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THE SEMEN OF THE BULL WITH
SPECIAL REFERENCE TO ITS STORAGE
AND TRANSPORT FOR ARTIFICIAL
INSEMINATION

Thesis for the Degree of M. S.

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This is to certify that the

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"The Semen of the Bull with Special
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R. H. Nelson

Major professor

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**THE SEMEN OF THE BULL
WITH SPECIAL REFERENCE TO ITS
STORAGE AND TRANSPORT
FOR
ARTIFICIAL INSEMINATION**

By

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1. INTRODUCTION

Although the practical application of artificial insemination is comparatively new, the concept is a very old one. Arabs are reported to have used artificial insemination successfully, in about 1322. An Arab chief is said to have inseminated his prize mare from the semen collected from a stallion belonging to his enemy. Scientific research in artificial insemination of domestic animals was conducted by the Italian physiologist, L. Spallanzani, in 1780 who conducted successful experiments in bitches. Soon after the beginning of the present century, the practical potentialities of artificial insemination began to be recognized. It was Russia who initiated and took up the method of insemination seriously, as a practical means of obtaining improvement in farm animals by using the sperm of valuable sires. The next drive towards artificial insemination was initiated by Danish people who organized first cooperative artificial breeding association in 1937.

The first cooperative artificial breeding in the United States was organized in 1938 by the Extension Service of the New Jersey State College of Agriculture. Since this new era, interest in the artificial insemination of farm animals has increased tremendously, especially in dairy cattle. New and better techniques have been devised for collection of semen, its evaluation, preservation and dilution, storage and transportation, preservation after transportation till used for effective

insemination. Hand in hand with these developments, many facts have been established concerning the biology of sperm cells, the secretions of the glands of the reproductive tract of the male, and of oestrus and its related phenomena in the female. The growing realization of its genetic and economic possibilities is now increasing and its use will be made in various countries as time goes on. Thus the key to success in artificial insemination is the use of superior bulls and a high conception rate. Its main purpose is to promote herd improvement by getting semen from good bulls and enough cows be made available to use it upon. Therefore it is a great task to keep a sufficient quantity and quality of semen in the field.

To accomplish this, correct measures should be adopted in techniques in collection of semen, its evaluation and injection of the semen into the female.

Techniques in collection of semen have improved very greatly during the past few years. The invention of artificial vagina marked a new mile-stone in the practice of artificial insemination; and most of the methods contrived for semen collection have been abandoned in favor of its use. It has greatly contributed to the development of large scale breeding by mechanical means. In this method practically the entire ejaculate is collected and the semen is free from extraneous secretions, thus danger of disease spread is minimized. Besides that the volume of the ejaculate is easily measured and its characteristics easily determined; and the viability of the spermatozoa in semen so obtained is higher than when other methods of collection are used. The

only limitation of this way of making collections is that a few males refuse to serve the vagina. Therefore collection of semen must be engineered with considerable degree of judgment and patience.

Research and experience have revealed a lot of information about insemination. In practice it has been shown that relatively small amounts of viable semen effect better conception than large quantities. In this connection Salisbury continues to extend the frontier where five years ago a dilution rate of one part of semen to 5 parts diluter was thought to be considerable, a rate of 1:100 is now found by Salisbury to give results as satisfactory as lower rates of dilution. The matter of concern is the required number of viable healthy sperm per implantation rather than the amount of semen to use.

The use of egg yolk as a main constituent of diluting medium remains one of the most important advances in sperm storage. Many diluters have been tried and discarded. The most satisfactory diluters to date are yolk-citrate and yolk-phosphate. The fertility of the semen preserved in the phosphate and in the citrate diluent is similar but citrate diluent is recommended for use in the artificial breeding associations as it disperses the substances in the egg yolk and enables the spermatozoa to be seen readily under microscope, which can only be done with difficulty in case of phosphate buffer. Addition of dyes to egg yolk diluter have been found to help in identifying semen of bulls of different breeds. Sufficient neutral red in 5% solution has been used with no adverse effects on motility of spermatozoa. Penicillin, sulphanilamide and streptomycin, to the diluted semen has been found to

control bacterial and fungal growth without harming the sperm. Methylene blue reduction test have been found successful in semen evaluation.

Much progress has been made as to the maintenance of storage temperatures after collection of semen and during shipment. Today as a routine practice the semen is not only shipped long distances but is often used for at least four days and occasionally longer. This change has been brought about by appropriate diluters, proper cooling of semen, and development of satisfactory shipping containers.

Advantages and Limitations

Uses.

Artificial insemination has been used relatively very little in the past and it is only during the last few years that the practice has been expanded to a large extent and its real potentialities for animal improvement have been discussed by many writers. The developments of improved techniques, the percentage of successful inseminations is increasing. The following are the uses in comparison with natural mating.

1. Increasing the Use of Valuable Sires.

It provides means of impregnating large numbers of females with semen from one male whose influence can thus be greatly extended. Full utilization of sires that have been proved can be advanced for transmission of desirable characteristics as butterfat and milk production in case of dairy cows. With the improvement in the technique of preservation of semen and the rapid extension of air transportation,

the stocks of cattle having best genetic make-up may be spread widely throughout various countries at a very small expense compared with the system of conveyance of breeding animals by rail or sea. 1,000 cows can be bred by a single bull during the season.

2. Making Advantageous Use of Young Sires.

The reproductive life of sires in most species of live stock is short. Therefore one of the problems in animal breeding is to prove sires in early life so that information can be used to discard the poor sires and to recognize and use the best sires as soon as possible. In artificial insemination young bulls might be proved at an earlier age than with natural mating due to the progeny tests on a more adequate scale.

3. Extending the period of usefulness of valuable sires.

A male is sometimes unable to mate normally, or may tire quickly, because of age and crippled condition and may serve only a very limited number of females. Thus artificial insemination may serve as an effective means of extending the period of usefulness of valuable sires which would otherwise have to be slaughtered.

4. Overcoming deficiencies due to difference in size.

If natural service is impossible because of marked difference in size or weight, artificial insemination can be used for effecting conception, especially in the breeding of young females to old heavy bulls. Seasonal production can be controlled better by artificial insemination than by the practice of allowing males to run with the herds or flocks and to mate at will.

5. An effective aid in disease control.

The dissemination of certain diseases has been one of the chief handicaps in breeding problems. Artificial insemination properly used, can be instrumental in preventing exposure of sires to genital disease and thus minimizing the spread of the disease. The indiscriminate use of bulls among several herds encourages the spread of genital disease and sterility. In herds of cows there are several important diseases such as contagious abortion, Trichomoniasis, Contagious vaginitis, *Corynebacterium pyogenes*, which can be prevented by artificial insemination.

6. Maintenance of satisfactory fertility.

Artificial insemination can only be expected to give best results when compared to natural breeding, and to achieve this above object certain procedures must be adopted which assist in obtaining and maintaining a satisfactory conception rate. These include: (a) Regular microscopic examination of the semen and use of high quality semen for insemination; (b) Keeping of good records and close attention to breeding performance as it makes more accurate control of dates and prevents the overwork of males; (c) Examination of cows for pregnancy; (d) Early detection of cases of sterility and their treatment.

7. Effecting Conception in females that do not conceive after copulation or that refuse the male.

In cases of temporary disturbances of function in valuable females artificial insemination brings about conception when normal mating proves ineffective. Care should be taken in cases where the

impaired fertility may have an inherent basis. In such cases breeding of these females should be avoided, or their offspring should be used for purposes other than breeding.

8. Benefits to small herds.

The greatest immediate value of artificial insemination lies with its use, particularly in small herds, where a distinct saving can be effected. Thus it makes possible for individual farmers to dispense with their own bulls, and to many dairy farmers it is of great advantage because dairy bulls are frequently very dangerous and always require some barn space and labor. Therefore additional income can be secured by using that barn space for housing productive females.

9. Obtaining species crosses when natural mating is impracticable.

In cross breeding where it is sometimes desirable to use breeds quite dissimilar in size, anatomical structure or psychological characteristics, natural mating has proved unpracticable. In this way it has proved useful as a tool for hybridization experiments.

10. Use in breeding associations and in large herds.

The greatest promise artificial insemination holds for livestock improvement is through the large scale use of valuable sires; and makes possible the development of large families of superior animals within a particular area which attracts the outside buyers due to uniform merits of the animals.

11. General.

It eliminates the need for taking females to the sire and vice-versa, thus matings may be made between animals located at distant

points at less expense or hazard. Participation in a better breeding program and mutual study of problems brings forth the best thought and spirit for advancement of breeding business.

Limitations of artificial insemination.

1. Artificial insemination is harmful if the influence of low quality males is widely disseminated.
2. Problem of disease transmission always remains as a result of careless technique. To be successful the method must be practiced by skilled persons such as veterinarians who have had special, adequate training. A good knowledge of the reproductive organs and of the proper methods of collection and handling of semen are fundamental to the efficient execution of the job.
3. There is likelihood of the injury to the reproductive system of both males and females therefore greatest care should be taken to avoid injury to the genital organs.
4. Instances are said to be existing where inseminators are not properly trained and where instruments are not properly cleaned and are roughly or improperly used. These above instances will probably always tend to limit the full usefulness of artificial insemination.
5. A large financial loss to farmers may occur if artificial insemination organizations are improperly organized or managed. The individual farmer is interested in improving his herd and getting his cows settled and if the herd owner's cows are not settled within the limit of normal expectations, the resulting financial loss may offset the benefits of the farmer. Therefore it is imperative that each step in

artificial insemination program should be done accurately and with the utmost care.

6. Due to carelessness of the inseminator exchange of semen may occur and he may inseminate a cow of one breed with the semen of some other breed or vice-versa.

II. REVIEW OF LITERATURE

A. Properties of Semen

Technical procedures. Changes in technical procedures of artificial insemination have had an important bearing on its developments in the period under review.

More progress has been made in the preparations and use of diluters than in any other aspect of the work. Previous to 1939 the diluents available served only to increase the bulk of ejaculate and storage for periods over twenty-four hours was accomplished by dilution. Phillips as stated by Henderson (17) introduced egg yolk-phosphate medium, which increased both bulk and storage life of sperm samples. The egg yolk-citrate introduced by Salisbury (27) has the important advantage, that samples could be examined microscopically with great ease. An addition of glucose and gelatin has been found to increase sperm survival (19).

Chick embryo diluter by Frank and others described by Anderson (1, p. 72) has significantly increased the survival time of sperm. The rate of dilution has been increasing steadily, and in the field the work of Salisbury and his associates (31) has been outstanding. Citrate buffer excelled the phosphate buffer when used with egg yolk-citrate for the preservation of semen (36). It has been shown that the egg yolk-citrate maintained motility at a higher level for extended periods of

storage than did the egg yolk-phosphate diluent. One serious disadvantage of the egg yolk-phosphate diluent is the presence of fat globules which makes it impossible to see the individual spermatozoa under the microscope. Salisbury (1941) introduced the diluent in which microscopic examination was done satisfactorily, and they were also able to show no significant decrease in fertility from the use of semen diluted up to 1:50 parts of egg yolk-citrate diluter. Salisbury (1946) found no difference in fertility between dilution rates of one part semen and up to 100 parts of egg yolk-citrate diluter. A recent method used by Danes is interesting and is apparently very successful. It consists of placing semen diluted in a gelatinous base in small soluble wax tubes which are slowly cooled, until the gelatin base is firm. In the field the technician cuts off the solid portion of the tube equivalent to 1 cc. of semen and introduces it into the vagina by ejector. This cool gelatin warms up slowly in the uterine tract and thus allows the sperm to return to normal activity rather slowly. It is reported that high conception rate is attained (9).

Addition of various dyes to egg yolk diluter in coloring samples for identification purposes have been reported by Phillips (23). This procedure does not affect the livability and fertility of the sperm.

Survival of sperm on storage is dependent on appropriate storage temperatures and on the avoidance of temperature shock shown by Davis (7).

The storage temperature for bull semen is 5° C. Reduction of temperature to 15° C. can be accomplished rapidly, but further

reduction to storage temperature should cover about one hour. If semen is to be stored at temperatures below 5° C., a slow cooling method should be adopted (17). While Rawson's observations by Henderson (17) observed that using egg yolk-phosphate diluter and storing the semen at 5° C. found little difference between slow and rapid cooling. Slow cooling is an inexpensive precaution and is worth while particularly if storage over three days is contemplated.

In general it can be said that good quality semen if properly cooled and diluted can be preserved up to four days, and gradual warming of semen before use is not thought to be beneficial (17).

Citrate buffer containing sulphanilamide was protected from direct sun light rays and used. An increase in the fertility by the use of sulphanilamide was obtained. These results are said to be due to the beneficial effect of the drug due to metabolic changes rather than due to bacterial control alone (18). Schultz and Davis (1947) showed that six out of twenty-seven samples showed retardation of oxygen consumption by the addition of thyroxine. Salisbury (31) showed that semen handled in the usual way decreases in ability to live under low temperature storage conditions with each increase in dilution rate, this so-called "Dilution effect" has been observed under a variety of handling techniques, and a method of treatment has not yet been devised to prevent it.

Methods of collection of semen have improved very greatly during the past few years. In the early days the practice of withdrawing the semen from the vagina of a mated cow was followed. Only a small portion of the ejaculate could be recovered from the vagina. It was

mixed with vaginal secretions which were injurious to the sperm, if it was to be stored (9).

Mechanical massaging of the ampullae by hand through the rectum has also been practiced. This method is useful for injured or lame sires and is still being practiced.

For collection of semen the artificial vagina has almost supplanted other methods. Great care is taken to insure that the semen is not subjected to a sudden drop in temperature due to contact with a cold collecting tube. A longer type of vagina with the collecting test tube enclosed in the water jacket has been introduced by Salisbury and Willett (37). Walton observed by Henderson (17) has designed a double wall collecting cup for use with vagina. Both models serve the purpose. Sufficient care is taken to protect the test tube and collecting cone.

Dummy cows are said to be satisfactory by some operators; while others find certain bulls unresponsive to them (3). It has been recognized that stages of the process, as time and place of collection and temperature and pressure of artificial vagina should be standardized as much as possible for each individual bull. Variety has been found to be necessary in some cases.

Maintenance of storage temperatures during shipment is accomplished by utilization of ice-filled balloons or thermos flasks well packed in cardboard boxes (35). For actual transport any form of conveyance suitable can be used. Airlines are commonly used for long distances. Russians in 1938 were delivering semen by plane to outlying stations, the packages were dropped without landing. The

packages were dropped from a distance of 300 meters and this did not harm the sperm cells (17).

In Russia and England pigeon carriers have been used for transport of semen for short distances.

In actual insemination, the method made popular in the United States by Larson, whereby the cervix is fixed through the rectal wall, has almost entirely supplanted the use of speculums, but some individual operators do use speculum method. In Russia the speculum method is still being used (17).

The practice is to place the semen through the cervix into the body of the uterus and this can only be accomplished by fixing the cervix either through the rectal wall or directly through the vagina.

In this connection results have been reported from New Zealand (1945) which say over-all conception rate of 24.6 per cent on 4226 inseminations using a speculum, where as in one herd, where the semen was deposited through the cervix, the conception rate was 60 per cent on 141 inseminations. In one English association, two inseminators used different methods, a difference of about 15 per cent in their conception rate was observed in favour of cervix method as compared to the use of speculum (19). Yet the success of the operation depends upon the operator as to how far he is efficient in his technical job.

Various inseminating instruments have been devised but the ordinary glass capillary tube attached to a 2 cc. glass syringe by a rubber connection is still in general use. The diluting semen is drawn only into the inseminating tube and has no contact with the rubber

connection. The syringe being used as a pump and having no contact with the semen, can be used for different bulls without danger of mixing semen, and problem to clean rubber becomes unimportant.

Viability of sperm in female reproductive tract.

The life of the sperm is generally regarded short, inside the vagina, especially in higher animals, about 36 to 48 hours in most cases, while it differs widely in human beings and lower animals. Live sperm have been recovered in a woman 7 days after coition. In bats sperms are capable of fertilizing the egg even after some months. In fowls fertilization is known to occur 32 days after insemination of semen (26). Observations of Brewster, May, and Cole by Anderson (1, p. 83) found that the minimum time required for sperm to reach the upper third of fallopian tube was $5\frac{1}{2}$ hours for mature cows and $4\frac{1}{2}$ hours for heifers. Experiments conducted with dead sperms, indicated that the uterine contractions have no influence on sperm travel in the genital tract. Sergin and co-workers observations by Anderson (1, p. 83) state that the life of sperm depends upon the conditions prevailing in the female genital tract of the cow. The alkaline reaction of the genital tract and high action of vaginal secretions stimulate the activity of the sperm and usually have unfavorable action on the survival of the sperm. Therefore they die off rapidly in vagina and uterus. The cervix is the most favorable place for sperm life due to weakly acid reaction and absence of leucocytes. The sperm can survive for two to three days in cervix and from here constantly pass towards the horns of uterus. Counts of sperms in the cervix after mating showed that the

1

number reached maximum in $2\frac{1}{2}$ to 3 hours and then decreased at 5 hours to about half the number.

Bacterial contamination of semen.

Several investigations have been made into the significance of bacteria contained in bovine semen. Gilman's observations by Anderson (1, p. 66) that twenty-five years ago Gilman isolated a number of organisms including streptococci and *Brucella abortus*, and demonstrated that, in the bulls studied, the lowered fertility was associated with increased number of bacteria in their semen. He also isolated *Pseudomonas pyocyaneus* and unidentified rods and showed that the genital tracts of normal healthy bulls were either free from bacteria or contained very low numbers. Gunsalus, Salisbury, and Willett (16) showed number of organisms except streptococci. Diptheroid organisms were found in large numbers. Presence of heavy number of contaminants did not seem to have any marked adverse effect on semen storage at the temperatures now employed. Anyhow contamination should be kept to a minimum by strict attention in collection, dilution and cleaning equipment. Slight purulent discharge which sometimes occurs after insemination may be due to semen contamination of the inseminating tube from the vulva. It has also been shown that trichomonads can be transmitted by artificial insemination from infected bulls and the importance of using bulls free from contagious abortion has been emphasized, which indicates high percentage of positive blood reaction following insemination to an infected bull (17). A method for preparation of sterile yolk-phosphate diluent have been described (16). Unless rigid

precautions are taken, diluents may be responsible for large number of bacteria to semen samples. During storage bacterial growth is held at minimum by storing at 5° C. or lower.

Sulpha, penicillin, and streptomycin have recently been added to reduce bacterial growth during storage. Sulphanilamide 1:300 of semen and penicillin 1:1200 seems to be effective (15). A lot of work is yet to be done in this connection of using these drugs in preservation of semen against bacteria.

Metabolism.

The most important biological reactions of spermatozoa are glycolysis and respiration. Spermatozoa derive energy through both respiration and glycolysis, but glycolysis is chief source of energy for it. Although motility of bovine spermatozoa is not dependent on a supply of oxygen, there is ample evidence to show that respiration rate is important as a measure of potential fertility (17). High quality semen contains a high percentage of vigorous, active, spermatozoa and strong spermatozoa show a definitely higher glycolytic and respiratory rate than do weak or immotile sperm (15). Glycolysis of spermatozoa is the reduction of glucose, thus changes in the pH of semen have been looked upon as a rough measure of the metabolic activity of the spermatozoa. The increase in the acidity of semen during storage is a good example of the effect of accumulated metabolic production (15).

Effects of temperature on viability of sperm in storage.

Spermatozoa carry certain biological reactions during the time they are in storage. These reactions depend upon the concentration and

activity of the sperm. There are several factors which are responsible for the decrease of sperm motility. Such factors are temperature, vigor of sperm, food supply to sperm (15). Salisbury (31) observed that the usual handling of semen decreased the ability under low temperature storage conditions with each increase in dilution rate called "dilution effect." Dilution rate of 1 part of semen to 8, 12, 16, 24, 50 parts of yolk-citrate diluter were compared following number of spermatozoa were contained in 1 ml. of diluted semen used in each insemination. 150, 104, 80, 54, 26 million.

Willet and Salisbury (36) found the effects of low temperatures from sudden cooling had a harmful effect on the subsequent respiratory activity of the sperm, and lower the temperature to which the semen is cooled, the greater is the harm done, and also it was shown that the sperm suffered if ejaculation was received in a cold receiver even at a temperature of 15° C. Spermatozoa, whether in undiluted semen or in yolk-phosphate diluent, suffer from temperature shock caused by rapid cooling of the semen. Anderson (1) also shows that the sperm cooled suddenly at 5, 10, 15° C. showed further reduction of activity. Sperm which had been subjected to temperature shock perished rapidly on further storage of 38° C. to 39° C. Green and Winters (14) showed that sperm are sensitive to low as well as high temperatures. They are usually more sensitive to high temperatures and are injured at temperatures above body heat. Outside body they live relatively short periods.

Effects of exercise on semen production.

Very little work has been done in this connection. Bartlett and Perry referred to by Anderson (1, p. 8) found that the regular exercise of bulls for one hour at the rate of $2\frac{1}{2}$ miles per hour increased the output 51 per cent. Anderson also gives reference of Kelly (1940) and Hamilton and Symington (1939) who showed a great increase in the quality of semen with exercise. The motility was 5 to 45 per cent before exercise, it was 60 per cent to 100 per cent after exercise. Without exercise motility ceased at 3 to 24 hours. They also showed that daily exercise increased the volume of the ejaculate, sperm concentration and motility.

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B. FACTORS AFFECTING SEMEN PRODUCTION

Hypoplasia or Testicular degeneration.

In testicular hypoplasia normal spermatogenesis does not occur. The epithelial layers of the seminal ducts are less developed with low percentage of fertilization occurring. It has been noted by several workers that it is usually unilateral and of a permanent nature. But when it is of bilateral nature the animal is completely sterile producing no viable gametes while one-sided hypoplasia may have irregular production from very good to sterile. The hypothesis of two recessive genes has been said to be the cause of hypoplasia, which when separate cause single sided hypoplasia and together double hypoplasia.

It is probable that scrotal temperatures and disturbance in the heat regulating function of the scrotum may adversely affect sperm production. It has also been observed that under colder conditions bulls are less keen to serve and produced poorer semen. Certain diseases affect the testicular substance. It has been noted by Anderson (1) that decrease in semen quality in the bull occurs after anaplasmosis and foot and mouth disease; this may be due to increased body temperature which affect spermatogenesis.

Endocrine functions.

The function of the testis is controlled by the reciprocal action of the pituitary and the testis. The internal secretion of testis controls

the development and activity of spermatozoa and a fluid vehicle for their transport. The accessory glands and functions of the ducts is quickly affected if the secretion from the testis fails to occur (1). Spermatozoan motility test is the physiological indicator of the hormone activity. Moore's observations of Anderson (1, p. 24) that when both testes are removed motility is lost in 20 to 23 days in guinea pigs. Thus the hormone of testes is responsible for prolonging the life of spermatozoa within the epididymis. While the degree to which the testes hormone and secretion from epididymis, may be responsible for poor motility of spermatozoa in ejaculates, and perhaps to some extent for variation in different ejaculates is not known as yet.

There is general relationship between the production of germ cells and hormone secretion but in case of some males that mate throughout the year, there is constant rate of gonadotrophic hormone secretion, while in seasonal breeders the rate of secretion of the gonadotropic hormone ceases rapidly after the breeding season and remains low till the next breeding season.

Nutrition.

It is believed that ordinary bull ration fed at slightly more than maintenance level is adequate for the special demands of reproduction, but in order to maintain fertility it is suggested that with increased protein feeding an increase in concentration, volume and sexual interest results, while low protein feed, frequently exerts a depressing influence. It has also been suggested by Branton (5) that dairy bulls should be kept

in vigorous physical condition but not too fat, since high finish is believed to make them sluggish.

An experiment was performed on 19 bulls, 10 Holstein-Friesians and 9 Guernseys. Studies were conducted with regard to semen characteristics, relative fertility, and body weight changes to total digestible nutrients. Concentrate mixtures containing 12, 16 and 20 per cent total protein were fed simultaneously. Under the condition of experiment, 12 per cent protein in the concentrate mixture fed with mixed hay containing approximately 10 per cent of legumes, supplied enough protein for semen production and maintenance of fertility.

Deficiency of vitamin A is said to cause degeneration of the germinal epithelium with consequent depression of fertility. This usually results from poor feeding. Subcutaneous administration of ascorbic acid improves semen quality. Vitamin E is said to be of doubtful importance in reproduction in the bull. An experiment was conducted by Salisbury in which 10 bulls were each fed one ounce daily of wheat germ oil in addition to standard ration for a period of one year. No beneficial effect was found. Pasture has a beneficial effect on fertility, it corrects amino acids and vitamin deficiencies, adds succulence, and provides exercise. Various minerals are important in reproductive requirements.

Temperature and season.

High atmospheric temperature, humidity and quality and quantity of light, has a deleterious effect on the reproductive processes of the male. It has been demonstrated that external heat applied to the

scrotum can affect both spermatogenesis and mature sperm. Studies of the influence of season upon reproduction in dairy cattle made at the Louisiana Experimental Station (12) show that more services per conception were required during the summer months than at any other period of the year. Phillips (23) noted a high abnormal count consisting mainly of tailless and coiled tail spermatozoa during the first few collections after a period of some months of sexual inactivity; after a few services these degenerate spermatozoa were cleared out and normal spermatozoa were ejaculated. It thus seems that heat can induce morphological changes in spermatozoa in the epididymis, as shown by coiling of tails and separation of tails from heads.

Frequency of copulation.

There is much individual variation in the number of times that males can be used without impairing their fertility. It has been shown that when ejaculates were taken from the bull once daily for 57 days, the volume of the semen was well maintained, but sperm concentration varied from 2,179 million sperm per cc. in the fourth week to none at the beginning of the ninth week. The longevity of the sperm increased slightly until the fifth week and then declined rapidly (3). Collections after about 10 days, taking two ejaculations are found to give best results, although some individual bulls may be capable of heavier service. Extremely long periods between services may adversely affect semen quality (17). It has been observed that too many valuable sires have been rendered infertile by over use. They will last longer in a program of moderate service at reasonably regular intervals than under a system

of intensive artificial or natural service for short periods.

Bogart's observations by Anderson (1, p. 137) that, as the interval between the collections increased from one up to 10 days, semen volume, total sperm per ejaculate, percentage of abnormal sperm, and fertility increased, sperm concentration increased for five days, then decreased.

Sexual behaviour.

Among the most perplexing problems which have to be faced in artificial insemination is impotence or inability to serve in the bull. It is therefore necessary to distinguish between inability and unwillingness to serve, or between pathological and psychological causes.

Anderson (1) states that different types and degrees of sexual activity may be on the following lines: (1) Normal service, (2) mounts female, complete erection and intromission, but failure at ejaculation, (3) mounts female but failure of intromission, (4) mounts female, no erection, (5) failure to mount female, (6) no interest other than standing near female, (7) fails to approach female.

Most common condition affecting ability to serve is foot infection. Either injury or infection affecting the hind feet may soon inhibit sexual desire, and, if accompanied by an increase in temperature, may adversely affect spermatogenesis. Contraction of extensor penis muscle can make mating impossible. Trichomonad infection is said to cause slowness in serving.

Anderson (1) has noted that bulls of all grades of fertility exhibit great variation in their ability and desire to serve, and sometimes a completely sterile bull will be much keener and more capable

of service than a highly fertile one. Slowness in service may be due to strange surroundings and to the nature of the control to which the animal is subjected. Over-exertion, or inability to copulate or when the bull mounts the cow feels pain, may cause diminished sexual desire. Acute infection of testes and seminal vesicles may incapacitate the bull to copulate.

C. CLINICAL EXAMINATION OF SEMEN

Great variation exists in the quantity and quality of the semen obtained from different males and also from same males at different times. Therefore, when examining an ejaculate, the typical characteristics of the particular species must be kept in mind. Consequently the semen of any male should be carefully examined before it is used for insemination. No single method is entirely adequate for evaluation of semen, therefore a combination of methods is employed for thorough examination. The following points are considered in examination of semen: (1) appearance, as color and cloudiness, (2) the volume of the semen, (3) concentration or consistency, (4) the motility of the sperm, (5) the number of normal sperm per cubic millimeter of semen, (6) freedom from bacteria, parasites, or cells that would indicate the pathological condition of the genital organs of the male from which the sample is obtained, (7) the respiration rate of the sperm, (8) proportion of abnormal spermatozoa, (9) hydrogen-ion concentration of the semen, (10) resistance of sperm, (11) longevity of sperm. In males intended for extensive use it is desirable that semen examination should be complete, for it has been shown that there is a relationship between certain of the above characteristics and fertility. In large breeding programs sires should be subjected to such examinations prior to and at intervals throughout the breeding season, because without fertile sperm the whole program for better breeding will break down. An examination based on one particular ejaculate refers to that ejaculate

only. It is therefore desirable to base the estimate of fertility on examination of as many ejaculates as possible, in order to obtain the average and range of variations in semen characteristics for the particular male. As considerable time is required for the collection of number of ejaculates, it may be said that a single good specimen would indicate that the male is of good fertility, but a poor ejaculate by no means condemns the male. However, succession of poor ejaculates from the same animal for over a period of time would certainly indicate a low breeding efficiency.

In extreme cases the sperm may be dead or absent in which case the animal would be declared completely sterile. While in other cases it can only be said the animal's fertility is "reduced" or "low." Thus the reproductive capacity of the male may be measured not on a single ejaculate, but on the total ejaculate produced in a given interval of time upon several different occasions.

Appearance and physical properties.

The general appearance of the semen, the color, consistency, opaqueness, and any abnormal qualities should be noted. The color and consistency varies with the concentration of spermatozoa; the thicker the semen, the higher the concentration. Normal semen of most species has the appearance of whole milk and its opacity is due to the dense mass of sperm. Samples of semen of lesser density are characterized by a more watery appearance and in such an ejaculate spermatozoa may be few or absent. A yellowish colour may indicate the presence of pus or urine, which may often be also detected by smell. Pinkish

or redish color indicates an admixture of fresh blood, while a deeper red or brownish coloration probably indicates the presence of degenerating blood and tissues, and greenish coloration may indicate purulent degeneration. Normal semen has little or no smell, but in abnormal condition of the male genital organs it may have a highly offensive smell. Such abnormal coloration of the semen may indicate abnormal conditions in the genital organs of the male, or possibly of the female in case the sample was collected directly from vagina. A sire from which abnormal semen is collected should not be used for insemination or breeding purposes until the cause of the abnormalities has been established and removed. If semen is collected from vagina care should be taken to see that the female is healthy. Because of admixture with vaginal secretions semen so collected will have somewhat different appearance than that collected in other ways. Early investigators have found that the cloudy formation of bull semen, due to masses of sperms, was usually associated with high rate of conception. Experiments conducted (1) showed that out of 206 inseminations, 86 per cent conceptions resulted when marked cloudiness was marked in the semen. When there was little cloudiness, 55 per cent conception occurred in 47 inseminations. When cloudiness was slight, 25 per cent conceptions were obtained in 20 cases; and when there was no cloudiness, one pregnancy in 19 inseminations was obtained, i.e., 5.3 per cent conception.

The volume of the semen.

As the number of inseminations that can be made from any one male is dependent upon the volume of semen produced, it is therefore

important to use males that produce large quantities of semen containing large number of active, viable spermatozoa. Large volume of semen does not necessarily mean a high sperm count, there is a reasonably high correlation for most animals between the total quantity of semen in the ejaculate and the number of spermatozoa contained therein, provided that the ejaculate is complete. In attempting the measurement of the volume of semen production for any given sire, one should not rely on a single ejaculate but upon a total ejaculate produced in a given interval of time upon several different occasions. In some species semen production varies slightly with the seasons. It is usually lowest in July, August, and September for dairy bulls. The amount of semen per ejaculate is roughly proportionate to age up to maturity, but varies markedly between the sires of the same age and size. Frequency of collection influences volume to some extent in individual males, especially those which are regularly used throughout the year. While some sires yield a constant amount under even heavy service. Little difference is found in the volume of first, second, and third ejaculates. Difference in volume is sometimes due to the thrust at the time of collection. It is therefore important that the artificial vagina be at the right temperature and suited to the male handled. Precautions should also be taken about the noises or other interferences when collection is made.

The average volume of the ejaculate in bulls is about 4 ml., and it may range from approximately 0.5 to 12.0 ml. Accessory glands greatly contribute to the volume of semen in (1) efficiency of the

ejaculatory reflex, (2) concentration of spermatozoa, (3) the amount of accessory secretions. Pathological as well as physiological conditions may influence the above three factors which may alter the volume of the ejaculate. The bulk of the semen is formed by the accessory secretions but if these accessory glands are abnormal the volume may be reduced.

There are indications of breed differences, of dairy bulls giving larger ejaculates than beef bulls. (17).

The motility of the sperm.

Microscopical examination of semen for motility is highly important, because without active movement spermatozoa cannot reach the fallopian tube for fertilization of ova. The essential points to be observed in the estimation of motility are (1) examination of semen immediately after collection of semen, (2) maintenance of optimum temperature, and (3) prevention of drying of the semen. If the semen is not examined immediately after collection a fall in temperature may reduce the activity of the sperm. Initial motility is believed to be the best evidence of viability of semen of a fertile male. Determination of motility, number of spermatozoa per cubic millimeter of ejaculate, abnormalities of the sperm, and examination for pathological cells should be conducted immediately after collection of semen. Samples should be taken and care exercised to see that the sample is representative of the whole ejaculate. The examination for motility is best made by means of hanging drop method. This is accomplished by placing a well mixed drop of semen on a cover slip with a pipette and a hollow

ground slide inverted over the cover slip. The slide is then turned over and placed on a microscope stage. If cover slips and hollow glass slides are not available, a drop of semen can be placed on an ordinary glass slide and drop of medicinal paraffin placed on the semen to prevent it drying up. For routine examination hollow-ground slides should be used. To accomplish this, the examination should be made in a warm room or by providing some means for keeping the sample at a constant temperature of 38°C . to 40°C .; such as slide incubator, a heating pad or hot water bottle. But at the same time care should be taken that the temperature should not be high as the sperm is killed at 46°C . A fairly satisfactory examination can be made by placing a drop of semen on a cover slip, and then inverted on the glass slide and cover slip supported by other cover slip to provide space for the semen drop with pressure on the sperm and examined immediately for motility using the low power objective on the microscope. Care should be taken to avoid the use of cold cover slips and slides. While examining the semen under the microscope, observations should be made at the edge of the semen to ascertain an approximation of the percentage of live, active spermatozoa. This should be done before the edges have become dry.

The areas near the center of the drop should be examined to observe the waves and all the movement of the drop. If the motility is poor on the first examination a fresh drop of semen should be examined after insuring that the temperature conditions are optimum.

Different types of motility have been investigated by different investigators. As the progressive motion by which the sperm move in

in a straight line, a rotary motion by which the sperm move in circles with diameters of about their own length, and an oscillatory movement which are convulsive in nature and without progress.

The relative number of sperm showing progressive movement may be expressed, as a percentage of the total number of sperm.

The method recommended by Herman (15) for classifying motility is as follows:

5 = Excellent motility - About 80 per cent or more of the sperm-atozoa exhibit energetic progressive motion. Movement is so vigorous that it is impossible to observe individual spermatozoa in undiluted semen.

4 = Very good motility - About 70 to 80 per cent of the spermatozoa are in vigorous rapid motion.

3 = Good motility - about 50 to 75 per cent of the spermatozoa are in progressive motion.

2 = Fair motility - From 20 to 50 per cent of the sperm are in motion. Movements are vigorous but no waves.

1 = Poor motility - Less than 30 per cent of the sperm are in motion. Motion is mostly weak and less progressive.

0 = No sperm are present.

Some investigators use the plan of estimating the motility by following classification: Very actively motile, actively motile, motile, sluggishly motile, and non-motile, and are given values of 100, 75, 50, 25, 0, respectively. It is very necessary to rate the motility with some degree of accuracy.

Another method of microscopic motility examination is the evaluation of semen by dead-alive staining of sperms. This is based upon the difference between dead cells and live cells in absorbing certain dyes. In this examination the sperm that are dead at the time of making slides will absorb the stain and appear blue, and those that were alive will remain white.

Preparation of stains: Stain A - 2% water soluble eosin in M/8 phosphate buffer. Stain B - 1 part opal blue, 1 part M/8 buffer. Equal parts of above stains to be mixed, heated and filtered. One to two drops of this stain to be placed on clean glass slide and then small amount of mixed semen spread on the slide. Second slide should be dropped over the smear and excess of the stain removed by wiping edges of slides with the cloth. The slides then should be made apart and placed on a hot place at about 150° to 200° F. till it gets dried. Sperm may be counted by oil immersion objective of microscope. At the most 333 sperms in all are generally counted on the slide.

Percentage of live sperm is computed by the following formula:

$$\text{Percentage} = \frac{\text{Number of live sperm counted} \times 3}{10}$$

Suppose 260 live sperms are counted on the slide; percentage of live sperm will be:

$$\frac{260 \times 3}{10} = 78\% \text{ live sperms.}$$

Hydrogen-ion concentration.

The fact that spermatozoa viability is limited to narrow pH range has emphasized the need for the consideration of hydrogen-ion concentration semen studies. However little investigation has been conducted to determine the relationship of pH to other pertinent data on semen characteristics. Webster's observation by Raps and others (24) that they first reported in 1932 that hydrogen-ion concentration of bull semen varied from pH 7.0 to 7.5. Milovanov's early observations quoted by Anderson (1, p. 4) that if the semen of the bull be slightly acid, pH usually would range from 6.5 to 6.8, but in one case it was 5.5. Presence of abnormal amounts of necessary gland secretions may be responsible for an alkaline reaction (pH 7.0 to 7.5). Davis (7) found that collection techniques modified the hydrogen-ion concentration of samples to a marked degree. Rectal massage of the ampullae and the accessory organs yielded semen of pH 7.5 to 8.0, while the use of artificial vagina produced semen whose pH usually was below 7.0. Sergen's observation by Anderson (1, p. 4) reported an average pH of 6.74 when using quino-hydrone electrode for the determination. Anderson (1) from the studies of 11 fertile bulls reported the mean pH of semen to be $6.73 \pm .02$. He further observed that in conditions involving pathological conditions of the genital organs, the reaction was characteristically alkaline. This observation was constant in hypogonadism and epididymitis, where consecutive samples have been studied, he states that, second ejaculate was more acid and was of better quality than the first. Anderson (1) further showed that sperm concentration up to 1.8 billion

sperm per cubic millimeter existed in ejaculates having a pH of 5.8 to 6.0 while samples of pH 7.0 contained an average of .6 billion sperm per cubic millimeter. Relation between long survival time and more acid pH has been described. Willett and Salisbury (36) showed that the effect of initial pH level on spermatozoa motility during storage indicated that the range was not narrow. Equal results were obtained when semen yolk phosphate mixture was adjusted to a pH level of 6.5 to 6.75 or 7.0 before storage; they further showed that the pH of highly buffered semen ranges from 4.0 to 5.0 and 9.5 to 10. Storage for three days increased the buffer capacity of semen in the region of pH 8.0 to 10.0 due to increase in carbonates present. Raps, Greg and Cannon (1947) studied the pH of 371 semen samples from 66 bulls in five studies. Highly significant correlation was found between the pH levels of individual studs. Conditions of the stud exerted marked effect upon the pH level of semen produced. They showed that the semen of individual bulls, irrespective of breeds, tended to maintain constant pH level and definite seasonal fluctuations were recognized during the study.

Concentration and enumeration of spermatozoa.

The concentration of sperm, both among the ejaculates of the same male and from male to male of the same species varies widely. It is very important to know the approximate number of sperm in a cubic centimeter as this helps to gauge its quality and determine the rate for dilution for routine examination. There are several methods of calculating sperm count. One is by means of a Haemocytometer, or cell counting apparatus. For this examination semen is diluted 100 or more times

with 0.9 per cent physiological saline solution by means of a special pipette. After diluting and thorough mixing, a drop of solution is placed on the counting chamber, and spermatozoa are counted for a definite number of squares on the counting chamber. When this number is obtained, the number per cubic millimeter may be calculated.

The apparatus used for this examination must be clean, dry and free from grease, and the semen should be well mixed, before a sample is taken. After the semen and saline solution are drawn up in the pipette, they should be well shaken and first few drops discarded. Procedures in enumeration of spermatozoa: (1) Sample should be stirred well. (2) Take 0.1 cc. of sample in a graduated pipette. (3) Add 9.9 cc. of 0.9 per cent solution of sodium chloride to make dilution of 1 in 100. (4) Again add 9.9 cc. of 0.9 per cent solution of sodium chloride to 0.1 cc. of 1:100 solution to make dilution of 1 in 10,000. (5) This should be mixed well and a drop placed on the slide and wait till the sperm settle. (6) Three such counting slides should be made. (7) In each slide 16 to 64 squares should be counted. The number of squares counted depending upon whether or not the sample is concentrated.

Calculation: $0.2 \text{ mm. (marked on slide)} \times 1/16 \text{ sq. mm.} = 1/80$ cubic mm. Number of sperm per square = a/b where a = number of sperm, and b = number of squares counted. Number of sperm per cubic millimeter = $a/(b \times 1/80) = (a \times 80)/b$. This should be multiplied by 10,000 to get the number of sperm per cubic millimeter, because dilution is 1 in 10,000.

Now to calculate the exact number of sperm per c.mm.:

Slide Number I there are 25 sperm in 16 squares.

Slide Number II there are 28 sperm in 16 squares.

Slide Number III there are $\frac{30}{83}$ sperm in $\frac{16}{48}$ squares.

Therefore, the concentration will be equal to: $(83 \times 80)/48 \times 10,000 = 1,380,000$ sperm per cubic millimeter.

Preparation of slides.

The slide should be well cleaned and sterilized. A small drop of well mixed undiluted semen is placed at one end of the glass slide. The edge of another glass slide is applied to the semen on the first slide which lies on the flat smooth surface. The semen is spread across the edge of second slide, which is held at an angle of 45° , and is pushed along the first slide, drawing the semen after it, in the same manner as the blood smears are made.

Another method consists in placing the drop of semen on the slide and gently dropping another slide on to the semen and then separating the two slides, the slide is then labeled and set aside in a clean, dust-free place to dry, after which it is stained for examination.

When using the two slides for spread of semen care should be taken that the layer of semen is sufficiently left over the slide to protect the sperm from maceration in which case the spermatozoa will be damaged, for the tails break very easily.

Staining methods.

Herman and Swanson (1941) introduced the Rose Bengal method of staining bull semen which is as follows: Methods: (1) 2 or 3 drops of buffer solution to be placed on the clean slide and then 1 drop of mixed semen is added and spread over the slide with second slide. The slide should be dried thoroughly and then stained with Rose Bengal stain and dried for 5-10 minutes, then rinse off excess stain by dipping in distilled water. Slide is dried and examined under microscope. Rose Bengal stain is made up as follows: 3 grams powdered Rose Bengal, 99 cc. distilled water, 1 cc. 40% formalin. (2) Dried semen smears may be stained satisfactorily with 0.5 per cent alcohol solution of gentian violet for three minutes. (3) The slide is air dried, and put in a saturated solution of chlorazene for 5 to 10 minutes, rinsed in distilled water, fixed in 10 per cent formalin for 3 to 5 minutes, stained with Ziehl's carbolfuchsin for two minutes, washed gently in running tap water, dried and examined.

D. TECHNICAL PROCEDURES FOR COLLECTION OF SEMEN

Equipment required.

The equipment and supplies for collection of semen and insemination vary considerably for different species but the procedures to be followed are generally the same.

The following is the complete list of equipment needed for collection of semen, semen examination, and insemination regardless of the methods used:

- (1) Supply of 65 per cent alcohol (not denatured).
- (2) Several rolls of absorbent cotton in sterile packages.
- (3) A good lubricant.
- (4) Rubber gloves.
- (5) A flashlight and heifer size speculum.
- (6) Good quality hand soap.
- (7) Supply of test tubes and hand bottles with rubber stoppers.
- (8) A stand for bottles and test tubes.
- (9) Glass test tube holder.
- (10) Insulated container in which to put the semen after collection.
- (11) A glass (wax) pencil or gummed labels for labeling the tubes.
- (12) An enameled tray and pan for keeping equipment after use.
- (13) Supply of distilled water and sodium chloride tablets for normal saline solution.
- (14) Two graduated beakers or flasks of 100 cc. and 500 cc. for preparing solutions, and several others ungraduated.

- (15) Box of glass slides with cover glasses.
 - (16) One hollow-ground slide for making motility observations.
 - (17) Slide box for storing slides after preparation.
 - (18) Thermometer for measuring the temperature of water for the artificial vagina.
 - (19) About a dozen of medicinal droppers.
 - (20) Two glass funnels, preferably of pyrex.
 - (21) A test tube brush with medium soft bristles.
 - (22) A 2 cc. graduated glass syringe.
 - (23) An 18'' ebonite nozzle equipped for attaching to the syringe.
- A glass tube with 1/8'' inner diameter may be substituted for the nozzle.
- (24) Artificial vagina.
 - (25) It is well to have a surgical and first aid kit for use in case of injury to man or animals.
 - (26) A microscope with magnifications up to 300 to 400 diameter and Hemocytometer, pipette and a supply of stains are needed for clinical examination of the semen.

Control of the bull and cow.

For collection of semen a cow in heat should be made available, preferably an old cow which may have been used several times and stands quietly. A nymphomaniac cow may be used for the purpose. The cow should be tethered to the post with halter or with a rope around the horns. The ground should be level and not slippery. The bull should be handled as for normal mating. He should be led up to the cow with a staff or halter. The safety of the operator should always be assured.

The paddock should be sufficiently large to allow plenty of room for turning and preferably the same yard should be used for all services as a bull soon learns to anticipate service when led into a yard which is used for that purpose.

Preparation of apparatus.

As spermatozoa are easily injured or killed by the toxic agents of many sorts, it is necessary that all the apparatus be free from harmful chemical agents as well as free from harmful bacteria and molds. Therefore, all apparatus must be thoroughly washed and disinfected before and after each use; and exacting sanitary precautions must be taken or artificial insemination may prove more of a liability than an asset to the live stock industry.

Rigid adherence to sanitary practice will insure the best viability of spermatozoa.

All new apparatus should be washed thoroughly with hot water and soap in order to remove all grease and other chemical substances that may be adhering to it. After being washed it should be carefully rinsed in several changes of clean water, preferably distilled water and then allowed to stand for about 10 minutes in 70 per cent ethyl alcohol. It is then kept in a clean, dust-free place, or, if required for immediate use, it may be rinsed in several changes of normal saline solution to remove the alcohol. Dry heat and flaming are excellent means for sterilizing glassware where facilities are available, but precautions are taken against too rapid heating or cooling, which may result in breakage.

The use of soap subsequent to the cleaning of new apparatus is not recommended, for traces of soap are injurious to spermatozoa and rubber apparatus. Rubber material is scrubbed with a paste of sodium bicarbonate and water, rinsed with distilled water, and dried well between periods of use.

All vials and centrifuge tubes should be well-corked.

Artificial vagina technique.

This is the most satisfactory method for collection of semen from the bull. The artificial vagina consists of a rubber cylinder $16\frac{1}{2}$ " long and $2\text{-}3/4$ " in diameter, fitted with an inner rubber lining, the ends of which are turned back over the ends of cylinder and held in place with heavy rubber bands. Near one end of this cylinder is a hole to admit warm water. The proper pressure is obtained by filling the outer jacket about one-half to two-thirds full of water at a temperature of 125° to 160° F. The whole inner lining is evenly and thoroughly smeared with a thin coat of lubricant as vaseline, mineral oil, or gum tragacanth to prevent the semen from contacting the rubber. The temperature is determined by inserting a thermometer in the open end of the artificial vagina. The temperature on the inside of vagina should be 105° to 110° F. when the apparatus is ready for use. If the water is too warm, time should be allowed for cooling; if the water is cold, some of it is emptied out and replaced with hot water. After the proper temperature is attained pressure is adjusted by the quantity of water used or by the application of air pressure in addition to water pressure. Then the bull is led up slowly behind the cow and the collector stands on the right side, holding

the apparatus in the right hand. The bull is allowed to mount the cow, the artificial vagina raised close to the flank of the cow behind the bull's fore leg with the opening directed toward the penis at an angle of about 45° . The penis is directed into the opening of the artificial vagina by applying the left hand to the sheath. When the penis comes into contact with the warm lubricated surface of the artificial vagina, the bull thrusts upward and ejaculates.

Points to be taken into consideration while using artificial vagina.

(1) Care should be taken, not to touch the penis as it may cause the bull to retract the organ and dismount.

(2) Too much lubricant should not be used. It may cause contamination of semen.

(3) Semen should be protected against shock after collection.

(4) After use water should be removed from vagina and the vagina cleaned by a thorough scrubbing with a paste made of sodium bicarbonate and water. It is then rinsed well, disinfected and allowed to dry.

(5) Care should be used to collect semen aseptically.

(6) Semen is labeled as soon as collected with regard to name and number of the bull, breed, date, and volume of semen.

Rectal massage of ampullae.

A trained operator can readily collect semen from bulls by rectal massage of ampullae of the sperm duct. In this method knowledge of the bull's anatomy, or instruction in the technique, is necessary. This method has the advantage of being rapid and requires little special equipment, and is practiced when a male cannot use the artificial vagina

due to lameness or some other condition. If proper care is taken a bull can be used by this method regularly over a long period of time without injury to the bull or his reproductive tract. The sire used for this purpose should be trained, as some difficulty may be encountered in securing samples from some untrained animals. Details of the methods are as follows:

The bull is tied in a place in a stall in such a manner that he cannot shift from side to side, and the sheath is carefully clipped and stroked two or three times towards the opening to "strip" out any residual urine to prevent urine contamination of semen. Then the sheath is washed with soft brush and warm water. After finishing this cleaning procedure, the assistant should hold a funnel, leading to the test tube at the end of sheath of penis. Several test tubes should be made available during the collection of the sample so that tubes may be substituted if one tube becomes dirty. The finger nails of the operator should be clipped very short. A rubber glove is placed on the hand and the arm, and anus of the bull lubricated with vaseline, mineral oil or mild soap. The hand is then inserted in the bull's rectum and all feces removed and the hand inserted enough forward into the rectum so that the fingers drop out the anterior border of the pelvic bone; then the hand is pulled backward with a gentle downward pressure by the hand, the fingers will come into a triangular space formed by the two ampullae. At the apex of this triangle the two seminal vesicles join the urethra. The ampullae are massaged with "stripping" action and backward strokes and the second finger of the hand is run between the ampullae and the

index and third fingers are placed on the outer side of the respective ampullae. A slow, rhythmic motion is used while massaging, and, if done correctly a sperm sample free from accessory secretions may be secured. Sometimes the ejaculate is retained in the sigmoid flexure of the penis. To avoid this, the operator should straighten this flexure with his other hand after massaging the ampullae. The semen is collected from the end of the penis or sheath by means of a funnel and test tube held by an assistant and after collection it is handled in the same manner as in other methods.

Collection of semen from vagina.

This is the oldest and simplest method for collection of semen from the vagina of a cow that has been served by the bull. The vagina is washed free from mucous by repeated flushings. The semen is collected from the floor of vagina, following copulation by means of a pipette, syringe, or special vaginal spoon. The use of speculum facilitates more complete collection of the ejaculate and there is less chance of injury to the cow from insertion of the nozzle, and the chances of the cows breaking the tube by sudden movement are minimized. In case of pipette coating of lubricant should be applied prior to use to prevent irritation of the lining of the vagina.

It has to be ascertained that the male and female used in this method are free from disease. It is necessary to wash the vagina thoroughly between services of different bulls, if the pedigree of the offspring are desired.

This above method is not recommended by several authors because the semen becomes contaminated with mucous and urine and there is an increased danger of spreading genital diseases.

E. STORAGE AND TRANSPORT OF SEMEN

Care and storage of semen.

It is absolutely necessary that the temperature of semen be carefully regulated. Sperm are injured at temperatures above body heat and during the time they are in storage, carry on certain biological reactions. The rates of these reactions are largely dependent upon the concentration and the activity of the sperm. Therefore it is of utmost importance to estimate the length of time the sperm will remain viable under proper storage conditions.

Two essential conditions for the successful storage of bull semen are:

- (1) Gradual cooling.**
- (2) The additions of proper diluents as egg yolk-phosphate or egg yolk-citrate buffer.**

After collection of semen its temperature should be carefully checked. The semen bottle and all glassware used for handling the semen, as well as liquid paraffin, should be warmed to a temperature of 30° C. Semen remains in the bottle at room temperature for few minutes during the estimation of motility. All the glassware that holds the semen must be at the same temperature as the semen; and when mixed with diluters, the two fluids must be at the same temperature. Then the semen is diluted with the preservative and mixed by gently inverting or shaking the container. A wide range of dilution rates have

been used. Dilutions of one part of semen to 100 parts of diluter have been used with satisfactory results, but as a general rule such large volumes of diluted semen are not necessary and dilutions of 1:10 to 1:50 are most commonly practiced. The diluted semen is placed in test tubes which are wrapped in cotton wool. Liquid paraffin is added to the tubes which are corked and sealed with candle wax.

The temperature of 40° F. is considered ideal for storage of semen. This is not cold enough to freeze the semen but it is sufficiently cool to keep the cells in a dormant state, thus preventing the exhaustion of their energy. The gradual cooling to this temperature may be accomplished by immersing the tube of semen in a body of water of 75° F. to 80° F. After this the semen should be so handled that the gradual cooling should take place at the approximate rate of 1° F. per minute up to 40° F. If the temperature is to be reduced to 33 to 35° F. great care is to be taken for cold shock to the spermatozoa. The cooling should be so gradual that it should take two additional hours to get the above temperatures. The sperm will die if they are subjected to temperatures below 32° F.

Preparation of diluting fluids.

The main object of diluting fluids for semen is to increase the volume of the ejaculate so that many cows can be inseminated from a single collection from a healthy and valuable bull than would be possible in natural mating.

Diluents, are used not only for dilution but also for providing favorable media which should aid in the preservation and longevity of

the spermatozoa. Diluters should improve the media surrounding the sperm by supplying nutritive requirements and undergo as little change as possible during storage and protect spermatozoa against adverse conditions and changes. Such chemical and physical characteristics as

(1) Buffering capacity: to protect against marked changes in the hydrogen-ion concentration.

(2) Osmotic pressure: The diluting fluid must be isotonic with the sperm of given species.

(3) The diluter must not be toxic to spermatozoa.

Pure salts have an injurious effect on vitality of sperm, for in such solutions sperm move very actively, but rapidly lose motility and die. Physiological saline solution is said to have destructive action on the capsule.

(4) It should be inexpensive and easy to prepare. Economy must be considered in formulating a diluter. It should be readily available.

(5) It should contain sufficient food nutrients and resistant factors for sperm viability. Diluters are being improved as more information in that connection becomes available.

The most widely used diluters for bull semen to date are yolk-citrate, yolk-phosphate and chick embryo; and investigators do not agree as to which of these is the most desirable for storage and breeding (15). Many other diluters are being tried and discarded, many other diluters are not satisfactory but are still being tried with various modifications.

Egg-yolk-citrate diluter

Salisbury Method as prepared at the Michigan Artificial
Breeding Co-operative at East Lansing.

Equipment Required:

A. (1) Sodium citrate - $\text{Na}_2\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$. Chemically pure reagent must be used. Other grades as technical or commercial are not satisfactory.

(2) Double distilled water should be used. It must be free from traces of acids or mineral salts.

(3) Balance and weights.

(4) Clean weighing pans.

(5) Volumetric flasks (such as 1 or ml or 1000 ml).

(6) Funnels.

(7) Clean graduated bottles with screw tops to store diluter.

(8) Auto-clave or pressure cooker.

(9) Refrigerator

B. Procedure

(1) Exact amount of citrate needed should be weighed to prepare 3.6% solution of $\text{Na}_2\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ or 36 grams in 1000 ml of redistilled water.

(2) Transfer weighed citrate to volumetric flask and fill to mark with redistilled water. Mix till all traces of sodium citrate have disappeared. Shake for 5 minutes more to insure proper dispersion of citrate molecule.

(3) Pour citrate solution in suitable bottle. It should not be filled too full, about $4/5$ from top for maximum.

(4) Heat solution in bottle under 15 pounds pressure for 20 minutes in auto-clave or pressure cooker. Use precautions to prevent loss of liquid or breaking of bottles.

(5) Remove from sterilizer. Close bottle securely. Discard any bottle if too much liquid has been lost. Store in refrigerator at 40° F. The pH of the buffer solution should be 6.7 to 6.8.

C. Precautions

(1) Prepare sodium citrate solution often enough so that it is not stored for more than three months when autoclaved.

(2) No attempt should be made to use un-autoclaved solution for more than two weeks. Solution must be kept refrigerated at 40° F. Even under low temperatures some mold may grow in citrate solution which will produce toxins that are harmful to sperm. More molds will grow at higher temperature and there would be more chance of toxin to be present.

D. Preparation of dilution as used for diluting semen.

Taking all aseptic precautions, equal volumes of fresh egg yolk and prepared sodium citrate solution should be mixed at the same temperature. Desired volume of citrate solution is poured into graduated cylinder and warmed at room temperature when preparing egg yolk.

(2) Fresh sterile eggs should be cleaned in water and alcohol and allowed to dry. White should be separated from the yolk by means of clean half shell and yolk held just over top of second graduated

cylinder, then puncture egg yolk membrane with sterile glass rod. All yolk contents should be allowed to flow into graduated cylinder and be kept absolutely free from white of egg or yolk membrane. Egg yolk should be poured into citrate solution to equalize the volumes. Clean cylinder or test tubes should be used for pouring the contents from one container to another until thoroughly mixed. The temperature of this diluent is adjusted to the temperature of semen and mixed and kept in the refrigerator at 40° F. Great care should be taken to avoid temperature shock. Sample semen may be diluted with saline on a microscope slide in order to check motility.

Fertilizing capacity of stored semen is estimated to be four days. The main advantages of the citrate buffer are; first, that it is easily prepared as it requires the weighing of only one chemical, secondly it disperses the globules in the egg yolk and improves microscopic examination, and the sperm tends to live longer than in other diluents. Dilution should be made as soon as possible after the semen sample is secured.

Egg-yolk phosphate diluter.

This diluter was first developed by Phillips in 1939 and at present is known as Phillips' Buffer or Wisconsin buffer.

Formulae for the solution of phosphate buffer. All the equipment used for citrate buffer is required.

0.2 gram KH_2PO_4 (Pot: Dihydrogen phosphate)
 2.0 @ $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (Sodium phosphate)
 100 cc. of H_2O double distilled.

The pH of the solution should be 6.7 to 6.8. All precautions should be taken as in preparation of citrate buffer. If the pH is lower than 6.75, more of the buffer solution should be added. Crystalline material should be dissolved and mixed in boiling water and then kept in cool place at 15° C. Fresh egg yolk should be prepared by taking all aseptic precautions. All white from the egg is removed and the yolk poured into a sterile container. Equal volumes of fresh egg yolk and phosphate buffer are mixed thoroughly. It should be diluted with semen when both are at the same temperature and then kept in refrigerator and cooled at 40° F. till used.

This diluter is also used at present and has proved to give a good conception rate. It aids in protecting the sperm against adverse conditions. Its high viscosity, dark color and presence of fat globules from the egg yolk, oppose convenient microscopical examination until and unless Na_2SO_4 , S. G. C.-2 or glucose solution is added to it.

Chick-Embryo diluter

10 to 12 days incubated egg should be used. Contents mixed in waring blender for 15 minutes and centrifuged at 2750 revolutions per minute for $1\frac{1}{2}$ to 2 hours. Then supernatant fluid removed with pipette and used for diluter.

Many investigators vary as to its better usefulness than citrate and phosphate buffer. Most trials have shown that the chick embryo is very unsatisfactory and far inferior to both the phosphate and citrate diluters.

Glucose diluter.

This diluter was introduced by Swanson and Herman in 1941 in which 3% and 5% glucose solutions were first developed to provide a diluent which would supply the spermatozoa with food nutrients, but the later investigations have shown that there is no beneficial factor in this diluter. Hence it is not widely used at present time because of its inferiority to egg yolk buffer diluters. It is prepared by mixing 3 grams of glucose in 100 cc. of glass-distilled water or 5 grams glucose in 100 cc. of glass-distilled water. It has been shown that dilutions of 1:3 were definitely harmful to sperm as measured by their effect upon motility and maintenance.

Temperature control, packing and transport.

When the semen of the bull or any other animal is to be shipped a long distance more elaborate precautions are taken. Cooling takes place promptly after examination and then ultimately cooled to 40° F. Cooling should be gradual prior to packing by changing the semen from one container to another. To maintain proper temperature while in transit, generally, each shipment is accompanied by a balloon or tin can of ice as a refrigerant. The vial containing the semen is wrapped in a layer of cotton and placed along a small can filled with solidly frozen ice or the package should be placed in quart vacuum bottle and tightly packed with chipped ice without salt. To avoid danger of breakage, it is best to place a layer of cotton in the bottom of the vacuum bottle before packing with ice and to add layer of cotton before stoppering. The can or the vacuum bottle containing the vial and the ice are wrapped

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tightly in a heavy insulating paper commonly referred to as "Jiffy paper." The wrapped package is then inserted in a shipping container and sufficient paper is used in packing to hold the whole container firmly. Under these above conditions semen may be kept at the required temperature of 40° F. for about 30 hours. For long shipments for 3 or 4 days, it is very important that adequate refrigeration of package should be maintained. The semen should be protected from intense cold of the refrigerant and insulation of the refrigerant against the external temperature.

Transport

In about the last seven or eight years considerable interest has been developed in the transportation of semen to distant herds. It is therefore very necessary to have careful co-ordination between the persons shipping the semen and those doing the insemination. It is necessary to plan the shipments so that a minimum of time will elapse between collection and insemination; it is also necessary that the females to be inseminated be in heat; and at the proper stage of heat, if large percentage of conception is to be obtained. The shipment may be mailed daily by special delivery to assure arrival in the field the morning before collection, at the latest. In case of short distances, semen may be transported by car, bus, or rail. Before shipment it should be placed in the insulated vials in a thermos bottle filled with cracked ice, to maintain the constant temperature, package should be labeled "Glass - handle with care."

The 40° F. temperature should be maintained until the semen is used. Immediately upon its arrival the temperature should be checked. If it is found to be 40° F., it should be immediately transferred to a refrigerator or vacuum bottle of the same temperature. If the temperature is higher, it should be immersed in a body of water of equal temperature and exposed to 40° F. allowing it to cool gradually to this degree.

Long distance shipment of semen seems practical, and several foreign governments as well as individual breeders are attempting to secure the inheritance of valuable sires by this practice. Thus the wide spread development of air services for transport of semen would be more effective at large experimental stations, on ranches, or in organized groups to spread the services of a sire over a large number of females.

F. FACTORS AFFECTING STORAGE OF SEMEN

Interaction of storage temperatures:

Sperm are sensitive to both high and low temperatures. The fall of motility is associated with a fall of temperature and it may be reversible, partially reversible or irreversible in accordance with the rate of cooling. Rapid cooling causes more or less irreversible changes which might lead to death of sperm. This harmful effect of sudden cooling, called "Temperature Shock," is of great practical importance and necessitates careful regulation of temperature of artificial insemination laboratories. It has been shown that a fall in temperature from 40° C. to 0° C. within 30 to 60 minutes caused rapid decrease of resistance and the loss of ability to be reactivated on being reheated to body temperature (36). If cooling was continued over two hours, the temperature shock was less severe and all samples reactivated on reheating; thus the susceptibility of bull sperm to temperature shock depends on the degree and rate of cooling. It was also noted by Anderson (1) that on rapid cooling from body temperature to 5° to 10° C., activity decreased and was not restored on re-heating.

Temperature shock is more severe at low temperatures than at higher ones, and while sudden cooling is harmful at all temperatures and the effect is more marked at the lower ones. Rapid warming has no harmful effect upon the subsequent respiratory activity of the sperm.

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An experiment at Cornell University, in connection with temperature shock from too rapid cooling was conducted by Salisbury and others (36) to check the findings.

After collection semen was transported to laboratory in water at 37° C. Each sample consisted of 0.5 cc. of undiluted semen. One-half of the samples were cooled from room temperature in un-insulated test tubes by placing them directly in the water bath at 5° C.; this treatment cooled the semen from the room temperature to the storage temperature of 5° C. in less than 1 minute.

The other half of the samples, in large insulated test tubes were cooled from room temperature at the rate of 10 degrees per 10 minutes. The semen was covered with mineral oil.

The results are given as below in table Nos. 1 and 2.

Table No. 1 - Comparison of motility of spermatozoa cooled slowly before storage at 5° C. under mineral oil, and those cooled rapidly.

Number days Stored	Number of Sample Pairs	Motility - mean percentage			Probabili- ties for Differences between means
		Before Storage	After storage		
			Rate 10 deg. /10 min.	Rate 30 deg. /1 min.	
2	9	79	29	14	< 0.01
4	8	79	24	12	< 0.01
6	8	79	24	7	< 0.01
8	6	78	17	8	> 0.05

It is seen that the rapid cooling was detrimental for the spermatozoa maintained greater motility after being cooled at the slower rate. In addition there was highly significant difference in pH values after storage with the two rates of cooling as is shown in table No. 2.

Table No. 2. Comparison of pH of Semen Cooled Slowly Before Storage at 5° C. under Mineral oil, and of that Cooled Rapidly.

Number days Stored	Number of Sample Pairs	pH mean			Probabilities for Differences between means
		Before Storage	After storage		
			Rate 10 deg. /10 min.	Rate 30 deg. /1 min.	
2	9	6.27	6.10	6.33	< 0.01
4	8	6.27	6.07	6.32	< 0.01
6	8	6.28	6.01	6.29	< 0.01
8	6	6.26	6.00	6.33	< 0.01

Apparently the spermatozoa in the semen that were cooled more rapidly were rather inactive during storage, as indicated by the constant pH level. From the above figures it is seen that the injury to the spermatozoa due to rapid cooling was severe and retarded metabolic activity as concluded from the pH values.

Effects of diluting fluids on storage and fertility of semen.

Several studies have been made by various investigators in which the semen have been diluted with yolk phosphate and yolk citrate diluters composed of 1 part fresh egg yolk and 1 part of a buffer solution. .2 grams of KH_2PO_4 and 2 grams of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$; and 3.6 grams $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ per 100 ml of distilled water. It was suggested by

Phillips and Lardy (23) that the rate of cooling had no influence on the fertility of bull semen when the semen is stored in a nutrient buffer solution such as the yolk-phosphate or yolk-citrate diluent.

Studies at the Cornell University were made to test the effect of the rate of cooling on the survival of spermatozoa diluted with the yolk-phosphate and yolk-citrate diluents.

In the experiment with yolk-phosphate, semen was diluted in the ratio of 1:4. One-third of the samples were cooled directly from the incubator into the 5° C. water bath without using the insulated tubes while other two samples were cooled at the rate of 5° C. per 5 minutes and 5° C. per 20 minutes respectively and taking about one hour to cool from room temperature to 5° C. Mineral oil was not used. The results are given on the next page in (Table No. 3).

Table 3. Comparison of Motility of Spermatozoa in the Yolk Phosphate Diluent Cooled at Three Different Rates Before Storage at 5° C. without mineral oil.

Number of days Stored	Number of Sample Pairs	Motility percentage				Probabilities of differences between means		
		Before Storage	After storage			Rate 30 deg. / 1 min. and 5 deg. / 5 min.	Rate 30 deg. / 1 min. and 5 deg. / 5 min.	Rate 5 deg. / 5 min. and 5 deg. / 5 min.
			Rate 30 deg. / 1 min.	Rate 5 deg. / 5 min.	Rate 5 deg. / 30 min.			
6	12	74	13	22	27	0.01	< 0.01	> 0.05
8	10	76	6	19	30	< 0.01	< 0.01	< 0.01
10	9	77	2	13	27	< 0.01	< 0.01	< 0.01
12	5	78	2	16	16	< 0.01	< 0.01	> 0.05
14	5	80	2	14	18	< 0.01	0.01	> 0.05

From the figure on the preceding page it will be seen that the significant differences are found in the motility percentage in all the three samples treated. This indicates that even in nutrient medium the spermatozoa suffered from temperature shock and that the slowest rate of cooling, 5 degrees per 20 minutes, was the most satisfactory. Similar experiment was conducted for pH. It was shown that there was no significant difference between the average of pH values for the samples cooled.

Therefore, it can be concluded that spermatozoa, whether in undiluted semen or in yolk phosphate diluent, suffer from temperature shock caused by rapid cooling of semen. The rate of chemical reaction doubles with increase of 10° C. Salisbury (Personal Discussions) recommends that the semen may be stored at 0° C. to 1° C. but greater care is to be taken with rate of cooling between 5° C. to 0° or 1° C. At least 2 hours time should elapse to keep the temperature from 5° C. to 0° or 1° C. Such studies were carried out at Cornell University with egg yolk citrate diluent by keeping the samples at various temperatures at the dilution rate of 1:4. Samples consisting of 0.5 cc. of the mixture were placed in small vials and kept in large insulated tubes. One-fourth of these samples were stored at 5° C. and were cooled at the rate of 5° C. per 10 minutes. The remaining three-fourth of samples were stored at 1° C. but were cooled at different rates: 5° C. per 10 minutes; 5° C. per 20 minutes; 5° C. per 30 minutes. Mineral oil was not used. The results of motility are shown in (Table No. 4).

Table No. 4. Comparison of Motility of Spermatozoa in the yolk citrate diluent in stored supply. Mineral oil was not used.

Number of days Stored	Number of Ejaculates	Motility Means (per cent)					Probabilities for Differences between means A and B
		Before Storage	After storage				
			Treatment				
			A	B	C	D	
2	6	77	40	24	33	28	$\angle 0.01$
4	6	77	30	18	27	26	$\angle 0.01$
6	6	77	19	15	23	22	$\gt 0.05$
8	6	77	13	10	18	16	$\gt 0.05$
10	6	77	11	9	10	15	$\gt 0.05$

Note:

A = Stored at 5° C. and cooled at the rate of 5 degrees per 10 minutes.

B = Stored at 1° C. and cooled at the rate of 5 degrees per 10 minutes.

C = 5 degrees per 20 minutes.

D = 5 degrees per 30 minutes.

From the figures it will be seen that significant differences were found only in favor of treatment 5 degrees per 10 min. over 5° C. per 10 minutes at 1° C. In addition it has been suggested that for the longest storage interval the storage temperature of 1° C. was the most satisfactory at the slowest rate of cooling, 5 degrees per 30 minutes. Not much difference was noticed in pH values.

Effect of dilution on fertility.

The rate of diluting bull semen has ranged all the way from one part semen and one part diluter to one part semen and one hundred parts diluter. Early studies conducted by Willett and others (36) showed a high

rate of conception for diluted semen used during the first 24 hours after collection, which rate was continued without important deviation up to 96 hours. Later Salisbury (9) studied to determine if dilution effect would influence the fertility of semen, testing dilution rates of 1:4, 1:8, 1:10, 1:12, and 1:16, 1:24, and 1:50 parts of egg yolk-citrate-diluent. All semen samples containing motile spermatozoa were used regardless of concentration. The latest investigation on this problem by Salisbury (32) has been made with dilution rates of 1:40, 1:60, 1:80, and 1:100 parts of the yolk citrate diluent. The results of all the above experiments are given on the next page in Table No. 5.

Table No. 5. Summary of the Fertility Data of the Dilution experiments.

Rate of Dilution	1:4	1:6	1:8	1:10	1:12	1:16	1:24	1:40	1:50	1:60	1:80	1:100
Number of Services	289	331	544	405	565	589	796	1328	694	1387	1502	1543
Per cent Conception	51.2	55.0	52.2	50.1	48.1	55.3	46.7	68.7	48.7	67.6	69.6	70.3

From the figure on the preceding page no difference in fertility was found between all the dilution rates. At the highest rate of dilution on the average 12,836,000 spermatozoa were introduced in each 1.0 ml. of diluted semen used for insemination. And also it will be seen from the figures that the highest average efficiency of conception was obtained with the highest dilution rate. 1:100; and the small differences shown between dilution rates are not significant.

III. STUDY OF SEASONAL AND BREED DIFFERENCES IN SEMEN PRODUCTION OF DAIRY BULLS

It has long been recognized that in many animals spermatogenesis is not continuous but tends to occur at one rather definite period of the year as in case of mon-oestrus animals. Cyclic spermatogenic activity is characteristic of some animals. Domesticated animals such as the bull, stallion, ram, etc., have been classified as continuous breeders, but the evidence is now accumulating (3) to show that although spermatozoa are produced throughout the year certain seasonal trends in the rate of spermatogenesis and the degree of fertility are evident.

It was therefore decided to study and determine the influence of season and breeds upon semen quality in the dairy bull with regard to its volume, motility and concentration.

Review of literature.

Moore's observation by Anderson (1) that the testis is regulated by the gonadotropic hormone of the anterior pituitary gland and to some extent by other endocrine glands, by light, by environmental testicular temperatures and the plane of body nutrition. In extensive studies of semen production of sheep was reported (12) that although definite seasonal trends in semen quality were apparent, maximum quality semen was observed from October to January and maximum efficiency was said to have been obtained in May. Studies made by Erb (13) showed that except for a noticeable increase in concentration in the spring season,

seasons other than summer had little effect on semen quality. During July, August and September motility and semen volume was lowered and the percentage of abnormal forms was increased. Bartlett (3) reported monthly averages of semen volume, concentration and longevity for six bulls and found it to be correlated with fertility. Anderson (1) presented monthly average semen volumes and motility observations made on bulls in Kenya which indicated distinct seasonal tendency.

Materials and Methods

The bulls were all pure bred animals used as regular sires by the Michigan Artificial Breeding Cooperative unit at Michigan State College. Four Holstein-Friesian and four Guernsey bulls were used in this study. They varied in age from 3 to 8 years but the majority of them were aged bulls, five years old or older. The animals had no access to pastures and were maintained on usual grain rations containing 15-16% protein composed of 1000 pounds oats, 250 pounds wheat middlings, 350 pounds wheat, 300 pounds soybean oat meal, 100 pounds meat scrap, 100 pounds poultry concentrate, 36 pounds salt. Of this mixture 4 to 5 pounds were fed to each bull with one ounce of mixed minerals as cobalt, iron, copper, etc. All the animals were in good breeding condition. First cutting of alfalfa and brome grass was fed at liberty.

Semen was collected from all bulls with an artificial vagina once each week and with few exceptions after two weeks. Two ejaculates were obtained within a ten minute interval at each collection period.

The methods of semen evaluation which are commonly used in investigations of this type and have been fully described under the chapter "Clinical Examination of Semen." Semen volume, initial motility, and concentration of spermatozoa were immediately recorded.

The degree of motility of the spermatozoa have already been expressed.

Concentration of spermatozoa per unit volume of semen was determined by haemocytometer using the technique which is ordinarily applied for the counting of red blood cells; and total motility rating score was obtained by summation of the individual motility ratings during the period for examination of smears of each ejaculate. Average records of semen examination of 8 bulls for winter and summer are shown in Tables 6 and 7.

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**Table No. 6. Average Record of Semen Examination
of 8 Bulls During January, February and March.**

Name of Bulls	Volume in ml	Initial Motility Percentage	Concentration of sperm /mm³
<u>Holsteins</u>			
Kbop	6.8	80	1312
Silver	5.4	74	1100
Rainbow	7.7	70	1053
Barber	7.3	71	1330
<u>Guernseys</u>			
Maxim	6.2	77	1283
Master	8.3	71	766
Neptune	8.2	80	1050
Mars	3.3	79	1511

**Table No. 7. Average Record of Semen Examination
of 8 Bulls During July, August and September.**

Name of Bulls	Volume in ml	Initial Motility Percentage	Concentration of sperm /mm³
<u>Holsteins</u>			
Kbop	5.9	71	1333
Silver	5.1	71	1100
Rainbow	6.7	61	1464
Barber	5.9	68	686
<u>Guernseys</u>			
Maxim	6.5	75	1328
Master	7.7	74	1022
Neptune	10.0	72	900
Mars	3.1	71	1590

Results

The result of examination of 179 ejaculates produced by 8 bulls between January, February, March and July, August and September are presented in this study. Significance of variation has been tested, and analysis of variance on samples collected from bulls during winter and summer has been performed and the details are given on the following tables. These bulls were normal, young, healthy animals with good breeding records and were maintained under the same system of management throughout the period.

Bull	Age	Breed
1. K. Bop	8 years	Holstein
2. Silver	7 years	"
3. Rainbow	6 years	"
4. Barber	3 years	"
5. Maxim	6 years	Guernsey
6. Master	6 years	"
7. Neptune	8 years	"
8. Mars	4 years	"

Summary of factors studied is shown in Table Nos. 8, 9, and 10. Significant differences occurred in initial motility between seasons only while no significant difference occurred in semen volume and concentration with regard to season and breeds. These results suggest that seasonal trends in the total motility rating show significant variation while no significant variation occurred in volume and concentration.

1

Data shows that semen of lower motility was produced during the months of July, August and September. No appreciable differences in semen quality occurred between the seasons and the breeds.

Initial Motility.

Two bulls produced spermatozoa with the lowest initial motility during the summer months.

The variation of these two bulls, Rainbow and Barber, may be due to individual differences. The average volume of semen per ejaculate did not show much variation with the exception of Mars, whose average semen volume was least in the summer.

Concentration of sperm.

No difference in sperm concentration in winter and summer and no difference between Holsteins and Guernseys was noticed.

Temperature as a factor in seasonal variation.

Although temperature, humidity and light vary during the seasons of the year and the conditions during the same months of different years are often dissimilar, it is believed that temperature changes are at least partially responsible for the differences in semen quality which were observed in total motility at the times when maximum temperatures were observed. Results of the Analysis of Variance are shown on table No. 11.

Table 8. Average Volume of Semen Collected from Eight Holstein and Guernsey Bulls During Winter and Summer.

	Winter	Summer	
Guernseys			
1	6.2	6.5	
2	8.3	7.7	
3	8.2	10.0	
4	3.3	3.1	
Total	26.0	27.3	53.3
Holsteins			
1	6.8	5.9	
2	5.4	5.1	
3	7.7	6.7	
4	7.3	5.9	
Total	27.2	23.6	50.8
Grand Total	53.2	50.9	104.1

$$\text{Interaction S.S} = \frac{(\text{Total})^2}{16} = 725.11 - 677.3 = 47.81$$

$$\text{Between Seasons} = \frac{(53.2)^2}{8} + \frac{(50.9)^2}{8} - 677.3 = 0.33$$

$$\text{Between Breeds} = \frac{(53.3)^2}{8} + \frac{(50.8)^2}{8} - 677.3 = 0.43$$

$$\text{Sub Classes} = 679.52 - 677.3 = 2.22$$

$$\text{Interaction} = 2.22 - (0.33 + .39) = 1.50$$

Analysis of Variance

Source of Variation	D/F	$\sum$ of Squares	Mean Squares
Total	15	47.81	
Between Seasons	1	0.33	0.33
Between Breeds	1	0.39	0.39
Breed & Season Interaction	1	1.50	1.50
Within Sub Classes	12	44.59	3.72
Season	$F = \frac{.33}{3.72}$	= 0.09	Non Significant
Breeds	$F = \frac{.39}{3.72}$	= 0.1	Non Significant
Breeds & Season	$F = \frac{1.50}{3.72}$	= 0.4	Non Significant

Table 9. Average Initial Motility of Semen Collected from Eight Holstein and Guernsey Bulls During Winter and Summer.

	Winter	Summer	
Guernseys			
1	77	75	
2	71	74	
3	80	72	
4	79	71	
Total	307	292	599
Holsteins			
1	80	71	
2	74	71	
3	70	61	
4	71	68	
Total	295	271	566
Grand Total	602	563	1165

$$\text{Interaction} = \frac{(1165)^2}{16} = 84827$$

$$\text{S.S.} - \frac{(\text{Total})^2}{16} = 85181 - 84827 = 354 \quad (\text{S.S.} = \text{Summation of Squares})$$

$$\text{Between Seasons} = \frac{(602)^2 + (563)^2}{8} = 84922 \quad 84922 - 84827 = 95$$

$$\text{Between Breeds} = \frac{(599)^2 + (566)^2}{8} = 84895 \quad 84895 - 84827 = 68$$

$$\text{Sub Classes} = \frac{(307)^2 + (292)^2 + (295)^2 + (271)^2}{4} = 84995 \quad 84995 - 84827 = 168$$

Analysis of Variance

Source of Variation	D/F	Σ of Squares	Mean Squares
Total	15	354	
Between Seasons	1	95	95*
Between Breeds	1	68	68
Breed & Season Interaction	1	5	5
Within Sub Classes	12	186	15.5
Season	$F = \frac{95}{15.5}$	= 6.13	Significant
Breeds	$F = \frac{68}{15.5}$	= 4.39	Non Significant
Breeds & Season	$F = \frac{5}{15.5}$	= 0.32	Non Significant

Table 10. Average Concentration of Sperm /mm³ from Semen Collected from Eight Holstein and Guernsey Bulls During Winter and Summer.

	Winter	Summer	
Guernseys			
1	1283	1328	
2	766	1022	
3	1050	900	
4	1511	1590	
Total	4610	4840	9450
Holsteins			
1	1312	1333	
2	1100	1100	
3	1053	1464	
4	1330	686	
Total	4795	4583	9378
Grand Total	9405	9423	18828

$$\text{Interaction S.S} = \frac{(\text{Total})^2}{16} = 23123468 - 22155849 = 967619$$

$$\text{Between Seasons} = \frac{(9405)^2}{8} + \frac{(9423)^2}{8} - 22155849 = 17$$

$$\text{Between Breeds} = \frac{(9450)^2}{8} + \frac{(9378)^2}{8} - 22155849 = 300$$

$$\text{Sub Classes} = \frac{(4610)^2}{4} + \frac{(4840)^2}{4} + \frac{(4795)^2}{4} + \frac{(4583)^2}{4} - 22155849 = 12554$$

$$\text{Interaction} = 12554 - (17 + 300) = 12237$$

11

Analysis of Variance

Source of Variation	D/F	$\sum$ of Squares	Mean Squares
Total	15	967619	
Between Season	1	17	17
Between Breeds	1	300	300
Breeds and Season Interaction	1	12237	12237
Sub Classes	12	955065	79589
Season	$F = \frac{17}{79589} =$	0.0002	Non Significant
Breed	$\frac{300}{79589} =$	0.004	Non Significant
Interaction	$\frac{12237}{79589} =$	0.15	Non Significant

**Table 11. Summary of the Analysis of Variance of the Semen
Characteristic of Eight Bulls.**

Factor Analyzed for Variance	Source of Variation		
	Between season	Between Breeds	Breed and Season Interaction
Volume in ml	-	-	-
Initial motility percentages	+	-	-
Concentration of sperm /mm³	-	-	-

- = Non Significant

*+ = Significant

Discussion

The cause of sterility and the factors which influence it are numerous and the investigators continue in that line of research.

Those species which are strictly seasonal breeders are sterile during the non-breeding season, whereas such species as man, cow, horse, fowl and others, although classified as continuous breeders, tend to produce at different intensities throughout the year.

Some critical studies of the influence of season upon reproduction in dairy cattle have already been made, evidence is that such influence does exist. It has been reported at Louisiana Experimental Station (2) that more services per conception were required during the summer months than at any other period of the year.

Since the management and plane of nutrition of the bulls were similar throughout the period, it is concluded that the changes observed as in motility rating were the result of those factors which characterize the season, temperature, and relative humidity.

Seasonal changes in semen volume and concentration were not significant as observed by Erb and others (12). Environmental temperature may be of considerable importance; as it has been shown that the direct application of heat to the testis, the insulation of the scrotum, the replacement of the testis in the abdominal cavity or continued febrile condition cause partial or complete inhibition of spermatogenesis. However, the number of bulls used in this study was too small to draw any definite conclusions. Results are merely an indication.

Summary

1. Analysis of variance in characteristics of semen production between seasons and breeds did not reveal highly significant differences.

2. Average semen volume did not show much difference except in one bull in which it was least in July, August, and September.

3. Average initial motility was least in July, August, and September.

4. Average concentration and volume of semen showed no significant differences between seasons and breeds.

IV. SUMMARY

The various advantages of artificial insemination over natural mating, mentioned, lead one to be too enthusiastic about its use to speed up the rate of live stock improvement. It provides means for increased use of sires, for evaluation of young sires, for overcoming difficulties due to size difference in males and females, for maintenance of satisfactory conception rate, for control of genital diseases and economic service, particularly to small herds.

There has been considerable progress with regard to semen collection and its evaluation, dilution, storage and transport. As to the collection of the semen, artificial vagina technique remains outstanding.

The accurate evaluation of the semen samples at the time of collection is important for obtaining satisfactory conception rate.

Progress has been made in preparation and use of diluters. In this connection, Salisbury's work is outstanding. Dilution rates varying from 1:4 up to 1 part of semen to 100 parts of yolk-citrate have had no influence on the fertility of bull semen stored for a period of four days.

The highest average efficiency of conception was obtained with the highest dilution rate of 1:100.

Refrigeration is the most effective method of preserving bull semen. A temperature of 40° F. is considered ideal, as it is sufficiently cool to keep the cells in dormant state without harming spermatozoa.

The optimum rate of cooling for storage at 5° C. is found to be 5 degrees per 10 minutes.

The optimum rate of cooling for storage at 1° C. is found to be 5 degrees per 2 hours.

Rapid cooling is found to be detrimental to the viability of sperm-atozoa.

1. Anders
1945

2. Ashev
1946

3. Bartle
184

4. Beck
194

5. Cee
19

6. Col
19

7. Dav
1
1

8. Ed
1

9. E

10. E

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