THE INFLUENCE OF GLYCEROL AND VARIOUS DILUENTS ON LOW TEMPERATURE SURVIVAL OF RAM SPERMATOZOA

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
Neal Lloyd First
1957

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By

Neal Lloyd First

A THESIS

Submitted to the College of Agriculture,
Michigan State University of Agriculture and Applied Science
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Animal Husbandry

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ACKNOWLEDGMENTS

The author desires to express his sincere appreciation to his Major Professor, Dr. Harold A. Henneman of the Department of Animal Husbandry, for his considerate guidance, counsel and criticism. His genuine interest and encouragement have been of great value during the course of this study.

Much is owed to Dr. James Williams of the Department of Animal Pathology for his constructive suggestions and criticism which aided considerably in developing some of the ideas expressed in this manuscript.

Grateful acknowledgments are extended to Dr. William T. Magee of the Department of Animal Husbandry for his assistance with the statistical analysis. Sincere thanks and appreciation are due Dr. Elwin Miller of the Animal Husbandry Department for his editorial advice. To Lee Bell, the Shepard, and his assistant, Neil Pifer, are due a grateful appreciation for their cooperation and help in handling the sheep used in this experiment.

The author wishes to thank the Michigan State University Animal Husbandry Department for the facilities provided to carry out this research, also, Mr. Fred Dombrosky and the Michigan Artificial Breeders Inc. for their cooperation in supplying the milk and antibiotics used.

In addition, he is deeply indebted to Mrs. Laurence Eichelberger for an excellent job in typing and arranging the manuscript.

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ABSTRACT

THE INFLUENCE OF GLYCEROL AND VARIOUS DILUENTS ON

LOW TEMPERATURE SURVIVAL OF RAM SPERMATOZOA

Neal Lloyd First

A study of the influence of glycerol and various diluents on low temperature survival (-79°C) of ram spermatozoa was conducted employing 35 ejaculates of ram semen in three experiments.

Split ejaculates were used to study the effect on ram sperm survival after freezing to -79°C of the following factors: (1) levels of glycerol, (2) egg yolk level in egg yolk phosphate citrate extenders, (3) arabinose, (4) penicillin and streptomycin, and (5) comparison of extenders of milk and egg yolk phosphate citrate.

All semen was diluted 1/10 and frozen in one ml. portions. The percent survival was based on progressive motility of all treatments just prior to and immediately after freezing. An analysis of variance was applied to all data.

The survival of milk extended ram spermatozoa frozen in levels of 0, 2, 4, 6, 8, 10, 12 and 15 percent glycerol was curvilinear with the highest survivals from six and eight percent glycerol.

The addition of 1.25 percent arabinose improved ram sperm survival in both milk and egg yolk phosphate citrate extenders.

Ram sperm survival was greater when heated whole milk was used as a semen extender than when extended with egg yolk phosphate citrate.

Egg yolk levels of 50, 37.5 and 30 percent resulted in greater sperm survival than 25 percent in egg yolk phosphate citrate extenders. However, there were no differences in survival between 50, 37.5 or 30 percent egg yolk.

The addition of 500 Oxford units penicillin and 500 micrograms dihydrostreptomycin sulphate per milliliter of extended semen did not significantly improve sperm survival in this study.

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INTRODUCTION

In the past five years, extensive research work has resulted in the development and perfection of a technique for the low temperature storage of bull spermatozoa. The technique has aided in extending the use of superior sires to more breeders and in providing a reliable source of semen in case of injury or illness of the sire.

Attempts have been made to adapt the technique to ram semen but the fertility of frozen ram semen has been disappointing. This research has been designed to discover techniques which will improve viability of ram sperm stored at low temperatures.

OBJECTIVES

(1) To develop a technique for the low temperature storage of ram spermatozoa which will result in a satisfactory sperm survival after freezing and consequent higher fertility and conception rates.

(2) To determine the effect of glycerol levels, milk and egg yolk phosphate citrate diluters, antibiotics, level of egg yolk and the addition of arabinose on the survival of ram spermatozoa after freezing.

REVIEW OF LITERATURE

Davenport (1897) reported that human spermatozoa would survive freezing at -17°C. Luyett and Hodapp (1938) successfully froze frog semen after dehydration with two M. sucrose and Jahnel (1938) showed that human sperm survived prolonged exposure to -196°C and -269°C, but not shorter exposures to -10°C and -20°C.

Luyett and Gehenio (1940) reported that the preservation of life at low temperatures depends on preventing the formation of introcellular ice crystals. Lovelock (1953) has refuted this theory and states that the damage to life from freezing is due to increased salt concentration in the cells.

Parkes (1945) obtained successful results by freezing human sperm to temperatures of -79°C and -196°C. His success was attributed to the use of larger freezing tubes than the ones formerly used in this type of experiment.

The addition of Glycerol

Polge, Smith and Parkes (1949) found that glycerol had a protective action when added to semen before freezing. They noted this in fowl and human sperm stored at -79°C. Smith and Polge (1950) reported that glycerol had a damaging effect on fowl sperm but this seemed to be true only when the glycerol was not removed before insemination.

Polge (1953) reported that when glycerol levels were varied from one to twenty percent, motility after freezing increased proportionately with increased glycerol levels until ten percent glycerol by weight was

reached, beyond this optimum level spermatozoa survival decreased with increased glycerol. It should be noted that ten percent glycerol by weight is equivalent to eight percent by volume.

Miller and Vandemark (1954) found with semen frozen at four, six, eight and ten percent glycerol by volume in an egg yolk citrate extender that eight percent glycerol gave the highest survival immediately after freezing while the highest survival after two days storage was from semen frozen in six percent glycerol.

Mixner and Saroff (1954) with a similar experiment report that 7.5 percent glycerol resulted in significantly greater survival when either egg yolk citrate or heated whole milk were used as extenders. Cragle and Myers (1954) reported highest survival using 7.6 percent glycerol with 25 percent egg yolk.

Saroff and Mixner (1955) stated that as the level of egg yolk in an extender is increased, correspondingly higher levels of glycerol are required for greater sperm survival. With 20 percent egg yolk, seven percent glycerol was found to result in highest sperm survival.

Odell and Almquist (1954) found that the optimum level of glycerol varied with the diluter employed. With heated homogenized milk, ten percent glycerol by volume resulted in the highest survival. However, with an egg yolk citrate extender, seven and ten percent glycerol both resulted in highest survival rates, while 13 percent glycerol was superior in a skim milk extender. This is not in agreement with Odell and Hurst (1955) who reported a higher recovery 24 and 48 hours after freezing bull semen with eight percent glycerol in a skim milk extender.

Graham and Marion (1953) found ten percent glycerol by volume to be superior to other levels in an egg yolk citrate extender.

The addition of Arabinose Sugars

Emmens and Blackshaw (1950) first reported that the addition of a pentose sugar, arabinose, to bull and ram semen greatly improved sperm survival after freezing with seven and one-half percent glycerol. This has been supported by White et al. (1954) and Blackshaw (1955 a, b).

Blackshaw (1955a) reported ram semen frozen with 7.5 percent glycerol resulted in only 13 percent survival, whereas when 1.25 persent arabinose was added along with the glycerol, 36 percent of the sperm survived. Likewise with bull semen, 42 percent survived when only glycerol was added, while 53 percent survived when treated with 7.5 percent glycerol plus 1.25 percent arabinose.

Other sugars have also been used. Blackshaw (1955a) in six treatments supplemented an egg yolk citrate extender containing 7.5 percent glycerol with 1.25 percent arabinose dulcitol, galactose, fructose, mannose, and 2.5 percent sucrose respectively. They found arabinose and fructose brought about the greatest increase in ram and bull sperm survival. All five sugars studied increased sperm survival over the control.

Hafs and Elliott (1955) added one percent each of three monosaccharides; fructose, glucose and xylose to bull semen containing glycerol. They found that one percent fructose resulted in 27.9 percent motile sperm after freezing, whereas glucose resulted in 26.4 percent and xylose 24.2 percent motile sperm. When these sugars were added entirely in the nonglycerol fraction, sperm motility after freezing was significantly less.

Egg Yolk Levels and Methods of Adding Egg Yolk

Polge (1952b) employed an egg yolk citrate extender plus ten or fifteen percent glycerol. The semen was diluted as two fractions. The first contained 50 percent egg yolk and the other 20 to 30 percent glycerol, such that the final extended semen contained 25 percent egg yolk and ten to fifteen percent glycerol. This same procedure has been used by Polge and Lovelock (1952), Rowson and Polge (1953), Polge (1953) and Blackshaw (1955a, b).

Dunn and Hafs (1953) suggested that the relative composition of the two dilution fractions normally used may influence the tolerance of spermatozoa to the freezing process as much as the composition of the final mixture.

Recently, Hafs and Elliot (1955) have compared the inclusion of 50 percent egg yolk, in the first dilution, with 25 percent egg yolk in both the first and second dilutions. They found that twenty five percent egg yolk in both fractions resulted in a significantly higher percentage of motile sperm surviving and a higher 60-90 day non-return rate.

In 1949, Swanson found that 20 percent egg yolk in unfrozen bull semen resulted in motility equal to that obtained with 50 percent egg yolk in a yolk citrate extender, however, motility was reduced when only ten percent egg yolk was employed. This has been substantiated by Olds et al. (1951) in a field trial involving over 1,000 cows per treatment.

Almquist (1951), however, found no differences in fertility with egg yolk levels ranging from 50 percent to 12.5 percent. This trial involved 8,222 cows.

Dunn and Hafs (1953) found that egg yolk diluters containing 25 percent egg yolk were superior to other diluters of 27.5 and 50 percent egg yolk. While Kinny and Vandemark (1954) found 16 and 24 percent egg yolk superior to 32 percent.

Saroff and Mixner (1955) reported that highly significant increases in bull sperm survival after freezing resulted when 20 percent egg yolk was compared with levels of 15 and 30 percent.

Cragle et al. (1955) in a series of experiments involving graduated levels of sodium citrate, glycerol and various equilibration times have established that when 24 percent egg yolk is employed, the highest survival rates are achieved with 2.9 percent sodium citrate and 7.6 percent glycerol with an equilibration period of 14.9 hours.

Egg Yolk Citrate or Milk

The use of heated whole milk as a semen diluter was first mentioned by Michajilov (1950). Prior to this, yolk citrate or phosphate were the chief extenders employed.

Thacker and Almquist (1951) have found heated homogenized milk equal to egg yolk citrate as a semen extender. This has been corroborated by Collins (1953), Almquist et al. (1954), Thacker and Almquist (1953) and Almquist (1954).

Williams et al. (1954) compared heated homogenized milk with an egg yolk citrate extender containing 50 percent egg yolk. The non-return rates from 1,070 first services were, 70.9 percent from the use of homogenized milk and 65.1 percent from the egg yolk citrate diluter. These differences were highly significant.

Almquist (1954) reported an eight percent increase in non-return rates in favor of heated homogenized milk as compared with a yolk citrate extender. Similar increases in favor of a milk extender have been reported by Dreher and Webb (1953) and by Flerchinger et al. (1953)

Perkins et al. (1955) found yolk citrate and heated homogenized milk diluters equal when the diluted semen was used within one day. However, highly significant differences resulted in favor of the heated milk after two days storage and in favor of the yolk citrate extender after three days storage at five degrees centigrade.

Odell and Almquist (1954) stated that heated homogenized milk and skim milk diluters gave spermatozoa revival rates equal to an egg yolk citrate diluter for freezing bull spermatozoa and that spermatozoa frozen in milk diluters maintained a higher motility after thawing than did spermatozoa frozen in the egg yolk citrate diluter.

Blackshaw (1955b) found heated homogenized milk inferior to an egg yolk, phosphate, citrate extender for low temperature preservation of bull spermatozoa. The two diluters contained 1.25 percent arabinose plus 7.5 percent glycerol.

Antibiotics

Antibiotics have been used commercially for some time as a supplement to various diluents for extending bull semen.

Salisbury, Willett and Gonsalus (1939) first suggested that bacteria in semen used for artificial insemination might reduce the fertilizing capacity of the semen.

Several antibiotics have been studied. Almquist et al. (1946) found that 1,000 to 2,000 Oxford units of penicillin had a highly significant ability to retard the decline in motility of stored bovine spermatozoa, while it also significantly reduced glucose utilization by the spermatozoa. Bacterial growth was retarded by levels of penicillin as low as 250 Oxford units, but 500 and 1,000 units had no appreciable effect on fertility of bull semen. This was confirmed by Mixner (1949). Almquist (1948) reported that 1,000 Oxford units of penicillin per milliliter in bull semen diluted with yolk citrate brought about highly significant increases in fertility. This was confirmed by Almquist (1949a).

Erb and Flerchinger (1954) had lower non-return rates with high fertility bulls from the addition of penicillin than without it.

The effect of various concentrations of penicillin was studied by Almquist et al. (1948). They concluded that concentrations in excess of 1,000 Oxford units per milliliter resulted in a significant decrease in viability during long term storage. They confirmed the findings of Almquist et al. (1946) and Branton and Prather (1954) that 500 and 1,000 Oxford units of penicillin decreased spermatozoa glucose utilization, while 1,000 Oxford units of penicillin effectively retarded bacterial growth.

Elliott et al. (1954) reported that 500 Oxford units of penicillin gave a higher percent survival for bull semen frozen in an egg yolk citrate extender than did 1,000 or 2,000 units.

Almquist (1949a) reported a 27 percent increase in fertility when streptomycin was added to the semen of relatively infertile bulls.

Easterbrooks et al. (1950a) reported similar findings with 100 micrograms streptomycin sulphate per milliliter of diluted semen. They found an inverse linear relationship between response to streptomycin sulphate and the initial level of fertility.

An optimum level of dihydrostreptomycin sulphate was found by Easterbrooks et al. (1951) to be between 100 and 900 micrograms per milliliter. They suggested the use of 500 micrograms since this quantity was required to prevent in-vitro growth of vibrio fetus cells. Almquist et al. (1949) reported that concentrations greater than 1,000 micrograms per milliliter increased sperm viability but concentrations below 100 micrograms permitted excellent bacterial growth.

Elliott et al. (1954) in studying bull semen extended with egg yolk citrate and frozen at -79°C, found no significant differences between no streptomycin and the addition of 500, 1,000 or 2,000 micrograms streptomycin per milliliter of extended semen.

Sulfanilamide is another antibacterial agent sometimes added to bull semen. Knodt and Salisbury (1946) found that 0.3 milligrams percent sulfanilamide in a yolk citrate diluent gave a significant improvement in the livability of ejaculated bull spermatozoa over a 20 day storage period and also prevented bacterial growth. They report that glucose and oxygen utilization were depressed and lactic acid accumulation was increased for all levels of sulfanilamide from 50 to 500 milligrams percent.

Salisbury and Knodt (1947) report varied results in a series of three experiments involving the addition of 0.3 milligrams percent sulfanilamide.

The first showed no beneficial effect on fertility, whereas, the second and third resulted in a 4.5 and 6.1 percent increase in fertility. Semen samples from both high and low fertility bulls were influenced similarly. Foote and Salisbury (1948) found penicillin more effective in controlling semen bacteria than sulfanilamide.

A decrease in bull semen fertility has been ascribed by some investigators to the addition of sulfanilamide. Almquist (1949a) found that 0.3 milligrams percent sulfanilamide decreased fertility 2.9 percent. Branton and Prather (1954) found sulfanilamide had a detrimental effect on viability of bull spermatozoa and that it depressed fructose utilization. Dunn et al. (1953) attributed a reduction in viability of spermatozoa ranging from 29 to 43 percent, to the presence of 0.3 milligrams percent sulfanilamide in diluted semen stored at -79°C. They suggested that sulfanilamide should not be included in egg yolk citrate diluents used for freezing.

Almquist et al. (1949) reported that combinations of penicillin and dihydrostreptomycin sulphate ranging from 100 to 1,000 units of each per milliliter of diluted semen did not significantly effect the livability of bull spermatozoa during a 20 day storage period, although bacterial growth was effectively controlled in diluted semen by the use of these two antibiotics.

Almquist (1949a) found that 1,000 units each of penicillin and streptomycin increased the fertility of diluted bull semen 21.3 percent. This was less, however, than the increase from each singly.

Easterbrooks et al. (1951) added streptomycin and penicillin in combinations and reported a trend favoring the addition of streptomycin alone.

Branton and Prather (1954) found that 500 units each of penicillin and streptomycin singly or in combination had beneficial effects on spermatozoa livability. This was corroborated by Erb and Flerchinger (1954).

Almquist (1954), Almquist and Prince (1950), Campbell and Edwards (1955), Easterbrooks et al. (1951) and Erb et al. (1954) have found a higher non-return rate with streptomycin than with a streptomycin, penicillin combination.

Campbell and Edwards (1955) had a lower non-return rate when sulfanilamide and dihydrostreptomycin sulfate were added to a bull semen extender than when these two were in combination with penicillin.

Almquist and Prince (1950) and Erb et al. (1954) found that adding 0.3 milligrams percent sulfanilamide to a penicillin plus streptomycin antibiotic mixture increased fertility of bull semen diluted with egg yolk citrate.

Willett and Ohms (1955) found that the addition of 500 micrograms streptomycin to a yolk citrate extender containing 0.3 milligrams percent sulfanilamide brought about a highly significant increase in non-return rate. This was also superior to a combination of streptomycin, penicillin and sulfanilamide.

Easterbrooks et al. (1951) studying combinations of 0.6 milligrams percent sulfanilamide plus penicillin and streptomycin found no significant difference between any of the combinations.

Myers et al. (1950) studied six levels of aureomycin hydrochloride from 50 to 1,000 micrograms per milliliter of diluted semen and stated that all six caused significant toxic effects on sperm motility.

Stallcup and McCartney (1953) found that 20 milligrams terramycin hydrochloride could be added per 100 milliliters of diluted semen without spermicidal effects and still be bactericidal.

Easterbrooks et al. (1950b) reported that the addition of a calcium chloride complex of streptomycin to a phosphate buffer resulted in an injurious calcium phosphate precipitate.

Sykes and Mixner (1950) studied the toxicity of two salts of penicillin, three salts of streptomycin, two salts of aureomycin and chloromycetin. The base and hydrochloride of both aureomycin and chloromycetin were found to be toxic to spermatozoa. No toxicity was observed with the salts of penicillin or streptomycin.

PROCEDURES

Standard Procedures:

Many of the procedures used in this study are standard for the three experiments and thus will be mentioned only once. These standard procedures are as follows.

Ram semen was collected with an artificial vagina using a ewe held in a chute. The artificial vagina used is shown in Figure 1.

Semen was examined immediately after collection for total volume, undiluted motility, progressive motility and sperm concentration. The microscopic motility and concentration determinations were made with a Bausch and Lomb microscope model number C12611 equipped with a C.S. and E. type 7 stage warmer. (1) The volume of the ejaculate was obtained by pipetting into a 2.5 ml. tube a column of water of the same height as the column of semen in an identical tube. (2) The raw motility was estimated by placing a drop of semen on a slide and examining it under 100X magnification. The sample was rated on a zero to five scale as reported by Herman and Swanson (1941). (3) Progressive motility was estimated by adding sufficient sodium citrate buffer to a small drop of semen such that a total of ten to 20 sperm were visible in the microscope field at 430X magnification. In each field, both the total number of sperm and the number moving in a straight forward manner were counted. Sufficient fields were counted so that in all approximately 100 sperm were counted. From this information, the percent of progressive motility was determined. (4) Sperm concentration was determined with a Neubaur hemocytometer as described by Laing (1955).

The ejaculate was divided into two equal portions immediately after removing a semen sample for examination. One portion was diluted to five times its volume with a whole milk extender, while the second was diluted in a like manner with sodium citrate phosphate extender. These extenders were added at a temperature of 95°C. This was nine degrees higher than recommended by Anderson (1945) but was found necessary to prevent temperature shock. The tubes of diluted semen were placed in water bath at 95°C. The bath was allowed to cool in a refrigerator for three hours to a temperature of five degrees centigrade, after which time the cooled semen was divided into one ml. portions. Each sample was then diluted further with one ml. of an extender containing the particular materials for each treatment. Each one ml. of extender containing the individual treatment materials was added in three portions at ten minute intervals until each tube contained two ml. of extended semen diluted one to ten from the original concentration.

All treatments were allowed to equilibrate for 12 to 15 hours at five degrees centigrade. This is near the optimum period of 14.9 hours for bull semen reported by Cragle and Myers (1955). Each treatment was then split so that duplicate one ml. samples were frozen.

The semen samples to be frozen were placed in a wire rack which was suspended in a Dewar flask containing ethyl alcohol at a temperature of five degrees centigrade. A low temperature thermometer was placed in the alcohol bath. The temperature of the bath was further lowered by adding small pieces of dry ice to the alcohol so that it

dropped 1°C per minute from +5° to -10, then 2° per minute from -10 to -18 and 4° per minute from -18 to -35 and 5° per minute from -35 to -75°C. The temperature drop was recorded each minute on graph paper.

The semen samples were removed immediately, upon reaching -79°C, thawed and examined individually. Each sample was thawed by immersing it in water at 40°C as described by Lovelock (1953c). The temperature was maintained for ten minutes after which duplicate slides were examined for progressive motility and the highest value recorded. A percent survival was calculated for each one ml. sample based on the motility immediately prior to freezing.

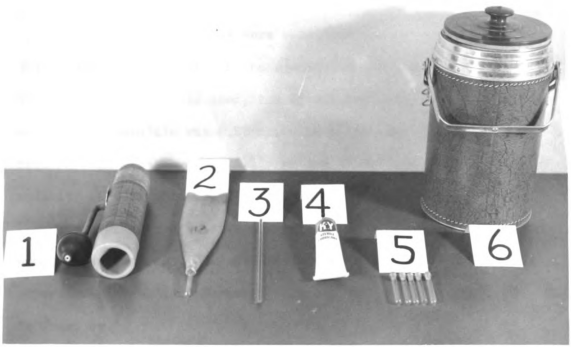

Figure 1.

(1) Artificial Vagina

(2) Artificial vagina inner liner and 2.5 cc collecting tube

(3) Glass stirring rod

(4) Sterile lubricant

(5) 2.5 cc glass tubes used for collecting and freezing semen

(6) One gallon Dewar flask

Materials

Thirty five ejaculates were collected from a Hampshire and a Shropshire ram. The majority of the ejaculates used were from the Hampshire. When the Shropshire was used, the ejaculates were pooled. The average volume per ejaculate was 0.57 ml. with an average concentration of 2.1 billion, an average raw motility rating of 3.8 and an average progressive motility of 69.1 percent.

The average progressive motilities for each of the three experiments were; experiment one, 60.4 percent; experiment two, 66.3 percent; and experiment three, 63.0 percent. The ratings were obtained just prior to freezing.

The milk extender used in these studies was obtained from the laboratory of the Michigan Artificial Breeders Cooperative. The extender was prepared by heating homogenized fresh whole milk to a temperature of 197°F for ten minutes, and storing at five degrees centigrade as described by Thacker and Almquist (1953). Five hundred Oxford units of penicillin and 500 micrograms of streptomycin were added per milliliter of milk.

The yolk phosphate citrate extender contained 50 percent fresh egg yolk and 50 percent phosphate citrate buffer at a P.H. of 6.95. The buffer was prepared by the method of Emmens and Blackshaw (1950) but modified to contain only one phosphate source. The buffer consisted of 42 ml. of 0.1 molar sodium dihydrogen phosphate and 958 ml. of a three percent sodium citrate (dihydrate) solution.

L-Arabinose was used for these trials. Since then, the author has found d-Arabinose to be equally satisfactory. The arabinose to be used in each experiment was first prepared in a ten percent solution to facilitate more accurate measurement of the low level used. In experiment two, a ten percent solution of arabinose in the sodium citrate phosphate buffer was employed for the addition of 1.25 percent arabinose to both the milk and the egg yolk phosphate citrate diluters. Whereas, in experiment three the ten percent arabinose solution was prepared in milk whenever it was to be added to a milk diluter.

The antibiotics used throughout the experiment consisted of the sodium salt of crystalline penicillin "G" and dihydrostreptomycin sulphate.

Design of the experiments

This study was composed of three separate experiments.

Experiment one was designed to determine a level of glycerol to be added to ram semen which would promote the greatest survival after freezing. Split ejaculates containing eight levels of glycerol in milk diluted semen were studied. All groups were handled in the manner described previously. The levels studied were zero, two, four, six, eight, ten, twelve and fifteen percent glycerol. Duplicate one ml. samples were prepared. There were five different freezings so that in all, 80 samples of semen were examined.

Experiments two and three were run concurrently with experiment one before any conclusions were drawn concerning glycerol level. Therefore, 7.5 percent glycerol was used so that the treatments containing arabinose would conform with that of Emmens and Blackshaw (1950).

Experiment two was composed of five different treatment groups as shown in Table 1.

Split ejaculates were employed and duplicate samples for each group were studied after each freezing. In all, four freezing repetitions were performed with a total of 40 semen samples studied.

Experiment three was a continuation of experiment two. The treatments received by groups one, two, three and five were the same for both experiments. However, experiment three differed from experiment two in that the arabinose used in group four of experiment three had been previously diluted with milk instead of the citrate phosphate buffer of experiment two. Four additional treatments were also studied as shown in Table 2. Split ejaculates were used again for this study. Treatment samples were duplicated for each of three separate freezing trials making a total of 54 semen samples studied. All statistical analysis was by conventional method since the data was Orthogonal.

Table 1.

Experiment II Treatments

Group	Semen 10% by vol.	Milk Diluter	Phosphate Citrate Buffer plus Egg Yolk 50%	Phosphate Citrate Buffer plus Egg Yolk 37.5%	1.25% Arabinose	Glycerol 7.5%	Penicillin (500 Oxford units) Streptomycin (500 micrograms)
1	X	X				X	X
2	X		X			X	
3	X		X		X	X	
4	X	X			X	X	X
5	X			X	X	X	

Table 2.

Experiment III Treatments

Group	Semen 10% by vol.	Milk Diluter	Phosphate Citrate Buffer plus Egg Yolk 50%	37.5%	30%	25%	1.25% Arabinose	Glycerol 7.5%	Penicillin (500 Oxford units) Streptomycin (500 micrograms)
1	X	X						X	X
2	X		X					X	
3	X		X				X	X	
4	X	X					X	X	X
5	X			X			X	X	
6	X				X		X	X	
7	X					X	X	X	
8	X		X				X	X	X
9	X					X	X	X	X

RESULTS AND DISCUSSION

Experiment I

Eight levels of glycerol ranging from zero to fifteen percent were studied to determine the influence of glycerol levels on sperm survival and to find a level of glycerol which would permit the greatest sperm survival. The results of this experiment are contained in Table 3 where the individual survival figures as well as average values for each glycerol level are reported.

The average values are also shown in graph form on page 28. The average values plotted appear curvilinear with a constant increase until the six percent level is reached after which the sperm survival declines as glycerol concentration increases. This trend occurred in four of five trials. In the fifth freezing, the highest survival was reached at the eight percent glycerol level.

To determine whether the differences in glycerol level were significant and to decide whether the differences between the six and eight percent levels were great enough to say that the six percent level could be expected to result in higher survival rates, an analysis of variance was used as illustrated in Table 4.

The interaction mean square was used in calculating the "F" values of the main effects since the interaction of glycerol and freezings was found to be significant ($P < .01$). The "F" value $\frac{1676.3}{111.3} = 73.1^{**}$ indicates a significant difference ($P < .01$) between the average values.

Table 3

Percent Sperm Survival after Freezing Ram Semen with Graduated Levels of Glycerol.

Percent Glycerol	Freezings					Average Values
	12/31	1/4	4/14a	4/14b	4/15	
0	0	0	0	0	0	0
	0	0	0	0	0	
2	1.5	6.0	0	12.3	13.3	6.5
	1.5	6.0	0	10.0	15.0	
4	11.5	30.0	14.8	16.9	29.1	19.5
	9.2	30.0	12.5	15.3	25.8	
6	46.1	33.7	37.5	35.9	33.3	36.3
	40.0	35.0	34.3	32.8	35.8	
8	40.7	13.3	30.0	32.3	44.1	32.9
	39.2	13.3	32.3	31.5	52.5	
10	36.9	7.0	27.7	27.7	32.5	25.6
	37.6	9.0	26.1	25.3	26.6	
12	8.4	2.5	19.5	20.3	25.8	15.3
	6.9	2.5	20.3	16.4	30.3	
15	3.8	0	11.5	10.7	17.5	8.5
	1.5	0	10.7	10.7	18.3	

NOTE: The least significant mean difference is 6.83

Figure 2.

The Percent Survival of Ram Spermatozoa Frozen in Graduated Levels of Glycerol.

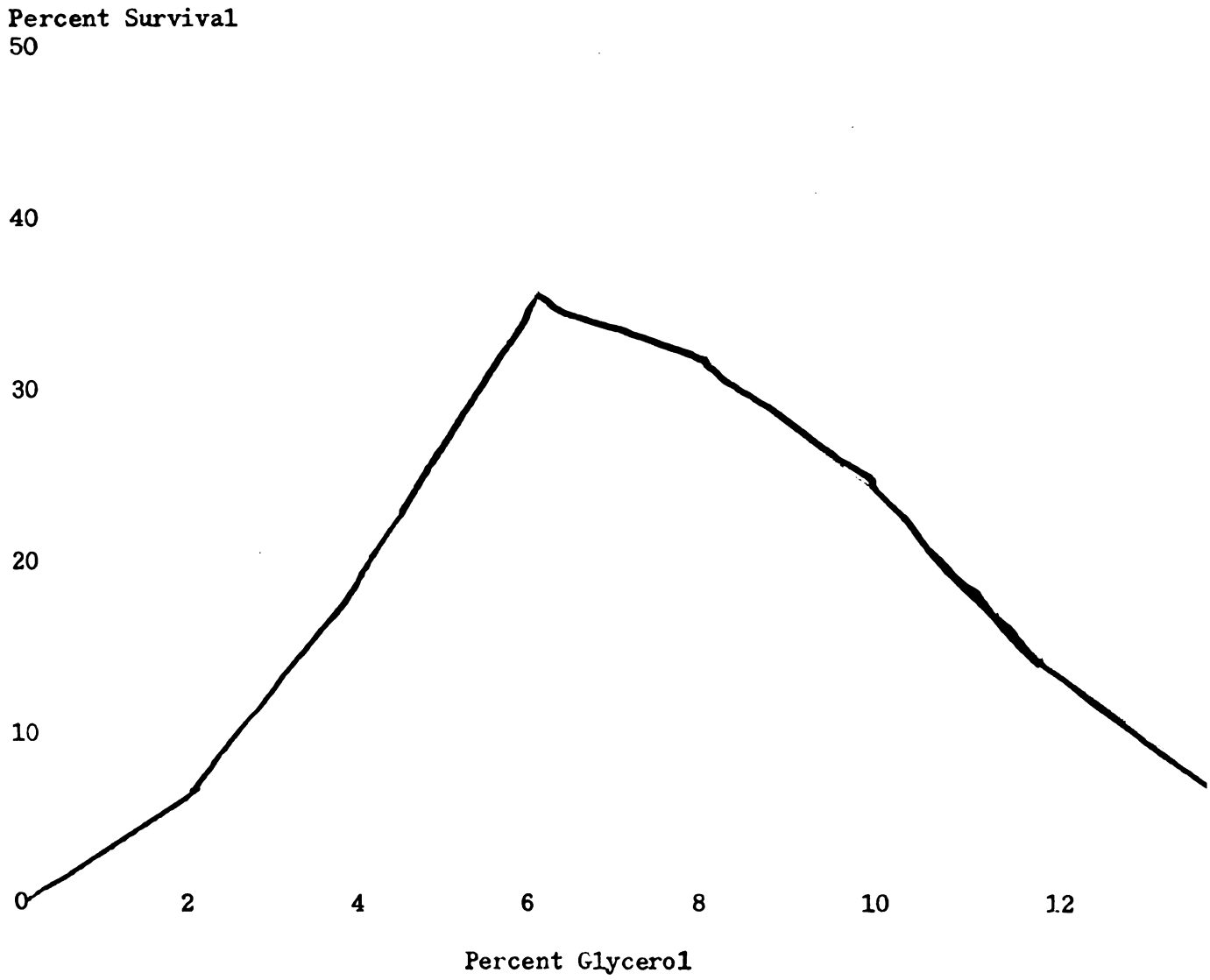

Table 4

Analysis of Variance

Source	Degree of Freedom	Sum of Squares	Mean Squares	F value
Percent Glycerol	7	11,734.1	1676.30	73.1**
Freezings	4	1,433.1	358.30	3.22*
Interaction (G x F)	28	3,117.5	111.30	32.3**
Error	40	137.8	3.45	
Total	79	16,422.5		

Using the "t" test, it was found that the average sperm survival of 36.3 percent for six percent glycerol was not significantly greater than the 32.9 percent survival obtained with eight percent glycerol.

Based on these data, the optimum glycerol level lies between six and eight percent. It is unfortunate that further studies were not made using many levels between the six and eight percent level. However, the high survivals at six and eight percent do suggest that the optimum glycerol level for ram semen may not be greatly different than that reported for bull semen. Mixner and Saroff (1954) found 7.5 percent glycerol gave the highest survival. Miller and vandemark (1954) reported the greatest survival from six percent glycerol. Polge (1953b), Cragle and Myers (1954), Odell and Hurst (1954) and Saroff and Mixner (1955) report optimum glycerol levels between six and eight percent.

The results of this experiment do not appear to be in agreement with Graham and Marion (1953) or Odell and Almquist (1954) who found

* indicates significance at .05 level
** indicates significance at .01 level

ten percent equal or superior to other glycerol levels. Odell and Almquist (1954) observed that the optimum level of glycerol depends on the extender employed and, therefore, only the findings of Mixner and Saroff (1954) and Odell and Almquist (1954) should be compared with the present experiment, since these were the studies using a heated whole milk extender.

Results of Experiment II

This experiment was designed to test milk and egg yolk extenders, the addition of arabinose to these extenders and two levels of egg yolk.

The results are found in Table 5 where average values are shown for each treatment.

An analysis of variance was applied to these data (Table 6) to determine if a significant difference occurred between any of the treatments.

Table 6

Analysis of Variance

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F value
Diluters	4	4,679.98	1,169.99	26.39**
Freezings	3	194.75	64.92	1.46
Interaction (D x F)	12	532.10	44.34	2.88*
Error	20	307.46	15.37	
Total	39	5,714.29		

A significant ($P<.05$) interaction of diluters x freezings was found and was used for computing the "F" values of the main effects instead of the error term. The "F" value $\frac{1169.95}{44.34} = 26.39$ indicates a significant difference ($P<.01$) between the treatments.

A "t" test was used to evaluate the differences between treatments. An average survival rate of 40.33 obtained with heated whole milk containing

Table 5

Percent Sperm Survival After Freezing with Five Different Diluters

Diluter	Freezings 3/26	3/27	3/28	3/29	Average Values
1. 82.5%[a] Milk 7.5% Glycerol 500 Oxford Units Penicillin 500 micrograms Streptomycin	29.2 36.1	39.5 39.5	22.5 24.1	28.4 25.3	30.58
2. 82.5% (50%[b] yolk + 50% Buffer) 7.5% Glycerol	8.4 16.3	7.1 5.6	12.0 11.2	14.1 21.6	14.61
3. 81.25% (50% yolk + 50% Buffer) 7.5% Glycerol 1.25% Arabinose	48.4 47.6	35.0 38.5	33.8 35.3	35.3 31.3	38.15
4. 81.25% Milk 7.5% Glycerol 1.25% Arabinose 500 Oxford Units Penicillin 500 micrograms Streptomycin	41.4 45.3	43.4 45.4	42.3 43.8	28.0 33.3	40.33
5. 81.25% (37.5% yolk + 62.5% Buffer) 7.5% Glycerol 1.25% Arabinose	46.6 45.2	23.5 41.9	41.5 42.3	31.3 31.3	37.98

[a] Percent of final volume which includes 10% semen.
[b] Percent of egg yolk in mixture of egg yolk and buffer only.

NOTE: The least significant mean difference is 5.14

1.25 percent arabinose was significantly greater ($P < .01$) than 30.58 percent survival obtained from heated whole milk alone.

Heated whole milk without arabinose gave an average sperm survival of 30.58 percent which was significantly greater ($P < .01$) than 14.61 percent survival for the egg yolk citrate phosphate extender containing 50 percent egg yolk.

In comparing two different 50 percent egg yolk, phosphate citrate extenders, the addition of 1.25 percent arabinose to one brought about a significant ($P < .01$) increase in sperm survival of 23.4 percentage units. When the milk and 50 percent egg yolk diluters both containing 1.25 percent arabinose were compared, there was no significant difference between the average sperm survival rates of 40.33 percent for the milk and 38.15 for 50 percent egg yolk. When the 38.15 percent survival for the 50 percent egg yolk + 1.25 percent arabinose extender was compared with a 37.98 percent survival from a similar extender containing 37.5 percent egg yolk, no significant difference was found.

Results of Experiment III

Experiment three is a continuation of experiment two. It is concerned with observations on milk and egg yolk phosphate citrate diluters with and without 1.25 percent arabinose. The egg yolk phosphate citrate diluters are of four different levels of egg yolk, some of which contain streptomycin and penicillin. The results of this experiment are contained in Table 8. The average percent survival for each treatment is shown in the last column. An analysis of variance was applied to these data. Table 7 contains this analysis.

Table 7

Analysis of Variance

Source	Degrees of Freedom	Sum of Squares	Mean Squares	F value
Diluters	8	14,323.0	1,790.4	22.9**
Freezings	2	170.6	85.3	1.09
Interaction (D x F)	16	1,249.42	78.09	17.6**
Error	27	119.80	4.44	
Total	53	15,862.82		

The error term of 78.09 was used since a significant ($P < .01$) interaction occurred between diluters and the three separate freezings. The F value $\frac{1790.40}{78.09}$ = 22.9** indicates a significant difference ($P < .01$) between treatments.

Table 8

Percent Sperm Survival After Freezing in Nine Different Diluters

Diluters	Freezings 4/14a/56	4/14b/56	4/15/56	Average Values
1. 82.5%[a] Milk 7.5% Glycerol	30.	32.3	44.1	37.12
500 U.ea. Penicillin & Streptomycin	32.3	31.8	52.5	
2. 82.5% (50%[b] yolk + 50% Buffer) 7.5% Glycerol	11.6	12.5	12.5	12.88
	10.8	18.3	11.6	
3. 81.25% (50% yolk + 50% Buffer) 7.5% Glycerol	43.8	41.5	39.1	41.20
1.25% Arabinose	45.3	39.2	38.3	
4. 81.25% Milk 7.5% Glycerol	68.4	81.5	60.8	67.67
1.25% Arabinose	70.0	75.3	50.0	
500 U.ea. Penicillin & Streptomycin				
5. 81.25% (37.5% yolk + 62.5% Buffer) 7.5% Glycerol	35.9	41.4	41.6	39.90
1.25% Arabinose	35.1	42.1	43.3	
6. 81.25% (30% yolk + 70% Buffer) 7.5% Glycerol	48.4	49.2	44.1	46.2
1.25% Arabinose	47.6	42.1	45.8	
7. 81.25% (25% yolk + 75% Buffer) 7.5% Glycerol	18.5	29.0	8.3	17.82
1.25% Arabinose	14.5	25.8	10.8	
8. 81.25% (50% yolk + 50% Buffer) 7.5% Glycerol	31.5	30.0	29.1	30.43
1.25% Arabinose	33.0	30.7	28.3	
500 U. ea. Penicillin & Streptomycin				
9. 81.25% (25% yolk + 75% Buffer) 7.5% Glycerol	17.4	24.6	15.0	16.98
1.25% Arabinose	13.4	17.4	14.1	
500 U. ea. Penicillin & Streptomycin				

[a] Percent of final volume which includes 10% semen.
[b] Percent of egg yolk in mixture of egg yolk and buffer only.

NOTE: The least significant mean difference is 7.63

In comparing groups one and four, the difference in survival rate of 67.67 percent for milk plus 1.25 percent arabinose as compared with 37.12 for milk alone was significant ($P < .01$). These results were in agreement with experiment two. If one compares the average survival of 67.67 percent from the milk plus arabinose group in experiment three with 40.33 percent survival from the same group in experiment two, the survival in experiment three is 27.34 percentage points greater. Perhaps this difference is the result of using l-arabinose in experiment two, which had been diluted previously with a phosphate citrate buffer instead of the whole milk of experiment three. These data do not agree with the findings of Blackshaw (1955b) who found an egg yolk phosphate citrate extender containing glycerol, arabinose and 25% egg yolk superior to heated homogenized milk.

There was a significant ($P < .01$) difference between the sperm survival rates of groups two and three. The 50 percent egg yolk plus arabinose group had a survival rate of 41.20 percent whereas only 12.88 percent of group two without arabinose survived. These results likewise agree with experiment two. These data agree with the findings of Emmens and Blackshaw (1950), White *et al.* (1954), and Blackshaw (1955a, b) although their extender differed slightly in that it contained 25 percent egg yolk. The magnitude of the difference observed between groups three and two in both experiments is very similar to that reported by Blackshaw (1955a) where a yolk phosphate citrate extender containing 1.25 percent arabinose resulted in a 36.4 percent survival rate as compared with 13 percent for the same diluter without arabinose.

A comparison between the milk extender containing arabinose (group four) and the 30 percent egg yolk phosphate citrate extender containing arabinose (group six) showed a significant difference ($P < .01$) in favor of the milk plus arabinose treatment. The average survival rates were 67.67 percent for the milk diluter and 46.2 for the 30 percent egg yolk diluter.

A significant difference ($P < .01$) occurred between groups one and two where group one contained milk only and had a survival of 31.2 percent. The survival for group two was only 12.88 percent. These results are in agreement with the differences found for these two treatments in experiment two, and substantiate the work of Williams et al. (1954), Almquist (1954), Dreher and Webb (1953) and Flerchinger et al. (1953).

A comparisos between groups one and six shows a significant difference ($P < .05$) between the milk extender without arabinose and a 30 percent egg yolk extender containing arabinose in favor of the extender containing arabinose.

When the egg yolk phosphate citrate extenders containing arabinose and four different levels of egg yolk were compared, no significant differences occurred between the 50, 37.5 or 30 percent levels, however, each of these three diluters brought about sperm survival that was greater than that occurring with 25 percent egg yolk in the same diluter. These differences were all significant ($P < .01$). The low survival from the 25 percent egg yolk treatment is not in agreement with the results of Dunn and Hafs (1953) who found 25 percent egg yolk superior to 27.5 and 50 percent. It is not in agreement with Kinny and Vandemark (1954) nor Cragle et al. (1955) or Saroff and Mixner (1955) who report highly significant increases in sperm survival when 20 percent egg yolk is compared

to 30 percent. Dunn and Hafs (1953) suggested that the relative composition of the two dilution fractions, namely, the egg yolk and glycerol bearing fractions may influence the tolerance of spermatozoa to freezing. Hafs and Elliott (1955) found that this was in fact true for when they included 25 percent egg yolk in both the first and second dilutions sperm survival was significantly greater than if 50 percent egg yolk were added all in one dilution as had been the common practice in preparing a 25 percent final dilution. The egg yolk for the 25 percent dilution in this experiment was added all in one 50 percent fraction.

These data shows a significantly lower sperm survival ($P < .05$) in a 50 percent egg yolk plus arabinose diluter containing streptomycin and penicillin, than in the same diluter without antibiotics. Diluters seven and nine differed from the two diluters just mentioned in that they contained only 25 percent egg yolk. When seven and nine were compared, there was no significant difference in sperm survival even though the average survival value for nine, the diluter containing antibiotics, was lower than that from seven.

The lower sperm survival from an egg yolk citrate phosphate extender containing 500 Oxford units of penicillin and 500 micrograms streptomycin does not agree with the findings of Branton and Prather (1954) or Erb and Flerchinger (1954) who found improved bull sperm livability after adding the same quantities of these two antibiotics. The extenders employed by both differed from that of this experiment since their extender contained no phosphate or arabinose and their results were based on livability of unfrozen semen.

Several authors report improved maintenance of sperm viability with semen stored at 5°C even though different levels of penicillin and streptomycin, singly or in combination, were used with different extenders. These reports were by Almquist et al. (1946), Almquist (1948), Almquist (1949a) and Easterbrooks et al. (1950a).

A few other workers, however, have found a decrease or no effect on sperm livability from the addition of penicillin and/or streptomycin. Erb and Flerchinger (1954) found that the fertility of semen from high fertility bulls was reduced by the addition of penicillin. This semen was not frozen. Elliott et al. (1954) froze bull semen in a diluter very similar to that of this experiment, which contained streptomycin only and at the 500 microgram level which was used for experiment three. They found no significant differences due to the addition of streptomycin. Almquist et al. (1949) found that levels of penicillin and streptomycin ranging from 100 to 1,000 units each had no effect on the livability of bull sperm.

While the results of most workers seem to favor the addition of penicillin and streptomycin to egg yolk citrate diluters, it is evident that some workers have found either no response or even a detrimental effect as was the case in experiment three. Since the same antibiotics were used in all the milk diluters of experiment three with satisfactory results, it would seem likely if the lower survival of group eight is a true difference, that it then might be due to some peculiar effect from the combination of the egg yolk phosphate citrate diluter and either one or both of the antibiotics. The phosphate in the diluter probably cannot

be considered the source of the difference since Easterbrooks et al. (1950b) found that it was the calcium complex of streptomycin and not the dihydrostreptomycin sulphate of the present experiment which had a detrimental effect on sperm livability when combined with phosphates.

SUMMARY AND CONCLUSIONS

Thirty-five ejaculates of ram semen were divided and used in three separate experiments employing split ejaculates to study the effects of the following factors on ram sperm survival after freezing to -79°C: (1) levels of glycerol, (2) egg yolk level, (3) arabinose, (4) penicillin and streptomycin, and (5) comparison of extenders of milk and egg yolk phosphate citrate. The results from these three experiments were analyzed statistically by analysis of variance. From this analysis, the following conclusions are drawn.

The survival of ram spermatozoa after freezing with graduated levels of glycerol from zero to 15 percent is curvilinear with the greatest survival occuring at or between six and eight percent. However, there is no significant difference between the survival at six and eight percent.

The addition of 1.25 percent arabinose improved ram sperm survival in both milk and egg yolk phosphate citrate diluters. These differences were significant in experiment three ($P < .01$) and in experiment two ($P < .05$).

Ram sperm survival is greater when heated whole milk is used as a semen extender than with egg yolk phosphate citrate. With arabinose in both extenders, the differences were significant ($P < .01$) in experiment three but not significant in experiment two. In the absence of arabinose for both extenders, the differences were significant ($P < .01$) in experiment two and three.

Based on these data, there was no significant difference between egg yolk levels of 50, 37.5 and 30 percent. The 25 percent egg yolk

treatment had a significantly ($P<.01$) lower sperm survival than the other egg yolk levels tested, however, no valid conclusions could be drawn since it has been previously shown that adding the egg yolk all in one fraction at the 25 percent level reduces sperm survival.

The addition of 500 Oxford units of penicillin and 500 micrograms of dihydrostreptomycin to a yolk citrate phosphate diluter resulted in a significantly lower sperm survival after freezing ($P<.01$) in experiment three while in experiment two there was no significant difference in sperm survival with or without these two antibiotics in the same diluter. Therefore, experiment two and three are in disagreement. The results of experiment three also disagree with much of the literature reviewed which reported favorable responses from the addition of these two antibiotics. In view of this, no conclusions concerning the addition of penicillin and streptomycin to ram semen were drawn from this study.

The author does not wish to conclude that these conclusions are final. Other diluters which have been used to extend bull sperm need to be studied. Further work is needed on diluters containing the lower levels of egg yolk. Egg yolk levels of 20 and 25 percent which are contained in both dilution fractions need to be studied.

Further research is needed on antibiotic levels and the antibiotics which should be used for both milk and egg yolk citrate diluters for low temperature preservation of ram spermatozoa. Investigation of the sperm survival response occurring from levels of glycerol between six and eight percent is needed. Such investigation should be attempted concerning glycerol level for extenders containing arabinose, various levels of egg yolk and citrate as well as the milk extender.

BIBLIOGRAPHY

Almquist, J. O. 1948. The Effect of Penicillin Upon the Fertility of Semen from Relatively Infertile Bulls. J. Dairy Sci., 31:681.

Almquist, J. O. 1949. A Comparison of Penicillin Streptomycin and Sulfanilamide for Improving the Fertility of Semen from Relatively Infertile Bulls. J. Dairy Sci., 32:722.

Almquist, J. O. 1951. The Fertility of Bovine Semen in Diluters Containing Varying Amounts of Egg Yolk. J. Dairy Sci., 34:763.

Almquist, J. O. 1954. A Comparison of Penicillin, Streptomycin and Sulfanilamide for Improving the Fertility of Semen from Bulls of Low Fertility. J. Dairy Sci., 34:819.

Almquist, J. O., P. J. Glantz and H. E. Shaffer. 1949. The Effect of a Combination of Penicillin and Streptomycin upon the Livability and Bacterial Content of Bovine Semen. J. Dairy Sci., 32:183.

Almquist, J. O., R. J. Flipse and D. L. Thacker. 1954. Diluters for Bovine Semen. IV Fertility of Bovine Spermatozoa in Heated Homogenized Milk and Skimmilk. J. Dairy Sci., 37:1303.

Almquist, J. O. and P. W. Prince. 1950. The effect of a Combination of Penicillin, Streptomycin and Sulfanilamide Upon the Fertility of Bull Semen. J. Dairy Sci., 33:393.

Almquist, J. O., W. T. S. Thorp and C. B. Knodt. 1946. The Effect of Penicillin Upon the Livability, Fertility and Bacterial Content of Bull Semen. J. Animal Sci., 5:400.

Almquist, J. O., W. T. S. Thorp and C. B. Knodt. 1948. The Effect of Penicillin Upon the Livability, Glycolysis and Bacterial Content of Bovine Semen. J. Dairy Sci., 31:11.

Anderson, James. 1945. The Semen of Animals and Its use for Artificial Insemination. Technical Communication, Imperial Bureau of Animal Breeding and Genetics, Edinburgh.

Blackshaw, A. W. 1955a. The Effect of Equilibration and the Addition of Various Sugars on the Revival of Spermatozoa from -79°C. The Aust. Vet. J., 31:124.

Blackshaw, A. W. 1955b. Factors Affecting the Revival of Bull and Ram Spermatozoa after Freezing to -79°C. Aust. Vet. J., 31:238.

Branton, C. and W. B. Prather. 1954. Metabolic Responses of Bovine Spermatozoa to Antibacterial Agents. J. Dairy Sci., 37:228.

Campbell, R. C. and J. Edwards. 1955. The Effect on Conception Rates of Semen Diluents Containing Citrate or Phosphate Buffer With all Combinations of Sulphanilamide, Streptomycin and Penicillin. J. Agr. Sci., 46:44.

Collins, W. J. 1953. Evaporated Milk as a Semen Extender. J. Dairy Sci., 36:578. (Abstract)

Cragle, R. G. and R. M. Myers. 1954. Effects of Levels of Sodium Citrate, Glycerol and Equilibrating Time on Recovery of Bovine Spermatozoa After Storage at -79°C. J. Dairy Sci., 37:652.

Cragle, R. G., R. M. Myers, R. K. Waugh, J. S. Hunter and R. L. Anderson. 1955. The Effects of Various Levels of Sodium Citrate, Glycerol and Equilibration Time on Survival of Bovine Spermatozoa After Storage at -79°C. J. Dairy Sci., 38:508.

Davenport, C. B. 1938. Experimental Morphology: Part I. Effect of Chemical and Physical Agents Upon Protoplasm. MacMillan Co., New York.

Dreher, W. H. and J. H. Webb. 1953. A Comparison of Homogenized Whole Milk and Egg Yolk Phosphate on Diluters for Bull Semen. J. Dairy Sci., 36:673.

Dunn, H. O. and H. D. Hafs. 1953. Extenders and Techniques for Freezing Bovine Spermatozoa. J. Dairy Sci., 36:557.

Dunn, H. O., G. L. Larson, E. L. Willet. 1953. The Effects of Freezing Bovine Spermatozoa in Extenders Containing Antibacterial Agents. J. Dairy Sci., 36:728.

Easterbrooks, H. L., P. Heller, W. N. Plasteridge, E. L. Jungherr and F. J. Elliott. 1950a. Fertility of Diluted Bull Semen Containing 100 Micrograms of Streptomycin per Milliliter. J. Dairy Sci., 33:737.

Easterbrooks, H. L., P. Heller, W. N. Plasteridge and E. L. Jungherr. 1950b. Comparative Fertility of Diluted Bull Semen Treated with Calcium Chloride Complex Streptomycin or Dihydrostreptomycin Sulfate. J. Dairy Sci., 33:851.

Easterbrooks, H. L., P. Heller, W. N. Plasteridge and E. L. Jungherr. 1951. Streptomycin and Streptomycin Plus Penicillin for Increasing the Fertility of Bull Semen. North Am. Vet., 32:394.

Elliott, F. I., E. J. Elliott, Harold D. Hafs and E. O. Conrad. 1954. Current Results with Frozen Semen. Proc. Nat. Assoc. Artif. Brd.

Emmens, C. W. and A. W. Blackshaw. 1950. The Low Temperature Storage of Ram, Bull and Rabbit Spermatozoa. Aust. Vet. J., 26:226.

Erb., R. E. and F. H. Flerchinger. 1954. Influence of Fertility Level and Treatment of Semen on Nonreturn Decline from 29 to 180 Days Following Artificial Service. J. Dairy Sci., 37:938.

Erb., R. E., L. E. Mikata, F. H. Flerchinger and M. H. Ehlers. 1954. Influence of Levels and Combinations of Antibiotics in Bull Semen Diluted with Heated Homogenized Milk on Nonreturn and Nonreturn Decline. J. Animal Sci., 13:1020.

Flerchinger, F. H., R. E. Erb and M. H. Ehlers. 1953. The Use of Heated Homogenized Milk as a Diluter for Bull Semen. J. Dairy Sci., 36:1016.

Foote, R. H. and G. W. Salisbury. 1948. The Effect of Sulfanilamides Upon the Livability of Spermatozoa and Upon the Control of Bacteria in Diluted Bull Semen. J. Dairy Sci., 31:763.

Graham, E. F. and G. B. Marion. 1953. A Technique of Freezing and Factors Affecting the Revival of Bovine Spermatozoa. J. Dairy Sci., 36:597.

Hafs., H. D. and F. I. Elliott. 1955. The Effect of Methods of Adding Egg Yolk and Monosaccharides on the Survival of Frozen Bull Spermatozoa. J. Dairy Sci., 38:811.

Herman, H. A. and E. W. Swanson. 1941. Variation in Bull Semen with Respect to its use in Artificial Insemination. Univ. Mo. Exp. Sta. Bul. 326.

Johnel, F. 1938. viber die Wider slandsfolugkigheit von menschlichen Spermatozoen gigenicher. starker Kalte, Wiederanftrebn der Beweglichkeit voch abkuhlung auf -196°C (Flussigen Stickstaffund -269.5°C etwa 3.7° absolutesi. Nullpunkt entfempt (flussiges Helium) Klin Wschr., 17:1273. (An. Brd. Abstr. 7:72.)

Kinny, W. C. and N. L. Vandemark. 1954. The Effect of Yolk and Citrate Levels and the Stage of Maturity on the Survival of Bull Sperm at Sub-Zero Storage Temperatures. J. Dairy Sci., 37:650.

Knodt, C. B. and G. W. Salisbury. 1946. The Effect of Sulfanilamide Upon the Livability and Metabolism of Bovine Spermatozoa. J. Dairy Sci., 29:285.

Laing, J. A. 1955. Fertility and Infertility in the Domestic Animals. Bailliere, Tindall and Cox W. C. 2 England.

Lovelock, J. B. 1953. Biophysical Aspects of the Freezing of Living Cells in the Preservation of Normal Tissues for Transportation. Cibia Foundation Symposium.

Luyet, B. J. and P. M. Gehenio. 1940. The Mechanism of Drying and Death by Low Temperatures. Biodynamica, 3:33.

Luyet, B. J. and E. L. Hodapp. 1938. Revival of Frog Spermatozoa Vitrified in Liquid Air. Proc. Soc. Exp. Biol., 39:433.

Miller, W. J. and N. L. Vandemark. 1954. The Influence of Glycerol Level Various Temperature Aspects and Certain Other Factors on the Survival of Bull Spermatozoa at Sub Zero Temperatures. J. Dairy Sci., 37:45.

Michajilov, N. N. 1950. Sperm Dilution in the Milk. J. American Med. Assoc., 117:337 (Abstract)

Mixner, J. P. 1949. The Effect of a Combination of Penicillin and Streptomycin in Semen Diluters on the Fertility of Dairy Bulls. J. Animal Sci., 8:642. (Abstract)

Mixner, J. P. and Jack Saroff. 1954. Interference by Glycerol with Differential Staining of Bull Spermatozoa as used with Semen Thawed from the Frozen State. J. Dairy Sci., 37:1094.

Myers, R. M., J. O. Almquist and P. W. Prince. 1950. The Influence of Aureomycin Upon the Livability and Bacterial Content of Bull Semen. J. Dairy Sci., 33:394.

O'Dell, W. T. and L. O. Almquist. 1954. Techniques for Freezing Bull Spermatozoa in Milk Diluter and Preliminary Breeding Results. Proc. Nat. Assoc. Artif. Brd.

O'Dell, Glen D. and Victor Hurst. 1955. The Effect of Glycerol Equilibration Time on the Freezing of Bovine Spermatozoa in Egg Yolk Sodium Citrate and Skimmilk Diluters. Unpublished data presented to the American Dairy Science Association Annual Meeting.

Olds, Durward, Levi Oliver, D. N. Seath and Marshal C. Carpenter. 1951. The Fertility of Bull Semen Extended with Yolk Citrate Containing Varying Proportions of Egg Yolk. J. Dairy Sci., 34:1081.

Parkes, A. S. 1945. Preservation of Human Spermatozoa at Low Temperatures. British Med. J., 2:212.

Perkins, J. R., M. C. Carpenter and D. M. Seath. 1955. A Comparison of the Fertility of Bull Semen Diluted in Egg Yolk Citrate and Homogenized Milk. J. Dairy Sci., 38:155.

Polge, C. 1953. The Storage of Bull Semen at Low Temperatures. Vet. Rec., 65:557.

Polge, C. and J. E. Lovelock. 1952. Preservation of Bull Semen at -79°C. Vet. Rec., 64:396.

Polge, C. and L. E. A. Rowson. 1952. Fertilizing Capacity of Bull Spermatozoa After Freezing at -79°C. Nature, 169:626.

Polge, C., A. W. Smith and A. S. Parkes. 1949. Revival of Spermatozoa After Vitrification and Dehydration at Low Temperatures. Nature, 164:666

Rowson, L. E. A. and C. Polge. 1953. Storage of Bull Semen at -79°C and Fertility Results for Up to 12 months. The Vet. Rec., 65:677.

Salisbury, G. W. and C. B. Knodt. 1947. The Effect of Sulfanilamide in the Diluent Upon Fertility of Bull Semen. J. Dairy Sci., 30:361.

Salisbury, G. W., E. L. Killett and I. C. Gonsalus. 1939. Some Problems in Bull Semen Storage. Proc. Amer. Soc. Animal Prod., 32:210.

Saroff, Jack and J. P. Mixner. 1955. The Relationship of Egg Yolk and Glycerol Content of Diluters and Glycerol Equilibration Time to Survival of Bull Spermatozoa after Low Temperature Freezing. J. Dairy Sci., 38:292.

Smith, A. U. and C. Polge. 1950. Storage of Bull Spermatozoa at Low Temperatures. Vet. Rec., 62:115.

Stallcup, O. T. and N. K. McCartney. 1953. Toxicity to Bull Spermatozoa of Terramycin Hydrochloride and Its Use as an Antibacterial Agent in Diluents. J. Dairy Sci., 36:293.

Swanson, Eric.W. 1949. The Effect of Varying Proportions of Egg Yolk and Sodium Citrate Buffer in Bull Semen Diluters Upon Sperm Motility. J. Dairy Sci., 32:345.

Sykes, James G. and John P. Mixner. 1950. Bull Semen Toxicity of Various Salts, Brands and Lots of Penicillin, Streptomycin, Aureomycin and Chloromycetin. J. Dairy Sci., 33:394.

Thacker, D. L. and J. O. Almquist. 1951. Milk and Milk Products as Diluters for Bovine Semen. J. Animal Sci., 10:1082. (Abstract)

Thacker, D. L. and J. O. Almquist. 1953. Diluters for Bovine Semen I. Fertility and Motility of Bovine Spermatozoa in Boiled Milk. J. Dairy Sci., 36:173.

Williams, James A., Richard W. Green and A. C. Baltzer. 1954. Heated Homogenized Milk vs. Egg Yolk Citrate as a Bull Semen Extender. J. Animal Sci., 13:1035 (Abstract)

Willett, H. L. and J. I. Ohms. 1955. Field Trials with Semen Containing Several Combinations of Antibacterial Agents. J. Dairy Sci., 38:1360.

White, I. G., A. W. Blackshaw and C. W. Emmens. 1954. Metabolic and Motility Studies Relating to the Low Temperature Storage of Ram and Bull Spermatozoa. Aust. Vet. J., 30:85.