PRELIMINARY STUDIES IN THE DEVELOPMENT OF A FERTILITY INDEX IN THE DAIRY BULL

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CHAPTER I

INTRODUCTION

Artificial insemination has been used significantly by animal breeders for many years. Initially, the dairy industry received the greatest attention, but today, the use of artificial insemination in beef cattle is becoming widespread.

The rapid growth of artificial insemination and the ever-increasing demand for superior livestock has focused some concern on the fertility of the genetically superior sires of the artificial insemination industry. Therefore, knowledge of the characteristics of semen, its evaluation, dilution, storage, insemination, and the factors that affect fertility continues to be critically important in sire utilization.

Development of laboratory tests which accurately predict the performance of semen in the field, as well as predict the fertility level of a given bull, has been the goal of numerous research efforts. This is an important goal in the artificial insemination industry, and once reached, would provide both livestock breeders and A. I. organizations with significant economic gains derived from improved breeding efficiency. The possibility of being able to distinguish the low fertility bull from higher fertility bulls, and to be able to do so in a matter of a few weeks, is an objective worth pursuing.

The reports of many researchers over several years have shown a wide variety of factors to be associated with the breeding performance of the male. However, there is no one simple test or series of tests that will suffice in accurately evaluating fertility levels.

Recent research has indicated the importance of the acrosomal cap in the prediction of the fertility level of the bull. For years, numerous semen characteristics have been used in attempts to predict semen fertility, with disappointing results. However, the use of acrosomal characteristics in combination with previously used criteria has added a new facet to the possibility of predicting the fertility level of a given male or a given semen sample.

This work was initiated in an endeavor to evaluate a number of semen characteristics and to determine to what degree they are useful in the prediction of the fertility level of the bovine male.

CHAPTER II

LITERATURE REVIEW

Semen Characteristics and Fertility Evaluation

Work published in 1925 by Williams and Savage opened the way for research involving the presence of abnormal spermatozoa in subfertile and sterile bulls. Even with the advent of refined instrumentation and techniques of today, the basic observations noted by Williams and Savage early in this century have stood the test of time. At that time these men made the following statements: "1) the dimensions of sperm heads from good fertility bulls are remarkably uniform; 2) we have observed no highly efficient bulls which emitted more than 17% abnormal sperm; and 3) permissible numbers of abnormal sperm in an ejaculate depend largely on the types of abnormalities present."

From that time to the present, researchers have continued to investigate ways of evaluating the fertility potential of semen. The widespread use of artificial insemination of dairy cattle and the need for highly fertile spermatozoa has caused the investigation of many semen quality tests. Some of the earlier more widely used tests were motility rating, concentration of spermatozoa, longevity of acceptable usability in storage, morphological examination, cold shock, and determination of the percent of live spermatozoa by use of a differential stain.

Among the various laboratory tests, initial motility rate has been the most widely used and has frequently served as a basis for evaluating other semen quality tests. Reynolds (1916) was one of the first to describe the various types of motility. He emphasized even then that vigor of motion was necessary for high fertility. Donham, et al. (1931) examined semen of bulls after natural services and found a significant relationship between motility and conception rate. Anderson (1946) found no significant correlation between motility and breeding efficiency within the range of 70 to 100% motile sperm. However, Swanson and Herman (1944) found a significant curvilinear relationship between motility and fertility when working with a wider range of difference in conception rate among samples having 45% motility and those higher.

Ellenberger and Lohmann (1946) found little relationship between type of motility, or percent of motile spermatozoa at time of collection, and fertility. Lindley, et al. (1959) found a significant negative correlation between percent motile sperm cells and number of services per conception in cattle.

Unfortunately, even with the many highly significant correlations, motility alone is not indicative of performance of that semen in the field. Many semen samples that possess good motility also have high abnormal counts.

In 1943, Lasley and Bogart compared the percentage of live spermatozoa with the percentage of conceptions and found a linear correlation up to about 50% live spermatozoa and little difference in conception rate with higher percentages of live sperm.

Shaffer and Almquist (1949) reported that the proportion of living spermatozoa (determined by staining with eosin aniline-blue) showed a highly significant cirvilinear regression with fertility. Stone, et al. (1950) used the fast green FCF-eosin Y differential stain and found no within-bull correlation for percentage initial living spermatozoa compared with fertility on 81 semen samples from fourteen bulls with 2,753 first and second services. Erb, et al. (1951) reported that the percentage of spermatozoa alive at time of collection of the semen, as determined by the eosin-aniline blue technique, showed a highly significant correlation of +0.212 with fertilizing capacity.

Bishop, et al. (1954) 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 time. 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 spermatozoa and the measurement of the impedence change frequency. Methylene-blue reduction test, fructolysis rate and oxygen uptake added little to the information gained by the above three tests. These workers and many others suggested that measurements of certain characteristics of ejaculates can be used as criteria of the fertilizing ability of bulls.

More recent work reported by Munroe (1961) showed that fertility was reduced when either the incidence of live spermatozoa fell below 70% or the incidence of primary and secondary abnormal spermatozoa rose above 23%, with head defects appearing to be the most important abnormalities. Prior to this time, Bratton and co-workers (1956) similarly considered that it was only the unusually high incidence of abnormal spermatozoa, averaging 24%, that accounted for a significant contribution to the prediction of fertility.

In 1954, Bishop, et al. found a low incidence of primary and secondary abnormalities averaging 6% in their study of 76 bulls, with no relationship appearing between this characteristic and fertility. Much earlier reports by Swanson and Herman (1944), Trimberger and Davis (1942), and Donham and co-workers (1931) indicated that there was no significant correlation between average morphological abnormalities and conception rate. They also found no correlation between concentration of spermatozoa and conception rate.

Working with sheep, Goerke, Thrift and Dutt (1970) found a negative correlation between percentage of abnormal sperm cells and percent ewes lambing, lambs per ewe exposed and lambs born per ewe lambing.

Volume of ejaculate has been used as a criterion of fertility. It is important in determining the total number of spermatozoa produced, but no correlation with fertility appears to exist (Ellenberger and Lohmann, 1946; and Swanson and Hermann, 1941).

Concentration has been widely used as an indication of fertility but has shown little predictive value in studies on semen of average or above average quality (Dougherty and Ewalt, 1941; Ellenberger and Lohmann, 1946; and Swanson and Hermann, 1941 and 1944). Herman and

Swanson (1941) showed that the length of time a desirable motility rating was maintained in storage of several different ejaculates gave a good index of the fertility of a bull.

From the results of years of research, it had become increasingly clear that no single test would accurately predict fertility, and that the study of semen by as many tests as possible was necessary.

Buckner, Willett and Bayley (1954) did a series of trials comparing correlations between nonreturn rates and single tests or combination of semen quality tests. The only among bulls correlations with a magnitude of 0.9 or higher were between nonreturn rates and a) motility of spermatozoa after incubation at 38°C in yoke-citrate plus antibacterial agents and b) the combination of methylene blue reduction time, drop in progressive motility after 120 minutes in 3% aniline blue solution at 4°C, and initial percent motile cells in yolk-citrate solution. However, the correlation for this combination in a later trial was only 0.73 and when done under field conditions, 0.55. Throughout their studies, they found the among-bulls correlations to be higher, in most instances, than the total of within-bulls correlations. This indicated that the possibility of estimating differences in fertility among bulls was more promising than estimating differences among individual semen samples.

Early work reported by Wiggins, Terrill, Emik (1953) using large numbers of sheep found that the only traits both appreciably and significantly correlated with fertility were percentage of normal sperm ($r = 0.43$) and to a lesser extent, the related traits, percentage of abnormal heads and percentage of live normal spermatozoa. In 1956, extensive work published by Bratton and co-workers indicated the

importance of being able to determine what combination of laboratory tests can be most useful for predicting the fertility of bovine semen. Even though this work provided only preliminary quantitative estimates, it did furnish insight as to what needed to be accomplished. The results of their works revealed that only ejaculate concentration and percent morphologically abnormal spermatozoa contributed significantly to the prediction of fertility.

Presently, although no one characteristic is highly correlated with fertility and no usable fertility index has been developed, 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.

Much dismay has been shown due to such contradictory findings and to the small correlations between different characteristics and fertility. However, it must be kept in mind that when working with bulls from artificial breeding organizations, that artificial insemination has permitted easier recognition and subsequent removal of subfertile and sterile males, which in itself would tend to lower the relationship of sperm abnormalities and fertility in many of the studies. Also, a literature review by Erb and co-workers (1955) showed that variations in methods of determining quality, the lack of objectivity of some determinations, geographic location of the bulls, fertility level of bulls used, dilution methods and treatment of semen, skill of technicians collecting, processing and using the semen for insemination, and variations in the cow population all contribute to errors in statistical evaluation of quality measurements and of fertility.

The experimental design plays a very vital role in the development of reliable and usable correlations between semen characteristics and fertility. Studies by Erb, et al. (1950) and Schultze, et al. (1948) show that in order to accurately measure the fertilizing capacity of an individual semen sample that an excess of 300 services is required. Also, attention must be given in the experimental design to the fact that there is daily variation in abnormal sperm within bulls and, therefore, differences in numbers of abnormal sperm among ejaculates of the same male tend to mask any relationship between percent abnormal sperm and fertility. Literature reveals that many of these factors are ignored when discussing the merits of semen quality. From these facts, Saacke (1970) concluded that neither reproductive history nor potential fertility of males can be judged on the basis of one or two ejaculates.

The Acrosome - Its Role in Fertility

A new perspective in semen evaluation was brought about by the use of the electron microscope through its help in better understanding sperm cell and acrosome cap morphology. Phase-contrast and ultraviolet microscopy and improved histochemical techniques have also added much to this understanding. More recently, the use of differential interference contrast microscopy by Saacke and Marshall (1968) and the development by Wells and Awa (1970) of a new differential acrosome stain has made acrosomal evaluation possible as part of routine semen evaluation. Differential interference contrast microscopy permits the detection of the apical ridge of the intact acrosome in unfixed live semen smears thereby eliminating the fear of fixation

artifacts. The Wells-Awa differential acrosome stain permits the easy detection of the acrosomal cap in both fresh and frozen semen using the conventional light microscope. It was shown to be superior to the Giemsa stain and to phase contrast microscopy for detecting the different kinds of acrosomal anomalies (Wells and Awa, 1970). Until this time, routine acrosomal evaluation was difficult due to either lengthy staining procedure, or the complexity of the equipment involved.

The structure of spermatozoa has been the subject of much extensive research and has led to the publication of voluminous literature relating to its ultrastructure. A comprehensive review of the literature relating to the ultrastructure of the normal sperm cell was published by Saacke and Almquist in 1964. Blom and Birch-Anderson (1965) present recent information relating specifically to the ultrastructure of the bull sperm head, the principle area of importance in this study. Figure 1, from Blom and Birch-Anderson (1965), presents a schematic drawing of the normal bull sperm head. The left drawing is a median sagittal section of the sperm head with the right drawing being a true coronal section. In the latter, the upper and lower limits of the equatorial segment are indicated by dashed lines. The abbreviations used in Figure 1 to describe the ultrastructures of the sperm head are as follows:

c.m.: cell membrane

a.v.: apical vacuole with the apical body

p.: perforatorium

o.m.: outer membrane of the acrosome cap (compare galea capitis)

i.m.: inner membrane of the acrosome cap

a.c.: acrosome cap (contents)

i.l.p.: intermediate layer (dense material found between e.s. and n.m.

e.s.: equatorial segment of pars intermedia

p.c.: post-nuclear cap

i.l.: intermediate layer between p.c. and n.m.

n.: nucleus

n.m.: nuclear membrane

b.p.: basal plate on basal lamella lining the implantation groove

b.k.: basal knob

$e.n.m._{I}$ evaginated nuclear membranes (more simple type)

$e.n.m._{II}$ evaginated nuclear membranes (more convoluted type)

c.: centriole

Of special interest to this study are the acrosomal membranes and the equatorial segment, as well as the general shape of the sperm cell head.

The familiarization with the normal structure of the sperm cell as well as alterations of the sperm cell due to aging and injury (Saacke and Marshall, 1968 and Saacke, 1970) will provide a much more sound basis for recognition of abnormal sperm cells and, consequently, their respective association with fertility.

Before the development of more refined staining and specimen preparation techniques, many sperm abnormalities causing subfertility had possibly gone undetected. Using a Giemsa staining technique, Hancock (1953) found an abnormality of the sperm acrosomal cap that

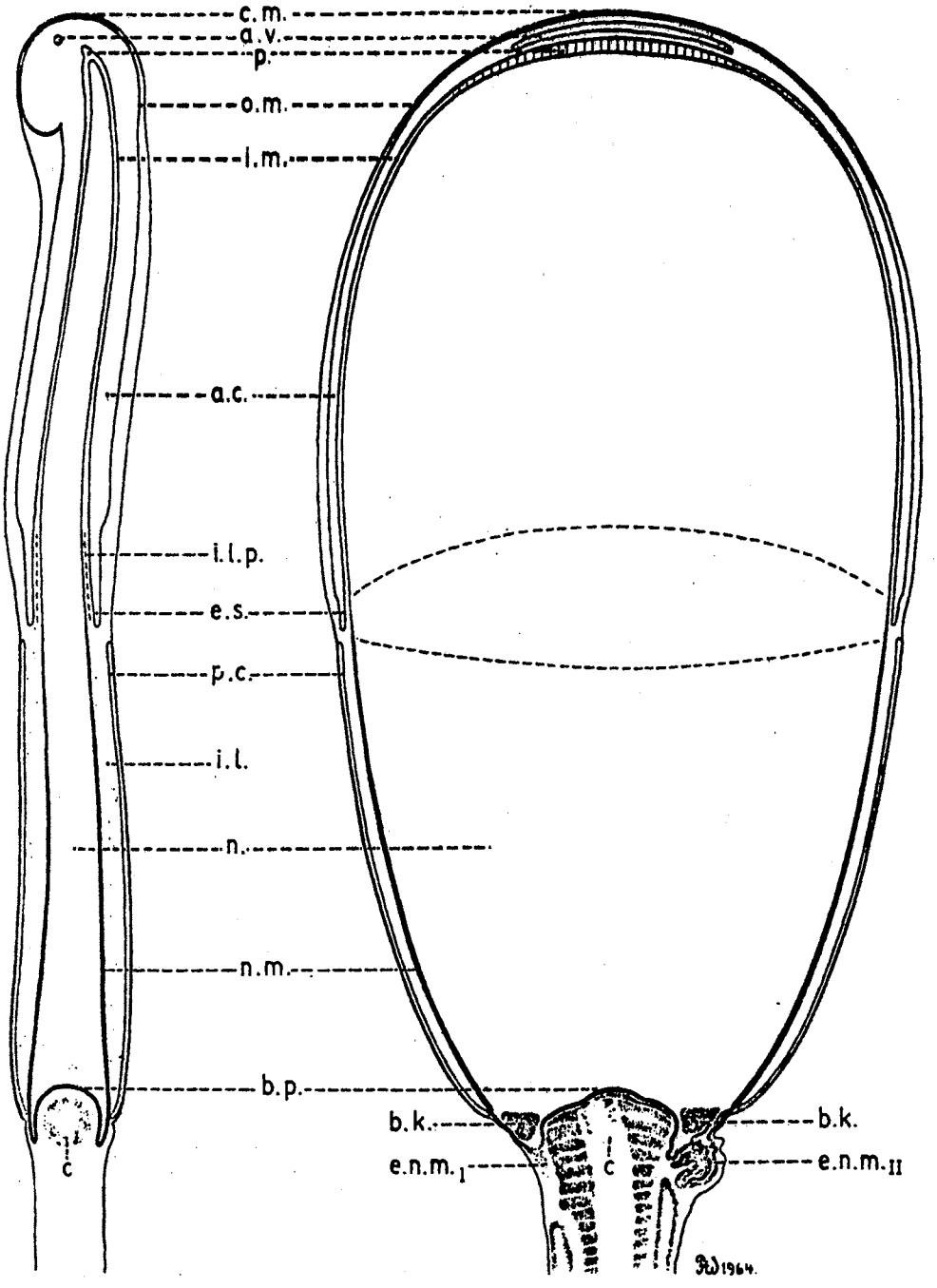

Figure 1. Morphology of the normal bovine sperm head

was later shown to be an inherited cause of sterility in Holsteins (Donald and Hancock, 1953). This abnormality was described as a "knobbed sperm" and was characterized by a head or knob located on the apical edge of the sperm head. In Hancock's study, nearly all spermatozoa of each bull were affected and sterility appeared absolute.

In 1968, Saacke, et al. found a variety of acrosomal alterations which included the previously described inherited knobbed sperm along with those having ruffled and incomplete acrosomes. In these cases, however, only a portion of the total sperm in ejaculates were affected and the bulls were subfertile rather than sterile.

Prior to this time, numerous other reports had indicated that acrosomal abnormalities can contribute to lowered fertility or complete sterility (Saacke, Almquist and Amann, 1966; Bane and Nicander, 1966; Hancock, 1949; Rollinson, 1951; and Teunissen, 1947).

The evaluation of abnormal acrosomes took on a new aspect when Saacke and Marshall (1968), utilizing differential interference contrast microscopy on unfixed live semen smears, described the aging sequence in acrosomal alteration without the fear of fixation artifacts. These observations brought about two major points relative to semen evaluation; "First, in order to evaluate acrosomal or head anomalies, it is now apparent that the aging sequence must be recognized as normal and minimized using optimum fixation and staining techniques on fresh ejaculates." The second point was that "the sequence of acrosomal alterations due to all aging or injury appears constant, but the rate at which alteration occurs is dependent upon the ejaculate as well as semen handling techniques or sperm environment" (Saacke, 1970).

Once extensive research had indicated a relationship of acrosomal abnormalities (genetic and aging) with fertility (Marx, 1964; Rollinson and MacKinson, 1949: Saacke, Amann and Marshall, 1968), the relationship among kinds and percentages of acrosomal abnormalities and fertility was to be established.

Saacke, Amann and Marshall (1968) and Wells and Awa (1970) found wide difference in acrosomal abnormalities among bulls and ejaculates and suggested that acrosomal evaluation be added to the routinely used ejaculate evaluation criteria.

The results of a study by Awa (1970) showed a significant correlation ($P < .01$) between the level of capless sperm and semen pH. However, he found no significant correlations between acrosomal characteristics and other ejaculate criteria. However, significant correlations were found between capless sperm and certain environmental conditions and concluded by suggesting that the acrosome cap state was essentially independent of other ejaculate characteristics. Further, he concluded that the acrosome was quite susceptible to internal and/or external environmental influences. Saacke (1970), in a study comparing ejaculates from five different bulls, showed that the correlation between post-thaw ($37^{o}C$) motile life and maintenance of the acrosome was significant, but relatively low ($r = .56$). This study showed that there was a highly significant difference among bulls as well as ejaculates within bulls relative to maintenance of the acrosomal cap at 37^{o} post-thaw.

The most recently reported work relating acrosomal state to fertility was by Saacke and White (1972). They used 156 ejaculates from 15 Holstein bulls with an average of 271 first services per

ejaculate. Thawed semen was incubated at 37°C for up to 10 hours and percent intact acrosomes was determined as noted by the presence of an apical ridge using differential interference contrast microscopy. Also, percent motility and percent abnormal cells were determined. The acrosomal measurement having the highest correlation with fertility was mean percent intact acrosomes for the 10-hour incubation ($r = .60$). Correlations of percent intact acrosomes with fertility at 2, 4, 10, and 8 hours of incubation were $r = .60$, .58, .51, and .48, respectively. Motility had the highest correlation to fertility at 0 hour, $r = .46$. They found a negative correlation with fertility and abnormal heads ($r = -0.34$) and protoplasmic droplets ($r = -0.37$). The study also showed abnormal tails to be unrelated to fertility.

Factors Affecting Semen Characteristics

The unique process of spermatogenesis has been the object of much research for countless years. The role of genetics as well as the influence of internal and external environmental factors has been of prime interest to many. The age and size of the bull, breed, frequency of semen collection, the level of sexual preparation and season of the year exert an influence on male performance. 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, 1200×10^6 per ml. (400-2,000 million per ml.); and initial sperm motility, 65 percent (50-80 percent).

Van Demark (1956), using Holstein bulls, found that volume of the ejaculate and concentration of spermatozoa both increased when analyzed on quarter-year periods following puberty. Baker, et al. (1955)

reported that sperm concentration per ejaculation was significantly correlated with age ($4 = 0.514$) and with body weight ($r = 0.533$). 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. They also found significant differences in sperm concentration associated with age, breed and bulls within breeds and ejaculation frequency.

The season of the year that collections are made has been shown by numerous reports to exert an influence on semen characteristics (Anderson, 1941; Erb, et al., 1942; Phillips, et al., 1943; Swanson and Herman, 1944; Salisbury, 1944; and Awa, 1970). Some reports are conflicting and the disagreement among the results obtained by these investigators indicates that the location, the age, and the management of the bulls may be factors that should be taken into consideration when determining the effect of season on various semen characteristics.

Since the beginning of artificial insemination, the frequency at which a male could be collected with no detrimental effects on the bull or semen quality has been the concern of several research studies. Baker, et al. (1955), working with young Holstein bulls for one year following puberty, concluded that there appears to be no harmful effects of repeated ejaculations up to three times per week except the decrease in libido. They found no significant differences in semen volume, sperm output, percent motile sperm, semen pH, total sperm per ejaculation, total motile sperm per ejaculation, or percent abnormal cells when frequencies of once, twice and three times per week were compared. Almquist and Cunningham (1967) reported that young beef bulls could be worked up to six times per week. They 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.

Thomson (1950) 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 month period. This did not occur with mature bulls.

Hafez and Darwish (1956) reported that when the interval between collections was short, detached acrosome caps and enlarged midpieces were observed. There was no 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 ejaculates.

Blom (1946) and Wells and Awa (1970) found that long periods of sexual rest resulted in 18% to 19% acrosomal abnormalities per ejaculate. Also, they observed that regular collection of semen was effective in reducing the percentage of acrosomal abnormalities. Further research by Awa (1970) and Wells, et al. (1971) indicates that a period of sexual rest of four weeks or more results in large increases in aged acrosomes. They found that in ejaculation frequency of four times weekly reduces aged acrosomes and maintains significantly fewer aged acrosomes than will a frequency of once weekly. Since these studies indicate the great susceptibility of the acrosome cap to internal and/or external environmental influences while in the epididymis, then increased frequency of ejaculation would allow the cells to spend less time in the epididymis and consequently less opportunity for acrosomal aging. Since it has been indicated that

the aging of the acrosome is related to fertility, then the influence of increased frequency of ejaculation is apparent.

Other reports directed to the effects of sexual rest on semen characteristics are numerous. 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. He suggested that sexual rest probably caused a gradual decrease in the activity of semen production due to the accumulation and resorption or spermatozoa in the cauda epididymis which thus causes a secondary gradual reduction in spermatogenesis.

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.

Wondafrash (1968), characterizing the acrosome quality in the first ejaculate of 22 Hereford and Angus bulls collected after a nine month period of sexual rest, found that the initial ejaculates contained 21.2 percent abnormal acrosome caps. He also found that 16.5 percent of the normal spermatozoa, 44.5 percent of the abnormal cells, and 77.0 percent of the tailless sperm had abnromal 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.

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.

Methods for extension of semen (Berndston and Foote, 1969; Bratton, et al. 1957; Foote, 1970; Gebauer, et al. 1970), rate of freezing (Erickson and Graham, 1959; Graham and Marion, 1953; Hodson, 1972; O'Dell, et al. 1958; Van Demark and Kinney, 1954; Pickett, et al. 1961; Bartlett and Van Demark, 1962; and Rapatz and Luyet, 1966), and rate of thawing (Rowson, 1953; Van Demark and Sharma, 1957; Dunn and Hafs, 1953; Hafs and Elliott, 1954; Pickett, et al. 1965; and Robbins, et al. 1972) have all been shown to exert an influence on semen characteristics. As reported by Saacke (1970), freeze-thawing, even under optimum conditions, is injurious and can be recognized by fewer sperm having intact acrosomes. However, he reports that loss of acrosomal caps post-thawing is not as dramatic as loss of motility.

In review of the literature, it is apparent that no one simple test or series of tests have been developed that will suffice in accurately evaluating fertility. Recent research has indicated that the state of the acrosome is important in the prediction of fertility and that this characteristic should be routinely used in combination with other semen characteristics to evaluate fertility. Therefore, this study was initiated in order to evaluate a number of semen characteristics, including the state of the acrosome, and to determine which of these characteristics are significant in the prediction of fertility in the bovine male, and to develop a fertility index utilizing these characteristics.

CHAPTER III

MATERIALS AND METHODS

Semen Collection and Distribution

Forty-three Holstein bulls in the Progeny Test Unit of the American Breeders Service, De Forest, Wisconsin, were utilized in this study. All bulls were approximately one year of age at the time of initial ejaculate collection. Bulls in this age range were utilized in the study since older proven males in a bull stud have been highly selected and culled on basis of performance, and consequently would result a population of decreased variation. The only selection placed on the bulls in this study was that they be desirable, genetically superior males and not be sterile. As far as possible, the bulls were started by quarter years with the initial bulls being started in February, 1970.

Prior consultation with ABS had set up standards and procedures that were to be employed in this study. All bulls were ejaculated utilizing routine procedures normally employed by ABS. Two ejaculates were collected from each bull once a week by artificial vagina and combined for processing. The first two collections were test collections which were used by ABS to establish a post-freeze motility level. From this they determined the concentration of cells that was necessary to obtain 10×10^6 (± 3, 7 to 13 inclusive) motile cells per $\frac{1}{2}$ cc. ampule post-freeze. Any collections outside of this range

were rejected in order to control some of the variation in the number of live cells per ampule.

A maximum of 104 ampules was frozen from each individual collection. In addition to this, two full racks from each collection were frozen and shipped to OSU. Ejaculate collections were continued on each bull for a minimum of five acceptable collections of 104 ampules each (a total of 520 ampules on each bull). Some bulls required as many as eight collections before the desired number of ampules were in storage. Six ampules of each collection were shipped to OSU for determination of post-freeze characteristics. The 520 ampules were frozen on each bull in hope that enough of the ampules would be distributed and utilized so that non-return information on at least 150 to 180 services per bull could be accumulated.

The frozen semen of each bull was randomly distributed by ABS to approximately 60,000 associate herds across the country. Normal distribution and insemination procedures were utilized, except that no bull was to be used more than four times in any given herd.

Non-return data for each bull was compiled by ABS and sent to OSU as it became available.

Initial Ejaculate Evaluations

Pre-freeze acrosomal and live-dead slides were prepared from the fresh semen immediately upon collection by ABS. Slides were made from each individual ejaculate and then shipped to OSU where the following data was obtained from each slide:

a) Percent Live Spermatozoa: This was determined using the nigrosin-eosin live-dead differential stain.

b) Acrosome Characteristics: These were determined for each ejaculate utilizing the Wells-Awa staining procedure.

c) Abnormal Spermatozoa: The proportion of morphologically abnormal sperm cells was also determined from slides prepared with the Wells-Awa acrosome stain.

Staining and Evaluation Procedures

Acrosome Staining Techniques

Duplicate slides were prepared using the Wells-Awa acrosome stain (Wells and Awa, 1970) in order to study the state of the acrosome. The stain was prepared as follows:

Solution "A": consisting of a 1% solution of water-soluble eosin B (total dye content 88%) in glass-distilled water.

Solution "B": consisting of a 1% solution of water-soluble Fast Green FCF (total dye content 90%) 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. Stock solutions "A" and "B" were prepared monthly and stored at $5^{o}C$.

Sperm cell smears from the initial fresh ejaculates were made and stained by ABS as follows:

1. One-tenth milliliter of fresh semen was diluted in 0.9 ml of 2.9% solution of sodium citrate dihydrate in a pre-warmed 17 mm diameter tubular absorption cell. The dilution rate

was narrowed in certain cases when the concentration of spermatozoa in the ejaculate was low.

2. Two drops of this suspension were withdrawn with a pre-warmed dropper and added to four drops of the Wells-Awa stain in another similar pre-warmed tubular absorption cell.
3. One to two minutes 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 smears were air dried at 37°C, and a glass cover slip was mounted with diaphane.

Live-Dead Staining

The use of differential staining techniques has enabled one to distinguish living sperm cells from those that are dead. 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 cells, 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 and 5 grams of eosin Y (total dye content 92%) in 300 milliliters of double distilled water. The stain has a pH of 9.4, is very stable and can be kept in the refrigerator for several weeks.

Spermatozoal smears were prepared by placing one drop of semen into five to eight drops of nigrosin-eosin stain and mixed gently.

Duplicate slides were made by smearing one drop of the suspension on pre-warmed glass slides.

Live-dead stains were made on each pre-freeze sample. Two hundred sperm cells were examined on each slide to determine the percent live. This was accomplished with the light microscope at a magnification of 430X.

Post-freeze Determination

Sperm cell smears were made by OSU from the semen samples shipped to them by ABS. The samples had been stored in liquid nitrogen during this time. The recovery and acrosome staining procedure used was as follows:

1. Ampule was removed from liquid nitrogen and completely thawed in ice water.
2. Contents of ampule was transferred to a 10 ml plastic centrifuge tube and was centrifuged for 5 minutes.
3. Supernatant was discarded and the pellet was washed with 0.5 ml of 2.9% sodium citrate dihydrate.
4. Above was centrifuged for 3 minutes at the same speed, decanted supernatant discarded and pellet was washed with 0.25 ml of sodium citrate.
5. Solution was then centrifuged for 2 minutes at the same speed, decanted supernatant discarded and pellet re-suspended in 0.2 ml sodium citrate and mixed thoroughly.
6. Two drops of the washed semen sample were added to four drops of the acrosome stain in a pre-warmed 17 mm diameter tubular absorption tube.

7. Four to seven minutes later, one small drop of stained cells was smeared on a pre-warmed, clean microscopic slide in a thin layer.
8. The smears were quickly air-dried at 37°C, and a glass cover slip was mounted with diaphane.

Slide Evaluation

Once the slides of the pre- and post-freeze samples had been properly prepared, 200 sperm cells were examined on each slide to determine acrosome characteristics and morphological abnormalities. This was accomplished with the light microscope under oil, at a magnification of 970X, and using a blue filter. All slides were read by the same individual in such a way as to be completely unaware of what bull the slide belonged to.

A data sheet was set up that included the state of the acrosome as distributed across each cell type. The data sheet consisted of the following information:

1. Bull number
2. Collection date
3. Live cells
4. Aged acrosomes
5. Normal morphology
6. Normal cells with aged acrosomes
7. Normal cells with nonaged acrosomes
8. Abnormal cells with nonaged acrosomes
9. Abnormal cells with aged acrosome
10. Remarks

Categories 3 through 9 were based on percentages as determined from the 200 cells read from each slide.

The following guideline was used to make the above determinations:

A) The acrosome condition was recorded as nonaged or aged. The nonaged sperm cells were those that displayed a uniformly smooth acrosome that was closely adherent to the nucleus. Aged acrosomes were those that exhibited any of the following morphological changes:

a) Elevated or thickened acrosome: This is characterized by a partial or complete swelling of the outer membrane of the acrosome cap and, thus, an enlargement in acrosomal size.

b) Ruffled acrosomal cap: This is characterized by a wrinkled or irregular surface either over the anterior portion of the cap or spreading over the entire surface of the acrosome.

c) Enlarged equatorial segment: This is characterized by the typical half moon shape of the equatorial segment becoming exaggerated as the acrosome becomes loosened from the sperm cell head.

d) Loosened acrosome cap: The acrosome is in the process of loosening from the sperm head, eventually leading to the capless sperm state.

e) Disintegrating acrosome cap: This indicates the latter stage of aging.

f) Capless sperm: This is characterized by the lack of the acrosome and is considered the terminal stage of the

aging and/or deterioration sequence. The upper portion of the nucleus stains a light pink instead of the pale green which is typical when the acrosome is present.

B) Morphological abnormalities of the sperm cell were recorded and included head, neck and tail defects. The basic guideline used for the reading of abnormal sperm cells was that discussed by Salisbury and Van Demark (1961). Slight modifications were made especially in relation to neck and neck abnormalities. The neck or midpiece was considered to be abnormal if it deviated more than 45° from its normal axis or if any part of it actually paralleled itself. Also, if the midpiece was kinky it was considered to be abnormal. Tails were considered to be abnormal if at any point the tail ran parallel to itself or if when a horizontal line was drawn across the point of initial bending and extended to transect a second point on the tail and at this point a vertical line drawn such that the angle made above the horizontal line and the tail was greater than 45°. Any distinct coiling of the tail, kinky tails or terminal coiled tips of the tail were all considered abnormal. Tailess sperm or vestigial tails also fit into this category of abnormal sperm cells.

Also, included in this study was the determination of post-freeze motility after one month storage in liquid nitrogen. This was accomplished by thawing the ampules of semen in ice water and removing a drop of the semen and allowing it to warm to 37°C. Using the conventional light microscope, the percentage of progressively motile cells was determined and expressed to the nearest whole percent. The same

individual made all the above determinations in such a way as to be completely unaware of what bull the sample had been taken from or of any other information relating to the semen samples.

Statistical Analysis

Semen samples were collected from 43 bulls over a period of 11 months. Collections were made once a week with two ejaculates per collection. The total number of collections per bull ranged from five to eight, depending on if the yield per collection was sufficient enough to secure the required number of ampules per bull with the desired post-freeze range of 7 to 13 million motile cells per ampule. The 43 bulls were collected in such a way that four distinct collection groups were present. Once processed, the samples were distributed by ABS for routine artificial insemination. To get as random a sample of cows as possible, the semen was distributed across 60,000 associate herds throughout the United States in such a way that no one bull was used in a given herd more than four times. ABS collected 60- to 90-day nonreturn data on as many of the breedings as possible.

Differences among bulls within a group and differences among groups on all measured characteristics were examined for significance with an analysis of variance procedure using four collections per bull.

In order to determine the degree of relationship among different sperm cell characteristics, linear correlation coefficients among these characteristics were obtained across all collection and all bulls. Also, correlation coefficients were determined between the sperm cell characteristics and 60- to 90-day non-return to first service.

The major goal of this project was to develop a fertility index using the different sperm cell characteristics measured. A stepwise multiple regression analysis was used to derive the equation. This computer program was set up to derive a sequence of multiple linear regression equations in a stepwise manner. Also, the procedure was programmed so that a variable could only be included in the equation that was significant at the .05 level. This was done by the selection of a significance or tolerance level and letting this value be the end-point for inclusion of variables into the equation. At each step, one additional variable was added to the regression equation. The variable added was the one which made the greatest reduction in the error sum of squares. Equivalently, it was the variable which had the highest partial correlation with the dependent variable partialed on the variables which had already been added; or it was the variable which, if it were added, would have the highest F value. Also, variables could be forced into the equation with the non-forced variables being automatically removed when their F values became too low.

CHAPTER IV

RESULTS AND DISCUSSION

Preliminary Bull Grouping and Analyses

The reviewed literature reporting efforts to derive a means to determine the fertility level of the bovine male or to determine the quality of a given semen sample is voluminous and has varied in semen characteristics utilized. However, all researchers are in agreement in that no one single semen characteristic or test is adequate in predicting fertility. This subsequently led to the use of several characteristics in attempting to predict fertility. However, the use of acrosomal characteristics has been largely ignored. Presently there is no doubt that the acrosome cap is an important feature of the sperm cell and that abnormalities of this structure can result in decreased fertility, or, dependent on the kind of abnormality, total sterility. This study, therefore, represents preliminary investigations undertaken to determine which spermatozoan characteristics are the most important in determining the fertility level of a given bull and to determine to what degree these factors are utilizable in predicting fertility.

American Breeders Service supplied 43 Holstein bulls averaging approximately one year in age for the study. These bulls were distributed over a one year period with new bulls being added to the project on a quarter-year basis.

Initially in February of 1970, eight bulls were started on a collection regime consisting of two ejaculates once weekly. Ejaculates were rejected that were determined by ABS not to have a post-freeze motile cell concentration of $10 \pm 3 \times 10^6$ motile cells per ampule. This factor, along with an occasional failure of a bull to work, prevented a consistent week-by-week collection sequence for all the bulls. Also, if the number of ampules frozen on a given collection was below 104, then additional collections were made on that bull so that at the end of the bull's period there were five to eight collections with a total of 520 ampules per bull. This resulted in a number of bulls having more than the initially planned five collections, which caused some bulls in one collection period to overlap with those in another period. The bulls were divided into four collection groups so that each bull was in a group that most closely fit an apparent pattern. Figure 2 shows the bulls and the month each collection was made on that bull.

Ideally, it would have been better not to have to consider grouping the bulls at all. But, in accord with the literature, the season of the year the bulls are collected has an apparent influence on semen characteristics. To confirm this point in respect to this population of bulls, analyses of variance among groups were computed for both pre- and post-freeze semen characteristics and are presented in Tables I and II. Table I shows very large significant differences ($P < .005$) between groups for all pre-freeze characteristics studied except for percentage of live spermatozoa. Table II shows similar relationships ($P < .005$) between many of the same characteristics as in Table I. From these results, it was apparent that grouping the bulls should be considered in order to allow for possible significant seasonal differences

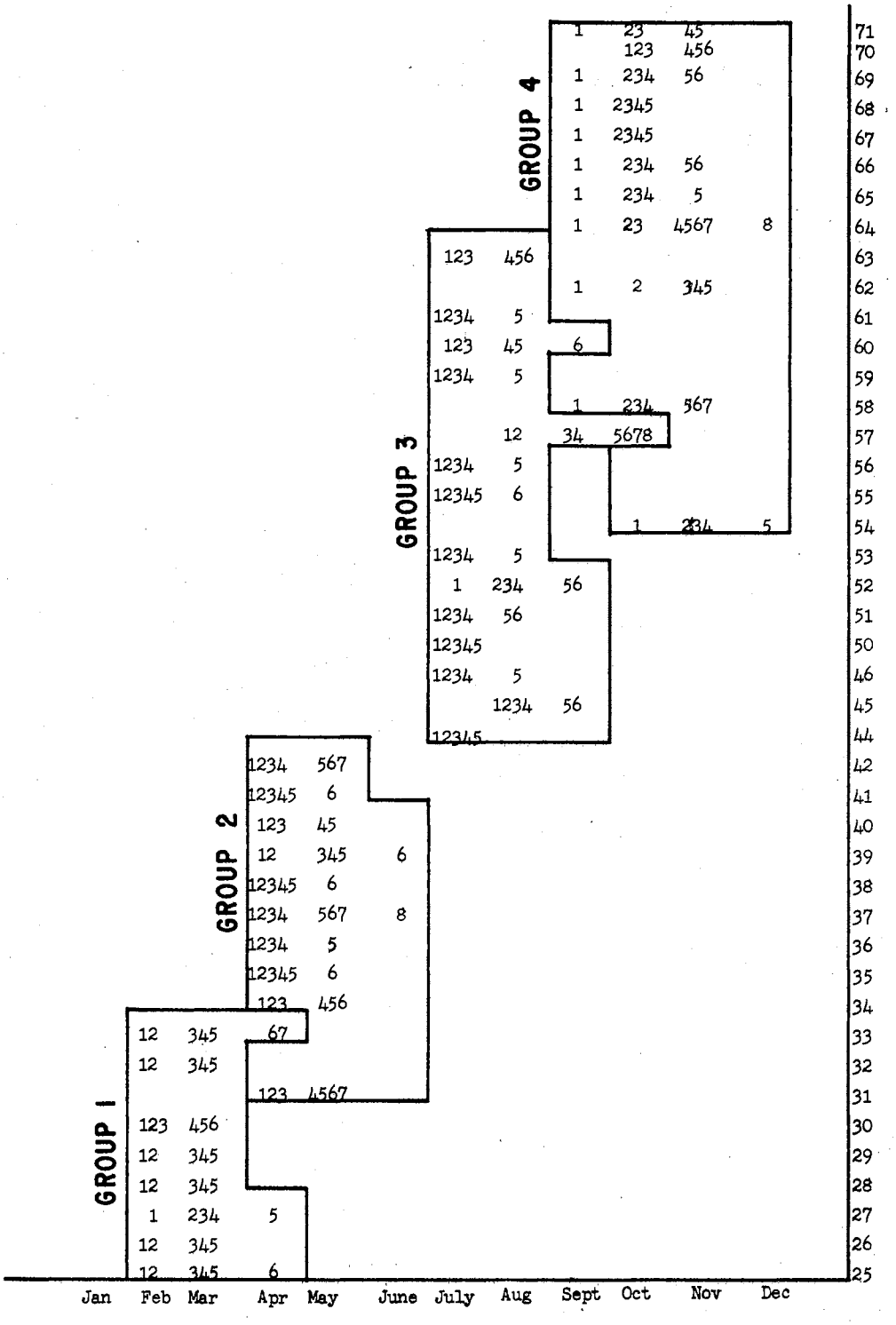

Figure 2. The distribution of the collection dates of 43 bulls and their classification into four different collection groups

TABLE I

ANALYSES OF VARIANCE FOR PRE-FREEZE SPERM CELL CHARACTERISTICS OF 43 BULLS IN FOUR DIFFERENT COLLECTION GROUPS

	Source of Variation	d.f.	Mean Square	F	Level of Significance
Percentage live	Groups	3	110.93	0.608	
	Bull (groups)	39	182.32		
Aged Acrosomes	Groups	3	1764.70	5.302	$P < .005$
	Bull (groups)	39	332.85		
Percent Morphologically Normal Sperm Cells	Groups	3	3938.33	27.612	$P < .005$
	Bull (groups)	39	142.63		
Percent Normal Cells with Aged Acrosomes	Groups	3	1428.05	6.624	$P < .005$
	Bull (groups)	39	215.57		
Percent Normal Cells with Nonaged Acrosomes	Groups	3	3420.44	14.330	$P < .005$
	Bull (groups)	39	238.68		
Percent Abnormal Cells with Nonaged Acrosomes	Groups	3	1529.08	20.759	$P < .005$
	Bull (groups)	39	73.66		
Percent Abnormal Cells with Aged Acrosomes	Groups	3	747.49	8.908	$P < .005$
	Bull (groups)	39	83.91		
Percent Nonaged Acrosomes	Groups	3	2327.19	6.771	$P < .005$
	Bull (groups)	39	343.67		

TABLE II

ANALYSES OF VARIANCE FOR POST-FREEZE SPERM CELL CHARACTERISTICS OF 43 BULLS IN FOUR DIFFERENT COLLECTION GROUPS

	Source of Variation	d.f.	Mean Square	F	Level of Significance
Percent Motility (0 storage time)	Groups	3	161.88	0.655	
	Bull (group)	39	248.49		
Percent Aged Acrosomes	Groups	3	8074.70	38.205	$P < .005$
	Bull (group)	39	211.39		
Percent Morphologically Normal Sperm Cells	Groups	3	10565.84	77.572	$P < .005$
	Bull (group)	39	136.22		
Percent Normal Cells with Aged Acrosomes	Groups	3	95.06	1.167	
	Bull (group)	39	82.29		
Percent Normal Cells with Nonaged Acrosomes	Groups	3	9527.40	65.474	$P < .005$
	Bull (group)	39	145.53		
Percent Abnormal Cells with Nonaged Acrosomes	Groups	3	106.95	1.433	
	Bull (group)	39	75.33		
Percent Abnormal Cells with Aged Acrosomes	Groups	3	8894.22	63.145	$P < .005$
	Bull (group)	39	140.87		

when computing the final regression analysis over all 43 bulls. However, the validity of testing for differences between groups is perhaps questionable due to the lack of a sufficient number of bulls in each group.

The evaluation criteria utilized in this study are described in Table III. The table includes a listing of all the sperm cell characteristics studied from 486 ejaculates of 43 bulls and their corresponding means, standard deviations, coefficients of variation and standard errors. Of possible concern are the relatively high coefficients of variation for some of the criteria. However, other research (Saacke, 1970; Erb, et al., 1951; Hafs, et al., 1958; Bishop and Hancock, 1955) reports several coefficients of variation for many of the same characteristics under basically the same conditions and indicates that the values reported in this study are very much within reported ranges and in many cases actually considerably lower. Therefore, in comparison with reported coefficients from other bull studs, it is suggestive that these estimates may be reasonably representative of bull studs in general and, consequently, are factors that must be presently accepted when working with such criteria.

The analyses of variance on pre-freeze semen characteristics (Table IV) revealed highly significant differences among bulls within a group for most of the characteristics studied. However, Group I showed no significant differences between bulls for five of the eight characteristics studied. This in part may be attributed to the fact that there were only eight bulls in this group as compared to a larger number of bulls in the other three groups. Excluding Group 1, the analyses of variance showed a significant difference ($P < .05$) among bulls on all acrosomal characteristics.

TABLE III

OVERALL MEANS, STANDARD DEVIATIONS, COEFFICIENTS OF VARIATIONS, AND STANDARD ERROR FOR THE SPERMATOZOAN CHARACTERISTICS STUDIED

Characteristic	$\bar{X}$ (%)	S.D. (%)	C.V. (%)	S.E. (%)
Pre-freeze Evaluation				
Live Spermatozoa	81.5	6.4	7	0.9
Aged Acrosomes	34.7	10.9	31	1.6
Normal Spermatozoa	74.1	10.3	13	1.6
Normal Cells with Aged Acrosomes	21.1	8.9	42	1.3
Normal Cells with Nonaged Acrosomes	53.0	10.8	20	1.6
Abnormal Cells with Nonaged Acrosomes	11.9	6.7	56	1.0
Abnormal Cells with Aged Acrosomes	13.6	5.8	42	0.9
Nonaged Acrosomes	65.0	11.3	17	1.7
Post-freeze Evaluation				
Motility (0-storage time)	32.5	7.3	22	1.1
Motility (1 mo. storage time)	31.2	5.3	16	0.8
Aged Acrosomes	47.9	12.4	25	1.9
Normal Spermatozoa	59.1	15.3	25	2.3
Normal Cells with Aged Acrosomes	20.2	3.9	19	0.6
Normal Cells with Nonaged Acrosomes	38.9	14.3	36	2.2
Abnormal Cells with Nonaged Acrosomes	11.9	4.4	36	0.7
Abnormal Cells with Aged Acrosomes	27.7	13.1	47	2.0
Nonaged Acrosomes	50.8	14.4	28	2.2

The analyses of variance on post-freeze sperm cell characteristics (Table V) for all four groups indicate highly significant differences ($P < .01$) among bulls within a group for the percentage of motile cells post-thaw at zero storage time. However, with post-freeze data, the uniformity across groups in relation to the state of the acrosome is not as apparent as it is for the pre-freeze data. Post-freeze differences in acrosomal characteristics among bulls within a group range from highly significant ($P < .01$) to insignificant. This in part can be explained by Table VI which shows the influence freezing has on the sperm cell characteristics. These means were tabulated over all 43 bulls across all groups and involved 243 collections with two ejaculates per collection. The results from these bulls indicated approximately a 14 percent increase in the percent of spermatozoa with aged acrosomes and a 14 percent decrease in the percent of normal sperm cells. It is feasible that freezing has different quantitative effects on sperm cells from different bulls and consequently, there is a lack of uniformity in the effect freezing has on acrosomal characteristics.

Tables VII and VIII show the different linear correlation coefficients among the sperm cell characteristics studied. These coefficients are based upon the grouped data from all 43 bulls in this study. Examination of these correlation coefficients reveals a large number of expected significant correlations. However, there are relatively numerous reports in the literature indicating correlations between the same two characteristics varying from one extreme to the other. Therefore, the importance of these actual correlation coefficients is questionable except in this given population of bulls under the specified conditions.

TABLE IV

ANALYSES OF VARIANCE FOR PRE-FREEZE SPERM CELL CHARACTERISTICS

	Source of Variation	Degrees of Freedom Groups 1	2	3	4
% Live Cells	Bull	7*	9	13**	10**
	Collection	3	3	3	3
	Bull x Coll.	21	27	39	30
% Aged Acrosome	Bull	*	*	**	**
% Morph. Normal Sperm Cells	Bull	—	—	**	**
% Normal Cells with Aged Acrosomes	Bull	—	**	**	**
% Normal Cells with Nonaged Acrosomes	Bull	—	*	**	**
% Abnormal Cells with Nonaged Acrosomes	Bull	—	*	**	**
% Abnormal Cells with Aged Acrosomes	Bull	*	*	**	**
% Nonaged Acrosomes	Bull	—	*	**	**

* $P < .05$
** $P < .01$

TABLE V

ANALYSES OF VARIANCE FOR POST-FREEZE SPERM CELL CHARACTERISTICS

	Source of Variation	Degrees of Freedom Groups 1	2	3	4
% Motility (0 storage time)	Bull	7**	9**	13**	10**
	Collection	3	3	3	3
	Bull x Coll.	21	27	39	30
% Aged Acrosomes	Bull	—	**	—	**
% Morph. Normal Sperm Cells	Bull	*	*	—	*
% Normal Cells with Aged Acrosomes	Bull	—	*	—	**
% Normal Cells with Nonaged Acrosomes	Bull	—	—	—	*
% Abnormal Cells with Nonaged Acrosomes	Bull	—	**	—	*
% Abnormal Cells with Aged Acrosomes	Bull	—	**	—	*

* $P < .05$
** $P < .01$

TABLE VI

INFLUENCE OF FREEZING ON VARIOUS
SPERM CELL CHARACTERISTICS

Sperm Cell Characteristic	Mean-% (243 collections)		
	Pre-freeze	Post-freeze	Change
Percent Normal Cells	74.5	60.2	-14.3
% Aged Acrosomes	34.2	48.2	+14.0
% Normal Cells with Aged Acrosomes	20.8	20.5	-0.3
% Normal Cells with Nonaged Acrosomes	53.7	39.7	-14.0
% Abnormal Cells with Nonaged Acrosomes	11.8	12.0	+0.2
% Abnormal Cells with Aged Acrosomes	13.4	27.7	+14.3
% Nonaged Acrosomes	65.5	50.9	-14.6

TABLE VII

VARIABLES USED TO IDENTIFY THE MEASURED SPERM CELL CHARACTER

X_1 :	Live spermatozoa (Pre-freeze)
X_2 :	Spermatozoa with aged acrosomes (Pre-freeze)
X_3 :	Spermatozoa with nonaged acrosomes (Pre-freeze)
X_4 :	Morphologically normal cells (Pre-freeze)
X_5 :	Normal cells with aged acrosomes (Pre-freeze)
X_6 :	Normal cells with nonaged acrosomes (Pre-freeze)
X_7 :	Abnormal cells with nonaged acrosomes (Pre-freeze)
X_8 :	Abnormal cells with aged acrosomes (Pre-freeze)
X_9 :	Motility (0 storage time) (Post-freeze)
X_{10}:	Motility (1 month storage time) (Post-freeze)
X_{11}:	Spermatozoa with aged acrosomes (Post-freeze)
X_{13}:	Morphologically normal cells (Post-freeze)
X_{14}:	Normal cells with aged acrosomes (Post-freeze)
X_{15}:	Normal cells with nonaged acrosomes (Post-freeze)
X_{16}:	Abnormal cells with nonaged acrosomes (Post-freeze)
X_{17}:	Abnormal cells with aged acrosomes (Post-freeze)

TABLE VIII

INTERRELATIONSHIPS OF SPERM CELL CHARACTERISTICS***

	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	X_{13}	X_{14}	X_{15}	X_{16}	X_{17}
X_1	1.00	0.10	-0.17	0.08	0.25	-0.13	0.05	-0.21	0.53**	0.50**	0.19	-0.09	-0.16	0.14	-0.21	0.14	0.14
X_2		1.00	-0.38*	-0.13	0.85**	-0.81**	-0.34*	0.58	0.08	0.14	0.54**	-0.99**	-0.35	0.15	-0.42**	0.11	0.47**
X_3			1.00	0.66**	-0.08	0.69**	-0.42**	-0.60**	0.12	0.20	-0.91**	0.42**	0.88**	-0.03	0.95**	0.16	-0.85**
X_4				1.00	0.36*	0.66**	-0.80**	-0.81**	0.12	0.33*	-0.63**	0.15	0.66**	0.16	0.66**	0.01	-0.65**
X_5					1.00	-0.47**	-0.64**	0.06	0.19	0.33*	0.22	-0.83**	-0.04	0.27	-0.11	0.09	0.13
X_6						1.00	-0.25	-0.82**	-0.04	0.04	-0.77**	0.82**	0.65**	-0.06	-0.71**	-0.07	-0.72**
X_7							1.00	0.34*	-0.05	-0.27	0.30	0.36*	-0.39*	-0.08	-0.39**	-0.08	0.31
X_8								1.00	-0.13	-0.25	0.69**	-0.58**	-0.62**	-0.14	-0.63**	0.09	0.70**
X_9									1.00	0.76**	-0.04	-0.06	0.18	0.27	0.12	-0.02	-0.12
X_{10}										1.00	-0.04	-0.13	0.17	0.14	0.14	0.20	-0.08
X_{11}											1.00	-0.57**	-0.88**	-0.01	-0.94**	0.08	0.96**
X_{12}												1.00	0.39**	-0.11	0.45**	-0.12	-0.51**
X_{13}													1.00	0.39*	0.97**	-0.28	-0.95**
X_{14}														1.00	0.14	-0.57**	-0.31*
X_{15}															1.00	-0.14	-0.93**
X_{16}																1.00	0.25
X_{17}																	1.00

* $P < .05$

** $P < .01$

*** 486 ejaculates from 43 Holstein bulls of approximately one year of age

The Relationship Between Fertility and Individual Spermatozoan Characteristics

Approximately 520 ampules of semen per bull were distributed across 60,000 associate herds by ABS. Normal distribution procedures were employed except that no one bull was used more than four times in a given herd. There was a total of 5976 first services with an average of 142 services for each of the 42 bulls. The number of services per bull ranged from a low of 110 to a high of 182 services.

The range for the 60 to 90-day non-return to first service was from 53.3% to 82.6% with an average of 70.7% for the 42 bulls.

As proposed, the final statistical analysis involved in this study was the development of a multiple linear equation, and since the dependent variable, which was 60 to 90-day non-return rate in this study, must meet the requirement of being normally distributed, Figure 3 was constructed to indicate such a distribution for the population of bulls used. There was slightly over a 29% difference between the two non-return extremes and, theoretically, this was a wide enough range to make a detection of differences in non-return rates feasible.

Table IX is a listing of all sperm cell characteristics measured in this study and their relationship to 60 to 90-day non-return rates. Only one variable, the percent of abnormal sperm cells with nonaged acrosomes, had a statistically significant correlation with fertility ($r = 0.349$, $P < .05$). All the other correlations were of relatively small magnitude with none of the single characteristics accounting for over 8% of the variation in fertility. Even the above characteristic that was significantly correlated with fertility could only account for slightly over 12% of the variation in fertility among bulls.

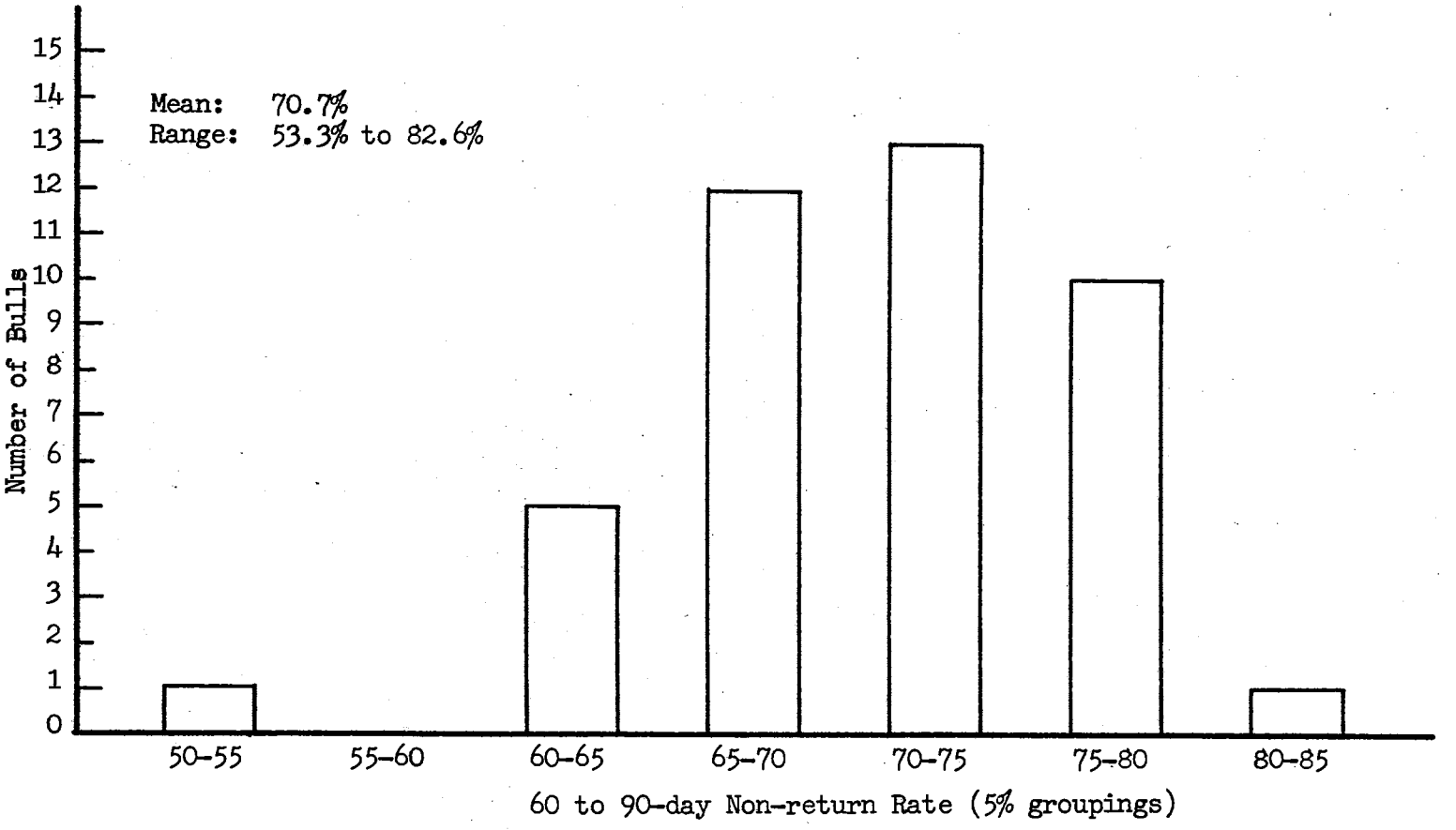

Figure 3. Distribution of the 60 to 90-day non-return rates of 42 Holstein bulls

TABLE IX

CORRELATION BETWEEN MEASURED SPERM CELL CHARACTERISTICS AND 60 TO 90-DAY NON-RETURN RATE**

Variable (%)	Correlation	Rerun
Pre-freeze Characteristics		
Live Sperm	0.10[1]	0.10[2]
Aged Acrosome	-0.18	-0.17
Normal Sperm	-0.28	-0.15
Normal Sperm with Aged Acrosome	-0.28	-0.18
Normal Sperm with Nonaged Acrosome	-0.03	0.08
Abnormal Sperm with Nonaged Acrosome	0.35*	0.23
Abnormal Sperm with Aged Acrosome	0.09	-0.04
Nonaged Acrosome	0.18	0.17
Post-freeze Characteristics		
Motility (0-storage time)	0.29	0.29
Motility (1 mo. storage time)	0.05	0.13
Aged Acrosomes	0.11	0.06
Normal Sperm	-0.07	0.06
Normal Sperm with Aged Acrosomes	0.05	0.05
Normal Sperm with Nonaged Acrosomes	-0.09	0.03
Abnormal Sperm with Nonaged Acrosomes	0.09	-0.16
Abnormal Sperm with Aged Acrosomes	0.09	-0.04
Nonaged Acrosomes	-0.06	———

* $P < .05$

** 474 ejaculates from 42 bulls

[1] value determined using means across all bulls

[2] values determined using pooled within group means

The reported linear correlations between fertility and various sperm cell characteristics in this study are much lower than many such values reported in the literature. Most recently Saacke and White (1972) reported such correlations ranging as high as 0.60. However, comparisons to such correlations is relatively meaningless since such values are dependent on a countless number of factors which vary extremely from one population of bulls to the next. One very important factor is the degree of selection that has been placed on the bulls prior to being utilized in the study. Normally, the variation among the bulls in bull studs is considerably less due to prior selection resulting in many of the correlations being relatively small. Optimum conditions of feeding, management, light and temperature the year round are also factors which tend to decrease the variation within the population of a bull stud. In general, as the variation of the population decreases, one should expect to find a decrease in correlation values.

Also, it must be kept in mind that correlations may be low when the optimum for a semen characteristic is reached which is considerably less than the maximum for that measure. When such an optimum value is reached, further increased values of the characteristic do not result in further increases in fertility.

Many of the differences in reported correlations between semen characteristics and fertility may be due to differences in age, size, health, environmental stress and many other factors involving individual bulls or groups of bulls. Also, handling procedures and environmental conditions to which the semen is subjected during dilution, processing, storage, shipment and insemination vary widely and, consequently, may account for much of the reported differences in correlation values.

Therefore, it is important to keep all of the above factors in mind before any correlation values are criticized as being either too high or too low.

Relationship Between Fertility and Multiple Spermatozoan Characteristics

For years researchers have sought readily measurable characteristics of semen quality which could be used to distinguish with precision the fertility differences among bulls. Even far more challenging, attempts to develop measurements of precision that would differentiate ejaculates of varying fertility produced by each bull have been the topic of many research efforts. However, this latter challenge appears almost impossible to meet with present day techniques. The physiological situation prevailing with any bull used for artificial insemination is dynamic and constantly changing, and the techniques for measuring ejaculate characteristics apparently do not reflect such changes. Chapter II gives an account of many such efforts and the problems that were encountered when the results were applied to actual field conditions.

This section reports on the results of the research on semen characteristics of 42 Holstein bulls and their combined relationship to 60 to 90-day non-return rate. A stepwise multiple regression analysis was utilized as described in Chapter III. The program was set up such that variables could be forced into the regression equation with the non-forced variables being added to the equation in order as to which one added the most to the prediction of fertility when in combination with the variables already in the equation.

Initially, the first series of multiple linear regression equations were computed separately on the pre-freeze variables and post-freeze

variables. The results of these computations were compared to results where both pre- and post-freeze variables were included in the equation. Under unrestricted conditions, the analyses resulted in a multiple correlation value of 0.387 (R^2 = 0.150) when utilizing only pre-freeze characteristics, a value of 0.455 (R^2 = 0.207) for post-freeze characters, and a value of 0.602 (R^2 = 0.363) when utilizing the combined pre- and post-freeze characteristics. It was evident at this point that both pre- and post-freeze variables must be considered in order to account for the most variation in fertility among bulls.

Group factors were computed to consider the fact that the bulls were collected at different times of the year. This was accomplished by forcing computed group variables into the regression equation. Another equation was developed where the above group variables were deleted from the equation and the two were compared to see if addition of the group variables resulted in a more adequate prediction equation.

Table X gives a list of the variables that were available for the stepwise regression analysis. After the initial regression run, various variables were forced or deleted from the equation as seen necessary. The variables used and the reasons for employing them are discussed in detail later in this section.

The first computer run of the stepwise regression analysis involving both pre- and post-freeze variables had the three group code variables deleted from the equation. All other variables listed in Table V were then available for selection into the program as previously described. A summary of the results is presented in Table XI. This initial analysis utilized ten variables with multiple correlation value of 0.602, therefore, accounting for approximately 36% of the variation in fertility among bulls.

TABLE X

VARIABLES UTILIZED FOR THE MULTIPLE REGRESSION ANALYSES

Number	Variable (%)
	Pre-freeze
1	Live spermatozoa
2	Spermatozoa with aged acrosomes
3	Spermatozoa with nonaged acrosomes
4	Morphologically normal cells
5	Normal cells with aged acrosomes
6	Normal cells with nonaged acrosomes
7	Abnormal cells with nonaged acrosomes
8	Abnormal cells with aged acrosomes
	Post-freeze
9	Motility (0 storage time)
10	Motility (1 month storage time)
11	Spermatozoa with aged acrosomes
12	Spermatozoa with nonaged acrosomes
13	Morphologically normal cells
14	Normal cells with aged acrosomes
15	Normal cells with nonaged acrosomes
16	Abnormal cells with nonaged acrosomes
17	Abnormal cells with aged acrosomes
A	Group 1 Code
B	Group 2 Code
C	Group 3 Code

TABLE XI

RESULTS OF THE STEPWISE MULTIPLE REGRESSION ANALYSIS WITH GROUP VARIABLES BEING DELETED FROM THE EQUATION

Step Number	Variable[1,2] Entered	Multiple R	Multiple R^2	Increase in R^2
1	# 9	0.2870	0.0824	0.0824
2	5	0.4442	0.1973	0.1149
3	4	0.4929	0.2430	0.0457
4	10	0.5052	0.2552	0.0122
5	11	0.5125	0.2627	0.0075
6	12	0.5474	0.2997	0.0370
7	2	0.5760	0.3318	0.0321
8	1	0.5816	0.3382	0.0064
9	14	0.5838	0.3408	0.0026
10	13	0.6021	0.3626	0.0218

[1]Number code to variables listed in Table X

[2]$P < .05$ for all variables

To determine if grouping the bulls into the four different collection groups added significantly to the prediction equation, another analysis was run with the group variables being forced into the equation. The results indicated no significant increase in the multiple correlation value ($R = 0.604$, $R^2 = 0.365$) as compared to the equation where the group variables had been deleted from the computations. This factor led to the conclusion that further analyses would be computed across all bulls ignoring collection time or groups.

Table XII lists the ten variables that added significantly ($P < .05$) to the prediction of 60 to 90-day non-return rate. Progressive motility and the morphology of the sperm cell are indicated to be two of the more important variables in the regression equation as far as prediction of fertility is concerned. This is in agreement with the literature which has for years reported motility and morphology to be important aspects of semen evaluation. Five of the ten variables that add significantly ($P < .05$) to the prediction of fertility involve the acrosome. This indicates that the acrosome is too important in semen evaluation to be ignored and should play a significant role in routine fertility evaluation.

Table XII also indicates that for all characteristics except motility, the initial pre-freeze state is important. In addition, for all pre-freeze characteristics in the equation that are substantially affected by freezing (Table VI), there are also present post-freeze representatives of the same characteristics. This possibly indicates that the variation in susceptibility of different bulls to stress is important. This was also indicated by motility one month post-freeze adding significantly to the prediction of fertility. For the characteristic of normal cells with aged acrosomes, a variable that showed very

TABLE XII

MULTIPLE LINEAR REGRESSION EQUATION USING TEN SPERMATOZOAN CHARACTERISTICS TO PREDICT 60 TO 90-DAY NON-RETURN RATE

Equation: $Y = a + b_1X_1 + b_2X_2 + b_3X_3 \cdots b_{10}X_{10}$

a = constant
b = regression coefficient
X = variable (%)

		Variable	Regression[1] Coefficient
		Constant ---- 76.132	
X_1:	#9	Post-freeze Motility (0-storage time)	0.469
X_2:	#5	Pre-freeze Normal Cells with Aged Acrosomes	0.339
X_3:	#4	Pre-freeze Normal Cells	-0.244
X_4:	#10	Post-freeze Motility (1 mo. storage time)	-0.416
X_5:	#11	Post-freeze Aged Acrosomes	0.264
X_6:	#12	Post-freeze Nonaged Acrosomes	0.473
X_7:	#2	Pre-freeze Aged Acrosomes	-0.455
X_8:	#1	Pre-freeze live cells	-0.135
X_9:	#14	Post-freeze Normal Cells with Aged Acrosomes	0.569
X_{10}:	#13	Post-freeze Normal Cells	-0.307

[1] $P < .05$ for all variables

little change due to freezing, the pre-freeze value adds considerably to the equation and is considered to be very important, whereas, its post-freeze value adds a relatively minor contribution to the prediction of fertility.

Table XII presents a list of the variables and their regression coefficients utilized in the multiple linear regression equation to predict 60 to 90-day non-return rates. The regression equation model was computed so that actual non-return rates are calculated instead of deviations from the mean of the population. This adjustment is made in the regression constant, a, and is also presented in Table XII.

Table XVII in the appendix presents a list of the 42 bulls used in this study and gives their individual observed 60 to 90-day non-return rates along with the computed or predicted non-return rates as determined using the regression equation from Table XII. Of the 42 bulls, the equation predicted the non-return rate of 33 of the bulls within five percent of their actual observed non-return values. The table also indicates that there is a tendency to overrate lower fertility bulls as opposed to underrating the higher fertility bulls when using this regression equation. However, with the above situation there would be little chance of culling out a very high fertility bull due to a low predicted fertility value since this predicted value would still be within ten percent of the actual value.

Figure 4 is a plot of the observed 60 to 90-day non-return rate versus the computed non-return rate, derived with different variables in the equation. Arbitrary fertility ranges were set at 71%, the mean of the population rounded to the nearest one percent and at 73% which is the mean plus one half of a standard deviation also rounded to the nearest one percent.

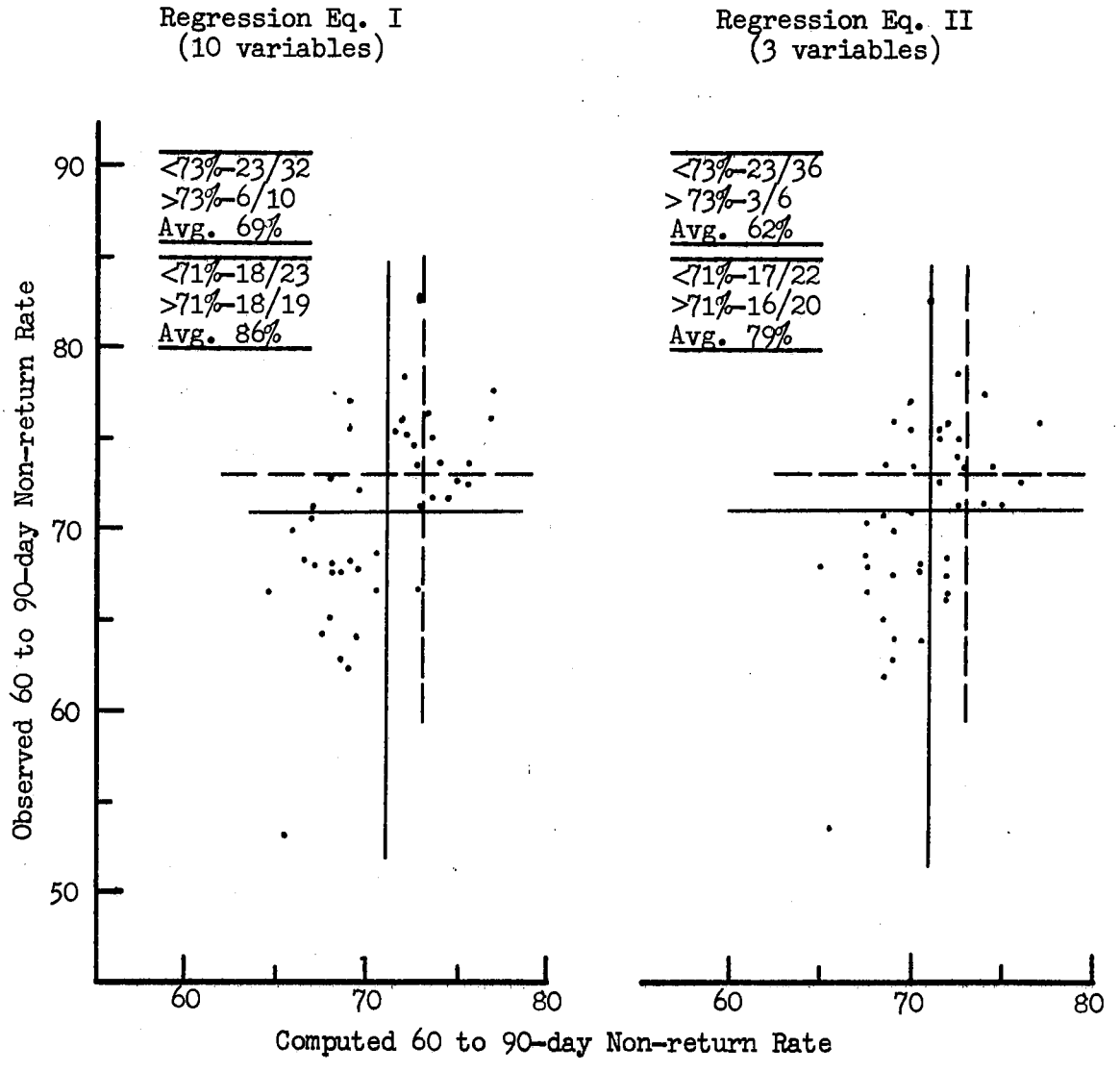

Figure 4. Scatter diagrams indicating the number of bulls that actually are in the range as designated by regression equations I and II

When the average was taken to be 71% with prediction equation I, Figure 4 indicates that of the 42 bulls that were categorized to be either above or below average, 36, or 86% of the bulls were classified correctly. When the average was set at 73%, then only 29 out of 42 or 69% of the bulls were actually above or below 73% as predicted.

In Table XII, it is apparent that the variables, percent post-freeze motility (0-storage time), percent pre-freeze normal cells with aged acrosomes and percent pre-freeze normal cells, account for the greatest amount of variance in fertility among these bulls. The other seven variables do contribute significantly ($P < .05$) to the prediction equation, but possibly not to the extent to make their measurements feasible. Therefore, another multiple regression equation was computed forcing these first three variables into the equation while deleting all other variables. This resulted in a multiple correlation value of 0.491 ($R^2 = 0.43$) as compared to a R-value of 0.602 ($R^2 = 0.363$) when using all ten variables. The equation and regression coefficients for using only three variables are presented in Table XIII.

Figure 4 also graphically represents the computed versus the observed 60 to 90-day non-return rates when using only three variables in prediction equation II. When the average non-return rate was set at 71%, of the 42 bulls classified as being above or below average, 33 or 69% of the bulls were classified correctly. However, when using 73% as the average, then only 26 out of 42 or 62% of the bulls were categorized correctly. Table XVIII in the appendix presents for each of the bulls the observed non-return rate as compared to the computed non-return rate as determined from the above equation.

TABLE XIII

MULTIPLE LINEAR REGRESSION EQUATION USING
ONLY THREE VARIABLES TO PREDICT
60 TO 90-DAY NON-RETURN RATES

Equation: $Y = a + b_1X_1 + b_2X_2 + b_3X_3$

a = constant
b = regression coefficient
X = variable (%)

		Variable	Regression[1] Coefficient
		Constant ——74.267	
X_1:	#9	Post-freeze Motility (0-storage time)	0.266
X_2:	#5	Pre-freeze Normal Cells with Aged Acrosomes	-0.159
X_3:	#4	Pre-freeze Normal Cells	-0.119

[1] $P < .05$ for all variables

Further study of the results of the regression analysis consisting of ten variables indicated the possibility of combining some of the original variables resulting in more generalized categories for the regression equation. Instead of being restricted to only normal cells with aged acrosomes, a combined category was added that also included the abnormal cells with aged acrosomes. This variable was then denoted as percent pre-freeze aged acrosomes. The percent post-freeze nonaged acrosomes had been one of the original ten variables to add significantly to the prediction equation and since it possibly indicates the "resistance" of the acrosome to be altered by stress, it was also forced into the new regression analysis. In addition, post-freeze motility at zero storage time and percent pre-freeze normal cells were forced into the equation. All other variables were completely deleted from the computations. The results of the regression analysis utilizing these four variables are presented in Table XIV. The multiple correlation value is 0.501 (R^2 = 0.251) when using four variables to predict fertility.

Figure 5 represents graphically the situation as previously described for the other regression equation. When the average was set to be 71%, then 31 of 42 bulls or 74% of the bulls designated to be above or below average were as described. However, when the average was set to be 73%, only 64% of the bulls were appropriately classified. Table XIX in the appendix gives the observed and computed non-return rates for each bull using the above equation.

The final multiple regression computations consisted of six variables. The first variables forced into the equation were the three variables that had previously been indicated to account for the greatest amount of variation in fertility among these bulls. The percent

TABLE XIV

MULTIPLE LINEAR REGRESSION EQUATION USING FOUR VARIABLES TO PREDICT 60 TO 90-DAY NON-RETURN RATE

Equation: $Y = a + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4$

a = constant
b = regression coefficient
X = variable (%)

		Variable	Regression[1] Coefficient
		Constant —— 80.623	
X_1:	#9	Post-freeze Motility (0-storage time)	0.253
X_2:	#4	Pre-freeze Normal Cells	-0.209
X_3:	#2	Pre-freeze Aged Acrosomes	-0.116
X_4:	#12	Post-freeze Nonaged Acrosomes	0.028

[1] $P < .05$ for all variables

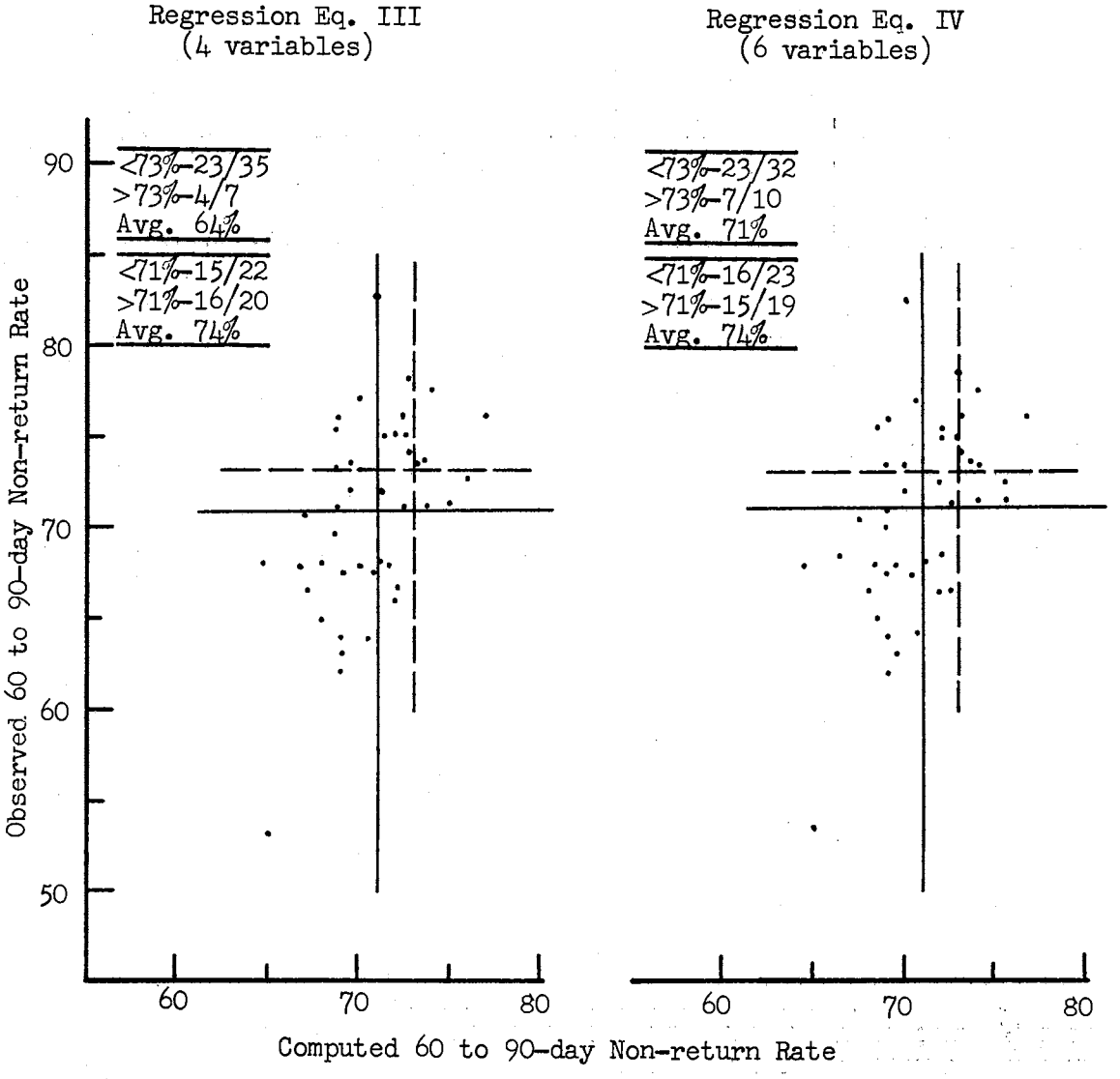

Figure 5. Scatter diagrams indicating the number of bulls that actually are in the range as designated by regression equations III and IV

pre-freeze aged acrosomes and percent post-freeze nonaged acrosomes were again included for the same reasons that they were utilized in the previous regression equation. The sixth variable to be included was percent post-freeze normal cells. This factor was included since its pre-freeze counterpart added very significantly to the equation. Since freezing affected the percent normal cells substantially, it was felt that this may account for a substantial amount of variation.

Table XV presents the multiple regression equation and regression coefficients for the six variables involved. Figure 5 also indicates that when the average was taken to be 71%, 31 out of 42 or 74% of the bulls were classified correctly when using this equation. Also, 71% were classified correctly when 73 percent was selected to be the average. The multiple correlation value is 0.504 (R^2 = 0.254) when using this regression equation. Table XX in the appendix gives a listing of each bull with his observed non-return rate and the computed non-return rate using the above regression equation consisting of six variables.

TABLE XV

MULTIPLE LINEAR REGRESSION EQUATION USING SIX VARIABLES TO PREDICT 60 TO 90-DAY NON-RETURN RATE

Equation: $Y = a + B_1X_1 + b_2X_2 + \ldots b_6X_6$

a = constant
b = regression coefficient
X = variable (%)

		Variable	Regression[1] Coefficient
		Constant ---- 84.447	
X_1:	#9	Post-freeze Motility (0-storage time)	0.251
X_2:	#5	Pre-freeze Normal Cells with Aged Acrosomes	0.103
X_3:	#4	Pre-freeze Normal Cells	-0.251
X_4:	#2	Pre-freeze Aged Acrosomes	-0.192
X_5:	#12	Post-freeze Nonaged Acrosomes	0.060
X_6:	#13	Post-freeze Normal Cells	-0.031

[1] $P < .05$ for all variables

CHAPTER V

SUMMARY AND CONCLUSION

It has been pointed out in the preceeding chapters that the state of the acrosome should be of vital concern when semen is being evaluated and that this characteristic should be introduced into routine semen evaluation by bull studs. Therefore, it was the purpose of this research to initiate studies in the determination as to which sperm cell characteristics account for the greatest amount of variation in fertility among bulls and to determine if the state of the acrosome actually does enter in significantly in the prediction of fertility.

Forty-two bulls were utilized in the study and two ejaculates were collected once a week for five to eight collections. As previously described in Chapter III, certain standards were required of a collection before it was considered to be acceptable. A total of 486 ejaculates were involved in the study with both pre- and post-freeze determinations being made on the samples. Frozen semen from each bull was distributed by ABS with 60 to 90-day non-return data being collected on a total of 5976 first services. The average non-return rate for the 42 bulls utilized in this study was 70.7 percent.

A total of 17 different categories were established to characterize the various sperm cell samples. Included in these characteristics were the number of live cells, progressive motility, morphology of the sperm cell and the state of the acrosomal cap.

A series of multiple linear regression equations were computed utilizing only the variables that added significantly to the prediction of fertility at the .05 level. Basically, there were ten variables that fitted the above specification with five of them involving the state of the acrosome. There were four regression equations computed with the number of variables in the equation varying from ten to three.

Table XVI presents a summary of the results obtained from each of the four equations. The results and characteristics involved in the regression equation utilizing ten variables are listed in Table XII. Of the ten variables involved, post-freeze motility (0-storage time), pre-freeze normal cells with aged acrosomes and pre-freeze normal cells accounted for the greatest degree of variation in fertility for the 42 bulls studied. The second regression equation involved only these three variables, but a certain degree of the precision was lost due to the elimination of the other seven variables. The ten variables accounted for approximately 36 percent of the variation in fertility among bulls as compared to only 24 percent when using the three variables.

When attempting to categorize the bulls as being above or below average using 71 and 73 percent as average fertility values, the ten variables were able to place approximately seven percent more of the bulls into a correct category when comparing observed and computed non-return values than was the equation utilizing only three variables. The practical question is whether or not this increase in precision can merit the use of seven additional variables to predict fertility. This would depend upon the availability of necessary personnel in the bull evaluation. Actually once an individual has become well acquainted with the evaluation procedure, ten variables as opposed to three variables

TABLE XVI

SUMMARY TABLE FOR FOUR MULTIPLE LINEAR REGRESSION EQUATIONS WITH A DIFFERENT NUMBER OF VARIABLES IN EACH EQUATION

Regression Equation	$\frac{R}{R^2}$	Bulls classified correctly when average set at 71%	Bulls classified correctly when average set at 73%	No. bulls where computed value less than 5% from observed fertility	No. bulls where computed value less than 7% from observed fertility
Equation I (10 variables)	$\frac{0.601}{0.365}$	$\frac{36}{42} = 86\%$	$\frac{29}{42} = 69\%$	$\frac{32}{42} = 76\%$	$\frac{39}{42} = 93\%$
Equation II (3 variables)	$\frac{0.493}{0.243}$	$\frac{33}{42} = 79\%$	$\frac{26}{42} = 62\%$	$\frac{30}{42} = 71\%$	$\frac{40}{42} = 95\%$
Equation III (4 variables)	$\frac{0.501}{0.251}$	$\frac{31}{42} = 74\%$	$\frac{27}{42} = 64\%$	$\frac{31}{42} = 74\%$	$\frac{39}{42} = 93\%$
Equation IV (6 variables)	$\frac{0.504}{0.254}$	$\frac{31}{42} = 74\%$	$\frac{30}{42} = 71\%$	$\frac{31}{42} = 74\%$	$\frac{39}{42} = 93\%$

requires very little additional time and requires no additional staining or preparation procedures except for the live-dead staining procedure. This procedure is not very involved and requires little skill to differentiate the live cells from the dead cells.

In addition to the two previously discussed regression equations, two other equations were computed. One of the equations invloved only four variables (Table XIV) and the other equation six variables (Table XV). When comparing the results of these two equations to the results involving the three variable equation (Table XIII), very little difference in precision was apparent.

The decision as to which of the four reported regression equations that should be used to categorize a group of bulls as to fertility rate is a question of precision versus personnel and time.

An important facet of this study is the knowledge of what semen characteristics are important in relationship to fertility. With this information available, bull studs can take a genetically superior male that has an undesirably low non-return rate and determine which of these characteristics are causing the problem in the individual. Once this has been determined, revision in handling of the bull or his semen samples can possible be made to favorably alter the necessary sperm cell characteristics. This preliminary study utilizing 42 bulls indicates that post-freeze motility (0-storage time), pre-freeze normal cells with aged acrosomes and pre-freeze normal cells are all relatively important in the evaluation of semen.

However, it must be pointed out that this research project is a pilot project and that additional work and re-evaluation needs to be done before its applicability can really be tested. Once this project

had been completed, two factors became apparent that may play some significance in fertility prediction and may consequently alter the prediction equations presented in this publication. Work published subsequent to this project indicated that tail abnormalities play little, if any, role in the prediction of fertility and that tail abnormalities possibly should not be considered as morphological abnormalities as was done in this study. Also, initially in this study, correlations of sperm cell characteristics to 60 to 90-day non-return rates were determined using values as determined across all bulls instead of pooling bulls within groups. To determine if this made a significant difference in the correlation values, a new statistical program was run using means pooled within groups and is presented in Table IX for comparison purposes. In most cases there is very little difference in the two correlation values. Also, those factors that are most highly correlated with non-return rates initially are still the same factors that are most highly correlated with non-return rates in the analyses rerun. Therefore, the complete regression analyses were not redone using pooled within group means.

It must be emphasized that if these equations are used for culling purposes or used for improvement of low fertility bulls, all characteristics must be read according to the outline presented in Chapter III in order to get comparable results. Also, the environment that the bulls are subjected to must be as similar as possible to the environmental conditions these experimental bulls had been subjected to.

In conclusion, this study indicates that motility, morphology, and the state of the acrosome are all important spermatozoan characteristics in the prediction of fertility. Presently, post-freeze motility and

morphology of the sperm cell are two of the characteristics routinely used in semen evaluation by bull studs. However, before the artificial insemination industry can profit to the greatest extent from semen evaluation, they must consider the state of the acrosome as an added criterion to semen evaluation.

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APPENDIX

TABLE XVII

COMPARISON OF OBSERVED VERSUS PREDICTED NON-RETURN RATES UTILIZING A MULTIPLE LINEAR EQUATION CONSISTING OF TEN VARIABLES

Bull Number	Non-return Rate (%)		Difference	Bull Number	Non-return Rate (%)		Difference
	Observed	Predicted			Observed	Predicted	
25	75.3	69.2	6.1	51	70.7	67.5	3.2
26	82.6	72.9	9.7	52	67.5	68.5	-1.0
27	72.6	75.1	-2.5	53	65.0	68.0	-2.8
28	64.0	67.5	-3.5	54	68.5	70.5	-2.0
29	73.6	73.6	0.0	55	66.5	64.6	1.9
31	67.8	69.5	-1.7	56	53.3	65.5	-12.2
32	64.0	69.7	5.7	57	69.8	65.8	4.2
33	62.2	69.1	-6.9	58	75.0	73.4	1.6
34	66.7	73.1	-6.4	59	72.4	75.5	-3.1
35	73.9	72.8	1.1	60	71.9	69.7	2.2
36	75.9	71.7	4.2	61	66.5	70.6	-4.1
37	67.9	66.9	1.0	62	71.4	73.6	-2.2
38	67.9	67.9	0.0	63	73.6	75.6	2.0
39	62.8	68.5	-5.7	64	75.0	72.4	2.6
40	73.3	68.0	5.3	65	77.6	76.8	0.7
41	68.1	69.1	-1.0	66	67.5	68.1	-0.6
42	78.3	72.3	6.0	67	75.3	71.4	3.9
44	68.3	66.8	1.5	68	71.2	73.1	-1.9
45	76.2	73.2	3.0	69	77.0	69.2	7.8
46	71.2	67.2	4.0	70	71.4	74.3	-2.9
50	73.6	74.0	-0.4	71	75.8	76.8	-1.0

TABLE XVIII

COMPARISON OF OBSERVED VERSUS PREDICTED NON-RETURN RATES UTILIZING A MULTIPLE LINEAR EQUATION CONSISTING OF THREE VARIABLES

Bull Number	Non-return Rate (%) Observed	Predicted	Difference	Bull Number	Non-return rate (%) Observed	Predicted	Difference
25	75.3	69.0	6.3	51	70.7	67.4	3.3
26	82.6	71.0	11.6	52	67.5	68.9	-1.4
27	72.6	76	-3.4	53	65.0	68.3	-3.3
28	64.0	70.8	-6.8	54	68.5	72.2	-3.7
29	73.6	74.4	-0.8	55	66.5	67.7	-1.2
31	67.8	70.5	-2.7	56	53.3	65.4	-12.1
32	64.0	69.1	-5.1	57	69.8	69.0	0.8
33	62.2	68.4	-6.2	58	75.0	71.4	3.6
34	66.7	72.0	-5.3	59	72.4	71.3	1.1
35	73.9	72.6	1.3	60	72.0	70.2	1.8
36	75.9	72.2	3.7	61	66.5	71.9	-5.4
37	67.9	64.8	3.1	62	71.4	74.1	-1.7
38	68.0	67.6	0.4	63	73.6	73.0	0.6
39	62.8	69.1	-6.3	64	75.0	72.3	2.7
40	73.3	68.3	5.0	65	77.6	74.0	3.6
41	68.1	70.9	-2.8	66	67.5	71.8	-4.3
42	78.3	72.3	6.0	67	75.3	71.6	3.7
44	68.3	67.6	0.7	68	71.2	72.4	-1.2
45	76.2	69.2	7.0	69	77.0	70.2	6.8
46	71.2	68.7	2.5	70	71.4	74.8	-3.4
50	73.6	69.9	3.7	71	75.8	76.9	-1.1

TABLE XIX

COMPARISON OF OBSERVED VERSUS PREDICTED NON-RETURN RATES UTILIZING A MULTIPLE LINEAR EQUATION CONSISTING OF FOUR VARIABLES

Bull Number	Non-Return Rate (%) Observed	Predicted	Difference	Bull Number	Non-return Rate (%) Observed	Predicted	Difference
25	75.3	68.9	6.4	51	70.7	67.2	3.4
26	83.6	70.7	11.9	52	67.5	69.0	-1.5
27	72.5	76.0	-3.5	53	65.0	68.3	-3.3
28	64.0	70.7	-6.7	54	68.5	71.9	-3.4
29	73.6	73.8	-0.2	55	66.5	67.3	-0.8
31	67.8	70.1	-2.3	56	53.3	65.1	-11.8
32	64.0	69.0	-5.0	57	69.8	68.7	1.1
33	62.2	68.9	-6.7	58	75.0	71.6	3.4
34	66.7	72.1	-5.4	59	72.4	71.4	1.0
35	73.9	72.8	1.1	60	71.9	69.7	2.2
36	75.9	72.6	3.3	61	66.5	72.0	-5.5
37	67.9	65.0	2.9	62	71.4	73.8	-2.4
38	67.9	68.2	-0.3	63	73.6	73.4	0.2
39	62.8	69.2	-6.4	64	75.0	72.6	2.4
40	73.3	68.8	4.5	65	77.6	74.0	3.6
41	68.1	71.3	-3.2	66	67.5	71.0	-3.5
42	78.3	72.8	5.5	67	75.3	71.9	3.4
44	68.3	66.8	1.5	68	71.2	72.6	-1.4
45	76.2	68.9	7.3	69	77.0	70.2	6.8
46	71.2	68.7	2.5	70	71.4	75.2	-3.8
50	73.6	69.8	3.8	71	75.8	76.7	-0.9

TABLE XX

COMPARISON OF OBSERVED VERSUS PREDICTED NON-RETURN RATES UTILIZING A MULTIPLE LINEAR EQUATION CONSISTING OF SIX VARIABLES

Bull Number	Non-return Rate (%) Observed	Predicted	Difference	Bull Number	Non-return Rate (%) Observed	Predicted	Difference
25	75.3	68.7	6.6	51	70.7	67.3	3.4
26	82.6	70.2	12.4	52	67.5	69.2	-1.7
27	72.6	75.7	-3.1	53	65.0	68.4	-3.4
28	64.0	70.6	-6.6	54	68.5	61.8	-3.3
29	73.6	73.3	0.3	55	66.5	67.5	-1.0
31	67.8	69.7	-1.9	56	53.3	65.2	-11.9
32	64.0	68.8	-4.8	57	69.8	68.8	1.0
33	62.2	68.8	-6.6	58	75.0	72.1	2.9
34	66.7	71.9	-5.2	59	72.4	72.0	0.4
35	73.9	72.8	1.1	60	72.0	69.8	2.2
36	75.9	72.8	3.1	61	66.5	72.3	-5.8
37	67.9	64.7	3.2	62	71.4	73.8	-2.4
38	68.0	68.4	-0.4	63	73.6	73.8	-0.2
39	62.8	69.5	-6.7	64	75.0	73.0	2.0
40	73.3	68.8	4.5	65	77.6	73.9	3.7
41	68.1	71.2	-3.1	66	67.5	70.4	-2.9
42	78.3	72.9	5.4	67	75.3	72.2	3.1
44	68.3	66.2	2.1	68	71.2	72.7	-1.5
45	76.2	68.9	7.3	69	77.0	70.4	6.6
46	71.2	68.9	2.3	70	71.4	75.3	-3.9
50	73.6	70.0	3.6	71	75.8	76.4	-0.6

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