2.9% citrate and IVT-CO2 extenders. For semen stored for three

days at 25C, the percentages of NAR were higher in BL1, PE and IVT+CO2 extenders than in GB and IVT-CO2 extenders. When semen was stored at 15C for 7 days the percentages of NAR were significantly higher for samples extended with BL1, PE, IVT+CO2, and IVT-CO2 than for samples extended with GB (Pursel et al., 1974). The percentage NAR was higher for the BL1 than for the PE extender. When samples that were stored at 15C for 7 days were incubated at 37C for 3 hr before acrosome evaluation the BL1, PE, IVT-CO2 and IVT+CO2 extenders had higher percentages of NAR than the GB extender. Also, the percentage NAR was higher for the BL1 extender than for PE, IVT+CO2 and IVT-CO2 extenders. Acrosome morphology decreased as storage time increased.

Effects of Storage Time on Sperm Motility

When sperm cells were diluted in either yolk-glycine or yolk-glucose extenders, Polge (1956a) reported that 60 to 70% of the spermatozoa demonstrated active motility after 5 or 6 days at a 5C storage temperature. However, Aamdal and Hogset (1957) found an average of 1.6% of extended spermatozoa living after storage at 15 to 20C for 120 hours. Stratman et al. (1958) reported a range in sperm motility of 30 to 65% after storage at 9C for 12 hours. The mean percent motility for fresh semen was 72.2% and 47.2% for stored semen. These authors stated that even though there was a reduction in sperm motility the fertilization rate remained

approximately the same for both fresh and stored semen (Stratman et al., 1958). First et al. (1963) found that the average percent motility of sperm cells was 79% and 33% for semen stored at 6 to 8C for 6 and 54 hr, respectively. Boender (1966) reported that approximately 20% of undiluted spermatozoa were motile after storage at 15C for 120 hours. Pursel et al. (1973c) found that there were no significant differences in the percentage of motile spermatozoa after 120 hours storage at 5C between undiluted semen and semen extended with egg-glucose-bicarbonate.

According to Pursel et al. (1974), sperm motility decreased at a faster rate over 72 hr when semen was extended in .9% saline and 2.9% citrate than in Tris-lactose-citric acid and IVT-CO2. This decline in sperm motility was exhibited at both 15C and 25C storage temperatures. For semen held for 3 days at 25C, the percentage of motile sperm cells was significantly higher in BL1, PE and IVT+CO2 extenders than in GB or IVT-CO2 extenders (for each comparison). When semen was stored at 15C for seven days, the percentage of motile spermatozoa was higher in IVT+CO2 extender than in BL1, PE, IVT-CO2 and GB extenders. At the same storage time and storage temperature, the percentage of motile sperm was higher in BL1 and PE extender than for GB and IVT-CO2 (Pursel et al., 1974). When the samples were incubated at 37C for three hours prior to evaluation, sperm

motility was higher in samples extended in BL1, PE and IVT+CO2 than those extended in GB and IVT-CO2. Sperm motility decreased as storage time increased.

Effects of Storage Time on Fertility

Ito et al. (1948a) reported that the pregnancy rate was 88.7, 71.4 and 40.0% for semen stored up to 24 hr, 24 to 48 hr and 48 to 72 hr, respectively. First et al. (1963) reported a higher average fertilization rate for semen stored 6 hr (61%) than for semen stored 54 hr (39.3%). Dziuk and Henshaw (1958) inseminated females with semen that was fresh or extended and stored for one, 2 or 3 days. The semen stored for three days was extended with antibiotic or without antibiotics. These authors found that the pregnancy rates for fresh semen and semen stored for one, two and three days with and without antibiotics were 56, 55, 5, 56 and 32%, respectively. These authors stated that the higher pregnancy rates for semen stored for 3 days as compared with 2 day storage might have been due to the addition of antibiotics to the semen stored for three days (Dziuk and Henshaw, 1958). Stratman et al. (1958) inseminated freshly collected extended semen and extended semen stored for 12 hours. The average fertilization rate for all fresh semen samples was 76.9% compared with 83.6% for the stored samples. Boender (1966) reported that the percentages of females pregnant after the first insemination were 69%, 67%,

68%, 66% and 50%, when semen was diluted and stored for 2, 24, 48, 72 and 96 hours respectively. Kabishima et al. (1975) stored semen at either a constant 15C or from 8C to 12C for 96 to 120 hours. These authors compared fresh semen and both types of stored semen and reported pregnancy rates of 94% for the fresh semen, 83% for the constant 15C stored semen and 85% for the 8 to 12C stored semen.

Pursel et al. (1973b) reported that the percentages of normally fertilized ova were 87.2, 91.4 and 92.6% for semen that was stored for 1, 3 and 5 days, respectively. Johnson et al. (1980) found that the percentages of females farrowing were 70.2, 65.9 and 58.7% for semen stored for 1, 2 and 3 days, respectively. The fertility of liquid semen decreased as storage time increased.

Incubation Time and Temperature

Effects of Incubation on Acrosome Morphology

Sperm acrosomes develop cold shock resistance, as indicated by the higher percentage NAR, during 2.5-hr and 4.5-hr incubations at 30C (Pursel et al. 1972a). These authors reported that acrosomes of whole ejaculates were more susceptible to cold shock than were acrosomes of the sperm-rich fraction. The percent NAR after cold shock was significantly lower for the 1 hr incubation period than for the 3

and 5 hr incubations of sperm cells extended with Tris-lactose-citric acid, 2.9% citrate and .9% saline extenders. Cold shock resistance was not developed when sperm were incubated at 30C in GB and IVT extenders for up to 5 hours (Pursel et al. 1972a). When semen extended in Tris-lactose-citric acid, 2.9% citrate and .9% saline extenders were incubated for 3 and 5 hr at 30C, spermatozoa developed more cold shock resistance than spermatozoa extended in GB and IVT extenders (Pursel et al. 1972a).

Pursel et al. (1972b) reported no significant differences in the percentage of NAR due to extender pH changes when samples were cold shocked after incubation at 30C for two hours. However, the percentage of NAR after cold shock was significantly higher after 4.5 hr incubation than after 2-hr incubation regardless of the pH of the extender. The percentages of NAR for pH 5.9, 6.6 and 7.3 extended samples were significantly higher after cold shock for samples incubated for 7-hr than those incubated for 4.5 hours. The percentages of NAR for pH 8.0 and 8.3 extended samples remained at nearly the same level after a 7-hr incubation as for samples incubated for 4.5 hours (Pursel et al., 1972b).

Sugar composition did not significantly affect the percentage of NAR after cold shock following incubation at 30C for 1 hour. However, for samples incubated for 5 hr, cold shock resulted in significantly lower percentages of NAR in

the extenders containing glucose or fructose than in the extenders containing lactose, sucrose or raffinose. The extender containing fructose had a significantly higher percent NAR than the extender containing glucose (Pursel et al. 1972b). A significantly higher percentage of NAR was observed after 5 hr incubation at 30C for samples extended with 0% egg yolk added to Tris-lactose extender than when 30% egg yolk was added or when 20% egg yolk was added (Pursel et al. 1972b).

Pursel et al. (1973a) found that washed boar spermatozoa had significantly higher percentages of NAR after 5 hr incubation than after 3 hr incubation and a higher percentage after 3 hr incubation than 1 hr incubation. These authors stated that the presence of seminal plasma provided additional protection against cold shock when incubated qt 30C for both 3 and 5 hours. Pursel et al. (1973a) proposed that the additional protection was due to an interaction between the spermatozoa and the seminal plasma during incubation.

The percentages of NAR were higher for undiluted semen incubated at 24 to 26C for 6.25 hr and 7.25 hr than for .25, 2.25 and 4.25 hours (Pursel et al. 1973c).

Extender pH, sugar composition and percent egg yolk did not significantly affec the acrosome morphology during incubation. The optimum incubation time appeared to be about 6 hr at a 30C incubation temperature.

Effect of Incubation on Sperm Motility

Several preliminary trials conducted by Dziuk (1958) indicated that either raw semen or diluted for less than four hours semen could be satisfactorily incubated at room temperature. However, incubation at room temperature for 8 hr or more prior to cooling reduced subsequent sperm motility.

Benson et al. (1967) incubated diluted semen for 5 hours. Samples for percent progressively motile sperm were estimated initially and at hourly intervals during the incubation period. The mean percent motility decreased from 79% to 53% during the first hour of incubation; decreased slowly from 53% to 40% from 1 to 4 hr; and then decreased rapidly from 40% to 28% during the final hour of the incubation (Benson et al., 1967). Similar results were reported by Singleton and Shelby (1972). Those authors found that the mean percent progressive motility decreased from 79% to 49% during the 3 hr incubation period at 38C.

Pursel et al. (1972a) reported that the percent motility of extended boar spermatozoa after cold shock was significantly lower for samples incubated at 30C for 1 hr than for the samples incubated for 3 and 5 hours. Pursel et al. (1972b) found that extenders with pH of 7.3, 8.0 and 8.3 had significantly higher percentages of motile spermatozoa when cold shocked after 2 hr incubation than extenders with pH of

5.9 and 6.6. However, the percent motility after cold shock was significantly higher after 4.5 hour incubations than after two hour incubations for all extender pH's. These authors also found no significant differences in percent motility among semen extenders due to sugar composition. However, percent motility was significantly higher when cold shocked after 5 hr incubations than after 1 hr incubations for all extenders (Pursel et al., 1972b). In the same study, Pursel et al. (1972b) reported that the addition of 0 to 30% egg yolk to a Tris-lactose-citric acid had no effect on percent motility after cold shock following either a 1 or 5 hr incubation. The percent motility was significantly higher when samples were cold shocked after 5 hr incubations than after 1 hr incubations for all levels of egg yolk (Pursel et al., 1972b). Similar effects of incubation at 30C on sperm motility have been reported by Pursel et al. (1973a,c). In both studies the percentages of sperm motility increased as the incubation time at 30C increased.

The optimum incubation conditions appear to be 30C for 6 hours. Extender pH, sugar composition and percent egg yolk did not significantly affect the sperm motility during incubation.

Cooling Rate

Polge (1956a) stated that when whole undiluted semen was cooled from 20C to 5C over a 6 hr period, very few sperm cells were alive after 24 hr storage. Dziuk (1958) cooled raw semen to room temperature in approximately 1 hr and diluted it at room temperature. Semen that was to be stored at 7C was cooled from room temperature to 7C at a rate of less than 5C/hour. This cooling period was based on results of preliminary trials which indicated that sperm motility was reduced after storage if semen was cooled at a faster rate (Dziuk 1958).

Boender (1966) reported the rapid decrease in viable spermatozoa at temperatures below 15C even when semen was cooled at 1C/5 minutes. He advised that a cryoprotectant such as egg yolk be added and the temperature of the semen be reduced to 15 to 20C as soon as possible. The cooling of the extended semen was then done at a rate of 1C/1 or 2 minutes. Boender (1966) stated that if the above procedures were followed there were no cold shock effects. However, Pursel *et al.* (1973c) reported that cold damage was an important factor during storage at 5C even though cooling from room temperature to 5C was done over a 2 hr period.

Antibiotics

History of Antibiotic Usage

The most commonly used antibiotics in boar semen extenders are a combination of penicillin and streptomycin (Polge 1956a; Aamdal and Hogset, 1957; Dziuk, 1958; Dziuk and Henshaw, 1958; Stratman et al. 1958; du Mesnil du Buisson and Dauzier, 1958; Stratman and Self, 1961; Benson et al., 1967; Foley et al., 1967, Pursel et al., 1973bc). The antibiotic sulphanilamide was added to a boar semen extender according to Noll (1950). In addition to the combination of pencillin and streptomycin sulphanilamide was added to the IVT extender (du Mensil du Buisson and Dauzier, 1958).

Effects of Antibiotics on Sperm Motility

Dziuk (1958) diluted 23 semen samples with extender containing penicillin and streptomycin. Of the semen samples stored at 16C the samples which contained antibiotics had higher motility than those not containing antibiotics (Dziuk, 1958).

Waltz et al. (1968) found and identified eight genera of bacteria present in fresh boar semen. The bacteria staphylococci were found most frequently. These organisms were found in the semen of all boars. When 16 antibacterial agents were tested with extended semen stored at 16C for 72 hr, erythromycin, polymixin B, neomycin, tylosin and sulfa-

diazine were most effective in bacterial control. Sperm motility was highest after storage in extenders containing neomycin, polymixin B and sulfadiazine (Waltz et al. 1968).

Effects of Antibiotics on Fertility

Polge (1956a) extended boar semen in a yolk-glycine extender with and without the antibiotic combination of penicillin and streptomycin. These samples were stored at 5C for 2 to 6 hr, 24 to 30 hr and 3 to 5 days. When gilts were inseminated with semen stored for 2 to 6 hr, the semen diluted with extender containing the antibiotics resulted in a 50% pregnancy rate compared with a 35.7% pregnancy rate for semen diluted without the antibiotics (Polge 1956a). When sows were inseminated with semen processed the same way, the semen which contained the antibiotics resulted in a 79% pregnancy rate compared with a 45.5% pregnancy rate for semen without antibiotics. When semen was stored for 24 to 30 hr, the pregnancy rate for samples containing antibiotics decreased to 33.3% and to 28.6% for samples without antibiotics. After storage for 3 to 5 days, Polge (1956a) reported a 0% pregnancy rate when gilts were bred with semen containing antibiotics compared to 21.4% for semen without antibiotics.

Dziuk and Henshaw (1958) stored diluted semen at 7C for 1, 2 and 3 days. These authors reported pregnancy rates of 56, 5, 5% and 42% for fresh, undiluted semen and diluted

semen stored for 1, 2 and 3 days, respectively. They proposed that the increased pregnancy rate which resulted from semen stored for 3 days compared with semen stored for 2 days might have been due to the additions of penicillin and streptomycin to the semen stored for 3 days.

The addition of antibiotics to semen extenders appears to have a beneficial effect on fertility.

Sperm Enzymes

Basic Aspects of Fertilization

Mammalian fertilization requires sperm penetration through the investments surrounding the ovum. These investments may include some or all of the following: cumulus oophorus; corona radiata; zona pellucida; and vitelline membrane. The penetration of these investments is facilitated by the hydrolytic enzymes from the acrosome.

During spermiogenesis, the Golgi apparatus produces membrane bound vesicles called proacrosomal granules. These proacrosomal vesicles coalesce to form a single acrosomic granule which is located outside the nuclear membrane in the anterior region of the sperm cell. With further development, the acrosomic granule spreads over the anterior half to two-thirds of the nucleus forming a cap-like structure known as the acrosome. This acrosome consists of an inner

and an outer acrosomal membrane enclosing the acrosomal matrix. The entire acrosome is enclosed within the sperm plasma membrane.

Before ejaculated spermatozoa are capable of fertilization, they must undergo a process known as capacitation. Capacitation was first described independently by Austin (1951) and by Chang (1951). These authors reported that the sperm of fertile rabbits and rats must first reside in the female reproductive tract for some hours before the sperm were able to penetrate the zona pellucida. Cornett et al. (1979) defined capacitation as those changes, whose nature is unknown, that must have taken place in the sperm cell before the cells could undergo the "true" acrosome reaction. The "true" acrosome reaction involves the progressive membrane fusion of the sperm plasma membrane and the underlying outer acrosomal membrane which leads to vesiculation and the exposure of the acrosomal enzymes (Bedford, 1970). The "false" acrosome reaction is a random loss of the sperm plasma membrane and the outer acrosomal membrane usually seen as a feature of degenerative breakdown (Bedford, 1970). The "true" acrosome reaction allows for a gradual release of the enzymes required for penetration of the cumulus oophorus and the corona radiata. The outer vesicules are lost before the zona pellucida is penetrated. The sperm cell, now bound anteriorly by the inner acrosomal membrane, penetrates the

zona pellucida and aligns with the vitelline membrane. The microvilli on the ovum plasma membrane surround the sperm head and the plasma membrane of the sperm and the ovum fuse beginning along the postacrosomal region of the sperm head. At the time of sperm penetration the ovum is activated and responds by the resumption of the second meiotic division and emission of the second polar body and by the induction of blocks to polyspermy, the zona reaction and the vitelline block. The blocks to polyspermy involve the loss of permeability at the zona pellucida and alterations in the surface of the ovum.

The ovum now contains a male pronucleus and a female pronucleus which undergo growth and enlargement. The two pronuclei move toward each other and come into contact. The two pronuclei undergo the process of syngamy. The nuclear envelopes of each pronucleus break down and disappear resulting in the condensation and assembly of the two groups of chromosomes. This ends the process of fertilization with the development of the zygote.

Acrosomal Hyaluronidase

In a review on the biochemistry of fertilization, McRorie and Williams (1974) reported that hyaluronidase was the most readily released sperm enzyme. Hyaluronidase functions to digest the hyaluronic acid found between the cells of the cumulus oophorus (McRorie and Williams 1974). The function

of acrosomal hyaluronidase was supported by the results of Metz (1973), who reported that the penetration of cumulus oophorus cells of the rabbit ova was blocked by antibodies to rabbit hyaluronidase.

McRorie and Williams (1974) reported that intact rabbit sperm agglutinated in the presence of anti-hyaluronidase antibody. This suggested that a portion of the hyaluronidase was located on the plasma membrane. Morton (1975) used an immunochemical, monospecific antibody for purified ram sperm hyaluronidase to determine the location of this enzyme. Following treatment with the anti-hyaluronidase antibody, intact ram spermatozoa were stained over the anterior region of the head, with the periphery of this region more densely stained (Morton, 1975). Ram sperm were subjected to hypotonic shock following freezing and thawing to disrupt the plasma membrane and the outer acrosomal membrane and to release the acrosomal contents. When these cells were exposed to the anti-hyaluronidase antibody there was a marked reduction in the staining of the sperm head. These results suggested that the major portion of hyaluronidase was located within the acrosomal contents and outer membranes (Morton, 1975). Gould and Bernstein (1973) localized hyaluronidase with the electron microscope using immunochemical techniques. They suggested that the major portion of sperm hyaluronidase was located in the acrosomal matrix.

Zaneveld et al. (1973) demonstrated that bull sperm acrosomal hyaluronidase was identical to bull testicular hyaluronidase and both were different from lysososomal hyaluronidase present in organs other than the testes. Bull sperm hyaluronidase had an optimum pH of 3.75 and was unstable at a pH below 3.0 or temperatures above 50C. The enzyme required salt for stability and activity (Zaneveld et al. 1973). Using a Sephadex G-100 column, Zaneveld et al. (1973) estimated that the molecular weight of the bull sperm acrosomal hyaluronidase was 110,000 daltons. In contrast, Yang and Srivastava (1974) reported that the pH optimum of ram acrosomal hyaluronidase was 4.3 and the molecular weight was estimated at 62,000 daltons. Yang and Srivastava (1974) proposed that Zaneveld et al. (1973) had estimated the molecular weight of a dimer of the bull acrosomal hyaluronidase used in that study.

Acrosomal hyaluronidase is a readily released sperm enzyme that is located on the outer acrosomal membrane and in the acrosomal matrix. It functions by digesting the hyaluranic acid found between the cells of the cumulus oophorus.

Corona Penetrating Enzyme

Initially, Zaneveld et al. (1968, 1969) reported the discovery of a sperm enzyme that dispersed the cells of the corona radiata but had no effect on the zona pellucida.

These early reports designated this enzyme corona-removing enzyme. However, Zaneveld and Williams (1970) changed the name to corona penetrating enzyme (CPE) to more properly describe the function of the enzyme in fertilization. They stated that CPE functioned by dissolving the intercellular material between the cells of the corona radiata in a manner similar to the action of hyaluronidase on the cells of the cumulus oophorus.

McRorie and Williams (1974) stated that Tillman (1972) found CPE activity in the acrosomes of the rabbit, bull, human, stallion and boar. McRorie and Williams (1974) also stated that CPE was demonstrated to be distinct from hyaluronidase and acrosin.

Proacrosin

Meizel (1972) identified an inactive zymogen precursor to acrosin in rabbit testis extracts. This precursor was termed proacrosin. Meizel and Mukerji (1975) extracted proacrosin from rabbit cauda epididymal spermatozoa. These authors reported that rabbit proacrosin had a molecular weight of 73,000 daltons and complete autoactivation of proacrosin resulted in the production of acrosin with a molecular weight of 38,000 daltons. The autoactivation of proacrosin to acrosin was stimulated by Ca^{++} and inhibited by Zn^{++} (Meizel and Mukerji 1975).

Harrison and Brown (1979) reported that the inactive zymogen, proacrosin, was the only form of acrosin within intact mature ram spermatozoa. The molecular weight of ram proacrosin was estimated at 60,000, using gel chromatography and at 51,300, using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. When the molecular weights of ram proacrosin were compared with the molecular weights of ram acrosin (44,000 and 40,000 daltons, using the two methods, respectively), Harrison and Brown (1979) indicated that a single acrosin molecule was derived from a single proacrosin molecule.

Polakoski (1974) reported that proacrosin accounted for almost all of the acrosin in freshly ejaculated boar spermatozoa. In contrast to the findings reported for other species, Polakoski and Parrish (1977) reported that two forms of proacrosin were purified from ejaculated boar sperm. Using sodium dodecyl sulfate gel electrophoresis they estimated that the molecular weights of the two forms were 55,000 and 53,000 daltons.

Conversion of Proacrosin to Acrosin

Polakoski and Parrish (1977) reported a subsequent degradation of the two forms of boar proacrosin prior to the detection of acrosin activity. Boar acrosin was found in three active forms; α-acrosin, B-acrosin and γ-acrosin. The molecular weights for the three forms of acrosin were

49,000, 34,000 and 25,000 daltons, respectively (Polakoski and Parrish, 1977). McRorie et al. (1976) reported the presence of a non-acrosin metalloproteinase in sperm extracts called acrolysin that initiated the conversion of the larger form of proacrosin to the smaller form of boar proacrosin. However, results reported by Harrison and Brown (1979) indicated that ram proacrosin was autoactivatable and involved no auxiliary enzyme.

Polakoski et al. (1979) found that 70 to 80% of the proacrosin was converted to acrosin in sperm cells incubated in utero for 120 min, while less than 3% of the proacrosin was converted to acrosin in the sperm incubated in the calcium-free ringer-fructose buffer at 37C. The first form of active acrosin obtained from sperm incubated in utero was the α-acrosin (molecular weight 49,000) which was followed by the appearance of B-acrosin (molecular weight, 34,000 daltons, Polakoski et al. 1979). Since only 3% of the proacrosin was converted to acrosin in sperm cells incubated in vitro, Polakoski et al. (1979) suggested that the female reproductive tract or its fluids played an active role in the conversion of proacrosin to acrosin.

Wincek et al. (1979) isolated a factor present in uterine flushings of gilts that stimulated the conversion of highly purified boar proacrosin to acrosin in vitro. Characterization of this factor from uterine flushings indicated

that the active component was a glycosaminoglycan (Wincek *et al.*, 1979). Parrish *et al.* (1979b) obtained glycosaminoglycans from shark cartilage, whale cartilage, porcine intestinal mucosa, porcine skin and human umbilical cord. Glycosaminoglycans from each source stimulated the *in vitro* conversion of highly purified boar proacrosin to α-acrosin. These results demonstrated that the glycosaminoglycan stimulation of the proacrosin to acrosin conversion was a general phenomenon that was not species or organ specific (Parrish *et al.*, 1979b).

Parrish and Polakoski (1977) demonstrated that polyamines, particularly spermine, stabilized and stimulated the enzymatic activity of boar acrosin and inhibited the autoactivation of boar proacrosin to acrosin. Spermine protected acrosin against autoproteolysis. The order of effectiveness, in decreasing rank, of the polyamines added was spermine, spermidine, cadaverine, putresceine and 1,3-diaminopropane. Parrish and Polakoski (1977) suggested that polyamines could serve as *in vivo* modulators of the proteolytic activity of acrosin and the activation of proacrosin. Similar results were reported by Parrish *et al.* (1979a) for the effect of polyamines on the conversion of human proacrosin to acrosin.

The conversion of proacrosin to acrosin is stimulated by glycosaminoglycans and is inhibited by polyamines, especially spermine.

Acrosin

Bedford (1970) discussed the existence of a factor that facilitated the passage of sperm through the zona pellucida. This factor was termed zona lysin. However the definite nature of zona lysin was unknown.

Zaneveld et al. (1972) obtained a highly purified proteolytic enzyme from rabbit sperm acrosomes. This proteolytic enzyme was termed acrosin and was found to be essential for penetration of the zona pellucida by spermatozoa. Working in the same laboratory, Polakoski et al. (1972) estimated that the molecular weight of the highly purified rabbit acrosin was 55,000 daltons by Sephadex chromatography.

Zaneveld et al. (1975) reported that acrosin hydrolyzed the amine and ester derivations of both arginine and lysine. Substrates that contained arginine were hydrolyzed at a faster rate than those that contained lysine.

Polakoski and McRorie (1973) also reported that boar acrosin exhibited amidase, esterase and proteinase activity on substrates that contained arginyl and lysyl residues. They determined that highly purified preparations of boar acrosin had endopeptidase activity and cleaved only the carboxyl bonds of arginine and lysine, with a strong preference for arginine bonds. Acrosin was inhibited by diisopropyl fluorophosphate and tosyl lysine chloromethyl ketone. Pola-

koski and McRorie (1973) proposed that this inhibition indicated that serine and histidine residues were present in the active site.

In 1978, Parrish and Polakoski characterized the properties of boar α-acrosin, the initial active enzyme that resulted from the conversion of proacrosin to acrosin. They indicated that the properties of α-acrosin and B-acrosin were very similar even though the estimated molecular weights differed by approximately 15,000 daltons. Parrish and Polakoski (1978) suggested that the portion of the molecule lost during the conversion contributed little to the spacial arrangement of either the active site or regulatory sites of the enzyme.

Srivastava et al. (1974) selectively removed the acrosomal membranes and acrosomal enzymes of ram spermatozoa. These authors reported that vesiculation and fragmentation of the plasma membrane and outer acrosomal membrane released hyaluronidase and corona penetrating enzyme. Subsequent detergent extraction of the inner acrosomal membrane and the electron dense material from the equatorial segment dissolved the zona pellucida. Their results indicated that the location of the acrosin of ram sperm was the inner acrosomal membrane or the equatorial segment.

Morton (1975) used an immunochemical antibody technique to locate acrosin in ram spermatozoa. When whole sperm cell

preparations were treated with antiacrosin antibody, the sperm cells were more densely stained in the equatorial region of the head and staining extended further down the sperm head. A more exact location of acrosin was obtained by removing the plasma membrane and the outer acrosomal membrane with a subsequent release of the acrosomal contents. Following removal of these membranes, sperm cells were treated with antiacrosin antibody and approximately 80% of the acrosin activity remained bound to the inner acrosomal membrane (Morton, 1975).

In contrast to the findings of Srivastava et al. (1974) and Morton (1975), Shams-Borhan et al. (1979) reported that acrosin was not bound to the inner acrosomal membrane of bull spermatozoa. Shams-Borhan et al. (1979) used a ferritin-conjugated soybean trypsin inhibitor to locate acrosin following acrosomal disruption. The ferritin label was not found on intact sperm cells. Ferritin particles were found uniformly amid the acrosomal matrix. When the acrosomal matrix was not present, ferritin label was observed on the external surface of the outer acrosomal membrane. However, these workers reported that at no time was the ferritin label found on the inner acrosomal membrane.

Acrosin is a proteolytic ensyme which penetrates the zona pellucida of the ova. It is believed to be located bound to the inner acrosomal membrane.

Naturally Occurring Acrosin Inhibitors

Zaneveld et al. (1969) reported that at the time of ejaculation mammalian sperm interacted with seminal plasma and bound proteinase inhibitors. These inhibitors were removed or inactivated and allowed sperm to digest a path through the zona pelucida of the ovum. These inhibitors were not present in capacitated spermatozoa, therefore, these inhibitors were removed while sperm were in the female reproductive tract (Zaneveld et al. 1969).

Polakoski and Williams (1974) isolated five proteinase inhibitors from boar seminal plasma and purified three of these inhibitors to homogeneity. They estimated that the molecular weights were 1600, 13400, 6800 and 5800 daltons for the second, third, fourth and fifth inhibitors, respectively. The molecular weight of the first inhibitor was not determined.

In the same study, Polakoski and Williams (1974) demonstrated the presence of three proteinase inhibitors in boar sperm acrosomal preparations. The molecular weight of these inhibitors were estimated to be 13400, 6800 and 1600 daltons. However, further characterization of these inhibitors was not undertaken due to the very small amount of starting material.

The third seminal plasma inhibitor was used to determine the effect of proteinase inhibitors on fertility.

Capacitated rabbit sperm were treated with the inhibitor and their ability to fertilize ova was determined *in vivo* and *in vitro*. Polakoski and Williams (1974) reported that the pure inhibitor prevented fertilization by capacitated sperm. No treated sperm cells were found in the perivitelline space. This indicated that the block to fertilization prevented sperm passage through the zona pellucida. However, ova exposed to the untreated sperm had sperm cells in the perivitelline space (Polakoski and Williams, 1974).

Polakoski *et al.* (1971) reported that *in vitro* incubation of ejaculated boar sperm at pH 3 dissociated the acrosin-acrosin inhibitor complex.

Fritz *et al.* (1975) proposed that the *in vivo* function of acrosin inhibitors in the male was to rapidly neutralize the enzymatic potential of acrosin if acrosin was released before mating or capacitation in the female reproductive tract.

Chapter III. EXPERIMENTAL PROCEDURES

This study consisted of two phases. Phase I involved the evaluation of extenders and the development of extender systems. In phase II we evaluated the fertilizing capacity for that extender and extender system from Phase I that maintained the highest level of cellular integrity.

Preliminary Studies

In a preliminary study, five boars trained for semen collection from a "dummy sow" were collected using the gloved-hand technique (Hancock and Hovell, 1959). Eighty-three ejaculates were collected and used to calibrate a Bausch and Lomb photoelectric colorimeter (Spectrophotometer 20) for the determination of sperm concentration, using the procedures outlined by Crabo (1978, personal communication).

This preliminary study was conducted to: determine the total measurable amount of acrosin activity released; and to evaluate the effect of freezing on acrosin activity. The sperm-rich fraction of an ejaculate was extended in both Beltsville L2 and Purdue extender to approximately 1×10^9 cells/milliliter. These samples were sonicated for 1 min to disrupt the cells. The samples were then centrifuged at 10,000G for 10 min. to remove the cellular debris. The

supernatants were then split into four aliquots and either assayed the same day or frozenn and assayed at weekly intervals. The samples were processed and assayed according to the procedures described by Polakoski et al. (1971) and Parrish and Polakoski (1978).

Phase I

Experimental Design

The factorial treatments consisted of: 1) extenders (Beltsville L2, Pursel et al., 1973c versus Purdue extender, Foley et al., 1967 as modified by Pursel et al., 1974); 2) extender antibiotics (potassium penicillin G and dihydrostreptomycin sulfate versus gentamicin); 3) cooling rate (two versus four hours); and 4) storage temperature (5C versus 15C). Each of the 16 treatment combinations was sampled at ten 12 hr intervals for each ejaculate. The effects of boar and ejaculate were random and all remaining effects were fixed. The composition of extenders used in this study is presented in table 1.

Experimental Procedures

Three mature Yorkshire boars were used in this study. The sperm-rich fraction of each ejaculate was collected into an insulated bottle by the method previously described and strained through two layers of cheesecloth into a beaker to

remove the gel particles. The beaker was then placed in a 37C waterbath. The ejaculate was evaluated for volume, concentration and the percentage of sperm with progressive forward motility. Equal amounts of semen were extended at 37C in each of the 16 treatment combinations and held in 250 ml Erlenmeyer flasks with rubber stoppers. The temperature difference between the semen and the extenders was not greater than ±1C. Prior to cooling the extended semen was incubated at 30C for 6 hours.

After the incubation period, the semen was cooled in either 2 or 4 hr and stored at either 5C or 15C for 108 hours. The evaluation of stored semen consisted of an initial evaluation for: 1) the percentage of acrosomes with normal apical ridges (Pursel et al. 1972a)

2) the percentage of cells with either progressive forward motility or vibrational and/or rotational motility; and 3) the acrosin activity of the supernatant at the end of the cooling period. The same parameters were measured at 12-hr intervals for 108 hours.

At each sampling interval three samples were taken. The sample for motility estimates was placed in a 37C waterbath for one hour prior to evaluation. Motility estimates were made using an American Optical 160 light microscope. Each estimate was an average based on a composite judgement of the motility observed from five fields of view. Sperm

TABLE 1

COMPOSITION OF EXTENDERS

Ingredient[a]	Beltsville L2[bcd]	Purdue[bce]
Glucose (g)	25.0	13.0
Tris (hydroxymethyl) aminomethante (g)	20.0	
Citric acid monohydrate (g)	11.0	
Sodium citrate dihydrate (g)		14.0
Sodium bicarbonate (g)		1.5
Potassium chloride (g)		.3

[a]Ingredients dissolved and brought to one liter with deionized water.

[b]Dihydrostreptomycin sulfate and potassium penicillin G were added to the "original" extenders at 1 gram and 1 x 10^6 IU per liter, respectively.

[c]Gentamicin sulfate was substituted for the combination of streptomycin and penicillin at .05 g/l, respectively.

[d]The pH of semen extended with Beltsville L2 was 7.1.

[e]The pH of semen extended with Purdue was 8.3.

motility was classified as either progressive forward motility or as vibrational and/or rotational motility. Motility estimates were not conducted by the same person at each sampling interval.

The sample for evaluating acrosomal integrity was fixed in a .1% glutaraldehyde solution according to the procedures described by Pursel and Johnson (1974). Two counts of 100 acrosomes each were done within fourteen days on fixed samples. Acrosomes were classified according to the presence or absence of a normal apical ridge using a Zeiss differential interference microscope at 1250x magnification. The percentage of acrosomes with normal apical ridges was determined by the same person for all samples.

A third sample was taken at each interval, centrifuged and the supernatant removed. Approximately 1 ml of a 1 mg/ml solution of bovine serum albumin (Grade V; Sigma Chemical Co., St. Louis, MO) was added to the supernatant to prevent autocatalysis of acrosin. The supernatant was adjusted to pH 2.5 to 3.0 using 1.0 M HCl, and frozen for later acrosin analysis. Prior to acrosin analysis samples were thawed and incubated in a waterbath at 37C for 15 minutes. This acidic incubation of these samples dissociated the acrosin-acrosin inhibitor complex in ejaculated boar semen (Polakoski et al. 1971). Following this incubation samples were placed on ice.

Acrosin activity was measured spectrophotometrically using a Gilford model 150 kinetic recording spectrophotometer and a 30C waterbath according to the procedures described by Parrish and Polakoski (1978). The assays were performed at pH 8.0 in a 1 ml reaction volume that contained .05 ml of 1 molar Tris/HCl, .5 milliliters of .1 M calcium chloride and .01 ml of .1 M N-α-benzoyl-L-arginine ethylester HCl. Fifty ul of the supernatant was added to the reaction mixture and the hydrolysis of the substrate was monitored at 253 nanometers.

Phase II

Experimental Design

Twenty prepubertal crossbred gilts, approximately 6 months of age, were used to evaluate the fertilizing capacity for that extender and extender system from Phase I that maintained the highest level of cellular integrity. Four gilts were assigned to each of five semen age groups. These age groups consisted of semen that was processed and stored for either: 1) 0 and 12 hours; 2) 24 and 36 hours; 3) 48 and 60 hours; 4) 72 and 84 hours; and 5)96 and 108 hours.

Experimental Procedures

The time of ovulation was induced to facilitate the breeding of gilts with semen of five age groups. Gilts were

injected with 750 IU of pregnant mare serum gonadotropin (PMSG; Sigma Chemical Co., St. Louis, MO) designated as time zero and with 500 IU of human chorionic gonadotropin (HCG; Henry Schein Inc.; Port Washington, NY) 80 hours later. Following the injection of PMSG, gilts were checked twice daily for estrus with an intact mature boar. If a gilt was detected in estrus after the PMSG injection and before 80 hours, HCG was given at the time that estrus was detected and bred 12 and 24 hours following HCG injection. If estrus was not detected before 80 hours, HCG was administered at 80 hours and the gilt was bred 24 and 36 hours following HCG injection. Ovulation was assumed to occur at 40 hr following HCG injection. All females were inseminated twice with approximately $4x10^9$ sperm cells in a volume of 100 milliliters. The day of the first breeding was designated day zero.

Ova were surgically flushed from the uterus using .9% saline at five days following the first insemination. Eggs were recovered and placed on a microscope slide under a coverslip supported by two beads of paraffin-vaseline mixture. Ova were cleared for 12 to 24 hours, using a solution of one part glacial acetic acid and three parts absolute ethanol. Ova were then stained with a small amount of 1% orcein in 45% acetic acid for 12 to 24 hours. Prior to examination, excess stain was removed with 45% acetic acid (Duane David

1981, personal communication). Ova were examined for the presence of a single nucleus in each blastomere using a Zeiss differential interference contrast microscope.

The percentage of ova recovered was determined based upon the number of ova found in the flushing medium divided by the number of corpora lutea on the ovaries. The fertilization rate was determined by the number of normally fertilized ova divided by the total number of ova recovered. Normally fertilized ova were defined as having a single nucleus in each blastomere and a symetrical stage of cleavage.

Statistical Analysis

Data in this study were analyzed statistically using appropriate tests. These included means and standard deviations, analysis of variance, simple and partial correlations and regression analysis (Sokal and Rohlf, 1969; and Barr et al., 1976 and 1979).

Chapter IV. RESULTS

Phase I In Vitro Semen Traits

Acrosome Morphology

Tables 2 through 9 present the mean values for the percentage of normal apical ridge acrosomes (NAR) for the 16 treatment combinations defined by the four treatment effects; namely, extender, antibiotic, cooling rate and storage temperature. The means for NAR show that within each treatment combination there was a decrease ($P<.05$) in percent NAR as storage time increased. Within an extender, antibiotic and cooling rate, the percent NAR was generally higher at each storage time for semen stored at 15C than at 5C.

The analysis of variance for normal apical ridge acrosomes is presented in appendix table 1. Least-squares means and standard errors of the treatment effects and selected two-way treatment effect interactions for NAR are presented in table 10. Extender, antibiotic, cooling rate and storage temperature did not significantly affect the percentage of NAR ($P<.05$). The interaction between antibiotic and storage temperature was significant ($P<.01$). At 15C, the combination of penicillin/streptomycin was higher than gentamicin

TABLE 2
MEAN VALUES FOR PERCENT NORMAL APICAL RIDGE ACROSOMES EXTENDED WITH BELTSVILLE L2 CONTAINING PENICILLIN/STREPTOMYCIN WITH A TWO HOUR COOLING RATE

Storage Time (Hours)	Storage Temperature			
	5 C		15 C	
	Mean	Standard Deviation	Mean	Standard Deviation
0	69.2[a]	10.8	74.7[a]	7.9
12	54.1	17.4	75.7	9.9
24	45.6	15.4	74.2	8.4
36	42.5	15.2	65.9	14.1
48	44.5	14.7	66.0	17.6
60	34.3	11.8	53.0	18.1
72	33.3	17.9	53.2	17.6
84	31.8	14.0	47.8	12.9
96	31.8	12.1	43.7	14.2
108	24.3	6.8	39.6	14.7

[a]Number of observations = 6.

TABLE 3
MEAN VALUES FOR PERCENT NORMAL APICAL RIDGE ACROSOMES EXTENDED WITH BELTSVILLE L2 CONTAINING PENCILLIN/STREPTOMYCIN WITH A FOUR HOUR COOLING RATE

Storage Time (Hours)	Storage Temperature			
	5 C		15 C	
	Mean	Standard Deviation	Mean	Standard Deviation
0	68.7[a]	16.0	74.1[a]	14.7
12	53.5	20.1	70.0	8.2
24	50.6	17.4	64.7	15.6
36	47.0	18.1	67.1	16.3
48	45.7	12.7	59.2	15.3
60	38.2	15.7	55.7	19.5
72	41.3	15.4	47.3	21.0
84	28.7	14.2	39.3	18.5
96	29.8	8.6	40.6	17.3
108	21.9	9.4	33.8	13.3

[a]Number of observations = 6.

TABLE 4

MEAN VALUES FOR PERCENT NORMAL APICAL RIDGE ACROSOMES EXTENDED WITH BELTSVILLE L2 CONTAINING GENTAMICIN WITH A TWO HOUR COOLING RATE

Storage Time (Hours)	Storage Temperature			
	5 C		15 C	
	Mean	Standard Deviation	Mean	Standard Deviation
0	67.5[a]	12.3	71.3[a]	11.0
12	56.8	17.9	73.2	12.1
24	49.3	17.2	67.8	12.5
36	49.5	15.3	63.8	16.4
48	49.2	17.8	60.3	16.4
60	44.4	20.1	51.0	17.8
72	35.3	16.9	49.9	19.2
84	37.8	17.9	47.0	12.1
96	32.0	11.5	40.8	14.2
108	25.3	13.4	31.0	14.3

[a]Number of observations = 6.

TABLE 5

MEAN VALUES FOR PERCENT NORMAL APICAL RIDGE ACROSOMES EXTENDED WITH BELTSVILLE L2 CONTAINING GENTAMICIN WITH A FOUR HOUR COOLING RATE

Storage Time (Hours)	Storage Temperature			
	5 C		15 C	
	Mean	Standard Deviation	Mean	Standard Deviation
0	68.6[a]	16.1	71.9[a]	17.3
12	53.9	14.3	74.9	11.4
24	49.3	18.3	68.5	13.0
36	44.9	19.0	61.3	19.4
48	45.3	25.9	55.8	14.0
60	39.2	17.9	51.5	16.8
72	35.8	10.5	51.0	13.1
84	32.5	12.0	40.7	8.4
96	28.3	13.1	37.8	11.6
108	27.8	17.3	32.7	12.0

[a]Number of observations = 6.

TABLE 6

MEAN VALUES FOR PERCENT NORMAL APICAL RIDGE ACROSOMES EXTENDED WITH PURDUE CONTAINING PENICILLIN/STREPTOMYCIN WITH A TWO HOUR COOLING RATE

Storage Time (Hours)	Storage Temperature			
	5 C		15 C	
	Mean	Standard Deviation	Mean	Standard Deviation
0	71.5[a]	13.5	81.2[a]	6.0
12	52.2	18.0	76.3	13.8
24	47.0	17.5	72.8	12.8
36	45.6	17.4	72.9	7.0
48	47.3	19.7	75.4	8.4
60	41.3	17.9	68.5	12.6
72	38.6	15.1	67.5	12.8
84	37.1	13.6	58.9	8.3
96	34.1	9.6	54.7	8.7
108	28.8	10.5	50.2	12.9

[a]Number of observations = 6.

TABLE 7

MEAN VALUES FOR PERCENT NORMAL APICAL RIDGE ACROSOMES EXTENDED WITH PURDUE CONTAINING PENICILLIN/STREPTOMYCIN WITH A FOUR HOUR COOLING RATE

Storage Time (Hours)	Storage Temperature			
	5 C		15 C	
	Mean	Standard Deviation	Mean	Standard Deviation
0	72.8[a]	10.7	81.1[a]	8.9
12	53.8	22.2	79.3	8.2
24	51.8	11.9	78.2	6.9
36	50.8	16.5	76.3	7.5
48	49.0	17.4	73.6	8.6
60	44.7	12.3	66.3	13.1
72	38.3	11.6	67.8	14.3
84	37.4	10.1	61.4	9.8
96	37.6	9.0	58.5	10.1
108	33.0	10.2	46.1	12.3

[a]Number of observations = 6.

TABLE 8

MEAN VALUES FOR PERCENT NORMAL APICAL RIDGE ACROSOMES EXTENDED WITH PURDUE CONTAINING GENTAMICIN WITH A TWO HOUR COOLING RATE

Storage Time (Hours)	Storage Temperature			
	5 C		15 C	
	Mean	Standard Deviation	Mean	Standard Deviation
0	77.3[a]	9.2	80.7[a]	9.2
12	57.7	13.1	77.4	9.4
24	54.4	12.5	76.4	9.6
36	49.8	16.5	73.3	9.9
48	47.3	19.3	68.9	11.6
60	42.4	19.8	64.6	16.2
72	40.3	19.9	51.6	20.2
84	36.0	18.7	51.0	14.9
96	33.7	14.4	44.9	14.1
108	28.7	10.0	36.2	14.1

[a]Number of observations = 6.

TABLE 9

MEAN VALUE FOR PERCENT NORMAL APICAL RIDGE ACROSOMES EXTENDED WITH PURDUE CONTAINING GENTAMICIN WITH A FOUR HOUR COOLING RATE

Storage Time	Storage Temperature			
	5 C		15 C	
	Mean	Standard Deviation	Mean	Standard Deviation
0	76.8[a]	11.0	80.5[a]	8.7
12	56.1	15.5	77.9	9.8
24	50.3	18.6	76.4	11.5
36	54.9	16.0	73.7	12.1
48	49.6	17.4	69.2	14.8
60	45.7	15.2	63.8	20.4
72	38.9	15.8	56.8	18.4
84	37.5	14.0	46.5	15.3
96	37.2	8.4	43.2	13.9
108	29.8	12.2	36.6	20.4

[a]Number of observations = 6.

TABLE 10

LEAST-SQUARES MEANS AND STANDARD ERRORS FOR NORMAL APICAL RIDGE ACROSOMES

Item	Acrosomes (%) LS Mean	Standard Error
Extender		
Beltsville L2	49.42	.46
Purdue	55.92	
Antibiotic		
Penicillin/Streptomycin	53.27	.46
Gentamicin	52.07	
Cooling Rate		
Two Hours	52.74	.46
Four Hours	52.59	
Storage Temperature		
5 C	44.58	.46
15 C	60.76	
Antibiotic x Storage Temperature**		
Pen/Strep x 5 C	43.73	.65
Pen/Strep x 15 C	62.80	
Gentamicin x 5 C	45.42	
Gentamicin x 15 C	58.71	
Extender x Cooling Rate*		
BL-2 x Two Hours	50.13	.65
BL-2 x Four Hours	48.71	
PE x Two Hours	55.36	
PE x Four Hours	56.48	
Extender x Storage Temperature**		
BL-2 x 5 C	42.73	.65
BL-2 x 15 C	56.10	
PE x 5 C	46.42	
PE x 15 C	65.41	

* $P < .05$

** $P < .01$

for NAR (figure 1); whereas, gentamicin was higher at 5C than the penicillin/streptomycin combination for NAR. The interaction between extender and cooling rate was significant ($P<.05$). A slightly higher percentage of NAR was found when PE was cooled in 4 hr versus 2 hr (figure 2). The converse was observed for the interaction between BL2 and cooling rate. The interaction (figure 3) between extender and storage temperature was significant ($P<.01$). At 5C and 15C the percentage of NAR was higher for PE than BL2. Within each extender, a storage temperature of 15C was superior to 5C for percent NAR.

There was a significant affect of ejaculate within boar on the percentage of NAR ($P<.001$). The second ejaculate from each boar had a higher percentage of NAR than that of the first ejaculate. The interactions between boar and extender ($P<.05$) and boar and storage temperature ($P<.01$) were significant. There was a higher percentage of NAR for semen extended in PE than for semen extended in BL2 for each boar. A higher percentage of NAR was observed when semen was stored at 15C than at 5C for each boar.

The regression of storage time on NAR was significant ($P<.05$). The prediction equation is $Y = 71.4267 - .1923\ X + .0001\ X^2$ where Y is the predicted NAR; and X is the storage time in hours. The regression of storage time x storage temperature on NAR was significant ($P<.001$). The prediction

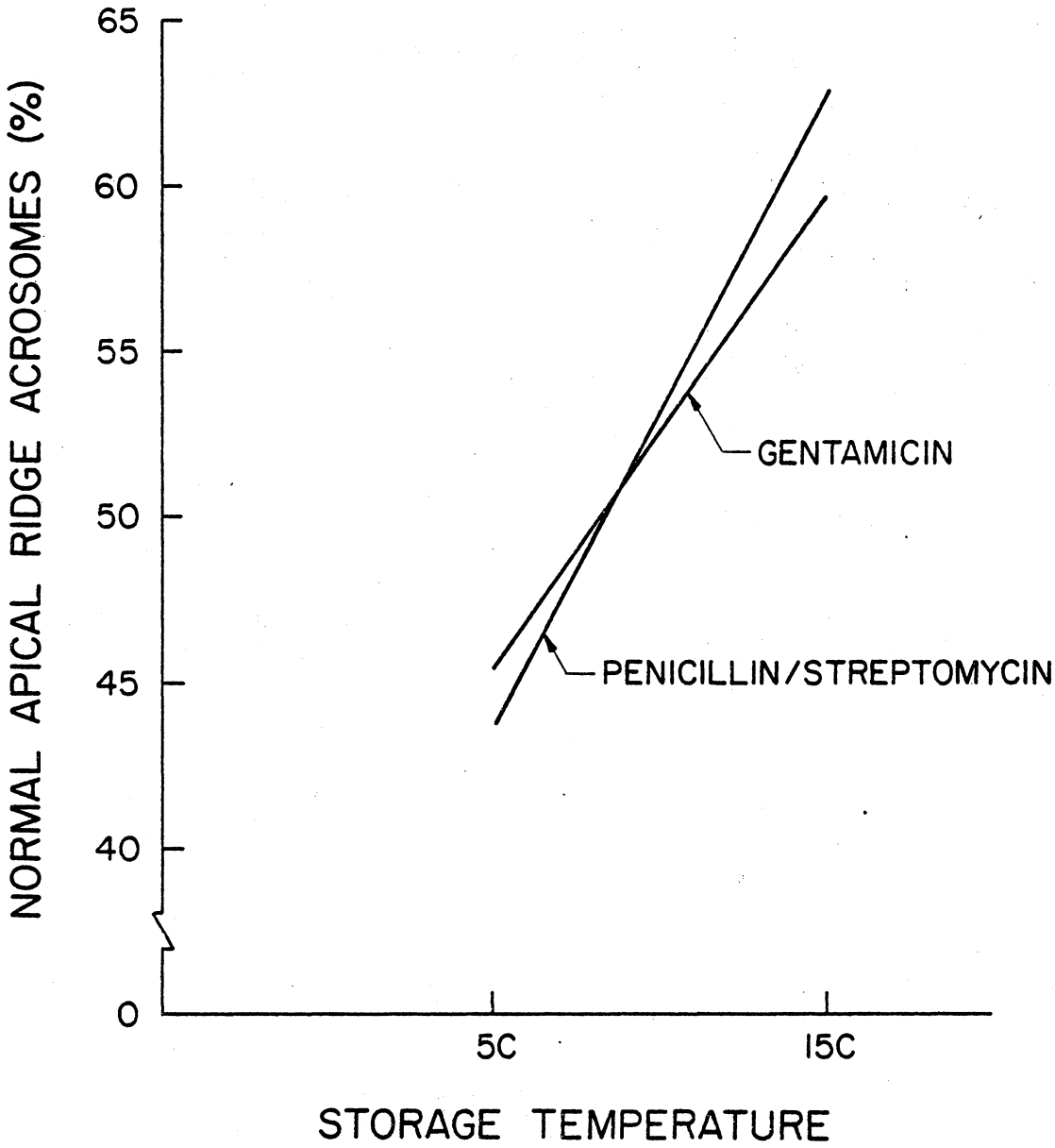

Figure 1. Effects of antibiotic and storage temperature on normal apical ridge acrosomes

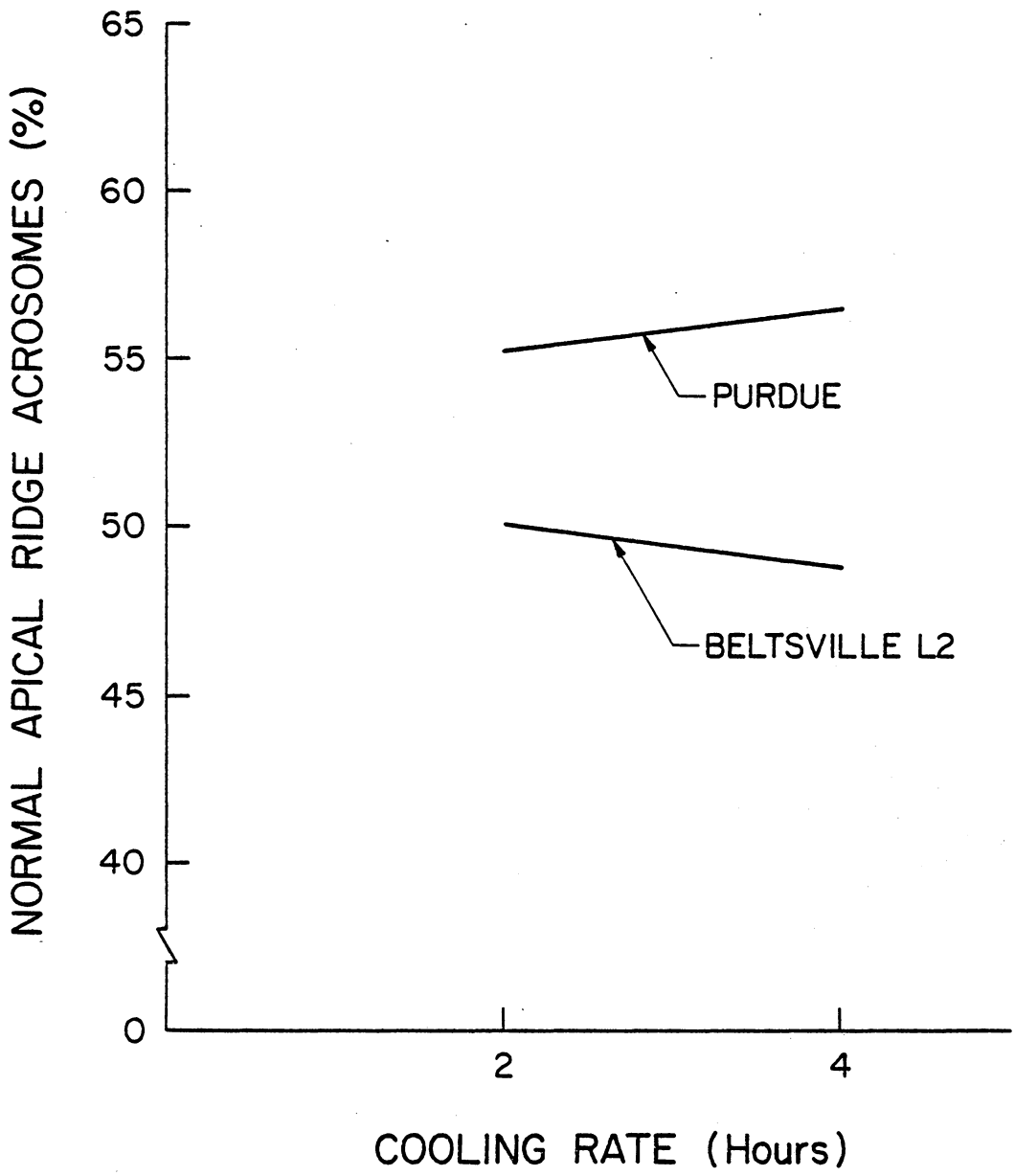

Figure 2. Effects of extender and cooling rate on normal apical ridge acrosomes

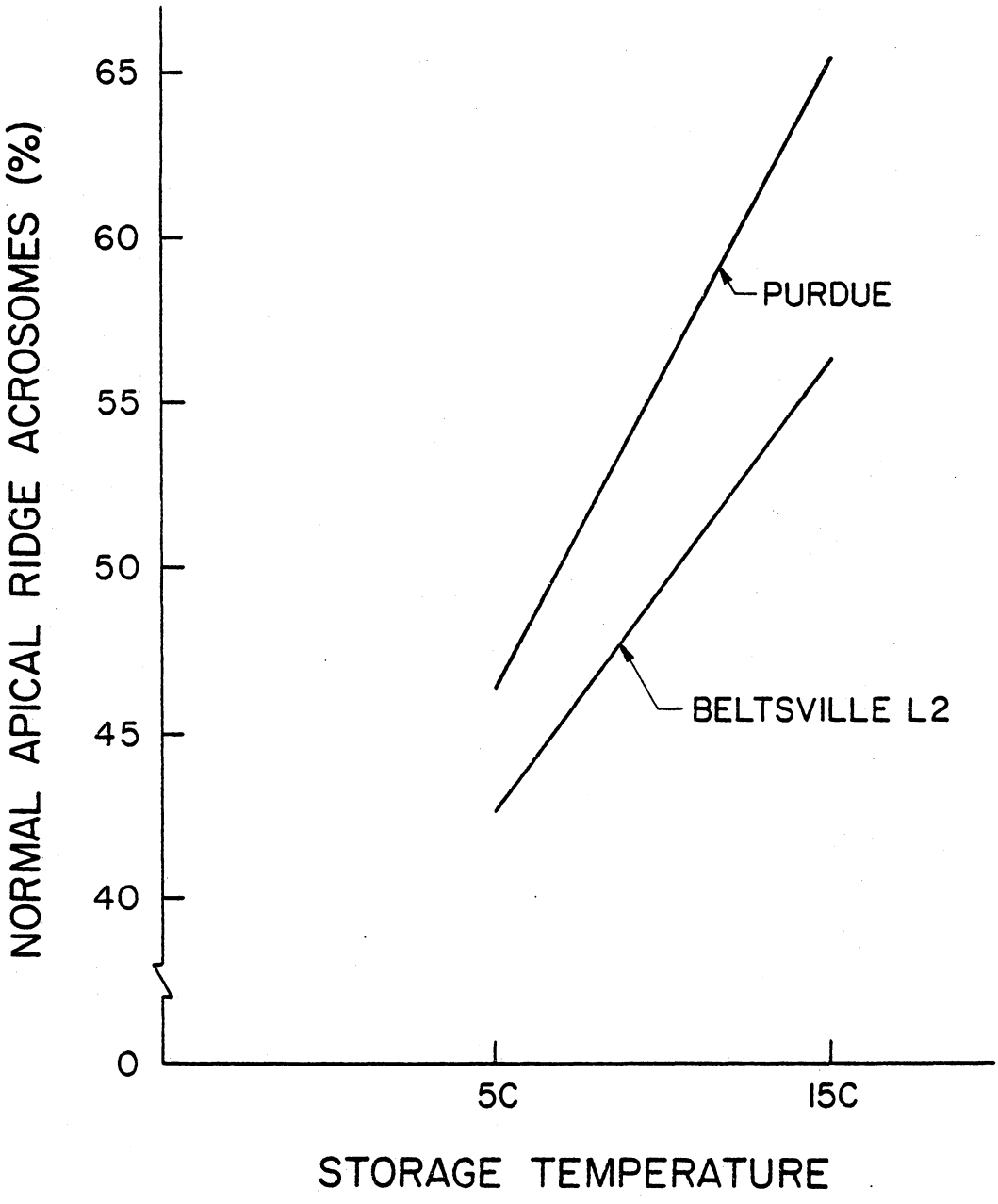

Figure 3. Effects of extender and storage temperature on normal apical ridge acrosomes

TABLE 11

MEAN VALUES FOR ACROSIN ACTIVITY EXTENDED WITH BELTSVILLE L2 CONTAINING PENICILLIN/STREPTOMYCIN WITH A TWO HOUR COOLING RATE[a]

Storage Time (Hours)	Storage Temperature			
	5 C		15 C	
	Mean	Standard Deviation	Mean	Standard Deviation
0	.0087[b]	.0057	.0092[b]	.0045
24	.0113	10084	.0085	.0051
48	.0111	.0064	.0096	.0052
72	.0128	.0080	.0086	.0037
96	.0119	.0071	.0082	.0036

[a]Activity = micromoles/ml/min/1 x 10^9 sperm cells.

[b]Number of observations = 6.

TABLE 12

MEAN VALUES FOR ACROSIN ACTIVITY EXTENDED WITH BELTSVILLE L2 CONTAINING PENICILLIN/STREPTOMYCIN WITH A FOUR HOUR COOLING RATE[a]

Storage Time (Hours)	Storage Temperature			
	5 C		15 C	
	Mean	Standard Deviation	Mean	Standard Deviation
0	.0096[b]	.0072	.0080[b]	.0036
24	.0115	.0093	.0087	.0042
48	.0117	.0080	.0087	.0050
72	.0106	.0066	.0089	.0039
96	.0110	.0077	.0081	.0030

[a]Activity = micromoles/ml/min/1 x 10^9 sperm cells.

[b]Number of observations = 6.

TABLE 13

MEAN VALUES FOR ACROSIN ACTIVITY EXTENDED WITH BELTSVILLE L2 CONTAINING GENTAMICIN WITH A TWO HOUR COOLING RATE[a]

Storage Time (Hours)	Storage Temperature			
	5 C		15 C	
	Mean	Standard Deviation	Mean	Standard Deviation
0	.0098[b]	.0054	.0096[b]	.0044
24	.0102	.0060	.0086	.0049
48	.0106	.0061	.0081	.0049
72	.0101	.0055	.0087	.0037
96	.0109	.0056	.0097	.0049

[a]Activity = micromoles/ml/min/l x 10^9 sperm cells.

[b]Number of observations = 6.

TABLE 14

MEAN VALUES FOR ACROSIN ACTIVITY EXTENDED WITH BELTSVILLE L2 CONTAINING GENTAMICIN WITH A FOUR HOUR COOLING RATE[a]

Storage Time (Hours)	Storage Temperature			
	5 C		15 C	
	Mean	Standard Deviation	Mean	Standard Deviation
0	.0098[b]	.0069	.0102[b]	.0061
24	.0103	.0051	.0087	.0050
48	.0106	.0055	.0089	.0046
72	.0010	.0056	.0085	.0041
96	.0111	.0057	.0079	.0033

[a]Activity = micromoles/ml/min/l x 10^9 sperm cells.

[b]Number of observations = 6.

TABLE 15

MEAN VALUES FOR ACROSIN ACTIVITY EXTENDED WITH PURDUE CONTAINING PENICILLIN/STREPTOMYCIN WITH A TWO HOUR COOLING RATE[a]

Storage Time (Hours)	Storage Temperature			
	5 C		15 C	
	Mean	Standard Deviation	Mean	Standard Deviation
0	.0091[b]	.0052	.0127[b]	.0090
24	.0113	.0066	.0109	.0070
48	.0126	.0068	.0111	.0074
72	.0131	.0080	.0103	.0061
96	.0130	.0048	.0105	.0057

[a]Activity = micromoles/ml/min/l x 10^9 sperm cells.

[b]Number of observations = 6.

TABLE 16

MEAN VALUES FOR ACROSIN ACTIVITY EXTENDED WITH PURDUE CONTAINING PENICILLIN/STREPTOMYCIN WITH A FOUR HOUR COOLING RATE[a]

Storage Time (Hours)	Storage Temperature			
	5 C		15 C	
	Mean	Standard Deviation	Mean	Standard Deviation
0	.0098[b]	.0058	.0119[b]	.0094
24	.0094	.0055	.0112	.0078
48	.0107	.0073	.0108	.0068
72	.0010	.0039	.0096	.0053
96	.0129	.0071	.0106	.0061

[a]Activity = micromoles/ml/min/l x 10^9 sperm cells.

[b]Number of observations = 6.

TABLE 17

MEAN VALUES FOR ACROSIN ACTIVITY EXTENDED WITH PURDUE CONTAINING GENTAMICIN WITH A TWO HOUR COOLING RATE[a]

Storage Time (Hours)	Storage Temperature			
	5 C		15 C	
	Mean	Standard Deviation	Mean	Standard Deviation
0	.0120[b]	.0084	.0133[b]	.0102
24	.0116	.0060	.0100	.0053
48	.0113	.0071	.0123	.0114
72	.0119	.0070	.0107	.0082
96	.0135	.0063	.0121	.0082

[a]Activity = micromoles/ml/min/l x 10^9 sperm cells.

[b]Number of observations = 6.

TABLE 18

MEAN VALUES FOR ACROSIN ACTIVITY EXTENDED WITH PURDUE CONTAINING GENTAMICIN WITH A FOUR HOUR COOLING RATE[a]

Storage Time (Hours)	Storage Temperature			
	5 C		15 C	
	Mean	Standard Deviation	Mean	Standard Deviation
0	.0100[b]	.0063	.0110[b]	.0085
24	.0114	.0078	.0097	.0065
48	.0117	.0064	.0118	.0078
72	.0111	.0068	.0117	.0086
96	.0127	.0073	.0117	.0081

[a]Activity = micromoles/ml/min/l x 10^9 sperm cells.

[b]Number of observations = 6.

equation for the 5C storage temperature is $Y = 77.5514 - .1867 X - .0017X^2$. For both equations, Y was the predicted percentge NAR and X was the storage time, in hours.

The regression of storage time x ejaculate within boar was significant for the percentage of NAR acrosomes. The prediction equations were: $Y = 85.5069 + .045 X - .0053X^2$ and $Y = 75.1094 - .9211 X + .0039 X^2$ for ejaculates 1 and 2 from the first boar, respectively; $Y = 70.6446 - .2946 X - .009 X^2$ and $Y = 54.6554 - .3932 X + .0023 X^2$ for ejaculates 1 and 2 from the second boar, respectively; and $Y = 77.1951 - .2664 X - .0001 X^2$ and $Y = 65.4296 - .3582 X - .2654 X^2$ for ejaculates 1 and 2 from the third boar, respectively. In the above equations, Y is the predicted percentage of NAR and X is the storage time, in hours.

Acrosin Activity

Means for acrosin activity for the 16 treatment combinations are presented in tables 11 through 18. The acrosin activity was determined for all samples in one replicate and the inclusion of the 12 hr storage times did not increase the fit of the model. Therefore, acrosin activity is reported for 24 hr storage times only. All activity values are adjusted to a concentration of $1x10^9$ sperm cells. Acrosin activity within each treatment combination generally increased with increased storage time. However, this trend was not significant ($P<.10$). The mean acrosin activity was

usually slightly higher for the same treatment combinations stored at 5C than when stored at 15C, for each storage time.

The analysis of variance for acrosin activity is presented in appendix table 2. Least-squares means and standard errors for the treatment effects and selected two-way treatment effect interactions are presented in table 19. Extender, antibiotic, cooling rate and storate temperature did not significantly affect the acrosin activity ($P>.05$). The interaction between extender and storage temperature was significant ($P<.01$) for acrosin activity. At both 5C and 15C, the acrosin activity was higher when semen was extended in PE than in BL2 extender.

There was a significant affect of ejaculate within boar on acrosin activity ($P<.001$). The second ejaculate from each boar had a higher amount of acrosin activity than that of the first ejaculate. the interactions between ejaculate within boar x antibiotic ($P<.05$), ejaculate within boar x extender ($P<.001$) and ejaculate within boar x storage temperature ($P<.001$) were significant. For the interaction of ejaculate within boar x antibiotic acrosin activity was higher in four ejaculates that were extended with the combination of penicillin/streptomycin while acrosin activity was higher in 2 ejaculates that were extended with gentamicin. Acrosin activity was higher for five ejaculates extended in PE while acrosin activity was higher for one ejaculate

TABLE 19

LEAST-SQUARES MEANS AND STANDARD ERRORS FOR ACROSIN ACTIVITY

Item	Acrosin Activity (micromoles/ml/min)	
	LS Means	Standard Errors
Extenders		
Beltsville L2	.0097	.0001
Purdue	.0113	
Antibiotic		
Penicillin/Streptomycin	.0105	.0001
Gentamicin	.0106	
Cooling Rate		
Two Hours	.0107	.0001
Four Hours	.0103	
Storage Temperature		
5 C	.0111	.0001
15 C	.0010	
Extender x Storage Temperature**		
BL-2 x 5 C	.0107	.0002
BL-2 x 15 C	.0088	
PE x 5 C	.0115	
PE x 15 C	.0112	

**P < .01

extended in Beltsville L2. For the interaction of ejaculate within boar x storage temperature, acrosin activity was higher for four ejaculates at 5 while storage at 15C resulted in higher acrosin activity in two ejaculates.

The regression of storage time x storage temperature on acrosin activity was significant ($P<.05$). The prediction equations for acrosin activity are: $y = .0141 + (5.8x10^{-5}) X + (1x10^{-8})X^2$ for the 5C storage temperature; and $Y = .0116 + (3x10^{-6}) X + (3.1x10^{-7}) X^2$ for the 15C storage temperature where Y is the predicted activity and X is the storage time, in hours.

Progressive Forward Motility

Mean values for percent progressive forward motility (PROMOT) were essentially zero at each storage time for the treatment combinations with BL2, either penicillin/streptomycin or gentamicin, two hour cooling rate and either 5C or 15C storage temperature. The treatment combination BL2 with penicillin/streptomycin and a four hour cooling rate had a mean of 1.7 and a standard deviation of 4.1 at the 12 hr storage time for the 5C storage temperature. Means for this treatment combination were zero for the 15C storage temperature. The PROMOT means for the treatment combination BL2 with gentamicin and a four hour cooling rate were 1.7% with a standard deviation of 4.1. This mean was reported at the 0 hr storage time for the 5C

storage temperature and at the 12 and 60 hr storage times for the 15C storage temperature. The mean values for percent PROMOT in any of the treatment combinations containing PE and at the 5C storage temperature were very low initially and zero after the 36 hr storage time. Mean values for percent PROMOT were highest for the treatment combination PE with penicillin/streptomycin, a 4 hr cooling rate and a 15C storage temperature. At the 0 hr storage time the mean was 28.3% with a standard deviation of 25.6. The 12 and 24 hr means and standard deviations were 25.0% ± 18.7 and 6.5% ± 16.3, respectively. After 24 hr storage, the means were not higher than those reported for the 24 hr. storage time. All other treatment combinations containing PE and at a storage temperature of 15C had lower mean values for the percent PROMOT than the combination PE with penicillin/streptomycin and a 4 hr cooling rate.

The analysis of variance for the percent progressive forward motility is presented in appendix table 3. Least-squares means and standard errors for the treatment effects and selected two-way treatment effect interactions for PROMOT are presented in table 20. Purdue extender had a higher ($P<.05$) percentage of PROMOT than BL2. Samples that contained penicillin/streptomycin had a significantly higher ($P<.05$) percent PROMOT than samples that contained gentamicin. Cooling rate and storate temperature did not signifi-

TABLE 20

LEAST-SQUARES MEANS AND STANDARD ERRORS FOR PROGRESSIVE FORWARD MOTILITY

Item	Progressive Forward Motility (%) LS Means	Standard Errors
Extender*		
Beltsville L2	.08	.30
Purdue	2.96	
Antibiotic*		
Penicillin/Streptomycin	1.94	.30
Gentamicin	1.10	
Cooling Rate		
Two Hours	1.29	.30
Four Hours	1.75	
Storage Temperature		
5 C	.67	.30
15 C	2.38	
Antibiotic x Extender*		
Pen/Strep x BL-2	.04	.42
Pen/Strep x PE	3.83	
Gentamicin x BL-2	.13	
Gentamicin x PE	2.08	
Extender x Storage Temperature**		
BL-2 x 5 C	.08	.42
BL-2 x 15 C	.08	
PE x 5 C	1.25	
PE x 15 C	4.66	

*P < .05

**P < .01

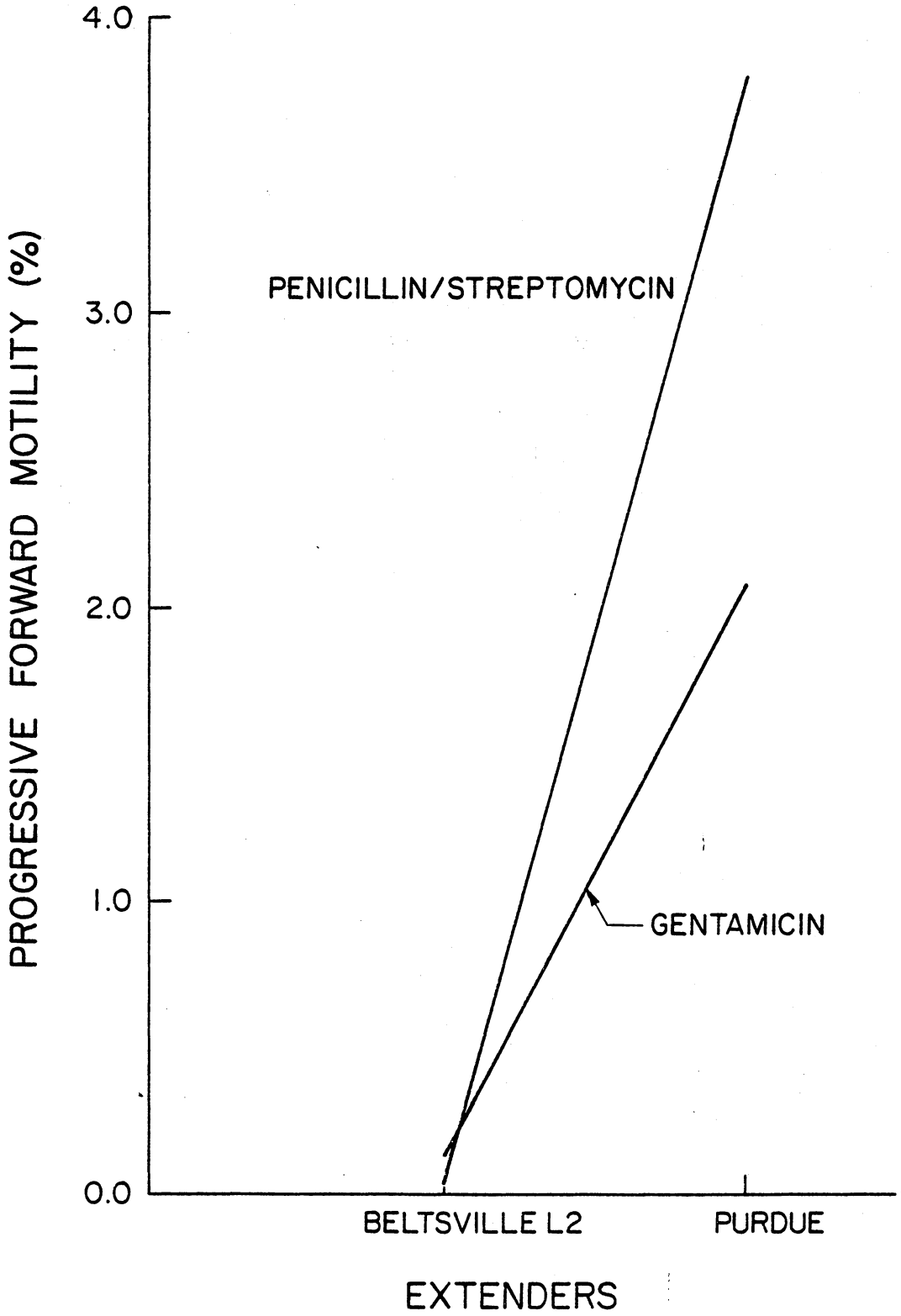

Figure 4. Effects of antibiotic and extender on progressive forward motility

cantly affect the percent PROMOT (P>.10). The interaction between antibiotic and extender (figure 4) was significant for PROMOT (P<.05). When penicillin/streptomycin or genetamicin were combined with BL2, the percent PROMOT was low. However, PROMOT in the combination of penicillin/streptomycin and PE produced greater PROMOT than the PE which contained gentamicin. The interaction between extender and storage temperature (figure 5) was significant (P<.01) for the percent PROMOT. The LS means for PROMOT were the same for BL2 at both the 5C and 15C storage temperatures. The mean percent PROMOT for semen extended in PE was higher than for semen extended in BL2 at both the 5C and 15C storage temperatures.

There was a significant affect of ejaculate within boar on the percentage of PROMOT (P<.001). The first ejaculate from each boar had a higher mean PROMOT value than that of the second ejaculate. The interaction between boar and cooling rate was significant for PROMOT (P<.01). The mean percent PROMOT was higher for two boars when semen was cooled in 4 hr, while PROMOT was higher for one boar when semen was cooled in 2 hours.

The regression of storage time x extender on PROMOT was significant (P<.001). The prediction equations were: $Y = -7.540 - .006 X + .0069 X^2$ for BL2; and $Y = 13.6177 - .3755 X + .0094 X^2$ for PE when Y was the predicted percentage PRO-

MOT and X was the storage time. The regression of storage time x storage temperature on PROMOT was significant ($P<.05$). The prediction equation for the 5C storage temperature was $Y = 1.8032 - .118\ X + .0004\ X^2$. At 15C, the equation was $Y = 9.2629 - .2634\ X + .0001\ X^2$. For these equations, Y was the predicted percentage PROMOT and X was the storage time, in hours.

The regression of storage time x ejaculate within boar on PROMOT was significant ($P<.001$). The prediction equations were: $Y = 15.5796 - .4289\ X + .0027\ X^2$ and $Y = 1.1189 - .0405\ X + .0002\ X^2$ for ejaculates 1 and 2 from the first boar; $Y = 12.709 - .4017\ X + .0027\ X^2$ and $Y = 4.225 - .1371\ X + .0009\ X^2$ for ejaculates 1 and 2 from the second boar; and $Y = 4.1399 - .1166\ X + .007\ X^2$ and $Y = .6059 - .0193\ X + .0001\ X^2$ for ejaculates 1 and 2 from the third boar. In each of these equations Y was the predicted PROMOT and X was the storage time, in hours.

Vibrational and/or Rotational Motility

Mean values for percent vibrational and/or rotational motility (VIBROT) are presented in tables 21 through 28 for each of the 16 treatment combinations. Means for percent VIBROT decreased ($P<.01$) as storage time increased.

The analysis of variance for percent vibrational and/or rotational motility is presented in appendix 4. Least-squares means for the treatment effects and selected two-way

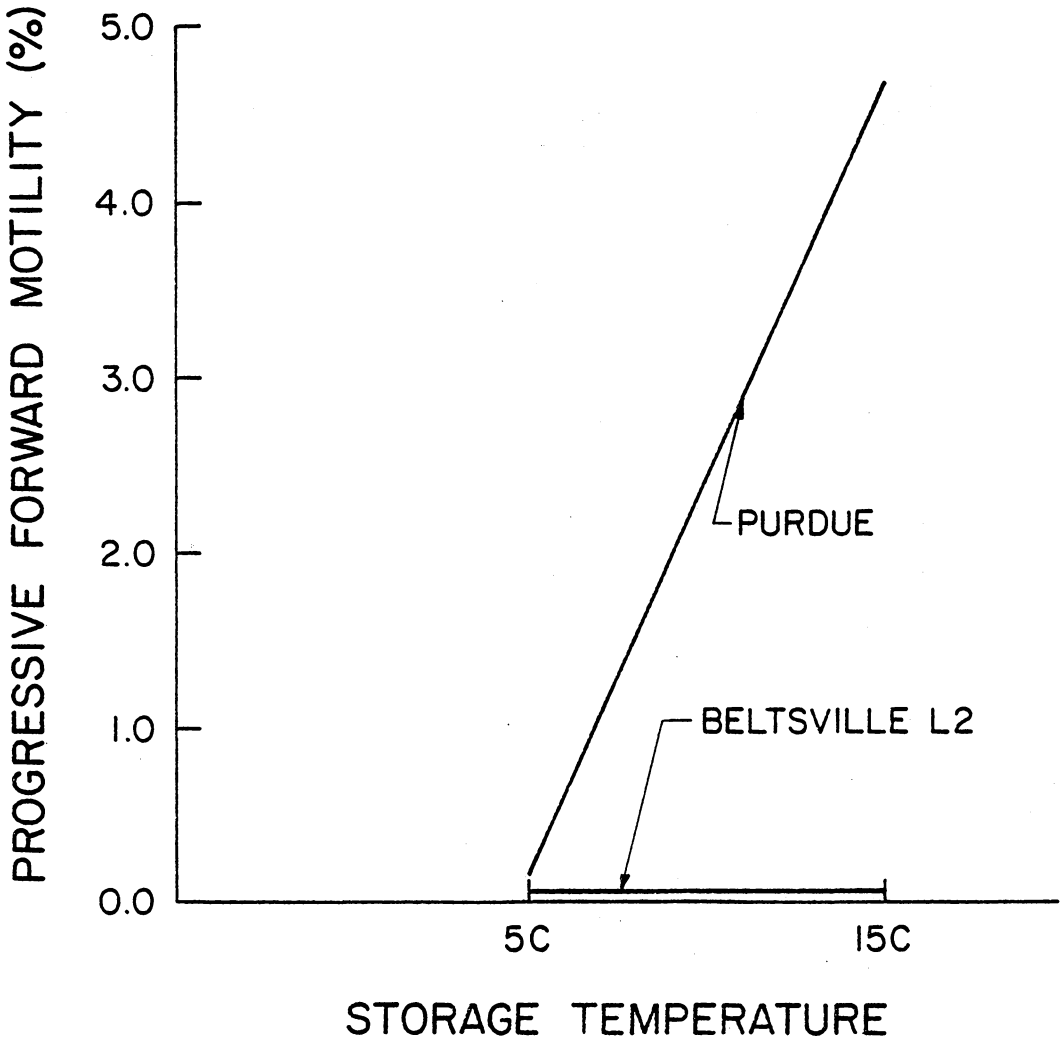

Figure 5. Effects of extender and storage temperature on progressive forward motility

TABLE 21

MEAN VALUES FOR PERCENT VIBRATIONAL AND/OR ROTATIONAL MOTILITY EXTENDED WITH BELTSVILLE L2 CONTAINING PENICILLIN/STREPTOMYCIN WITH A TWO HOUR COOLING RATE

Storage Time (Hours)	Storage Temperature			
	5 C		15 C	
	Mean	Standard Deviation	Mean	Standard Deviation
0	43.3[a]	25.0	48.3[a]	28.6
12	50.0	16.7	58.3	9.8
24	21.7	19.4	33.3	30.8
36	16.7	10.3	46.7	20.7
48	21.7	20.4	45.0	25.1
60	6.7	8.2	25.0	25.9
72	11.7	11.7	21.7	34.9
84	5.0	5.5	13.3	21.6
96	3.3	5.2	0	0
108	3.3	5.2	0	0

[a]Number of observations = 6.

TABLE 22

MEAN VALUES FOR PERCENT VIBRATIONAL AND/OR ROTATIONAL MOTILITY EXTENDED WITH BELTSVILLE L2 CONTAINING PENICILLIN/STREPTOMYCIN WITH A FOUR HOUR COOLING RATE

Storage Time (Hours)	Storage Temperature			
	5 C		15 C	
	Mean	Standard Deviation	Mean	Standard Deviation
0	48.3[a]	25.6	60.0[a]	17.9
12	35.0	16.4	45.0	25.1
24	21.7	24.0	21.7	21.4
36	13.3	15.1	36.7	42.3
48	13.3	15.1	20.0	17.9
60	6.7	12.1	25.0	32.1
72	15.0	27.4	16.7	20.7
84	1.7	4.1	18.3	20.4
96	1.7	4.1	0	0
108	1.7	4.1	0	0

[a]Number of observations = 6.

TABLE 23

MEAN VALUES FOR PERCENT VIBRATIONAL AND/OR ROTATIONAL MOTILITY EXTENDED WITH BELTSVILLE L2 CONTAINING GENTAMICIN WITH A TWO HOUR COOLING RATE

Storage Time (Hours)	Storage Temperature			
	5 C		15 C	
	Mean	Standard Deviation	Mean	Standard Deviation
0	36.7[a]	25.0	38.3[a]	35.4
12	20.0	16.7	66.7	10.3
24	23.3	22.5	30.0	28.3
36	16.7	15.1	50.0	28.3
48	16.7	16.3	36.7	18.6
60	10.0	12.6	31.7	29.3
72	23.3	23.4	45.0	36.7
84	8.3	13.3	16.7	22.5
96	0	0	20.0	31.0
108	1.7	4.1	13.3	16.3

[a]Number of observations = 6.

TABLE 24

MEAN VALUES FOR PERCENT VIBRATIONAL AND/OR ROTATIONAL MOTILITY EXTENDED WITH BELTSVILLE L2 CONTAINING GENTAMICIN WITH A FOUR HOUR COOLING RATE

Storage Time (Hours)	Storage Temperature			
	5 C		15 C	
	Mean	Standard Deviation	Mean	Standard Deviation
0	56.7[a]	10.3	63.3[a]	12.1
12	30.0	8.9	56.7	22.5
24	18.3	19.4	31.7	23.2
36	8.3	9.8	50.0	17.9
48	18.3	14.7	41.7	31.9
60	8.3	7.5	26.7	33.3
72	15.0	18.7	31.7	36.0
84	5.0	5.5	23.3	25.8
96	8.3	13.3	16.7	18.6
108	6.7	8.2	20.0	24.5

[a]Number of observations = 6.

TABLE 25

MEAN VALUES FOR PERCENT VIBRATIONAL AND/OR ROTATIONAL MOTILITY EXTENEDED WITH PURDUE CONTAINING PENICILLIN/STREPTOMYCIN WITH A TWO HOUR COOLING RATE

Storage Time (Hours)	Storage Temperature			
	5 C		15 C	
	Mean	Standard Deviation	Mean	Standard Deviation
0	18.3[a]	14.7	16.7[a]	13.7
12	8.3	16.0	23.3	18.6
24	8.3	9.8	8.3	13.3
36	0	0	10.0	15.5
48	1.7	4.1	10.0	16.7
60	0	0	3.3	8.2
72	1.7	4.1	3.3	5.2
84	0	0	3.3	8.2
96	0	0	1.7	4.1
108	0	0	0	0

[a]Number of observations = 6.

TABLE 26

MEAN VALUES FOR PERCENT VIBRATIONAL AND/OR ROTATIONAL MOTILITY EXTENDED WITH PURDUE CONTAINING PENICILLIN/STREPTOMYCIN WITH A FOUR HOUR COOLING RATE

Storage Time (Hours)	Storage Temperature			
	5 C		15 C	
	Mean	Standard Deviation	Mean	Standard Deviation
0	20.0[a]	17.9	23.3[a]	25.0
12	3.3	5.2	13.3	12.1
24	3.3	8.2	1.7	4.1
36	1.7	4.1	15.0	15.2
48	1.7	4.1	5.0	8.4
60	1.7	4.1	5.0	8.4
72	0	0	1.7	4.1
84	1.7	4.1	5.0	12.2
96	0	0	1.7	4.1
108	0	0	0	0

[a]Number of observations = 6.

TABLE 27

MEAN VALUES FOR PERCENT VIBRATIONAL AND/OR ROTATIONAL MOTILITY EXTENDED WITH PURDUE CONTAINING GENTAMICIN WITH A TWO HOUR COOLING RATE

Storage Time (Hours)	Storage Temperature			
	5 C		15 C	
	Mean	Standard Deviation	Mean	Standard Deviation
0	13.3[a]	20.0	8.3[a]	7.5
12	3.3	5.2	11.7	19.4
24	0	0	10.0	24.5
36	0	0	11.7	20.4
48	0	0	3.3	8.2
60	1.7	4.1	1.7	4.1
72	0	0	0	0
84	0	0	0	0
96	0	0	0	0
108	0	0	0	0

[a]Number of observations = 6.

TABLE 28

MEAN VALUES FOR PERCENT VIBRATIONAL AND/OR ROTATIONAL MOTILITY EXTENDED WITH PURDUE CONTAINING GENTAMICIN WITH A FOUR HOUR COOLING RATE

Storage Time (Hours)	Storage Temperature			
	5 C		15 C	
	Mean	Standard Deviation	Mean	Standard Deviation
0	15.0[a]	19.7	6.7[a]	8.2
12	6.7	12.1	5.0	12.2
24	0	0	0	0
36	0	0	0	0
48	0	0	0	0
60	0	0	1.7	4.1
72	0	0	0	0
84	0	0	0	0
96	0	0	0	0
108	0	0	0	0

[a]Number of observations = 6.

treatment effect interactions are presented in table 29. VIBROT values than treatments stored at 5C. Extender, antibiotic, cooling rate and storage temperature did not significantly affect the percent VIBROT (P>.05). However, the interaction of antibiotic and extender was significant (P<.01). Figure 6 illustrates the interaction of antibiotic and extender for percent VIBROT. Beltsville L2 extender had higher percent VIBROT when combined with gentamicin than with penicillin/streptomycin. Purdue extender had a higher percent VIBROT when combined with penicillin/streptomycin than with gentamicin. The extender and storage temperature interaction was also significant (P<.01) for percent VIBROT (figure 7). At both storage temperatures percent VIBROT values were higher for BL2 than PE.

There was a significant affect of ejaculate within boar on the percent VIBROT (P<.001). The first ejaculate from each boar had a higher percent VIBROT than the second ejaculate. The interactions between ejaculate within boar x extender (P<.001) and ejaculates within boar x cooling rate (P<.05) were significant. The percent VIBROT was higher for all six ejaculates when semen was extended in BL2 than when semen was extended in Purdue extender. For the second interaction, the percent VIBROT was higher for three ejaculates cooled in 2 hr, while three ejaculates had higher VIBROT values when cooled in 4 hours.

TABLE 29

LEAST-SQUARES MEANS AND STANDARD ERRORS FOR VIBRATIONAL AND/OR ROTATIONAL MOTILITY

Item	Vibrational and/or Rotational Motility (%) LS Means	Standard Errors
Extender		
Beltsville L2	23.98	.69
Purdue	4.04	
Antibiotic		
Penicillin/Streptomycin	13.75	.69
Gentamicin	14.27	
Cooling Rate		
Two Hours	14.54	.69
Four Hours	13.48	
Storage Temperature		
5 C	9.81	.69
15 C	18.21	
Antibiotic x Extender**		
Pen/Strep x BL2	21.92	.98
Pen/Strep x PE	5.58	
Gentamicin x BL2	26.04	
Gentamicin x PE	2.50	
Extender x Storage Temperature**		
BL2 x 5 C	16.83	.98
BL2 x 15 C	31.13	
PE x 5 C	2.79	
PE x 15 C	5.29	

**P < .01

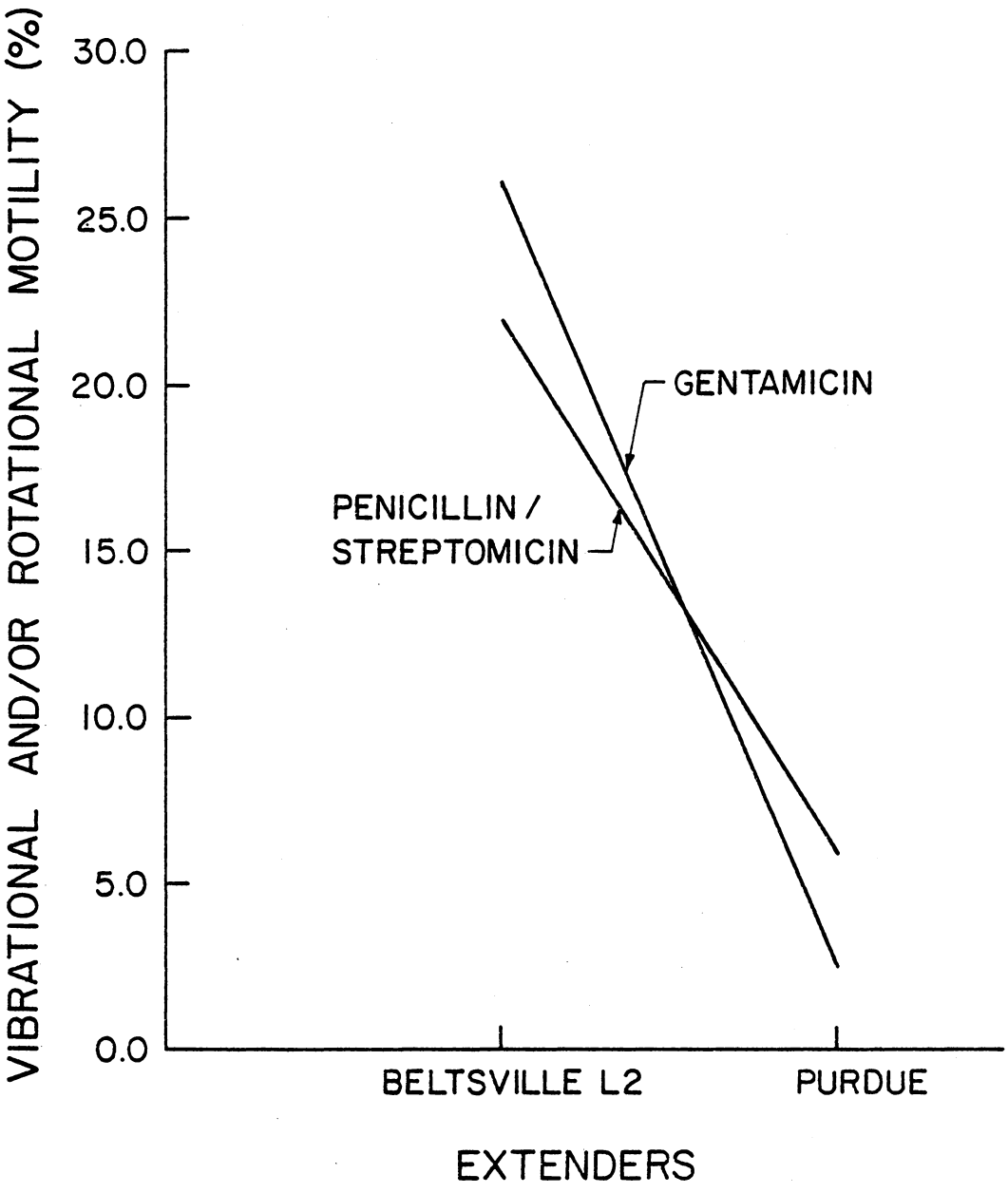

Figure 6. Effects of antibiotic and extender on vibrational and/or rotational motility

The regression of storage time on percent VIBROT was significant ($P<.01$). The prediction equation was $Y = 9.2102 - .1879 X + .001 X^2$, where Y is the predicted VIBROT and X is the storage time, in hours. The Y and X definitions are the same for the remaining regression equations presented in this section. The prediction equations for the regression of storage time x extender for VIBROT ($P<.01$) were $Y = 46.7731 - .5676 X + .0018 X^2$ for BL2 extender and $Y = 18.6484 - .3143 X + .0020 X^2$ for the Purdue extender. The regression of storage time x cooling rate on VIBROT ($P<.01$) yielded the following equations: $Y = 42.1214 - .3112 X + .007 X^2$ for the 2 hr cooling rate; and $Y = 40.6278 - .5705 X + .0031 X^2$ for the 4 hr cooling rate. The prediction equations for the regression of storage time x storage temperature on VIBROT ($P<.001$) were $Y = 21.5004 - .5809 X + .0034 X^2$ and $Y = 40.5408 - .3007 X + .003 X^2$, for the 5C and 15C storage temperature, respectively. The regression of storage time x ejaculates within boar on VIBROT was significant ($P<.001$). The prediction equations were: $Y = 16.2759 - .2268 X + .0008 X^2$ and $Y = 29.4656 - .8429 X + .0055 X^2$ for ejaculates 1 and 2 from the first boar; $Y = 45.0502 - .5386 X + .0017 X^2$ and $Y = 32.1840 - .3990 X + .0016 X^2$ for ejaculates 1 and 2 from the second boar; and $Y = 37.2878 - .0820 X - .0015 X^2$ and $Y = 34.1309 - .5558 X + .0029 X^2$ for ejaculates 1 and 2 from the third boar, respectively.

Relationship Among Semen Traits

Table 30 presents the correlation coefficients among the *in vitro* semen traits measured. The coefficients were very low and all were non-significant (P>.10).

Optimum Extender and Extender System

The optimum extender and extender system that was chosen from the *in vitro* data consisted of Purdue extender containing penicillin/streptomycin. This extender was cooled to a 15C storage temperature in 4 hours. The fertilization rate of semen processed in this extender and extender system is reported in the following section.

Phase II In Vivo Fertilization Rate

The analyses of variance for the normally fertilized and abnormally fertilized ova are presented in appendix tables 5 and 6, respectively. The analysis of variance for the effect of semen age on the percentage of ova recovered was not significant (P>.10). Table 31 presents the least-squares means for the percentage of ova recovered and the fertilization rate of recovered ova. The overall mean percentage of ova recovered was 83.09% and ranged from 65.78% (72 and 84 hr) to 96.92% (0 and 12 hr). The mean percentages of normally fertilized ova (NORM) were 71.50, 98.08, 95.55, 70.83 and 36.79 for the 0 and 12, 24 and 36, 48 and

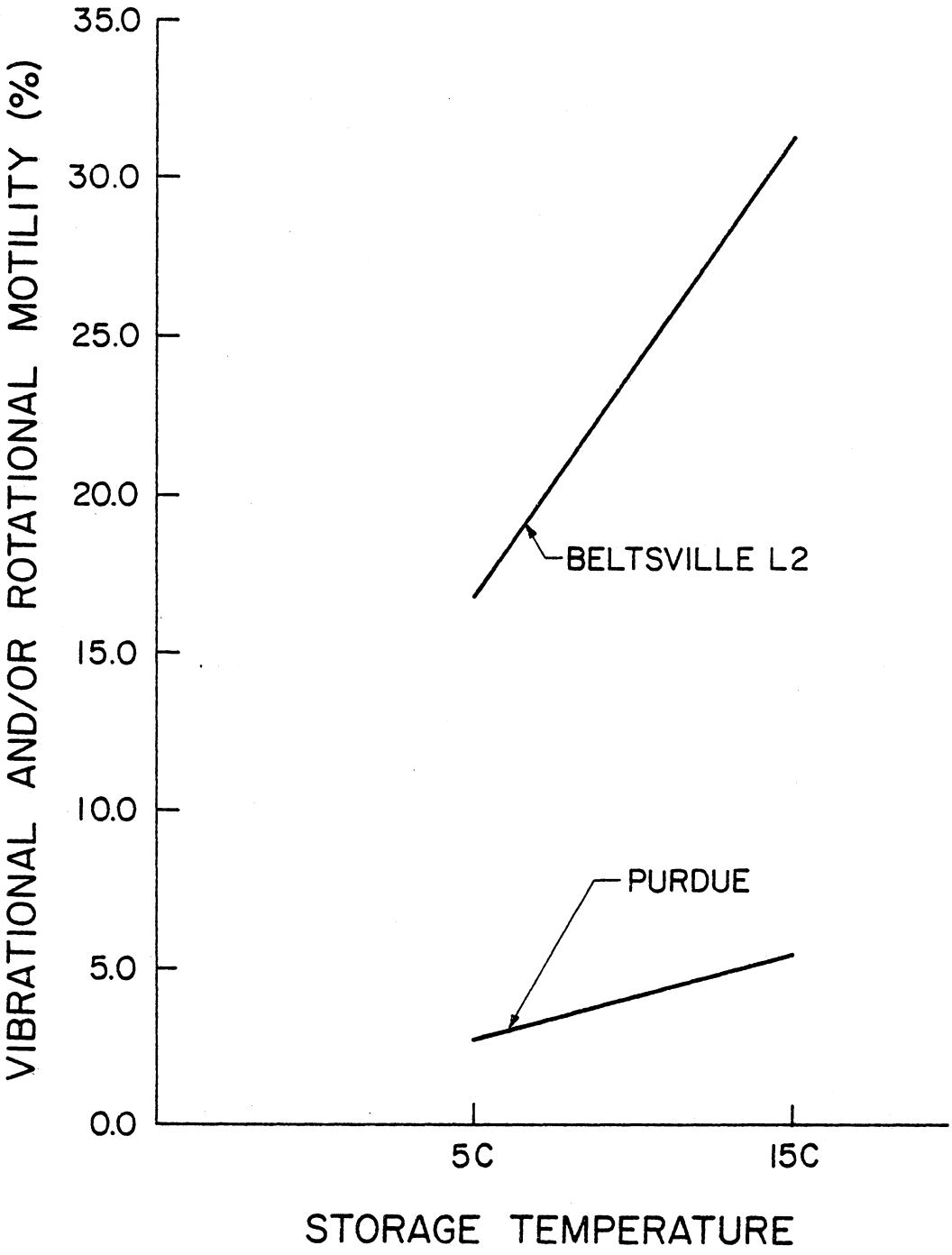

Figure 7. Effects of extender and storage temperature on vibrational and/or rotational motility

TABLE 30

CORRELATION COEFFIEIENTS AMONG SEMEN TRAITS

Traits	NAR[a]	PROMOT[b]	VIBROT[c]
Acrosin (423)[d]	-.013	-.056	-.009
NAR (423)		-.050	-.022
PROMOT (423)			-.038

[a]Normal apical ridge acrosomes.

[b]Progressive forward motility.

[c]Vibrational and/or rotational motility.

( )[d]Degrees of freedom from error mean square of general linear model.

TABLE 31

LEAST-SQUARES MEANS FOR OVA RECOVERY AND FERTILIZATION RATE[a]

Semen Age (Hours)	Total Number of Ova	Ova Recovery (%)[a]	Normal Fertilization (%)[b]	Abnormal Fertilization (%)[c]
0 and 12	55	96.92	71.50	28.50
24 and 36	55	81.15	98.08	1.92
48 and 60	87	96.15	95.55	4.45
72 and 84	68	65.78	70.83	29.17
96 and 108	46	75.45	36.79	63.21

[a]A total of four females in each semen age group.

[b]Standard error for all LS means = ± 12.59.

[c]Standard error for all LS means = ± 12.11.

60, 72 and 84 and the 96 and 108 hr semen age groups, respectively. The overall mean percentage of normally fertilized ova was 74.55. The quadratic regression of semen age on NORM was significant ($P<.01$). The distribution of normally fertilized ova was 23, 131, 37 and 3 for 4-cell, 8-cell, morula and blastocyst stage of development, respectively. The mean percentages of abnormally fertilized ova (ABN) were 28.50, 1.92, 4.45, 29.17 and 63.21 for the 0 and 12, 24 and 36, 48 and 60, 72 and 84 and the 96 and 108 hr semen age groups, respectively. The overall mean percentage of ABN was 25.45. The quadratic regression of semen age on ABN was significant ($P<.01$).

Chapter V. GENERAL DISCUSSION

Acrosome Morphology

Ejaculates within boars significantly effected the percentage of NAR acrosomes ($P<.001$). This demonstrated the variation among ejaculates within males. All boars were collected 2 days prior to the day that semen was collected and processed to minimize this source of variation. The interactions between boar x extender ($P<.05$) and boar x storage temperature ($P<.01$) were significant. These interactions demonstrated the variation among males for these two treatment effects.

There was a linear decrease ($P<.05$) in the percentage of NAR with an increase in storage time. This decrease in NAR occurred at a decreased rate. The regression of storage time x storage temperature on NAR decreased at a decreasing rate for the 5C temperatures while at the 15C storage temperature the NAR decreased at an increasing rate.

The regression of storage time x ejaculate within boar on NAR was significant ($P<.001$). Within males, the intercept for the first ejaculate was higher than the intercept for the second ejaculate. The first ejaculate NAR from the first boar increased at a decreasing rate while the second ejaculate NAR decreased at a decreasing rate. For the sec-

ond boar, the first ejaculate NAR decreased over time but at an increased rate, whereas the NAR of the second ejaculate decreased over time but at a decreased rate. The percentage of NAR for both ejaculates from the third boar decreased over time at an increased rate. However, the decrease in NAR was higher in the second ejaculate and was also at a faster rate than in the first ejaculate.

Three two-way treatment effect in interactions were significant for NAR acrosomes. The interactions were; antibiotic x storage temperature ($P<.01$); extender x cooling rate ($P<.05$); and extender x storage temperature ($P<.01$). The percentage of NAR was higher at the 15C storage temperature for penicillin/streptomycin and gentamicin. Purdue extender had a higher percentge of NAR at the 15C storage temperature while BL-2 had a higher NAR value at the 5C storage temperature. The percentage of NAR was higher at the 15C storage temperature for both extenders than at the 5C storage temperature. Purdue extender had a higher NAR value at both storage temperatures than that of BL2 extender. From these two interactions, the optimum extender system included PE and a 15C storage temperature. At 15C the antibiotic of choice was penicillin/streptomycin. The percentage of NAR was higher for PE than for BL2 at both cooling rates. There was a slightly higher NAR value for PE extended semen cooled in 4 hr than for PE cooled in 2 hours. Therefore, the 4 hr cooling rate was included in the system.

Acrosin Activity

The variation among ejaculates within males was demonstrated by the affect of ejaculate within males ($P<.001$) and by the interactions between ejaculates within males and antibiotic; extender; and storage temperature. For these interactions, acrosin activity was higher in the majority of ejaculates for samples: extended in PE; containing penicillin/streptomycin; and stored at the 5C storage temperature.

The mean values for acrosin activity appeared to increase with increased storage time, however this relationship was not significant ($P>.05$). From the preliminary study described earlier, the total amount of acrosin activity detected averaged .137 micromoles/ml/min for samples extended with BL2 and .248 micromoles/ml/min for samples extended with Purdue extender. The mean acrosin activity reported in this study accounts for approximately 6% to 9% of the activity observed for samples extended in BL2 and for approximately .4% to 5% of the activity observed for samples extended in Purdue extender. These percentages are in agreement with values reported by Goodpasture et al. (1979, 1980).

The interaction between extender and storage temperature was significant ($P<.01$). At 5C, acrosin activity was slightly higher in samples extended with PE than in samples

in BL2 extender. The activity was also higher in samples extended with PE than in samples extended with BL2 at the 15C storage temperature. The acrosin activity was slightly higher in samples stored at 15C than in samples stored at 5C for Purdue extender.

Since acrosin is involved in the penetration of the zona pellucida, the amount of acrosin activity found in the supernatant of extended semen is inversely related to te fertilizing potential of the extended semen. Therefore, the treatment effects with the lower acrosin activity are the more desirable treatments. The optimum extender system based on lower activity would include BL2 containing gentamicin and stored at a 15C storage temperature. However, since the activity throughout the preliminary study were always higher in samples extended with PE than in samples extended with BL2 extender, this affect may be due to the extender composition and not to a beneficial affect attributable to the BL2 extender.

The regression of storage time x storage temperature on acrosin activity was significant ($P<.05$). At both storage temperatures acrosin activity increased with increased time at an increasing rate. At 5C, the increase with increased time was higher than the increase at 15C, however the rate of increase was greater at the 15C storage temperature.

Progressive Forward Motility

Means reported for progressive forward motility (PROMOT) were extremely low. When samples exhibited PROMOT for more than one storage time, estimates were seldom consistant. In this study, samples were held in a water bath at 37C for one hour before PROMOT was estimated. However, Polge (1956b) stated that motility was practically indistinguishable from fresh semen if samples were held in a 37C water bath for two hours and shaken. These difference in processing samples may account for part of the inconsistency in PROMOT estimates. Another possible explanation for the inconsistant results is that several persons were involved in estimating PROMOT.

The ejaculate within boar affect ($P<.001$) demonstrated the variation among ejaculates within males. The interaction between boar and cooling rate ($P<.01$) demonstrated the variation among males for this treatment effect.

The percent PROMOT was significantly higher ($P<.05$) for samples extended with PE than BL2. Samples with penicillin/streptomycin had a higher ($P<.05$) percentage PROMOT than did samples with gentamicin. The antibiotic x extender ($P<.05$) and extender x storage temperature ($P<.01$) interactions were significant. From these treatment effects and treatment effect interactions, the optimum extender system

for the percentage of PROMOT included PE containing penicillin/streptomycin with a 15C storage temperature.

The regression of storage time x extender on the percentage PROMOT was significant ($P<.001$). PROMOT values for the BL2 extender, were essentially zero and did not increase with a change in time, whereas PROMOT values for the PE decreased with increased time but at a decreasing rate. The regression of storage time x storage temperature on the percentage PROMOT was significant ($P<.001$). The regression line for the 15C storage temperature begins at a higher intercept has a greater decrease over time than the line for the 5C storage temperature. However, the rate of decrease over time is faster for the 5C storage temperature. The regression lines predicted by the storage time x ejaculate within boar on the percentage PROMOT were significant ($P<.001$). The first ejaculates for all three boars have higher intercepts than the second ejaculates and the first ejaculates decrease with time faster and at a faster decreasing rate than do the same values for the second ejaculates.

Vibrational and/or Rotational Motility

Vibrational and/or rotational motility (VIBROT) was estimated using the same samples of PROMOT estimates employing the technique described above.

Ejaculates within boar significantly effected the percentage of VIBROT ($P<.001$). The interactions between ejaculates within boar and extender ($P<.01$) and cooling rate ($P<.05$) were also significant. These factors demonstrate the variation among ejaculates within males and the variation among males for these two treatment effects. Within each ejaculate the samples extended with BL2 had higher VIBROT values than did the samples extended with Purdue extender. Tso et al. (1980) reported that Tris was the best medium for maintaining motility and since BL2 contains Tris this may account for the higher VIBROT values observed in the samples extended with BL2 extender. In addition, boar spermatozoa adhere to glass microscope slide causing a cell that would normally exhibit PROMOT to exhibit a VIBROT type of movement. This situation would inflate the VIBROT estimates.

The interactions between antibiotic and extender ($P<.01$) and extender and storage temeprature ($P<.01$) were significant. The BL2 samples with gentamicin had a higher percent VIBROT than the BL2 samples with penicillin/streptomycin. The effect of antibiotic within the PE samples was reversed. Schafer et al. (1972) reported that gentamicin was a broad spectrum antibiotic that was stable over a range of 2 to 10 pH units. The higher VIBROT values for the BL2 samples containing gentamicin may be due to the

ability of gentamicin to maintain a more stable environment than BL2 with penicillin/streptomycin. The percent VIBROT was higher at both 5C and 15C for the samples extended with BL2 extender. The VIBROT values were higher at 15C than 5C within both extenders.

The regression of storage time x extender on the percent VIBROT was significant ($P<.01$). The equation for BL2 extender had a higher intercept and decreased with time faster than the equation for Purdue extender. However, the equation for PE had a higher quadratic coefficient and therefore decreased at a faster rate. The regression of storage time x cooling rate on VIBROT was significant ($P<.01$). The 4 hr cooling rate had a lower intercept but decreased with time faster and at a higher decreasing rate than the 2 hr cooling rate. The regression of storage time x storage temperature on VIBROT was significant ($P<.01$). The 15C storage temperature had a larger intercept and decreased less with time and at a slower rate than the 5C storage temperature.

The regression of storage time x ejaculate within boar on VIBROT was significant ($P<.001$). For the first boar, the second ejaculate had a higher intercept and decreased faster with time and at a greater decreasing rate than the first ejaculate. For the second boar, the first ejaculate had a higher intercept and decreased faster with time at a greater

decreasing rate. For the third boar, the second ejaculate had a lower intercept but decreased faster with time and at a greater decreasing rate than the first ejaculate.

Ova Recovery and Fertilization Rate

The overall mean of 83.09% for ova recovery was slightly lower than the 86% and 88.3% ova recoveries reported by Hunter and Dziuk (1968) and Stratman and Self (1960), respectively.

The percent NORM for the 0 and 12 hour age group was lower than the percent NORM for the 24 and 36 hour and 48 and 60 hour age groups. The 71.50% NORM reported in this study was lower than the 87.2% NORM reported by Pursel et al. (1973b) for semen that was stored at 15C for six hours and the 91% reported by Wiggins et al. (1951) for fresh semen. However, the percent NORM for the 0 and 12 hour semen age group was superior to the fertilization rate reported by Stratman and Self (1960) for fresh semen. The low percent NORM for the 0 and 12 hour age group was due to one female with a 4.17% NORM. Another female in the same age group was bred with semen from the same ejaculate and had a 90.9% NORM. Since both females were bred with semen from the same ejaculate, the difference in percent NORM may be attributed to the female. The NORM was increased to 93.94% when the female with the low percent NORM was omit-

ted. The percent NORM for the 48 and 60 hour age group was 95.55 which is slightly higher than the 54 hour value reported by Pursel et al. (1973b). However, the 102 hour value reported by Pursel et al. (1973b) was higher than the 36.79% reported for the 96 and 108 hour semen age group. The percentages of ABN is the complement for the percentage values reported for NORM.

The linear and quadratic contrasts of semen age on NORM and ABN were significant ($P<.05$, linear; and $P<.01$, quadratic).

The ova recovery and fertilization rate data were transformed into acrosins and tested by analysis of variance. However, the transformed data did not change the analysis of variance on the observed data, therefore the transformed data was not reported.

Chapter VI. SUMMARY AND CONCLUSIONS

This study was conducted to: (a) determine the optimum extender system for two extenders that will maintain the highest level of cellular integrity when stored at either 5C or 15C for a minimum of 72 hr; (b) evaluate the fertilizing capacity of stored spermatozoa using the extender system that maintained the highest level of cellular integrity; (c) critically analyze enzymatic and morphological changes associated with storage and aging of boar spermatozoa; and (d) characterize properties of boar spermatozoa important to fertilization. The treatment effects were extender, antibiotic, cooling rate and storage temperature with two level per effect.

The in vitro semen traits measured in this study were the percentage of normal apical ridge acrosomes (NAR), progressive forward motility (PROMOT), vibrational and/or rotational motility (VIBROT) and acrosin activity.

There was a decrease ($P<.05$) in the mean NAR within each treatment combination as storage time increased. Ejaculate within boars ($P<.001$); boar x extender ($P<.05$); boar x storage temperature ($P<.01$) were significant. The follownng regressions were significant: storage time x storage temperature ($P<.001$) and storage time x ejaculate within

boar (P<.001). The interactions of antibiotic x storage temperature (P<.01), extender x cooling rate (P<.05) and extender x storage temperature (P<.01) were significant for NAR.

Acrosin activity generally increased with increased storage time, within each treatment combination. However, this trend was not significant (P<.10). Ejaculate within boar (P<.001); ejaculate within boar x antibiotic (P<.05); ejaculate within boar x extender (P<.001); and ejaculate within boar x storage temperature (P<.001) were significant. The storage time x storage temperature regression was significant (P<.05). The interaction of extender x storage temperature was significant (P<.01) for acrosin activity.

Mean values for PROMOT were either non-estimatable or very low initially and decreased rapidly for all treatment combinations. Purdue extender had a higher (P<.05) percent PROMOT than BL2. Treatments that contained penicillin/streptomycin had a significantly higher (P<.05) PROMOT than treatments that contained gentamicin. Ejaculates within boar was significant (P<.001). The storage time x extender (P<.001); storage time x storage temperature (P<.05); and storage time x ejaculate within boar (P<.001) regressions were significant. The interactions of antibiotic x extender and extender x storage temperature were significant (P<.05 and P<.01, respectively).

Mean values for percent VIBROT generally decreased ($P<.01$) as storage time increased. Ejacualte within boar ($P<.001$); ejaculate within boar x extender ($P<.001$); ejaculate within boar x cooling rate ($P<.05$) were significant. The regressions of storage time x extender ($P<.01$); storage time x cooling rate ($P<.01$); storage time x storage temperature ($P<.01$); and storage time x ejaculate within boar ($P<.001$) were significant. The interactions of antibiotic x extender and extender x storage temperature were significant for VIBROT ($P<.01$).

The correlations between the semen traits were low and non-significant ($P<.10$).

The optimum extender and extender system consisted of Purdue extender containing penicillin/streptomycin. This extender was cooled to a 15C storage temperature in 4 hours.

The fertilization rate of the optimum extender and extender system resulted in an overall mean of 74.55% normally fertilized ova. The percentages of normally fertilized ova were 71.50, 98.08, 95.55, 70.83 and 36.79 for the 0 and 12, 24 and 36, 48 and 60, 72 and 84 and the 96 and 108 hr semen age groups, respectively.

In conclusion, the extender and extender system that maintained the highest level of cellular integrity consisted of Purdue extender containing penicillin/streptomycin and cooled to a 15C storage temperature in 4 hours. This exten-

der system maintained a 70% minimum fertilization rate for 84 hours.

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APPENDIX

TABLE 1

ANALYSIS OF VARIANCE FOR NORMAL APICAL RIDGE ACROSOMES

General Linear Model Procedure

Source	DF	Sum of Squares	Mean Square	F Value	R-Square	Mean	Std. Dev.
Model	55	344637.518	6266.137	99.95***	.859	52.667	7.918
Error	904	56675.340	62.694				
Corrected Total	959	401312.858					

Source	DF	Sum of Squares	F Value
Boar[a]	2	8159.914	1.97
Ejaculate (Boar)[b]	3	6186.344	32.89***
Antibiotic[c]	1	51.632	.94
Extender[c]	1	785.118	.69
Cooling Rate[c]	1	.255	.00
Storage Temperature[c]	1	5312.832	1.03
Boar x Antibiotic[d]	2	109.689	.36
Boar x Extender[d]	2	2265.476	11.43*
Boar x Cooling Rate[d]	2	616.026	2.34
Boar x Storage Temperature[d]	2	10327.627	43.59**
Ejaculate (Boar) x Antibiotic[b]	3	456.676	2.43
Ejaculate (Boar) x Extender[b]	3	297.410	1.58
Ejaculate (Boar) x Cooling Rate[b]	3	395.741	2.10
Ejaculate (Boar) x Storage Temperature[b]	3	355.376	1.89
Antibiotic x Extender[b]	1	214.232	3.42
Antibiotic x Cooling Rate[b]	1	27.846	.44
Antibiotic x Storage Temperature[b]	1	2011.157	32.08***
Extender x Cooling Rate[b]	1	385.700	6.15*
Extender x Storage Temperature[b]	1	1894.221	30.21***
Cooling Rate x Stoarage Temperature[b]	1	167.919	2.68

(Continued)

TABLE 1 (Cont.)

ANALYSIS OF VARIANCE FOR NORMAL APICAL RIDGE ACROSOMES

Source	DF	Sum of Squares	F Value
Sample Hour (linear)[e]	1	11103.169	93.30*
Sample Hour (quadratic)[e]	1	20.205	.10
Sample Hour (linear) x Antibiotic[b]	1	16.157	.26
Sample Hour (quadratic) x Antibiotic[b]	1	11.216	.18
Sample Hour (linear) x Extender[b]	1	93.894	1.50
Sample Hour (quadratic) x Extender[b]	1	40.909	.65
Sample Hour (linear) x Cooling Rate[b]	1	.868	.01
Sample Hour (quadratic) x Cooling Rate[b]	1	5.253	.08
Sample Hour (linear) x Storage Temperature[b]	1	2645.024	42.19***
Sample Hour (quadratic) x Storage Temperature[b]	1	3389.750	54.07***
Sample Hour (linear) x Boar[f]	2	238.021	.05
Sample Hour (quadratic) x Boar[f]	2	400.000	.07
Sample Hour (linear) x Ejaculate (Boar)[b]	3	6622.493	35.21***
Sample Hour (quadratic) x Ejaculate (Boar)[b]	3	8479.098	45.08***

[a]The F-test denominator is the ejaculate (boar) mean square.

[b]The F-test denominator is the model error mean square.

[c]The F-test denominator is the appropriate boar x treatment mean square.

[d]The F-test denominator is the appropriate ejaculate (boar) x treatment mean square.

[e]The F-test denominator is the appropriate sample hour x boar mean square.

[f]The F-test denominator is the appropriate sample hour x ejaculate (boar) mean square.

*P < .05
**P < .01
***P < .001

TABLE 2

ANALYSIS OF VARIANCE FOR ACROSIN ACTIVITY

General Linear Model Procedure

Source	DF	Sum of Squares	Mean Square	F Value	R-Square	Mean	Std. Dev.
Model	55	1.58×10^{-2}	2.88×10^{-4}	57.19***	.881	1.05×10^{-2}	2.24×10^{-3}
Error	424	2.13×10^{-3}	5.03×10^{-6}				
Corrected Total	479	1.79×10^{2}					

Source	DF	Sum of Squares	F Value
Boar[a]	2	3.42×10^{-4}	.16
Ejaculate (Boar)[b]	3	3.15×10^{-3}	208.76***
Antibiotic[c]	1	1.42×10^{-5}	9.46
Extender[c]	1	8.46×10^{-5}	1.28
Cooling Rate[c]	1	4.22×10^{-6}	.69
Storage Temperature[c]	1	8.11×10^{-6}	.13
Boar x Antibiotic[d]	2	3.00×10^{-5}	8.22
Boar x Extender[d]	2	1.32×10^{-4}	.55
Boar x Cooling Rate[d]	2	1.23×10^{-5}	.53
Boar x Storage Temperature[d]	2	1.24×10^{-4}	1.67
Ejaculate (Boar) x Antibiotic[b]	3	5.48×10^{-5}	3.63*
Ejaculate (Boar) x Extender[b]	3	3.61×10^{-4}	23.95***
Ejaculate (Boar) x Cooling Rate[b]	3	3.50×10^{-7}	.02
Ejaculate (Boar) x Storage Temperature[b]	3	1.11×10^{-4}	7.35***
Antibiotic x Extender[b]	1	1.58×10^{-5}	3.15
Antibiotic x Cooling Rate[b]	1	1.73×10^{-6}	.34
Antibiotic x Storage Temperature[b]	1	4.94×10^{-6}	.98
Extender x Cooling Rate[b]	1	7.71×10^{-6}	1.53
Extender x Storage Temperature[b]	1	8.16×10^{-5}	16.22***
Cooling Rate x Storage Temperature[b]	1	1.49×10^{-6}	.30

(Continued)

TABLE 2 (Cont.)

ANALYSIS OF VARIANCE FOR ACROSIN ACTIVITY

Source	DF	Sum of Squares	F Value
Sample Hour (linear)[e]	1	4.00×10^{-8}	.00
Sample Hour (quadratic)[e]	1	1.57×10^{-6}	2.02
Sample Hour (linear) x Antibiotic[b]	1	1.88×10^{-5}	3.74
Sample Hour (quadratic) x Antibiotic[b]	1	1.80×10^{-5}	3.58
Sample Hour (linear) x Extender[b]	1	8.66×10^{-6}	1.72
Sample Hour (quadratic) x Extender[b]	1	1.29×10^{-5}	2.57
Sample Hour (linear) x Cooling Rate[b]	1	2.40×10^{-7}	.05
Sample Hour (quadratic) x Cooling Rate[b]	1	5.70×10^{-7}	.11
Sample Hour (linear) x Storage Temperature[b]	1	3.36×10^{-5}	6.68*
Sample Hour (quadratic) x Storage Temperature[b]	1	9.24×10^{-6}	1.84
Sample Hour (linear) x Boar[f]	2	1.79×10^{-5}	3.77
Sample Hour (quadratic) x Boar[f]	2	1.56×10^{-5}	3.50
Sample Hour (linear) x Ejaculate (Boar)[b]	3	7.11×10^{-6}	.47
Sample Hour (quadratic) x Ejaculate (Boar)[b]	3	6.66×10^{-6}	.44

[a]The F-test denominator is the ejaculate (boar) mean square.

[b]The F-test denominator is the model error mean square.

[c]The F-test denominator is the appropriate boar x treatment mean square.

[d]The F-test denominator is the appropriate ejaculate (boar) x treatment mean square.

[e]The F-test denominator is the appropriate sample hour x boar mean square.

[f]The F-test denominator is the appropriate sample hour x ejaculate (boar) mean square.

*P < .05

***P < .001

TABLE 3

ANALYSIS OF VARIANCE FOR PROGRESSIVE FORWARD MOTILITY

General Linear Model Procedure

Source	DF	Sum of Squares	Mean Square	F Value	R-Square	Mean	Std. Dev.
Model	55	23320.073	424.001	13.56***	.452	1.521	5.591
Error	904	28259.511	31.261				
Corrected Total	959	51579.583					

Source	DF	Sum of Squares	F Value
Boar[a]	2	1335.202	.50
Ejaculate (Boar)[b]	3	4012.622	42.79***
Antibiotic[c]	1	229.950	88.30*
Extender[c]	1	6204.827	29.68*
Cooling Rate[c]	1	118.640	3.32
Storage Temperature[c]	1	1368.681	12.72
Boar x Antibiotic[d]	2	5.208	.01
Boar x Extender[d]	2	418.125	.39
Boar x Cooling Rate[d]	2	71.458	57.17**
Boar x Storage Temperature[d]	2	215.208	.64
Ejaculate (Boar) x Antibiotic[b]	3	79.375	.85
Ejaculate (Boar) x Extender[b]	3	1601.875	17.08***
Ejaculate (Boar) x Cooling Rate[b]	3	1.875	.02
Ejaculate (Boar) x Storage Temperature[b]	3	503.125	5.36**
Antibiotic x Extender[b]	1	201.667	6.45*
Antibiotic x Cooling Rate[b]	1	1.667	.05
Antibiotic x Storage Temperature[b]	1	15.000	.48
Extender x Cooling Rate[b]	1	20.417	.65
Extender x Storage Temperature[b]	1	700.417	22.41***
Cooling Rate x Storage Temperature[b]	1	70.417	2.25

(Continued)

TABLE 3 (Cont.)

ANALYSIS OF VARIANCE FOR PROGRESSIVE FORWARD MOTILITY

Source	DF	Sum of Squares	F Value
Sample Hour (linear)[e]	1	3035.078	9.40
Sample Hour (quadratic)[e]	1	1706.321	8.83
Sample Hour (linear) x Antibiotic[b]	1	47.198	1.51
Sample Hour (quadratic) x Antibiotic[b]	1	14.591	.47
Sample Hour (linear) x Extender[b]	1	2851.171	91.21***
Sample Hour (quadratic) x Extender[b]	1	1619.389	51.80***
Sample Hour (linear) x Cooling Rate[b]	1	57.815	1.85
Sample Hour (quadratic) x Cooling Rate[b]	1	37.571	1.20
Sample Hour (linear) x Storage Temperature[b]	1	441.229	14.11***
Sample Hour (quadratic) x Storage Temperature[b]	1	204.553	6.54*
Sample Hour (linear) x Boar[f]	2	645.924	.60
Sample Hour (quadratic) x Boar[f]	2	386.553	.68
Sample Hour (linear) x Ejaculate (Boar)[b]	3	1602.675	17.09***
Sample Hour (quadratic) x Ejaculate (Boar)[b]	3	853.480	9.10***

[a]The F-test denominator is the ejaculate (boar) mean square.

[b]The F-test denominator is the model error mean square.

[c]The F-test denominator is the appropriate boar x treatment mean square.

[d]The F-test denominator is the appropriate ejaculate (boar) x treatment mean square.

[e]The F-test denominator is the appropriate sample hour x boar mean square.

[f]The F-test denominator is the appropriate sample hour x ejaculate (boar) mean square.

*P < .05
**P < .01
***P < .001

TABLE 4

ANALYSIS OF VARIANCE FOR VIBRATIONAL AND/OR ROTATIONAL MOTILITY

General Linear Model Procedure

Source	DF	Sum of Squares	Mean Square	F Value	R-Square	Mean	Std. Dev.
Model	55	294732.879	5358.780	28.99***	.638	14.010	13.597
Error	904	167127.017	184.875				
Corrected Total	959	461859.896					

Source	DF	Sum of Squares	F Value
Boar[a]	2	3799.523	1.20
Ejaculate (Boar)[a]	3	4747.364	8.56*
Antibiotic[c]	1	1061.598	.94
Extender[c]	1	45759.265	6.67
Cooling Rate[c]	1	333.279	4.32
Storage Temperature[c]	1	1384.443	2.15
Boar x Antibiotic[d]	2	2250.208	5.03
Boar x Extender[d]	2	13714.375	4.38
Boar x Cooling Rate[d]	2	154.375	.13
Boar x Storage Temperature[d]	2	1287.708	1.47
Ejaculate (Boar) x Antibiotic[b]	3	671.563	1.21
Ejaculate (Boar) x Extender[b]	3	4696.563	8.47***
Ejaculate (Boar) x Cooling Rate[b]	3	1791.563	3.23*
Ejaculate (Boar) x Storage Temperature[b]	3	1314.063	2.37
Antibiotic x Extender[b]	1	3117.604	16.86***
Antibiotic x Cooling Rate[b]	1	292.604	1.58
Antibiotic x Storage Temperature[b]	1	585.938	3.17
Extender x Cooling Rate[b]	1	.104	.00
Extender x Storage Temperature[b]	1	8342.604	45.13***
Cooling Rate x Storage Temperature[b]	1	175.104	.95

(Continued)

TABLE 4 (Cont.)

ANALYSIS OF VARIANCE FOR VIBRATIONAL AND/OR ROTATIONAL MOTILITY

Source	DF	Sum of Squares	F Value
Sample Hour (linear)[e]	1	16236.335	47.62**
Sample Hour (quadratic)[e]	1	3283.026	6.33
Sample Hour (linear) x Antibiotic[b]	1	455.554	2.46
Sample Hour (quadratic) x Antibiotic[b]	1	77.344	.42
Sample Hour (linear) x Extender[b]	1	1343.206	7.27**
Sample Hour (quadratic) x Extender[b]	1	7.584	.04
Sample Hour (linear) x Cooling Rate[b]	1	1403.488	7.59**
Sample Hour (quadratic) x Cooling Rate[b]	1	1562.697	8.45**
Sample Hour (linear) x Storage Temperature[b]	1	1639.330	8.87**
Sample Hour (quadratic) x Storage Temperature[b]	1	2519.137	13.63***
Sample Hour (linear) x Boar[f]	2	681.976	.23
Sample Hour (quadratic) x Boar[f]	2	1037.121	.43
Sample Hour (linear) x Ejaculate (Boar)[b]	3	4340.121	7.83***
Sample Hour (quadratic) x Ejaculate (Boar)[b]	3	3610.298	6.51***

[a]The F-test denominator is the ejaculate (boar) mean square.

[b]The F-test denominator is the model error mean square.

[c]The F-test denominator is the appropriate boar x treatment mean square.

[d]The F-test denominator is the appropriate ejaculate (boar) x treatment mean square.

[e]The F-test denominator is the appropriate sample hour x ejaculate (boar) mean square.

*P < .05
**P < .01
***P < .001

TABLE 5

ANALYSIS OF VARIANCE FOR NORMALLY FERTILIZED OVA

Source	DF	Sum of Squares	Mean Square	F Value	R-Square	Mean	Std. Dev.
Model	4	9775.751	2443.938	4.17*	.526	74.549	24.222
Error	15	8800.552	586.703				
Corrected Total	19	18576.303					

Source	DF	Sum of Squares	F Value
Semen Age	4	9775.751	4.17*

Contrast	DF	Sum of Squares	F Value
Semen Age (linear)	1	3737.617	6.37*
Semen Age (quadratic)	1	5879.657	10.02**

*P < .05

**P < .01

TABLE 6

ANALYSIS OF VARIANCE FOR ABNORMALLY FERTILIZED OVA

Source	DF	Sum of Squares	Mean Square	F Value	R-Square	Mean	Std. Dev.
Model	4	9775.751	2443.938	4.17*	.526	25.451	24.222
Error	15	8800.552	586.703				
Corrected Total	19	18576.303					

Source	DF	Sum of Squares	F Value
Semen Age	4	9775.751	4.17*

Contrast	DF	Sum of Squares	F Value
Semen Age (linear)	1	3737.617	6.37*
Semen Age (quadratic)	1	5879.657	10.02**

*P < .05

**P < .01

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LONG-TERM STORAGE OF LIQUID BOAR SPERMATOZOA

by

Charles Raymond Underwood III

(ABSTRACT)

This study was conducted to: (a) determine the optimum extender system for two extenders that will maintain the highest level of cellular integrity when stored at either 5C or 15C for a minimum of 72 hr; (b) evaluate the fertilizing capacity of stored spermatozoa using the optimum extender system; (c) critically analyze enzymatic and morphological changes associated with storage and aging of boar spermatozoa; and (d) characterize properties of boar spermatozoa important to fertilization.

The least-squares means for the percentage of normal apical ridge acrosomes were significantly affected by ejaculate within boar ($P<.001$), boar x extender ($P<.05$), boar x storage temperature ($P<.01$), extender x storage temperature ($P<.01$), extender x cooling rate ($P<.05$) and antibiotic x storage temperature ($P<.01$). The regression equations of storage time x treatment effect were reported.

The least-squares means for acrosin activity were significantly affected by ejaculate within boar ($P<.001$), ejaculate within boar x antibiotic ($P<.05$), ejaculate within

boar x extender ($P<.001$), ejacualte within boar x storage temperature and extender x storage temperature ($P<.05$). The regression of storage time x storage temperature was reported.

The least-squares means for progressive forward motility were significantly affected by extender ($P<.05$), antibiotic ($P<.05$), ejaculate within boar ($P<.001$), antibiotic x extender ($P<.05$) and extender x storage temperature ($P<.01$). The regressions of storage time x treatment effects were reported.

The least-squares means for vibrational and/or rotational motility were affected by ejaculate within boar ($P<.001$), ejacualte within boar x extender ($P<.001$), ejaculate within boar x cooling rate ($P<.05$), antibiotic x extender and extender x storage temperature ($P<.01$).

The optimum extender system consisted of Purdue extender containing penicillin/streptomycin and cooled to a 15C storage temperature in 4 hours. This extender system maintained a 70% minimum fertilization rate for 84 hours.