STUDIES ON THE ACROSOME

OF BOVINE SPERMATOZOA

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CHAPTER I

INTRODUCTION

Artificial insemination in farm animals and particularly in dairy cattle has become a big industry in the United States and many other countries. Perry (1968) reported that in 1967 there were 33 bull studs in the United States with 2,028 dairy sires in service, 6,259,425 dairy cows and heifers artificially inseminated and 3,303 cows bred per sire. These figures indicate that approximately 48.1 per cent of the dairy cows and heifers in the United States were artificially inseminated.

The rapid growth of the business of artificial insemination and the development of advanced techniques for handling and storing bovine semen have greatly increased the influence of certain sires on the cattle population. This is one of the major advantages of artificial breeding, that is, increasing and extending the influence of genetically superior sires. This in turn necessitates that these genetically proven sires possess a high degree of fertility. Knowledge of the characteristics of semen, its evaluation, dilution, storage, insemination, and the factors that affect fertility is, therefore, of a great importance.

The spermatozoon is a unique cell containing a haploid chromosomal number and very little, if any, cytoplasmic material. The sperm cell was first seen under the microscope by Johan Hamm and this was first reported by Leeuwenhoek in 1677. An important component of the sperm

cell is the acrosome cap which is now known to play an important role in the male's fertility. At the present time, many studies on the structural and functional aspects of the acrosome cap can be found in the recent literature. Abnormalities of the acrosome cap of the bull and other farm animals have been confirmed as causes of the impaired fertility of the male. Anomalies of this cap are classified among the primary abnormalities of spermatozoa that may result in complete sterility. However, not all the functional aspects of this tiny structure are known with certainty and much more work is necessary for identifying more clearly and accurately the acrosome cap and its important role in fertilization.

The objectives of this investigation were the following:

1. To study the characteristics of the acrosome cap of functional dairy bulls receiving normal care and ejaculated at a frequency of once a week for 56 weeks.
2. To study the interrelationships of acrosomal characteristics with other semen criteria and with specific ambient temperature changes.
3. To determine the changes in the acrosomal characteristics of ejaculates of bulls emerging from sexual rest.
4. To study the effects of high levels of semen collection on the acrosome cap.

CHAPTER II

LITERATURE REVIEW

Semen Characteristics

Spermatogenesis in the male is a continuous process which ensues on puberty and the harvesting of the largest number of healthy and normal spermatozoa from improved males is of a great importance to the animal breeder.

Semen quality has received considerable attention throughout several decades and has led to the development of definitive methods for assessing semen quality. Certain semen characteristics are evaluated in assessing the sexual performance of the bull as well as other farm males.

The literature contains a large number of reports concerning the relationships between semen characteristics and fertility. Correlation coefficients between certain semen characteristics and fertility have been determined by several workers (Mercier and Salisbury, 1946; Ludwig et al., 1948; Bishop et al., 1954). Munroe (1961) reported that fertility was reduced when either the incidence of live spermatozoa fell below 70 per cent or the incidence of primary and secondary abnormal spermatozoa rose above 23 per cent, with head defects appearing to be the most important abnormalities. Bishop et al. (1954) also reported that there was a highly significant inverse relationship between fertility and the incidence of dead sperm cells and a highly

significant direct relationship between fertility and impedence change frequency. They also found evidence of relationships between fertility and resistance to temperature shock, age of the bull, ejaculate volume, fructolysis rate and methylene-blue reduction rate. These authors concluded that there is evidence of a relationship between the physical activity of bull semen and its fertilizing ability, and they suggested that semen quality could be measured by determination of the concentration of sperm cells in the ejaculate; the proportion of dead sperm and the measurement of the impedence change frequency. Methylene-blue reduction test, fructolysis rate and oxygen uptake add little to the information gained by the above three tests. These workers and many others have suggested that measurements of certain characteristics of ejaculates can be used as criteria of the fertilizing ability of bulls. Although no one characteristic is highly correlated with fertility, artificial insemination units typically use ejaculate volume, sperm concentration, the proportion of live cells in the ejaculate and the proportion of normal cells as indicators of ejaculate quality.

Factors Affecting Semen Characteristics

Sperm cell production starts at puberty, an event which is affected by genetic as well as by internal and external environmental factors. The first few ejaculates will typically be of low quality with gradual improvement being apparent with repeated ejaculations and age. Almquist (1968) stated that the average semen characteristics of normal, mature bulls are: ejaculate volume, 8 ml. (range 2 to 15 ml.); initial sperm concentration, 1200 x 10^6 per ml (400-2,000 million per ml.); and initial sperm motility, 65 per cent (50-80 per cent). Age and size of the

bull, breed, frequency of semen collection, the level of sexual preparation and season of the year are known to exert their influence on male performance.

VanDemark (1956), using Holstein bulls, found that the volume of the ejaculate increased from 2.34 to 3.21, 3.51, and 3.36 ml. as analyzed on quarter-year periods following puberty. The concentration of spermatozoa increased from 429 x 10^6 to 735 x 10^6, 916 x 10^6, and 987 x 10^6 and the total sperm number per ejaculate from 1,255 x 10^6 to 2,690 x 10^6, 3,592 x 10^6 and 3,668 x 10^6 as analyzed on the same basis. The average body weight of the 15 Holstein bulls used in the study increased from 366 to 446, 515, and 580 kg. in the same quarter-year periods.

Almquist and Cunningham (1967) working with Angus and Hereford bulls found that ejaculate volume varied significantly with age, ejaculation frequency and bulls within breed and frequency. Mean semen volume increased from 3.1 to 3.7 ml. between 53 to 68 and 89 to 104 weeks of age. They also found significant differences in sperm concentration associated with age, breed and bulls within breeds and ejaculation frequency. Differences in sperm density due to ejaculation frequency were not significant. Sperm concentration increased from 0.7 x 10^9 to 1.2 x 10^9 sperm cell per ml. between 53 to 68 and 89 to 104 weeks of age. Average weekly sperm output between one and two years of age increased from 4.5 x 10^9 at 56 weeks to 12.8 x 10^9 at 101 to 104 weeks of age. Weekly output of motile sperm showed an increase between one and two years of age.

Baker et al. (1955), working with Holstein bulls, reported that sperm concentration per ejaculation was significantly correlated with

age ($r = 0.514$) and with body weight ($r = 0.533$).

Lasely and Bogart (1943) stated that a considerable amount of variation in the characteristics of bull semen may be attributed to differences among bulls. They also suggested that animals reacted differently to external and internal environmental factors and that this resulted in differences in the rate of spermatogenesis and in the rate of secretory activity of the accessory sex glands. They also reported that sperm production varied with the age of the bull.

Frequency of Semen Collection

Repeated semen collection is a procedure used to obtain the largest possible harvest of spermatozoa and thus to increase the usefulness of genetically superior sires. The effect of frequency of semen collection on semen characteristics and fertility has been the subject of many studies. Such studies, in general, have shown greater sperm production by bulls to be associated with increased frequencies of collection.

Working with young Holstein bulls for one year following puberty, Baker et al. (1955) reported that no significant differences in semen volume, sperm output, per cent motile sperm, semen pH, total sperm per ejaculation, total motile sperm per ejaculation, or per cent abnormal cells were found attributable to frequency of ejaculations when frequencies of once, twice, and three times per week were compared. They found a highly significant reduction in libido in bulls assigned to thrice weekly collection. The authors concluded that there appeared to be no harmful effects of repeated ejaculations up to three times per week except the decrease in libido. Almquist and Cunningham (1967)

reported that young beef bulls could be worked up to six times per week. These authors found that as the frequency of ejaculation increased, there was a significant decrease in semen volume but there were significant increases in total number of sperm and total motile cells per week. No significant differences in sperm concentration were found. They also found that sperm density and the weekly total sperm output were significantly higher for Hereford than Angus bulls and there was significant bull variation within breeds and frequency groups.

Bratton and Foote (1954), working with dairy bulls, reported that one ejaculation every fourth day or two ejaculations every eighth day for one year were not detrimental to semen production or fertility and also stated that semen collection at a rate of two ejaculations per eight day interval would yield approximately 60 per cent more sperm cells than one ejaculate per eight day interval. Similarly, VanDemark et. al. (1955) found no harmful effects on libido or semen quality in a bull ejaculated three times weekly for three years. Other similar results were reported by Hafs et al. (1959) who stated that aged bulls (7.5 to 10 years) could be ejaculated daily for as long as eight months with no detrimental effects, either on the bulls or on the quality of semen they produced.

Differences between young and mature bulls with regard to the effects of increased ejaculation frequency were also reported by Thomson (1950) who found that the reduction of the interval between services from seven to four days reduced both the volume of semen and sperm motility of young bulls over a two to three months period. This did not occur with mature bulls.

The only report concerning the effect of increased frequency of

ejaculations on the acrosome cap of spermatozoa was that of Hafez and Darwish (1956). These authors, working with buffalo bulls, stated that when the interval between collections was short, detached acrosome caps and enlarged midpieces were observed. They did not specify clearly the "short" interval between collections nor the proportion of detached acrosome caps in the ejaculate. On the other hand, they did not find an adverse effect of short interval between semen collections on ejaculate volume, sperm concentration and the percentage of live spermatozoa. The percentage of abnormal sperm greatly increased with shorter periods between ejaculations.

Another factor significantly affecting semen production is the sexual preparation of the bull for service. Several reports have indicated that restraining the bull for a few minutes and allowing one or more false mounts increased semen production (Collins et al., 1951; Branton et al., 1952; Hale et al., 1953; Baker et al., 1955). Crombach and de Rover (1956) working with identical twin bulls reported that restraint for 10 minutes before the first ejaculate resulted in that ejaculate containing 2.9 times as many live sperm cells with better motility than that of the non-restrained bulls. The second ejaculate was also improved by sexual stimulation. Almquist and Hale (1956) stated that in the presence of a constant stimulus the bulls showed a gradual decrease in the number of ejaculations per unit of time.

Effect of Sexual Rest on Semen and Acrosome Cap Characteristics

Salisbury and VanDemark (1961) stated that bulls are frequently allowed a period of time during which no semen is collected. They also

stated that "There is a strong opinion that the practice is of value in recuperating the sexual interest of bulls and their fertility. However, objective data, free of the effect of season, supporting this contention are lacking."

Bonadonna (1956) stated that a prolonged period of sexual rest exceeding two or three weeks may cause a gradual deterioration of semen quality and production. He found that a period of sexual rest for bulls of about six weeks resulted in a great decrease in semen production which, in some instances, did not return to normal values until 20 or 30 days later. No specific data concerning the frequency of ejaculation or the characteristics of semen affected by this period were given by the author. He suggested that sexual rest probably caused a gradual decrease in the activity of semen production due to the accumulation and resorption of spermatozoa in the cauda epididymis which thus causes a secondary gradual reduction of spermatogenesis.

A report by Marting et al. (1966) stated that the first ejaculates collected from bulls after 44 weeks of sexual rest froze satisfactorily. The same was true with semen collected at a rate of six ejaculations per week for four years. Thus the authors suggested that neither continuous semen collection at high frequency for up to four years nor 44-week periods of sexual rest exerted a detrimental effect on semen freezability.

Schmidt et al. (1957) allowed bulls different periods of sexual rest (up to 13 days). They found that the best results for semen quantity (semen volume, sperm concentration, and total number of sperm per ejaculate) were obtained with a rest period of four days, and for semen quality (fertilizing ability and survival time) with a rest

period of eight days. Fertility as measured by the number of inseminations necessary for conception was best after a sexual rest period of seven or more days.

There is very little information in the literature concerning the effect of sexual rest on the quality of the acrosome cap. Blom (1946) reported that a period of sexual rest of three or more weeks resulted in up to 18 per cent detachment of the "galea capitis" of the bull's sperm cells. Regular semen collection thereafter reduced this detachment to zero, and he concluded that prolonged storage of spermatozoa in the epididymis resulted in the separation of the acrosome and that this was among the first stages of spermatozoal disintegration. Awa (1968) also reported a high percentage of detached acrosomes (8.06 per cent) in the initial ejaculate of a Hereford bull collected after 6 months of sexual rest, and also in the first ejaculate of a Jersey bull (19.49 per cent detached caps) collected after a six-month period of sexual rest. The detached cap can be seen in well-stained spermatozoal smears as a semilunar shaped structure which was called the galea capitis by Blom (1946). Recent studies have shown that this is a part of the acrosome cap. Blom (1964), Saacke and Almquist (1964) and Blom and Birch-Andersen (1965) reported that the galea capitis is the outer layer of the thicker part of the acrosome cap. Those workers agreed that the detachment of the acrosome cap involves only the outer membrane of this structure, while the inner membrane of the acrosome cap and both membranes of the equatorial segment are left intact.

Wondafrash (1968), characterizing the acrosome quality in the first ejaculate of 22 Hereford and Angus bulls collected after a nine months period of sexual rest found that the initial ejaculates contained

21.2 per cent abnormal acrosome caps. He also found that 16.5 per cent of the normal spermatozoa, 44.5 per cent of the abnormal cells, and 77.0 per cent of the tailless sperm had abnormal acrosomes. This suggested that acrosomal anomalies in the first ejaculate following a long period of abstinence was associated with malformed, deteriorating spermatozoa. However, a considerable proportion of the morphologically normal spermatozoa had abnormal acrosomes.

Seasonal Effects on Semen Characteristics

Lasley (1968) stated that most species of wild animals have a defined sexual season. However, thousands of years of domestication have resulted in gradual expansion of the breeding season so that most species, except certain breeds of sheep, will now breed in almost any period of the year. Seasonal variations in the reproductive efficiency of farm animals seem to exist.

There is good evidence that both the quantity and quality of semen vary with the season of the year and great variation in this regard is observed between species, breeds and animals within breeds.

Semen production of bulls and conception rates may vary by season. Semen quality has been shown to deteriorate during the summer in many areas, but Hafez (1967) states that it is not clear how much these changes are affected by temperature, day length, and other environmental factors. Erb et al. (1942) found that there were highly significant differences between bulls and between months in Indiana for all semen characteristics except semen pH. The average volume of the ejaculate was lowest in July, August, and September, and the same trend was

observed in initial rate of sperm motility. Sperm concentration was maximum during April, May and June. An increase of about 25 per cent in sperm abnormalities was observed in July, August and September. The authors thus concluded that semen quality was significantly lower during the summer. On the other hand, Swanson and Herman (1949) found semen quality in Missouri to be lower during winter and they suggested that the adverse effects of winter weather on the physical well being and sexual activity of the aged bulls they used caused the decline in semen quality.

Phillips et al. (1943) reported that there were significant differences between individuals within breeds (Beef Shorthorn and Milking Shorthorn) with regard to sperm initial motility, sperm concentration and abnormal cells in the ejaculate. These characteristics, except the initial rate of motility, showed significant differences between seasons. A low level of sperm production was found in June and July (Mercier and Salisbury, 1946). These workers also reported significant differences between bulls and between months of the year. Seasonal changes in semen quality of European bulls in Kenya has also been reported by Anderson (1945).

Lindley et al. (1959) stated that the yearly and seasonal differences in semen volume of beef bulls in Oklahoma were significant, with ejaculate volume being lower in the winter. In Louisiana, Johnston and Branton (1953) reported that rising temperatures resulted in an increase in the percentage of morphologically abnormal spermatozoa and a decrease in the per cent live cells.

Mukherjee and Singh (1966) found that the shape and the length of sperm cells of Hariana bulls varied significantly between seasons.

Working with Kumauni Hill bulls, Mukherjee and Battacharya (1952) found that initial rate of sperm motility was lower in the summer while sperm concentration was lower in the fall.

Sullivan and Elliott (1968) found a significant difference in the fertility level of nine Holstein bulls due to seasonal effect. The non-return rate of cows inseminated with semen produced during the spring and summer was significantly lower than that obtained with semen produced in the fall and winter. On the other hand, no significant difference was detected between the semen collected in the spring and summer, or in the fall and winter. However, since the bulls were housed at a constant temperature, they concluded that some other environmental or intrinsic factor also influences the fertility of the bulls.

This discussion indicates that seasonal variation in semen characteristics and fertility may be observed and that ambient temperature may affect semen production. This has also been shown experimentally by raising scrotal temperature in farm animals and man (Ortavant, 1954; Glover, 1955; Dutt and Hamm, 1957; Foote et al., 1957; Austin et al., 1961; Procope, 1965). The results of such studies and others have indicated that raising scrotal temperature resulted in a reduced semen production and increased sperm abnormalities.

The Acrosome Cap

It appears that the term acrosome has been used by Lenhossek in 1898 (Bishop and Walton, 1960). The most recent advances in the ultrastructural studies of sperm cells have been made possible by development and utilization of techniques for preparing very thin sections

of spermatozoa which at the same time maintain the integrity of the sperm components. The use of the electron microscope has resulted in better understanding of the sperm cell and its acrosome cap. Much information has also been gained by the use of the phase-contrast and ultraviolet microscopes, and improved histochemical techniques.

Since the original studies of Bowen (1924), several investigations have confirmed that the acrosome cap originates from the Golgi apparatus of the spermatid (Leblond and Clermont, 1950; Gresson, 1951; Schrader and Leuchtenberger, 1951; Nath, 1956; Gumbo, 1966). Bishop and Walton (1960) summarized the formation of the acrosome cap as follows: The early spermatid contains small granules, each within a small vacuole. These granules combine into one large granule within a large vacuole. This structure approaches the surface of the nucleus and causes a shallow depression in the nuclear surface. It spreads over the nuclear membrane until it covers about one-half or more of the nucleus. The next phase is an elongation of the nucleus. The posterior end of the acrosome spreads further so that it covers approximately two-thirds of the nucleus. A slow condensation of the nuclear chromatin occurs, in addition to more flattening of the nucleus. The acrosomal projection becomes broader and attaches to the anterior tip of the nucleus forming what Blom and Birch-Andersen (1961, 1965) called the apical body, which is a very small thickening on the anterior edge of the normal sperm cell.

The size of the acrosome cap differs among species. Austin and Bishop (1958) studied the acrosome in 10 mammalian species and found the acrosome cap of the bull to be the smallest while that of the guinea pig to be the largest. The shape of the cap also differs among

species, particularly in rodents. They suggested that the acrosome cap is a universal feature of the flagellate sperm, and Nath (1956) has stated that this structure is found in all animal sperm cells from the sponges upward in the animal kingdom. Early studies suggested that the human spermatozoon was devoid of an acrosome but Friedlaender (1952), Schnall (1952), and Anberg (1957) confirmed the existence of the acrosome in human spermatozoa. The human acrosome is, however, very small in size and closely adherent to the nucleus.

There has been much controversy concerning the structure of the acrosome. Hancock (1952) described it as a cytoplasmic cap consisting of two components, an "outer" and an "inner" acrosome, the latter being smaller and having a concave posterior border while the outer acrosome being larger and having a straight posterior border. The area between the two posterior borders was described as the equatorial segment. In the same sense, Bishop and Austin (1957) stated that the equatorial segment is that part where the outer acrosome overlaps the inner acrosome. Similar descriptions were given by Lovell and Getty (1960) and Karras (1959). On the other hand, Fawcett and Burgos (1956) stated that the inner acrosome lies within the outer acrosome and not beneath it. Fawcett (1958) stated that the inability of the electron beam to penetrate the dense head of the intact spermatozoa was one of the reasons for the inconclusive results obtained with the electron microscope before 1954. Recent electron microscope studies using thin-sectioning of the sperm cell head have shown the acrosome cap to have an external outer membrane continuous with an inner membrane. The space between these two membranes is filled by an electron-dense matrix. These components have been reported for the bull (Saacke and Almquist,

1964; Blom and Birch-Andersen, 1965), boar (Nicander and Bane, 1962), rabbit (Hadek, 1963 a, b), guinea pig (Fawcett and Hollenberg, 1963) and rat (Piko and Tyler, 1964). Blom and Birch-Andersen (1965) made thin sections of the bull's sperm cells, and their electron micrographs revealed that the nuclear membrane is covered by two membranes: (1) the acrosome cap and, (2) the cell membrane which covers the entire sperm. The acrosome cap was described by those authors to be about 0.1 μ in thickness and to cover about 60 per cent of the sperm head. The front part of the acrosome cap consisted of a double-layer sac and was continuous with the rear part which appears to be identical with the equatorial segment. The space between the two layers of the acrosome cap was occupied by a substance of low electron density. The perforatorium is found at the tip of the nucleus beneath the inner layer of the acrosome cap. The outer membrane of the front part of the acrosome shows a well-defined swelling at the tip of the sperm head which has been called the apical body (Blom, 1963). The equatorial segment was shown to be the part of the acrosome cap in which the inner and outer membranes are paralelled and close to each other, containing a material of high electron density. Saacke and Almquist (1964) have also shown that the equatorial segment is a part of the acrosome cap.

Abnormalities of the Acrosome Cap

It is well known that certain abnormalities of the acrosome cap of the bull and other male farm animals impairs the male's fertility or causes sterility. Teunissen (1947) was probably the first to report that 82-96 per cent of spermatozoa of a sterile bull had an abnormal formation on the head. This defect was also found in the sperm cells of

60 other bulls which suffered low fertility or sterility. Schnall (1952) described acrosomal abnormalities in sterile men, with the cap being smaller than normal or absent.

Evidence that the knobbed acrosome, which constitutes one kind of acrosomal abnormality, is inherited, and results in sterility or reduced fertility, has been reported by several workers in several different species (Hancock, 1949 and 1953; Rollinson, 1951; Rollinson and Makinson, 1949; Donald and Hancock, 1953; Bane, 1961; Wohlforth, 1962; Rob, 1964; and Buttle and Hancock, 1965). Rothschild (1959) stated that semen of bulls having the knobbed acrosome defect had normal metabolic activity.

Slizynska and Slizynski (1953) found that sperm head abnormality in the bull was associated with vacuole formation during spermatogenesis but not with structural changes in the chromosomes. Hollander et al. (1966) found that a recessive mutation, found in X-ray treated male mice, resulted in almost total sterility in homozygous males. This was partly because of the high incidence of abnormal acrosome formation and partly because of the failure of those males to copulate.

Bane and Nicander (1966) studied the development of defective acrosome caps in a sterile boar and found three types of acrosomal abnormalities, the most frequent being the persistance of a tongue-like protrusion of the acrosome, normally present only during the late stages of acrosome formation.

Saacke and Amann (1966) presented evidence of inherited abnormal acrosome caps in the sperm cells of bulls which exhibited low fertility. They found that ejaculates of a subfertile Holstein sire had 33.1 per cent total abnormal acrosome (Knobbed, 6.2%; ruffled, 7.9%, and incom-

plete acrosome, 19.0%). One of the sons of this bull showed 30.7 per cent and another son had 36.9 per cent total acrosomal abnormalities (knobbed, 3.6 and 5.9%; ruffled, 7.8 and 9.1%; and incomplete caps, 19.3% and 21.9%; in the first and second sons of that sire, respectively). Control ejaculates from 10 normal bulls had an average of 4.1 per cent total acrosomal abnormalities (0.3% knobbed, 1.7% ruffled, and 2.1% incomplete caps). They also stated that an asymmetrical equatorial segment was associated with many abnormal acrosomes.

Some unidentified environmental factors may have an adverse effect on the acrosome cap resulting in variable incidence of abnormal acrosome in the ejaculate. No controlled experiments concerning this aspect have been performed. Indirect evidence may be obtained from the study of Saacke et al. (1968) who determined the incidence of acrosomal anomalies in subfertile bulls and stated that there was a considerable day-to-day variation in the incidence of acrosomal abnormalities, and suggested, as did Awa (1968), that bull studs should consider acrosomal examination as an additional evaluation of ejaculate quality.

Methods for the Study of the Acrosome Cap

Despite the importance of the acrosome cap and its apparent relationship to the male's fertility, the acrosomal state is not typically evaluated by artificial insemination units. This is principally due to the lack of a simple and efficient technique for revealing the acrosomal state of an ejaculate. Many studies (Blandau, 1951; Friedlaender, 1952; Rahlmann, 1961; Nicander and Bane, 1962; Bedford, 1964; Piko and Tyler, 1964; Saacke and Almquist, 1964; Blom and Birch-Ander-

sen, 1965; Bane and Nicander, 1966) have utilized the electron microscope, the phase contrast, and ultraviolet microscopy but these are typically limited to use in research work rather than in artificial breeding units. The Giemsa stain has been used in certain studies for identifying the acrosome cap (Saacke et al., 1968). It is thus evident that there has been a need for a simple and efficient technique for observing the acrosome cap. Previous work at Oklahoma State University led to the development of the Wells-Awa stain for differentially staining the acrosome cap (Awa, 1968). This stain, employing a 5 minute staining procedure and the oil immersion objective of a light microscope, is a significant aid in visualizing the acrosome cap, its morphological state, changes and abnormalities. Wells and Awa (1970) described several tests made on this stain to determine its efficacy and showed its superiority to the Giemsa stain and to phase contrast microscopy for detecting the different kinds of acrosomal anomalies. Until the introduction of this stain, routine acrosomal evaluation was difficult due to either the lengthy staining procedure, or the complexity of the equipment involved.

The literature contains several studies, some of which have been referred to, on the changes in the usually measured semen characteristics as affected by age, frequency of ejaculation, environmental influences, and other factors. No comparable studies on the acrosome cap have been made and much research is needed to more fully define the relationship of the acrosome cap to other semen characteristics, the factors that influence the acrosomal state, and ultimately its role in fertility. Some of those factors and relationships, as described in chapter I, are the subject of this study.

CHAPTER III

MATERIALS AND METHODS

Semen Collection

Six dairy bulls from the Oklahoma State University herd were used in this study. The bulls were housed in the dairy bull barn under normal management and nutritional conditions. Four of the bulls were collected weekly for 56 weeks. Following this part of the study, all six bulls were utilized to determine the influence of sexual rest and ejaculation frequency on ejaculate characteristics.

Semen collection was carried out according to the procedure described by Wells (1962). A cow, sometimes in estrus, was tied in the collection stall. The bulls were led individually to an adjoining chute where the preputial area was thoroughly cleaned with warm water and dried. The bull was then led to the collection stall and restrained behind the cow for three to five minutes, with one false mount allowed, before serving the artificial vagina. The graduated tube of the artificial vagina was protected carefully against cold shock. Except during the summer and early fall, the graduated tube was protected by a plastic tube containing water at 95-100° F. The collecting funnel and tube were further covered with a special protecting jacket to avoid any cold shock to the sperm cells and particularly the acrosome cap. The temperature of the water used to fill the artificial vagina ranged between 120° to 140° F, according to the bull prefer-

ence. In certain infrequent instances, some bulls refused to serve the artificial vagina, and semen was collected with the electro-ejaculator as described by Wells (1962).

All glassware used in semen collection and tests was washed in soapy water, then in tap water, rinsed in glass-distilled water, and dried in a hot-air oven at 100° C. Rubber materials of the artificial vaginas were washed in soapy water and then stored in isopropyl alcohol until the following collection time.

Semen Tests

Several measurements were made on the semen of each bull after ejaculation. The semen tubes were kept in a water bath at 95-100° F throughout the time needed for making the initial tests and preparing the stained slides.

The following data were obtained for each ejaculate:

a) Semen Volume: Semen volume was obtained to the nearest 0.1 ml. from the 15 ml. graduated tubes in which semen was collected.

b) Sperm Concentration: The density of spermatozoa per milliliter of semen was made according to the method described by Wells (1962). 0.1 ml. of the fresh semen was diluted in 4.0 ml. of 2.9 per cent sodium citrate dihydrate solution at 37° C and mixed gently in the 17 mm. diameter tubular absorption cell. Colorimetric readings were obtained by a Cenco photelometer which had been standardized with hemocytometer counts. The concentration of spermatozoa per ml. of semen was obtained from the corresponding colorimetric reading.

c) Semen pH: The hydrogen ion concentration of each ejaculate

was obtained using a Beckman pH meter, Model H-2.

d) Sperm Motility: This was estimated immediately after semen collection. A very small drop of the semen was mixed gently with a drop of 2.9 per cent sodium citrate dihydrate solution at the same temperature on a prewarmed slide. The suspension was covered with a cover slip and examined with the light microscope (X430) with the following determinations being made:

(1) the percentage of motile cells, expressed in units of 5 from 0 to 100;

(2) the rate of motility, described according to the following rating:

0 Non-progressive motility-dead sperm cells.

1 Sluggish or rocking motility typically showing no progressive movement.

2 Progressive but slow and somewhat sluggish motility.

3 Intermediate, fairly rapid motility.

4 Maximum progressive motility, very rapid and vigorous.

e) Per cent Live Spermatozoa: This was determined for each ejaculate using the nigrosin-eosin live-dead differential stain.

f) Abnormal Spermatozoa: The proportion of abnormal sperm cells in the ejaculate was estimated by direct microscopic examination in the samples used for estimating sperm motility with an actual count of sperm abnormality being made later from the live-dead sperm preparations.

g) Acrosome Characteristics: These were determined for each eja-

culate using the Wells-Awa acrosome stain (Awa, 1968).

Staining Procedure

I. The Wells-Awa Staining Technique

Throughout the study, duplicate slides from each fresh ejaculate were prepared and stained immediately after semen collection with the Wells-Awa stain for studying acrosome characteristics. The stain was prepared as follows:

Solution (A): consisting of a one percent solution of water-soluble eosin B[1] (Total dye content 88 per cent) in glass-distilled water.

Solution (B): consisting of a one per cent solution of water-soluble fast green FCF[1] (Total dye content 90 per cent) in glass-distilled water.

On the day of semen collection one volume of solution (A) was mixed with two volumes of solution (B) and 1.7 volumes of ethyl alcohol. The stain has a pH of 6.6. Stock solutions (A) and (B) were prepared monthly and kept in the refrigerator.

Sperm cell smears were made, stained, and examined as follows:

1. One-tenth milliliter of fresh semen was diluted in nine-tenth milliliter of 2.9 per cent solution of sodium citrate dihydrate in a prewarmed 17 mm. diameter tubular absorption cell. The dilution rate was narrowed in certain cases when the concentration of spermatozoa in the ejaculate was low.

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[1]Allied Chemical and Dye Corporation, Pharmaceutical laboratories, National Aniline Division, New York.

2. Two drops of this suspension were withdrawn with a prewarmed dropper and added to four drops of the Wells-Awa stain in another similar prewarmed tubular absorption cell.
3. 30 to 60 seconds later, one small drop of the sperm-stain suspension was withdrawn and placed on a prewarmed clean microscopic slide, and then smeared in a thin layer.
4. The semen supply, diluting and staining solutions and slides were maintained at 37° C.
5. The smears were air-dried at 37° C and a glass cover slip was mounted with diaphane.[1]
6. 200 sperm cells were examined on each slide to determine the acrosome characteristics. This was accomplished with the light microscope under oil, at a magnification of 970X, and using a blue filter.
7. The acrosomal state was determined according to the following definitions:

   The normal acrosome cap is observed as a component of the sperm covering about two-thirds of the anterior part of the cell head, closely adherent to the nucleus, having a smooth and continuous surface, and free from any anomalies.

   Morphological abnormalities of the acrosome cap are of several sorts, the most observable being as follows:

   a) Elevated or thickened Acrosome Cap: This is characterized by a partial or complete swelling of the outer

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[1] Will Scientific, Inc., New York.

membrane of the acrosome cap and thus an enlargement in acrosomal size.

b) Knobbed Acrosome Cap: This is a tongue-like protrusion at the anterior portion of the cap. This can be of different sizes and may be seen either folding over the nuclear tip or protruding above it.

c) Ruffled Acrosome Cap: This is characterized by a wrinkled outer edge either over the anterior portion of the cap or spreading over the entire surface of the acrosome.

d) Detaching Acrosome Cap: Observed as a loose structure in the process of being removed from the sperm head, and eventually leading to the state of capless sperm.

e) Incomplete Acrosome Cap: Showing a part of the cap as being absent.

f) Disintegrating Acrosome Cap: Typically seen in aged or stressed samples. This usually indicates the latter stage of aging.

The above six kinds of abnormalities were grouped in one class termed "sperm with abnormal acrosome caps". Another kind of acrosomal abnormality was termed "capless sperm". This is characterized by the absence of the acrosome cap. On the stained slides, the upper portion of the nucleus could be clearly seen as a bright pale red elongated structure protruding above the postnuclear cap. The detached acrosomes could be seen as the semilunar structure described by Blom and Birch-Andersen (1965).

The remainder of spermatozoa were considered as having

morphologically normal caps and were termed "sperm with normal acrosome cap".

II. Live-Dead Staining of Spermatozoa

Differentiating living sperm cells from dead spermatozoa depends on their different reactions to certain stains. The technique most widely used for this purpose and which was utilized throughout this study was that of Hancock (1952). This technique depends on the fact that dead sperm, due to certain changes in cell membrane permeability, take up eosin Y, while live cells take no stain. Nigrosin is used to provide a suitable background to facilitate differentiating live from dead sperm cells.

The live-dead differential stain was prepared by dissolving 30 grams of water-soluble nigrosin[1] and 5 grams of eosin Y[1] (total dye content 92 per cent) in 300 milliliters of double distilled water. The stain has a pH of 9.4 and it is thus hypertonic to bull's semen. It has good stability and can be kept in the refrigerator for several weeks.

Spermatozoal smears were prepared within a few minutes following semen collection by diluting one drop of the semen with six drops of the nigrosin-eosin stain in a pre-warmed 17 mm. diameter tubular absorption cell and the suspension was allowed to stand for three minutes. Then one drop of the semen-stain mixture was withdrawn, placed on a clean microscopic slide, smeared and dried on a warm stage at 37° C. Duplicate smears for each ejaculate were prepared and the determination of the percentage of live sperm cells was made by counting the stained

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[1] Matheson Coleman and Bell, Norwood (Cincinati), Ohio.

and unstained cells per two hundred sperm per slide. Partially stained sperm were considered dead. The average of two slides was taken as the percentage of live spermatozoa in the ejaculate.

Statistical Analyses

Semen was collected from the bulls A, C, J and K for 56 weeks at weekly intervals. Differences among weeks and bulls in each ejaculate characteristic were examined for significance with an analysis of variance procedure using a randomized complete block design. Analyses of variance were also performed on the data grouped on a monthly basis to reveal possible seasonal influences and trends.

In order to determine the degree of relationship between the different semen characteristics and the acrosomal state, linear correlation coefficients between these characteristics were obtained on the basis of the weekly ejaculates. Also, correlation coefficients between acrosomal characteristics and certain temperature averages, as will be described in Chapter IV, were obtained. This portion of the study was started on October 2, 1968 and terminated on October 30, 1969.

Following the 13-months study described above, two bulls were added to the group and the six bulls were utilized to collect data on the effects of sexual rest and two frequencies of ejaculation on acrosomal characteristics. The procedure and analyses used will be discussed in Chapter IV.

CHAPTER IV

RESULTS AND DISCUSSION

Acrosomal and Other Characteristics of Weekly Ejaculates

It has been reported by several investigators that semen characteristics show considerable variation from ejaculate to ejaculate and that different males show marked differences in their semen quality. Seasonal changes in semen criteria of the bull and other species of farm animals have also been reported and a brief reference to some of the factors that affect semen criteria have been discussed in chapter II.

There is no doubt, at the time being, that the acrosome cap is an important feature of the spermatozoon and that the abnormalities of this structure can result in decreased fertility, or, dependent on the kind of abnormality, total sterility. There are many references which indicate that the acrosome cap is a very labile structure which is subject to morphological and functional alterations under several conditions. However, there are no detailed studies in the literature which deal with the changes that this cap undergoes under different situations. This study, therefore, represents preliminary investigations undertaken to detect the acrosomal changes that can be observed in ejaculates of bulls receiving routine treatment.

Figures 1 to 6 show the weekly averages of the semen characteristics studied. Those figures are plots of the average of the weekly

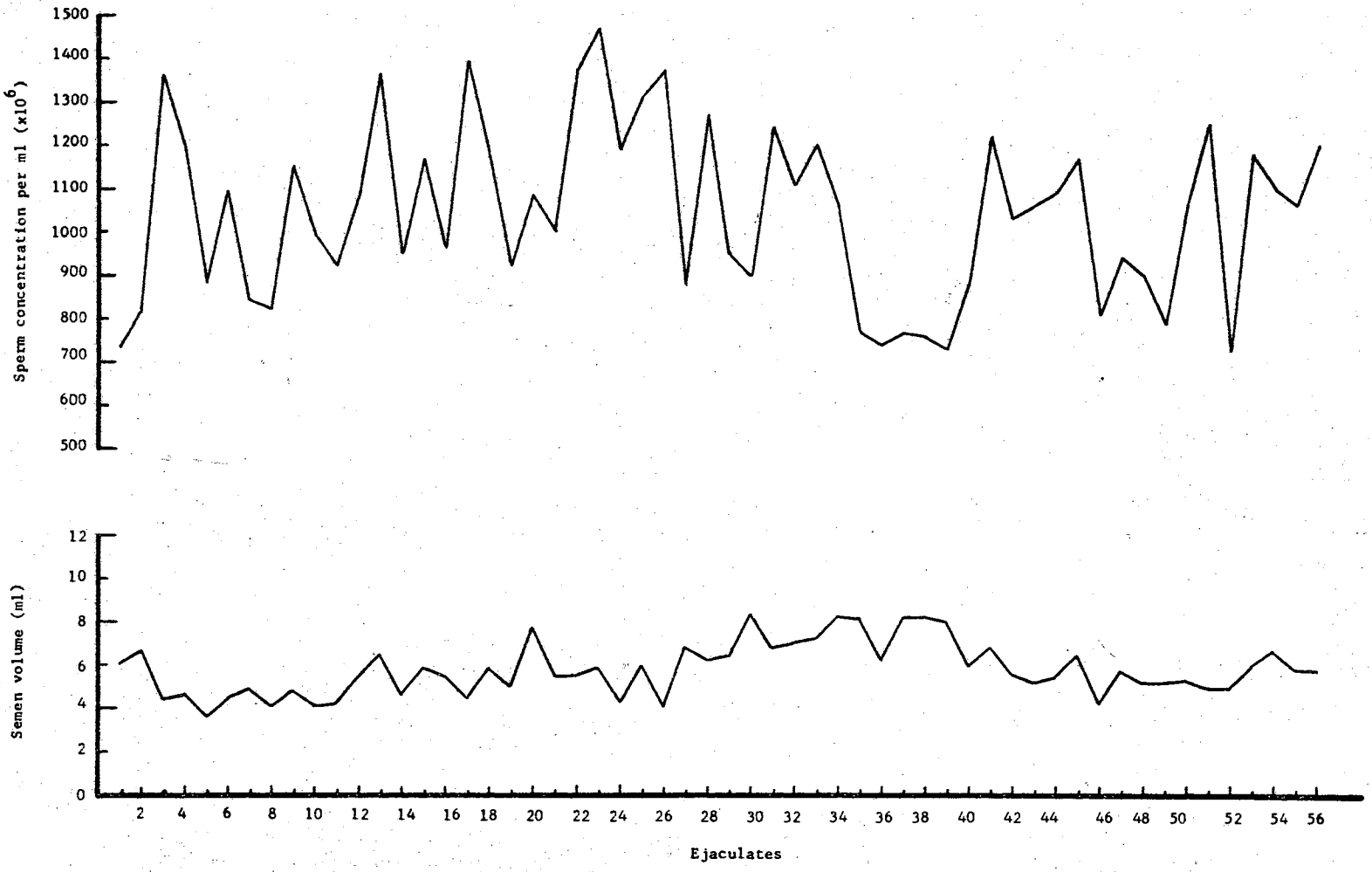


Figure 1. Weekly Averages of Ejaculate Volume and Sperm Concentration per ml. of Four Bulls Ejaculated at Weekly Intervals for 56 Weeks.

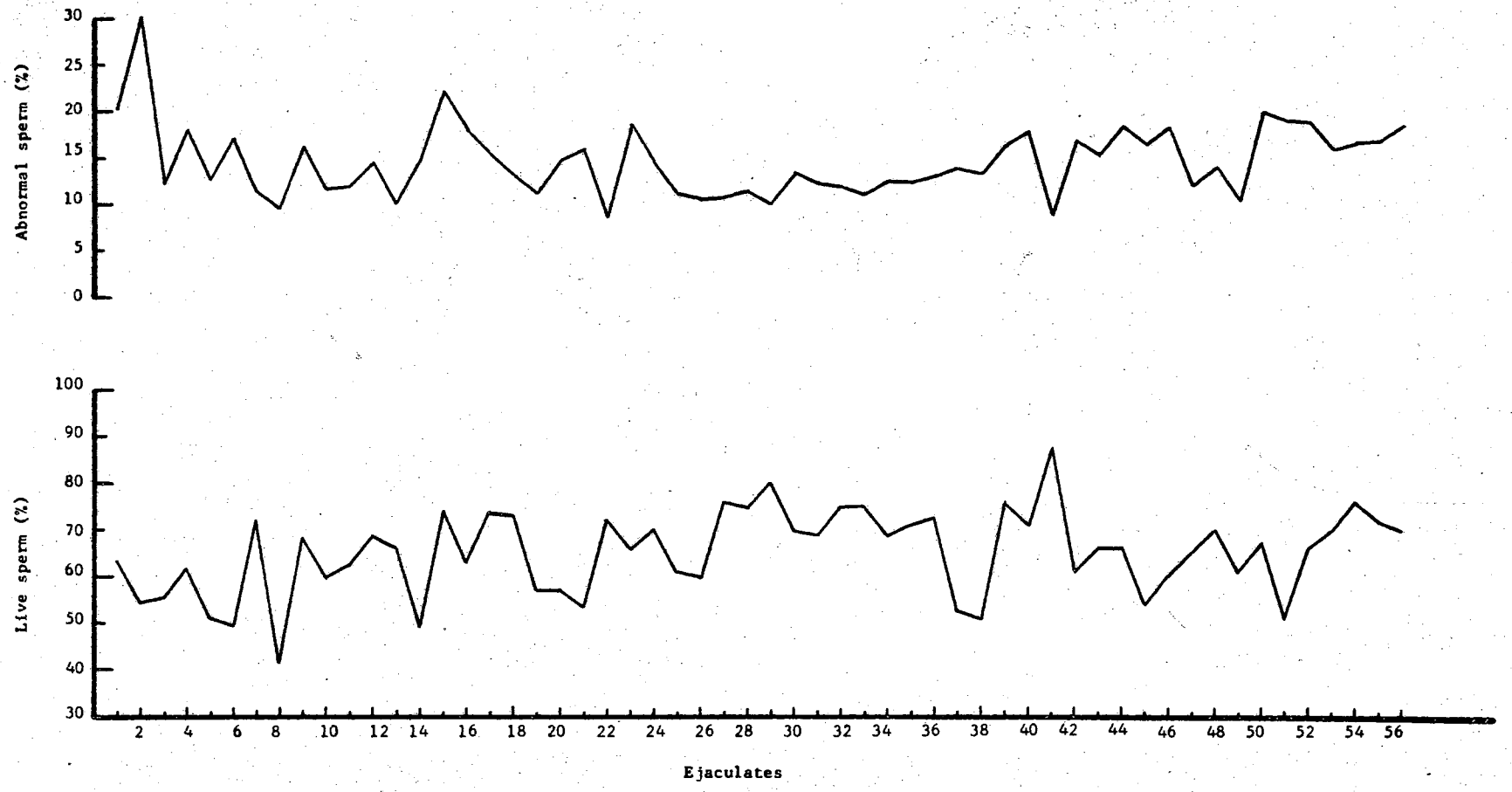


Figure 2. Weekly Averages of Per Cent Live Sperm and Per Cent Sperm Abnormalities in the Ejaculates of Four Bulls Ejaculated at Weekly Intervals for 56 Weeks.

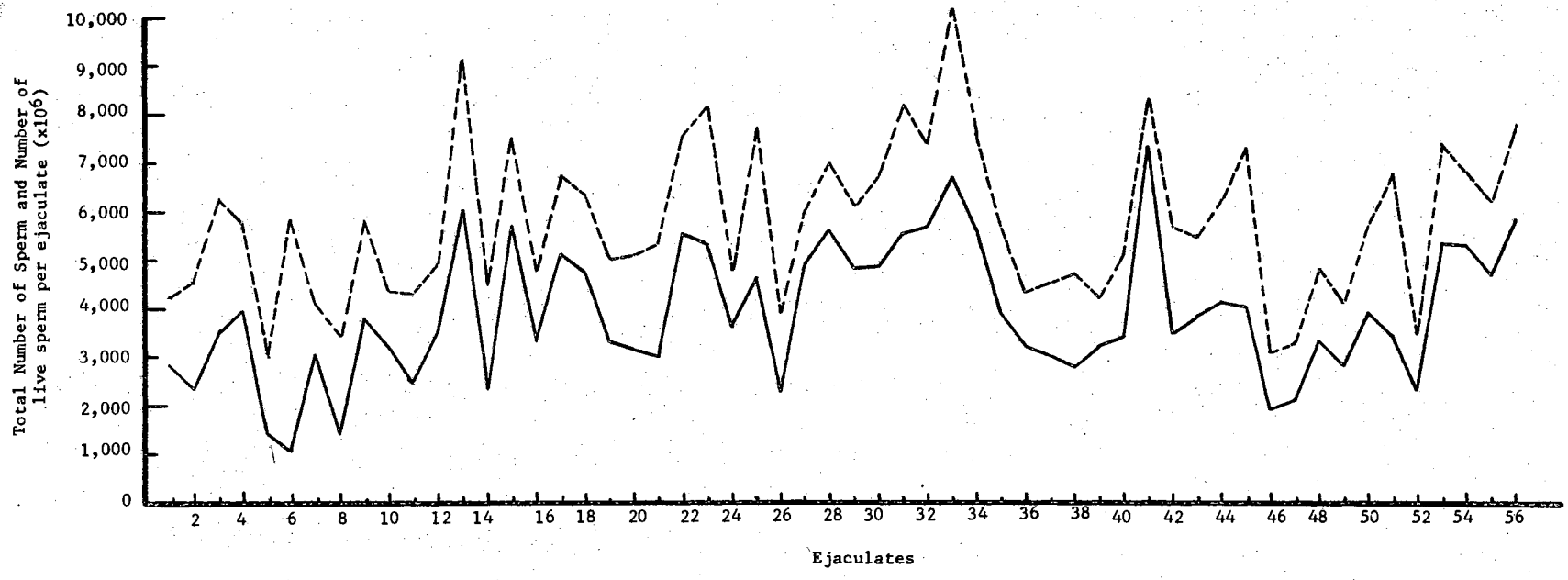


Figure 3. Weekly Averages of Total Number of Sperm (Broken Line) and Total Number of Live Sperm (Solid Line) in the Ejaculates of Four Bulls Ejaculated at Weekly Intervals for 56 Weeks.

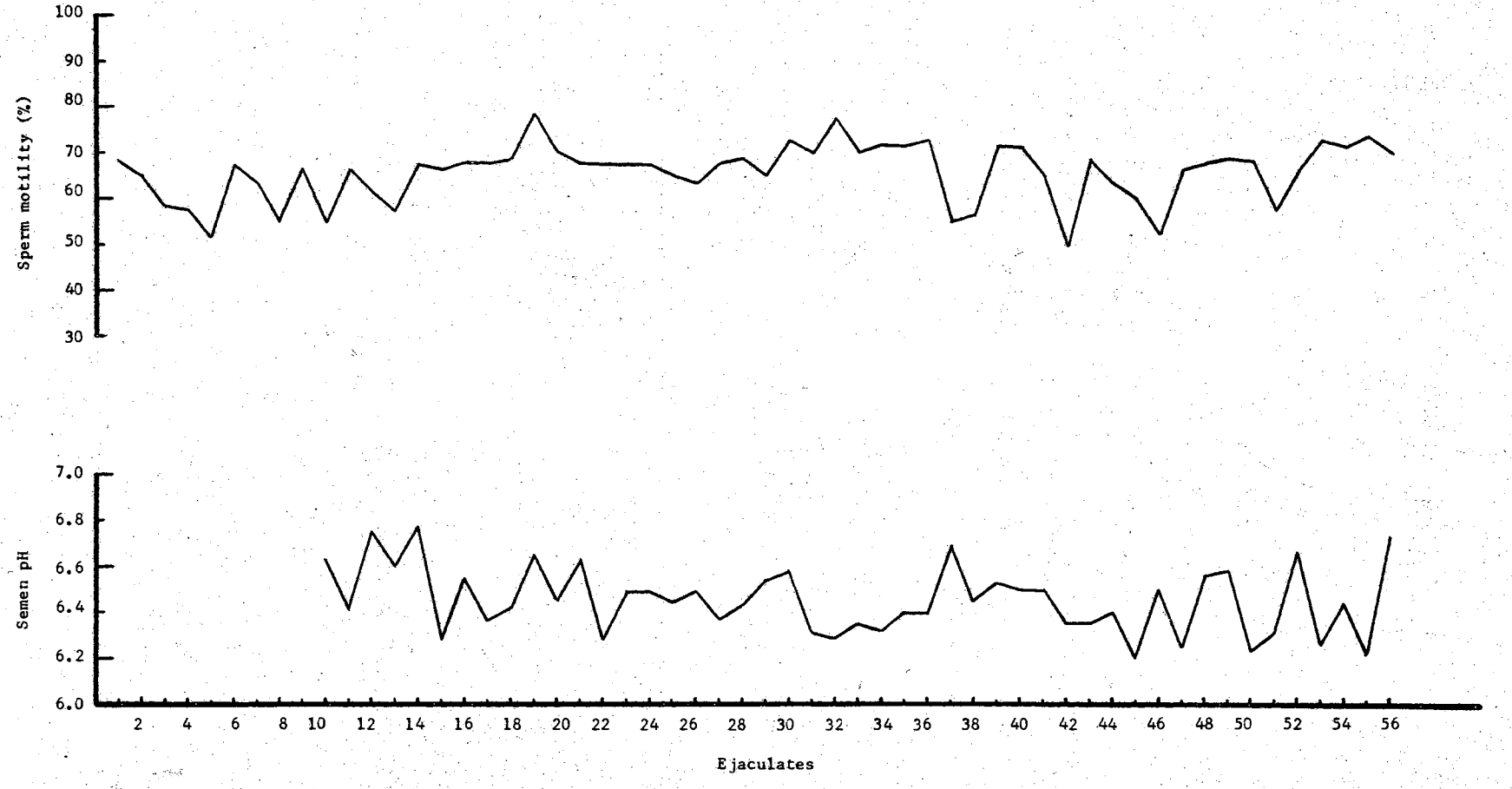


Figure 4. Weekly Averages of Sperm Motility and Semen pH in the Ejaculates of Four Bulls Ejaculated at Weekly Intervals for 56 Weeks.

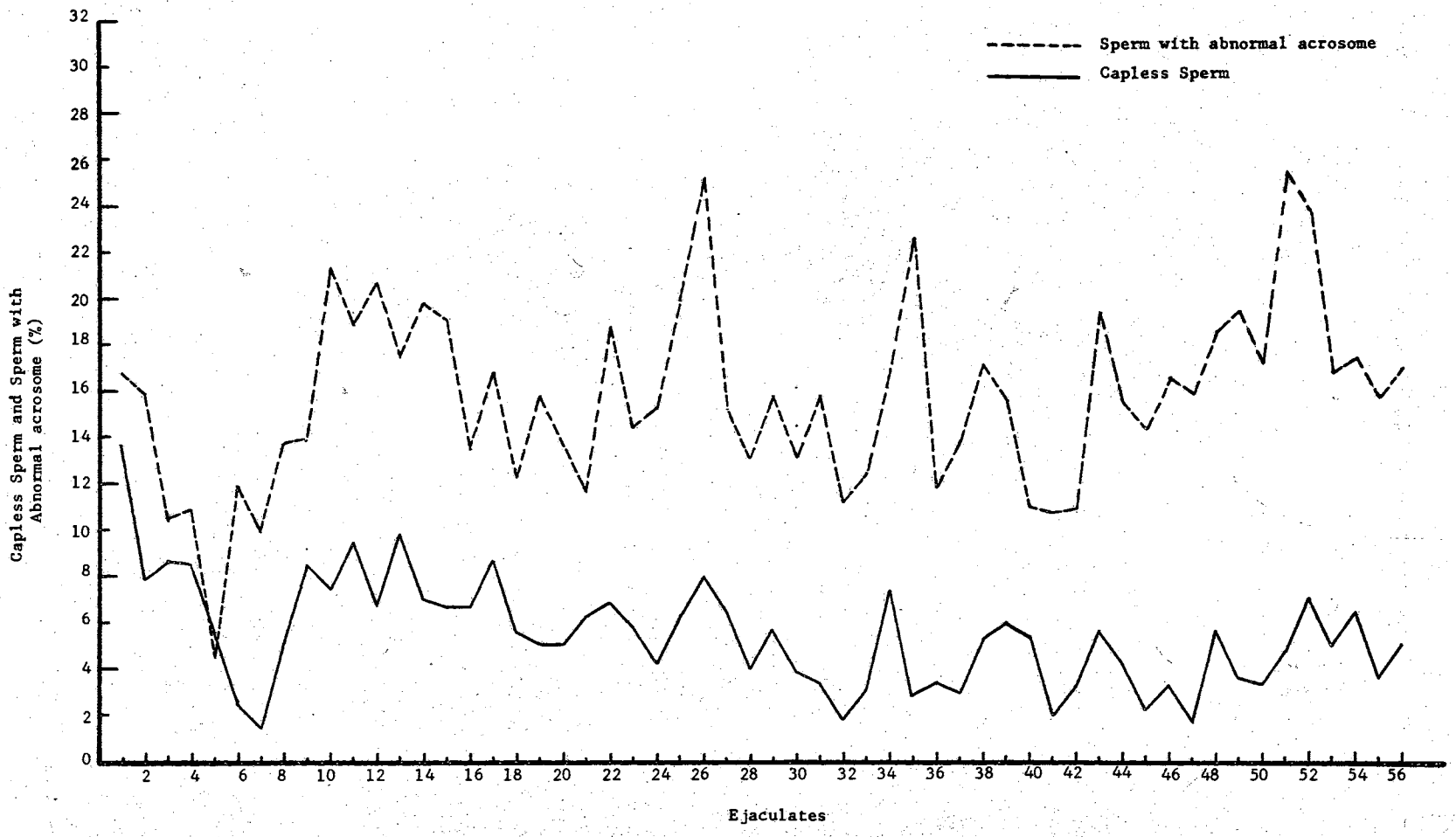


Figure 5. Weekly Averages of Capless Sperm and Sperm with Abnormal Acrosome Caps in the Ejaculates of Four Bulls Ejaculated at Weekly Intervals for 56 Weeks.

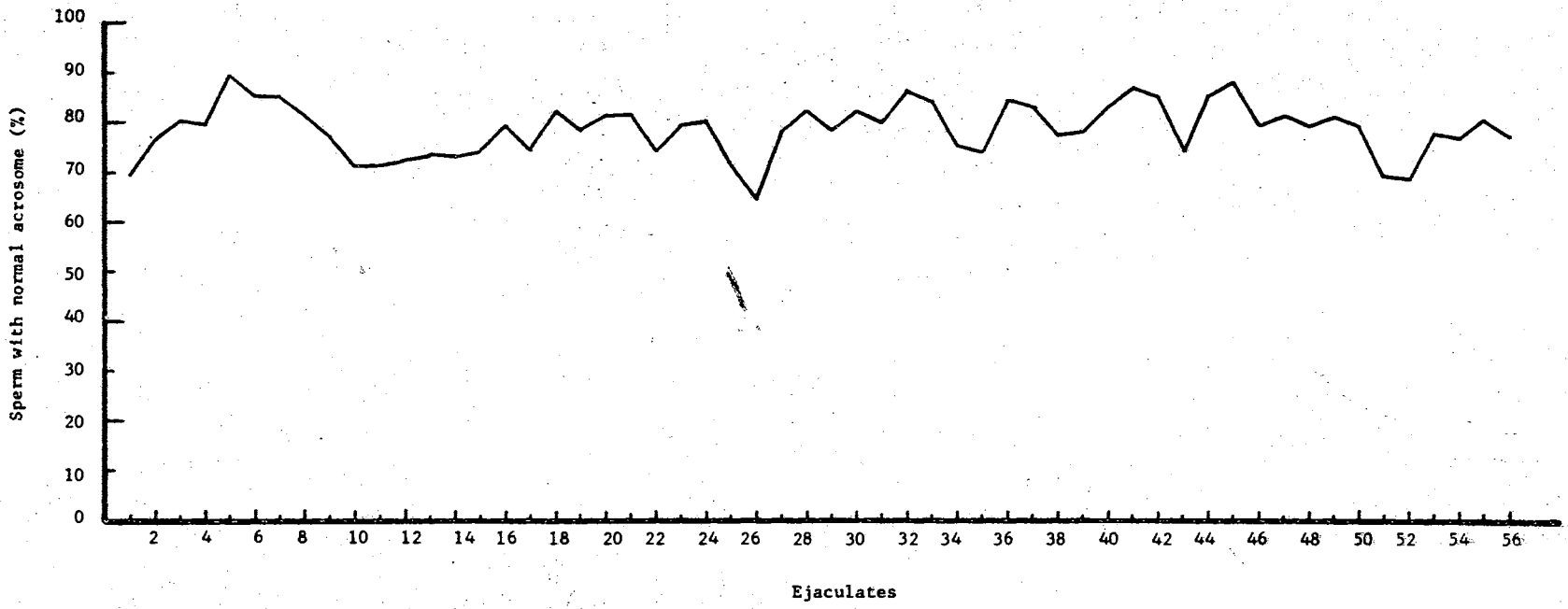


Figure 6. Weekly Averages of Sperm with Normal Acrosome Caps in the Ejaculates of Four Bulls Ejaculated at Weekly Intervals for 56 Weeks.

ejaculates of four bulls (A,C,J and K) for each semen criterion. Changes in the acrosomal state for each of the four bulls are shown in Figures 16 to 21. Acrosomal changes for the other two bulls (R and B) which were ejaculated for shorter periods of time are shown in Figures 22 and 23. The data obtained from the two latter bulls, however, were not included in the statistical analysis. The analyses of variance for each of the 12 semen characteristics studied are presented in Table I.

Examination of Figures 5 and 6, which present the average acrosomal changes during the 56 weeks of this study, and, of Figures 16 to 23 which show the individual changes in the acrosome state for each bull, clearly indicates that the acrosome cap is indeed a very labile structure that exhibits a great amount of variation from one ejaculate to the next. At this low level of semen collection, the overall average percentage of sperm cells having completely detached caps was 5.6. The overall average percentage of spermatozoa exhibiting morphological abnormalities of the acrosome cap was 15.9. Thus, an overall average of only 78.5 per cent of the sperm cells of these four bulls could be characterized as having apparently normal acrosome caps. It is evident from Figures 16 to 21 that not all bulls exhibited similar acrosomal changes from one week to another. This was confirmed by the analyses of variance (Table I) which showed that the differences in the levels of capless sperm and abnormal acrosomes among the four bulls were highly significant ($P < .005$) and that significant variation ($P < .01$) occurred among the weekly levels in these characteristics. Therefore, it appears that the suggestion of Saacke et al. (1968) of the existence of real week-to-week variation in the state of the acrosome cap is valid, and thus it is difficult to predict the acrosomal state of any particular

TABLE I

ANALYSES OF VARIANCE FOR SEMEN CHARACTERISTICS
OF FOUR BULLS EJACULATED AT WEEKLY INTERVALS
FOR 56 WEEKS

	Source of Variation	d.f.	Mean Squares	F	Level of Significance
Ejaculate Volume (ml.)	Bulls	3	96.08	22.497	$P < .005$
	Weeks	55	6.40	1.498	$P < .05$
	Bulls X Weeks	165	4.27		
Sperm Concentration per ml.	Bulls	3	5,142,640.15	34.292	$P < .005$
	Weeks	55	166,777.07	1.112	
	Bulls X Weeks	165	149,962.10		
Live Sperm (%)	Bulls	3	2,463.71	13.756	$P < .005$
	Weeks	55	346.70	1.936	$P < .01$
	Bulls X Weeks	165	179.09		
No. of Live Sperm per Ejaculate	Bulls	3	67,821,690.28	13.174	$P < .005$
	Weeks	55	7,439,845.18	1.445	$P < .05$
	Bulls X Weeks	165	5,147,877.12		

TABLE I (Continued)

	Source of Variation	d.f.	Mean Squares	F	Level of Significance
No. of Sperm per Ejaculate	Bulls	3	118,119,601.10	13.591	P < .005
	Weeks	55	9,882,114.99	1.137	
	Bulls X Weeks	165	8,690,823.98		
Sperm Motility (%)	Bulls	3	1,633.23	11.943	P < .005
	Weeks	55	144.79	1.059	
	Bulls X Weeks	165	136.75		
Sperm Motility (rate)	Bulls	3	1.23	5.009	P < .005
	Weeks	55	0.24	0.973	
	Bulls X Weeks	165	0.25		
Abnormal Sperm (%)	Bulls	3	7,171.37	203.133	P < .005
	Weeks	55	54.07	1.531	P < .05
	Bulls X Weeks	165	35.30		

TABLE I (Continued)

	Source of Variation	d.f.	Mean Squares	F	Level of Significance
Semen pH	Bulls	3	0.34	5.672	$P < .005$
	Weeks	46	0.09	1.427	$P < .05$
	Bulls X Weeks	138	0.06		
Capless Sperm (%)	Bulls	3	305.44	23.464	$P < .005$
	Weeks	55	21.69	1.666	$P < .01$
	Bulls X Weeks	165	13.02		
Sperm with Abnormal Acrosome (%)	Bulls	3	1,883.48	58.343	$P < .005$
	Weeks	55	58.68	1.818	$P < .01$
	Bulls X Weeks	165	32.28		
Sperm with Normal Acrosome (%)	Bulls	3	2,803.07	53.017	$P < .005$
	Weeks	55	102.40	1.939	$P < .01$
	Bulls X Weeks	165	52.87		

ejaculate at this level of sexual use. Although not tested, it can be assumed that the morphological state of the acrosome cap may be accompanied by changes in the physiologic and functional states of this structure and consequently with the functional state of the sperm cells. If so, this should also be reflected in the fertility level of the bull. This, of course, awaits further detailed investigations. The great weekly variations in the acrosomal state can be markedly reduced at higher levels of sexual use, and marked improvement in acrosomal characteristics can be obtained. This will be discussed further in a later section.

The weekly means of each semen characteristic studied are shown in Table II for each bull. As with the case of the acrosomal characteristics, most of the semen criteria studied showed considerable variation both among bulls and among weekly ejaculates. The differences among the four bulls were highly significant ($P < .005$) for every semen criterion measured. Significant differences in weekly ejaculate means were found for ejaculate volume ($P < .05$), per cent live sperm cells ($P < .01$), number of live spermatozoa in the ejaculate ($P < .05$), per cent abnormal sperm cells ($P < .05$), and semen pH ($P < .05$). No significant differences were observed among the means of weekly ejaculates of the four bulls with regard to sperm concentration per milliliter, total number of spermatozoa in the ejaculate, and sperm initial motility. With the exception of acrosomal characteristics, most of the criteria used in this study are typically used in artificial insemination units as indicators of semen quality. This mainly involves the determination of the volume of the ejaculate, the density of spermatozoa, initial sperm motility and the incidence of living spermatozoa. Many attempts

TABLE II

OVERALL AVERAGES OF SEMEN CHARACTERISTICS OF DAIRY BULLS COLLECTED ONCE WEEKLY

	Bulls					
	A[1]	B[2]	C[1]	J[1]	K[1]	R[3]
Ejaculate Volume (ml.)	7.0	4.5	6.4	4.0	6.0	5.9
Sperm Conc./ml. (X 10^6)	842.6	855.7	1346.6	1267.8	738.4	1391.5
Live Sperm (%)	67.6	69.8	66.6	55.8	71.1	77.7
No. Live Sperm/ Ejaculate (X 10^6)	4015.4	2715.3	5472.4	2976.9	3346.2	6141.2
No. Sperm/Ejaculate (X 10^6)	5800.5	3890.1	7861.1	5131.7	4520.9	7499.7
Sperm Motility (%)	68.7	75.0	63.0	59.8	71.8	76.8
Sperm Motility (rate)	3.6	3.8	3.3	3.4	3.6	3.8
Abnormal Sperm (%)	10.9	8.0	5.2	30.9	10.5	5.7
Semen pH	6.5	6.6	6.4	6.5	6.3	6.2
Capless Sperm (%)	4.3	2.4	4.5	9.1	4.4	3.2
Sperm with Abnormal Acrosome (%)	13.5	16.0	8.9	19.5	21.7	8.6
Sperm with Normal Acrosome (%)	82.2	81.6	86.6	71.4	73.9	88.2

[1]Each value represents the average of 56 ejaculates.

[2]Each value represents the average of 24 ejaculates.

[3]Each value represents the average of 38 ejaculates.

[4]Only the data on bulls A, C, J and K were used in the 56 week study. Bulls B and R were used in the short-term study on ejaculation frequency.

have been made to reveal how these characteristics are correlated with fertility. The results published are somewhat contradictory and are best summarized by Emmens and Blackshaw (1956) who indicated that no single ejaculate measurement was satisfactory for indicating ultimate fertility. In addition, many investigators have reported that several factors can affect the ejaculate characteristics, and that considerable differences among bulls and breeds exist. At the time being, it seems very probable that the acrosome cap should be evaluated in the course of the determination of quality of the semen and ultimately the level of fertility of the bull. This study has verified the existence of significant variation in acrosomal characteristics among bulls and among ejaculates of the same bull. Therefore, it appears that it is of a great importance to include the examination of the state of the acrosome cap in the routine tests of semen quality.

This portion of the study was also analyzed on a monthly basis to reveal possible seasonal effects on the ejaculate characteristics. Figure 7 shows the monthly averages of capless sperm cells and sperm with abnormal acrosomes in the ejaculates of the four bulls of the study. The monthly changes in the proportion of spermatozoa having morphologically normal acrosome caps are illustrated in Figure 8. Appendix Figures 24 to 27 show monthly changes in other semen characteristics of those bulls, and individual changes in acrosomal characteristics of each of those four bulls are shown graphically in appendix Figures 28 to 30. These figures also illustrate the great variation among months in the different ejaculate characteristics. These monthly differences were tested statistically, and the results are shown in Table IV. Highly significant differences ($P < .005$) among

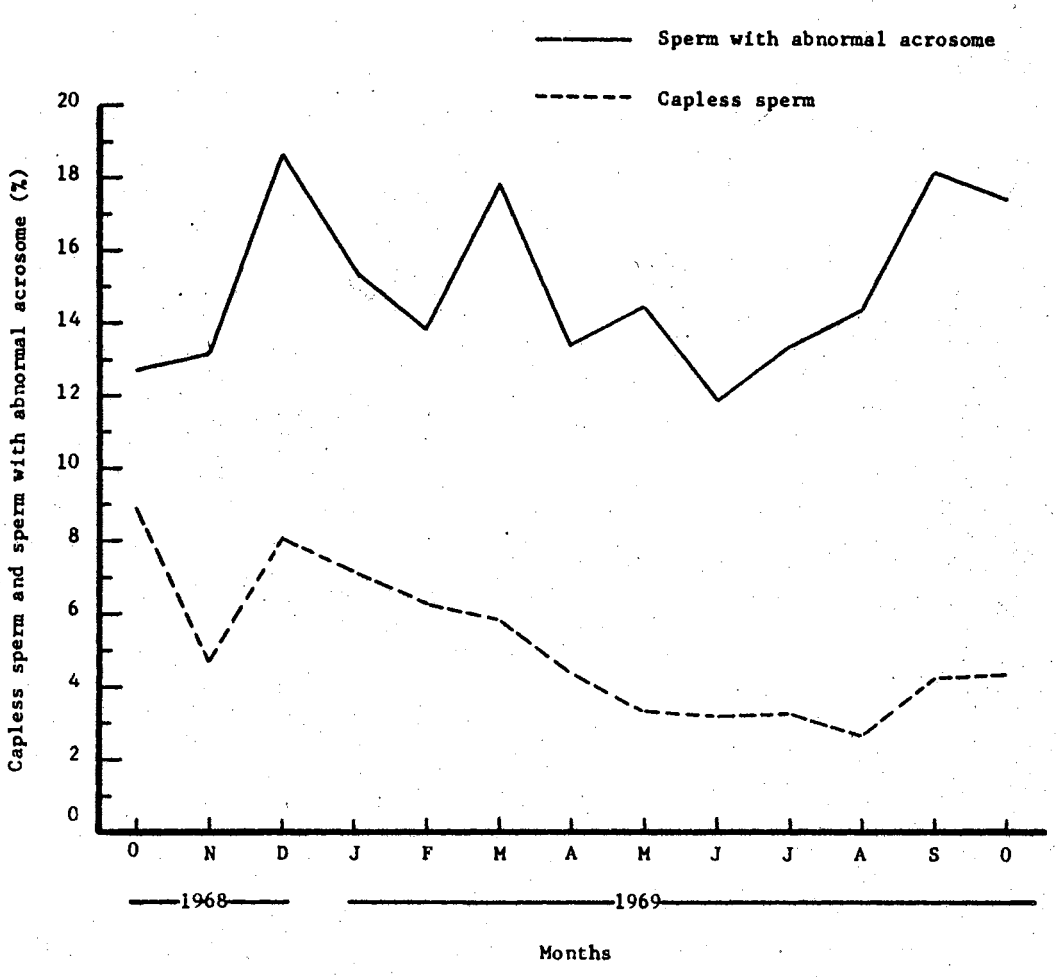


Figure 7. Monthly Averages of Acrosomal Anomalies in the Ejaculates of Four Bulls Ejaculated at Weekly Intervals.

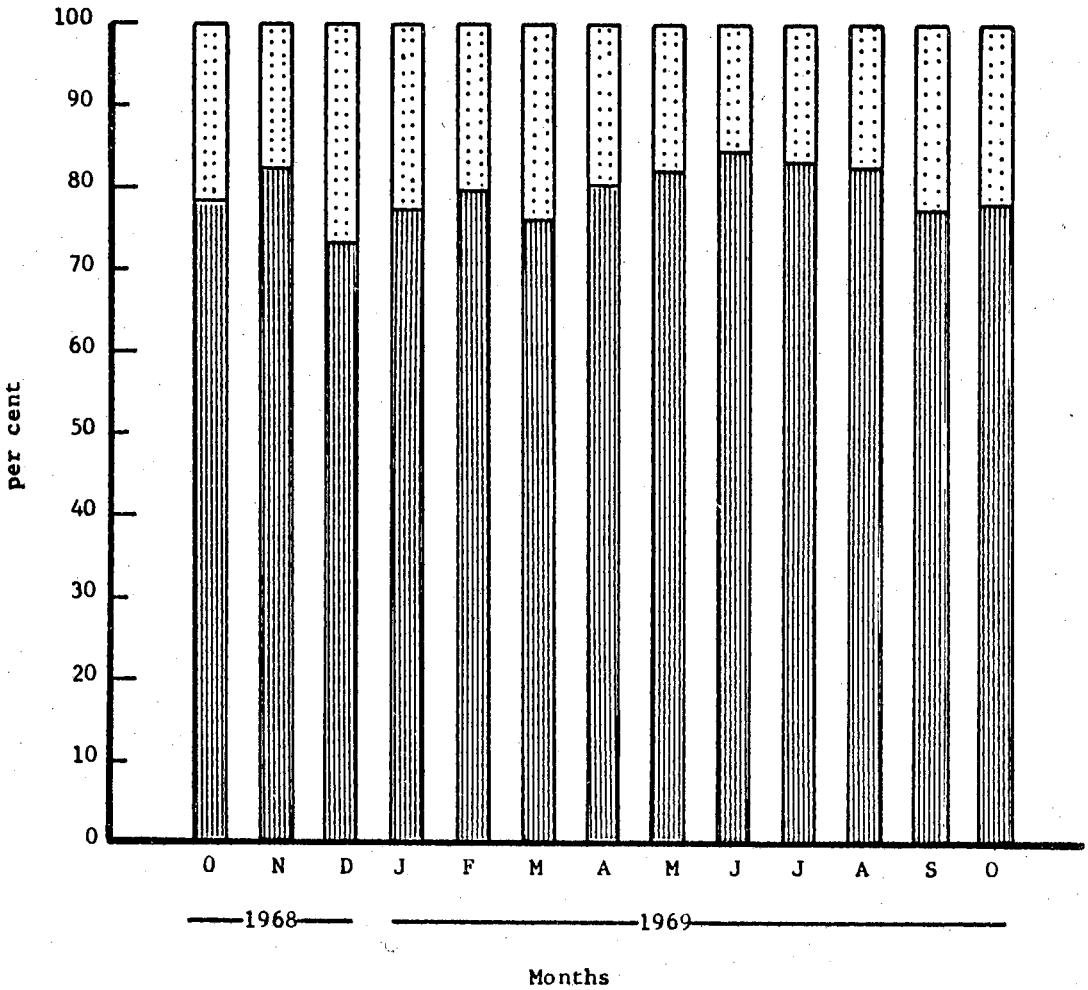


Figure 8. Monthly Averages of Sperm Cells Having Normal Acrosome Caps (Stripped bars) in the Ejaculates of Four Bulls Ejaculated at Weekly Intervals. Dotted Bars Indicate Total Acrosomal Abnormalities.

TABLE III

MONTHLY AVERAGES OF SEMEN CHARACTERISTICS OF FOUR BULLS
EJACULATED AT WEEKLY INTERVALS

	Oct. 68	Nov. 68	Dec. 68	Jan. 69	Feb. 69	March 69	April 69	May 69	June 69	July 69	August 69	Sept. 69	Oct. 69	Average ± S.E.
Ejaculate Volume (ml.)	4.3	4.6	5.0	5.2	5.5	5.1	6.9	7.8	8.1	6.0	5.6	5.4	5.9	5.8 ± 0.32
Sperm Conc./ml. (X 10^6)	1003.2	1034.6	1065.1	1191.0	1075.5	1339.3	1031.7	1038.8	798.0	1072.4	979.0	977.2	1060.9	1045.9 ± 37.70
Live Sperm (%)	57.3	58.4	61.4	72.8	60.3	64.5	74.4	73.1	60.5	71.7	60.9	55.9	71.2	64.8 ± 1.91
No. of Live Sperm/ejaculate (X 10^6)	2871.7	2758.4	3532.4	4731.5	3799.2	4036.8	5152.9	5502.2	2995.1	4579.5	2727.3	3483.7	4983.4	3934.9 ± 269.61
No. of Sperm/Ejaculate (X 10^6)	4750.5	4465.4	5471.3	6320.9	5788.7	6167.1	6902.5	7492.1	4430.8	6321.2	4600.8	5636.5	6383.8	5287.0 ± 705.32
Sperm Motility (%)	59.8	63.1	65.5	68.8	70.9	65.9	68.8	72.5	61.1	67.3	59.6	63.4	70.8	66.0 ± 1.17
Rate of Sperm Motility	3.2	3.2	3.4	3.6	3.6	3.6	3.6	3.6	3.2	3.4	3.2	3.4	3.7	3.4 ± 0.05
Abnormal Sperm (%)	16.7	16.2	10.8	17.4	12.5	14.0	10.3	12.2	13.7	14.2	14.1	15.4	17.0	14.2 ± 2.22
Semen pH	6.5	-	6.6	6.5	6.5	6.5	6.4	6.3	6.5	6.3	6.3	6.5	6.5	6.4 ± 0.17
Capless Sperm (%)	9.0	4.7	8.1	7.2	5.9	6.1	4.8	3.9	4.2	4.0	2.8	4.8	5.1	5.4 ± 0.49
Sperm with Abnormal Acrosome (%)	12.6	13.2	18.7	15.4	15.0	19.8	14.7	15.9	14.2	13.1	15.8	19.7	18.3	15.9 ± 0.69
Sperm with Normal Acrosome (%)	78.4	82.1	73.2	77.4	79.1	74.1	80.5	80.2	81.6	82.9	81.4	75.5	76.6	78.7 ± 0.87

TABLE IV

ANALYSES OF VARIANCE OF MONTHLY AVERAGES
OF SEMEN CHARACTERISTICS

	Source of Variation	d.f.	Mean Squares	F	Level of Significance
Ejaculate Volume	Bulls	3	21.15	12.98	$P < .005$
	Months	12	5.32	3.26	$P < .005$
	Bulls X Months	36	1.63		
Sperm Concentration	Bulls	3	1,131,978.28	20.41	$P < .005$
	Months	12	75,986.92	1.37	
	Bulls X Months	36	55,235.79		
Live Sperm (%)	Bulls	3	498.78	5.59	$P < .005$
	Months	12	184.68	2.07	$P < .05$
	Bulls X Months	36	89.18		
No. of Live Sperm per Ejaculate	Bulls	3	13,362,814.45	8.09	$P < .005$
	Months	12	3,624,439.88	2.19	$P < .05$
	Bulls X Months	36	1,650,716.30		

TABLE IV (Continued)

	Source of Variation	d.f.	Mean Squares	F	Level of Significance
No. of Sperm per Ejaculate	Bulls	3	23,682,422.72	9.16	$P < .005$
	Months	12	3,762,098.40	1.45	
	Bulls X Months	36	2,585,329.62		
Sperm Motility (%)	Bulls	3	402.94	6.29	$P < .005$
	Months	12	81.25	1.27	
	Bulls X Months	36	64.02		
Sperm Motility (rate)	Bulls	3	0.34	3.09	$P < .05$
	Months	12	0.15	1.36	
	Bulls X Months	36	0.11		
Abnormal Sperm (%)	Bulls	3	1,575.10	98.93	$P < .05$
	Months	12	9.54	0.58	
	Bulls X Months	36	15.92		
Semen pH	Bulls	3	0.08	3.07	$P < .05$
	Months	10	0.03	1.18	
	Bulls X Months	30	0.03		

TABLE IV (Continued)

	Source of Variation	d.f.	Mean Squares	F	Level of Significance
Capless Sperm (%)	Bulls	3	71.51	17.23	$P < .005$
	Months	12	12.70	3.06	$P < .005$
	Bulls X Months	36	4.15		
Sperm with Abnormal Acrosomes (%)	Bulls	3	54.83	4.38	$P < .025$
	Months	12	24.82	1.98	$P < .10$
	Bulls X Months	36	12.52		
Sperm with Normal Acrosomes (%)	Bulls	3	593.63	27.37	$P < .005$
	Months	12	44.92	2.06	$P < .05$
	Bulls X Months	36	21.72		

monthly averages of capless sperm cells were observed, with this defect showing a general decline with months. Significant differences ($P<.10$) were also observed between monthly averages of sperm cells having abnormal caps. However, no definite trend was observed and several monthly peaks were noticed. Conversely, significant differences ($P<.05$) in the average level of normal acrosome caps were also observed. Bull differences were also highly significant ($P < .025$) with regard to all these characteristics. Table III shows that the range of monthly averages of normal acrosomes varied between 73.2 and 82.9 per cent. The highest total acrosomal abnormalities (capless sperm and abnormal acrosome) were observed in December, 1968 and March, 1969 (26.8 and 25.9 per cent, respectively). Figure 8 indicates that spring and early summer are more favorable than fall and winter in their effect on the acrosome state.

Ejaculate volume, the percentage of live cells and the number of live sperm per ejaculate also exhibited statistical differences ($P<.05$) when grouped on a monthly basis (Table IV) with spring and early summer again appearing to be the more favorable time.

Interrelationships of Ejaculate Characteristics

Table V shows the different linear correlation coefficients among the semen characteristics studied. These coefficients are based on the 56 weekly averages obtained from the four bulls of this study.

Examination of the correlation coefficients presented in Table V reveals several expected significant correlations. The number of sperm per milliliter is negatively correlated with ejaculate volume ($r=-0.58$, $P < .01$). The percentage of live spermatozoa is significantly

TABLE V

INTERRELATIONSHIPS OF SEMEN CHARACTERISTICS IN THE EJACULATES OF FOUR BULLS COLLECTED AT WEEKLY INTERVALS

	Ejaculate Volume (ml)	Sperm Conc. per ml.	Live Sperm (%)	No. Live Sperm per Ejaculate	No. of Sperm per Ejaculate	Abnormal Sperm (%)	Semen pH	Capless Sperm (%)	Sperm with Abnormal Acrosome	Sperm with Normal Acrosome
Ejaculate Volume (ml.)										
Sperm Conc. per ml.	-0.58[a]									
Live Sperm (%)	0.34[a]	0.21								
No. Live Sperm per Ejaculate	0.45[a]	0.57[a]	0.71[a]							
No. Sperm per Ejaculate	0.40[a]	0.68[a]	0.46[a]	0.93[a]						
Abnormal Sperm (%)	-0.09	-0.08	-0.22	-0.21	-0.10					
Semen pH	-0.21	-0.29[b]	-0.31[b]	-0.37[a]	-0.38[a]	0.22				
Capless Sperm (%)	-0.27	0.15	-0.13	-0.14	-0.08	0.23	0.41[a]			
Sperm with Abnormal Acrosome (%)	-0.09	0.07	-0.09	-0.09	-0.05	0.09	0.16	0.29[b]		
Sperm with Normal Acrosome (%)	0.20	-0.11	0.12	0.13	0.08	-0.17	-0.29[b]	-0.68[a]	-0.89[a]	

a) $P < .01$
b) $P < .05$

correlated with ejaculate volume ($r = 0.45$, $P < .01$). The total number of live spermatozoa per ejaculate is also significantly correlated with ejaculate volume ($r = 0.45$, $P < .01$), with sperm concentration ($r=0.57$, $P < .01$) and percent live spermatozoa ($r = 0.71$, $P < .01$). This is in agreement with the results reported by Bishop et al. (1954), and other workers. Different correlations between semen volume and sperm density have been reported. A value of 0.355 was reported by Davis and Williams (1939); -0.235 (Rashwan, 1953); and -0.035 (Lasely and Bogart, 1943), as cited by Salisbury (1955). The collection procedures and frequency of collection can affect this relationship greatly. For instance, it is a known fact that electroejaculation is usually characterized by a larger ejaculate volume and decreased sperm density (Wells, 1962). The number of sperm cells per ejaculate was significantly correlated with semen volume ($r = 0.40$, $P < .01$) and the number of live spermatozoa per ejaculate ($r = 0.93$, $P < .01$). The incidence of abnormal sperm cells in the ejaculate seemed to be independent of other semen characteristics. This agrees with the results obtained by Rashwan (1953) and Van Demark et al. (1945) who found no significant correlation between per cent abnormal sperm and sperm concentration and semen volume (Cited by Salisbury, 1955).

Semen pH was negatively correlated with sperm concentration ($r = -0.29$, $P < .05$), the percentage of live sperm cells ($r = -0.31$, $P < .05$), the number of live sperm per ejaculate ($r = -0.37$, $P < .01$) and the number of sperm per ejaculate ($r = -0.37$, $P < .01$).

It is of particular interest to note that no significant correlation was detected between the state of the acrosome cap and any other ejaculate characteristics, except semen pH, a measurement not routinely

used in bull stud laboratories. There was a significant positive correlation between semen pH and the proportion of capless spermatozoa ($r = 0.41$, $P < .01$), and between the latter and the level of sperm cells having abnormal acrosome caps ($r = 0.28$, $P < .05$). Conversely, there was a significant negative correlation between semen pH and the level of normal acrosomes ($r = -0.29$, $P < .05$). This indicates that an increase in semen pH is associated with an increase in detached acrosomes and therefore a decrease in the proportion of normal acrosome in the ejaculate. No explanation is available at the present time for such a relationship. This suggests that the micro-environment surrounding the sperm cells at the time of ejaculation is important in determining the acrosomal state of the sperm cells. Semen pH is typically determined by the secretions of the accessory sex organs, with their functions in turn being influenced by the degree of stimulation, ejaculation frequency and other factors. Further definitive research is needed to reveal the interplay of these various factors. It is interesting to note that Salisbury (1955) has stated that semen pH appears to show a consistent relationship to other semen characteristics, and he quoted research results indicating negative correlations between semen pH and ejaculate volume, sperm concentration per milliliter, and initial sperm motility. It perhaps should be stated at this point that although the correlation between semen pH and acrosomal state in the ejaculates were statistically significant, they were not high enough to be used as predictors of acrosomal state.

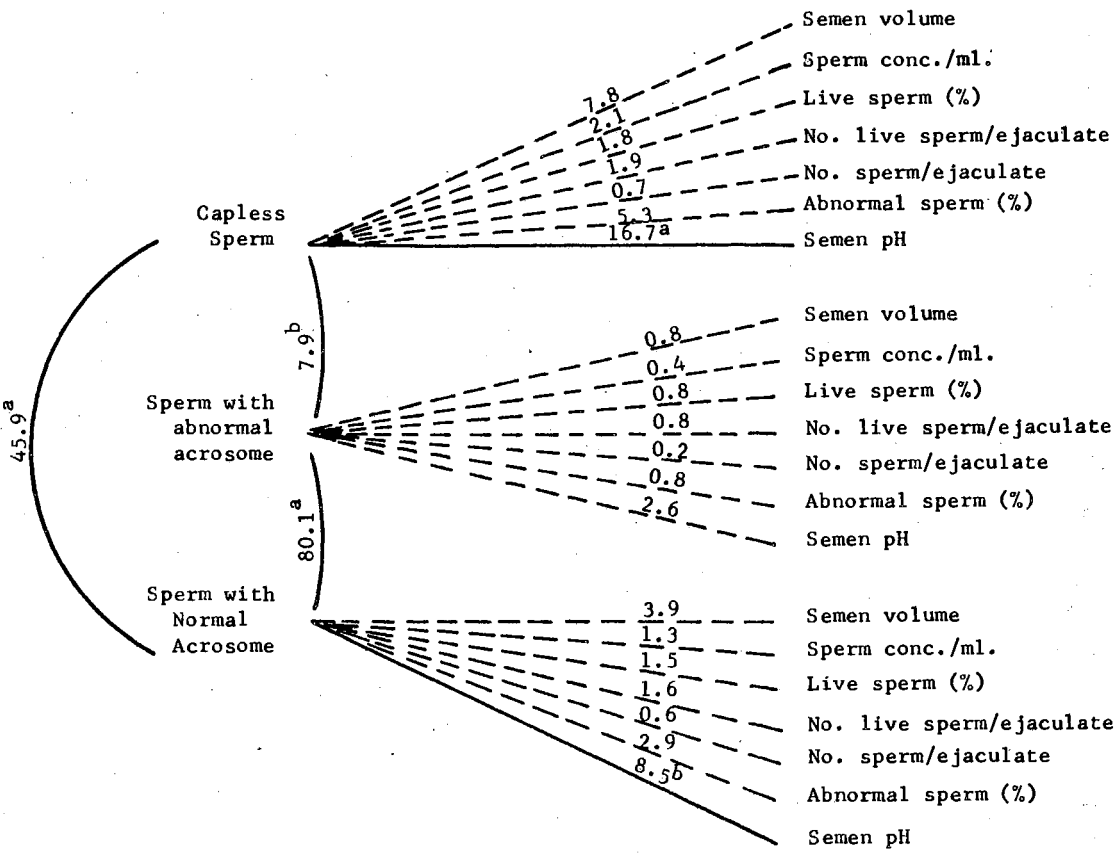
The measurements of the degree of correlation among different semen characteristics are of particular interest since this provides information on whether two measurements vary together or whether they are

independent of each other. Obviously, when two characters are highly correlated, one may measure one of them and disregard the other, with the measurements being obtained via the character that can easily and accurately be determined. As previously discussed, only semen pH had any significant degree of correlation with the acrosomal state of an ejaculate. This suggests the independence of acrosomal characteristics from other ejaculate criteria and indicates the necessity of obtaining precise measurements on the characteristics of the acrosome cap in ejaculated spermatozoa.

Another measure of the "closeness" of relationship is the coefficient of determination ($100\ r^2$). This index shows the amount of variation in one character explained by the variation in another correlated character. The coefficients of determination for acrosomal characteristics and other semen measurements are illustrated in Figure 9 which shows that 16.7 per cent of the variation in capless sperm and only 8.5 per cent of the variation in normal acrosomes is explained by variations in semen pH. The remainder of the indices relating to other ejaculate characteristics are shown in Figure 10. These values reflect the patterns and relationships previously discussed.

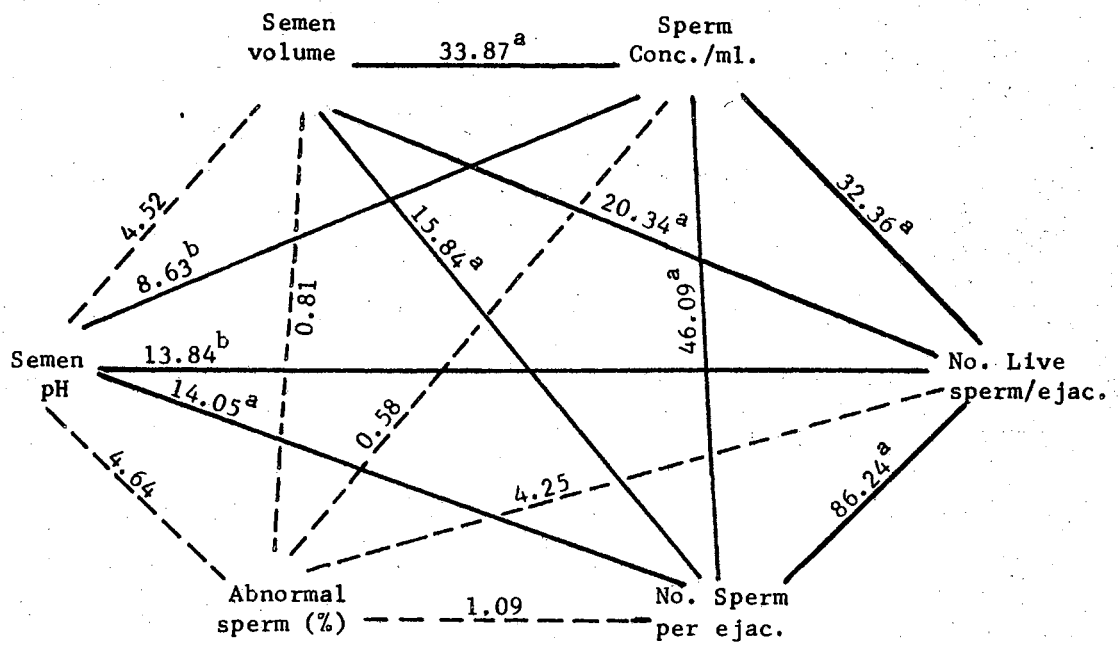
Effects of Ambient Temperature on Acrosomal Characteristics of Weekly Ejaculates

Salisbury and VanDemark (1961) stated that "it is a common experience for weather conditions on the day of collection to have striking effects on measured semen values." Also, Clegg and Ganong (1959) reported that very high temperatures were associated with a depression of spermatogenesis with an occasional lag of about one month before the



a: $P < .01$
b: $P < .05$

Figure 9. Coefficients of Determination Showing the Percentages of Variation in Acrosomal Characteristics Explained by the Variation in Other Ejaculate Criteria.



a: $P < .01$
b: $P < .05$

Figure 10. Coefficients of Determination Showing the Interrelationships Between Ejaculate Characteristics, Other than the Acrosome State.

reduction in semen quality was observed. This decrease in semen quality was observed to last for as long as two months after the hottest period of the year.

Using the 56 weeks of data on the 4 bulls collected once weekly, an attempt was made to relate acrosomal anomalies with:

1. Average temperature of the week preceding and including the collection day.
2. Average temperature of the day of semen collection and,
3. Average temperature two weeks prior to semen collection.

The correlation coefficients obtained are listed in Table VI which shows that there were significant negative correlations between capless sperm and the temperature averages studied. Stronger coefficients were

TABLE VI

CORRELATION BETWEEN TEMPERATURE AVERAGES

AND ACROSOMAL ANOMALIES

	Capless Sperm (%)	Sperm with Abnormal Acrosome (%)
Average Temperature (Collection Week)	-0.37	0.14
Average Temperature (Collection Day)	-0.30	0.11
Average Temperature (2 Weeks Prior to Semen Collection)	-0.38	0.19

obtained between the percentage capless sperm and the temperature average on collection week ($r = -0.37$, $P < .01$), and two weeks prior to

semen collection ($r = -0.38$, $P < .01$) than on collection day ($r = -0.30$, $P < .05$), probably indicating a lag period in the effect of low temperature on the incidence of capless sperm cells. No significant correlation was found between the level of abnormal acrosomes and temperature. These correlation values correspond well with the fact that the levels of capless sperm cells were highest in the fall and winter and lowest in spring and summer (Figure 7). In the winter months, it seems that the combination of low temperature and windy conditions led to increased levels of acrosomal anomalies. The available data were investigated for this possibility.

The wind chill factor is a numerical index and is descriptive terminology for expressing the combined effect of wind and cold temperature in cooling the exposed parts of the body (Siple and Passel, 1954). This index is known to be useful in cold climates as a quantitative measure of the cooling power of the environment consistent with subjective experience of cooling and discomfort. It was possible to calculate wind chill index values for 21 consecutive weeks from November, 1968 to March, 1969. Using these 21 weeks of data on the four bulls collected weekly, correlation coefficients were determined between acrosomal anomalies and:

1. Average wind chill index of the week preceding and including collection day;
2. Average wind chill index of the day of semen collection, and,
3. Average wind chill index two weeks prior to semen collection.

The correlation values obtained are presented in Table VII. It is interesting to observe that there was no significant correlation between acrosomal anomalies and any of the wind chill indices with the

TABLE VII

CORRELATION BETWEEN WIND CHILL INDEX

AND ACROSOMAL ANOMALIES

	Capless Sperm (%)	Sperm with Abnormal Acrosome (%)
Wind Chill Index (Collection Week)	0.01	-0.16
Wind Chill Index (Collection Day)	0.06	-0.01
Wind Chill Index (2 Weeks Prior to Semen Collection)	-0.63	-0.02

exception of a highly significant negative correlation between the wind chill index two weeks prior to semen collection and the incidence of capless sperm ($r = -0.63$, $P < .01$). This suggests that an increase in wind chill index values (towards positive values) is associated with a decrease in capless sperm and vice versa (Figure 11), and that there is a time lag in the effect of cold temperature on the acrosome cap. Since this two weeks period is approximately the length of time required for passage of sperm cells through the epididymis, it appears that the cells are vulnerable to the effect of wind and low temperature during their passage through the epididymis. These data also suggest that the incidence of abnormal acrosome caps is not associated with the effect of low temperature.

These results, however, should be taken as suggestive indications since the number of animals and observations involved were limited. The effect of ambient temperature and wind conditions on acrosomal and other semen characteristics may be associated with several other envi-

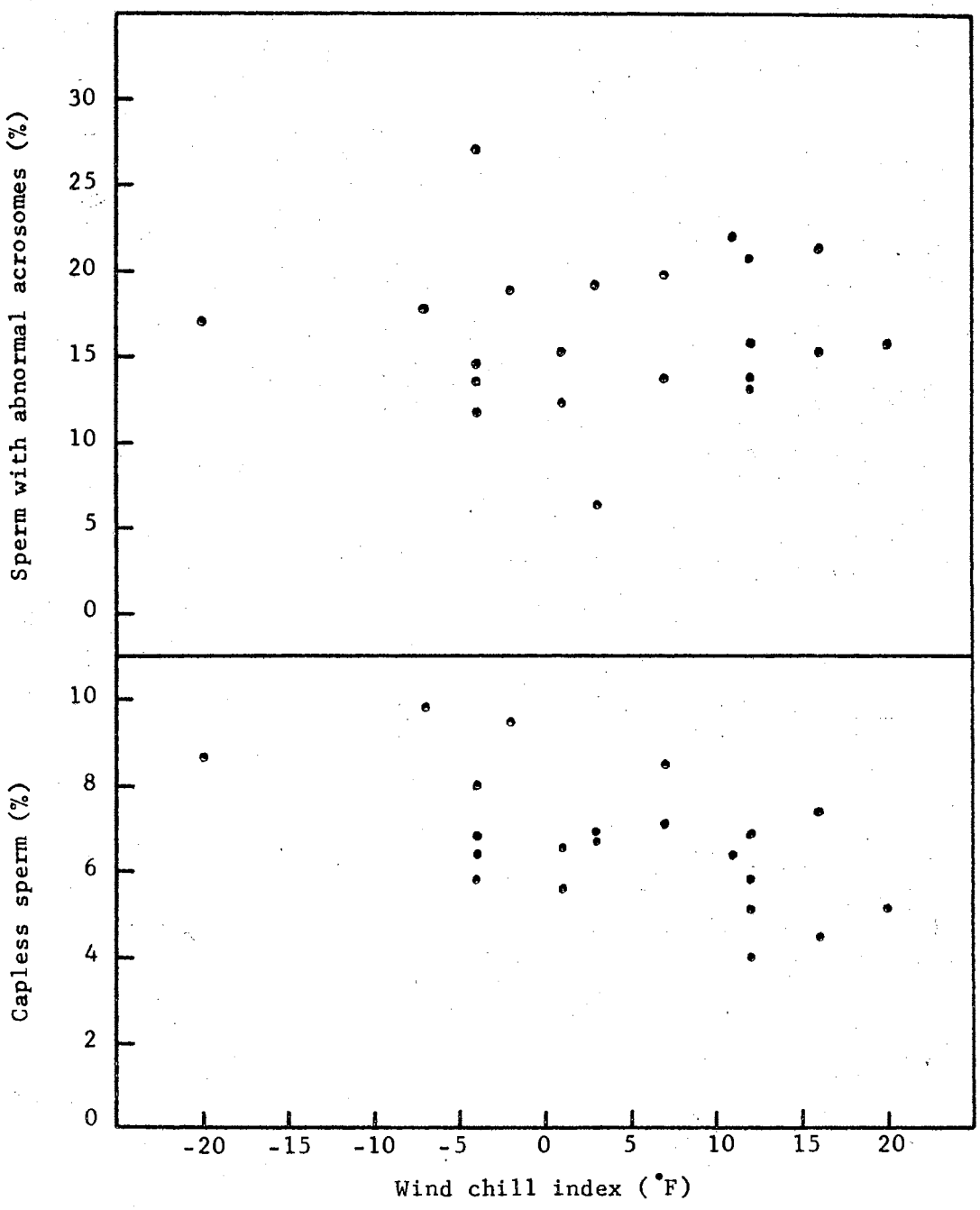


Figure 11. A scatter Diagram of the Levels of Acrosomal Anomalies at Certain Wind Chill Index Values, 2 Weeks Prior to Semen Collection.

ronmental factors. This study, however, indicates that acrosomal integrity is sensitive to ambient temperature. Further research is needed to determine the influence of environmental circumstances on acrosomal characteristics and define the stage of the occurance of this effect, i.e., whether such effect occurs at the spermatogenic level or during the transport and storage of the sperm cells through the epididymis.

Effects of Frequency of Ejaculation and Sexual Rest on the Acrosome Cap

Increasing the usefulness of outstanding sires may be accomplished by increasing the frequency of semen collection and consequently harvesting greater numbers of healthy spermatozoa from the male. Increasing the rate of semen dilution may aid in increasing the number of females that can be bred artificially from any one ejaculate of a sire. However, there are practical limitations to this procedure. In the usual routine of artificial breeding centers, semen is usually collected once or twice weekly. Increasing the frequency of semen collection would be, therefore, a useful means of increasing the usefulness of the sire, especially when one considers the short lifespan of the male. The literature contains many studies on the effect of high ejaculation frequency on the quality of semen. No study, however, is available on the effect of such treatment on the acrosome cap of spermatozoa, although the close relationship between the acrosome cap and the male's fertility has been confirmed in several studies. The difficulty, as has been mentioned elsewhere, appears to be the lack of an efficient procedure for evaluating acrosomal state and accurately detecting its characteristics and abnormalities.

There also exist the belief that a period of sexual rest may be beneficial to the bull. The practice of allowing bulls certain periods of sexual rest is used in some artificial breeding centers. This is probably used more in beef bulls. Previous work at Oklahoma State University has shown that the first ejaculates of beef bulls collected after prolonged sexual rest had low acrosomal quality (Wondafrash, 1968). Some other reports have indicated that sexual rest had a detrimental effect on semen characteristics. Thus, it was felt that this practice may be detrimental rather than beneficial.

In view of this, it was decided to carry out a preliminary investigation on the effects of ejaculation frequency and sexual rest on the acrosome cap of the bull. The six bulls available were divided into two equal groups. The bulls of group 1 were changed, without a sexual rest period, from once a week ejaculation frequency (1X/wk) to four times per week (4X/wk). In this group, semen was collected from each bull twice on each of two days per week, (Mondays and Thursdays). Ten first and ten second ejaculates were collected from each of the three bulls. The bulls of group 2 were placed in sexual rest for six weeks and then collected at a frequency of four times per week until their semen quality was similar to that of group 1. The mean effects of these treatments on the total acrosomal anomalies are shown graphically in Figure 12. This figure illustrates that sexual rest has a rather drastic effect on the acrosomal state. The averages for capless sperm, abnormal acrosomes and sperm with normal acrosome in the weekly ejaculates of the bulls in group 2 for the four weeks preceding sexual rest were 2.6 ± 0.13, 15.5 ± 0.84 and 81.9 ± 0.87 per cent, respectively. The same characteristics in the average of the ejaculates of the first

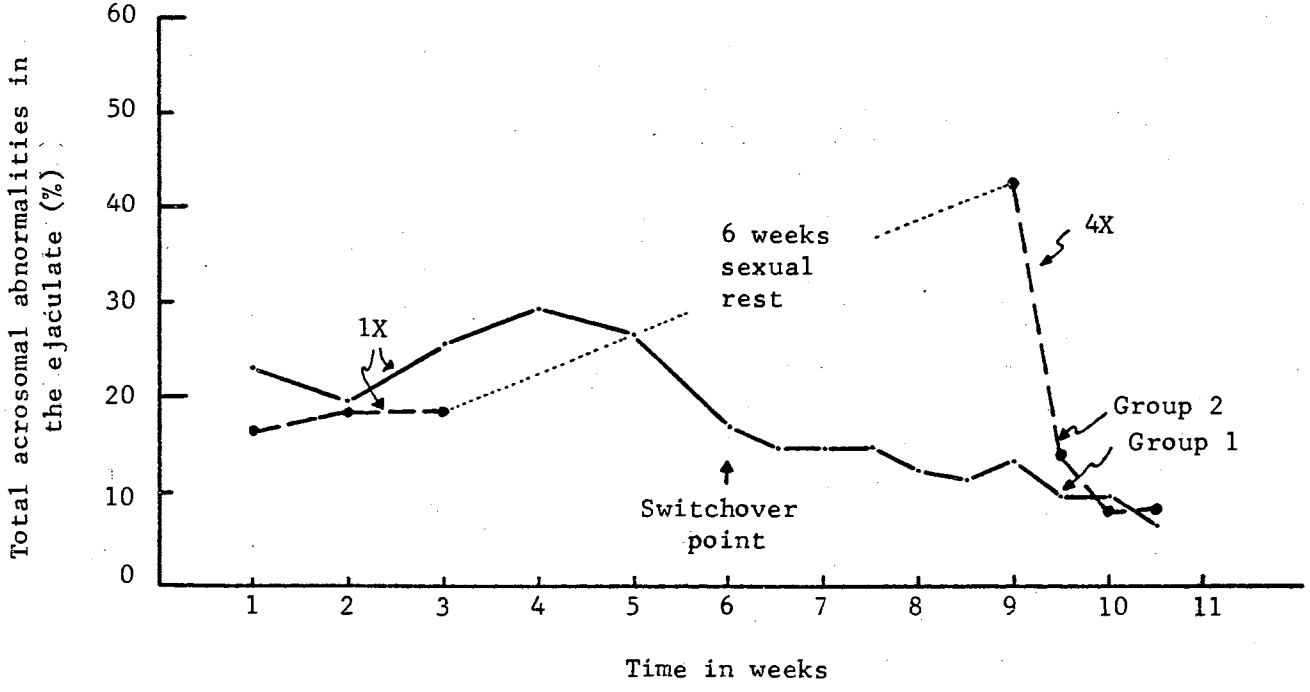


Figure 12. Effect of High Ejaculation Frequency on the Incidence of Total Acrosomal Anomalies With and Without a Period of Sexual Rest

collection following the six weeks sexual rest were 11.3, 30.9 and 57.8 per cent, respectively. However, the ejaculation frequency of 4X/wk. for two weeks (following the sexual rest) reduced these high levels of acrosomal anomalies to levels comparable to those of group 1 which had been on the 4X/wk. collection schedule for five weeks. No statistical difference was observed at the end of this period between the two groups. Following the increase in ejaculation frequency for the bulls of group 1 (from 1X to 4X/wk), with no sexual rest period, it was observed that acrosomal anomalies tended to decline and to show less ejaculate-to-ejaculate variation. The averages of 10 first and 10 second ejaculates obtained from group 1 within a five week period were 2.7 ± 0.20 per cent capless sperm, 9.1 ± 0.94 per cent sperm with abnormal acrosome caps, and 88.2 ± 1.08 per cent sperm with normal acrosomes. This was compared to averages of 5.0 ± 0.48, 18.7 ± 1.21, and 76.3 ± 1.10 per cent, for the same acrosomal characteristics in the weekly ejaculates of the same three bulls for the 10 weeks prior to increasing the frequency of ejaculation. Thus, there was a 46 per cent decrease in the incidence of capless sperm and a 51 per cent decrease in the proportion of abnormal acrosomes at the higher level of ejaculation. The differences between acrosomal characteristics of these two frequencies were statistically significant ($P < .005$), strongly indicating that there was an improvement in acrosomal characteristics upon increasing the frequency of ejaculation in this group of bulls.

The two groups of bulls were then allowed a sexual rest period for four weeks during which no semen collection was made. Two bulls were eliminated from the study following this period because of refusal to serve the artificial vagina, and the remaining four bulls, two within

each group, were assigned at random to either a frequency of 4X/wk. or 1X/wk. The groups were collected at these frequencies for one month and then the groups were switched, with no rest period, with the once weekly bulls changing to four times weekly and the four times weekly changing to the once weekly rate. The bulls were collected for another month at these rates. The results of these treatments are shown in Figures 13, 14, and 15. It was observed that this period of four weeks sexual rest again had detrimental effects on the acrosomal state. The percentage of capless sperm (Figure 13) for the two groups increased from 1.3 per cent and 1.4 per cent in the average of the last two ejaculates prior to sexual rest to 3.6 per cent and 4.6 per cent in the average of the first two ejaculates following that period. The proportion of abnormal caps for each group (Figure 14) also increased from 3.9 and 6.4 per cent to 33.2 and 31.5 per cent, respectively. Conversely, the proportions of normal acrosome (Figure 15) decreased from 94.8 and 92.2 per cent to 63.2 and 63.9 per cent, respectively. This clearly illustrates a deterioration in acrosomal quality due to this four week period of abstinence.

The effects of ejaculation frequency on this deteriorated state of the acrosome is obvious in Figures 13 and 14. Figure 13 presents the data relative to the effects on the levels of capless sperm cells. The groups were within 1 per cent of each other on the first ejaculate following sexual rest. However, the bulls collected 4X/wk. (group 1) dropped sharply from 3.6 per cent to 1.0 per cent in the four week collection period. Group 2, collected once weekly, also showed a decline, but were only down to 2.1 per cent after four weeks (4 ejaculates) of collection. This difference between the groups was statistically signi-

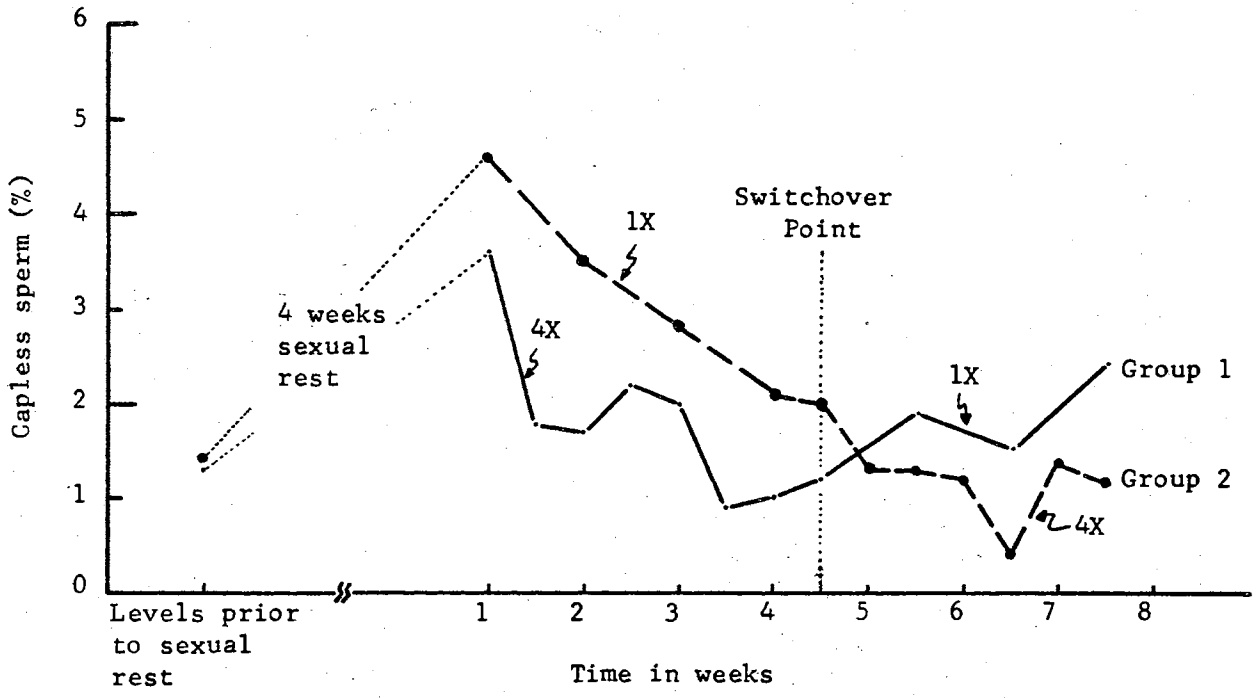


Figure 13. Level of Capless Sperm Cells at Two Frequencies of Ejaculation Following Four Weeks of Sexual Rest.

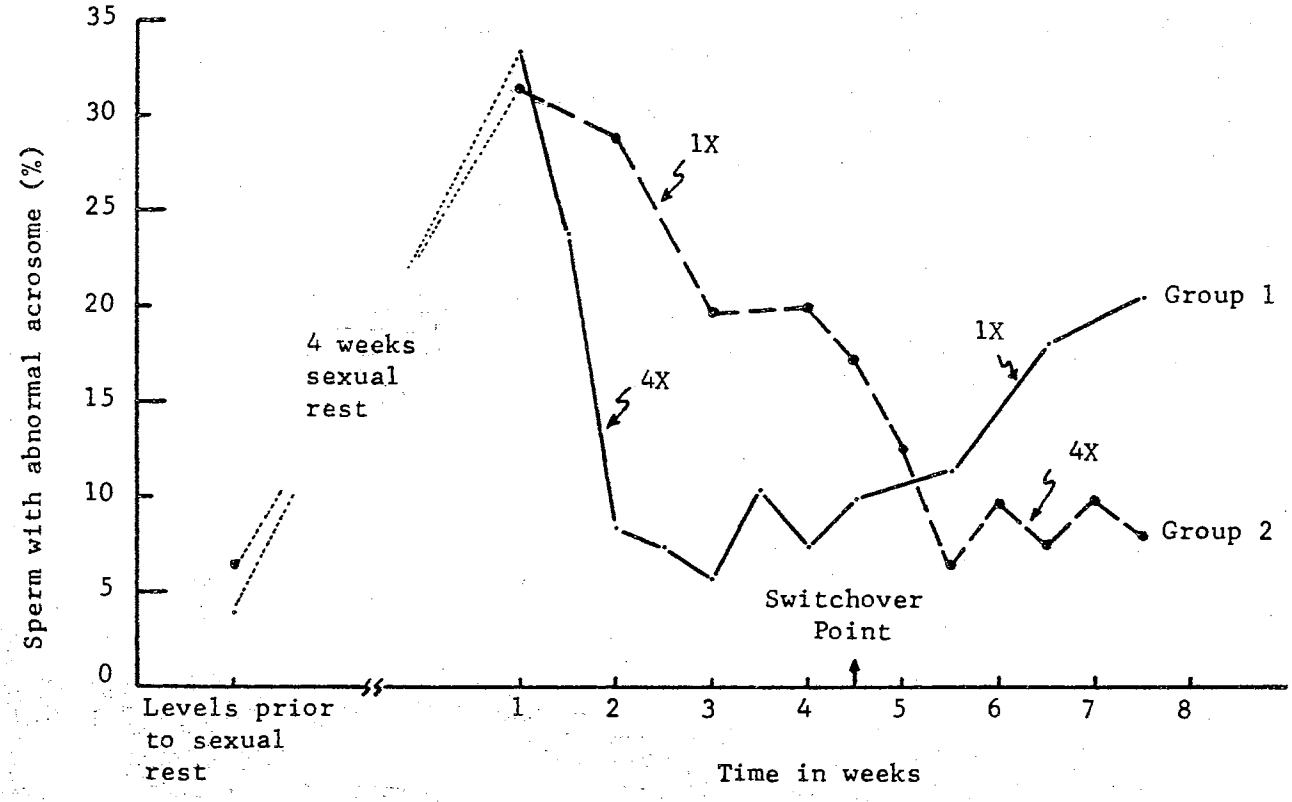


Figure 14. Levels of Sperm Cells Having Abnormal Acrosome Caps at Two Frequencies of Ejaculation Following Four Weeks of Sexual Rest.

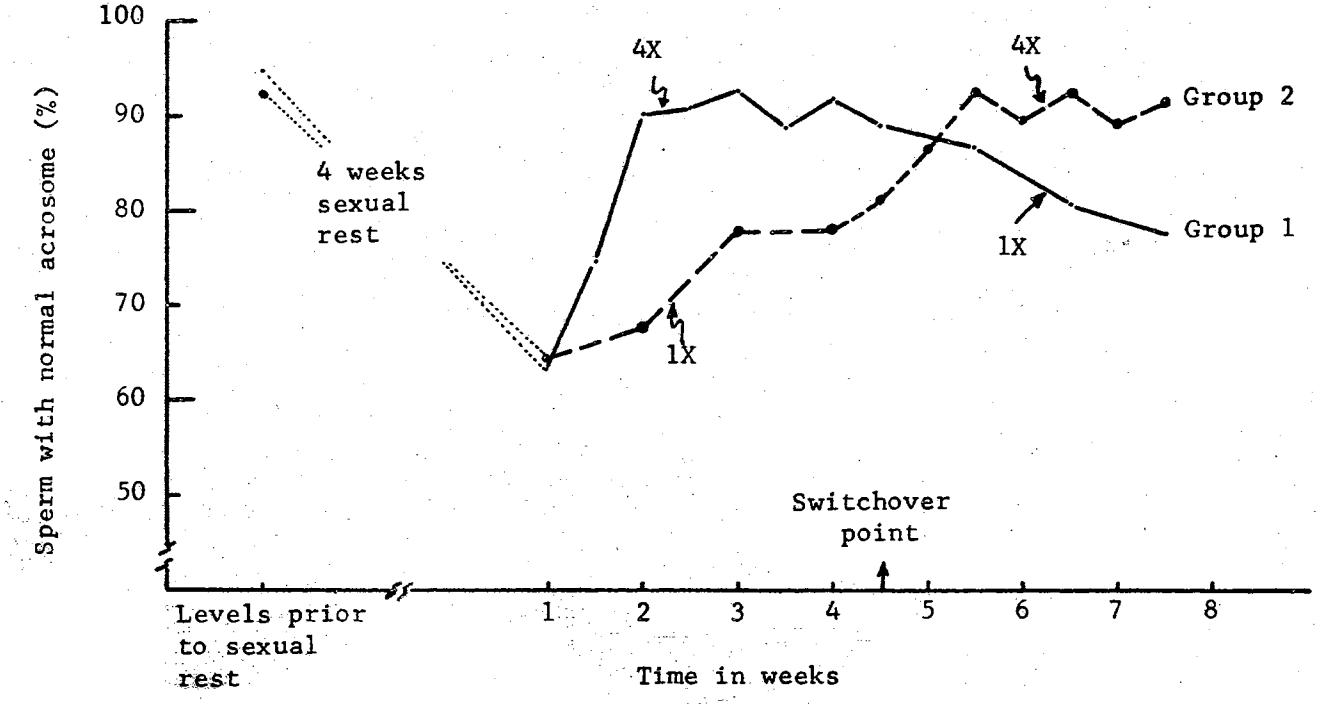


Figure 15. Levels of Sperm Cells Having Normal Acrosome Caps at Two Levels of Ejaculation Frequencies Following Four Weeks of Sexual Rest

ficant ($P < .05$). At this point ejaculation frequency changed. At the 4X/wk. level, the group previously collected once weekly (group 2) continued to decline in percentage capless sperm and finished the study four weeks later at about the 1.2 per cent level. The other bulls (group 1), switched from the high to the low level of ejaculation showed a reversal in their pattern with the percentage capless cells rising from 1.0 per cent to 2.4 per cent at the end of the study. The group means were not significant for this interval. However, the trends observed are more important than the lack of significance. The difference between the means of the last ejaculates of the two groups was statistically significant ($P < .05$) (Duncan's new multiple range test--Steel and Torrie, 1960).

The percentages of sperm with abnormal acrosome caps showed similar, but magnified trends in the two intervals following sexual rest (Figure 14). The group collected at 4X/wk. after the period of sexual rest (group 1) showed a marked decrease from 33.2 per cent to 7.3 per cent abnormal caps, and was significantly lower ($P < .10$) than the group collected 1X/wk. which exhibited a slow decline from 31.4 to 17.1 per cent abnormal acrosomes. The more striking effect of frequency of collection was observed when the groups were switched. At the 1X/wk. level, the bulls of group 1, previously collected 4X/wk. rose markedly to 20.1 per cent abnormal acrosomes. This group was significantly higher ($P < .005$) than the group switched from 1X/wk. to 4X/wk. which decreased to 7.8 per cent abnormal acrosomes at the end of the study.

A comparison of the sensitivity of the acrosome cap and other characteristics of the ejaculate to the level of ejaculation frequency is presented in Table VIII. This table shows the weekly averages of the bulls

TABLE VIII

WEEKLY AVERAGES OF EJACULATE CHARACTERISTICS
AT TWO LEVELS OF EJACULATION FREQUENCY*

	Average of Five Weekly Ejaculates Prior to Increasing Ejaculation Frequency	Average of Four Ejaculates per Week for Five Weeks Following the Increase in Ejaculation Frequency	Per Cent Increase (+) or Decrease (-) after Increasing Ejaculation Frequency	P
Ejaculate Volume (ml.)	5.0	3.9	-22.3	<.05
Sperm Concentration per ml. ($X10^6$)	1153.8	979.3	-15.4	<.05
Live Sperm (%)	68.3	65.7	- 3.8	N.S.
No. Live Sperm per Ejaculate ($X10^6$)	4116.6	2920.9	-29.0	<.005
No. Sperm per Ejaculate ($X10^6$)	5787.6	3756.2	-35.1	<.005
Sperm Motility (%)	69.9	67.0	- 4.2	N.S.
Abnormal Sperm (%)	16.3	14.8	- 8.9	N.S.
Semen pH	6.3	6.5	+ 3.1	<.05
Capless Sperm (%)	4.8	2.7	-43.7	<.05
Sperm with Abnormal Acrosomes (%)	20.0	9.1	-54.5	<.005
Sperm with Normal Acrosomes (%)	75.2	87.9	+16.9	<.005

* Each Value is the Average of Three Bulls

of group 1 for the five weeks prior to any increase in ejaculation frequency and the weekly averages of the four ejaculates obtained from those bulls after increasing the frequency of ejaculation to 4X/wk. It is evident that the sensitivity of the acrosome cap to the increased frequency exceeded any of the other ejaculate characteristics. Table IX shows that the level of capless sperm showed a 43.7 per cent decrease ($P < .05$) and a 54.5 per cent decrease ($P < .005$) in the proportion of sperm cells having abnormal acrosome caps upon increasing the level of ejaculation frequency. There also was a significant increase in semen pH ($P < .05$), while other semen characteristics showed different amounts of decline upon increasing ejaculate frequency, which agree in general with the results of similar studies reported in the literature (Baker et al., 1955; Hafs et al., 1959; Hupp et al., 1962; Almquist and Cunningham, 1967). The major declines were a 22.3 per cent decrease in ejaculate volume ($P < .05$), 15.4 per cent decrease in sperm density per milliliter ($P < .05$), 29.0 per cent decrease in the number of live sperm per ejaculate ($P < .005$), and a 35.1 per cent decrease in the total number of sperm per ejaculate ($P < .005$). However, considering the four ejaculates per bull per week, there were 310.8 per cent increase in the volume of semen secured, 283.8 per cent increase in the number of live spermatozoa, 259.6 per cent increase in the total number of sperm cells, and 324.0 per cent increase in the number of motile sperm obtained per bull per week following the increase in the level of ejaculation frequency from one to four ejaculates per week. Although the number of observations included in this comparison is small, these results are quite comparable with results reported on larger number of bulls. These results indicate that increasing the

level of ejaculation had beneficial effects on the acrosomal state as well as on other semen characteristics. The greatest effect appeared to be a dramatic reduction in the incidence of acrosomal anomalies. It is interesting to note that the percentages of live sperm and abnormal sperm showed no significant change in the same period of time, indicating that the increase in ejaculation frequency had no significant harmful effects on these criteria. It is certainly difficult to draw definite conclusions from the results obtained from a small number of experimental animals. However, the observations obtained during these preliminary manipulations of the frequency of semen collection and sexual rest indicate that certain effects may be observed. Sexual rest had dramatic detrimental effects on acrosomal characteristics, and the first few ejaculates following such a period had high levels of acrosomal abnormalities. High ejaculation frequency, however, had beneficial effects on acrosomal quality and reversed the harmful effects of sexual rest within a short period of time. This is supported by the observation that the 56 weekly averages of acrosomal anomalies of four bulls (A, C, J and K) were high, with averages of 5.6 per cent capless sperm and 15.9 per cent abnormal acrosomes.

No conclusive reasons can be offered explaining the high levels of acrosomal abnormalities at low levels of semen collection (weekly ejaculation) or following a period of sexual rest. However, considering the labile nature of the acrosome cap and its apparent susceptibility to internal and/or external environmental circumstances, it again is postulated that the acrosome cap is affected by epididymal conditions and/or ambient temperature during the period of time between semen collection. It is most likely that these acrosomal abnor-

malities and detached caps represent the first stages of sperm degeneration. Thus, at higher frequencies of ejaculation, the sperm cells will spend less time in the epididymis and therefore there will be less opportunity for acrosomal integrity to be altered by prevailing conditions.

Characteristics of First and Second Ejaculates

During this study, it was observed that the second ejaculate consistently contained less acrosomal anomalies than the first ejaculate at any particular day of semen collection. Excluding the first and second ejaculates of each bull immediately following any sexual rest, 25 first and 25 second ejaculates were compared, with the results shown in Table IX. The first ejaculate contained 2.5 per cent capless sperm and was significantly higher ($P < .005$) than the second ejaculate which averaged 1.6 per cent capless cells. Also, the second ejaculate contained less ($P < .10$) sperm with abnormal acrosome (8.2 per cent) than did the first ejaculate (10.2 per cent). Consequently, the second ejaculate contained significantly more sperm with normal acrosomes (90.2 per cent, $P < .025$) than did the first ejaculate (87.3 per cent). Table IX also shows that there were no significant differences between the first and second ejaculates concerning ejaculate volume, per cent live spermatozoa, initial sperm motility, and per cent abnormal sperm cells. First ejaculates were significantly higher ($P < .05$) in the number of sperm and number of live sperm per ejaculate. This agrees with the results reported by MacMillan et al. (1967) who found no significant difference in ejaculate volume between the first and second ejaculates but they reported significantly more sperm cells

TABLE IX

CHARACTERISTICS OF 25 FIRST AND
25 SECOND EJACULATES OF BULLS*

	First Ejaculate Mean ± S.E.	Second Ejaculate Mean ± S.E.	P
Ejaculate Volume (ml.)	4.6 ± 0.22	4.8 ± 0.24	N.S.
Sperm Conc./ml. (X 10^6)	858.7 ± 67.94	677.2 ± 65.74	<.10
Live Sperm (%)	68.1 ± 1.27	64.0 ± 2.46	N.S.
No. Live Sperm/Ejaculate (X 10^6)	3377.8 ± 243.51	2298.9 ± 274.79	<.05
No. Sperm/Ejaculate (X 10^6)	4485.4 ± 371.95	3451.4 ± 340.01	<.05
Initial Motility (%)	66.4 ± 1.16	65.7 ± 1.67	N.S.
Abnormal Sperm (%)	11.9 ± 0.71	12.6 ± 1.03	N.S.
Semen pH	6.5 ± 0.16	6.6 ± 0.27	<.10
Capless Sperm (%)	2.5 ± 0.16	1.6 ± 0.15	<.005
Sperm with Abnormal Acrosomes (%)	10.2 ± 0.86	8.2 ± 0.70	<.10
Sperm with Normal Acrosomes (%)	87.3 ± 0.92	90.2 ± 0.74	<.025

* Each Ejaculate Mean is the Average of 4 Bulls.

in the first ejaculate than in the second.

These data clearly indicate that multiple collection on each collection day will result in better average quality of the semen collected. This fact coupled with the gain in total sperm cells per week leaves little doubt that multiple ejaculations per collection day (within limits) will result in greater utilization of the bull's sperm cell producing ability.

CHAPTER V

SUMMARY AND CONCLUSIONS

It has been pointed out in the preceeding chapters that acrosomal anomalies may result in impaired fertility or even complete sterility, and that the acrosomal state is not typically evaluated in routine semen examination.

The Wells-Awa acrosome stain (Awa, 1968; Wells and Awa, 1970) was utilized in a series of preliminary investigations on the acrosome cap of bovine spermatozoa. Four bulls were ejaculated at weekly intervals for 56 weeks and the morphological state of the acrosome cap in their ejaculates was examined as well as certain other ejaculate characteristics. The results of this portion of the study indicated that the weekly ejaculates of these bulls contained an overall average of 5.6 per cent capless sperm cells and 15.9 per cent sperm with abnormal acrosome caps, and therefore only 78.5 per cent of the sperm cells were characterized by apparently normal acrosome caps. There were significant differences among the weekly ejaculates concerning the incidence of capless sperm and sperm with abnormal acrosome caps ($P<.01$). Significant difference also existed among bulls. Significant differences with regard to those acrosomal anomalies were also observed when the data were analyzed on a monthly basis. The percentage capless sperm showed a general decline in the spring and summer months while abnormal acrosomes had several peaks and did not exhibit an apparent

pattern. The percentage of sperm cells with normal caps was higher in the spring and early summer than in the fall and winter. This indicates that, at this level of sexual use, there is a great amount of variation from one ejaculate to the next with regard to acrosomal characteristics and most of the other semen criteria examined.

The incidence of acrosomal anomalies in the weekly ejaculates appeared to be independent of any other ejaculate characteristic except semen pH. There was a significant correlation between semen pH and capless sperm cells ($r = 0.41$, $P < .01$), and between semen pH and the percentage of sperm having normal acrosome caps ($r = -0.29$, $P < .05$). Significant correlation ($r = 0.28$, $P < .05$) was also detected between the two classes of acrosomal anomalies. Semen pH was also significantly correlated with sperm concentration per ml. ($r = -0.31$, $P < .05$), the number of live sperm per ejaculate ($r = -0.37$, $P < .01$) and the number of sperm per ejaculate ($r = -0.37$, $P < .01$). Other interrelationships of ejaculate characteristics, excluding the acrosomal state, agree in general with those reported in the literature.

No significant correlation was observed between the incidence of abnormal acrosome caps and the average temperature of the collection week, collection day, or two weeks prior to semen collection. On the other hand, there were significant correlations between the weekly levels of capless sperm cells and those temperature averages. The strongest correlation was between the level of capless sperm and the average temperature two weeks prior to semen collection ($r = -0.38$, $P < .01$). Also, it was observed that there was a highly significant correlation between this class of acrosomal anomalies and the wind chill index two weeks prior to semen collection ($r = -0.63$, $P < .01$). These

correlation coefficients between semen pH and the acrosomal state, and between ambient temperature and the acrosomal characteristics indicate that the acrosomal integrity at this level of sexual use is subject to the effects of the internal and/or external environment of the sperm cells. These also suggest that there may be a lag period in the effect of ambient temperature on the acrosome cap. Further research is needed to explore the nature of such effects and the time of its occurence.

The incidence of acrosomal abnormalities could be greatly decreased by increasing the level of ejaculation frequency. Several manipulations of two ejaculation levels (once weekly and four times per week) consistently showed that increasing the frequency to four times per week greatly reduced the proportions of both capless sperm and sperm with abnormal acrosomes, and, therefore, resulted in marked improvement in apparent acrosomal quality. Once a week ejaculation frequency, on the other hand, showed reverse effects and thus a decline in acrosomal quality comparable to that observed during the 56 weeks of weekly ejaculates. The acrosome cap appeared to be very sensitive to the level of ejaculation as compared to other semen characteristics. After increasing the frequency of semen collection it was also observed that the second ejaculate on any particular collection day contained less acrosomal anomalies than did the first ejaculate. A deterioration of the acrosomal state was observed after allowing the bulls periods of sexual rest, with both classes of acrosomal anomalies increasing. This effect could, however, be reversed by routine semen collection, with quicker recovery of acrosomal quality being obtained at four ejaculation frequency per week than at once weekly.

The apparent independence of acrosomal state from other ejaculate characteristics indicates the unique nature of the acrosome and consequently confirms the suggestions of Awa (1968) and Wells and Awa (1970) of the necessity of the examination of the acrosomal state in the ejaculate as an important part in routine semen evaluation.

This study indicates that the acrosomal state is labile and may change markedly from one ejaculate to the next. The apparent acrosomal susceptibility to internal and/or external environmental influences and management practices may well indicate the need for more useful manipulation of the environment in securing consistently higher levels of male fertility. The reported studies also indicate that the specific stain developed for assessing acrosomal anomalies is a simple and very useful means of detecting the acrosomal state in ejaculated spermatozoa.

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APPENDIX

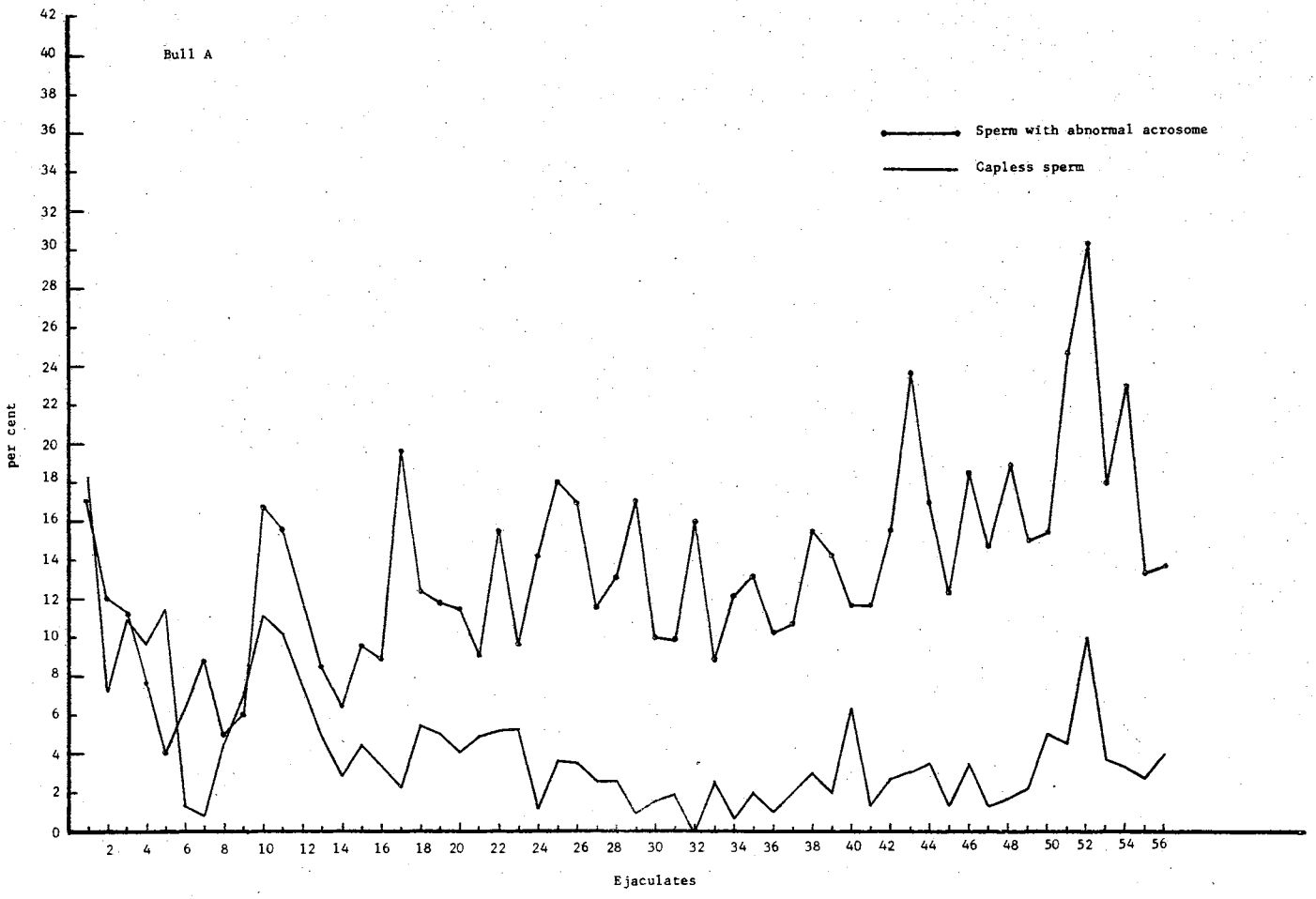

Figure 16. Weekly Averages of Capless Sperm and Sperm with Abnormal Acrosome Caps in the Ejaculates of Bull A.

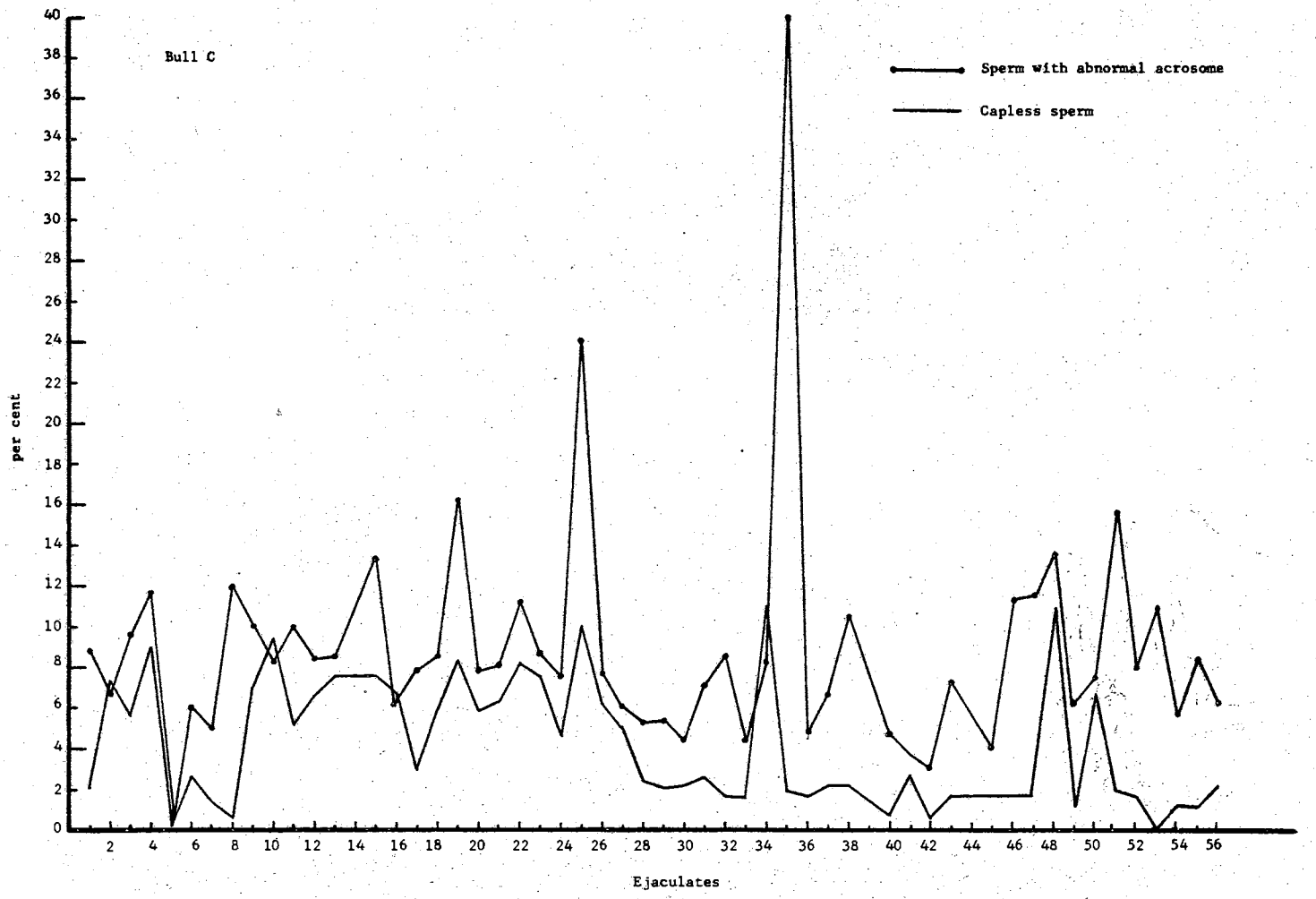

Figure 17. Weekly Averages of Capless Sperm and Sperm with Abnormal Acrosome Caps in the Ejaculates of Bull C.

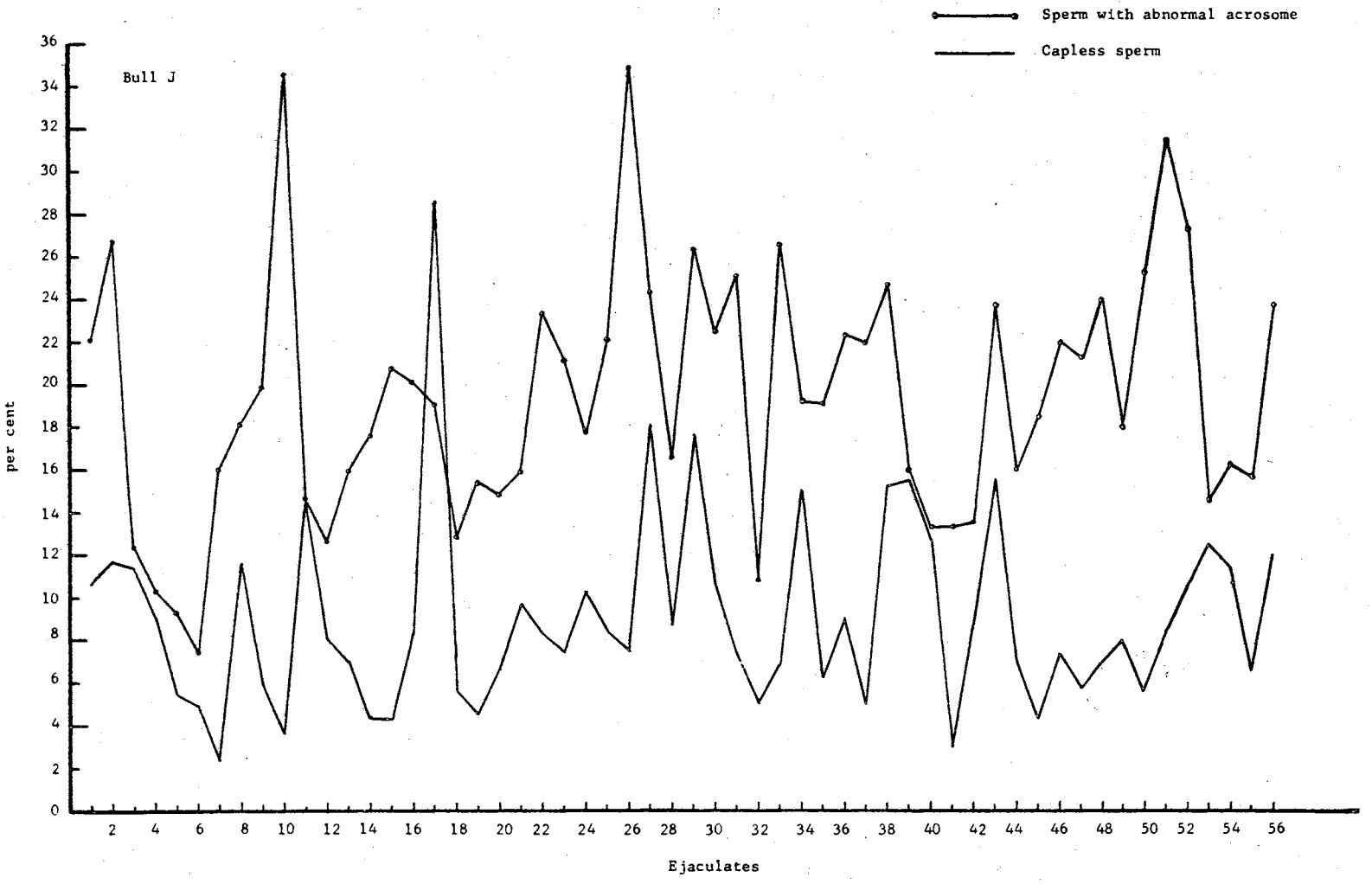

Figure 18. Weekly Averages of Capless Sperm and Sperm with Abnormal Acrosome Caps in the Ejaculates of Bull J.

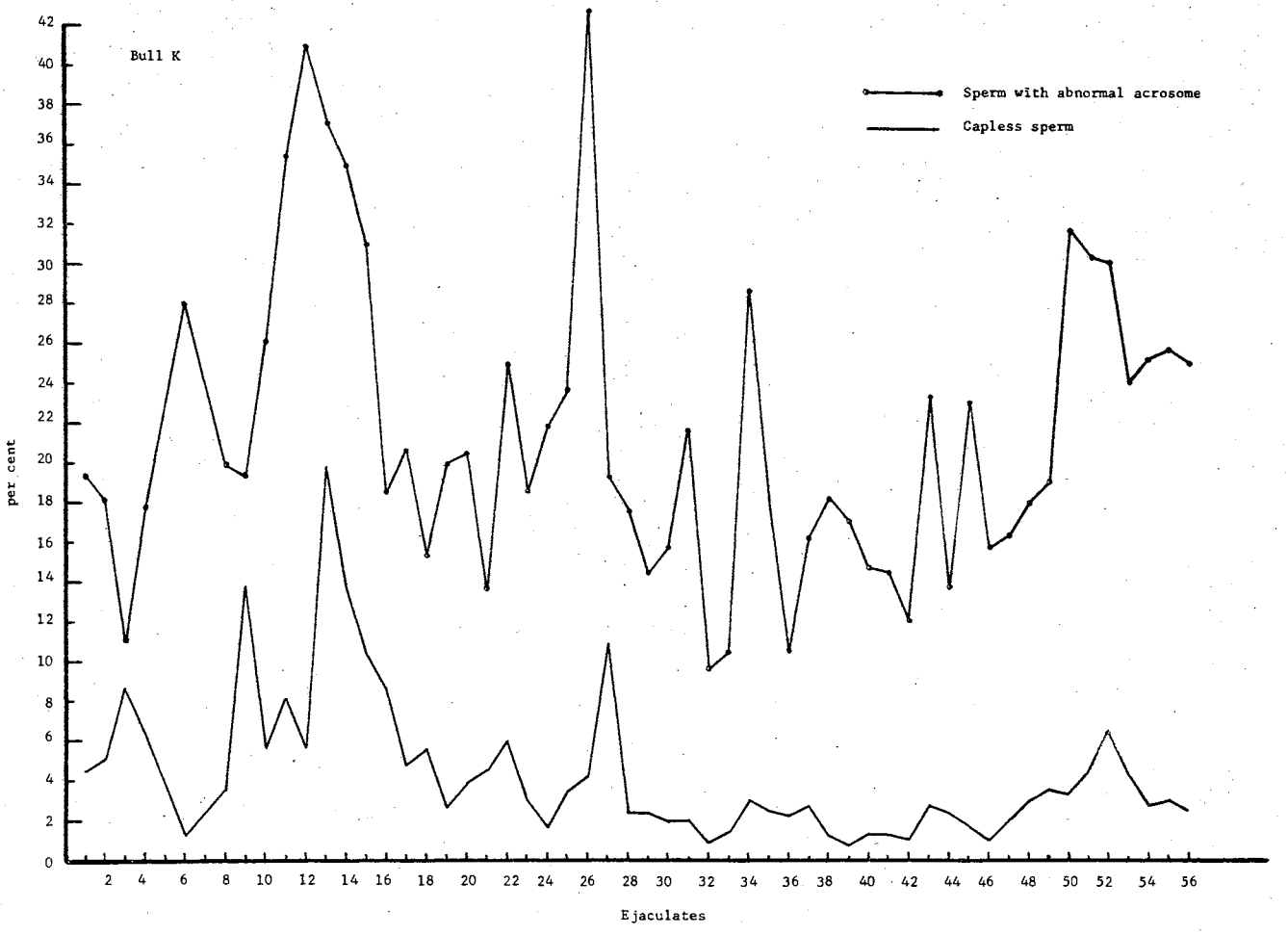

Figure 19. Weekly Averages of Capless Sperm and Sperm with Abnormal Acrosome Caps in the Ejaculates of Bull K.

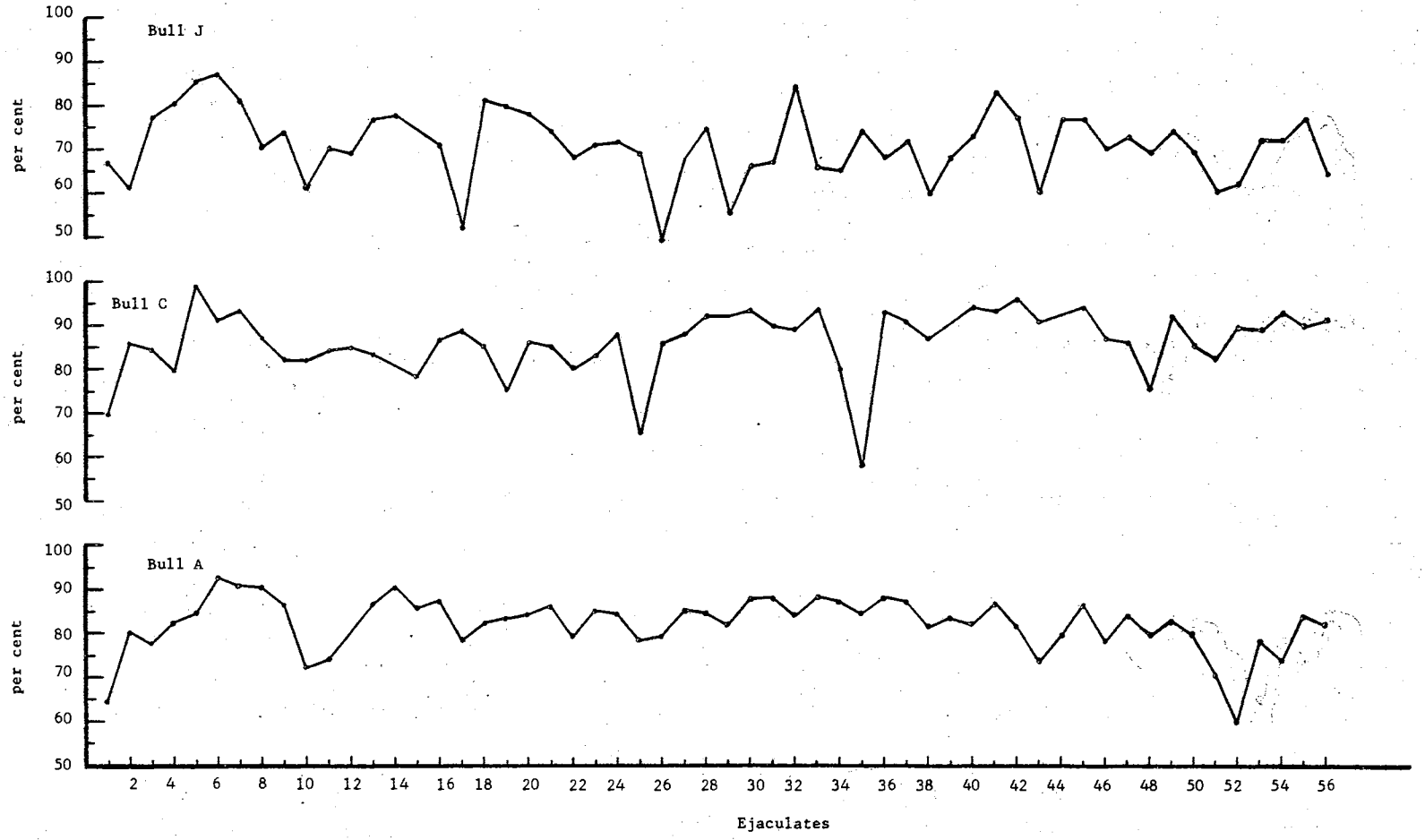

Figure 20. Weekly Averages of Sperm with Normal Acrosome Caps in the Individual Weekly Ejaculates of Bulls A, C and J.

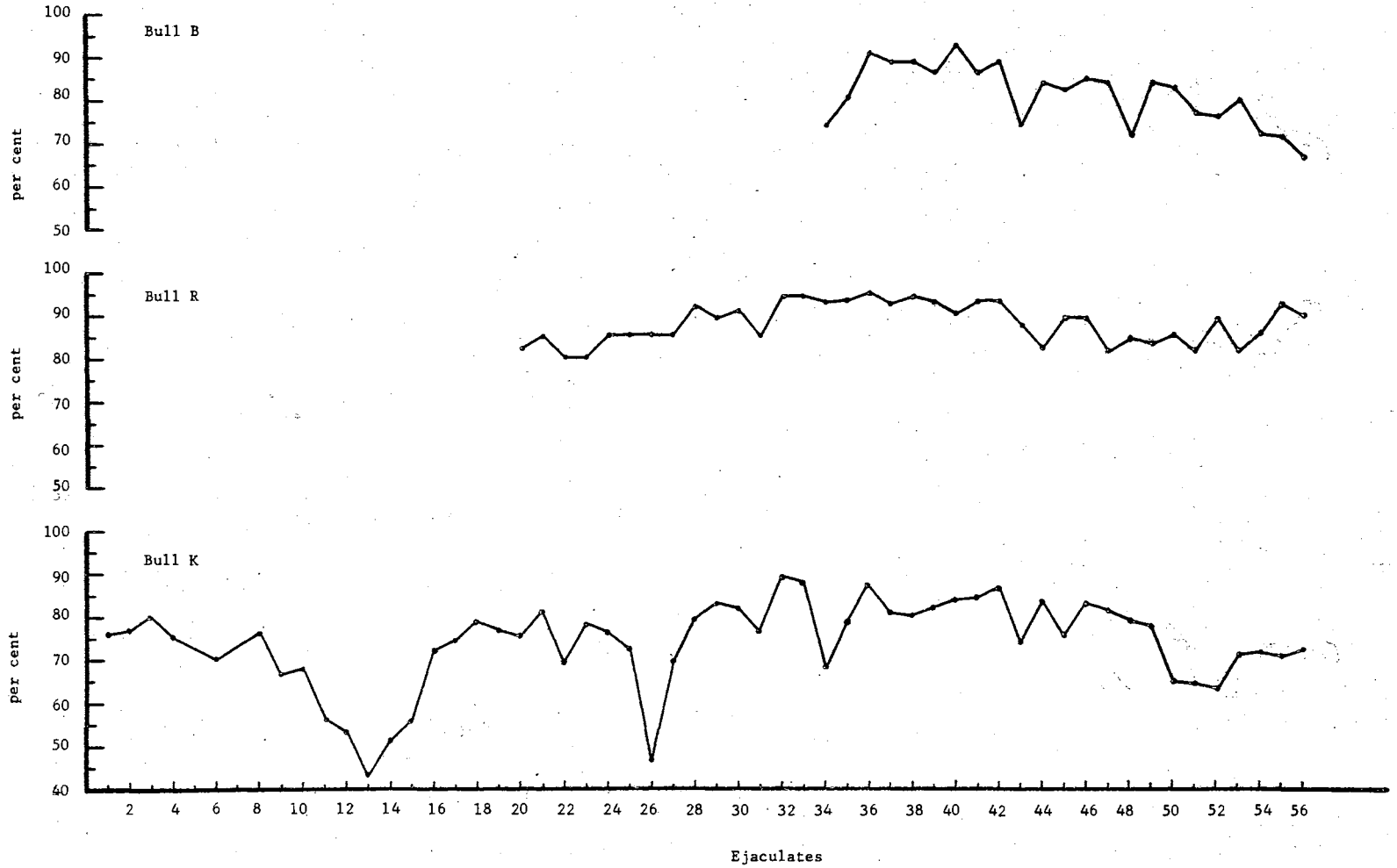

Figure 21. Weekly Averages of Sperm with Normal Acrosome Caps in the Individual Weekly Ejaculates of Bulls K, R and B.

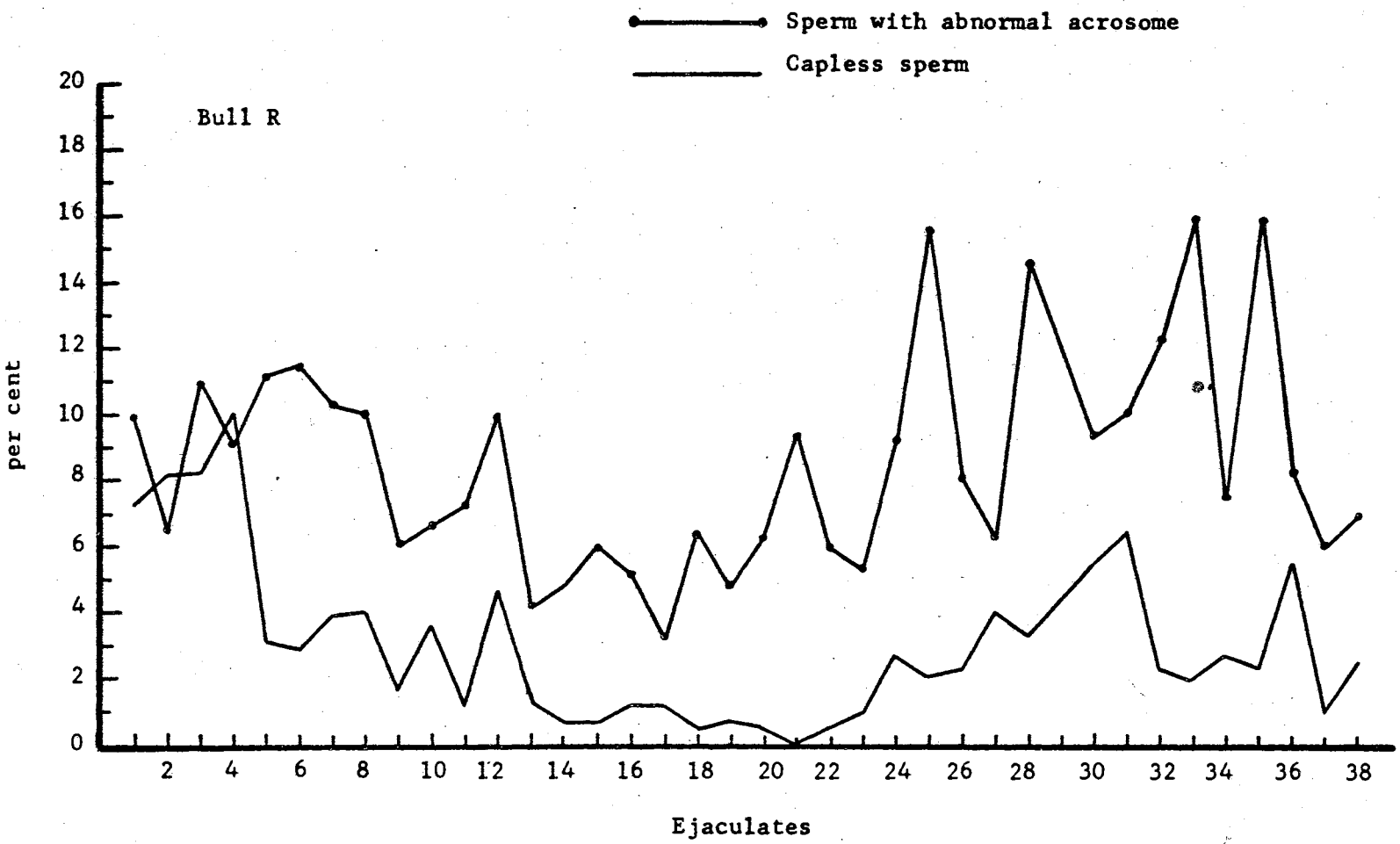

Figure 22. Weekly Averages of Capless Sperm and Sperm with Abnormal Acrosome Caps in the Semen of Bull R.

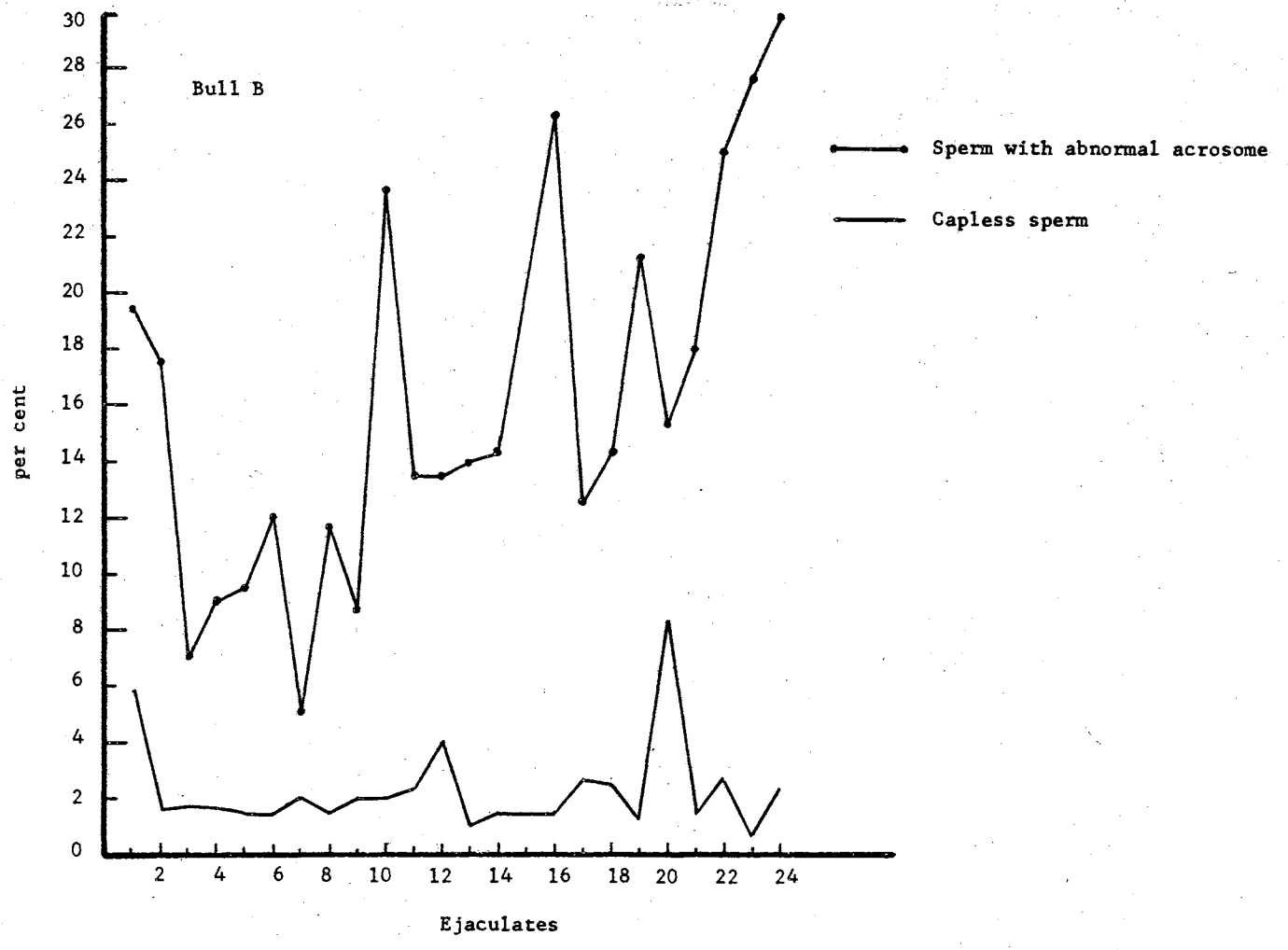

Figure 23. Weekly Averages of Capless Sperm and Sperm with Abnormal Acrosome Caps in the Semen of Bull B.

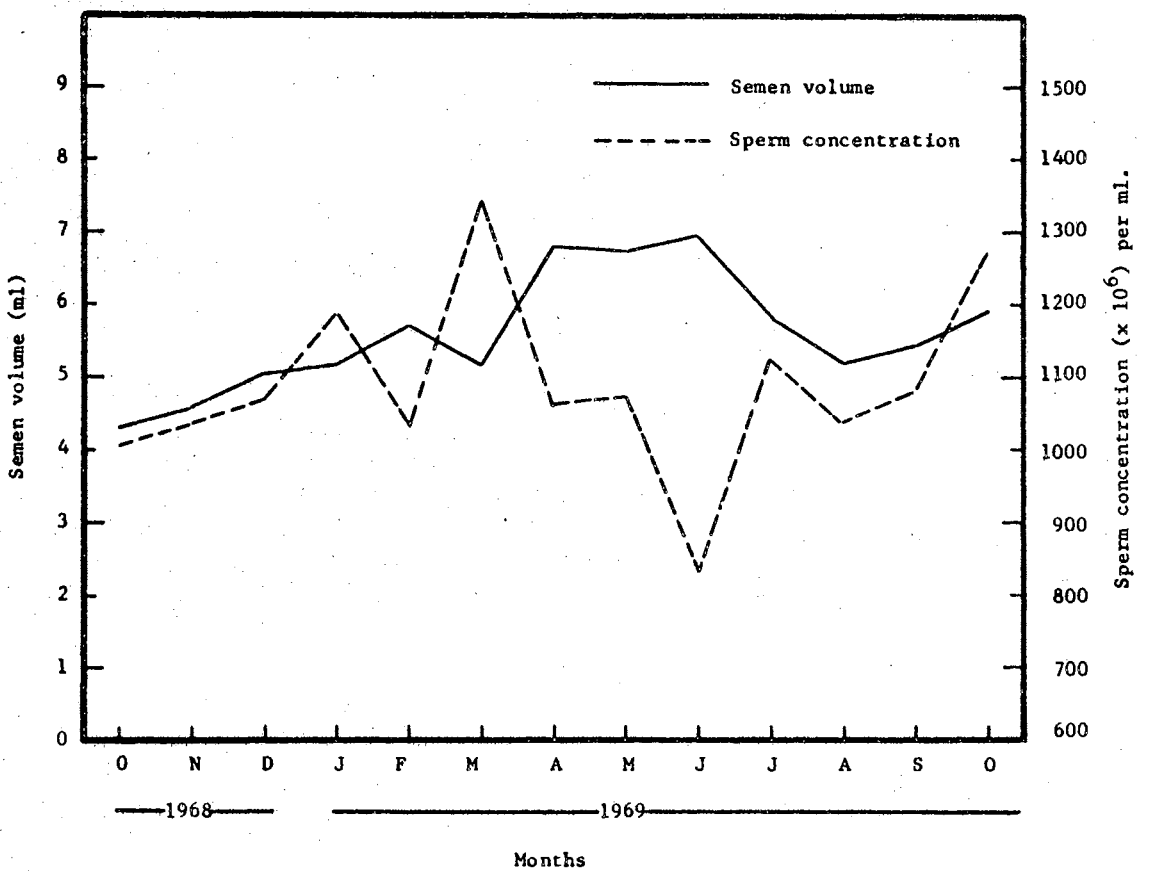

Figure 24. Monthly Averages of Ejaculate Volume and Sperm Concentration per ml. in the Ejaculates of Four Bulls Ejaculated at Weekly Intervals.

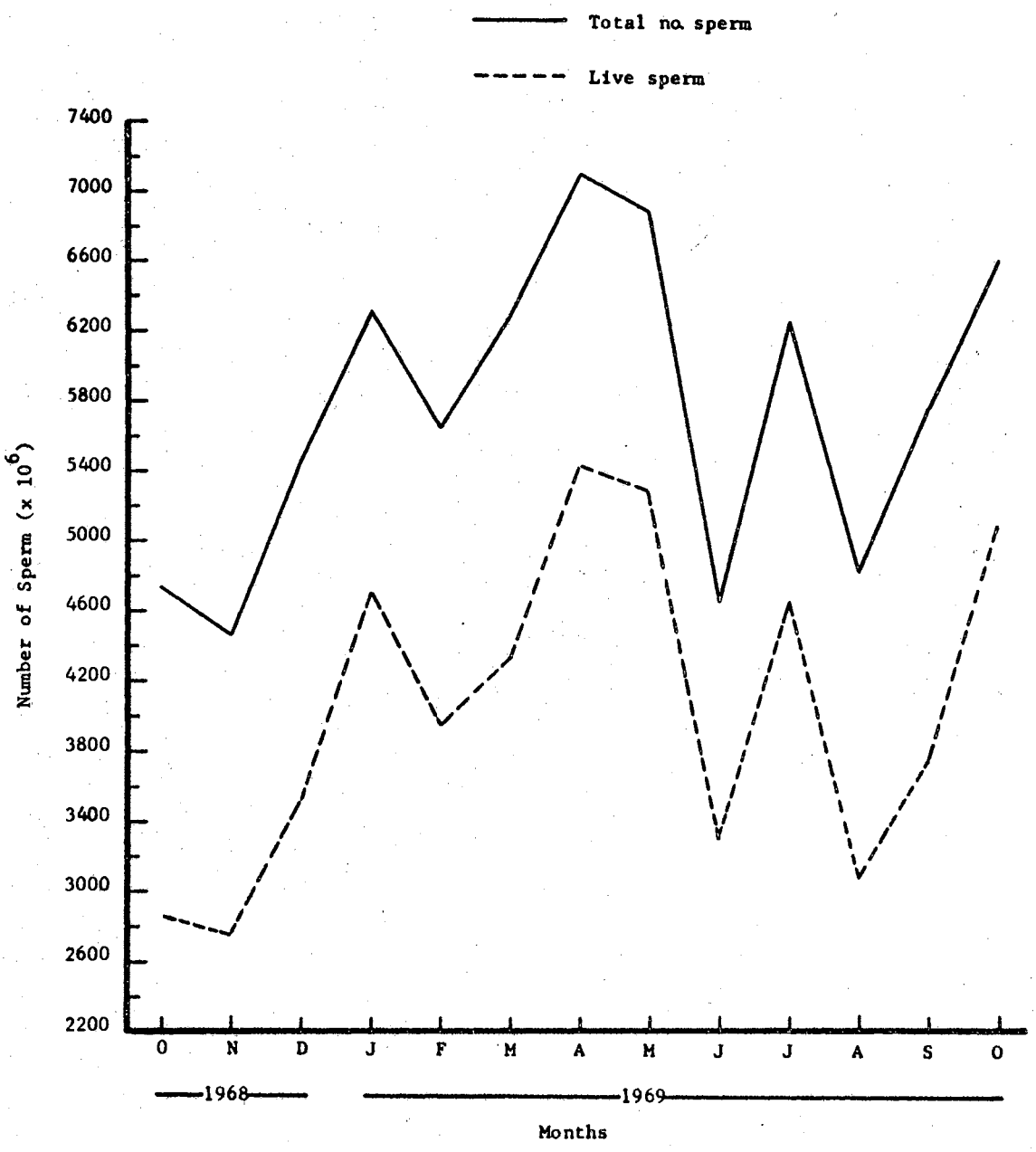

Figure 25. Monthly Averages of the Total Number of Sperm and the Number of Live Spermatozoa in the Ejaculates of Four Bulls Ejaculated at Weekly Intervals.

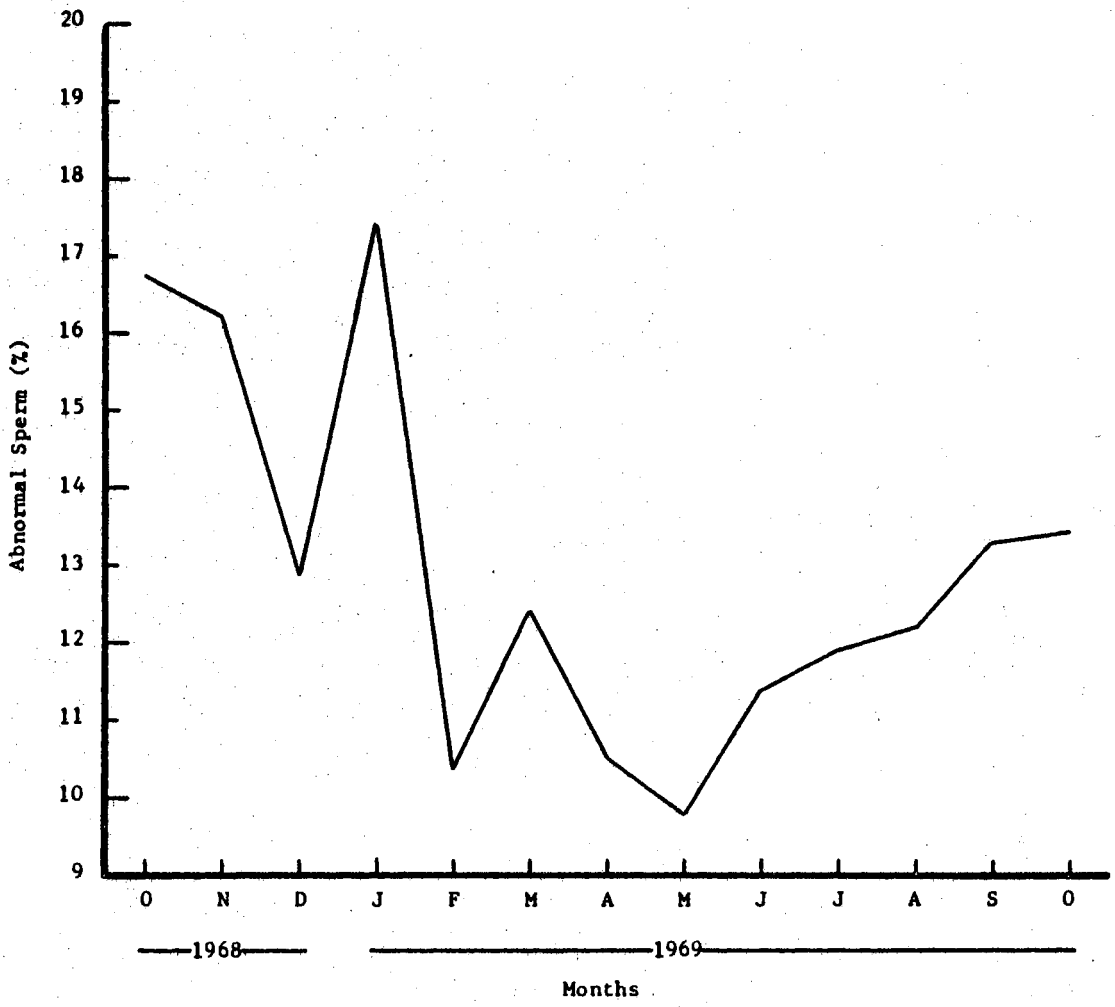

Figure 26. Monthly Averages of Abnormal Spermatozoa in the Ejaculates of Four Bulls Ejaculated at Weekly Intervals.

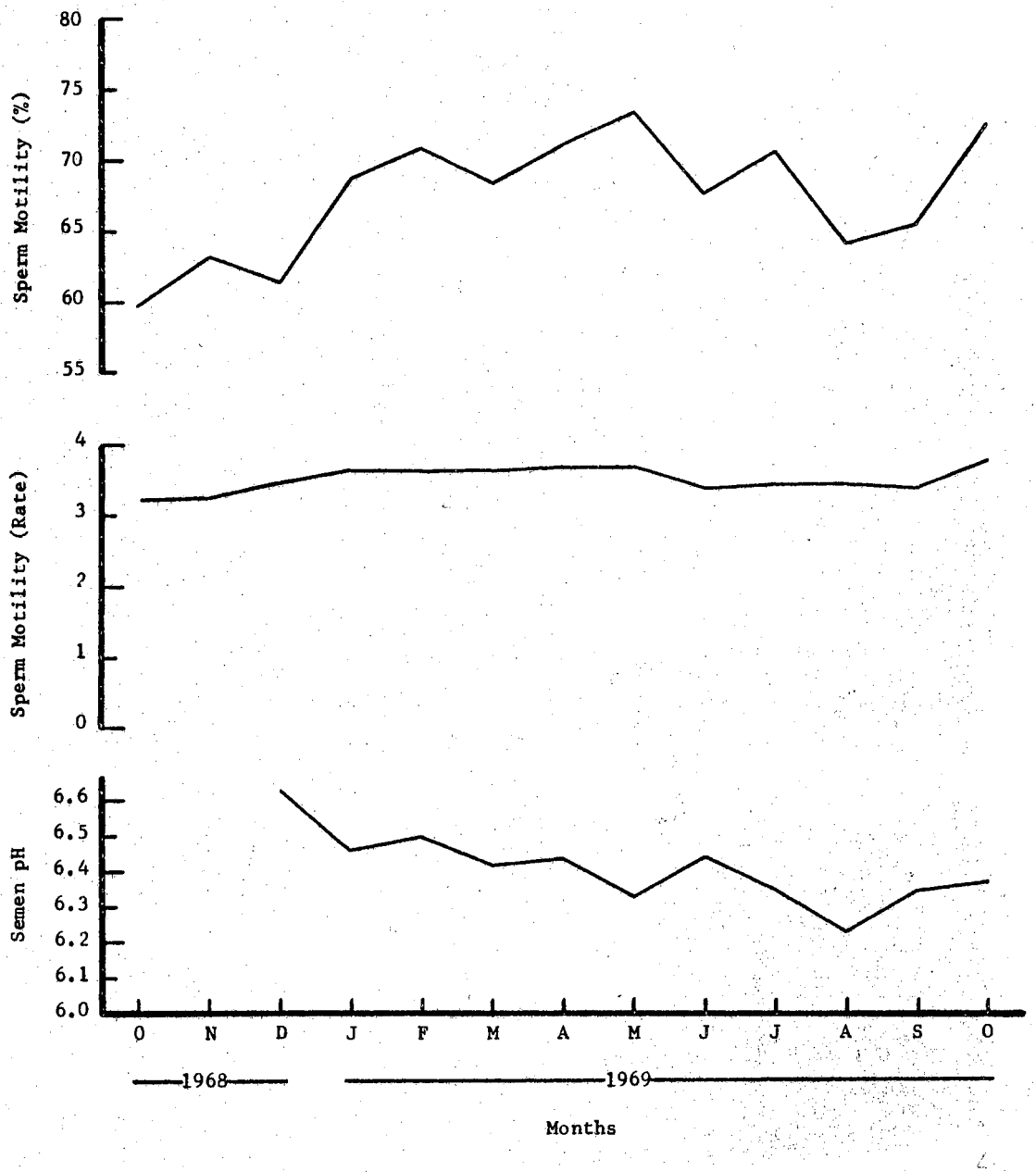

Figure 27. Monthly Averages of Semen pH, Rate of Sperm Motility and Per Cent Sperm Motility in the Ejaculates of Four Bulls Ejaculated at Weekly Intervals

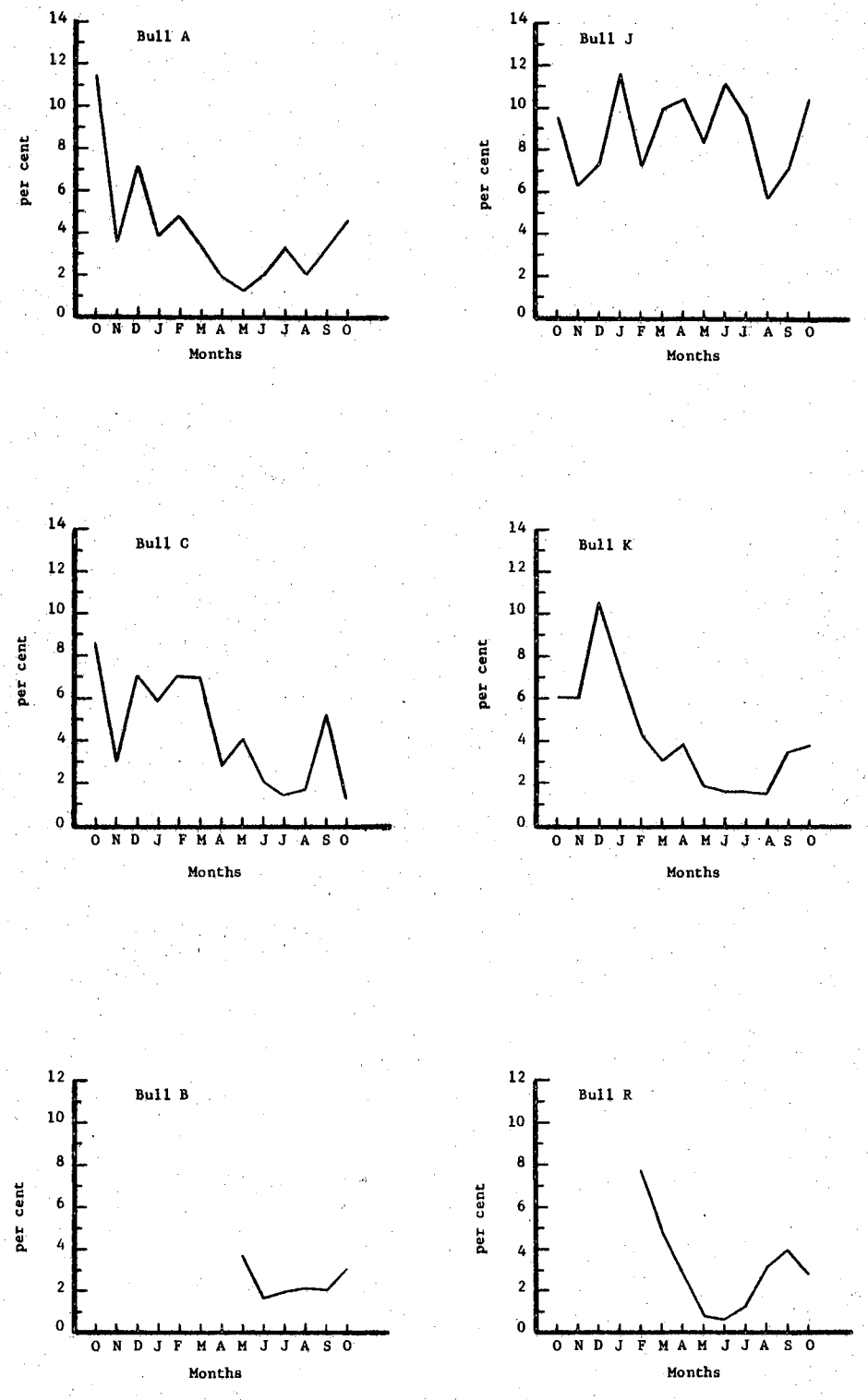

Figure 28. Monthly Averages of Capless Sperm in the Ejaculates of Individual Bulls Collected at Weekly Intervals.

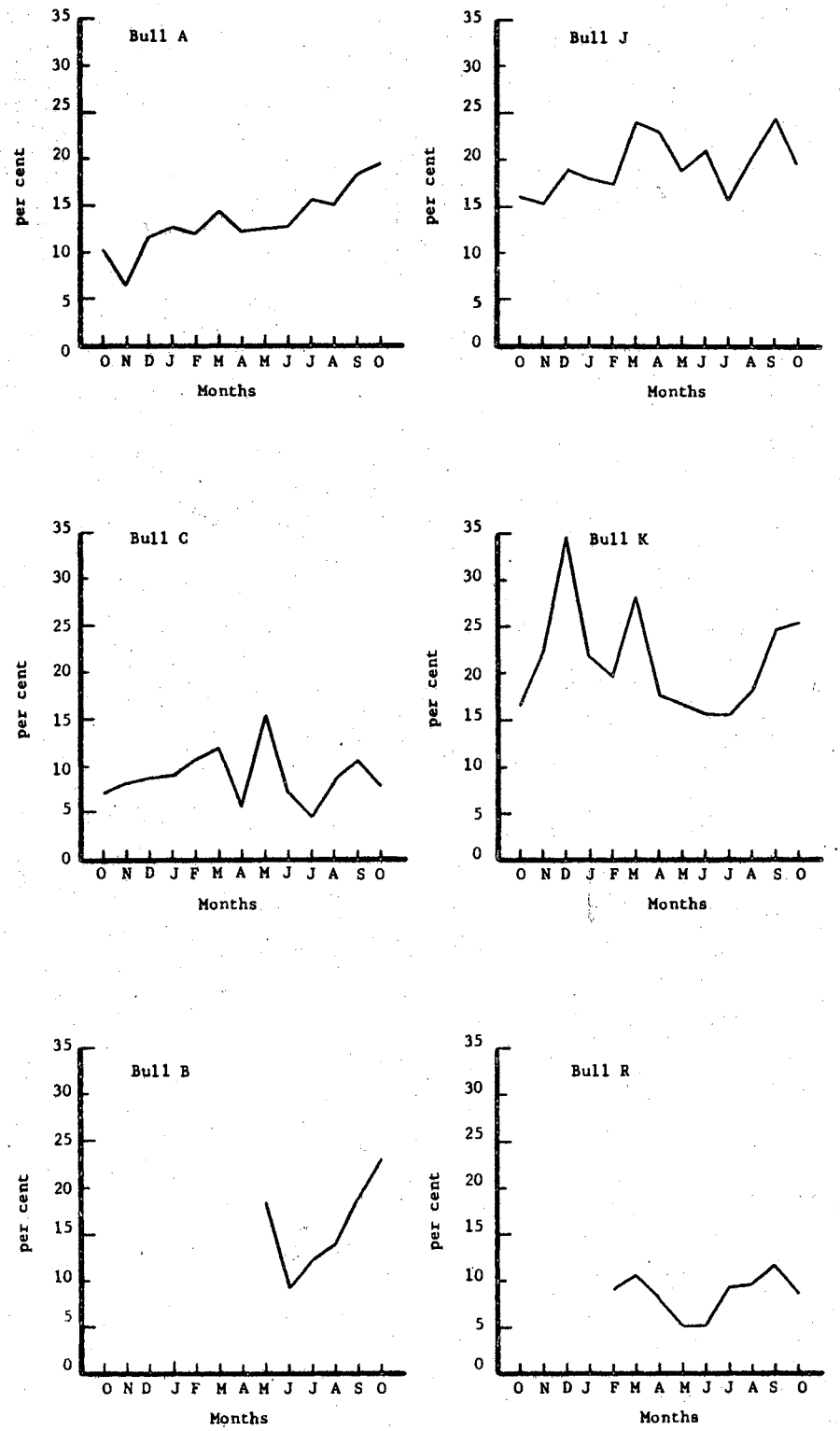

Figure 29. Monthly Averages of Sperm with Abnormal Acrosome Caps in the Ejaculates of Individual Bulls Collected at Weekly Intervals.

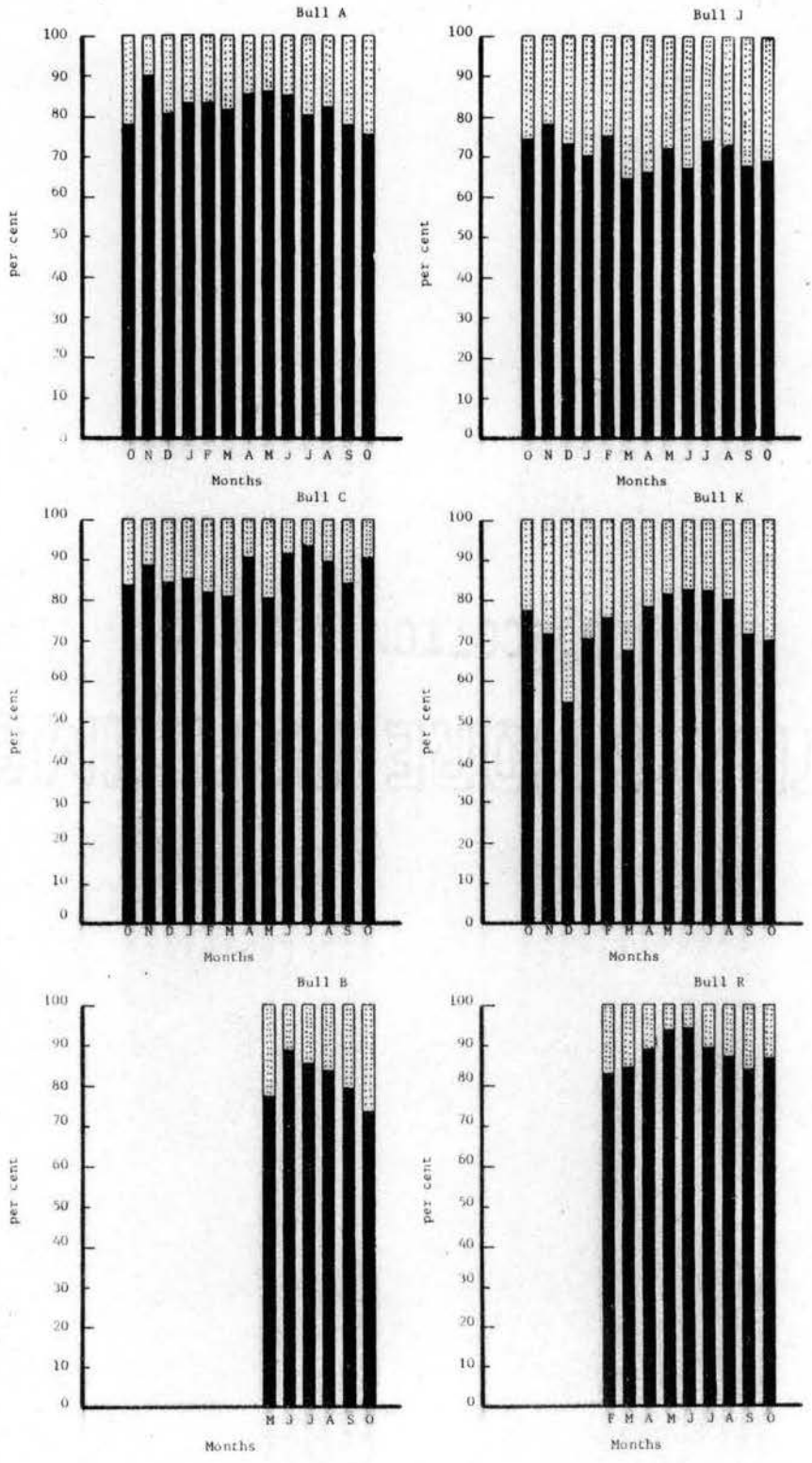

Figure 30. Monthly Averages of Sperm with Normal Acrosome Caps (Black Bars) and Total Acrosomal Anomalies (Dotted Bars).

VITA

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