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studies in GENETICS

I. a cytophotometric analysis of the deoxyribonucleic acid (DNA) content in germ cells from santa gertrudis bulls

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THE UNIVERSITY OF TEXAS : AUSTIN

The benefits of education and of useful knowledge, generally diffused through a community, are essential to the preservation of a free government.

SAM HOUSTON

Cultivated mind is the guardian genius of Democracy, and while guided and controlled by virtue, the noblest attribute of man. It is the only dictator that freemen acknowledge, and the only security which freemen desire.

MIRABEAU B. LAMAR

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Foreword

Within recent years the role of deoxyribonucleic acid (DNA) as the genetic substance of cells has been firmly established, and the development of the Feulgen staining technique has made it possible to measure the DNA content of individual cells rather precisely. For example, Dr. Cecilie Leuchtenberger and her collaborators have shown that the amount of DNA in sperm cells is more or less correlated with the fertility of the male producing them. The articles appearing in this bulletin are the culmination of several years' work and deal with the DNA content of bull sperm in relation to fertility and infertility.

After having reviewed the work of Dr. Leuchtenberger and her collaborators, Profs. C. P. Oliver, R. P. Wagner and W. S. Stone felt that further and more extensive work in this field of investigation might provide a better understanding of fertility and infertility in mammals as well as an insight into the role of DNA as a genetic material. Of the possible test animals for such work, the Santa Gertrudis breed of cattle, which had been developed only recently by the King Ranch, seemed most promising. Drs. Oliver and Stone visited the ranch, discussed the problem with the ranch personnel and obtained their very generous cooperation. In the several years that followed, they obtained and sent to our laboratories for testing a number of semen samples from many young bulls, most of which were of unknown fertility. Since Dr. R. M. Welch, postdoctoral research associate, was experienced with the use of the cytophotometric techniques necessary for DNA measurement, DNA analysis of semen spermatozoa appeared to be the best approach to this problem. These studies were begun in the summer of 1956 under the direction of Dr. Welch.

Several problems were immediately apparent. These were: (1) Is the cytophotometric determination of DNA valid; i.e., could any variation found in the DNA content be established as true variation within the cell above the variation incurred as part of the experimental method? (2) If the variation were established as true DNA fluctuation, could a line be drawn between the DNA content of sperm obtained from the few tested infertile animals and that obtained from the few tested fertile animals? (3) Could it be shown from the data obtained from the group of animals of unknown fertility, that some were probably infertile, and if so, just what reliability could be placed on this test? (4) Could a low amount of DNA be correlated with other criteria for fertility, such as morphology and motility of sperm as well as sperm count? (5) If such a correlation were found to exist, could DNA-content determination on sperm be established as another criterion for judging fertility; and if so, how? Finally, (6) if such a criterion could be established, then just how beneficial would the technical procedures be for the practical animal breeder? Moreover, from a theoretical view, it was desirable throughout the program to obtain as much information as possible about DNA and its function. These and other considerations of the project are described in Article I, the introduction to the series of articles in this publication.

Article II considers in great detail the experimental error of the cytophotometric technique and its resulting limitations. Article III presents the limited data obtained from the animals of known fertility and infertility gathered from both

semen samples and testes preparations. The large amount of data accumulated from bulls of unknown fertility is examined in Article IV. It is divided into three parts corresponding to the three divisions of the project. Article V considers the possible correlations among the DNA data and the previously used criteria for fertility—morphology, motility and numbers of spermatozoa. The greatest correlation was found to exist among these last three factors; i.e., if a sample contained sperm in large numbers, these sperm would usually be of normal morphology and show normal motility. However, results were obtained indicating rather strong correlations between DNA content and motility and sperm count, while a much weaker correlation existed between DNA and sperm morphology. Furthermore, it was found that the variation in DNA content of the Santa Gertrudis sperm was somewhat, if not considerably, less than that reported by Leuchtenberger for sperm of bulls and especially for human sperm.

Finally, a possible fertility rating system, which takes into account all four factors examined, is suggested in Article VI. A general discussion and summary which examines the possible practical and theoretical implications of the conclusions reached completes the bulletin.

We wish to express our especial indebtedness to Mr. Robert J. Kleberg, Jr., President; Mr. Richard M. Kleberg, Jr., Vice-president; Dr. J. K. Northway and Mr. Albert O. Rhoad, all of the King Ranch. The research was possible only because of their interest, direct financial support and extensive work necessary to obtain the many semen samples from the large number of bulls tested, as well as the special group of *U. T.* bulls set aside for our continued testing. Mr. Rhoad, as the King Ranch geneticist, helped us with our long range plans, fitting them into the ranch operations, and many other persons, named and unnamed, carried out the work on the ranch for us.

Dr. Robert M. Welch, a postdoctoral research associate, directed the work at The University of Texas. Dr. William C. Guest and Mr. E. W. Hanly assisted him as graduate students and are coauthors of some of the papers as is Miss Kathleen Resch, research assistant, who was most valuable in the final stages of the work and in the assembly of data and preparation of figures and tables. Miss Barbara Sutherland, Mrs. Caroline Alford and Mr. Bert Cromack were technical assistants for parts of the work. Mrs. Jane Hubby, the department artist, was kind enough to prepare most of the figures. We also wish to thank The University of Texas Computation Center for helping Mr. Hanly program the data and compute some of the correlations.

The necessary financial support was provided by the Alice G. K. Kleberg Fund from the King Ranch, the Rockefeller Foundation, and the U. S. Public Health Service, National Institutes of Health grants RG-5133 and RG-6492.

WILSON S. STONE
Director, Genetics Foundation
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Editor

I. Introduction

ROBERT M. WELCH

The extensive research of Leuchtenberger and collaborators (*6*, *7*, *8*, *11*) has implicated the deoxyribonucleic acid (DNA) content of the spermatozoa in certain types of human male infertility. Similar results were obtained in studies on sperm of morphologically normal bulls *(9)* as well as deviations found in dwarf cattle *(10, 32)*. Genetically, the possible inheritance of a biochemical abnormality interfering with the synthesis of DNA is of obvious interest; from the practical standpoint of animal breeding, a possible new index has been added to that list of criteria by which it has been attempted to detect infertility prior to breeding and thus avoid the costly trial-and-error process of the range and breeding pen.

There are two main approaches to this problem, logically related in that the second is properly sequential to the first. In the beginning, as was necessary, Leuchtenberger and collaborators demonstrated DNA constancy in men of known fertility and DNA deviation in men of suspected infertility and, less exhaustively, a similar situation in bulls. This is the more economical approach, as the screening process and the separation of the infertile men and bulls have already been accomplished. Next, however, it is necessary to find DNA constancy and deviation in animals of unknown fertility, and if possible, correlate these, respectively, with fertility and infertility. This step is not to be considered an application of an established theory but rather a research corollary.

The logical confirmation of the theory that DNA deviation is related to infertility because it is found in infertile animals is to find DNA deviation in animals of unknown fertility and correlate it with infertility. At the same time, the use of bulls as experimental material would not be subject to the weakness of human material, in which male infertility could not be conclusively established. Furthermore, the incidence and degree of DNA deviation in animals of unknown fertility and its possible correlation with motility, morphology and sperm count are in themselves of theoretical importance from the standpoint of DNA constancy or variation and the relation of DNA to cell function. In addition, DNA deviation is also of importance from the standpoint of animal breeding, since such data are necessary in determining the economic importance of the condition, the design and the application of testing programs. Finally, the geneticist would like to know whether this abnormal condition is present in young animals in order to rule out the possibility that it might be a phenomenon of testicular degeneration of environmental influence. This requirement, as well as those previously mentioned, is satisfied by the use of young animals of unknown fertility.

The possible extent of infertility in young bulls can be roughly approximated from such data as the estimate that "10 per cent of farm livestock is removed from herds annually because of infertility" and that "dairy herd improvement association records in Michigan show that more than 11 per cent of the animals in high producing herds were culled out each year during the period of 1950 to 1954 because of failure to conceive." (*29*) The first figure apparently refers to both

male and female infertility in herds in which the males are a highly selected group and thus understates male infertility among unselected bulls. The figure of 11 per cent in the second statement presumably refers to cows that could not conceive although mated with fertile bulls and thus is concerned with female infertility alone. If we assume that male and female infertility are of roughly equal incidence (a 10 to 20 per cent difference would not affect the general deductions of this paragraph) the 11 per cent figure might be taken as representative of male infertility, although in one respect it might be an underestimate since male infertility in an unselected group of bulls might well be greater than female infertility in a selected group of cows; in another respect it might be an overestimate since it would include all cases of infertility, both hereditary and degenerative. Without attempting an unnecessary precision, one might reasonably expect to find up to 10 per cent infertility in an unselected group of bulls. How many of these would be due to DNA deviation is another question. Although the Leuchtenberger group draws the conservative conclusion that DNA seems implicated in some cases of male infertility, from both seminal sperm and testis work, their data suggest a more general involvement. There is, for example, a clear separation between the DNA means for all the fifteen infertile bulls and all the fifteen fertile bulls examined, with the exception of only one overlapping mean in each category (*9*), although this conclusion is somewhat vitiated by the fact that many DNA means of the total group of 55 fertile bulls overlap those of the infertile group. Yet, over half the means of the infertile group are lower than the lowest mean of the fertile group. On the available data, it seems that one might reasonably expect to find DNA deviation in about 5 per cent of a group of unselected bulls, possibly a little more or possibly less. If the incidence and degree are sufficiently great, elimination of the bulls affected might be economically worthwhile, even for a group of unselected bulls intended for use on the range. If the incidence and degree are slight, practical application of the technique might be limited to highly selected bulls intended for use in single sire herds and artificial insemination programs. In any event, determination of the incidence and degree of the condition, whatever it may be, is both a theoretically desirable goal and a research prerequisite to any practical application.

MATERIAL AND METHODS

Samples of semen from Santa Gertrudis bulls of the King Ranch, which, with the comparatively few exceptions noted, were under two years of age and of unknown fertility, furnished the experimental material. Although these did not include the higher quality Santa Gertrudis bulls, they were sufficient for the purpose of the research. These could be assembled by ranch personnel at one convenient location, samples taken in one morning from up to twenty-odd animals and forwarded on the same day via air freight to this laboratory. Further details of collection, preservation, shipment, handling on arrival, preparation of slides, staining, analysis and definition of experimental error are included in Article II, which is devoted exclusively to method, but it is summarized here for those who are not interested in the details. Essentials of the method include Feulgen staining of smears prepared from centrifuged semen in the manner of Leuch-

tenberger (*6*) and subsequent determination of the DNA content of individual morphologically normal sperm by cytophotometric analysis using the EC^2/F method of Swift (*28*), with simplified calculations by Kasten (5). Adaptations and innovations originating in this laboratory are described in the paper.

The decision to devote a separate paper to method had its origin in the preliminary analysis described in preceding paragraphs. At an early stage it became apparent that, whatever DNA deviation existed, its incidence and degree might be comparatively slight, to such an extent that any true deviation might be blurred by the vagueness of experimental error limits. It would then be impossible to draw any firm conclusions concerning the existence of true deviation in the animals tested. On the other hand, the more closely the plus and minus limits of experimental error approached the mean, the smaller would be the variation that could be established as true deviation.

The main components of the total experimental error, analyzed in detail in the method paper, are the intra-slide error and the inter-slide error. The intra-slide error is chiefly expressed by the standard error, which includes, most importantly, observer error as in size measurements, and non-proportionality of staining among different sperm of the same sample smear. Distributional inhomogeneity, non-specific light loss or gain, and instrumental error from such as current fluctuation are slight, negligible, or nonexistent sources of error in bull sperm measurements, made with the equipment used here. The error implicit in the method used, to the extent that the shape of the sperm does not correspond to the sphere assumed, is also negligible, in relative measurements, because of the great uniformity in size and shape of the sperm. A component of the intra-slide error not included in the standard error is the non-proportionality of staining among sperm of different sample smears, a comparatively minor source of error that needs correction only when refinements of technique are desired to reduce the total experimental error below 10%, in which event an indirect method is available as described in the method paper. On the other hand, when it is necessary to stain material on different slides in the same or different containers, the slide to slide staining variation, defined as the inter-slide error, can lead to extremes of experimental error whenever the staining variation exceeds 5%. This may occur occasionally, infrequently, or perhaps not at all, depending somewhat on the kind of material and the number of staining events. Whenever it does occur, it should be corrected for, particularly when true DNA deviants among the animals tested may be a very small percentage of the total.

Since the paraffin technique was contra-indicated for reasons given in the method paper, it was necessary to devise effective means of correcting for the staining variation in smear preparations. Rat liver tissue was found ineffective as a control because of the fact that rat liver nuclei accept the Feulgen stain with considerably more stability than bull sperm smears, so that they do not reflect staining variation in bull sperm with enough sensitivity. A control system based on measurements on different slides made from the same sample of semen, corrected by measurements on different control slides made from part of a sample of semen retained from the previous batch was found to be too complicated as well as inexact but was an exploratory move in the right direction. During the early stage of the work, amounting to about two-thirds of the total for the first year, a

control system somewhat analogous to the "grade curve" system was used. Whenever there was an indication of high or low staining, all means of samples stained in the same batch were increased or decreased by a percentage that would bring the maximum number within a range 10 per cent on either side of the normal mean. This was based on the assumption that most of the animals tested would be normal in DNA, a valid assumption in view of the premises developed in preceding paragraphs. The normal mean was originally arrived at from tests of known fertile animals and was subsequently equated with that published by Leuchtenberger and collaborators (*9*) in order that results would be comparable. This method has much to recommend it from the standpoint of simplicity, and also precision, whenever deviations in DNA are expected to run 15 to 20 per cent or higher. It has the disadvantage that the experimental error cannot be determined exactly nor reduced below about 10 per cent with certainty. During most of the work a control system was used that provided for staining three sample smears on the same slide, together with a control smear made from the retained part of a sample of semen analyzed in the preceding batch. As described in detail in the method paper, a specially designed experiment was carried out to establish the validity of this method of control. It was established that the method limited the total experimental error to 10 per cent in 80 per cent of 184 separate corrections and to 15 per cent in all corrections. Refinements of technique are also described by which the total experimental error could be reduced still further. The main conclusions of the present inquiry are based upon the limits of error thus established.

SCOPE

Although the major effort of the program has been investigation of the DNA content of spermatozoa from young bulls of unknown fertility, some exploratory work was done in the beginning on a few bulls of high fertility and low fertility, determined through actual breeding experience. At the conclusion of this work the infertile bulls were killed and samples of the testes obtained, which were subsequently subjected to DNA analysis and the results compared with those obtained from a similar analysis of testis material from bulls, the DNA normality of which had been established by a series of tests extending over a period of a year or more. This work is presented in Article III. During the course of this preliminary work, positive results suggesting DNA deviation in the sperm of infertile bulls as well as men were published by the Leuchtenberger group (*9*), which provided sufficient encouragement for continuance of the program on its main course, irrespective of whatever degree of confirmation might be furnished by our own limited work on animals of known fertility and infertility. The latter work was therefore terminated and work on young bulls of unknown fertility begun, in accordance with the primary intent and emphasis of the particular program of research planned here.

The plan of attack of the main program has been predicated chiefly on conclusions reached by the Leuchtenberger group in human sperm research which indicated the possibility of "persistently low", "persistently high", and "fluctuating" types of DNA deviation in the sperm of infertile men as opposed to a high degree of constancy in the sperm of fertile men. The fluctuating type is obviously

the most difficult to detect under any circumstances since it requires numerous tests over an extended period of time. To detect it in bulls of unknown fertility, in less than five per cent of which it might occur, might require the application of the tests to at least one hundred animals (and to rule out the possibility with reasonable thoroughness would certainly require that number) involving an amount of work in excess of both practical ranch limitations and the research capacity of this laboratory. As a compromise, 21 young bulls of unknown fertility were given a series of tests over a period of about one year.

The program has been concentrated chiefly on over 200 bulls, from which, generally, from one to three samples were tested. These tests would be sufficient to detect the low and the high types, within the limits to be discussed, while the data would also have considerable bearing on the fluctuating type of DNA deviation. In all except a few of the earliest samples the DNA analysis has been preceded by motility, morphology and sperm count examinations. The results are presented in two main papers, the first (Article IV) concerned with the incidence and degree of DNA deviation in the samples, the second (Article V) with the interrelationships of this deviation with the well known indices of morphology, motility and sperm count. Part A of Article IV deals with the 98 prospective herd sires designated Group A bulls and tested during the fall and spring of 1956–1957, and Part B deals with the 135 prospective herd sires designated Group B bulls and tested during the fall and spring of 1957–1958. Part C deals with 21 reject bulls, designated Group C animals, which were tested over a comparatively extended period of time; it is concerned with the original tests given these bulls and includes an analysis of the follow-up tests on ten including nine doubtful bulls and one normal control. An analysis and summary of variation in Group C bulls concludes this particular paper. Article VI develops a rating system for bulls of unknown fertility, and is followed by a general discussion and summary of the entire program.

II. The Experimental Error of Feulgen Cytophotometry in the Analysis of Bull Spermatozoa Over an Extended Period of Time

ROBERT M. WELCH AND E. W. HANLY

It is common knowledge among experienced practitioners of quantitative Feulgen cytophotometry that the experimental error of the method is at its minimum when measurements are made on the same slide, principally because nonuniformity of staining due to causes other than variation in DNA is least under these circumstances. Yet, there are problems in which it is desirable or necessary to stain material on different slides and even at different times. An outstanding example of such a problem is the variation in the DNA content of spermatozoa of infertile men and other species, brought into prominence by the research of Leuchtenberger and collaborators (*6*, *7*, *8*, *9*, *11*).

In the course of testing this theory in the genetics laboratories of The University of Texas, 658 samples of semen from 275 Santa Gertrudis bulls of the King Ranch have been analyzed during a period of about two years. It is obvious that, if the data from these smears are to be comparable, the staining from batch to batch must be controlled within well defined limits of experimental error. Although staining variation due to experimental error can be reduced by meticulous attention to uniformity of procedure, it cannot be eliminated entirely. For this reason it has been recommended that, if the material is stained on different slides, a piece of "control" tissue be placed on each slide so that abnormally heavy or light staining can be adjusted in accordance with the control value, which, aside from other experimental errors, should be the same on all slides. Yet, no statistics seem to have been published that would permit a reasonably precise estimate of just how effective such a control system is. Consequently, one cannot really state just what the experimental error of Feulgen cytophotometry is under the circumstances of staining on different slides and at different dates.

In the comparison of data from the analysis of sperm over a considerable period of time, it is necessary to assess the error discussed above, as well as the sampling error, the error due to variations among individuals in size and extinction measurements, and the error in extinction measurements resulting from such variables as light scatter, inhomogeneity of DNA distribution, and instrumental imperfections, as discussed by Swift (*28*). The more precisely the total maximum error is determined, the more precisely one can define the margin by which the sperm must deviate in DNA in order to result in infertility. This paper presents statistical data from DNA analysis of bull spermatozoa, obtained from a testing program of about two years' duration, and from specially designed experiments on the material received during this time, and draws conclusions concerning the experimental error of Feulgen cytophotometry under the circumstances of analysis of spermatozoa over an extended period of time. It has particular importance with regard to DNA analysis of spermatozoa, made significant by the work of

Leuchtenberger and collaborators, and general importance with regard to the quantitative reliability of Feulgen cytophotometry when it is impossible or undesirable to confine all measurements to a single slide.

MATERIALS AND METHODS

Experimental material was obtained from the King Ranch and consisted of samples of semen from Santa Gertrudis bulls, most of which were under two years of age and of unknown fertility. King Ranch personnel obtained the semen routinely by electro-ejaculation, subjected it to a preliminary examination, and shipped it in a suitable container via air to this laboratory so that samples arrived here from four to eight hours after being taken at the ranch. As shown in Table 1, semen arrived in best state of preservation, judged from motility, if kept at a temperature between 15 and 20 degrees Centigrade, while the shock of near freezing and subfreezing temperatures resulted in marked reduction of motility, which was little protected by diluents of the kind and in the manner employed. As it was subsequently found that reduction of motility caused by temperature shock had no detectible effect on DNA analysis, it was unnecessary on the one hand to maintain the 15–20 degree range, or, on the other, to follow up the problem of use of diluents and restoration of motility after their use. Among other details, motility was recorded at the ranch, after which samples were customarily packed with sufficient refrigeration to keep the temperature between 5 and 10 degrees Centigrade.

Upon arrival here semen was examined for motility (usually reduced), morphology, and sperm count, and smears made by methods in general agreement with those used by Leuchtenberger (*6*), although, in the latter phase of the work, morphology was done by phase contrast microscopy. Staining with Schiff's reagent to produce the Feulgen stain was carried out according to the method of Stowell (*26*), with an hydrolysis time of 8 minutes. Slides were mounted in HSR, which has a refractive index of 1.5202 in solution and 1.5390 as the dry resin (*13*).

Cytophotometric equipment used in this laboratory has been described previously (*5*, *31*). During the latter part of the work certain changes were made, notably the substitution of a Sorensen model 1001 AC line voltage regulator and an American Optical Company Microstar for the corresponding equipment previously in use. The microscope was equipped with a trinocular with slide-out prism and with other items necessary to meet the standards of optical alignment and performance established for this kind of work (*22*, *28*). The entrance and exit-slits of the Bausch and Lomb 250 millimeter grating monochromator, with a dispersion of 6.6 millimicrons per millimeter, were set at from 0.30 to 0.40 millimeters; and analysis was carried out at 560 millimicrons, in the region of the absorption peak of the sperm DNA-Feulgen complex. Magnification in the plane of the phototube aperture was about 400×, increased to about 2000× by the 5× eyepiece used in selection and centering of sperm. The shape of the object to be measured and the distribution of absorbing material are the chief determinants of the method of analysis. In bull sperm, the distribution of DNA-Feulgen is exceptionally homogeneous and offers no difficulty. The shape, ovoid accord-

ing to Mann (*14*), provides some occasion for reflection and weighing of the merits of various methods. There are three chief possibilities: (1) the two-wavelength method, independent of shape (*18, 17, 19, 15*); (2) the ER^2 method based on the method of Ris and Mirsky (*23*), in which the sperm is considered a disc

TABLE 1

Preservation of bull semen during transportation

Sample	Ranch 15°–20° UT	D&T MPD UT	D&T MPD UT	D&T MPD UT	D&T MPD UT	D&T MPD UT
65	60G	PG1–1;5°	PG1–4;5°	RG1–1;5°	Undil;5°	
	10F	10S	10S	10S	...	
		5S	5S	5S	5F	
66	blank					
67	80G	PG1–1;5°	Undil;5°	Undil;25°		
	80G	10S	...	...		
		5S	10F	NM		
74	60G	PG1–1;5°	PG1–4;5°	RG1–1;5°	RG1–4;5°	Undil;5°
	40F	10S	10S	10S	10S	...
		5S	5S	5S	5S	5F
76	85E	Undil;25°				
	85E	...				
		50F				
77	90E	PG1–1;5°	PG1–4;5°	Undil;5°		
	60G	10S	10S	...		
		NM	NM	5S		

Sample	Ranch 15°–20° UT	Sample	Ranch 15°–20° UT	Sample	Ranch 15°–20° UT	Sample	Ranch 15°–20° UT
68	50F	71	60G	79	25F	94	10F
	80G		60G		5S		NM
69	10S	72	50G	88	80G	101	5F
	20F		50E		40F		5F
70	40F	73	90E	92	50G	103	50F
	40F		90E		30F		50F

Purpose of this experiment was to determine the best method of preservation of semen samples during the four to eight hours normally elapsing after collection and during transportation from King Ranch to The University of Texas via air, a distance of about 200 miles. Part of each sample was kept at a temperature of between 15 and 20 degrees Centigrade from the time of collection until examination at the University, eight to ten hours after collection. Some were diluted with a phosphate-glycerol or Ringer-glycerol solution (*14*), or not diluted at all; and these were placed in the "insemikit" shipping container together with cans of ice in the usual manner (subsequently, "Skotch Ice" or similar products have been used). The temperature in this box was between 5 and 10 degrees Centigrade. Two samples were brought back without any refrigeration at all.

In the table, the first column gives the motility at the ranch above the line and the motility recorded at the University following return below the line, samples having been kept between 15 and 20 degrees Centigrade. Succeeding columns give dilution and treatment at the ranch, motility immediately following dilution, and motility at the University. The second part of the table concerns those samples that received no treatment other than transport between 15 and 20 degrees Centigrade.

Abbreviations are as follows: PG: phosphate-glycerol diluent. RG: Ringer-glycerol diluent. 1–1: equal parts of semen and diluent. 1–4: one part semen and four parts diluent. NM: none motile. Motility is described by numerals and a letter, the former representing the percentage of forms motile and the latter the degree of activity, of which four categories are used, *sluggish*, *fair*, *good*, and *excellent* (in accordance with the method of Hotchkiss, used by Leuchtenberger (*6*)).

with a radius R, and a central core is measured; (3) the "plug" method, or EC^2/F, (*28*), C being the radius of the plug and F the fraction of total sperm volume contained in the measured plug, a method that assumes a spherical object unless the variation from the spherical is uniform in the objects measured and the C/R ratio is constant (*1*, *28*). Test measurements according to the two-wavelength method showed that the degree of DNA dispersion in bull sperm together with the large amount of blank space left on either side of the sperm when the entire sperm is circumscribed resulted in such high transmissions as to reduce severely the precision of the method obtainable with more favorable objects. An indication of this is that all the calculations fall on the last page of the tables provided by Mendelsohn (*15*). The coefficient of variation of twenty sperm measured by this method was 18% in contrast to the 8% obtained when the same sperm were measured and calculated according to methods (2) and (3). It is therefore necessary to compromise and make a choice between methods (2) and (3). If one uses method (2), the departure in sperm shape from that of a disc will lead to an error in one direction to the extent that the departure is variable among the sperm measured and the C/R ratio is not constant; similarly, any departure from the spherical, under the same conditions, will lead to an error in the other direction when method (3) is used. Making the plug diameter equal to or just short of the width of the sperm would eliminate the error arising from width variation in the plug, but would leave that due to the sperm outside of the plug along the long axis of the sperm and might possibly introduce some error due to marginal light diffraction, as well as lead to some error from variation in measurement of the plug radius and to some inconvenience in calculation if the EC^2/F method is used. When a central, constant plug is measured, whatever advantage in precision there is between the two methods seems to lie with method (3) as can be seen from Table 2. In eight of eleven samples calculated by both methods, the coefficient of variation was slightly less for method (3); the same in two samples; and in only one sample did method (2) show the greater precision. The high order of precision obtainable in sperm measurements despite

TABLE 2

Statistics of representative samples of spermatozoa from bulls of unknown fertility over 2 years of age calculated by two methods, ER^2 and EC^2/F

Number of bull	DNA-Feulgen mean		S. D.		S. E.		C. V.	
	ER^2	EC^2/F	ER^2	EC^2/F	ER^2	EC^2/F	ER^2	EC^2/F
12–F–1	131	1.49	12.3	0.14	2.46	0.03	9.4%	9.2%
12–$\frac{K}{4}$ 8–1	104	1.13	12.8	0.14	2.55	0.03	12.3	11.8
12–P–1	129	1.48	12.3	0.14	2.37	0.03	9.5	9.2
12–177–2	99	1.11	10.12	0.11	2.32	0.03	10.2	10.1
12–164–4	142	1.83	9.66	0.12	1.76	0.02	6.8	7.4
13–22–1	126	1.54	8.05	0.09	1.58	0.02	6.4	6.3
13–6–2	142	1.56	5.62	0.06	1.36	0.02	4.0	3.7
14–10–1	117	1.49	10.16	0.12	2.03	0.02	8.7	8.1
14–10–2	125	1.43	6.60	0.08	1.30	0.02	5.3	5.3
15–5–1	123	1.48	11.7	0.14	2.25	0.03	9.5	9.4
15–8–1	133	1.50	5.3	0.06	1.11	0.01	4.0	4.0

the necessity for compromise in the method used can be attributed to a number of reasons, chief of which are the homogeneous distribution of DNA-Feulgen and the uniformity of the sperm, not only in shape but also in length and width, which results in a C/R ratio that varies little from constancy. It is an interesting sidelight that the fact that methods (2) and (3) yield almost the same degree of precision is mathematical proof that the shape of the sperm is almost midway between a disc and a sphere, in other words, ovoid, as previously noted. The slightly greater precision obtained with method (3) would indicate that the shape of the sperm is somewhat closer to a sphere than a disc. Method (3) therefore appears to be the most precise method available and is the method used throughout this work. The measurements were made with a constant diaphragm of minimum width, slightly less than the short diameter of the sperm. Simplified calculations were carried out according to the method of Kasten (5).

EXPERIMENTAL ERROR OF THE METHOD

It should be borne in mind at the outset that a DNA deficiency in the sperm of young bulls, if it exists, may affect less than 5% of the animals tested. This means that it is the "tails" of the distribution in which we are interested—the maximum experimental error; in statistical terms, the experimental error must be expressed at a high level of confidence. We must be able to say, for example, that, in over 95% of the total animals tested, the maximum experimental error does not exceed a certain percentage, if we are to exclude experimental error as a possible cause of DNA deviation in excess of that percentage in the animals in which it occurs, if these are no more than 5% of the total. The method requirements of the problem are therefore exacting and would be rigorous if whatever DNA irregularities exist are only moderate in degree.

For convenience in analyzing this maximum experimental error, certain categories of experimental error may be established by more or less arbitrary definition, accompanied by an awareness of the interrelationships among the various types of error so classified. The "sampling error" is defined here as the variation among means of different series of sperm from the same sample of semen measured by one or more individuals. This includes variation in absorbance due to instrumental error, possible slight actual variation in DNA content in sperm, non-uniformity of staining on the same slide due to causes other than actual variation in DNA; it includes, further, actual size variations in sperm and apparent size variations due to inaccuracies of measurement by one or more individuals, the extent of which depends upon technique, instrumentation, and the training and experience of the observer. The second major category of experimental error is defined as the "staining error", arising from non-uniformity of staining due to causes other than actual variation in DNA, when measurements are made on two or more slides processed in the same or different containers on the same or different dates. This is over and above the sampling error, though not necessarily additive, and is the remainder when the maximum sampling error is subtracted from the maximum total error.

Since the conventional statistical approach to the sampling error is through the standard error, this and related statistics are presented in Table 3. These statistics

TABLE 3

Statistics of randomly selected samples of spermatozoa from bulls of unknown fertility under two years of age

	Size				DNA-Feulgen			
Number	Mean	S.D.	C.V.	S.E.	Mean	S.D.	C.V.	S.E.
1–1	5.61	0.16	2.8%	0.04	1.36	0.09	6.8%	0.02
1–3	5.55	0.23	4.1	0.05	1.34	0.10	7.4	0.02
8–2	5.82	0.19	3.3	0.05	1.30	0.05	3.5	0.001
9–3	5.70	0.15	2.5	0.03	1.23	0.11	9.2	0.02
10–1	5.63	0.17	2.9	0.04	1.12	0.07	6.5	0.02
14–2	5.63	0.17	3.0	0.03	1.39	0.15	10.8	0.03
49–2	5.66	0.12	2.2	0.02	1.44	0.08	5.3	0.02
50–1	5.46	0.10	1.8	0.03	1.39	0.07	5.2	0.02
50–2	5.54	0.17	3.0	0.04	1.41	0.14	10.1	0.03
55–1	6.24	0.17	2.7	0.03	1.34	0.16	11.9	0.03
56–1	5.54	0.17	3.1	0.04	1.36	0.06	4.1	0.01
59–3	6.24	0.22	3.5	0.04	1.36	0.18	13.4	0.04
61–2	5.68	0.16	2.7	0.03	1.53	0.16	10.3	0.03
67–1*	5.68	0.13	2.3	0.03	1.41	0.11	7.8	0.02
67–1†	6.05	0.09	1.5	0.02	1.69	0.12	7.0	0.02
80–1	5.80	0.14	2.4	0.03	1.67	0.09	5.2	0.02
92–3	6.38	0.11	1.7	0.02	1.94	0.08	4.3	0.02
94–2	6.00	0.22	3.6	0.04	1.00	0.07	6.6	0.01
97–2	6.00	0.19	3.1	0.04	1.62	0.09	5.5	0.02
98–3	5.78	0.18	3.2	0.04	1.67	0.08	4.9	0.02
108–1	6.18	0.15	2.4	0.03	1.91	0.12	6.5	0.02
734–2	5.69	0.19	3.4	0.04	1.71	0.10	5.6	0.02
03–4	6.03	0.19	3.1	0.04	1.39	0.08	5.5	0.02

* Group 5.
† Group 8.

Size mean is in microns computed by averaging the major and minor axes of the sperm.

obviously reflect the character of the sperm measured, the choice of which is random except for the rejection of abnormal sperm. As the latter usually amount to at least 20% of the total number (*14*), the central point of inquiry concerns the 80% or less of normal sperm, a percentage that leaves considerable margin for the rejection of abnormal sperm. Morphological abnormalities include irregularities in shape, tapering forms, disproportionate width in relation to length, and tail abnormalities that can be distinguished from fixation and staining artefacts. Also included are abnormally small and large sperm, to some extent a matter of definition. Examination of many samples of semen from bulls of varying ages has shown that, in 95% or more, at least 90% of the sperm, after fixation and staining, fall within the limits of 6.6 to 8.2 microns in length and 3.9 to 4.7 microns in width. The live sperm are up to two microns longer and up to 0.5 micron wider. These dimensions are used as wide limits of normality, any variation of samples within these limits being reflected in the size average calculated for each sample. Except for their effect on motility, biochemical abnormalities are ordinarily not detectible visually or photometrically after Feulgen staining, except for poorly staining sperm. So long as these fall within the usual percentage of abnormality, they may, and in fact should be rejected in measuring in order that the

DNA value of the usual normal percentage of sperm will be a truer one. Consequently, in samples yielding both normal and abnormal means, poorly staining sperm, up to a maximum of about 10% per sample and, on an average, about 5% or less, are rejected. Sometimes these can be detected visually; usually, however, they take the form of extremes of distribution, discontinuous by 5 per cent or more from the adjacent value. It follows that the size average of "normal" sperm may vary from sample to sample, as well as the percentage of the sample represented by the mean. The latter is closely related to the per cent morphological abnormality found in the sample, but the correspondence is not exact, as to name two outstanding exceptions, the per cent morphological abnormality includes tail abnormalities, which, of course, do not appear in the stained preparations, while it does not include possible abnormalities resulting from fixation and staining.

Turning again to Table 3, compiled from 23 randomly selected samples comprising about 10% of the total number of 246 analyzed, one notes from the respective coefficients of variation of the DNA means that each sample varies considerably around its mean. The question arises whether this variation is due to differences in size, either actual or the result of experimental error in individual measurements, to differences in extinction resulting from inconstancy of instrumentation, to actual variation in DNA, or to non-uniform staining due to causes other than actual variation of DNA. Both aspects of the size factor would appear to be ruled out by the fact that the size coefficients of variation are highly consistent in contrast to the fluctuating values obtained for the DNA means. In order to assess the importance of the extinction factor one must look ahead to Table 4. The coefficients of variation for the extinction means obtained by individuals A, B, and C measuring the same mapped sperm in sample 11–110–1 have been calculated to be 7.5%, 7.4%, and 7.4% respectively, values that seem to eliminate extinction variation caused by instrumental error as a factor in the variation in spread of DNA values for the individual sperm in a sample. We are then left with a choice between true and apparent variation of DNA from sperm to sperm to explain the variation within an individual sample.

Unless the variation is correlated with some factor peculiar to certain samples, one might expect it to be much the same from sample to sample if due to an actual difference in DNA. Certainly there appears to be no correlation of the variation with any level of the DNA mean, as can be seen from Fig. 1. The most likely explanation is that the variation within a sample is due to random non-uniformity of staining differing in degree from sample to sample, one of the components of the experimental error of the method. So far as the intra-sample variation is concerned, however, it is unnecessary to reach an unequivocal conclusion.

Understanding of the nature of the variation of sample size and DNA means, particularly the latter, is, on the other hand, critical to the solution of the problem. The experimental error must be separated from the true variation; and, in the course of accomplishing this, it is interesting and useful to determine what factors contribute in approximately what degree to the experimental error. A major component of the latter is, of course, the sampling error, including variation due to actual differences in size and possible differences in DNA content. Furthermore, non-uniformity of staining within the same sample, as well as size

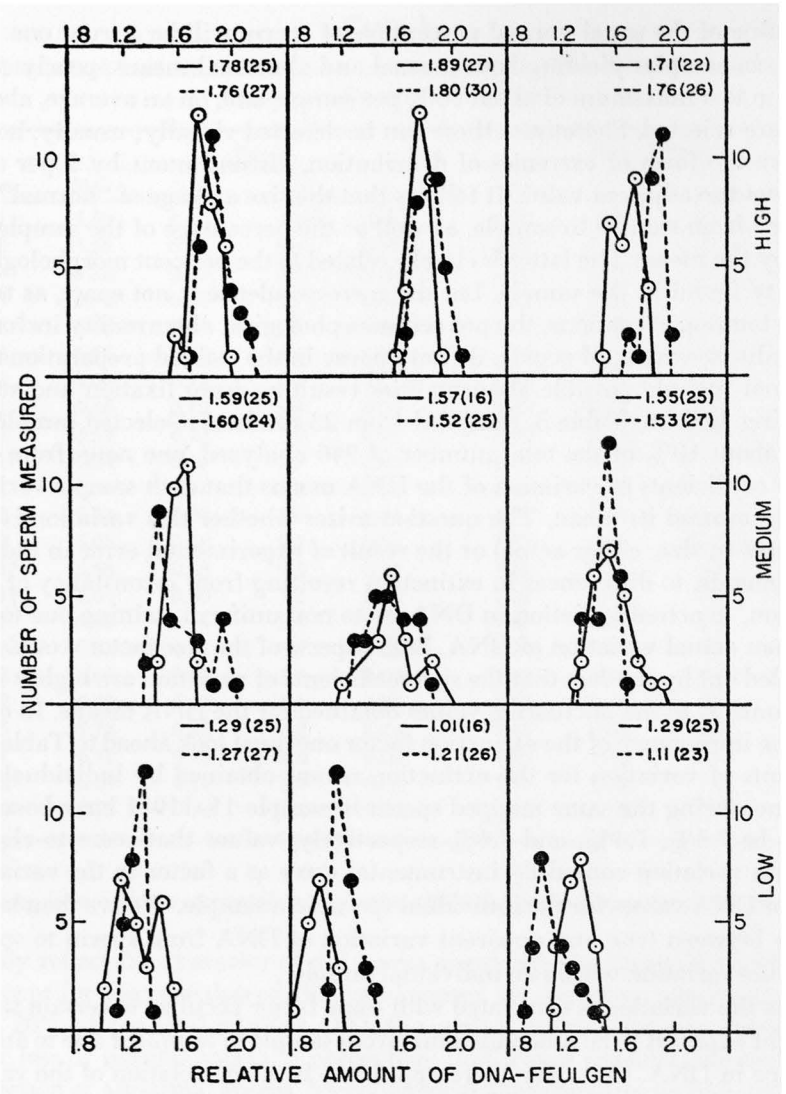


FIG. 1. Comparison of variation in samples of low, medium and high DNA means.

and extinction variations among individuals are included. These are defined statistically by the standard errors of the mean in Table 3.

An attempt to improve the precision of size measurements has resulted in the introduction of a slight innovation. In the experience of workers in this laboratory and of others (*5*, *28*), errors in size measurements arise from two chief causes, vagueness of outline of the object under the high magnification necessary in cytophotometric work and difficulty in reading precisely the scale of the ocular micrometer under the customary 5× magnification. In the work here the first of these is minimized by limiting the magnification so that the minimum aperture of the diaphragm falls just within the width boundaries of the sperm, forming a core with a radius of 1.58 microns. Some uncertainty, however, still remains,

particularly in the anterior portion of the sperm head, which has a tendency to "fade out". As regards the second factor, if one increases the magnification of the micrometer scale directly, one increases at the same time the magnification of the object and therefore its vagueness of outline, so that what is gained in one direction is lost in another. This dilemma, however, can be avoided by the indirect method of first circumscribing the sperm with the diaphragm opening under 5× magnification and then measuring the diameter of the field so obtained under 30× or similar high magnification.

Since one of the main causes of error in size measurements has been eliminated, one might expect an improvement in the degree of precision of measurements. Such appears to be true. The standard deviations of the size measurements in Table 3 form no more than from 1.5 to 4.1 per cent of the mean, with most falling around 2 or 3 per cent, while the standard errors vary from 0.02 to 0.05. Three times the standard error would give a total plus and minus range of experimental error, at a 99% level of confidence, of from about 0.1 to 0.3 micron, depending on the size of the standard error in each sample. This narrow range of variation is due both to the natural uniformity of sperm and to a high degree of precision in measurements.

The maximum standard error of the DNA means is 0.04, but this occurs only once in 23 samples. In 95% of the samples the maximum standard error does not exceed 0.03. This is similar to the standard errors obtained by Leuchtenberger in analysis of human sperm (*7*) and would seem to indicate that the increased standard errors resulting from Leuchtenberger's bull sperm data (*9*) are due to causes other than greater variability of bull sperm over human sperm, as the Leuchtenberger data might imply. Multiplying the maximum standard error of 0.04 by three, one obtains a maximum sampling error (in approximately 99% of samples) of 0.12, about 8% of the overall mean of 1.53 for 246 samples. This, however, is a liberal interpretation of the sampling error. Usually it is much less. For example, in "typical cytophotometric data" presented by Pollister and Ornstein (*22*), six extinction means obtained from 25 or more liver nuclei each range from 0.126 to 0.151, the maximum deviation between any two means being about 10%, approximately the same as the maximum deviation from the overall mean for 192 cells, although three times the standard error for these means would be 15 to 20% of the mean. On a reduced level of experimental error, a similar situation prevails in sperm measurements. In two series of sperm measurements, made by the same individual, on 64 sperm each, from different samples, the maximum deviation between any two DNA means calculated for blocks of 16 sperm is 2.8%. The maximum deviation from the overall mean is 1.8%.

It has possibly occurred to the reader that the standard error obtained from measurements made by one individual on a sample may be misleading when means of different samples measured by different individuals are compared. In order to establish the relative importance of extinction variation due to individual differences in circumscribing sperm, closing down on "core", taking blanks, reading the galvanometer, etc., and the importance of size variation due to individual differences, a special experiment was carried out in the course of which three individuals measured the same twenty mapped sperm on two occasions

from each of two samples, while two individuals of the three subsequently measured 27 randomly selected sperm from each of the two samples. The results are presented in Table 4. That individual bias in regard to the points previously mentioned plays no great part in the present work is evident from the close correspondence between size and extinction means obtained by different individuals measuring the same samples. The slight size and extinction variations among the three individuals are not statistically significant in comparison with the variation in measurements on one sample by one individual and with variation between means of duplicate measurements by one individual on the same sample (differences insignificant as measured by F-tests). The DNA means of both sets of random measurements on the other hand show a deviation of 7 to 8%, understandable, as these measurements contain all the various components of the sampling error previously discussed in addition to individual differences. At first glance the size of this deviation seems a perverse and inopportune contradiction to the argument advanced in the preceding paragraph. Fortunately, one can point to Table 5 below, in which the deviations among four individuals and from the overall mean do not exceed 5%.

In the second phase of the work, however, to offset any possible increase in the factor of individual variability in the course of routine measurements by a number of individuals as well as individual bias, if any, in the selection of sperm for measurement, it has been the practice to assign two or more individuals to measure one sample. In one sample at least, the precision seems improved over that obtained when comparisons are made of DNA means from measurements of only one individual per sample and approaches that of measurements of the

TABLE 4

Size, extinction, and DNA statistics of two samples of mapped and randomly selected bull sperm measured by three individuals

	Sample 11–110–1 Individuals			Sample 13–28–6 Individuals		
	A	B	C	A	B	C
Mapped sperm (1)						
Size (20)	6.09	6.04	6.16	6.19	6.10	6.22
Ext. (20)	0.238	0.250	0.248	0.208	0.218	0.212
DNA (20)	1.60	1.66	1.69	1.43	1.46	1.47
Mapped sperm (2)						
Size (20)	6.09	6.11	6.12	6.25	6.20	6.13
Ext. (20)	0.234	0.248	0.239	0.195	0.212	0.194
DNA (20)	1.57	1.62	1.61	1.34	1.47	1.31
Random sperm						
DNA (27)	...	1.69	1.45	...	1.39	1.21

Mapped sperm (20) from Sample 11–110–1 measured by five individuals over six months later:

A	B	C	D	E	F
5.95	6.14	...	6.10	5.95	5.97

Numbers in parentheses refer to number of sperm measured.
First and repeat measurements of mapped sperm were separated by at least one week.
Size mean is in microns computed by averaging the major and minor axes of the sperm.

TABLE 5

Size and DNA means obtained by four individuals, separately and in combination, from the same Feulgen stained bull sperm smear

Individual	Number sperm	Mean size*	Mean DNA
Total	70	5.9	1.49
A	18	6.1	1.42
B	17	5.8	1.60
C	18	5.9	1.59
D	17	5.8	1.36
A–B	18	6.0	1.51
A–C	18	6.0	1.51
A–D	18	6.0	1.38
B–C	18	5.9	1.58
B–D	18	5.9	1.45
C–D	18	5.9	1.44
A–B–C	27	6.0	1.48
A–B–D	27	5.9	1.44
A–C–D	27	6.0	1.44
B–C–D	27	5.9	1.49

* Mean of long and short diameters of sperm in microns.

same individual on different samples. In Table 5, for example, it is evident that, in the particular sample concerned, this practice results in less variation among the DNA means and practically irons out size variation. Whatever improvement in precision occurs, results from the averaging of individual variation in the same sample rather than in contrasting it in different samples. As variation between individuals measuring the same sample increases, so, of course, does the standard error. When this occurs, the practice has been to measure additional sperm until the standard error is reduced to a maximum of 0.03. Thus, the maximum sampling error is not increased by any increase in the standard error, as the statistics in Table 6 for the second phase of work show; while the averaging of slight individual variation in the same sample improves the overall precision. These statistics should be compared with those in Table 3 where a single individual measured each sample. Similar statistics from a group of 21 bulls tested periodically over a period of about one year will be presented in another article. It is premature, however, to draw final conclusions on the basis of the sampling error alone, as the problem of sperm analysis is complicated by the necessity of comparing DNA means of samples on different places on the same slide, on different slides of the batch, or in different batches in different processing jars. This introduces the experimental error of non-uniformity of staining due to cause or causes other than actual variation of DNA.

Most workers in this field, as, for example, Swift (*27*, *28*), prefer to avoid this problem by making all measurements on one slide. Although this is desirable whenever it can be done, yet, it restricts the general application of the method by ruling out all problems in which measurements must be made on different slides. It is the chief purpose of this paper to show that the experimental error of the method when measurements are made on sperm material stained on different slides on different dates can be closely comparable to that obtainable when all

measurements are made on the same slide, when the proper control system is used.

Throughout the work on bull sperm material, smears have been the method of choice rather than sections, although the latter would seem to have several advantages. If only a few sections of each sample are used, many more samples can be placed on one slide than is possible with the smear method, while one good sample of semen would provide enough material for embedding to last indefinitely as a control; furthermore, the material of all samples embedded would be preserved more or less permanently for later checks or comparison. Whatever the potentiality of this technique may be in sperm research, serious technical

TABLE 6

Statistics of randomly selected samples of spermatozoa from bulls of unknown fertility under two years of age

	Size				DNA-Feulgen			
Number	Mean	S.D.	C.V.	S.E.	Mean	S.D.	C.V.	S.E.
12–F–1	5.71	0.10	1.7%	0.02	1.49	0.14	9.2%	0.03
12–$\frac{K}{4}$ 8–1	5.78	0.11	1.9	0.02	1.13	0.14	11.8	0.03
12–P–1	5.70	0.10	1.7	0.02	1.48	0.14	9.2	0.03
12–175–2	5.58	0.13	2.2	0.03	1.29	0.12	9.8	0.03
12–177–2	5.45	0.09	1.6	0.02	1.11	0.11	10.1	0.03
12–169–3	5.96	0.15	2.5	0.03	1.56	0.13	8.4	0.03
12–164–4	5.67	0.13	2.3	0.02	1.83	0.12	7.4	0.02
13–8–1	5.62	0.08	1.5	0.02	1.51	0.12	7.9	0.02
13–9–1	5.83	0.12	2.1	0.02	1.69	0.11	6.1	0.02
13–22–1	5.91	0.13	2.3	0.03	1.54	0.09	6.3	0.02
13–6–2	5.80	0.08	1.3	0.02	1.56	0.06	3.7	0.02
13–16–2	5.78	0.08	1.4	0.02	1.51	0.09	5.4	0.02
13–25–2	5.58	0.11	2.0	0.02	1.58	0.09	5.5	0.02
14–10–1	5.73	0.20	3.5	0.04	1.49	0.12	8.1	0.02
14–13–1	5.61	0.16	2.9	0.04	1.55	0.07	4.6	0.02
14–19–1	5.61	0.19	3.4	0.04	1.61	0.11	6.8	0.02
14–10–2	5.94	0.33	5.6	0.07	1.43	0.08	5.3	0.02
15–5–1	5.71	0.11	2.0	0.02	1.48	0.07	4.9	0.01
15–8–1	5.86	0.13	2.2	0.03	1.50	0.06	4.0	0.01
15–13–1	5.77	0.12	2.1	0.03	1.41	0.10	7.4	0.02
15–15–1	5.78	0.10	1.7	0.03	1.49	0.06	3.9	0.01
16–17–1	5.71	0.12	2.1	0.03	1.49	0.11	7.2	0.03
16–20–1	5.74	0.19	3.2	0.05	1.54	0.06	3.9	0.01
16–21–1	5.95	0.15	2.6	0.04	1.55	0.12	7.6	0.03
17–31–1	5.70	0.15	2.6	0.04	1.59	0.10	6.3	0.02
17–32–1	5.68	0.16	2.9	0.04	1.36	0.08	5.8	0.02
17–42–1	5.67	0.09	1.6	0.02	1.40	0.09	6.2	0.02
18–46–1	5.97	0.19	3.2	0.05	1.46	0.08	5.1	0.02
18–51–1	5.61	0.11	2.0	0.03	1.59	0.10	6.0	0.02
18–54–1	5.73	0.13	2.2	0.03	1.56	0.06	4.0	0.02
19–63–1	5.63	0.11	2.0	0.03	1.60	0.11	6.7	0.03
19–66–1	5.71	0.10	1.8	0.03	1.41	0.11	8.1	0.03
19–71–1	5.57	0.14	2.5	0.02	1.59	0.15	9.7	0.03

Size mean is in microns computed by averaging the major and minor axes of the sperm.

difficulties argue against it. It is, of course, much more time-consuming than the smear method. Furthermore, some samples of bull semen are relatively dilute, thus making the centrifuged residues so slight that the paraffin technique can be performed only with great difficulty, if at all. Finally, although minimum times are allowed for the various processes, particularly infiltration, the resulting sectioned material, on the basis of a dozen or more trials, infrequently stains darkly enough for satisfactory measurement.*

A method employing features of both techniques would be to use smears for the samples and sections for the control. This suffers from the disadvantage of inconvenience in processing as well as the difficulty of light staining previously mentioned. The critical objection, however, is that one would be using a section to control a smear, whereas, *a priori*, a smear should be used to control a smear. If this is done, part of the semen sample must be preserved from the time of original staining to the time when it is stained as a control for new samples. At that time it will not serve the purpose of a control if put on a separate slide because of intra-container variation among slides, and averaging the results of several control slides is laborious and inexact. The control smear must be put on the same slide as the smear it is intended to control. The method used here is to divide the slide into four rectangles, leaving space at one end for identification, and put three new samples, together with the control smear previously analyzed, on the same slide. Each slide thus has its own control. Though unnecessary as an adhesive, a film of albumen fixative on the slide will serve to prevent the rapid drying out of material while four smears are being made on one slide. Successful use of this technique assumes that a sample of semen can be preserved from the time it is originally used to the time it is used as a control without deterioration with regard to DNA determination, and that the staining variability in four smears on one slide is of such similarity in degree that the variation in one smear can be used to calculate and correct the variation in another. These important questions cannot be satisfactorily answered by an optimistic guess but only by an exhaustive compilation of data, such as that summarized in Tables 7–11. These are the result of an experiment in which slides were made from three samples when originally received and subsequently after the samples had been kept for one week at 5° Centigrade. Table 7 is the master table, giving DNA means obtained for the various smears together with number of sperm analyzed in each instance and identifying the different slides in a footnote. The measurements were made by three individuals, although only one person worked on each smear. Some slides were selected that showed marked staining variation so that the control system could be tested under a variety of circumstances. An inspection of the table does not reveal any essential difference between the DNA means of smears made soon after receipt of semen and those of smears made from semen kept for one week at 5°. Thus, it appears that preservation at 5°, without dilution and without gradual cooling precautions, is adequate for the purpose of DNA analysis, although for longer periods preservation in dry ice would be indicated, since deterioration of samples with time would occur sooner at 5° than at the –78° temperature of dry ice.

* This result was first obtained by the senior author and subsequently confirmed by Mr. William Guest.

Tables 8–10, arranged according to type of material and time of staining, present a detailed application of the control hypothesis that the variation in one smear can be calculated and corrected by the variation in another. Smears of three samples are made on each slide of a number of duplicate slides, at the time the semen is received and after it has been kept one week at 5° Centigrade. Comparisons are then made between the means of samples processed on different slides in the same containers, in different containers, and between the means of smears made when the material was first received and after preservation for one week at 5° Centigrade. In each case, the deviation between means of the same sample on different slides is calculated; then the mean of each sample is corrected by the percentage variation of the mean of another sample, and the deviation of the former recalculated. Thus, when the staining varies from the first to the second slide, one can compare the uncorrected deviations, respectively, between the means on the two slides with the corrected deviations when the mean of a sample on the second slide is corrected by the percentage variation of the mean of each of the other two samples from its mean on the first slide. Two general observations suggest themselves. No matter what the original uncorrected deviation may be, the maximum corrected deviation does not exceed 15%. More fundamentally, the effectiveness of correction by this method is roughly proportional to the uncorrected deviation, being more effective at the higher uncorrected deviations and less at the lower until, in the neighborhood of 10% uncorrected deviation, the control system regresses to the point of becoming an additional source of experimental error. Few, if any, would claim, however, that Feulgen staining can be controlled even under the most uniform procedure at-

TABLE 7

DNA means of samples of bull sperm obtained by three individuals from smears processed on or in the same or different slides, containers, or dates*

Slide	Sample		
	28	110	734
11–1	1.45 (25)	1.57 (20)	1.76 (27)
11–2	1.32 (18)	1.42 (20)	1.38 (20)
11–4	1.52 (26)	1.74 (26)	1.83 (25)
11–5	1.48 (27)	1.72 (26)	1.77 (26)
11–9	1.09 (20)	1.15 (20)	1.38 (19)
12–1	1.60 (27)	1.47 (19)	1.52 (19)
12–4	1.94 (26)	1.73 (27)	1.83 (26)
13–3	1.63 (20)	1.61 (20)	1.71 (20)
13–4	1.58 (20)	1.51 (20)	1.57 (20)
13–5	1.57 (20)	1.90 (20)	1.85 (20)
13–6	1.30 (20)	1.52 (20)	1.45 (18)
14–2	1.62 (20)	1.63 (20)	1.56 (20)
15–6	1.51 (26)	1.48 (26)	
19–4	1.31 (27)	1.24 (27)	
19–7	1.06 (24)	1.21 (26)	

* Slides are designated according to group and series within the group. Slides 11, 13, and 15 were processed on receipt of samples, while slides 12, 14, and 19 were prepared from the same samples respectively after storage for one week at 5° Centigrade. Nine duplicate slides were made in each numbered group; and these were processed in batches of three, each batch in a different container.

Number of sperm analyzed in each sample is indicated in parentheses.

TABLE 8

Variation and correction of variation in DNA means of bull sperm samples processed on different slides in the same containers

Slide and sample comparison	Samples			Mean percent deviation	Slide and sample comparison	Samples			Mean percent deviation
	28	110	734			28	110	734	
11–1	1.45	1.57	1.76	..	11–4	1.52	1.74	1.83	..
11–2	1.32	1.42	1.38	..	11–5	1.48	1.72	1.77	..
%D–Un	4.7	5.0	12.1	7.3	%D–Un	1.3	0.6	1.7	1.2
%C	+9.9	+10.6	+27.5	..	%C	+2.7	+1.2	+3.4	..
CM–%D C–28	...	1.56–0.3	1.52–7.3	3.8	CM–%D C–28	...	1.77–0.9	1.82–0.3	0.6
CM–%D C–110	1.46–0.3	...	1.53–7.0	3.7	CM–%D C–110	1.50–0.7	...	1.79–1.1	0.9
CM–%D C–734	1.68–7.3	1.81–7.1	...	7.2	CM–%D C–734	1.53–0.3	1.78–1.1	...	0.7
13–4	1.58	1.51	1.57	..	13–4	1.58	1.51	1.57	..
13–5	1.57	1.90	1.85	..	13–6	1.30	1.52	1.45	..
%D–Un	0.3	11.4	8.2	6.6	%D–Un	9.7	0.3	4.0	4.7
%C	+0.6	–20.5	–15.1	..	%C	+21.5	–0.6	+8.3	..
CM–%D C–28	...	1.91–11.7	1.86–8.5	10.1	CM–%D C–28	...	1.85–10.1	1.76–5.7	7.9
CM–%D C–110	1.25–11.7	...	1.47–3.3	7.5	CM–%D C–110	1.29–10.1	...	1.44–4.3	7.2
CM–%D C–734	1.33–8.6	1.61–3.2	...	5.9	CM–%D C–734	1.41–5.7	1.65–4.4	...	5.1
13–5	1.57	1.90	1.85	..					
13–6	1.30	1.52	1.45	..					
%D–Un	9.4	11.1	12.1	10.9					
%C	+20.8	+25.0	+27.6	..					
CM–%D C–28	...	1.84–1.6	1.75–2.8	2.2					
CM–%D C–110	1.63–1.9	...	1.81–1.1	1.5					
CM–%D C–734	1.66–2.8	1.94–1.0	...	1.9					

Abbreviations:
%D–Un: % deviation between two means of smears from the same sample on different slides processed in the same containers before correction.
%C: % difference between means of one sample used to correct variation in other samples.
CM–%D
C–28 (C–110, C–734): corrected mean and % deviation when the mean of sample 110 or 734 is corrected by the % variation of sample 28, and similarly, when each sample is corrected by the variation in another.

TABLE 9

Variation and correction of variation in DNA means of bull sperm samples processed on slides in different containers

Slide and sample comparison	Samples			Mean percent deviation	Slide and sample comparison	Samples			Mean percent deviation
	28	110	734			28	110	734	
11–1	1.45	1.57	1.76	..	11–4	1.52	1.74	1.83	..
11–4	1.52	1.74	1.83	..	11–9	1.09	1.15	1.38	..
%D–Un	2.4	5.1	1.9	3.1	%D–Un	16.5	20.5	14.0	17.0
%C	—4.6	—9.8	—3.8	..	%C	+39.5	+51.3	+32.6	..
CM–%D C–28	...	1.66–2.8	1.75–0.3	1.6	CM–%D C–28	...	1.60–4.2	1.93–2.7	3.5
CM–%D C–110	1.37–2.8	...	1.65–3.2	3.0	CM–%D C–110	1.65–4.1	...	2.09–6.6	5.4
CM–%D C–734	1.46–0.3	1.67–3.1	...	1.7	CM–%D C–734	1.45–2.4	1.52–6.7	...	4.6
11–1	1.45	1.57	1.76	..	11–5	1.48	1.72	1.77	..
11–5	1.48	1.72	1.77	..	11–9	1.09	1.15	1.38	..
%D–Un	1.0	4.6	0.3	2.0	%D–Un	15.2	19.9	12.4	15.8
%C	—2.0	—8.7	—0.6	..	%C	+35.8	+49.6	+28.2	..
CM–%D C–28	...	1.69–3.7	1.73–0.9	2.3	CM–%D C–28	...	1.56–4.9	1.87–2.7	3.8
CM–%D C–110	1.35–3.6	...	1.62–4.1	3.9	CM–%D C–110	1.63–4.8	...	2.06–7.6	6.2
CM–%D C–734	1.47–0.7	1.71–4.3	...	2.5	CM–%D C–734	1.40–2.8	1.47–7.8	...	5.3
11–1	1.45	1.57	1.76	..	13–3	1.63	1.61	1.71	..
11–9	1.09	1.15	1.38	..	13–4	1.58	1.51	1.57	..
%D–Un	14.2	15.4	12.1	13.9	%D–Un	1.6	3.2	4.3	3.0
%C	+33.0	+36.5	+27.5	..	%C	+3.2	+6.6	+8.9	..
CM–%D C–28	...	1.53–1.3	1.84–2.2	1.8	CM–%D C–28	...	1.56–1.6	1.62–2.7	2.2
CM–%D C–110	1.49–1.4	...	1.88–3.3	2.4	CM–%D C–110	1.68–1.5	...	1.67–1.2	1.4
CM–%D	1.39–2.1	1.47–3.3	...	2.7	CM–%D	1.72–2.7	1.64–0.9	...	1.8

C–734				
11–2	1.32	1.42	1.38	..
11–4	1.52	1.74	1.83	..
%D–Un	7.0	10.1	14.0	10.4
%C	—13.2	—18.4	—24.6	..
CM–%D	...	1.51–3.1	1.59–7.1	5.1
C–28				
CM–%D	1.24–3.1	...	1.49–3.8	3.5
C–110				
CM–%D	1.15–6.9	1.31–4.0	...	5.5
C–734				
11–2	1.32	1.42	1.38	..
11–5	1.48	1.72	1.77	..
%D–Un	5.7	9.6	12.4	9.2
%C	—10.8	—17.4	—22.0	..
C–110				
CM–%D	...	1.53–3.7	1.58–6.8	5.3
C–28				
CM–%D	1.22–3.9	. .	1.46–2.8	3.4
C–110				
CM–%D	1.15–6.9	1.34–2.9	...	4.9
C–734				
11–2	1.32	1.42	1.38	..
11–9	1.09	1.15	1.38	..
%D–Un	9.5	10.5	0.0	6.7
%C	+21.0	+23.5	...	
CM–%D	...	1.39–1.1	1.67–9.5	5.3
C–28				
CM–%D	1.35–1.1	...	1.70–10.4	5.8
C–110				
CM–%D	1.09–9.5	1.15–10.5	...	10.0
C–734				

C–734				
13–3	1.63	1.61	1.71	..
13–5	1.57	1.90	1.85	..
%D–Un	1.9	8.3	3.9	4.7
%C	+3.8	—15.3	—7.6	..
CM–%D	...	1.97–10.1	1.92–5.8	8.0
C–28				
CM–%D	1.33–10.1	...	1.57–4.3	7.2
C–110				
CM–%D	1.45–5.8	1.76–4.5	...	5.2
C–734				
13–3	1.63	1.61	1.71	..
13–6	1.30	1.32	1.45	..
%D–Un	11.3	2.9	8.2	7.5
%C	+25.4	+6.0	+17.9	..
CM–%D	...	1.91–8.5	1.82–3.7	5.8
C–28				
CM–%D	1.38–8.3	...	1.54–5.2	6.8
C–110				
CM–%D	1.53–3.2	1.79–5.3	...	4.3
C–734				

Abbreviations:
%D–Un: % deviation between two means of smears from the same sample on different slides processed in the same containers before correction.
%C: % difference between means of one sample used to correct variation in other samples.
CM–%D
C–28 (C–110, C–734): corrected mean and % deviation when the mean of sample 110 or 734 is corrected by the % variation of sample 28, and similarly, when each sample is corrected by the variation in another.

TABLE 10

Variation and correction of variation in DNA means of bull sperm samples processed on receipt and after storage for one week at 5° Centigrade

Slide and sample comparison	Samples			Mean percent deviation	Slide and sample comparison	Samples			Mean percent deviation
	28	110	734			28	110	734	
11–1	1.45	1.57	1.76	..	11–5	1.48	1.72	1.77	..
12–1	1.60	1.47	1.52	..	12–4	1.94	1.73	1.83	..
%D–Un	4.9	3.3	7.3	5.2	%D–Un	13.5	0.3	1.7	5.2
%C	—9.4	+6.8	+15.8	..	%C	—23.7	—0.6	—3.3	..
CM–%D C–28	...	1.33–8.3	1.38–12.1	10.2	CM–%D C–28	...	1.32–13.2	1.40–11.7	12.5
CM–%D C–110	1.71–8.2	...	1.62–4.1	6.2	CM–%D C–110	1.93–13.2	...	1.82–1.4	7.3
CM–%D C–734	1.85–12.1	1.70–4.0	...	8.1	CM–%D C–734	1.88–11.9	1.67–1.5	...	6.7
11–2	1.32	1.42	1.38	..	11–9	1.09	1.15	1.38	..
12–1	1.60	1.47	1.52	..	12–4	1.94	1.73	1.83	..
%D–Un	9.6	1.7	4.8	5.4	%D–Un	28.1	20.1	14.0	20.7
%C	—17.5	—3.4	—9.2	..	%C	—43.8	—33.5	—24.6	..
CM–%D C–28	...	1.21–8.0	1.25–4.9	6.5	CM–%D C–28	...	0.97–8.5	1.03–14.5	11.5
CM–%D C–110	1.55–8.0	...	1.47–3.2	5.6	CM–%D C–110	1.29–8.4	...	1.22–6.2	7.3
CM–%D C–734	1.45–4.7	1.33–3.3	...	4.0	CM–%D C–734	1.46–14.5	1.30–6.1	...	10.3
11–4	1.52	1.74	1.83	..	13–3	1.63	1.61	1.71	..
12–1	1.60	1.47	1.52	..	14–2	1.62	1.63	1.56	..
%D–Un	2.6	8.4	9.3	6.8	%D–Un	0.3	0.6	4.6	1.8
%C	—5.0	+18.4	+20.4	..	%C	+0.6	—1.2	+9.6	..
CM–%D C–28	...	1.40–10.8	1.44–11.9	11.4	CM–%D C–28	...	1.64–0.9	1.57–4.3	2.6
CM–%D C–110	1.89–10.9	...	1.80–0.8	5.9	CM–%D C–110	1.60–0.9	...	1.54–5.2	3.1
CM–%D C–734	1.93–11.9	1.77–0.9	...	6.4	CM–%D C–734	1.78–4.4	1.79–5.3	...	4.9
11–5	1.48	1.72	1.77	..	13–4	1.58	1.51	1.57	..
12–1	1.60	1.47	1.52	..	14–2	1.62	1.63	1.56	..
%D–Un	3.9	7.8	7.6	6.4	%D–Un	1.3	3.8	0.3	1.8
%C	—7.5	+17.0	+16.5	..	%C	—2.5	—7.4	+0.6	..
CM–%D	...	1.36–11.7	1.41–11.3	11.5	CM–%D	...	1.59–2.6	1.52–1.6	2.1

C–28				
CM–%D	1.87–11.6	. . .	1.78–0.3	6.0
C–110				
CM–%D	1.86–11.4	1.71–0.3	. . .	5.9
C–734				
11–9	1.09	1.15	1.38	. .
12–1	1.60	1.47	1.52	. .
%D–Un	19.0	12.2	4.8	12.0
%C	—31.9	—21.8	—9.2	. .
CM–%D	. . .	1.00–7.0	1.04–14.0	10.5
C–28				
CM–%D	1.25–6.8	. . .	1.19–7.4	7.1
C–110				
CM–%D	1.45–14.2	1.33–7.3	. . .	10.8
C–734				
11–1	1.45	1.57	1.76	. .
12–4	1.94	1.73	1.83	. .
%D–Un	14.5	4.9	1.9	7.1
%C	—25.2	—9.2	—4.0	. .
CM–%D	. . .	1.29–9.8	1.36–12.8	11.3
C–28				
CM–%D	1.76–9.7	. . .	1.60–4.8	7.3
C–110				
CM–%D	1.86–12.4	1.66–2.8	. . .	7.6
C–734				
11–2	1.32	1.42	1.38	. .
12–4	1.94	1.73	1.83	. .
%D–Un	19.0	9.8	14.0	14.3
%C	—32.0	—17.9	—24.6	. .
CM–%D	. . .	1.18–9.2	1.24–5.3	7.3
C–28				
CM–%D	1.59–9.3	. . .	1.50–4.2	6.8
C–110				
CM–%D	1.46–5.0	1.30–4.4	. . .	4.7
C–734				
11–4	1.52	1.74	1.83	. .
12–4	1.94	1.73	1.83	. .
%D–Un	12.1	0.3	0.0	4.1
%C	—21.6	+0.6	. . .	. .
CM–%D	. . .	1.36–12.3	1.43–11.9	13.3
C–28				
CM–%D	1.50–2.6	. . .	1.44–4.3	3.5
C–110				
CM–%D	1.63–1.6	1.64–4.1	. . .	2.9
C–734				
13–5	1.57	1.90	1.85	. .
14–2	1.62	1.63	1.56	. .
%D–Un	1.6	7.6	8.5	5.9
%C	—3.1	+16.6	+18.6	. .
CM–%D	. . .	1.58–9.2	1.51–10.1	9.7
C–28				
CM–%D	1.89–9.2	. . .	1.82–0.8	5.0
C–110				
CM–%D	1.92–10.0	1.93–0.8	. .	5.4
C–734				
13–6	1.30	1.52	1.45	. .
14–2	1.62	1.63	1.56	. .
%D–Un	11.0	3.5	3.7	6.1
%C	—19.8	—6.8	—7.1	. .
CM–%D	. . .	1.31–7.4	1.25–7.4	7.4
C–28				
CM–%D	1.51–7.5	. . .	1.45–0.0	3.8
C–110				
CM–%D	1.50–7.1	1.51–0.3	. . .	3.7
C–734				
15–6	1.51	1.48	. . .	. .
19–4	1.31	1.24	. . .	. .
%D–Un	7.1	8.8	. . .	8.0
%C	+15.3	+19.4	. . .	. .
CM–%D	. . .	1.43–1.7	. . .	1.7
C–28				
CM–%D	1.56–1.6	. . .	. . .	1.6
C–110				
CM–%D	. . .	. . .	. . .	. .
C–734				
15–6	1.51	1.48	. . .	. .
19–7	1.06	1.21	. . .	. .
%D–Un	17.5	10.0	. . .	13.8
%C	+42.5	+22.3	. . .	. .
CM–%D	. . .	1.72–7.5	. . .	7.5

TABLE 10—Continued

Variation and correction of variation in DNA means of bull sperm samples processed on receipt and after storage for one week at 5° Centigrade.

Slide and sample comparison	Samples			Mean percent deviation	Slide and sample comparison	Samples			Mean percent deviation
	28	110	734			28	110	734	
C–28					C–28				
CM–%D	1.95–12.4	. . .	1.84–0.3	6.4	CM–%D	1.30–7.5	. . .	. . .	7.5
C–110					C–110				
CM–%D	1.94–12.1	1.73–0.3	. . .	6.2	CM–%D	. . .	. . .	. . .	. .
C–734					C–734				

Abbreviations:
%D–Un: % deviation between two means of smears from the same sample on different slides processed in the same containers before correction.
%C: % difference between means of one sample used to correct variation in other samples.
CM–%D
C–28 (C–110, C–734): corrected mean and % deviation when the mean of sample 110 or 734 is corrected by the % variation of sample 28, and similarly, when each sample is corrected by the variation in another.

TABLE 11

Summary of variation and correction of variation in DNA means of samples of bull sperm processed on or in the same or different slides, containers, or dates

Percent deviation uncorrected	No.	Percent deviation corrected				
		0–5	6–7	8–10	11–12	13–15
0–5	42	49	6	13	11	5
6–7	3	4	2	0	0	0
Summary	45	53	8	13	11	5
8–10	18	19	2	5	7	1
Summary	63	72	10	18	18	6
11–12	7	7	0	4	2	0
Summary	70	79	10	22	20	6
13–15	14	11	8	3	1	5
Summary	84	90	18	25	21	11
>15	10	8	5	4	0	2
Summary	94	98	23	29	21	13

tainable to better than a 5% deviation. Most will concede that, inevitably, the deviation on some occasions will exceed 5%. If one adds a maximum of 5–8% sampling error one reaches a level of deviation that is subject to effective control. The maximum deviation in the Leuchtenberger human sperm data of about 10% (*7*), if deviation in the determinations on repeat samples of semen from fertile men is taken as an indication of the experimental error, indicates a 5% staining deviation, assuming about a 5% sampling error from the standard errors given. Extensive experience in this laboratory with quantitative use of the Feulgen reaction indicates that such uniformity of staining is at, or close to the maximum attainable with the procedure developed to date; and that over a long period of time the staining deviation in some instances might easily be considerably in excess of this. This might vary with the material. The Leuchtenberger bull sperm data (*9*), for example, are far less consistent than that for human sperm (*7*); and in this laboratory staining uniformity from batch to batch has been found more difficult to maintain in bull sperm than in other types of nuclei.

A more precise and detailed analysis of the results is presented in Table 11. Consulting the summaries and keeping in mind that, in most cases, there are two corrections for every uncorrected deviation, one notes that 44.7% of the total uncorrected deviations does not exceed 5% and 47.9% does not exceed 7%; when corrected, in contrast, 53.3% does not exceed 5% and 65.7% does not exceed 7%. When only the uncorrected deviations up to 15% are considered, 50.0% does not exceed 5% and 53.6% does not exceed 7% ;corrected, 54.5% does not exceed 5% and 65.5% does not exceed 7%. Of the uncorrected deviations up to 12%, 60.0% does not exceed 5% and 64.3% does not exceed 7%; corrected, 59.1% does not exceed 5% and 65.0% does not exceed 7%. Up to 10% uncorrected, 66.7% does not exceed 5% and 71.4% does not exceed 7%; corrected, 58.1% does not exceed 5% and 66.1% does not exceed 7%. Up to 7% uncorrected, 93.3% does not exceed 5% and 100% does not exceed 7%; corrected, 58.9% does not exceed 5% and 67.8% does not exceed 7%. Up to 5% uncorrected, 100% does not exceed 5%; corrected, 58.3% does not exceed 5% and

65.5% does not exceed 7%. Thus, down to 12% uncorrected deviation, control and correction reduce the experimental error. This may be summed up as follows. Of 184 separate corrections, 53.3% had an experimental error not exceeding 5%, 65.8% not exceeding 7%, 81.5% not exceeding 10%, 92.9% not exceeding 12%; and, in not a single instance, did the experimental error exceed 15%. Although the skewness of distribution of the percentage deviations makes it impossible to calculate statistically valid levels of confidence, the size of the sample tested justifies acceptance of these percentage levels as a fair statement of the experimental error. After a few exceptions have been allowed for, any deviation in excess of these limits would be suspected of representing true variation.

A maximum of 15% error is by no means excessive for this type of work when measurements are made on different slides. A 10% error is given by Pollister and Ornstein (*22*) as a conveniently attainable standard of reproducibility when measurements are made on the same slide. When measurements are made on different slides and corrected by control tissue on each slide, it is necessary to add the error of the control to that of the sample, making a total of 20% at the minimum when the control mean is referred to an overall mean obtained from numerous measurements on the control tissue. If the control mean on one slide is compared with the control mean on another slide, the error can reach as high as 30%. In sperm measurements, it is possible to limit the error to 15% even when measurements are made on different slides because the sampling error in sperm is less than in other types of nuclei. When measurements are made on the same slide, the sampling error reaches a maximum of about 8%. The reason why the system does not work even better is not hard to find: it is the aberrant smear, the one that yields a mean out of line with the other two on the same slide. This has a disastrous effect when it produces a situation in which one sample has a mean in excess of that of its opposite number on the comparison slide, while the means of the other two samples are lower than their comparison smears; or, vice versa. The fact that this aberrant mean is the result of a sampling extreme or a possible variation in staining on a different part of the slide suggests a refinement of technique by which erroneous corrections can be detected. This is simply staining duplicate slides, which, of course, means additional work, though not excessive when three smears are placed on one slide together with the control. This will permit selecting a slide for measurement, in the first place, that has a control that appears to stain normally and it will provide also a second and a third slide to check any variations on the first that exceed the deviation representing the maximum experimental error desired during the course of the work. Thus, if the odds are one in three that the experimental error will exceed 7%, as above, the odds are one in nine that 7% will be exceeded by both of the two means, each of which has been calculated separately from measurements on different slides. The experimental error has thus become 7% at a level of confidence of about 90%. Measurements on a third slide of those yielding two means with deviations over 7% would raise the level of confidence to over 95%. This method is superior to increasing the sample number on the same slide in that it offers the additional advantage of detecting a possible intra-slide staining variation between sample and control.

The principle of control and correction of variation in sample smears by the

variation in a control smear placed on the same slide has been demonstrated by comparison of means unreferred to a normal standard. In actual practice, the mean of each sample selected from a new group to be used as a control for the following group has itself been corrected by the control from the previous group and so on back to the beginning of the work when a "normal" level of staining was arbitrarily defined in accordance with the mean value of 1.52 obtained by Leuchtenberger (*9*), so that data would be comparable. In 246 samples from 98 bulls under two years of age analyzed here, the sampling error was ironed out to such an extent that an overall mean of 1.53 ± 0.01 was obtained. Thus, the maximum experimental error in a single determination represents the maximum per cent deviation from 1.53 permitted in a normal sample. Now, accepting 1.53 as the normal mean value, one can select a normal sample from one batch, preserve part of it at 5° or in dry ice and use it as a control for the next batch, correcting all samples on one slide, usually three, according to the percentage variation from 1.53 of the control smear on that particular slide. This is the control system used in the second phase of the work, during which 155 samples from 135 bulls, most of which were under two years of age, have been analyzed.

In practice, when only one sample is taken, all those deviating by more than 7% from 1.53 would be measured on a duplicate slide and on a triplicate whenever the mean of the duplicate slide measurements also deviated by more than 7% from 1.53. Duplicate deviations in excess of 7% would be considered evidence of true DNA deviation in 90% and triplicate in 95% of samples. This means simply that in 100 samples there should not be more than ten that deviate from 1.53 by more than 7% after two separate determinations on different slides, not more than five of such nature after triplicate determinations. A normal value obtained at any time—first, duplicate or triplicate determinations—would imply, assuming a maximum experimental error of 15% on a single determination, that the bull does not deviate from the normal 1.53 by more than 20%. As the level of confidence is lowered and the maximum experimental error accordingly reduced, this maximum percentage deviation would diminish. Additional samples or determinations on additional slides would have a similar effect.

Should the problem demand it, the experimental error can be reduced still further. Measuring additional sperm in the same sample would reduce the sampling error a few per cent and, finally, the total error below 5%. Furthermore, if sufficient samples from the same bull are analyzed, or even if sufficient duplicate smears from the same sample but stained on different slides are analyzed, the over-all standard error of the mean could be calculated. Also, if only two or three determinations are made, one might reasonably estimate the reduction of the experimental error when two or three means are averaged by analogy with the halving of the standard error of the mean of a single sample when the number of measurements is quadrupled. Thus, one might expect the experimental error of the over-all mean of three samples to be about two-thirds that of a single sample. This assumes, of course, that the actual amount of DNA is constant in the samples analyzed. If the individual tests are separated by more than the experimental error of a single test, fluctuation of DNA would be suspected, the detection of which would require extensive tests over a considerable period of time, as would

also the proper study of a possible non-hereditary type of DNA deviation resulting from some form of testicular degeneration.

In contrast with the potential accuracy and precision of cytophotometric technique in sperm analysis, chemical techniques do not seem especially suitable for this type of work. As reported by Leuchtenberger (*7*), two groups of workers (*16*, *30*) have obtained values of 2.8 and 3.3×10^{-9} mg. for a single bull sperm, while more extensive tests of 11 samples of normal human semen (*3*) have yielded values ranging from 1.8 to 5.8×10^{-9} mg. per sperm. These results were obtained by "standard" chemical analysis. Less quantitative techniques of biochemistry, such as bioassay, would be expected to be even less reliable. Under such an overlay of experimental error any true difference in DNA amounts, unless of an unlikely magnitude, would be completely obscured. As explained by Leuchtenberger (*7*), the chemical value for a single sperm is an average derived from the total amount of DNA found in a suspension of sperm, which includes morphologically abnormal sperm as well as other cellular elements. Since cytophotometry does not labor under this handicap and, partly because of this fact, has a low experimental error, it seems preeminently adapted to detection of moderate differences in the DNA content of morphologically normal sperm, presumably related to infertility.

SUMMARY

Feulgen cytophotometry has been applied over a period of about two years to the analysis of deoxyribonucleic acid (DNA) content of 658 samples of spermatozoa from 275 Santa Gertrudis bulls of the King Ranch, most of which were under two years of age. Procedure is described, and the various components of the experimental error are distinguished and analyzed. A system of correction for smear to smear and slide to slide staining variation due to experimental error is presented that restricts the total maximum experimental error of a single determination to 15%, referred to the established normal mean of 1.53 in relative quantitative units, and to 10% in 80% of samples, when 18 to 25 sperm are measured per smear. Refinements of technique are described by which the maximum error can be reduced to 7% in 95% of the samples and to 5% by increase in the number of sperm measured per sample smear.

III. The Deoxyribonucleic Acid (DNA) Content of Semen Spermatozoa and Testis Germinal Cells From Santa Gertrudis Bulls of Known Fertility and Infertility

ROBERT M. WELCH AND KATHLEEN RESCH

The program was initiated during the summer of 1956 by analysis of samples of semen from five bulls of known fertility and six of known infertility. These were bulls three or more years in age taken from the breeding herds of the King Ranch. The main issue under investigation was not entirely clearcut here for two reasons. As stated, the six bulls were infertile rather than sterile; and three of the five shown in Table 1 had some degree of hypoplasia. Testes of the sixth bull, L 727, were not available for weighing. Thus, the question here was mainly whether infertile, hypoplastic bulls exhibit DNA abnormality in semen sperm and testis germinal cells in comparison with bulls of known fertility and testes of normal size. In slightly different words, does hypoplasia exert its effect through abnormal DNA as well as through lowered production of sperm? For only one bull was the issue of infertility and abnormal DNA uncomplicated by the additional factor of hypoplasia.

When the tests had been completed, five of the six infertile bulls were killed and samples taken of testis and, in some instances, of liver. DNA analysis was made on slides prepared from this material. Additional paraffin embedded material was retained for subsequent comparison with material obtained from certain of the Group C bulls, to be designated later, established as relatively normal in the DNA content of their semen sperm, on the reasonable assumption that the testis nuclei of such bulls would also be normal in DNA. This was in lieu of material from bulls of known fertility, unobtainable either through sacrifice or biopsy.

The tests were made at intervals of from one to two weeks. Handling of material, preparation of slides, staining and measuring were substantially as described previously, with exceptions as noted below. The final control system had not been elaborated at this time. Smears were made on separate slides; and, in two groups of samples, all means were reduced by 15% and 10% respectively because execessively dark staining was found to have occurred. Motility is that recorded at the ranch when the samples were taken. Sperm count was estimated as "low", "medium", or "high" from a qualitative examination of the semen at the laboratory and from the amount of centrifuged sediment present. No routine morphology examination was made at this time. Testis and liver material was obtained at the slaughterhouse immediately after the bulls were killed, fixed in acetic alcohol, and embedded routinely. Liver and testis sections were cut at 10 and 12 microns respectively, slides were stained as previously described, with an hydrolysis time of 12 minutes, and measurements were made at 570 millimicrons with the equipment already described and according to the EC^2/F method previously referred to. The first testis and liver measurements were made on several

different slides. The comparison measurements between the material from the infertile bulls and that from the bulls relatively normal in semen sperm were all made on the same slide.

RESULTS

Weights of the formalin fixed testes from the infertile bulls are given in Table 1. The extent of hypoplasia in each bull is evident by comparison with the weight of the testis from bull K-51, whose testes were of normal size. Fresh testis weights of the bulls established as relatively normal in DNA content of semen sperm are given in Table 2. All these had testes of normal size and appearance. DNA-Feulgen means for the samples of sperm from fertile and infertile bulls, together with other relevant data, are given in Table 3; statistics for these means are given in Table 4; and the DNA means are graphed in Fig. 1. When the analyses were first carried out, certain of the means were excluded from the tabulation of the results because of the possibility that drying out of the semen on the slide while the smear was being made, resulting from an extremely small amount of sediment being present after centrifuging, might have affected the degree of staining. It was subsequently found, however, as discussed in a paper to follow in this series, that a true correlation exists between low sperm count and low DNA, after proper measures have been taken to prevent any possible drying out of the smear. Accordingly, the DNA means previously eliminated have been restored and appear in both table and graph. To avoid confusion, the data for fertile bulls in Table 3 and Fig. 1 have been limited to those fertile bulls tested at the same time as the six bulls of known infertility. In addition, five bulls of known fertility were tested subsequently. Results for these are given in Table 5. Also, during November and

TABLE 1

Weight of testis of Santa Gertrudis bulls of known infertility

Bull	Weight in grams
C–322 (left)	238
C–15 (left)	323
K–51 (right)	615
LMD (left)	265
KMD (right)	400

Specimens were weighed after formalin preservation.
Right and left testes were of approximately equal size except for C–15, the right testis of which was somewhat larger.

TABLE 2

Fresh weight of testis of Group C bulls relatively normal in deoxyribonucleic acid (DNA) content of semen spermatozoa

Bull	Weight in grams
O6 (left)	392
U2 (left)	314
U14 (right)	320
U15 (left)	414
O5 (right)	467

TABLE 3

Relative amount of DNA-Feulgen, sperm count and motility in spermatozoa from semen of Santa Gertrudis bulls of known fertility and infertility

Fertile				Infertile			
Bull	Mean DNA-Feulgen	Motility	Sperm count	Bull	Mean DNA-Feulgen	Motility	Sperm count
C $\frac{1}{1}$ 34	1.19	F	L	K-51	1.55	VP	M
					1.72	NM	M
	1.32	VG	L		1.53	NM	L
	1.53	E	H				
	1.61	E	H	C–322	1.48	F	M
	1.56	E	M		1.30	P	L
	1.63	E	H		1.40	NM	L
					1.41	VP	L
=L ≈ 3	1.58	G	H				
	1.44	E	H	C–15	1.34	VP	L
	1.58	E	H		1.39	VP	L
	1.50	VG	M		1.41	G	L
	1.43	VP	L		1.42	NM	L
W956	1.57	E	H		1.65	G	M
	1.29	VG	L				
	1.32	G	L	L 727	1.45	G	M
	1.57	VG	M		1.52	F	M
	1.61	G	L		1.36	F	M
					1.56	VP	M
L 707	1.61	E	M		1.55	G	H
	1.73	E	M				
	1.44	G	L	KMD	1.45	P	M
	1.36	E	M		1.40	E	H
	1.42	F	L		1.57	E	H
K pt	1.41	G	L		1.52	E	M
	1.47	E	M		1.49	G	L
	1.54	VG	H				
	1.57	G	H	LMD	1.47	G	M
	1.66	VG	M				

Motility is that recorded at ranch in grades from "Excellent" to "Very Poor".
Sperm count is recorded as low, medium or high from ranch and laboratory examination of semen and amount of centrifuged sediment.

December of 1956, four bulls were tested, one of which was a doubtful hypoplastic and three of which were considered hypoplastic at the time of testing. The former gave four tests all well within the normal range. Of the latter, two gave two and one test respectively in the normal range, while the third gave three out of four tests within the normal range.

The first measurements on liver and testis of infertile animals are presented in Table 6. Comparison measurements between the animals of known infertility and those relatively normal in DNA content of semen sperm are given in Table 7. Of those bulls available for slaughter, the five most constant in DNA were selected; and comparison measurements were made on the three of these that yielded the most favorable material for analysis after the slides had been made. These bulls were U-2, U-14 and O5. DNA values for these can be found in Table

TABLE 4

Statistics of samples of spermatozoa from Santa Gertrudis bulls of known fertility and infertility

No. of bull	Mean DNA-Feulgen	S.D.	C.V.	S.E.	No. of bull	Mean DNA-Feulgen	S.D.	C.V.	S.E.
					K-51	1.55	0.12	8.0	0.028
C $\frac{1}{1}$ 34	1.19	0.09	7.5%	0.030		1.72	0.13	7.7	0.034
	1.32	0.07	5.5	0.014		1.53	0.05	3.2	0.012
	1.53	0.08	5.5	0.022					
	1.61	0.10	6.0	0.023	C–322	1.48	0.07	4.6	0.015
	1.56	0.07	4.4	0.017		1.30	0.02	1.4	0.005
=L ≈ 3	1.58	0.05	2.9	0.012	C–15	1.34	0.08	5.8	0.025
	1.44	0.07	5.0	0.014		1.39	0.08	5.9	0.018
	1.58	0.08	5.2	0.021		1.41	0.06	4.6	0.017
	1.50	0.07	4.6	0.017					
					L 727	1.45	0.06	4.5	0.022
W956	1.57	0.10	6.3	0.031		1.52	0.05	3.6	0.012
	1.29	0.06	4.9	0.014		1.36	0.10	7.2	0.025
	1.32	0.08	6.3	0.021					
	1.57	0.09	5.5	0.022	K pt	1.41	0.05	3.8	0.017
	1.61	0.10	6.0	0.024		1.47	0.06	4.0	0.013
						1.54	0.08	5.5	0.021
L 707	1.61	0.09	5.8	0.032		1.57	0.05	3.3	0.013
	1.73	0.10	5.7	0.022		1.66	0.09	5.1	0.021
	1.44	0.08	5.8	0.021					
	1.36	0.04	3.2	0.011	KMD	1.45	0.05	3.2	0.015
	1.42	0.08	5.5	0.019		1.40	0.10	7.1	0.026
						1.57	0.08	5.1	0.020
LMD	1.47	0.08	5.4	0.018		1.52	0.06	3.6	0.014

8, Part C of the following paper. The first had four of five means within 10% of the over-all normal mean; the second had one abnormal mean followed by five normal means; and the third had four means, all in the normal range. In addition, semen samples were taken from these bulls just prior to slaughter. The DNA mean for each of the three was normal.

ANALYSIS AND DISCUSSION

When the results of the semen sperm analyses were first considered on a basis of elimination of thirteen means derived from samples of extremely low sperm density for the reason previously mentioned, there was found a difference of about nine per cent between fertile and infertile collective means compared with about 34% in the Leuchtenberger report (*9*). Because, however, of a low spread of values, resulting in standard errors from 0.01 to 0.03 for means in the vicinity of 1.50 compared with the range in the Leuchtenberger report of 0.03 to 0.09 for means of 1.08 and 1.52, and the fact that the collective means were derived from a number of samples, the 9% difference was considered statistically more significant than an equal percentage would be in the Leuchtenberger report based on single samples showing a wide spread. When the means derived from samples of extremely low sperm density are restored, however, this difference, already

small, is greatly reduced or obliterated. One can no longer state that there is any significant difference between fertile and infertile collective means. If, however, one examines the individual data, presented in Table 3 and Fig. 1, one can still make a separation although there is considerable overlapping. For example,

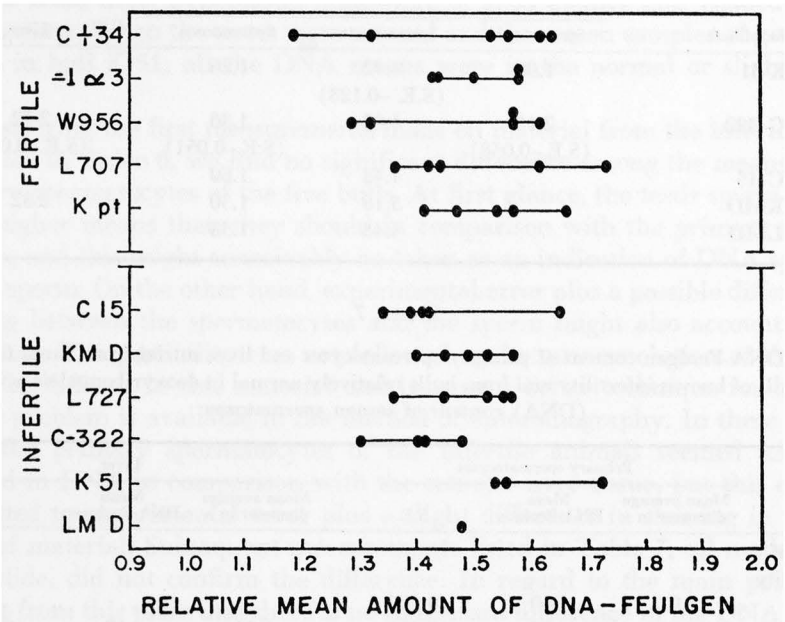


Fig. 1. Relative mean amount of DNA-Feulgen in semen spermatozoa from Santa Gertrudis bulls of known fertility and infertility.

Table 5

Relative DNA-Feulgen content of semen spermatozoa from Santa Gertrudis bulls of known fertility

No. of bull	Date	Mean avg. size in μ	Mean DNA-Feulgen	Motility[1]	Morphology[2]	Sperm count[3]
10–2	3/18/57	6.1	1.41 (26)	E	25	80
	3/25/57	6.0	1.61 (26)	VG	15	19
	4/1/57	6.1	1.78 (25)	E	10	84
10–22	3/18/57	6.3	1.86 (27)	VG	15	73
	3/25/57	6.1	1.53 (25)	F	20	180
	4/1/57	6.2	1.67 (25)	E	15	125
10–28	3/18/57	6.1	1.51 (27)	E	10	410
	3/25/57	6.0	1.90 (27)	E	15	100
	4/1/57	6.0	1.52 (26)	E	25	265
10–734	3/18/57	6.3	1.58 (23)	E	10	192
	3/25/57	5.7	1.71 (25)	G	10	110
	4/1/57	6.1	1.51 (27)	G	25	55
10–993	3/18/57	6.3	1.57 (25)	VG	15	300
	3/25/57	6.0	1.54 (27)	G	10	130
	4/1/57	6.1	1.39 (26)	E	10	630

[1] Motility in grades Excellent to Very Poor.
[2] Percentage of abnormal forms.
[3] In millions per cc.
Number of sperm analyzed given in parenthesis after DNA mean.

TABLE 6

Relative DNA-Feulgen content of nuclei and spermatozoa in testis and liver of Santa Gertrudis bulls of known infertility

No. of bull	Testis			Liver
	Spermatogonia	Spermatocytes	Spermatozoa	
K-51	1.98	4.42 (S.E. –0.128)	1.26	...
C–322	2.13 (S.E.–0.058)	4.80	1.36 (S.E.–0.051)	2.72 (S.E.–0.068)
C–15	...	4.48	1.60	...
KMD	...	5.18	1.30	2.82
LMD	...	4.48	1.38	...

TABLE 7

Relative DNA-Feulgen content of primary spermatocytes and liver nuclei from Santa Gertrudis bulls of known infertility and from bulls relatively normal in deoxyribonucleic acid (DNA) content of semen spermatozoa

No. of bull	Primary spermatocytes			Liver		
	Mean average diameter in μ	Mean DNA-Feulgen	S.E.	Mean average diameter in μ	Mean DNA-Feulgen	S.E.
Group I of low fertility						
K-51	7.87	4.49	0.106	...	...	....
C–322	8.03	4.09	0.082	6.44	2.19	0.077
C–15	7.73	4.32	0.076	...	...	....
Relatively normal in semen spermatozoa						
U2	7.28	4.43	0.085	...	...	....
U14	7.14	4.10	0.089	...	...	....
O5	7.46	4.26	0.104	6.36	2.05	0.054
U15	...	...	....	6.53	2.21	0.106

while the means for KMD, L 727 and K-51 do not appear significantly different from those of the fertile animals, if we look at C-15 and C-322, we see that four of five means of the former are below or in close proximity to the 10% deviation line from a normal mean of 1.52 and that three of four means of the latter can be similarly described. The fertile bull most comparable in this respect has only two of five values in this range. If we consider the five fertile bulls subsequently tested, listed in Table 5, the difference is further confirmed. Only two of the five bulls listed have a mean in the low range, and, in each case, the single low mean is accompanied by two means in the normal or high range. Thus, there does appear to be some difference in sperm DNA between the fertile bulls and at least some of the infertile bulls, although the fact that the fertile bulls give some low values on poor samples and the fact that the infertile bulls generally yielded poorer samples from the standpoint of motility and sperm count suggest that the DNA difference may be correlated with motility and sperm count. This possibility will be taken up more thoroughly in a subsequent paper. Hypoplasia, in

itself, does not seem to result in low DNA means but only through its effect on the quality of the sample. According to the figures previously given, out of seven samples from hypoplastic bulls tested in addition to the original bulls, only one gave a low value, and this sample had a low sperm count. The remaining six samples from these hypoplastic bulls were of good quality and yielded normal DNA means. When the testes were normal and the semen samples of good quality, as in bull K-51, all the DNA means were in the normal or slightly high ranges.

Considering the first measurements made on material from the infertile bulls, presented in Table 6, we find no significant difference among the means for the primary spermatocytes of the five bulls. At first glance, the testis sperm seem to yield higher means than they should in comparison with the primary spermatocytes; and this might conceivably be taken as an indication of DNA synthesis by the sperm. On the other hand, experimental error plus a possible difference in staining between the spermatocytes and the sperm might also account for the difference. The possibility was not followed up because resolution of the doubt would be difficult in this material and because a better technique for this particular problem is available in the method of autoradiography. In these first results, the primary spermatocytes of the infertile animals seemed somewhat reduced in DNA in comparison with the somatic liver tissue, but this could be attributed to experimental error plus a slight difference in staining in the two types of material. Subsequent measurements listed in Table 7, all made on the same slide, did not confirm the difference. In regard to the main point, it is evident from this table that there is no significant difference in the DNA content of the primary spermatocytes between the three infertile bulls and the three bulls relatively normal in DNA content of semen spermatozoa. The results would apply to all five infertile bulls, as Table 6 shows no significant difference among them with regard to the primary spermatocytes. If one asks the question why C-15 and C-322 can yield low values for semen sperm and yet give normal values for testis spermatocytes, the only answer that occurs is that the semen sperm differences are too small to be apparent except on the basis of a number of tests and are not reflected in a limited number of measurements made on a single testis sample. There is, of course, the possibility previously mentioned that sperm synthesize DNA and the sperm deficient in DNA have failed to synthesize the proper amount, but this cannot be taken seriously without additional evidence.

IV. The Deoxyribonucleic Acid (DNA) Content of Spermatozoa in Semen From Santa Gertrudis Bulls of Unknown Fertility Under Two Years of Age

ROBERT M. WELCH, E. W. HANLY, AND WILLIAM GUEST

Part A

During the fall and spring of 1956–1957, 246 samples were analyzed for DNA, three from each of 57 bulls, two of which had one and two additional samples respectively; two samples from each of 31 bulls, and one sample from each of 10 bulls, all these bulls being of unknown fertility and under two years of age. The distribution of the DNA means obtained is shown in the histogram of Fig. 1. Although controlled to the mean of 1.52 which Leuchtenberger obtained from 55 fertile bulls (*9*), the over-all mean of 1.53 yielded by these 246 samples is used as the normal DNA constant value from which to separate deviation possibly in excess of experimental error, on the assumption that a greater number of samples would result in a smaller sampling error, while any deviating samples

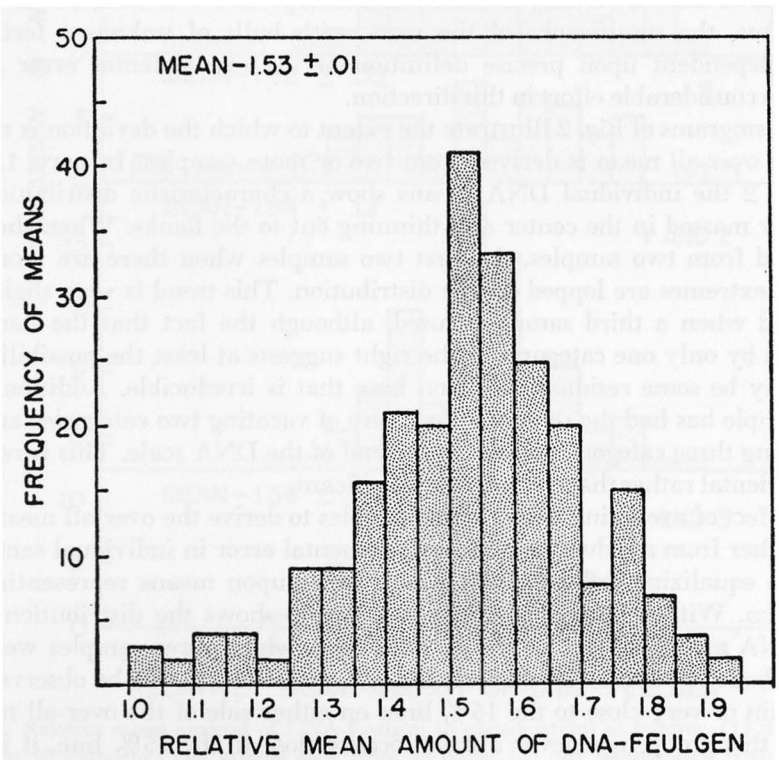


Fig. 1. Relative mean amount of DNA-Feulgen of 246 samples from 98 bulls of unknown fertility under two years of age.

would be so few, as well as being distributed on both sides of the mean, as to have a negligible effect on the final mean.

It is apparent from an examination of the histogram of Fig. 1 that, although the bulk of the DNA means falls within a comparatively narrow range, less than 10 or 15 per cent on either side of the mean of 1.53, there are still numerous values that fall outside this range. In the wide range of values obtained, the histogram obtained from these 98 bulls of unknown fertility is strikingly similar to that obtained by Leuchtenberger (*9*) from 55 bulls of known fertility. In the latter, two alternatives suggest themselves: either the amount of DNA is constant in the sperm and the experimental error is large, leading to widely distributed values; or the more extreme means represent actual DNA deviations, which yet have no effect on fertility. The alternative applicable to the Leuchtenberger data need not necessarily apply to the data of Fig. 1, the critical point being the extent of the experimental error in each case. A third interpretation is possible when the bulls are of unknown fertility, that the variations represent actual deviations in DNA, which do have an effect on fertility, although this would be unlikely in the event that the variation in bulls of known fertility results from actual DNA deviation. As the latter has not been established, nor even claimed, the presumption is that experimental error is responsible for the spread. In this event, it becomes necessary to show reasonable evidence why the experimental error of the work with bulls of unknown fertility is less than that with bulls of known fertility if a different interpretation is claimed for the results. Thus, the significance of the work with bulls of unknown fertility is largely dependent upon precise definition of the experimental error and so justifies a considerable effort in this direction.

The histograms of Fig. 2 illustrate the extent to which the deviation is reduced when an over-all mean is derived from two or more samples. In parts 1, 2 and 3 of Fig. 2 the individual DNA means show a characteristic distribution, numerically massed in the center and thinning out to the flanks. When the mean is derived from two samples, the first two samples when there are more than two, the extremes are lopped off the distribution. This trend is very slightly accentuated when a third sample is used, although the fact that the flanks are pulled in by only one category on the right suggests at least the possibility that there may be some residual deviation here that is irreducible. Addition of the third sample has had the striking effect here of vacating two categories and thus of isolating three categories on the lower end of the DNA scale. This is regarded as coincidental rather than typical and significant.

The effect of averaging two or more samples to derive the over-all mean could result either from a reduction of the experimental error in individual samples or from the equalizing influence of normal values upon means representing true fluctuation. With reference to this point, Fig. 3 shows the distribution of the three DNA means in each of the 57 bulls from which three samples were analyzed. About two-thirds of the bulls have three means, it will be observed, that fall within or very close to the 15% lines on either side of the over-all mean of 1.53. In this group, whenever a mean occurs close to the 15% line, it is offset by two means deviating considerably less from the normal. Then, there is a group in which a single mean in the 15% neighborhood is accompanied by two

other means that also show considerable deviation from the normal but not to the same degree, all three yielding an over-all mean in excess of 10% of the normal. Similar to the preceding is a small group with two means within or close to the 15% line and one less than 10%, the over-all mean deviating by more than 10%. Then, there are two groups with characteristics similar to the two above, except that the single aberrant mean is markedly in excess of the

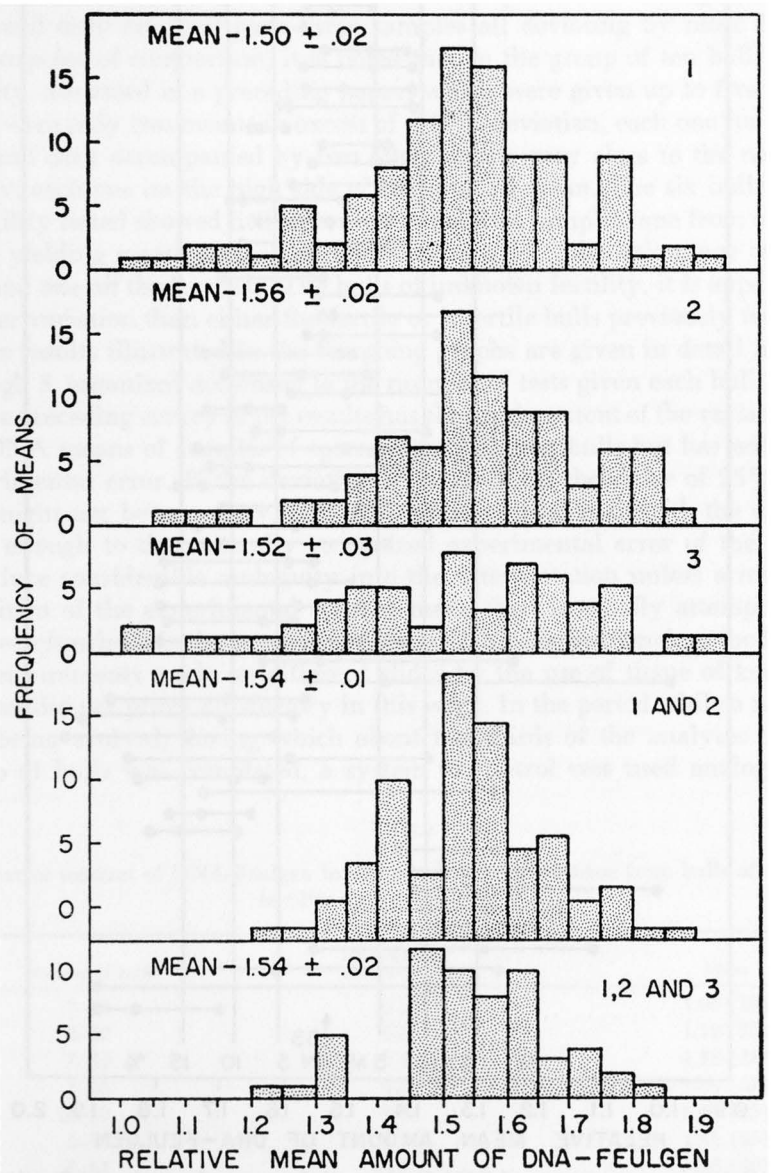


FIG. 2. Relative mean amount of DNA-Feulgen in classifications as follows: (1) one sample each from 98 bulls; (2) one sample each from 88 bulls; (3) one sample each from 57 bulls; (1 and 2) average of two samples from each of 88 bulls; (1, 2 and 3) average of three samples from each of 57 bulls.

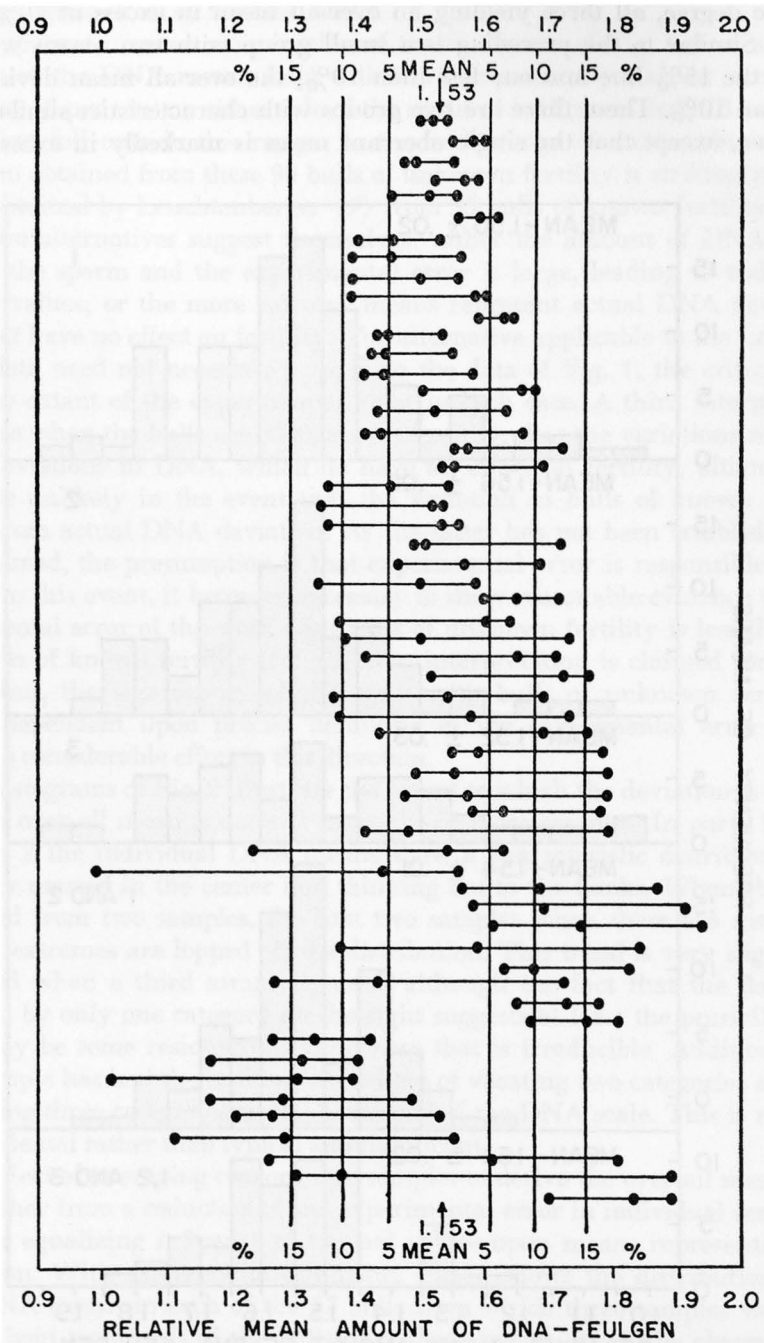


FIG. 3. Relative mean amount of DNA-Feulgen of three samples, individually plotted, from 57 bulls of unknown fertility under two years of age.

15% line. Next, there are two groups that have in common the fact that two means are in excess of 15%, while differing in that one group has a third mean close to normal, while the other group has a mean that deviates considerably, though less than 15%, from the normal. Especially to be noted is the extremely rare occurrence of those bulls that deviate on both sides of the normal mean. Only two can be placed in this category with one or two others showing a tendency in that direction. No bull gave three samples all deviating by more than 15% and only one bull gave three samples all deviating by more than 10%. For purposes of comparison, it is noted that in the group of ten bulls of known fertility, discussed in a preceding paper, which were given up to five tests each, there were only two means in excess of a 15% deviation, each one for a separate bull and each accompanied by two other means very close to the normal and, finally, each one on the high side of the over-all mean. The six bulls of known infertility tested showed little more variation, two samples, one from each of two bulls, yielding means in the neighborhood of a 15% deviation, one on the high side and one on the low side. The bulls of unknown fertility, it is apparent, show greater variation than either the fertile or infertile bulls previously tested.

The results illustrated in the foregoing graphs are given in detail in Tables 1 through 3, organized according to the number of tests given each bull.

The preceding survey of the results has shown the extent of the variation among the DNA means of samples of sperm from different bulls but has not separated experimental error. If the deviation were large, in the order of 25% or more, this might not be necessary, but, as the results have indicated, the deviation is close enough to the generally recognized experimental error of the method to introduce considerable ambiguity into the interpretation unless a more precise definition of the experimental error is made than is usually attempted. As explained elsewhere in the preceding Article II, the conventional method of control of measurements made on different slides by the use of tissue of known DNA content did not prove satisfactory in this work. In the period while a new system was being evolved, during which about two-thirds of the analyses of the first group of bulls was completed, a system of control was used analogous to the

TABLE 1

Relative amount of DNA-Feulgen in one sample of spermatozoa from bulls of unknown fertility under two years of age.

Number of bull	Mean average diameter in μ	Mean
7–54	6.3	1.08 (28)
4–57	5.5	1.12 (20)
7–52	6.4	1.19 (27)
7–45	5.7	1.34 (27)
10–19	5.8	1.49 (25)
3–5	5.5	1.51 (25)
5–67	5.7	1.55 (25)
8–85	6.2	1.56 (38)
7–51	5.9	1.62 (19)
9–93	5.9	1.69 (26)

Number of sperm analyzed in each sample is indicated in parentheses after the mean.

"curve grading" method. On the reasonable assumption that most if not all the bulls in a group of 10 to 20 animals of unknown fertility would be expected to have a normal, constant sperm DNA, all the DNA means of a given batch of slides were raised or lowered, if necessary, by an amount sufficient to bring the maximum number of animals within a range 10% plus or minus the Leuchtenberger normal mean of 1.52 (*9*). The experimental error component of the deviation from this mean in any individual bull would then be due either to the sampling error shown, in Article II, to reach a maximum of about 7%, and the staining error arising from random variation in staining from slide to slide in the same batch. An indication of the latter may be obtained by an examination of Table 6 in the method paper (Article II) which shows that, in three samples on

TABLE 2

Relative amount of DNA-Feulgen in two samples of spermatozoa from bulls of unknown fertility under two years of age

Number of bull	Mean average diameter in μ	DNA-Feulgen Sample means First	Second	Over-all mean
8-77	6.4	1.10 (27)	1.34 (26)	1.22
7-55	6.3	1.48 (28)	1.06 (28)	1.27
7-56	6.3	1.50 (27)	1.13 (27)	1.32
8-79	6.1	1.26 (27)	1.50 (21)	1.38
7-59	6.1	1.27 (28)	1.50 (27)	1.39
4-60	5.8	1.17 (22)	1.60 (23)	1.39
10-1003	6.0	1.25 (25)	1.56 (24)	1.40
8-94	6.1	1.00 (27)	1.80 (25)	1.40
4-48	5.7	1.39 (24)	1.44 (25)	1.42
2-67	5.9	1.45 (16)	1.43 (16)	1.44
7-62	6.2	1.52 (22)	1.39 (27)	1.46
10-50	6.0	1.48 (26)	1.78 (24)	1.63
2-68	5.6	1.46 (16)	1.54 (25)	1.50
2-66	5.9	1.53 (16)	1.49 (16)	1.51
7-60	6.3	1.45 (28)	1.56 (27)	1.51
8-65	6.2	1.53 (27)	1.51 (25)	1.52
10-02	6.0	1.53 (26)	1.54 (53)	1.54
4-61	5.6	1.44 (16)	1.64 (22)	1.54
9-82	6.0	1.38 (26)	1.69 (24)	1.54
9-91	6.1	1.58 (27)	1.51 (26)	1.55
7-46	6.2	1.51 (35)	1.60 (26)	1.55
7-57	5.9	1.63 (21)	1.49 (28)	1.56
7-47	5.9	1.50 (17)	1.61 (27)	1.56
7-50	5.8	1.50 (24)	1.65 (28)	1.58
7-44	6.0	1.40 (20)	1.75 (23)	1.58
8-73	6.0	1.50 (27)	1.67 (26)	1.59
7-48	6.0	1.58 (27)	1.66 (25)	1.62
9-105	6.0	1.79 (27)	1.52 (22)	1.66
9-98	5.8	1.64 (27)	1.67 (27)	1.66
9-87	5.9	1.87 (25)	1.51 (24)	1.69
9-89	6.0	1.83 (25)	1.54 (24)	1.69
3-6*	5.7	1.50 (16)	1.48 (25)	1.49

Number of sperm analyzed in each sample is indicated in parentheses after the mean.
* 3 year old bull of unknown fertility.

each of two slides in the same batch, and in three samples on each of three slides in the same batch, the maximum deviation is not more than 5% over and above a maximum 7% sampling error. And these slides were deliberately selected for maximum variation from slide to slide in the same batch. If the experimental error variation of normal samples is abnormally low, the "curve" control system might actually bring some instances of true deviation within the normal range. At any rate, it would seem reasonable to consider a deviation 15% or more in a single DNA mean as strong presumptive evidence of true DNA variation. If the sperm DNA is constant from sample to sample and all sample to sample variation is due to experimental error, one might expect the experimental error of one sample to be reduced by the square root of the total number of samples analyzed by analogy with the reduction of the standard error of a number of measurements. The experimental error of the over-all mean of three samples of the same bull would then be about 10%. This is on a level with the maximum experimental error of the final control system evolved, which applies to approximately one-third of the first group of bulls. The latter, however, was calculated from extensive experimental data specially assembled for the purpose, which make it possible to limit the error to 10% in 80% of means, to define the error precisely at other levels of confidence, and to reduce the error by a known amount by measurements on two or more slides. The conclusions of Part A, however, do not rely on these refinements but are based on the maximum 15 and 10 per cent experimental error, which would apply to all the data of Part A. For reasons previously explained, 1.53 is taken as the fiduciary mean from which to demarcate experimental error.

The histogram showing the distribution of the 246 samples of the first group of bulls is very similar to that obtained by Leuchtenberger for samples from 55 fertile bulls (*9*), but the standard errors of the Leuchtenberger measurements run about twice as high as in our determinations. In part, though not entirely, this may be due to the fact that in our measurements, marked extremes of staining, when they form only a small percentage of the total number of sperm measured, are considered in the category of abnormalities and eliminated from the calculation of the DNA mean for that particular sample, on the grounds that the DNA mean for the sample will then be more representative of the normal sperm in the sample. This technique is rationalized in the method article. Therefore, although the two graphs are superficially similar, the spread of values obtained here is much more significant. Of the 246 samples, 46 fall outside the maximum 15% deviation, 25 below and 21 above the fiduciary mean. At a 95% level of confidence, this would amount to deviations affecting 14% of the total number of samples. When the over-all means for 57 bulls are obtained from three samples each, there are 14 of the total number deviating more than the calculated experimental error, seven below and seven above the fiduciary mean, so that, on the basis of three tests, 19% of the bulls have some deviation in DNA. All the deviating over-all means have one or two samples in the normal range, as can be seen from Fig. 3. This could be either the result of experimental error in a truly deviating value, throwing it into the normal range, or it could possibly be an instance of fluctuation from the abnormal to the normal. It is impossible to answer this question definitely on the basis of three tests. To throw some light on

TABLE 3

Relative amount of DNA-Feulgen in three samples of spermatozoa from bulls of unknown fertility under two years of age

Number of bull	Mean average diameter in μ	DNA-Feulgen Sample means First	Second	Third	Over-all means Two samples	Three samples
3–7	5.7	1.30 (25)	1.38 (25)	1.01 (25)	1.34	1.23
4–52	5.7	1.40 (16)	1.16 (24)	1.31 (25)	1.28	1.29
4–56	5.6	1.49 (16)	1.28 (23)	1.17 (25)	1.39	1.31
3–12	5.6	1.55 (25)	1.29 (25)	1.11 (24)	1.42	1.32
3–4	5.7	1.56 (25)	1.44 (23)	0.99 (16)	1.50	1.33
8–69	6.1	1.27 (27)	1.42 (24)	1.33 (26)	1.35	1.34
8–68	6.2	1.27 (27)	1.53 (27)	1.21 (26)	1.40	1.34
10–109	6.3	1.45 (26)	1.35 (24)	1.54 (26)	1.40	1.45
6–N–1*	5.4	1.42 (21)	1.42 (25)	1.55 (22)	1.42	1.46
3–2	5.7	1.43 (16)	1.53 (25)	1.43 (25)	1.48	1.46
9–81	6.1	1.53 (27)	1.46 (25)	1.40 (24)	1.50	1.46
3–11	5.4	1.52 (16)	1.53 (25)	1.34 (24)	1.53	1.46
3–10	5.6	1.23 (16)	1.63 (25)	1.54 (25)	1.43	1.47
10–03	6.0	1.45 (27)	1.56 (25)	1.39 (26)	1.51	1.47
8–71	6.1	1.43 (24)	1.72 (23)	1.27 (27)	1.58	1.47
6–N–2	5.5	1.41 (10)	1.44 (24)	1.58 (24)	1.43	1.48
10–111	6.1	1.56 (25)	1.50 (25)	1.39 (25)	1.53	1.48
4–49	5.5	1.50 (16)	1.59 (25)	1.34 (25)	1.55	1.48
4–50	5.5	1.53 (16)	1.56 (23)	1.35 (25)	1.55	1.48
10–49	6.0	1.66 (24)	1.41 (25)	1.43 (25)	1.54	1.50
2–71	5.8	1.45 (16)	1.56 (19)	1.51 (16)	1.51	1.51
7–58	6.1	1.55 (28)	1.49 (19)	1.48 (27)	1.52	1.51
8–70	6.2	1.37 (27)	1.30 (25)	1.89 (26)	1.34	1.52
3–1	5.6	1.50 (16)	1.54 (25)	1.52 (25)	1.52	1.52
3–9	5.7	1.54 (25)	1.36 (23)	1.69 (20)	1.45	1.53
9–95	6.0	1.63 (25)	1.52 (26)	1.43 (24)	1.58	1.53
9–97	5.9	1.60 (27)	1.61 (27)	1.39 (24)	1.61	1.53
4–55	5.5	1.37 (16)	1.64 (25)	1.61 (25)	1.51	1.54
4–54	5.7	1.38 (16)	1.73 (22)	1.50 (25)	1.56	1.54
8–103	6.4	1.48 (25)	1.41 (26)	1.79 (23)	1.45	1.56
7–49	6.0	1.37 (28)	1.58 (20)	1.73 (28)	1.48	1.56
3–14	5.6	1.57 (16)	1.52 (25)	1.60 (24)	1.55	1.56
10–114	6.0	1.61 (26)	1.81 (40)	1.26 (23)	1.71	1.56
8–101	6.1	1.50 (27)	1.49 (24)	1.72 (25)	1.50	1.57
3–8	5.6	1.57 (16)	1.46 (25)	1.69 (25)	1.52	1.57
2–75	5.8	1.59 (16)	1.55 (16)	1.60 (25)	1.57	1.58
2–70	5.6	1.69 (16)	1.55 (24)	1.53 (25)	1.62	1.59
6–N–3†	5.8	1.56 (25)	1.60 (24)	1.63 (12)	1.58	1.60
10–113	6.0	1.59 (25)	1.84 (25)	1.37 (26)	1.72	1.60
8–76	6.2	1.49 (27)	1.55 (27)	1.78 (25)	1.52	1.61
10–110	6.0	1.41 (27)	1.65 (25)	1.76 (25)	1.53	1.61
7–61	6.0	1.50 (26)	1.68 (26)	1.66 (26)	1.59	1.61
8–67	6.1	1.71 (25)	1.57 (26)	1.55 (39)	1.64	1.61
8–88	6.2	1.57 (27)	1.79 (25)	1.48 (25)	1.68	1.61
9–78	5.9	1.64 (25)	1.63 (25)	1.60 (25)	1.64	1.62
9–96	5.7	1.76 (26)	1.69 (27)	1.44 (23)	1.73	1.63
9–90	5.8	1.59 (17)	1.55 (26)	1.78 (25)	1.57	1.64
9–99	6.0	1.76 (25)	1.51 (26)	1.72 (25)	1.64	1.66

10–48	6.2	1.61 (49)	1.71 (27)	1.65 (23)	1.66	1.66
9–84	6.0	1.58 (27)	1.78 (24)	1.63 (24)	1.68	1.66
9–80	5.9	1.67 (26)	1.82 (25)	1.63 (24)	1.75	1.71
9–100	6.1	1.72 (27)	1.77 (26)	1.65 (25)	1.75	1.71
8–74	6.2	1.69 (27)	1.87 (26)	1.56 (26)	1.78	1.71
10–108	6.0	1.91 (25)	1.69 (27)	1.58 (24)	1.80	1.73
9–102	5.9	1.76 (27)	1.81 (30)	1.67 (27)	1.79	1.75
8–92	6.2	1.61 (25)	1.75 (25)	1.94 (26)	1.68	1.77
8–72	6.2	1.89 (27)	1.83 (25)	1.75 (22)	1.86	1.82

Number of sperm analyzed in each sample is indicated in parentheses after the mean.
* Additional samples: 1.31 (12) and 1.43 (12).
† Additional sample: 1.36 (11).

this as well as the question of deviation, a graph, Fig. 4, has been drawn showing the probable chance distribution of 171 DNA means among 57 bulls, each having three samples, assuming that the only differences among them are due to experimental error, and contrasting this probable chance distribution with that actually obtained. Although it is not impossible to obtain a chance distribution diverging from probability to the degree shown, the fact that the extremes are numerically much greater than would be expected from chance is at least consistent with and supports the conclusion of true deviation, while the fact that those bulls having three DNA values distributed throughout the entire DNA range are far fewer than would be expected from chance, argues also against any considerable degree of fluctuation.

Although it would be premature at the present time to enter into any extended discussion of the results, one can at least state that, so far, the weight of the evidence is in favor of DNA variation in the spermatozoa of young bulls. Its incidence and degree, however, may be small. Certainly, nothing has been found in these bulls comparable to the marked deficiencies and extreme fluctuations discovered by Leuchtenberger and collaborators. It must be kept in mind, however, that we dealt with bulls of unknown fertility, while the Leuchtenberger research was concerned with men and bulls of known fertility or sterility.

Part B

During the second year a somewhat greater number of bulls was tested, but, in general, fewer tests were given each bull. This work represents an advance over that of the first year in two major respects: All work was carried out under a uniform control system with a calculated experimental error; and DNA means deviating more than about seven per cent were checked by an additional set of measurements made on a duplicate slide. During the first part of the work, the number of sperm analyzed was, with a few exceptions, between 20 and 30, usually 27. In the second part, a minimum of 18 was found sufficient to restrict the standard error to 0.03 except in a few samples, in which a greater number of sperm was measured. The basic idea was that the number of sperm measured should be adjusted to the variation among individual sperm in a particular sample, so as to keep the standard error, and so the sampling error, at or below a maximum level. The fiduciary mean in this part of the work was arrived at by combining the samples of the first and second years' work and calculating the over-all mean. This turned out to be 1.52 plus or minus 0.008, identical with

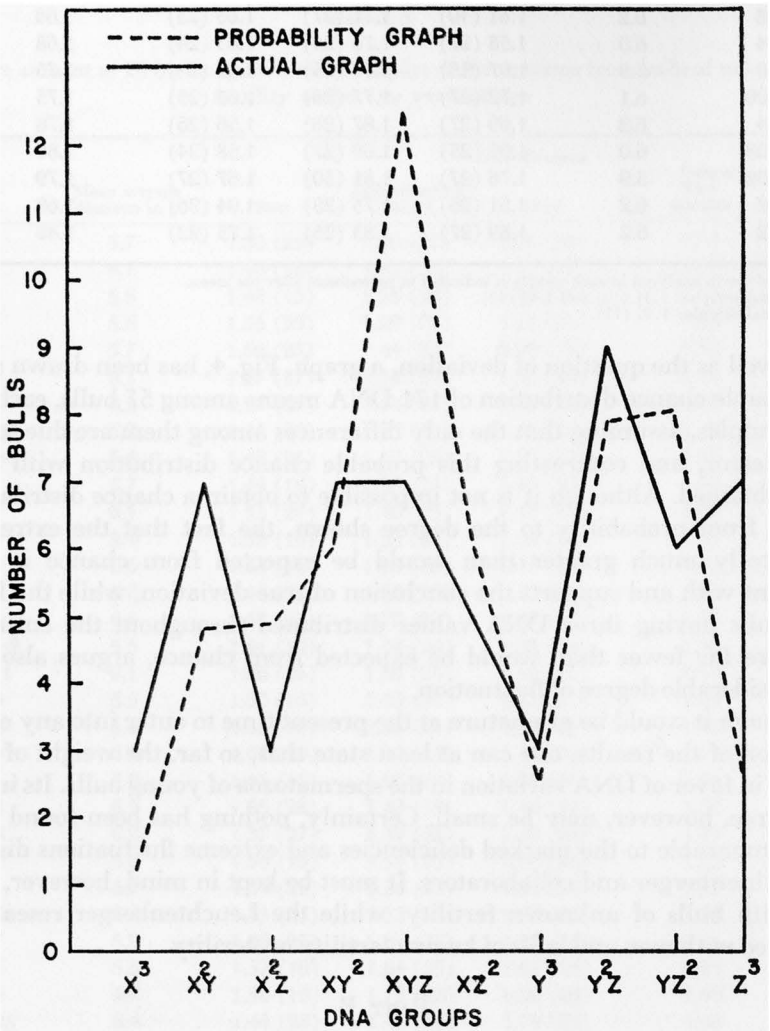


FIG. 4. Actual versus probable chance distribution of three DNA sample means from each of 57 bulls of unknown fertility under two years of age. $X < 1.45$; $Y = 1.45 - 1.59$; $Z > 1.59$.

the Leuchtenberger mean, although the sampling error is considerably less. In making adjustments for experimental variation in staining, the control smear on each slide was corrected to this over-all mean of 1.52, in contrast to the method used in the last one-third of the first year's work when the control smear was corrected to the value obtained on the slide originally stained on receipt of the samples. In theory, correction to an over-all mean is more accurate, since the experimental error involved is the sampling error of the sample plus that of the control, which is least when an over-all mean is used as the control mean. In practice, this advantage is offset by the fact that the sample chosen to be retained for control use in the next batch is one that gives a value close to the normal mean and also by the fact that a sample chosen for control might have some slight variation in DNA, which would not be taken into consideration when its

control value is corrected to an over-all mean. At any rate, the experimental error of correcting to an over-all mean cannot be any greater and is possibly less than correction to the previous mean. Accordingly, corrections to an over-all mean were used in drawing conclusions as to DNA deviation in the second part of the work.

Results

These are presented graphically in Figs. 5 and 6 and detailed in Tables 4 through 7. Table 7 lists the results of tests given a group of bulls of unknown fertility between two and three years of age, which are not included in the figures in order to preserve the consistency of approach to the problem by concentrating on comparatively young bulls of unknown fertility under two years of age. In Part B, because of the measurements on duplicate slides, there are two ways of assessing deviation in excess of experimental error: by the experimental error of the over-all mean, taking into consideration its reduction from that of a single mean; and by an increase in the level of confidence when the mean on the duplicate slide deviates from the fiduciary mean by a percentage equal to that of the original mean. We can estimate that, if the experimental error of a

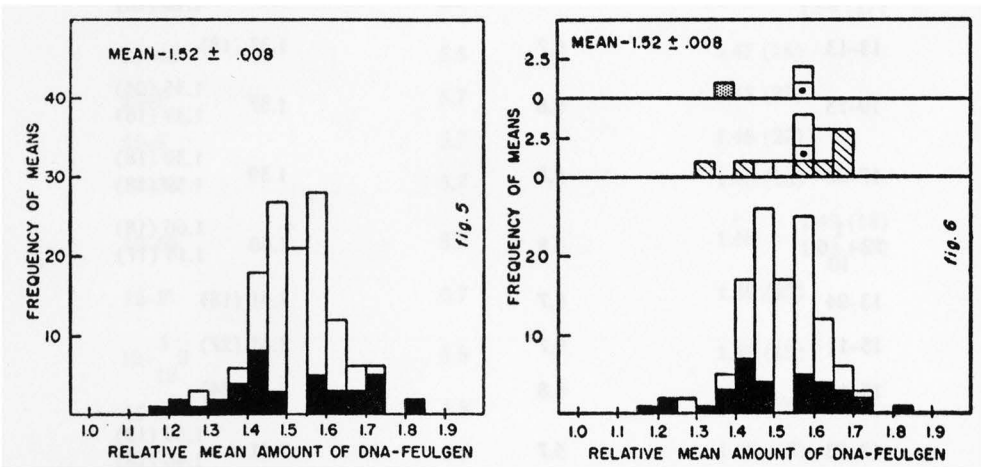


Fig. 5. Relative mean amount of DNA-Feulgen of 135 samples from 118 bulls of unknown fertility under two years of age. The shaded area represents those samples for which an over-all mean has been calculated from two means derived from measurements of duplicate slides made from the same sample.

Fig. 6. Relative mean amount of DNA-Feulgen in classifications as follows: lower—one sample each from 118 bulls; middle—two samples each from 14 bulls; upper—three samples each from three bulls. Duplicate slide determinations are indicated as follows:

Shaded squares: One sample plus one duplicate slide determination.

Squares containing diagonal lines: Two samples plus one duplicate slide determination.

Squares containing a central dot: Two samples plus two duplicate slide determinations, one for each sample.

Stippled squares: Three samples plus two duplicate slide determinations, one each for two samples.

Duplicate slide means are given equal weight with sample means in caluculation of the over-all mean for the bulls involved. The fiduciary mean of 1.52 ± 0.008 is calculated from all samples of both Group A and Group B.

TABLE 4

Relative amount of DNA-Feulgen in one sample of spermatozoa from bulls of unknown fertility under two years of age

Number of bull	Mean average diameter in μ	Mean	
12–177	5.5	1.18	1.11 (19) 1.25 (18)
12–$\frac{K}{4}$8	5.8	1.22	1.13 (26) 1.31 (17)
12–137	5.7	1.22	1.06 (28) 1.39 (16)
15–3	5.9	1.29 (70)	
15–4	5.8	1.29 (27)	
12–P_1	6.0	1.31	1.39 (25) 1.23 (18)
15–1	5.9	1.35 (18)	
17–40	5.6	1.35	1.24 (17) 1.46 (18)
13–13	5.7	1.37 (18)	
19–73	5.8	1.37	1.35 (24) 1.39 (18)
17–38	5.8	1.39	1.39 (18) 1.39 (18)
12–$\frac{1}{10}$6	5.8	1.40	1.66 (18) 1.15 (17)
13–24	5.7	1.41 (18)	
15–11	5.7	1.41 (27)	
15–13	5.8	1.41 (24)	
17–32	5.7	1.41	1.36 (18) 1.46 (18)
17–44	5.7	1.41	1.38 (18) 1.44 (17)
13–11	5.9	1.42 (18)	
13–23	6.0	1.42 (18)	
18–56	5.9	1.42	1.26 (27) 1.59 (17)
19–68A	5.7	1.42	1.43 (18) 1.42 (18)
15–2	5.6	1.43 (26)	
19–66	5.7	1.43	1.41 (18) 1.45 (18)

15–7	5.6	1.44 (17)
18–60	5.9	1.44 1.30 (18) 1.58 (18)
12–175	5.6	1.46 1.29 (18) 1.75 (16) 1.33 (19)
16–18	5.6	1.46 (18)
18–46	6.0	1.46 (18)
18–50	5.8	1.46 (18)
18–57	5.8	1.46 1.62 (18) 1.29 (18)
13–15	5.9	1.47 (18)
13–18	5.6	1.47 (18)
13–27	5.6	1.47 (21)
15–10	5.6	1.47 (26)
17–42	5.7	1.47 1.40 (18) 1.54 (17)
18–48	5.8	1.47 (18)
12–P	5.7	1.48 (27)
15–5	5.7	1.48 (27)
17–36	5.7	1.48 (18)
19–65	5.7	1.48 1.42 (18) 1.53 (17)
12–F	5.7	1.49 (25)
$12-\frac{1}{12}2$	5.8	1.49 (18)
15–9	5.8	1.49 (18)
15–15	5.8	1.49 (18)
16–17	5.7	1.49 (18)
17–39	5.5	1.49 (18)
17–43	5.8	1.49 (17)
18–47	5.9	1.49 (18)
18–55	5.9	1.49 (18)
12–174	5.6	1.50 (27)
13–(10–6)	5.7	1.50 (18)
13–19	5.6	1.50 (18)
15–8	5.9	1.50 (23)
17–45	5.7	1.50 (18)

TABLE 4—(Continued)

Relative amount of DNA-Feulgen in one sample of spermatozoa from bulls of unknown fertility under two years of age.

Number of bull	Mean average diameter in μ	Mean
18–59	5.8	1.52 (18)
19–74	5.8	1.52 (18)
16–27	5.9	1.53 (18)
16–30	5.9	1.53 (27)
19–62	5.7	1.53 (18)
19–64	5.6	1.53 (18)
13–2	5.7	1.54 (18)
13–22	5.9	1.54 (26)
16–19	5.8	1.54 (18)
16–20	5.7	1.54 (18)
12–X	5.6	1.55 1.40 (26) 1.70 (18)
12–168	5.8	1.55 1.74 (18) 1.36 (25)
16–29A	6.0	1.55 1.65 (18) 1.45 (23)
16–21	6.0	1.55 (18)
13–1	5.6	1.56 (18)
13–6	5.8	1.56 (17)
13–17	5.7	1.56 (36)
16–25	5.8	1.56 (18)
17–35	5.8	1.56 (18)
17–37	5.7	1.56 (17)
18–52	5.6	1.56 (18)
18–54	5.7	1.56 (18)
12–B1	5.9	1.57 (36)
$12-\frac{R}{1}3$	5.8	1.57 (16)
16–24	5.9	1.58 1.70 (18) 1.47 (18)
18–58	5.8	1.58 1.68 (27) 1.48 (18)
19–68B	5.9	1.58 (18)
$12-\frac{W}{1}14$	5.6	1.59 (26)

16–29B	5.8	1.59 (16)	
17–31	5.7	1.59 (18)	
17–33	5.7	1.59 (18)	
18–51	5.6	1.59 (18)	
19–70	5.8	1.59 (18)	
19–71	5.6	1.59 (27)	
12–J	5.7	1.60 (24)	
19–63	5.6	1.60 (18)	
19–72	5.8	1.60 (18)	
12–165	5.8	1.61 (26)	
19–69	5.9	1.61 (18)	
12–82	5.9	1.62 (27)	
12–166	5.8	1.62	1.65 (18) 1.58 (18)
13–28	5.9	1.62 (18)	
16–23	5.7	1.62	1.62 (18) 1.63 (18)
16–26	5.7	1.62	1.64 (18) 1.61 (18)
13–14	5.7	1.65 (18)	
18–49	5.8	1.65	1.72 (18) 1.58 (18)
13–3	5.6	1.66 (20)	
16–22	5.8	1.66	1.65 (18) 1.67 (18)
13–5	5.6	1.68	1.65 (17) 1.72 (18)
18–53	5.8	1.71	1.80 (18) 1.62 (18)

Number of sperm analyzed in each sample is indicated in parentheses after the mean.
When determinations are made on more than one slide, they are listed separately to the right of the mean.

single mean has a maximum of 15%, as has been calculated, the experimental error of the over-all mean of two means will be limited to about 12%, and to about 10% when the over-all mean is derived from three separate means. And if the odds are that in one of three samples the experimental error will exceed 7%, as has been calculated, the odds should be that in only one of nine samples will the experimental error exceed 7% in each of two means, each derived from measurements on a separate slide, as explained in Article II.

Applying these criteria first to all samples, as represented in Fig. 5, without regard for the particular bull to which each pertains, we note from the graph and

TABLE 5

Relative amount of DNA-Feulgen in two samples of spermatozoa from bulls of unknown fertility under two years of age

Number of bull	Mean average diameter in μ	DNA-Feulgen Sample means First	Second	Over-all mean
13–8	5.6	1.51 (30)	1.40 (18)	1.42
			1.36 (18)	
13–16	5.8	1.43 (17)	1.51 (25)	1.47
12–171	5.8	1.86 (18)	1.37 (17)	1.56
		1.77 (17)	1.22 (23)	
13–25	5.6	1.54 (18)	1.58 (26)	1.56
13–7	5.8	1.63 (18)	1.54 (32)	1.58
12–169	5.8	1.68 (24)	1.56 (27)	1.59
		1.54 (18)		
13–10	5.9	1.70 (18)	1.54 (18)	1.62
13–9	5.8	1.69 (26)	1.58 (18)	1.64
12–173	5.6	1.49 (16)	1.73 (22)	1.65
			1.74 (18)	
12–167	5.9	1.71 (17)	1.61 (16)	1.67
		1.70 (18)		
12–164	5.7	1.44 (18)	1.83 (30)	1.69
			1.81 (18)	

Number of sperm analyzed in each sample is indicated in parentheses after the mean.
Duplicate slide determinations of the same sample are listed below the original. In the calculation of the over-all mean, equal weight is given to original sample and duplicate slide determinations.

TABLE 6

Relative amount of DNA-Feulgen in three samples of spermatozoa from bulls of unknown fertility under two years of age

No. of bull	Mean average diameter in μ	DNA-Feulgen Sample means First	Second	Third	Over-all means Two samples	Three samples
12–176	5.6	1.46 (18)	1.23 (17)	1.24 (27)	1.33	1.37
			1.29 (16)	1.62 (18)		
13–20	5.3	1.58 (18)	1.48 (18)	1.51 (18)	1.53	1.52
13–21	5.6	1.43 (18)	1.70 (18)	1.46 (27)	1.62	1.58
			1.73 (18)			

Number of sperm analyzed in each sample is indicated in parenthesis after the mean.
Duplicate slide determinations of the same sample are listed below the original. In the calculation of the over-all mean, equal weight is given to original sample and duplicate slide determinations.

Tables 4 through 6 that, out of a total of 135 samples, twelve over-all means, each derived from two separate means, deviate by more than 12% from the fiduciary mean of 1.52, six of these below and six above. This would be approximately a 10% deviation in excess of experimental error of the total number of samples involved. This is to be considered a minimum, however, as some of the samples with original normal means, for which duplicate slide determinations were not made, might have yielded a second mean low or high enough to have produced an over-all mean in excess of the 12% deviation. Of the total number of 135 samples, 48 deviated from the fiduciary mean of 1.52 by more than 7%, allowing 0.01 for

TABLE 7

Relative amount of DNA-Feulgen in samples of spermatozoa from bulls of unknown fertility between two and three years of age

No. of bull	Mean average diameter in μ	Mean	
14–20	5.4	1.28	1.41 (26) 1.15 (18)
14–6	5.8	1.44	1.39 (27) 1.49 (18)
14–8*	5.8	1.48 (16)	
14–10†	5.8	1.49 (25)	
14–14‡	5.7	1.51 (18)	
14–9	5.9	1.52 (26)	
14–12	5.7	1.52 (26)	
14–16	5.9	1.52 (18)	
14–5	5.7	1.53 (18)	
14–7	5.6	1.53 (18)	
14–1	6.0	1.54 (35)	
14–4	5.8	1.54 (18)	
14–13	5.6	1.55 (18)	
14–3	5.9	1.56 (27)	
14–19	5.6	1.61 (26)	
14–15	5.6	1.74	1.75 (18) 1.58 (17) 1.88 (18)
14–2	5.6	1.78	1.68 (18) 1.88 (18)

Number of sperm analyzed in each sample is indicated in parentheses after the mean.
When determinations are made on more than one slide, they are listed separately to the right of the mean.
* Second sample: 5.9–1.56 (18).
† Second sample: 5.9–1.43 (26).
‡ Second sample: 5.8–1.50 (27).

sampling error, 26 below and 22 above. Of these 48, 12 were not checked by duplicate slide measurements, either because satisfactory preparations were not available or because they belonged to a group that was delayed in transit for several days with resultant depressed values. Duplicate slide determinations on a few samples deviating by less than 7% are not of sufficient relevance to justify the complication of including them in the final conclusions. One may consider, then, that one has to deal with 36 samples, 19 below and 17 above, of a total of 101 (subtracting from 135 not only the twelve in excess of 7% omitted but also their proportionate normal means at the ratio of 48 to 135) that deviate from the mean by more than 7% and that 36/101 × 36/101 should give the proportion of samples of the 101 that should yield a second mean, on a duplicate slide, still deviating by more than 7%, assuming that experimental error is responsible for all the original deviating means. This would be about one-eighth, or approximately 13 samples. Actually, 16 samples, 9 above and 7 below, gave a second mean deviating by more than 7%. Thus, 3 samples, or about 3% of the total of 101 samples on which the calculations are based, appear to deviate from the fiduciary mean by more than would be expected from experimental error.

Applying the same criteria but to individual bulls in groups as represented in Fig. 6,* we note from Table 4 that five bulls have an over-all mean derived from two means that is outside the 1.34–1.70 limits set by a 12% experimental error, 4 below and one above. This would be about 5% of the total number of 104 bulls involved, but, in the same manner as explained above, this must be considered a minimum. Thirty-five deviate in excess of the 7% error ± 0.01, 22 below and 13 above, of which 26 were checked by duplicate slide determinations. In accordance with procedure similar to that given above, the percentage of second deviants in excess of 7% to be expected, assuming experimental error is responsible for all the first deviations, would be $26/77 \times 26/77$, which is approximately equal to one-ninth. One would then expect about nine second deviants. Actually, there are only eight, 6 below and 2 above, so that in this particular group, this approach does not separate any true deviants, although, as explained above, it is a statistical concept and would not necessarily conflict with the minimum 5% deviation arrived at by the first method.

In the group of animals having two samples, listed in Table 5, together with a duplicate slide determination in most instances, there is only one bull, on the high side, that deviates beyond the maximum 10% to be expected from the over-all mean of three determinations, but, since there are only eleven animals involved, this would be about 10%. Ten samples exceeded the 7% error, 2 below and 8 above, of which seven received duplicate slide determinations. Six of these seven had a second deviant in excess of the 7% error, although the number expected would be, in the manner calculated above, $7/16 \times 7/16 \times 16$, which is approximately equal to three. Thus, there are in this group three more than would be expected from experimental error, about 20% of the total of 16, on which the calculations are based. There is no marked incompatibility between the first and second samples in this group except in one bull, 12–171. This could be a fluctuation, or, conceivably, since it is so isolated, it could be one of the rare examples of experimental error beyond even a high level of confidence. The results of a separate breakdown of the bulls according to groups suggests the possibility that a sampling error is involved, which is less a factor when the analysis is made of all samples combined.

This would be even more true of the third group, listed in Table 6, which had three samples each. Of the three bulls in this group, only one is particularly noteworthy. This one, after three samples, including two duplicate slide determinations, yielded an over-all mean of 1.37, still 10% below the fiduciary mean of 1.52.

Results for the bulls between two and three years of age are given in Table 7 for information but are not included in the preceding analysis and are not given a separate breakdown. In general, they resemble results obtained from the younger bulls.

The most reliable figures would seem to be those derived from all the Group B bulls, which, it will be remembered, gave 10% variation for the over-all means and 3% variation by the method of checking variation on a duplicate slide. These

*Fig. 6, starting from the bottom, first plots the individual bulls on a basis of one sample or the first sample of those having more than one, then on a basis of two samples or the first two of those having three, and finally on a basis of three samples for each bull.

are not necessarily in conflict. There are a number of borderline cases and, more important, a normal value on a duplicate slide does not necessarily exclude variation in DNA but simply limits its extent, according to the experimental error in a single determination. The 10% figure would then include all those with any DNA variation, while the 3% figure would include only those varying to such an extent that maximum experimental error on the plus side would not be sufficient to bring them into the normal range. In general, the results confirm those of Part A, although the percentage of variation is a little less. There continue to be DNA means that exceed the limits of a carefully calculated experimental error.

PART C

The present study is concerned particularly with the possibility of DNA fluctuation over a comparatively long period of time, but which might escape detection by the limited number of tests it has been found practical to give most of the bulls examined during the course of the work. Involved also is the question as to whether there was a possible seasonal fluctuation in the absolute amount of DNA per nucleus. It is conceivable that such a fluctuation could be caused by one or several factors, such as seasonal temperature variation, diet, and breeding activity. If such a variation exists, a correction of data used in a sterility study would be necessary.

Methods and Materials

Twenty-one Santa Gertrudis bulls of the King Ranch under two years of age formed the experimental group of animals. Personnel of the ranch secured, at their convenience, semen samples by electro-ejaculation. Consequently, it was impossible to maintain a specific interval of time between sampling. There is no evidence to indicate, however, that irregularity of sampling is undesirable.

General appearance of the samples—color and quantity of seminal fluid and motility of the sperm—was checked and recorded at the ranch. The collected samples were then shipped via air freight to this laboratory where they were immediately examined for motility which was usually lowered, morphology and sperm count. The methods used for these checks are in general agreement with those used by Leuchtenberger (*6*), except that during the latter phase of the work, phase contrast microscopy was used for morphology and motility examinations. Morphological abnormalities were of various types including the following: sperm without tails, relatively short sperm, relatively narrow sperm, relatively broad sperm, irregularly shaped sperm, tail abnormalities, and incomplete migration of the cytoplasmic droplet.

The method employed was substantially that described in the preceding article, with additions and modifications as noted below. For the most part smears of the samples were made the day following their arrival at this laboratory. In a few cases the seminal fluid was stored at 5° Centigrade before preparation. Under no conditions were the samples retained longer than three days.

Staining variation in the samples received during November and December, 1956, was controlled by correcting all values of samples stained in the same batch by the percentage necessary to bring the majority of them within the previously

determined normal limits on the assumption that the majority, at the least, would be normal in DNA in the manner of a "grade curve." This could conceivably lead to a greater error than that obtained under the more precise methods of control described below; whether it did or not will be considered in the discussion. Subsequently, the method of control was as follows. A slide was generally divided into four rectangles, three of these being filled by smears from "unknown" samples obtained in the most recent shipment of semen. The fourth rectangle contained a "control" smear—a sample from a previous shipment for which the DNA content had already been determined and which had been retained at the temperature of dry ice for the intervening period. The per cent deviation between the mean of the control smear and its previously determined value was then used to correct the "unknowns" on the same slide with the control smear. Slides were stained in triplicate to allow additional determinations on duplicate slides in doubtful cases. See Article II for extended explanation and discussion of this technique.

The last two shipments were controlled by using the identical "control smear" method on the same slide; but the control mean was corrected to 1.53* rather than its previously determined value, and the means for the three other samples on the slide were corrected by the same percentage.

In two cases the control showed lowered DNA values possibly because they had been stored in a freezer without dry ice. Consequently, in these groups the mean DNA values of the three samples on a slide other than the control were averaged, and this average was corrected to 1.53. The percentage required for this correction was then applied to the means of each of the three samples, resulting in the final DNA values.

The amount of DNA in the individual spermatozoa was determined microspectrophotometrically using equipment described by Kasten (*5*) and Welch (*31*). A minimum of eighteen morphologically normal sperm was measured from each sample over a wide area of the smear and a mean DNA value was computed. In some instances, only 16 or 17 values were used to calculate a mean for any one sample, in accordance with a technique of eliminating occasional aberrant values rationalized in the preceding method paper. In other instances additional measurements were required in order to validate a mean deviating from the normal value of 1.53 by an amount greater than 10 per cent, when the standard error of only eighteen measurements was large enough to bring a 10 per cent deviation within the range of the sampling error. Limits of error are discussed in Article II.

Results

Fig. 7 represents the DNA means calculated for all the samples obtained during the one year period; these are arranged to show the values for each bull individually. Bull U6 died after this laboratory had received only two semen samples. Several samples from other bulls were termed "blanks" when sperm present in the seminal fluid were two few to analyze. For example, bull 19

* "Normal" DNA values as determined by Leuchtenberger and co-workers (*9*) and from 246 samples analyzed in this laboratory are 1.52 and 1.53 respectively. The value of 1.53 will be used since it is based on many more measurements.

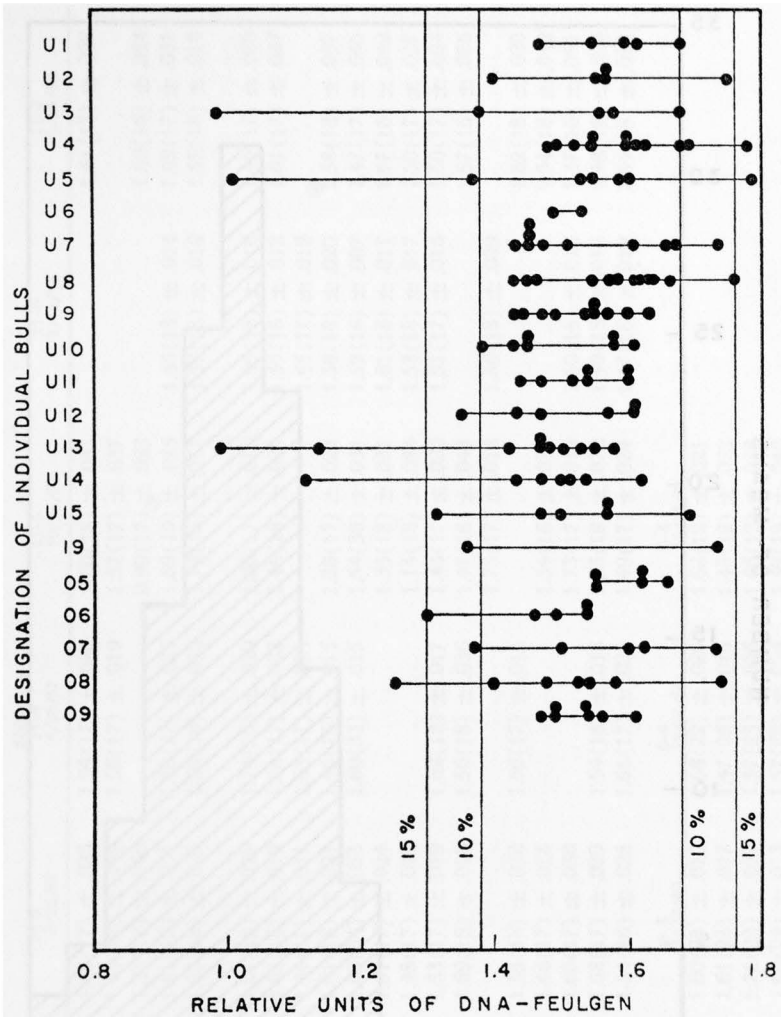


FIG. 7. Mean DNA values for each bull. Each circle represents a mean. Horizontal lines demonstrate the range of DNA values obtained for any particular animal. Vertical lines represent 10 and 15 per cent deviation from the normal value of 1.53.

produced only two samples capable of being analyzed. Table 8 lists the dates on which the samples were received which in all cases were the dates that the samples were taken at the King Ranch. Table 8 also shows the DNA means listed according to these shipments. Animals U4, U7, U8, U9 and U13 each had five additional samples taken several months prior to the beginning of this specific test. These samples were taken at one-week intervals and were analyzed as a part of another study. Since only five of the twenty-one bulls studied have these five extra samples, groups 6-1 through 6-5 have not been considered in the general statistical studies, although the data will be listed.

Fig. 8 is a histogram of all mean DNA values obtained during the one year period with a peak between 1.50 and 1.55. The curve is slightly skewed toward

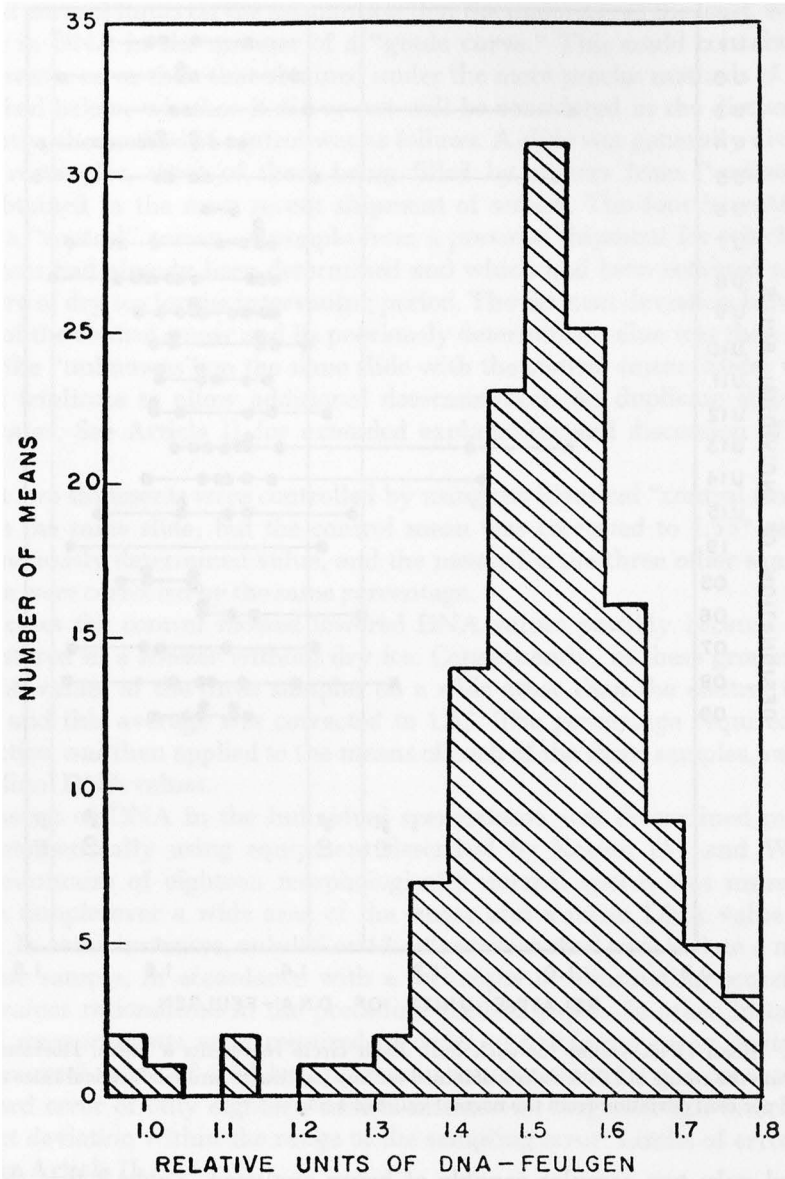


FIG. 8. All mean DNA values obtained in this study are represented in this figure. The histogram has a peak between 1.50 and 1.55 and is slightly skewed toward the low DNA side.

the low DNA side, similar to those published by Leuchtenberger and co-workers (*7*, *8*).

For each mean, a standard error has been calculated. The results are listed in Table 8. They range from 0.007 to 0.084. However, of the 143 means in the final data, only a few standard errors exceed 0.030.

Table 9 shows the mean size of the sperm in microns computed by averaging the long and short diameters of the sperm head. The standard error of each mean

TABLE 8

Mean DNA values

Bull no.	Shipments 11–1 5/5/57	11–2 6/19/57	11–3 7/22/57	11–4 8/20/57	11–5 10/28/57	11–6 2/11/58	11–7 5/14/58
U1	1.47(18) ± .033*	.....†	1.68(17) ± .023	1.55(17) ± .020	1.60(18) ± .041	.....	1.61(18) ± .022
U2	1.57(18) ± .023	1.40(16) ± .025	1.75(18) ± .049	1.56(17) ± .019	1.57(17) ± .037	.....	.....
U3	1.58(16) ± .017	1.68(15) ± .029	1.38(17) ± .040	.....	0.99(17) ± .023	.....	1.56(16) ± .024
U4	1.55(17) ± .014	1.52(18) ± .037	1.61(18) ± .031	1.78(27) ± .025	1.60(19) ± .023	1.55(18) ± .014	1.69(17) ± .031
U5	1.01(26) ± .022	1.60(17) ± .029	1.53(18) ± .025	1.59(18) ± .030	1.79(16) ± .017	1.37(18) ± .016	1.55(18) ± .018
U6	1.53(18) ± .020	1.49(18) ± .013	.....	.....	.....	.....	.....
U7	1.61(27) ± .014	.....	1.67(18) ± .028	1.74(25) ± .020	1.66(17) ± .019	1.45(18) ± .010	1.45(17) ± .026
U8	1.62(18) ± .020	1.64(18) ± .028	1.63(16) ± .019	1.58(17) ± .023	1.45(18) ± .024	1.55(18) ± .012	1.61(17) ± .027
U9	1.43(26) ± .025	1.47(17) ± .025	1.44(17) ± .031	1.63(17) ± .024	.....	1.55(17) ± .012	.....
U10	1.45(18) ± .028	1.43(17) ± .032	1.61(18) ± .021	1.45(18) ± .015	1.38(17) ± .028	1.58(18) ± .020	1.58(18) ± .037
U11	1.44(27) ± .018	1.54(17) ± .040	1.60(34) ± .033	1.60(33) ± .015	1.54(36) ± .031	1.52(18) ± .007	1.47(17) ± .045
U12	1.47(18) ± .017	1.43(17) ± .026	1.61(17) ± .024	.....	1.35(18) ± .031	1.61(18) ± .011	1.57(16) ± .042
U13	1.55(26) ± .016	1.05(53) ± .025	1.48(17) ± .013	.....	1.14(18) ± .084	1.53(18) ± .017	1.50(17) ± .027
U14	1.12(25) ± .038	1.47(18) ± .029	1.53(17) ± .019	1.62(18) ± .017	1.43(17) ± .026	1.51(17) ± .016	1.50(17) ± .024
U15	1.31(26) ± .024	1.57(17) ± .025	1.69(18) ± .016	1.50(18) ± .036	1.47(18) ± .043	.....	1.57(16) ± .026
19	.....	.....	.....	.....	1.73(17) ± .018	1.36(18) ± .009	.....
O5	1.55(24) ± .023	1.62(17) ± .031	1.55(18) ± .032	1.66(17) ± .015	.....	.....	1.62(18) ± .020
O6	1.49(25) ± .018	1.30(25) ± .017	1.46(17) ± .028	.....	1.54(16) ± .030	.....	1.54(18) ± .019
O7	.....	1.62(18) ± .029	1.60(17) ± .030	.....	1.73(17) ± .016	1.50(18) ± .016	1.37(16) ± .051
O8	1.53(16) ± .025	1.74(26) ± .015	1.58(17) ± .020	1.54(18) ± .018	1.25(18) ± .072	1.40(18) ± .018	1.48(16) ± .020
O9	1.56(16) ± .020	1.49(16) ± .037	1.54(16) ± .028	1.61(17) ± .025	1.49(17) ± .024	1.47(18) ± .019	1.54(18) ± .028
	6–1 11/19/56	6–2 11/27/56	6–3 12/3/56	6–4 12/10/56	6–5 12/17/56		
U4	1.48(34) ± .029	1.49(22) ± .024	1.60(23) ± .019	1.68(22) ± .025	1.62(12) ± .021		
U7	1.45(19) ± .033	1.51(24) ± .021	1.61(23) ± .017	1.47(25) ± .015	1.43(12) ± .022		
U8	1.46(23) ± .016	1.43(24) ± .019	1.76(22) ± .020	1.57(23) ± .026	1.66(12) ± .032		
U9	1.54(25) ± .021	1.55(25) ± .018	1.60(24) ± .013	1.57(22) ± .024	1.49(12) ± .048		
U13	1.42(10) ± .025	1.47(24) ± .021	.....	1.47(23) ± .020	1.58(12) ± .020		

* Numbers in parentheses represent the number of sperm analyzed per sample.
† Samples with too few sperm to be analyzed.

TABLE 9

Mean size values in microns

Bull no.	Shipments 11–1 5/5/57	11–2 6/19/57	11–3 7/22/57	11–4 8/20/57	11–5 10/28/57	11–6 2/11/58	11–7 5/14/58
U1	5.8(18) ± .047*	.....†	5.9(17) ± .029	5.8(17) ± .049	5.8(18) ± .042	.....	5.8(18) ± .045
U2	5.8(18) ± .035	5.6(16) ± .073	5.6(18) ± .045	5.6(17) ± .017	5.8(17) ± .034	.....	.....
U3	5.6(16) ± .030	5.6(15) ± .058	5.7(17) ± .049	.....	5.7(17) ± .049	.....	5.6(16) ± .030
U4	5.7(17) ± .038	5.8(18) ± .033	5.6(18) ± .042	5.6(27) ± .029	5.8(19) ± .037	5.5(18) ± .042	5.8(17) ± .036
U5	5.8(26) ± .037	5.5(17) ± .036	5.6(18) ± .045	5.7(18) ± .026	5.8(16) ± .040	5.5(18) ± .035	5.6(18) ± .026
U6	5.6(18) ± .038	5.6(18) ± .038	.....	.....	.....	.....	.....
U7	5.6(27) ± .038	.....	5.8(18) ± .052	5.7(25) ± .034	5.6(17) ± .044	5.6(18) ± .035	5.9(17) ± .034
U8	5.6(18) ± .033	5.5(18) ± .028	5.7(16) ± .040	5.7(17) ± .029	5.6(18) ± .038	5.5(18) ± .031	5.8(17) ± .029
U9	5.8(26) ± .039	5.9(17) ± .034	5.9(17) ± .051	5.9(17) ± .051	.....	5.6(17) ± .022	.....
U10	5.7(18) ± .038	5.9(17) ± .032	5.9(18) ± .026	5.8(18) ± .016	5.9(17) ± .044	5.7(18) ± .033	5.8(18) ± .038
U11	5.7(27) ± .033	5.7(17) ± .036	5.7(34) ± .043	5.7(33) ± .023	5.8(36) ± .032	5.8(18) ± .047	5.8(17) ± .046
U12	5.6(18) ± .021	5.5(17) ± .039	5.6(17) ± .041	.....	5.7(18) ± .027	5.6(18) ± .039	5.8(16) ± .025
U13	5.4(26) ± .024	5.5(53) ± .026	5.6(17) ± .027	.....	5.8(18) ± .028	5.6(18) ± .035	5.8(17) ± .065
U14	5.8(25) ± .038	5.5(18) ± .026	5.7(17) ± .032	5.9(18) ± .057	5.8(17) ± .051	5.6(17) ± .046	5.8(17) ± .041
U15	5.7(26) ± .041	5.6(17) ± .044	5.8(18) ± .021	5.7(18) ± .031	5.7(18) ± .031	.....	5.6(16) ± .028
19	.....	.....	.....	.....	5.5(17) ± .029	5.5(18) ± .031	.....
O5	5.8(24) ± .037	5.6(17) ± .040	5.6(18) ± .016	5.7(17) ± .027	.....	.....	5.6(18) ± .031
O6	5.8(25) ± .038	5.6(25) ± .030	5.7(17) ± .017	.....	5.7(16) ± .025	.....	5.5(18) ± .071
O7	.....	6.0(18) ± .040	5.6(17) ± .029	.....	5.9(17) ± .040	5.7(18) ± .026	6.0(16) ± .028
O8	5.6(16) ± .030	5.5(26) ± .027	5.6(17) ± .032	5.5(18) ± .021	5.7(18) ± .026	5.5(18) ± .026	5.5(16) ± .028
O9	5.6(16) ± .040	5.4(16) ± .048	5.7(16) ± .043	5.8(17) ± .029	5.8(17) ± .039	5.7(18) ± .038	5.8(18) ± .026

	6–1 11/19/56	6–2 11/27/56	6–3 12/3/56	6–4 12/10/56	6–5 12/17/56
U4	5.5(34) ± .033	5.5(22) ± .030	5.5(23) ± .027	5.7(22) ± .053	5.6(12) ± .046
U7	5.4(19) ± .023	5.4(24) ± .022	5.6(23) ± .046	6.0(25) ± .042	5.6(12) ± .038
U8	5.4(23) ± .021	5.4(24) ± .027	5.5(22) ± .047	5.7(23) ± .052	5.5(12) ± .061
U9	5.6(25) ± .027	5.6(25) ± .032	5.7(24) ± .041	5.9(22) ± .075	6.4(12) ± .078
U13	5.4(10) ± .044	5.4(24) ± .024	.....	5.8(23) ± .067	5.8(12) ± .081

* Numbers in parentheses represent the number of sperm analyzed per sample.
† Samples with too few sperm to be analyzed.

is also shown in this table. Only in a few cases does the standard error of a mean exceed 0.050.

Discussion

As established in the method paper, a reasonable estimate of the maximum experimental error is 15% and 10% in 80% of samples, with some reduction when additional measurements are made on duplicate slides. A possible exception to these limits of experimental error might be some of the samples received in November and December, 1956, and analyzed under a different control system. However, it can be seen in Table 8 that only one of the 24 means obtained from these groups falls outside the 10% confidence limits. This is actually less than would be expected, merely from the staining error. Consequently, from these data, one is not justified in considering this control system inadequate.

A second exception might be present in the samples controlled by averaging the three unknown values, as described above. This process was necessitated by the fact that the control values were much lowered and if they had been used, all DNA values for the unknowns would have been considerably above the normal. Both shipments 11–4 and 11–5 were controlled in this manner. By referring to Table 8 it can be seen that almost 50% of the means which are outside the 10% confidence limits fall within these two groups. This fact is merely a warning that any observed seasonal variation should not be based entirely on results from groups 11–4 and/or 11–5.

The correction of the control to 1.53 rather than to its previously determined value was an improvement on the entire project. Formerly, when the control was corrected to its previously determined value, all unknown values may have been either high or low, depending on the staining error, sampling error, and/or true DNA fluctuation of the control, both in its original determination and when it was used as the control. However, these elevations or depressions would have been limited by the fact that the value to which the control smear was referred was chosen in close proximity to 1.53. It is also true that the advantage of correcting to 1.53 instead of the previously determined value is somewhat offset by the fact that, when the experimental error of the control smear and that of the previously determined value are in the same direction they tend to cancel each other out. At any rate, conclusions are based not on the possible but uncertain greater precision of correction to 1.53, but on the experimental error limits previously given, which were calculated from an experiment in which sample means were corrected by the deviation of a control smear from its previous value.

Leuchtenberger *et al.*, have shown rather conclusive proof that many human males of suspected infertility have a lowered (*8*) or a fluctuating (*7*) amount of DNA in their sperm nuclei. However, she was working with magnitudes of DNA which might not be present in a seasonal fluctuation of the type described above. Consequently, if the confidence limits are arbitrarily set at 1.53 ± 10%, the variation being derived from a sampling error of at least five per cent and a staining error of about the same amount, no seasonal fluctuation above this 10 per cent can be discerned.

By comparing the mean DNA values for each sample with their respective standard errors, some interesting points arise (Table 8). At first glance, the

standard errors for shipment 11–5 seem to be especially large while those for shipment 11–6 seem to be exceedingly small. However, both of these anomalies can be explained: shipment 11–5 was controlled by a defective control sample, and the technique of averaging the three sample means was employed. Furthermore, at the time of shipment, new personnel were employed and the deviation between measurements recorded by the different individuals was greater. It has been the practice in this laboratory for each sample to be measured by at least two individuals with an over-all mean calculated from the combined data. This was done to offset any differences in the general measuring procedure which might result in different DNA values reported by different individuals. Those differences were extremely large when the new personnel were employed. After the new workers became more experienced, however, the differences became less and, consequently, the standard errors of the samples became smaller. In shipment 11–6, almost all difficulties due to deviations in the measurement of samples by the workers had been corrected. With these facts in mind, no conclusions should be drawn concerning these two shipments as representing seasonal variation of any kind.

As previously noted, about 20 per cent of the standard errors for DNA values exceed 0.030. If these standard errors are examined with their respective mean DNA values, it is seen that most standard errors of less than 0.020 have a corresponding DNA mean inside the 10 per cent normal range, although this is not true in reverse, for some normal DNA values have a standard error greater than 0.020.

Table 9 shows similar tabulations for the size data for individual sperm as measured in microns. Standard errors and means are shown according to individual bull and according to shipment. Statistical analyses show no significant seasonal variation of any kind.

The compiled data in Fig. 7 show two definite extremes. Bulls U9 and O9 have relatively constant values while animals U3 and U13 have widely varying values. Bulls U6 and 19 are omitted from the discussion since only two means for each were obtainable.

Only five of the bulls have DNA values which fall outside the 15 per cent level of confidence and only one of these five, U13, has two mean DNA values below 1.30. The fertility of these animals might be questioned. Subsequent study of these doubtful animals is presented in the following paragraphs.

Statistics, as given in Table 10, were compiled for the 21 bulls tested previously. On the basis of the standard errors and the coefficients of variation, both of which, of course, indicate the spread of values and therefore possible fluctuation, the following 9 bulls were selected for additional testing: U2, U3, U5, U13, U14, U15, O6, O7, and O8. Bull O5, which had a record of high constancy, was added to this group as a control. These ten bulls were given from one to three additional tests each at intervals of about two weeks. On the basis of these tests combined with the previous tests, five bulls, U2, U14, U15, O6, and the constant bull O5, were eliminated from further consideration and slaughtered to obtain testis material for comparison with testis material from bulls of known infertility, following the taking of one additional semen sample. The remaining five bulls, U3, U5, U13, O7, and O8 were given three additional tests each at intervals of

TABLE 10

Statistics for Group C bulls of unknown fertility originally under 2 years of age tested over a period of approximately 18 months

No. of bull	DNA-Feulgen			
	Mean	S.D.	C.V.	S.E.
U1	1.58	0.07	4.4%	0.03
U2	1.57	0.11	7.1	0.05
U3	1.44	0.24	16.9	0.11
U4	1.60	0.08	5.3	0.02
U5	1.49	0.23	15.3	0.09
U6	1.51	0.02	1.3	0.01
U7	1.55	0.11	6.8	0.03
U8	1.58	0.09	5.8	0.03
U9	1.53	0.06	4.2	0.02
U10	1.50	0.08	5.6	0.03
U11	1.53	0.06	3.6	0.02
U12	1.51	0.10	6.5	0.04
U13	1.42	0.17	11.9	0.05
U14	1.45	0.15	10.1	0.06
U15	1.52	0.12	7.6	0.05
O5	1.60	0.04	2.7	0.02
O6	1.47	0.09	6.0	0.04
O7	1.56	0.12	7.8	0.05
O8	1.50	0.14	9.2	0.05
O9	1.53	0.05	3.0	0.02
19	1.54	0.18	12.0	0.13

about one month, with the exception of bulls O7 and O8, which received only two additional tests. The bulls were not available for further testing or for testis analysis. Method of analysis was similar to that carried out on the Group B bulls, with additional determinations being made on duplicate slides in most doubtful cases. The results of these tests are presented in Fig. 9, together with previous means, original and additional tests being suitably designated. The five bulls showing the greatest evidence of fluctuation have been plotted as a group. For comparison the eleven bulls that did not receive additional tests are included. The five most constant of these have been plotted as a group.

ANALYSIS AND SUMMARY

Bull O5, the constant bull retained as a control, continued to give values well within the normal range, four additional tests making a total of nine. Of the nine bulls suspected of possible fluctuation, the five first eliminated gave all means of the follow-up tests within the normal range defined as the range exhibited by the five most constant of the 21 bulls, with the exception of O6, which gave one follow-up test outside the 15% deviation line, not confirmed, however, by the additional test on a duplicate slide. The evidence for variation in these bulls is so slight that they could be called essentially constant. The five bulls retained longest display no marked evidence of fluctuation, but they are not entirely satisfactory from the standpoint of constancy. There are two ways of assessing variation in this group. One may compute the over-all mean for all the samples analyzed and determine whether this deviates significantly from the

normal mean of 1.53 used here as a standard of comparison, considering the reduction of the maximum 15% experimental error brought about by multiple means. If it is assumed that deviation from the normal mean of 1.53 is caused entirely by experimental error, the deviation of the over-all mean of a number of individual means should decrease as the number of means increases; and the decrease should be proportional to the square root of the total number of means used in computing the over-all mean. Any deviation of the over-all mean in excess of the experimental error thus calculated would be attributed to true variation. By this criterion, only U13 has an over-all mean that might be considered suspect, as can be seen in Table 11, since 1.45 deviates from 1.53 by slightly more than the approximate 4% one might expect as the experimental error of 15 means, when 15% is the maximum error of a single mean. Even so, the margin of two points is too slight to be convincing in view of the many normal samples analyzed for this bull. In order, however, for this criterion to apply to all cases, it would obviously be necessary that the deviating means fall predominantly on the same side of the normal mean of 1.53; else, they would tend to cancel each other out. For U13, the deviating means are all on the lower side of 1.53, but this does not hold true in the case of the remaining four doubtful bulls plotted in Fig. 9, particularly U5 and O8. It is therefore necessary to consider the means individually.

If we first consider the individual means from the group standpoint, we find that there is a heavy concentration of the means deviating by more than 10% and 15% in the group of five bulls retained longest. Considering both original sample means and follow-up tests without a contradictory duplicate slide determination, one finds 7 out of 9 means deviating by more than 15% and 15 out of 28 means deviating by more than 10% concentrated in the group of five bulls considered most doubtful. In considering the individual means as a group from

TABLE 11

Statistics for 5 Group C bulls of unknown fertility originally under 2 years of age given additional tests after end of original period of approximately eighteen months

No. of bull	Number of samples	DNA-Feulgen			
		Over-all mean	S.D.	C.V.	S.E.
U3	10 (5)*	1.45	0.18	12.5%	0.06
U5	12 (5)*	1.52	0.19	12.3	0.05
U13	15 (5)*	1.45	0.15	10.4	0.04
O7	10 (5)*	1.52	0.14	9.4	0.05
O8	11 (4)*	1.49	0.16	10.4	0.05

* Number of additional tests in parentheses.

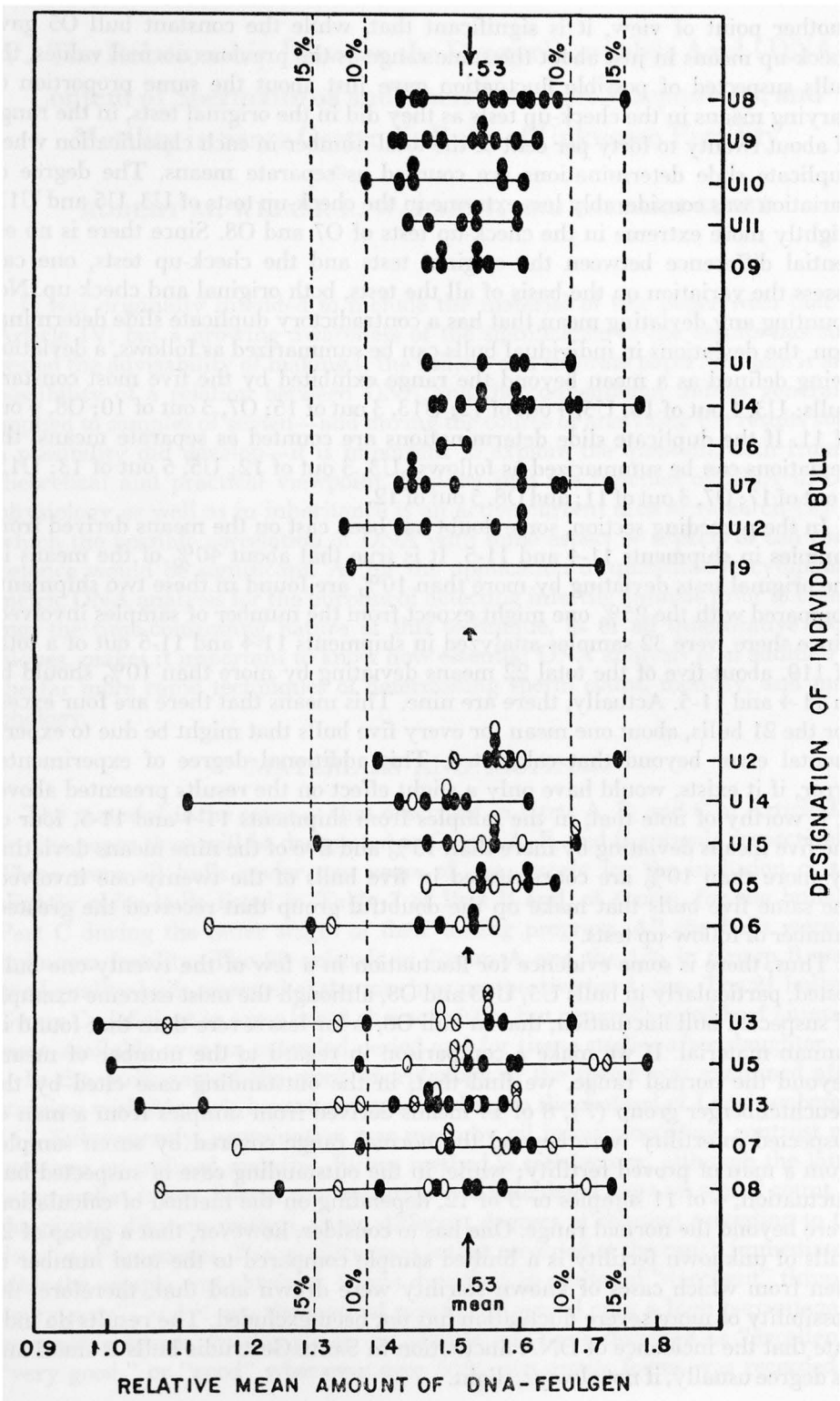
FIG. 9. Relative mean amount of DNA-Feulgen in 21 Santa Gertrudis bulls of unknown fertility originally under two years of age tested over a period of approximately 18 months, after which check-up tests were given bulls suspected of possible fluctuation.

Shaded oval: Original sample mean.

Open oval: Check-up sample mean without additional determination on duplicate slide.

Oval containing one diagonal line: Check-up sample mean followed by additional determination on duplicate slide.

Oval containing crossed diagonal lines: Second sample mean followed by additional determination on duplicate slide.


15 %
10 %
1.53
10 %
15 %
U8
U9
U10
U11
09
U1
U4
U6
U7
U12
19
U2
U14
U15
05
06
U3
U5
U13
07
08
15%
10 %
1.53
mean
10 %
15 %
0.9
1.0
1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8
RELATIVE MEAN AMOUNT OF DNA-FEULGEN
DESIGNATION OF INDIVIDUAL BULL

another point of view, it is significant that, while the constant bull O5 gave check-up means in just about the same range as the previous normal values, the bulls suspected of possible fluctuation gave just about the same proportion of varying means in the check-up tests as they did in the original tests, in the range of about twenty to forty per cent of the total number in each classification when duplicate slide determinations are counted as separate means. The degree of variation was considerably less extreme in the check-up tests of U3, U5 and U13, slightly more extreme in the check-up tests of O7 and O8. Since there is no essential difference between the original tests and the check-up tests, one can assess the variation on the basis of all the tests, both original and check-up. Not counting any deviating mean that has a contradictory duplicate slide determination, the deviations in individual bulls can be summarized as follows, a deviation being defined as a mean beyond the range exhibited by the five most constant bulls: U3, 1 out of 10; U5, 4 out of 12; U13, 3 out of 15; O7, 3 out of 10; O8, 4 out of 11. If the duplicate slide determinations are counted as separate means, the deviations can be summarized as follows: U3, 3 out of 12; U5, 5 out of 13; U13, 4 out of 17; O7, 3 out of 11; and O8, 5 out of 12.

In the preceding section, some doubt has been cast on the means derived from samples in shipments 11-4 and 11-5. It is true that about 40% of the means in the original tests deviating by more than 10% are found in these two shipments compared with the 25% one might expect from the number of samples involved. Since there were 32 samples analyzed in shipments 11-4 and 11-5 out of a total of 119, about five of the total 22 means deviating by more than 10% should be in 11-4 and 11-5. Actually, there are nine. This means that there are four excess for the 21 bulls, about one mean for every five bulls that might be due to experimental error beyond that calculated. This additional degree of experimental error, if it exists, would have only a slight effect on the results presented above. It is worthy of note that, in the samples from shipments 11-4 and 11-5, four of the five means deviating by more than 15% and five of the nine means deviating by more than 10% are concentrated in five bulls of the twenty-one involved, the same five bulls that make up the doubtful group that received the greatest number of follow-up tests.

Thus, there is some evidence for fluctuation in a few of the twenty-one bulls tested, particularly in bulls U5, U13 and O8, although the most extreme example of suspected bull fluctuation, that in bull O8, is far less severe than that found in human material. If we make a comparison in regard to the number of means beyond the normal range, we find that, in the outstanding case cited by the Leuchtenberger group (7), 8 of 14 means derived from samples from a man of suspected infertility were beyond the normal range covered by seven samples from a man of proved fertility; while, in the outstanding case of suspected bull fluctuation, 4 of 11 samples or 5 of 12, depending on the method of calculation, were beyond the normal range. One has to consider, however, that a group of 21 bulls of unknown fertility is a limited sample compared to the total number of men from which cases of known sterility were drawn and that, therefore, the possibility of more severe fluctuation has not been excluded. The results do indicate that the incidence of DNA fluctuation in Santa Gertrudis bulls is small and its degree usually, if not always, slight.

V. The Relationship Between the Deoxyribonucleic Acid (DNA) Content of Spermatozoa and Their Number, Morphology, and Motility in Santa Gertrudis Bulls of Unknown Fertility

ROBERT M. WELCH, E. W. HANLY, AND WILLIAM GUEST

It was originally intended to include the material of this paper with that of Article IV, but a growing awareness of the significance of the results has suggested the advisability of making it the subject of a separate paper. If there is any possibility of a relation between DNA and the conventional indices generally applied to samples of semen—and during the course of analyzing the results such a possibility did develop—it is important to explore the question, both from a theoretical and practical viewpoint. Inquiry into the function of DNA in cell physiology as well as in inheritance is an active current area of research (*2*) in which the possible correlation of DNA with some aspect or aspects of cell competence would be significant. As for the application of quantitative cytophotometric DNA analysis for the purpose of detecting infertile animals prior to breeding, the time-consuming nature of this technique, as of all quantitative techniques, makes it important to know how essential DNA analysis is in addition to the far more rapid techniques of determining sperm count, motility, and morphology.

MATERIALS AND METHODS

The material is the same as that described in Parts A, B, and C of Article IV. In this paper they will be designated as Group A, B, and C animals respectively. These were all bulls under two years of age, except for the inclusion of the slightly older bulls listed in Table 7 of Part B and, of course, for the bulls of Part C during the latter stages of their testing program. All the bulls were of unknown fertility. The 98 animals in Group A and the 135 in Group B were good quality bulls reserved at the ranch as prospective herd sires. The 21 bulls in Group C, deficient in some breed characteristics but generally good beef animals, were available over an extended period and for tissue studies after slaughter.

In the early part of the work, morphology of the sperm was examined after staining with Mayer's hematoxylin, according to the method of Leuchtenberger (*6*). Subsequently, examination was made by oil immersion phase contrast microscopy on unfixed material. Either method is satisfactory, although the latter is somewhat faster. Sperm concentration was calculated from an actual count by the pipette dilution method, using different degrees of dilution according to the density of the sperm. The motility used is that recorded at the ranch immediately after the sample was obtained. It was described in grades of "excellent" through "very poor"; and it was the general practice to record only a high percentage of non-motile sperm. In this paper, no sample has been classified as "excellent," "very good," or "good" whenever over 50% non-motile forms was recorded at

the ranch, and, similarly, no sample has been classified as "fair" whenever over 25% non-motile forms was recorded.

RESULTS

Gross evidence that sperm DNA acts or is acted upon in the arena of cell physiology was first noticed when over-all means were calculated separately for each of the motility, morphology, and sperm count classes. Figs. 1 through 9 show a breakdown according to the respective groups and categories. A DNA mean has been calculated for each category. Construction of the graphs has been influenced by their main purpose, which is to indicate the presence or absence of correlation between the familiar conventional indices of fertility and the possible new DNA index. Accordingly, all samples tested have been classified without reference to the individual bull concerned. Also, for convenience, the various categories have been expressed in relative rather than absolute terms, since the direction of change of motility, morphology, and count is sufficient to satisfy the purpose of the figures. A key is given in each figure legend. The total number of samples in each figure is not necessarily equal to the total number analyzed for DNA in that group, for various reasons, chief among which are: that (1) the morphology and/or sperm count were not done for some of the early samples, (2) in one instance, the motility classification is lacking for one sample analyzed here but recorded as a "blank" at the ranch, and (3) as previously explained,

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FIG. 1. Motility of 245 samples from 98 bulls of unknown fertility under two years of age. Motility is that recorded at ranch. In arbitrary units as follows: 1–2: very poor; 2–3: poor; 3–4: fair; 4–5: good; 5–6: very good; 6–7: excellent.

FIG. 2. Motility of 155 samples from 135 bulls of unknown fertility. All samples were from bulls under 2 years of age except 20 samples from 17 bulls between 2 and 3 years of age. Arbitrary units as in Fig. 1.

FIG. 3. Motility of 135 samples from 21 bulls of unknown fertility. Bulls from which samples were taken were originally 12 to 18 months of age; and samples were taken over a period of a year to a year and a half. Arbitrary units as in Fig. 1.

FIG. 4. Sperm count of 234 samples from 95 bulls of unknown fertility under two years of age. In arbitrary units as follows: In millions per cc 1–2: 0–5; 2–3; 5–10; 3–4: 10–25; 4–5: 25–50; 5–6: 50–100; 6–7: 100–200; 7–8: 200–400; 8–9: 400–800; 9–10: 800–1600.

FIG. 5. Sperm count of 155 samples from 135 bulls of unknown fertility. Age classification as in Fig. 2. Arbitrary units as in Fig. 4.

FIG. 6. Sperm count of 140 samples from 21 bulls of unknown fertility. Age classification as in Fig. 3. Arbitrary units as in Fig. 4.

FIG. 7. Morphology of 229 samples from 95 bulls of unknown fertility under two years of age. In arbitrary units as follows: 1–2: 65 and 70% abnormality; 2–3: 55 and 60%; 3–4: 45 and 50%; 4–5: 35 and 40%; 5–6: 25 and 30%; 6–7: 15 and 20%; 7–8: 5 and 10%.

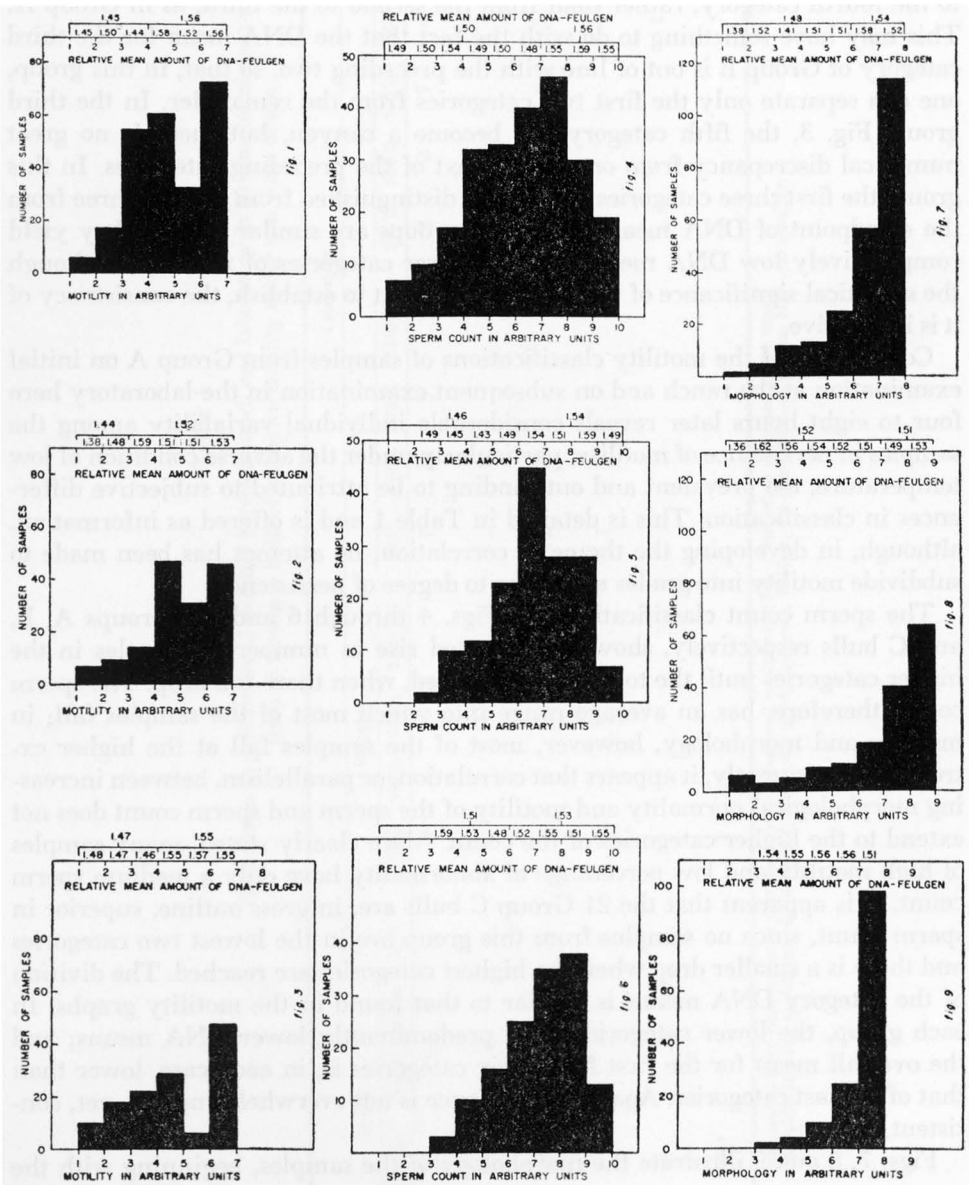
FIG. 8. Morphology of 155 samples from 135 bulls of unknown fertility. Age classification as in Fig. 2. Arbitrary units as in Fig. 7.

FIG. 9. Morphology of 140 samples from 21 bulls of unknown fertility. Age classification as in Fig. 3. Arbitrary units as in Fig. 7.

DNA means for each class on scale at top of each figure.

some samples were not used when too high a percentage of non-motile forms was recorded together with a high grade of activity of those motile.

From Fig. 1, pertaining to the 98 Group A bulls studied during the first year, it is evident that the number of samples in the various categories shows a general increase with improvement in motility, with the exception of the next-to-last category in which there is a rather precipitate drop from the previous. The increase, however, is irregular, notably cliff-like from the second to the third category and from the next-to-last to the last. Everyone of the DNA means of the first three categories, those with the worst motility, is lower than any DNA mean of



the last three categories; and the DNA mean of all samples in the first three categories (not the average of the three category means) is considerably lower than the DNA mean of all samples in the last three categories. Thus, there does appear to be some degree of correlation between inferior motility and low DNA in this group, although the mean of the first three groups is still within 10% of the over-all mean of 1.53 for 246 samples analyzed, and although the correlation in individual samples is by no means invariable. A similar situation prevails in the bulls of Group B, studied during the second year, as can be seen from Fig. 2, with one noteworthy difference. As in Group A, the fifth category shows a rather precipitate drop, but the cliff-like increase occurs in Group B from the third to the fourth category, rather than from the second to the third, as in Group A. This may have something to do with the fact that the DNA mean for the third category of Group B is out of line with the preceding two, so that, in this group, one can separate only the first two categories from the remainder. In the third group, Fig. 3, the fifth category has become a canyon, but there is no great numerical discrepancy from one to the next of the preceding categories. In this group, the first three categories are clearly distinguished from the last three from the standpoint of DNA means. All three groups are similar in that they yield comparatively low DNA means for the poorer categories of motility. Although the statistical significance of this might be difficult to establish, the consistency of it is impressive.

Comparison of the motility classifications of samples from Group A on initial examination at the ranch and on subsequent examination in the laboratory here four to eight hours later reveals considerable individual variability among the samples in persistence of motility, particularly under the adverse condition of low temperature, too prevalent and outstanding to be attributed to subjective differences in classification. This is detailed in Table 1 and is offered as information, although, in developing the theme of correlation, no attempt has been made to subdivide motility into grades according to degree of persistence.

The sperm count classifications, in Figs. 4 through 6 and for Groups A, B, and C bulls respectively, show the expected rise in number of samples in the higher categories until the topmost are reached, when there is a drop. The sperm count, therefore, has an average range into which most of the samples fall; in motility and morphology, however, most of the samples fall at the higher extremes. Consequently, it appears that correlation, or parallelism, between increasing morphological normality and motility of the sperm and sperm count does not extend to the higher categories of the count. More clearly stated, many samples of high motility and low percentage of abnormality have only a medium sperm count. It is apparent that the 21 Group C bulls are, in gross outline, superior in sperm count, since no samples from this group are in the lowest two categories and there is a smaller drop when the highest categories are reached. The division of the category DNA means is similar to that found in the motility graphs. In each group, the lower categories yield predominantly lower DNA means; and the over-all mean for the first five or six categories is, in each case, lower than that of the last categories. Again, the evidence is not overwhelming but, yet, consistent.

Figs. 7, 8 and 9 illustrate the morphology of the samples, beginning with the

TABLE 1

Differences in persistence of motility between ranch and laboratory in 80 samples of spermatozoa from 45 Group A bulls of unknown fertility under two years of age

Ranch		Laboratory															
			Sluggish				Fair				Good				Excellent			
Grade	No. of samples	NM	≤25%	≤50%	≤75%	≤100%	≤25%	≤50%	≤75%	≤100%	≤25%	≤50%	≤75%	≤100%	≤25%	≤50%	≤75%	≤100%
E	51	5	4	.	.	.	7	7	3	.	3	2	7	4	.	.	1	8
VG	6	2	.	.	.	.	.	1	.	.	1	1	.	.	.	.	.	1
G	13	3	2	.	.	.	2	2	.	.	1	1	1	1	.	.	.	.
F	6	3	1	.	.	.	1	1	.	.	.	.	.	.	.	.	.	.
P	3	2	.	.	.	.	.	.	.	.	.	.	.	1	.	.	.	.
VP	1	.	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

No samples have been included that were given a degree of motility of 75% or less when examined at the ranch.
Abbreviations are as follows: E—excellent; VG—very good; G—good; F—fair; P—poor; VP—very poor; NM—no motility.

highest percentage of abnormality and continuing to the lowest on the right. Types of abnormal sperm are described separately in Article II. In addition, during the latter part of the work, attention was given to tail abnormalities and immature forms, such as those having a "cytoplasmic droplet." The proportion of these found was recorded, but no break-down has been made in the graphs. The three figures for the three groups of bulls are strikingly similar. In all three, most of the sperm fall into the last three categories of least abnormality, each of these sharply demarcated numerically from the succeeding one.* The remainder of the sperm, a comparatively small proportion of the total, is distributed among the categories of greater abnormality in, for the most part, shallowly gradated blocks. Again, as in motility and sperm count, the Group C bulls appear to have an over-all superiority, since there are no samples in the first two categories of greatest abnormality, while a higher proportion of the samples than in Groups A and B fall into the last category of least abnormality. In Group A, the DNA means appear to be following the trend previously noted, as the over-all mean of the first five categories is distinctly less than that of the last two. In Groups B and C, however, the consistency is lost, clearly in B, as can be seen from the over-all means, and so markedly in C that it is unnecessary to calculate the over-all means. On the basis of the analysis thus far, one might suspect a relationship between motility, sperm count, and DNA, but there is little evidence that this relationship, if such it is, extends to morphology.

In general, the evidence presented suggests but does not prove a relationship between motility, sperm count, and DNA. The data need further analysis. Specifically, there are three fundamental questions that occur: (1) Are the low category DNA means the result of a comparatively few extremely low means pulling down the average of a group predominantly normal, or is the group as a whole predominantly abnormal? (2) To what extent is the relationship between low sperm count and DNA, for example, influenced by the presence in the same sample of poor motility, and vice-versa? (3) And, if the correlation is not entirely consistent in general, how consistent is it in the individual bulls? These three questions cannot be answered completely in one kind of graph. For each a series of figures has been designed to illustrate the particular point in question.

The degree of correlation between each factor of motility, morphology, and sperm count, can be determined in every group and on every respective level from Figs. 10 through 21 and from the summary Tables 2, 3, and 4. For convenience of summary and to establish the conclusions on as broad a base as possible, 1.52, the fiduciary mean obtained by calculating the over-all mean for all samples analyzed in all three groups, has been taken as the point of demarcation of the DNA means in the tables, but a breakdown can be made for any level of the DNA mean by reference to the charts. Also for convenience, the factors of motility, morphology, and sperm count have been graded in arbitrary, relative numerical values, explanations of which will be found in the figure legends and table notes.

In this series of graphs, as in the first already presented, morphology shows the weakest correlation. Using as a standard of comparison the ratio of those samples

* It is to be noted that Group B has an additional category on the abnormal end of the scale, the eighth category being the same as the seventh in A and C.

TABLE 2

Classification of DNA means according to morphology in 524 samples of spermatozoa from 256 bulls of unknown fertility

	A		B*		C		Totals	
Morphology	<1.52	≥1.52	<1.52	≥1.52	<1.52	≥1.52	<1.52	≥1.52
1	0	0	6(2)	0	0	0	6	0 = 6
2	2	0	1	2(2)	0	0	3	2 = 5
3	3	2	2(2)	3(1)	0	0	5	5 = 10
4	9	4	4(1)	5(1)	1	1	14	10 = 24
5	9	5	5(2)	5(2)	1	3	15	13 = 28
6	10	15	8(3)	10(2)	3	7	21	32 = 53
7	16	40	22(8)	18(3)	9	16	47	74 = 121
8	53	61	27(6)	37(9)	44	55	124	153 = 277
Totals	102	127	75(24)	80(20)	58	82	235	289 = 524

* Number of samples with duplicate slide determinations is entered in parentheses to the right of the total.
1. A, B, C: 95, 135 and 26 bulls of first year, second year and long term group respectively.
2. 1.52 is the fiduciary mean obtained by calculating the over-all mean of all samples analyzed in Groups A, B, and C.
3. 1—75–80 percent morphological abnormality; 2—65–70%; 3—55–60%; 4—45–50%; 5—35–40%; 6—25–30%; 7—15–20%; 8—5–10%.

TABLE 3

Classification of DNA means according to sperm count in 529 samples of spermatozoa from 256 bulls of unknown fertility

	A		B*		C		Totals	
Sperm count	<1.52	≥1.52	<1.52	≥1.52	<1.52	≥1.52	<1.52	≥1.52
1	10	7	2	0	0	0	12	7 = 19
2	6	11	5(3)	5(1)	1	2	12	18 = 30
3	14	18	9(3)	3	5	5	28	26 = 54
4	20	13	14(5)	10(2)	11	5	45	28 = 73
5	23	17	22(7)	22(8)	7	18	52	57 = 109
6	16	30	13(5)	15(4)	12	23	41	68 = 109
7	9	21	5(1)	23(5)	17	21	31	65 = 96
8	6	13	5	2	5	8	16	23 = 39
Totals	104	130	75(24)	80(20)	58	82	237	292 = 529

* Number of samples with duplicate slide determinations is entered in parentheses to the right of the total.
1. A, B, C: 95, 135 and 26 bulls of first year, second year and long term group respectively.
2. 1.52 is the fiduciary mean obtained by calculating the over-all mean of all samples analyzed in Groups A, B, and C.
3. 1—0–10 millions per cc; 2—10–25; 3—25–50; 4—50–100; 5—100–200; 6—200–400; 7—400–800; 8—800–1600.

yielding DNA means less than 1.52 to those yielding DNA means equal to or greater than 1.52, we note from Table 2 that those samples having a morphology of less than 7, (equivalent to a percentage of abnormal forms greater than 20) give a ratio of 64/62, while those in the upper levels, 7 or higher (equivalent to a percentage of abnormality 20% or less) yield a ratio of 171/227. If, in similar fashion, 5 is taken as the dividing line (less than 5 and 5 or over), the ratios are, respectively, 28/17 and 207/272. The ratio is 6/0 for the first category compared with 124/153 for the eighth. The correlation is strongest in Group A, somewhat weaker in Group B, and non-existent in Group C.

Although such data cannot be dismissed casually as without significance, the apparent correlation between DNA and sperm count is considerably stronger, as

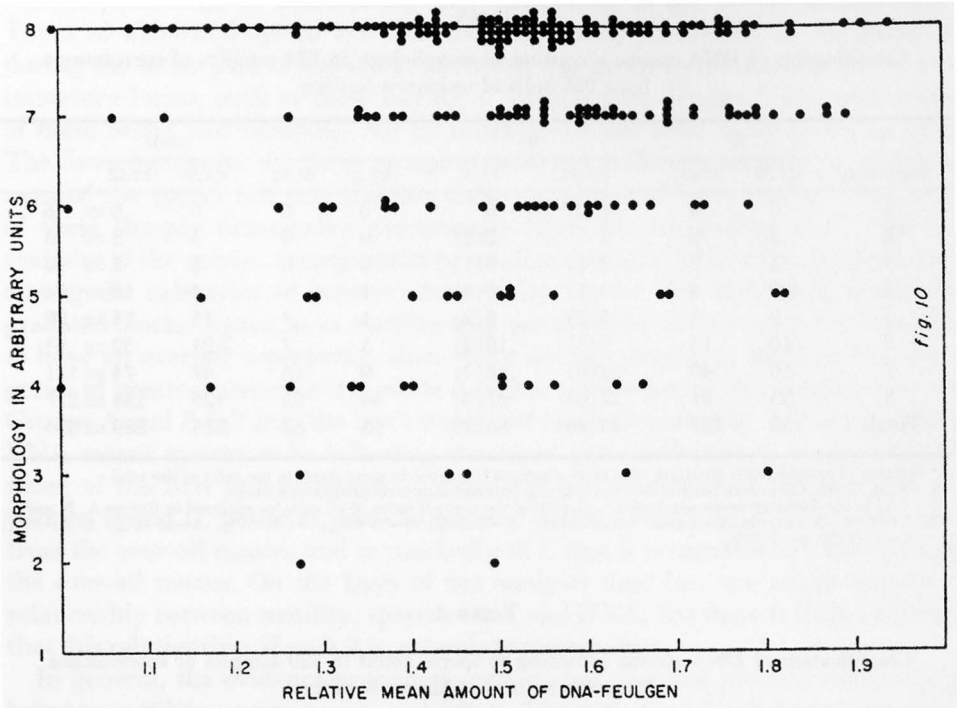


FIG. 10. Relationship between relative DNA means and morphology in 229 samples of spermatozoa from 95 Group A bulls of unknown fertility under two years of age. Arbitrary units as follows: 1: 75%–80% morphological abnormality; 2: 65%–70%; 3: 55%–60%; 4: 45%–50%; 5: 35%–40%; 6: 25%–30%; 7: 15%–20%; 8: 5%–10%.

shown in Table 3. Samples having a grade of less than 5, less than 100 million sperm per cc., yield a ratio of 97/79, while those with a grade of 5 or over yield a ratio of 140/213. The ratio for the first category is 12/7, for the eighth, 16/23, and for the seventh, 31/65. The apparent correlation holds in all groups, somewhat more firmly in Groups B and C than in Group A, although, in the latter, the borderline number five category has a ratio favoring the lower DNA division, which somewhat diminishes the degree of apparent correlation.

The strongest apparent correlation is that between DNA and motility, shown

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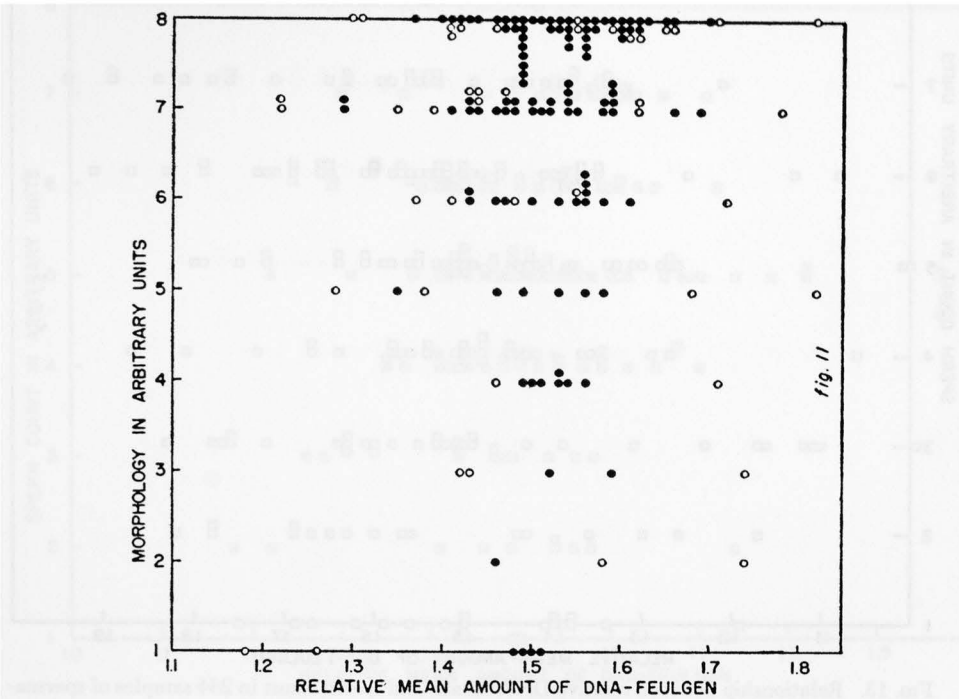
FIG. 11. Relationship between relative DNA means and morphology in 155 samples from 135 Group B bulls under 2 years of age except 20 samples from 17 bulls between 2 and 3 years of age. Arbitrary units as in Fig. 10.

Unshaded circles represent samples for which additional determinations were made on duplicate slides.

FIG. 12. Relationship between relative DNA means and morphology in 140 samples from 21 Group C bulls of unknown fertility. Bulls from which samples were taken were originally 12 to 18 months of age; and samples were taken over a period of a year to a year and a half. Arbitrary units as in Fig. 10.


MORPHOLOGY IN ARBITRARY UNITS
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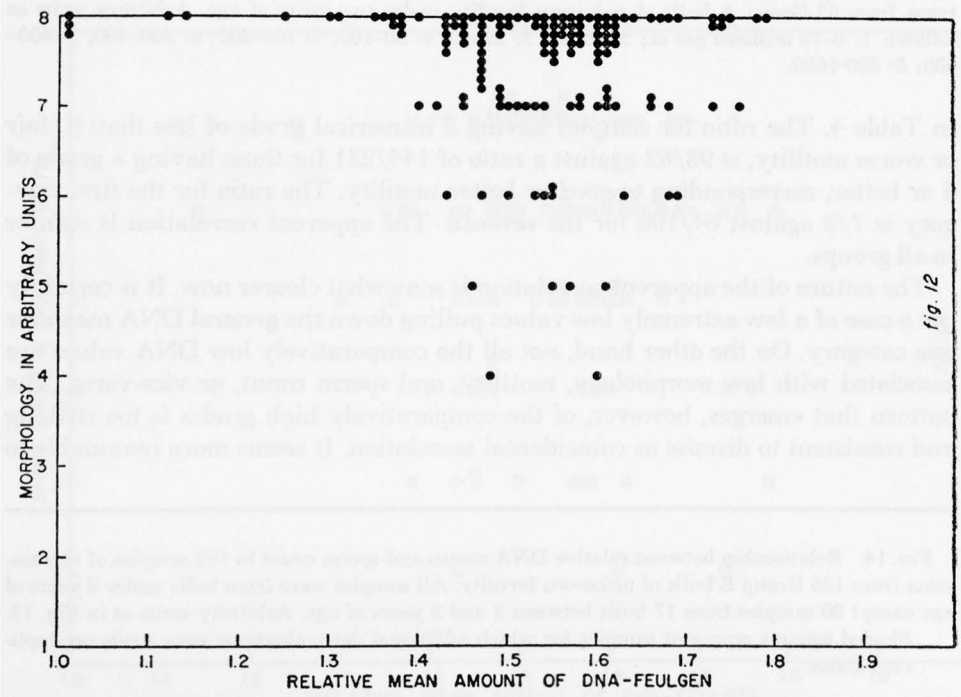
RELATIVE MEAN AMOUNT OF DNA-FEULGEN

fig. 11


MORPHOLOGY IN ARBITRARY UNITS
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RELATIVE MEAN AMOUNT OF DNA-FEULGEN

fig. 12

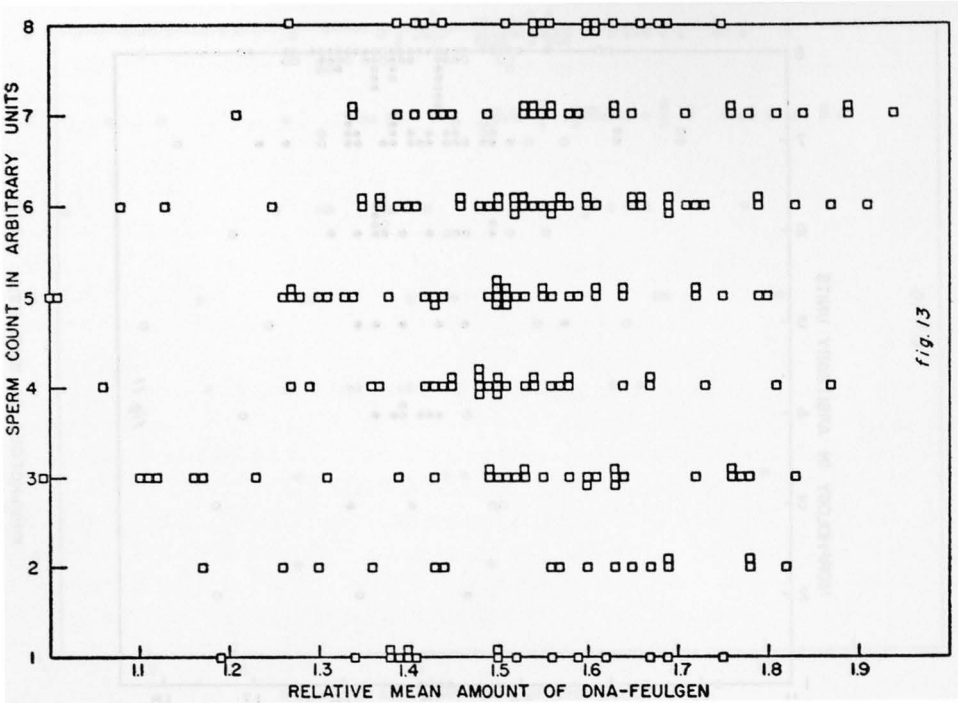

FIG. 13. Relationship between relative DNA means and sperm count in 234 samples of spermatozoa from 95 Group A bulls of unknown fertility under two years of age. Arbitrary units as follows: 1: 0–10 millions per cc; 2: 10–25; 3: 25–50; 4: 50–100; 5: 100–200; 6: 200–400; 7: 400–800; 8: 800–1600.

in Table 4. The ratio for samples having a numerical grade of less than 5, fair or worse motility, is 98/62 against a ratio of 144/231 for those having a grade of 5 or better, corresponding to good or better motility. The ratio for the first category is 7/2 against 64/106 for the seventh. The apparent correlation is similar in all groups.

The nature of the apparent correlation is somewhat clearer now. It is certainly not a case of a few extremely low values pulling down the general DNA mean for one category. On the other hand, not all the comparatively low DNA values are associated with low morphology, motility, and sperm count, or vice-versa. The pattern that emerges, however, of the comparatively high grades is too striking and consistent to dismiss as coincidental association. It seems more reasonable to

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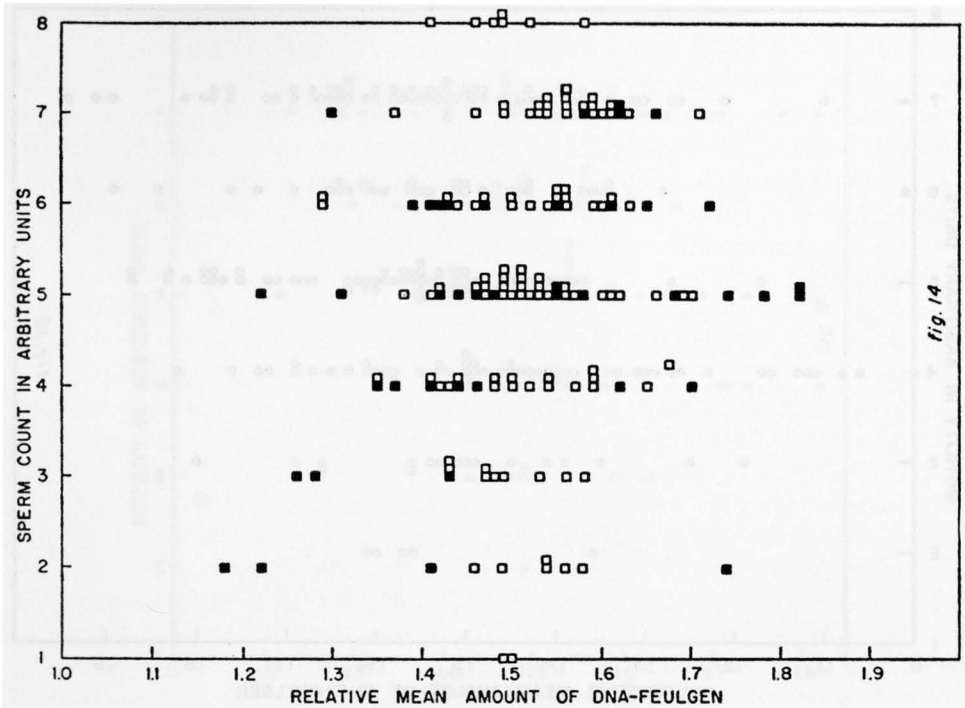
FIG. 14. Relationship between relative DNA means and sperm count in 155 samples of spermatozoa from 135 Group B bulls of unknown fertility. All samples were from bulls under 2 years of age except 20 samples from 17 bulls between 2 and 3 years of age. Arbitrary units as in Fig. 13. Shaded squares represent samples for which additional determinations were made on duplicate slides.

FIG. 15. Relationship between relative DNA means and sperm count in 140 samples of spermatozoa from 21 Group C bulls of unknown fertility. Bulls from which samples were taken were originally 12 to 18 months of age; and samples were taken over a period of a year to a year and a half. Arbitrary units as in Fig. 13.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RELATIVE MEAN AMOUNT OF DNA-FEULGEN
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fig. 14

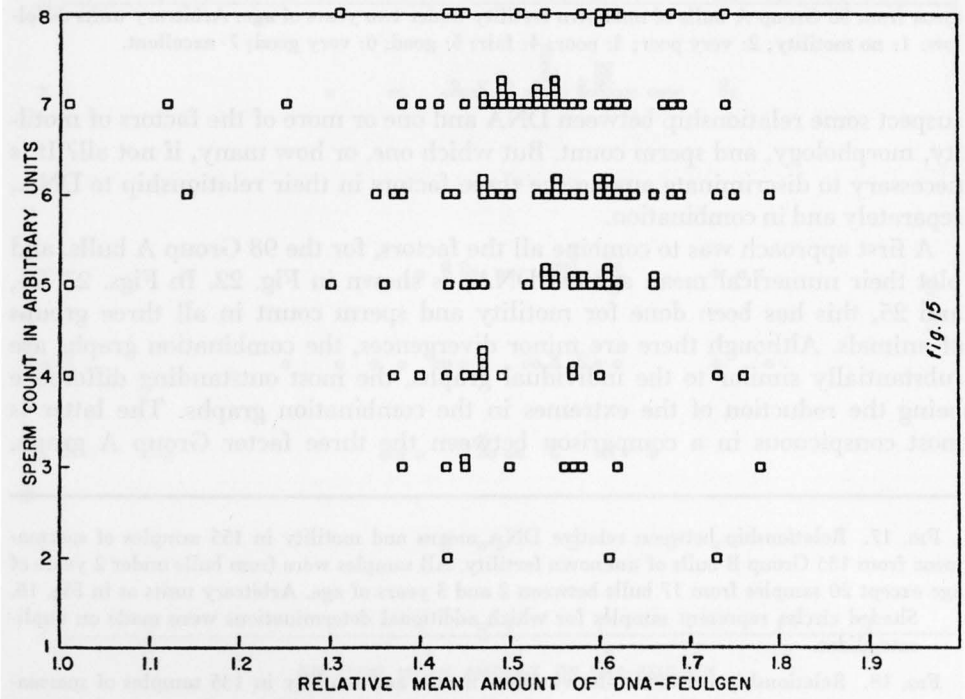
SPERM COUNT IN ARBITRARY UNITS
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fig. 15

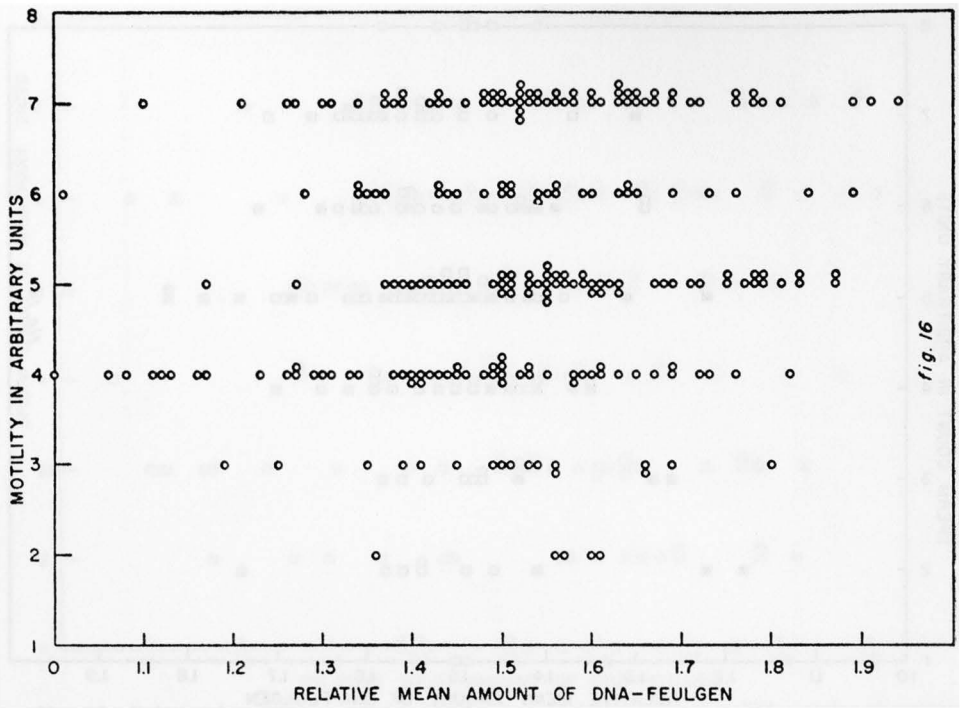

FIG. 16. Relationship between relative DNA means and motility in 245 samples of spermatozoa from 98 Group A bulls of unknown fertility under two years of age. Arbitrary units as follow: 1: no motility; 2: very poor; 3: poor; 4: fair; 5: good; 6: very good; 7: excellent.

suspect some relationship between DNA and one or more of the factors of motility, morphology, and sperm count. But which one, or how many, if not all? It is necessary to discriminate among the three factors in their relationship to DNA, separately and in combination.

A first approach was to combine all the factors, for the 98 Group A bulls, and plot their numerical mean against DNA, as shown in Fig. 22. In Figs. 23, 24, and 25, this has been done for motility and sperm count in all three groups of animals. Although there are minor divergences, the combination graphs are substantially similar to the individual graphs, the most outstanding difference being the reduction of the extremes in the combination graphs. The latter is most conspicuous in a comparison between the three factor Group A graph,

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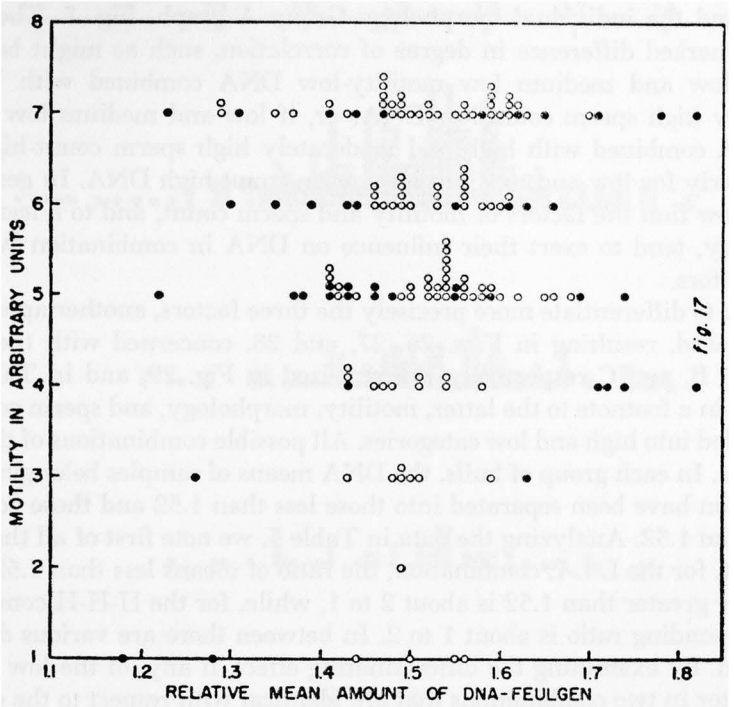
FIG. 17. Relationship between relative DNA means and motility in 155 samples of spermatozoa from 135 Group B bulls of unknown fertility. All samples were from bulls under 2 years of age except 20 samples from 17 bulls between 2 and 3 years of age. Arbitrary units as in Fig. 16. Shaded circles represent samples for which additional determinations were made on duplicate slides.

FIG. 18. Relationship between relative DNA means and motility in 135 samples of spermatozoa from 21 Group C bulls of unknown fertility. Bulls from which samples were taken were originally 12 to 18 months of age; and samples were taken over a period of a year to a year and a half. Arbitrary units as in Fig. 16.


MOTILITY IN ARBITRARY UNITS
RELATIVE MEAN AMOUNT OF DNA-FEULGEN
fig. 17

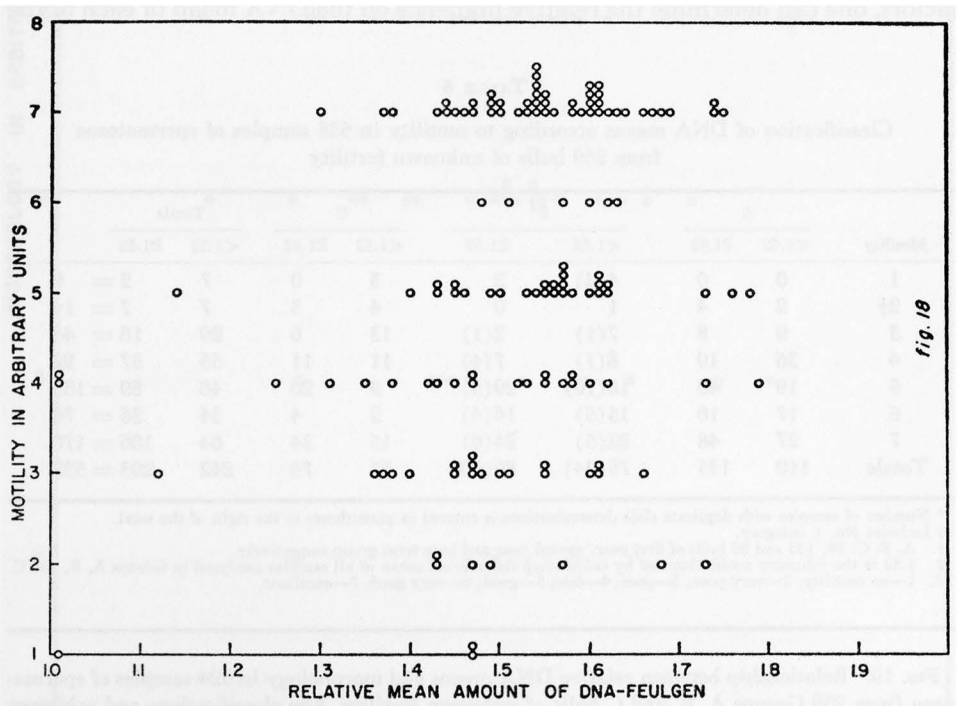

MOTILITY IN ARBITRARY UNITS
RELATIVE MEAN AMOUNT OF DNA-FEULGEN
fig. 18

Fig. 22, and the individual morphology Group A graph, Fig. 7. There seems to be no marked difference in degree of correlation, such as might be brought about if low and medium low motility-low DNA combined with high and moderately high sperm count-low DNA; or, if low and medium low motility-high DNA combined with high and moderately high sperm count-high DNA, and similarly for low and medium low sperm count-high DNA. In general, the graphs show that the factors of motility and sperm count, and to a lesser extent morphology, tend to exert their influence on DNA in combination with other similar factors.

In order to differentiate more precisely the three factors, another approach has been followed, resulting in Figs. 26, 27, and 28, concerned with the bulls of Groups A, B, and C respectively, summarized in Fig. 29, and in Table 5. As explained in a footnote to the latter, motility, morphology, and sperm count have been divided into high and low categories. All possible combinations of these have been made. In each group of bulls, the DNA means of samples belonging to each combination have been separated into those less than 1.52 and those equal to or greater than 1.52. Analyzing the data in Table 5, we note first of all the striking result that, for the L-L-L combination, the ratio of means less than 1.52 to those equal to or greater than 1.52 is about 2 to 1, while, for the H-H-H combination, the corresponding ratio is about 1 to 2. In between there are various degrees of correlation. By examining the differentiating effect, if any, of the low and high of one factor in two combinations that are identical with respect to the other two factors, one can determine the relative influence on the DNA mean of each of the

TABLE 4

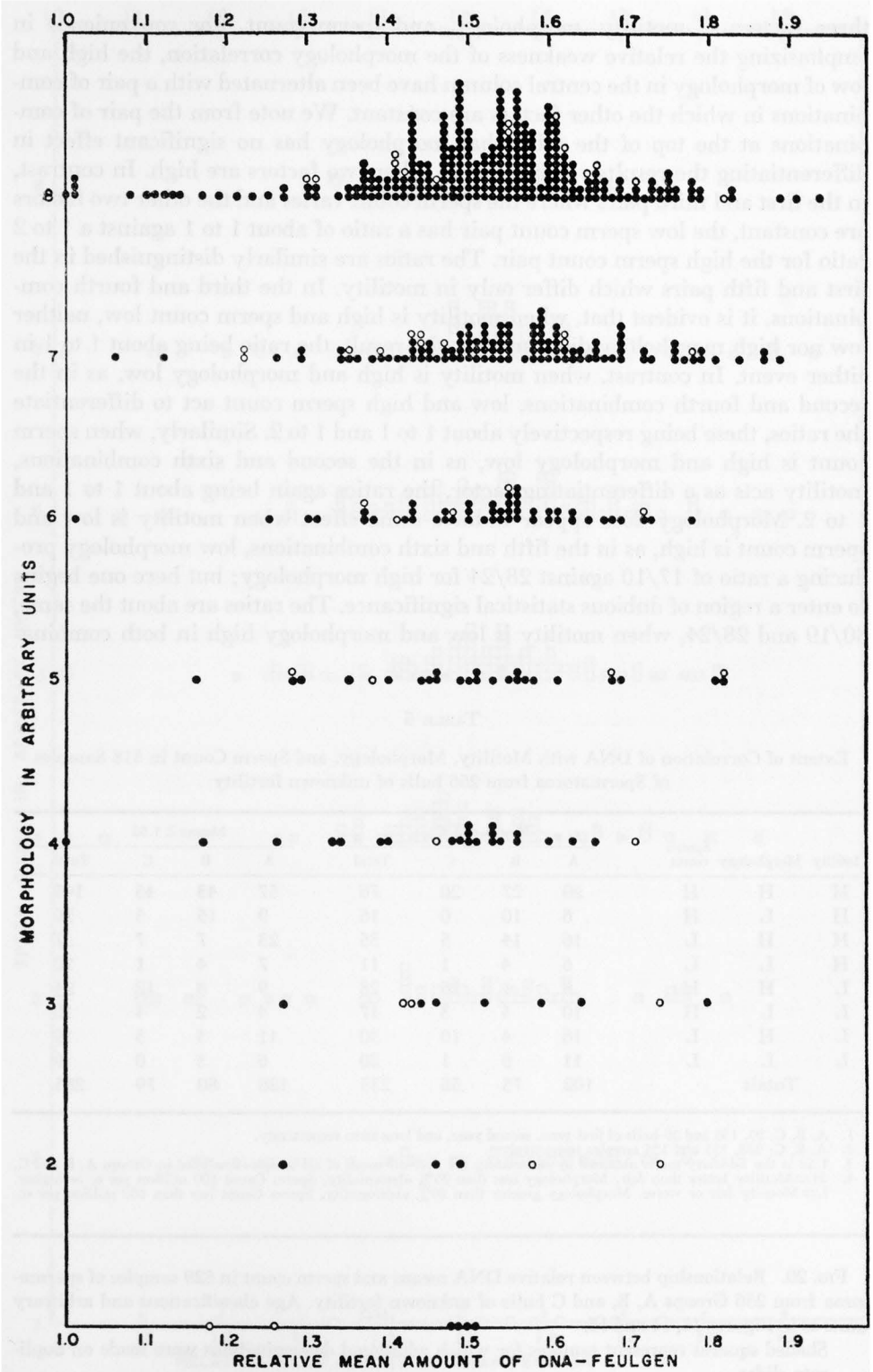
Classification of DNA means according to motility in 535 samples of spermatozoa from 259 bulls of unknown fertility

	A		B*		C		Totals	
Motility	<1.52	≥1.52	<1.52	≥1.52	<1.52	≥1.52	<1.52	≥1.52
1	0	0	4(2)	2	3	0	7	2 = 9
2†	2	4	1	0	4	3	7	7 = 14
3	9	8	7(1)	2(1)	13	6	29	16 = 45
4	36	19	8(1)	7(4)	11	11	55	37 = 92
5	19	40	18(10)	29(5)	9	20	46	89 = 135
6	17	16	15(5)	16(4)	2	4	34	36 = 70
7	27	48	22(5)	24(6)	15	34	64	106 = 170
Totals	110	135	75(24)	80(20)	57	78	242	293 = 535

* Number of samples with duplicate slide determinations is entered in parentheses to the right of the total.
† Includes No. 1 category.
1. A, B, C: 98, 135 and 26 bulls of first year, second year and long term group respectively.
2. 1.52 is the fiduciary mean obtained by calculating the over-all mean of all samples analyzed in Groups A, B, and C.
3. 1—no motility; 2—very poor; 3—poor; 4—fair; 5—good; 6—very good; 7—excellent.

FIG. 19. Relationship between relative DNA means and morphology in 524 samples of spermatozoa from 256 Groups A, B, and C bulls of unknown fertility. Age classifications and arbitrary units as in Figures 10, 11 and 12.

Unshaded circles represent samples for which additional determinations were made on duplicate slides.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MORPHOLOGY IN ARBITRARY UNITS
RELATIVE MEAN AMOUNT OF DNA-FEULGEN

three factors of motility, morphology, and sperm count. For convenience in emphasizing the relative weakness of the morphology correlation, the high and low of morphology in the central column have been alternated with a pair of combinations in which the other factors are constant. We note from the pair of combinations at the top of the table that morphology has no significant effect in differentiating the results when both the other two factors are high. In contrast, in the first and third pairs where the sperm count varies and the other two factors are constant, the low sperm count pair has a ratio of about 1 to 1 against a 1 to 2 ratio for the high sperm count pair. The ratios are similarly distinguished in the first and fifth pairs which differ only in motility. In the third and fourth combinations, it is evident that, when motility is high and sperm count low, neither low nor high morphology differentiates the result, the ratio being about 1 to 1 in either event. In contrast, when motility is high and morphology low, as in the second and fourth combinations, low and high sperm count act to differentiate the ratios, these being respectively about 1 to 1 and 1 to 2. Similarly, when sperm count is high and morphology low, as in the second and sixth combinations, motility acts as a differentiating factor, the ratios again being about 1 to 1 and 1 to 2. Morphology does appear to have some effect when motility is low and sperm count is high, as in the fifth and sixth combinations, low morphology producing a ratio of 17/10 against 28/24 for high morphology; but here one begins to enter a region of dubious statistical significance. The ratios are about the same, 30/19 and 28/24, when motility is low and morphology high in both combina-

TABLE 5

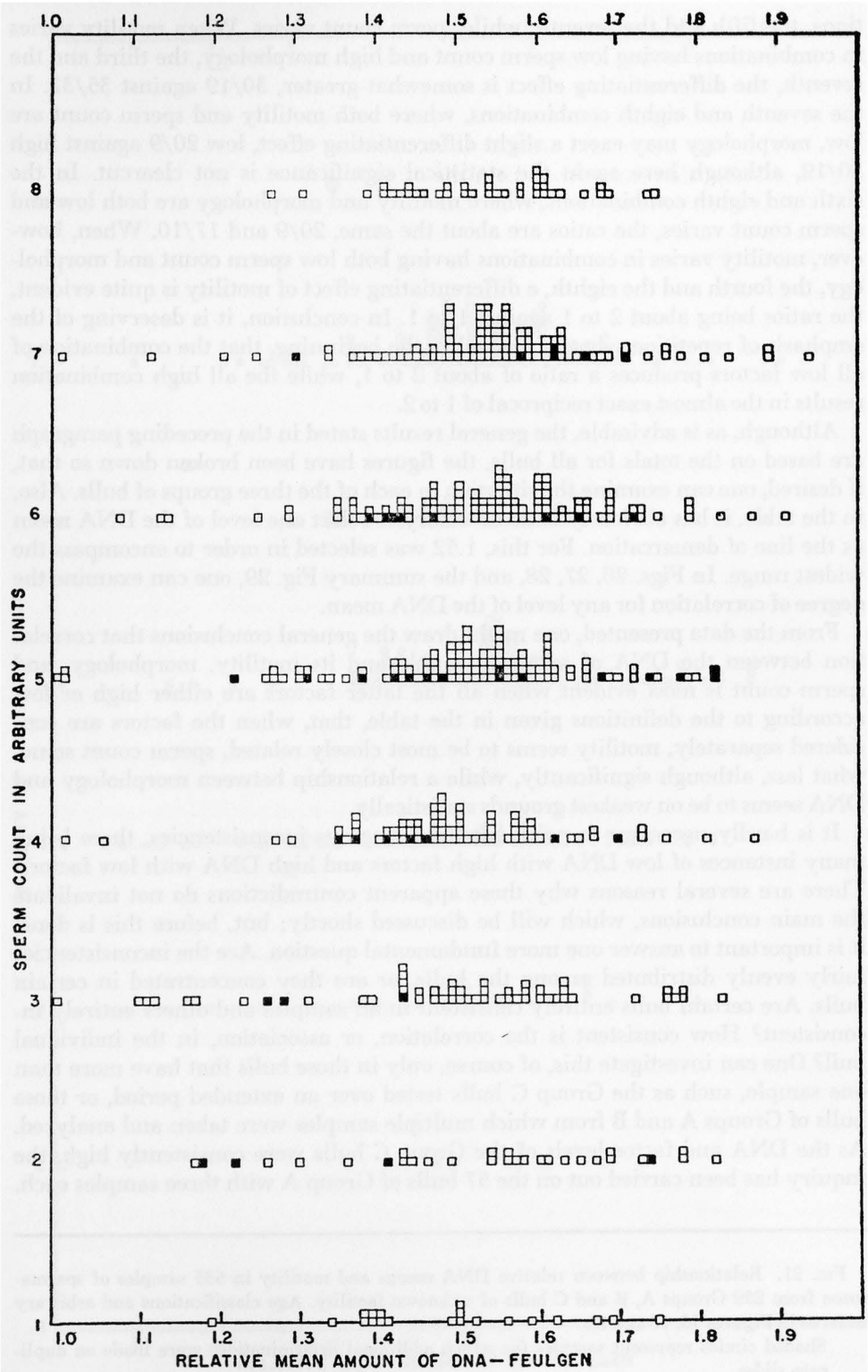
Extent of Correlation of DNA with Motility, Morphology, and Sperm Count in 518 Samples of Spermatozoa from 256 bulls of unknown fertility

Motility	Morphology	Sperm count	Means < 1.52				Means ≥ 1.52			
			A	B	C	Total	A	B	C	Total
H	H	H	29	27	20	76	57	43	45	145
H	L	H	6	10	0	16	9	15	5	29
H	H	L	16	14	5	35	23	7	7	37
H	L	L	6	4	1	11	7	4	1	12
L	H	H	8	4	16	28	9	3	12	24
L	L	H	10	4	3	17	4	2	4	10
L	H	L	16	4	10	30	11	3	5	19
L	L	L	11	8	1	20	6	3	0	9
Totals			102	75	56	233	126	80	79	285

1. A, B, C: 95, 135 and 26 bulls of first year, second year, and long term respectively.
2. A, B, C: 228, 155 and 135 samples respectively.
3. 1.52 is the fiduciary mean obtained by calculating the over-all mean of all samples analyzed in Groups A, B and C.
4. H=Motility better than fair, Morphology less than 25% abnormality, Sperm Count 100 million per cc or higher.
   L=Motility fair or worse, Morphology greater than 20% abnormality, Sperm Count less than 100 million per cc.

FIG. 20. Relationship between relative DNA means and sperm count in 529 samples of spermatozoa from 256 Groups A, B, and C bulls of unknown fertility. Age classifications and arbitrary units as in Figures 13, 14 and 15.

Shaded squares represent samples for which additional determinations were made on duplicate slides.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SPERM COUNT IN ARBITRARY UNITS
RELATIVE MEAN AMOUNT OF DNA—FEULGEN

tions, the fifth and the seventh, while sperm count varies. When motility varies in combinations having low sperm count and high morphology, the third and the seventh, the differentiating effect is somewhat greater, 30/19 against 35/37. In the seventh and eighth combinations, where both motility and sperm count are low, morphology may exert a slight differentiating effect, low 20/9 against high 30/19, although here again the statistical significance is not clearcut. In the sixth and eighth combinations, where motility and morphology are both low and sperm count varies, the ratios are about the same, 20/9 and 17/10. When, however, motility varies in combinations having both low sperm count and morphology, the fourth and the eighth, a differentiating effect of motility is quite evident, the ratios being about 2 to 1 against 1 to 1. In conclusion, it is deserving of the emphasis of repetition what was noted in the beginning, that the combination of all low factors produces a ratio of about 2 to 1, while the all high combination results in the almost exact reciprocal of 1 to 2.

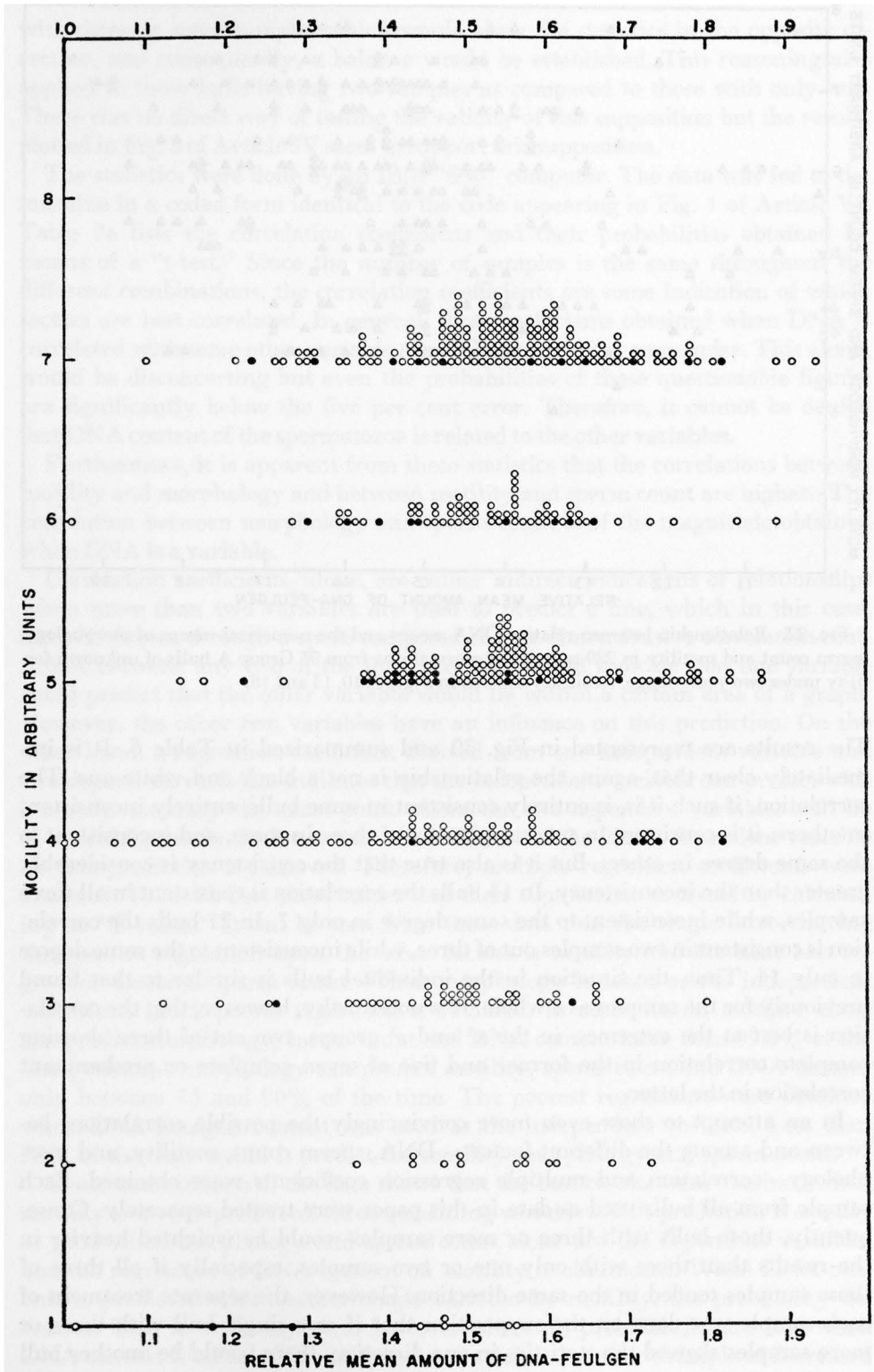
Although, as is advisable, the general results stated in the preceding paragraph are based on the totals for all bulls, the figures have been broken down so that, if desired, one can examine the situation in each of the three groups of bulls. Also, in the table, it has obviously been necessary to select one level of the DNA mean as the line of demarcation. For this, 1.52 was selected in order to encompass the widest range. In Figs. 26, 27, 28, and the summary Fig. 29, one can examine the degree of correlation for any level of the DNA mean.

From the data presented, one might draw the general conclusions that correlation between the DNA of a sperm sample and its motility, morphology, and sperm count is most evident when all the latter factors are either high or low according to the definitions given in the table, that, when the factors are considered separately, motility seems to be most closely related, sperm count somewhat less, although significantly, while a relationship between morphology and DNA seems to be on weakest grounds statistically.

It is hardly necessary to point out the numerous inconsistencies, there being many instances of low DNA with high factors and high DNA with low factors. There are several reasons why these apparent contradictions do not invalidate the main conclusions, which will be discussed shortly; but, before this is done, it is important to answer one more fundamental question. Are the inconsistencies fairly evenly distributed among the bulls, or are they concentrated in certain bulls. Are certain bulls entirely consistent in all samples and others entirely inconsistent? How consistent is the correlation, or association, in the individual bull? One can investigate this, of course, only in those bulls that have more than one sample, such as the Group C bulls tested over an extended period, or those bulls of Groups A and B from which multiple samples were taken and analyzed. As the DNA and factor levels of the Group C bulls were consistently high, the inquiry has been carried out on the 57 bulls of Group A with three samples each.

FIG. 21. Relationship between relative DNA means and motility in 535 samples of spermatozoa from 259 Groups A, B and C bulls of unknown fertility. Age classifications and arbitrary units as in Figures 16, 17 and 18.

Shaded circles represent samples for which additional determinations were made on duplicate slides.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MOTILITY IN ARBITRARY UNITS
RELATIVE MEAN AMOUNT OF DNA-FEULGEN

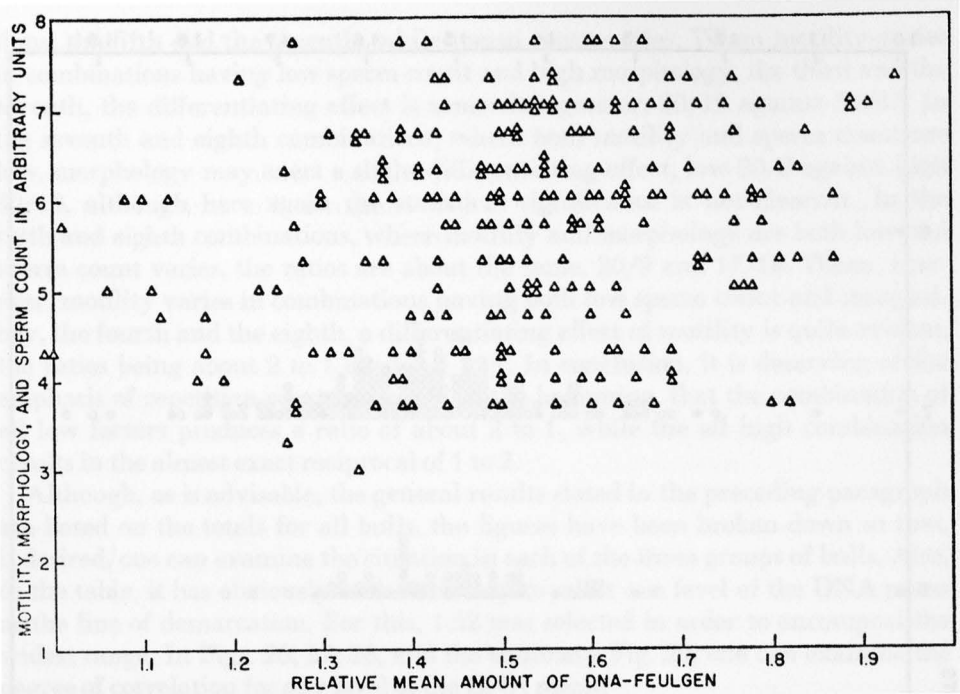


FIG. 22. Relationship between relative DNA means and the numerical means of morphology, sperm count and motility in 229 samples of spermatozoa from 95 Group A bulls of unknown fertility under two years of age. Arbitrary units as in Figures 10, 13 and 16.

The results are represented in Fig. 30 and summarized in Table 6. It is immediately clear that, again, the relationship is not a black and white one. The correlation, if such it is, is entirely consistent in some bulls, entirely inconsistent in others; it is consistent in two samples out of three in some, and inconsistent to the same degree in others. But it is also true that the consistency is considerably greater than the inconsistency. In 14 bulls the correlation is consistent in all three samples, while inconsistent to the same degree in only 7. In 21 bulls the correlation is consistent in two samples out of three, while inconsistent to the same degree in only 14. Thus, the situation in the individual bulls is similar to that found previously for the samples as a whole. It is noteworthy, however, that the correlation is best at the extremes, in the x^3 and z^3 groups, two out of three showing complete correlation in the former and five of seven complete or predominant correlation in the latter.

In an attempt to show even more convincingly the possible correlations between and among the different factors—DNA, sperm count, motility, and morphology—correlation and multiple regression coefficients were obtained. Each sample from all bulls used as data in this paper were treated separately. Consequently, those bulls with three or more samples would be weighted heavier in the results than those with only one or two samples, especially if all three of those samples tended in the same direction. However, the separate treatment of each sample was done on the supposition that if any single bull with three or more samples skewed the statistics in one direction, there would be another bull

with three or more samples which would skew the statistics in the opposite direction, and consequently, a balance would be established. This reasoning also applied to those bulls having two samples as compared to those with only one. There was no direct way of testing the validity of this supposition but the results plotted in Fig. 3 of Article IV seem to support this supposition.

The statistics were done by an IBM "650" computor. The data was fed to the machine in a coded form identical to the code appearing in Fig. 1 of Article VI. Table 7a lists the correlation coefficients and their probabilities obtained by means of a "t-test." Since the number of samples is the same throughout the different combinations, the correlation coefficients are some indication of which factors are best correlated. In general, those coefficients obtained when DNA is correlated with some other variable are all lower than the remainder. This alone, would be disconcerting but even the probabilities of these questionable figures are significantly below the five per cent error. Therefore, it cannot be denied that DNA content of the spermatozoa is related to the other variables.

Furthermore, it is apparent from these statistics that the correlations between motility and morphology and between motility and sperm count are highest. The correlation between morphology and sperm count is of the magnitude obtained when DNA is a variable.

Correlation coefficients, alone, are rather indirect indications of relationships when more than two variables are used to predict a line, which in this case, must be drawn through a space composed of four dimensions. A correlation coefficient between any two of the four variables would indicate that one variable could predict that the other variable would lie within a certain area of a graph. However, the other two variables have an influence on this prediction. On the other hand, a regression coefficient derived from one independent variable and two dependent variables indicates that the independent variable can predict with a certain accuracy the exact point where the two dependent variables will lie in a solid figure. In the latter case, the influence of the fourth dependent variable on this prediction is removed. Therefore, multiple regression coefficients were obtained. Table 7b lists these values and their significance as tested by calculating an "F-value." It can be seen from these data that the single factor having the greatest regression upon the other factors is motility and the next best regression factor is sperm count. When morphology is taken as the independent variable, a much poorer regression is obtained. In other words, motility alone can predict morphology, sperm count and DNA content more than 99.9% of the time; whereas morphology can predict motility, sperm count and DNA content only between 75 and 90% of the time. The poorest regressions were obtained when DNA-Feulgen content was used as the independent variable. Less than 75% of the time would it predict the motility, morphology and sperm count.

Closer examination of the data shows that the best results were obtained when motility is used to predict all three remaining variables. This prediction is equally as good when morphology and sperm count alone are the dependent variables and the regression of DNA content on motility is eliminated. When DNA content is predicted with another single variable by motility, the probability decreases. This is to be expected, however, since all probabilities are low when DNA content is taken as the independent variable. Another example of decreased

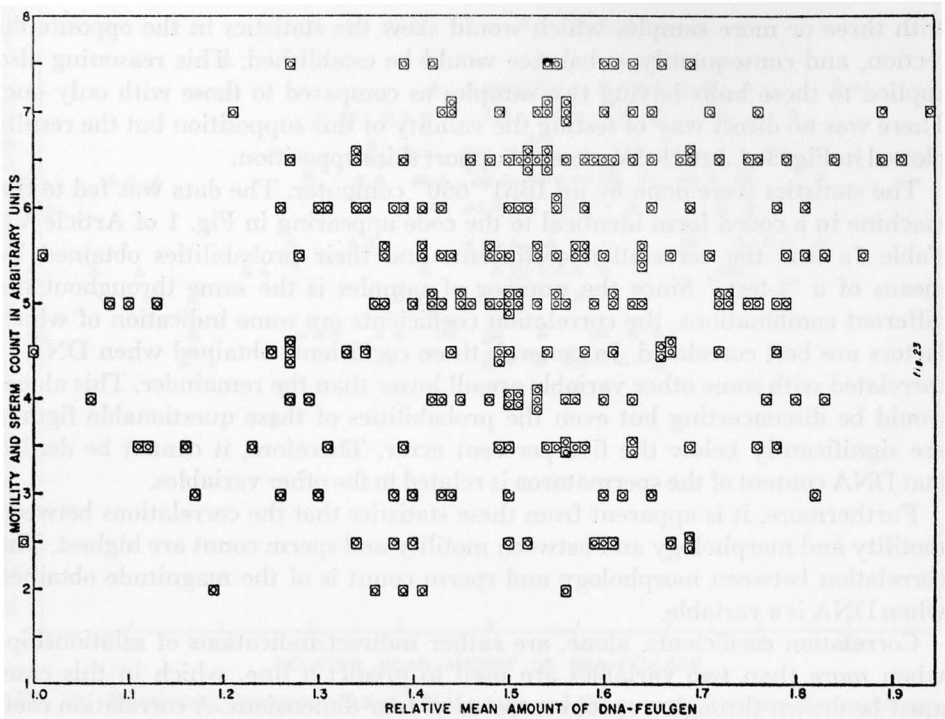

MOTILITY AND SPERM COUNT IN ARBITRARY UNITS
RELATIVE MEAN AMOUNT OF DNA-FEULGEN
1.0
1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8
1.9
1
2
3
4
5
6
7
8

Fig 23

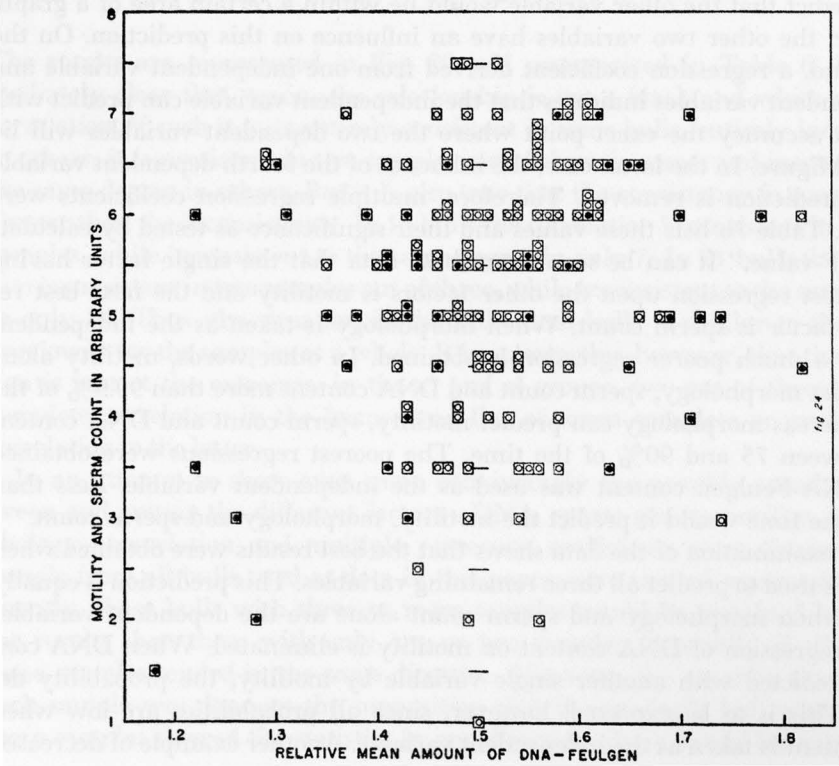
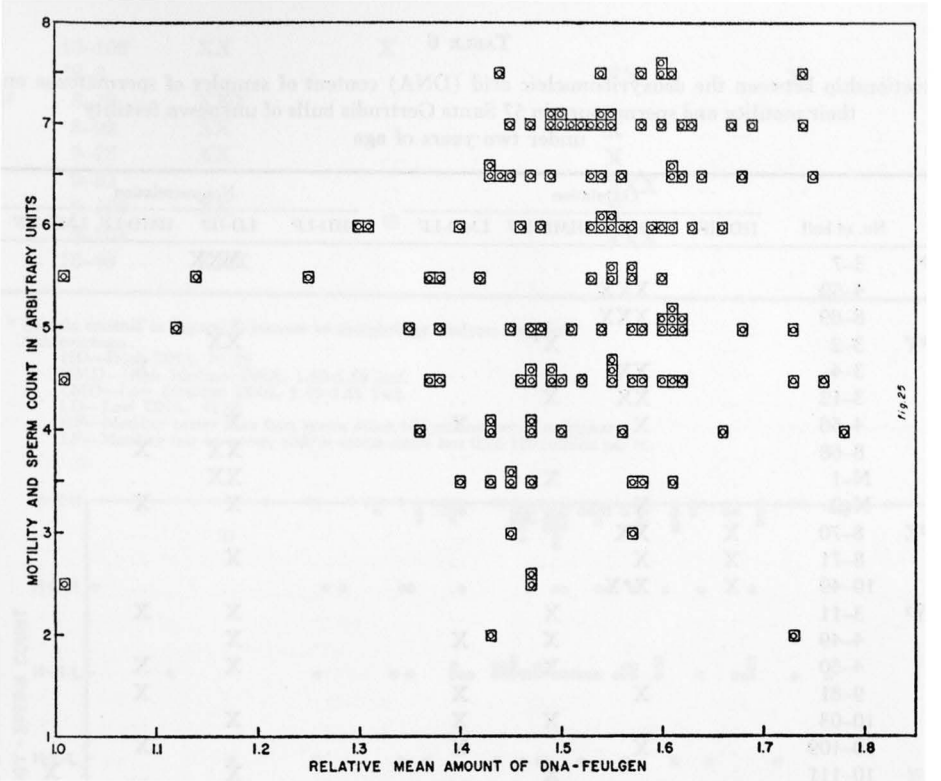

MOTILITY AND SPERM COUNT IN ARBITRARY UNITS
RELATIVE MEAN AMOUNT OF DNA-FEULGEN
1.2
1.3
1.4
1.5
1.6
1.7
1.8
1
2
3
4
5
6
7
8

Fig 24



probability is found when sperm count is used to predict DNA content and morphology with the regression of motility on sperm count eliminated. But this, too, is expected since DNA content and morphology show the lowest correlations. When sperm count is used to predict DNA content in combination with motility or with motility and morphology, a higher probability is obtained.

One of the conditions which has influenced these statistics should be called to the attention of the reader; *viz.*, the coding system used for the variables. As seen in Fig. 1, Article VI, a sperm count of 50 million per cubic centimeter would have the same coded value as a count of 99 million per cubic centimeter. A similiar situation is found in morphology scaling. Furthermore, the scales for rating DNA content and morphology are arithmetic while that for sperm count is

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FIG. 23. Relationship between DNA means and the numerical means of motility and sperm count in 234 samples of spermatozoa from 95 Group A bulls of unknown fertility under two years of age. Arbitrary units as in Figures 13 and 16.

FIG. 24. Relationship between relative DNA means and the numerical means of motility and sperm count in 155 samples of spermatozoa from 135 Group B bulls of unknown fertility. All samples were from bulls under two years of age except 20 samples from 17 bulls between 2 and 3 years of age. Arbitrary units as in Figures 13 and 16.

Shaded circles represent samples for which additional determinations were made on duplicate slides.

FIG. 25. Relationship between relative DNA means and the numerical means of motility and sperm count in 135 samples of spermatozoa from 21 Group C bulls of unknown fertility. Bulls from which samples were taken were originally 12 to 18 months of age; and samples were taken over a period of a year to a year and a half. Arbitrary units as in Figures 13 and 16.

TABLE 6

Relationship between the deoxyribonucleic acid (DNA) content of samples of spermatozoa and their motility and sperm count in 57 Santa Gertrudis bulls of unknown fertility under two years of age

		Correlation				Non-correlation			
	No. of bull	HD-HF	LD-LF	HMD-HF	LMD-LF	HD-LF	LD-HF	HMD-LF	LMD-HF
X^3	3–7	..	..	..	..	..	XXX	..	..
	4–52	..	XXX	..	..	..	..	..	..
	8–69	..	XXX	..	..	..	..	..	..
X^2Y	3–2	..	..	X*	..	..	XX	..	..
	3–4	..	XX	..	..	..	..	X	..
	3–12	..	XX	X	..	..	..	..	..
	4–56	..	X	..	X	..	X	..	..
	8–68	..	..	..	..	..	XX	X	..
	N–1	..	..	X	..	..	XX	..	..
	N–2	..	X	..	..	..	X	X	..
X^2Z	8–70	X	XX	..	..	..	..	..	..
	8–71	X	X	..	..	..	X	..	..
	10–49	X	X*X	..	..	..	..	..	..
XY^2	3–11	..	..	X	..	..	X	X	..
	4–49	..	..	X	X	..	X	..	..
	4–50	..	..	X	..	..	X	X	..
	9–81	..	X	..	X	..	..	X	..
	10–03	..	..	X	X	..	X	..	..
	10–109	..	X	..	X	..	..	X	..
	10–111	..	..	X	..	..	X	..	X
XYZ	3–9	X	X	X	..	..	..	..	..
	3–10	X	X	X	..	..	..	..	..
	4–54	..	X	..	..	X	..	..	X
	7–49	..	..	..	..	X	X	X	..
	8–103	X	X	..	X	..	..	..	..
	9–95	..	X	X	..	X	..	..	..
	10–113	X	..	X	..	..	X	..	..
XZ^2	4–55	XX	X	..	..	..	..	..	..
	9–96	X	..	..	..	X	X	..	..
	9–97	X	..	..	..	X	X	..	..
	10–110	XX	..	..	..	..	X	..	..
	10–114	XX	..	..	..	..	X	..	..
Y^3	2–71	..	..	X	X	..	..	..	X
	3–1	..	..	X*X	..	..	..	..	X
	7–58	..	..	X	XX	..	..	..	..
Y^2Z	2–70	..	..	X	..	X	..	X	..
	2–75	X	..	..	..	..	..	XX	..
	3–8	X	..	..	..	..	..	X	X*
	3–14	X	..	X	..	..	..	X*	..
	8–67	X	..	XX	..	..	..	..	..
	8–76	X	..	X	..	..	..	..	X
	8–88	X	..	..	..	..	..	X	X
	8–101	..	..	..	XX	X	..	..	..
	9–90	..	..	..	..	X	..	XX	..
YZ^2	7–61	X	..	..	X	X	..	..	..
	8–74	XX	..	X	..	..	..	..	..
	9–84	X	..	X	..	X	..	..	..
	9–99	..	..	..	X	XX	..	..	..

	10–108	XX	..	X	..	..	..	..	..	..	
	N–3	..	..	..	..	XX	..	X	..		
Z^3	8–72	XXX	..	..	..	..	..	..	..		
	8–92	XX	..	..	..	X	..	..	..		
	9–78	XX	..	..	..	X	..	..	..		
	9–80	..	..	..	..	XXX	..	..	..		
	9–100	XX	..	..	..	X	..	..	..		
	9–102	..	..	..	..	XXX	..	..	..		
	10–48	XXX	..	..	..	..	..	..	..		

* Sample omitted in Figure 30 because no morphology analyses recorded.

Abbreviations

HD—High DNA, >1.59
HMD—High Medium DNA, 1.52–1.59 incl.
LMD—Low Medium DNA, 1.45–1.51 incl.
LD—Low DNA, <1.45
HF—Motility better than fair; sperm count 100 million per cc or higher
LF—Motility fair or worse; and/or sperm count less than 100 million per cc.

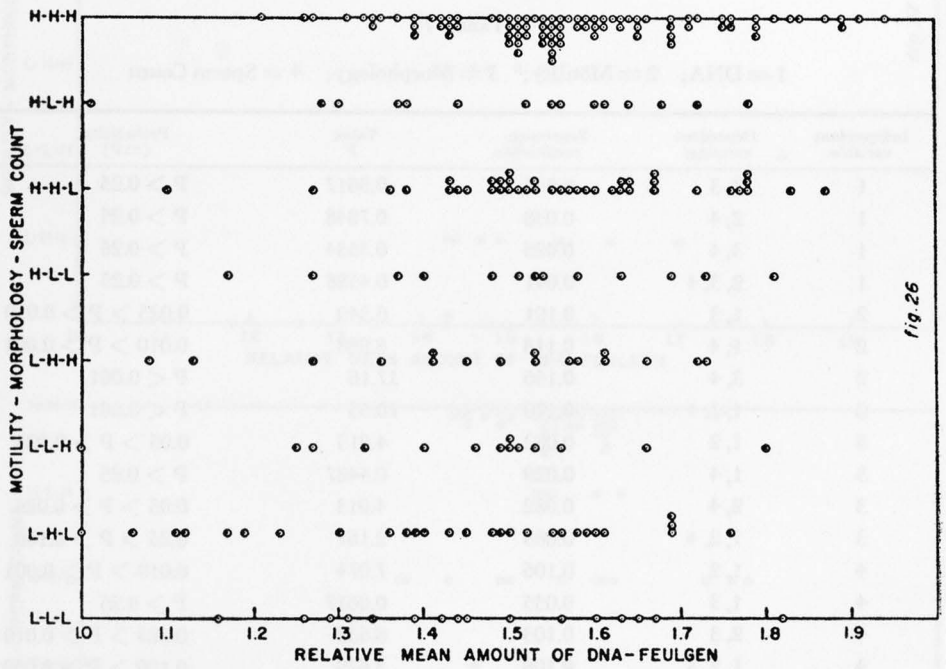


FIG. 26. Relationship between relative DNA means and combinations of 'low' and 'high' motility, morphology and sperm count in 228 samples of spermatozoa from 95 Group A bulls of unknown fertility under two years of age.

'H' is equivalent to a motility better than fair, morphology less than 25% abnormality, sperm count 100 million per cc or higher. 'L' is equivalent to a motility fair or worse, morphology greater than 20% abnormality, sperm count less than 100 million per cc.

not. Consequently, higher correlation and regression coefficients would be expected than would have been obtained if the uncoded data had been used. However, the method of coding is not completely arbitrary. For motility, sperm count and morphology, the values are based on a general idea of how much each would contribute to fertility or infertility. A high code value for DNA content is given at 1.60 or greater. This top value was chosen on the assumption that higher DNA

TABLE 7a

1 = DNA; 2 = Motility; 3 = Morphology; 4 = Sperm Count

Independent variable	Dependent variable	Correlation coefficient	Value t	Probability (=P)
1	2	0.1786	4.072	$P < 0.001$
1	3	0.1069	2.416	$0.025 > P > 0.010$
1	4	0.1334	3.028	$0.005 > P > 0.001$
2	3	0.2808	6.543	$P << 0.001$
2	4	0.3154	7.475	$P << 0.001$
3	4	0.1454	3.301	$P < 0.001$

TABLE 7b

1 = DNA; 2 = Motility; 3 = Morphology; 4 = Sperm Count

Independent variable	Dependent variables	Regression coefficient	Value F	Probability (=P)
1	2, 3	0.035	0.6617	$P > 0.25$
1	2, 4	0.038	0.7848	$P > 0.25$
1	3, 4	0.026	0.3584	$P > 0.25$
1	2, 3, 4	0.041	0.4588	$P > 0.25$
2	1, 3	0.101	6.349	$0.025 > P > 0.010$
2	1, 4	0.118	8.988	$0.010 > P > 0.001$
2	3, 4	0.156	17.16	$P < 0.001$
2	1, 3, 4	0.170	10.53	$P < 0.001$
3	1, 2	0.082	4.013	$0.05 > P > 0.025$
3	1, 4	0.029	0.4487	$P > 0.25$
3	2, 4	0.082	4.013	$0.05 > P > 0.025$
3	1, 2, 4	0.085	2.167	$0.25 > P > 0.100$
4	1, 2	0.106	7.074	$0.010 > P > 0.001$
4	1, 3	0.035	0.6617	$P > 0.25$
4	2, 3	0.103	6.633	$0.025 > P > 0.010$
4	1, 2, 3	0.108	3.679	$0.100 > P > 0.050$

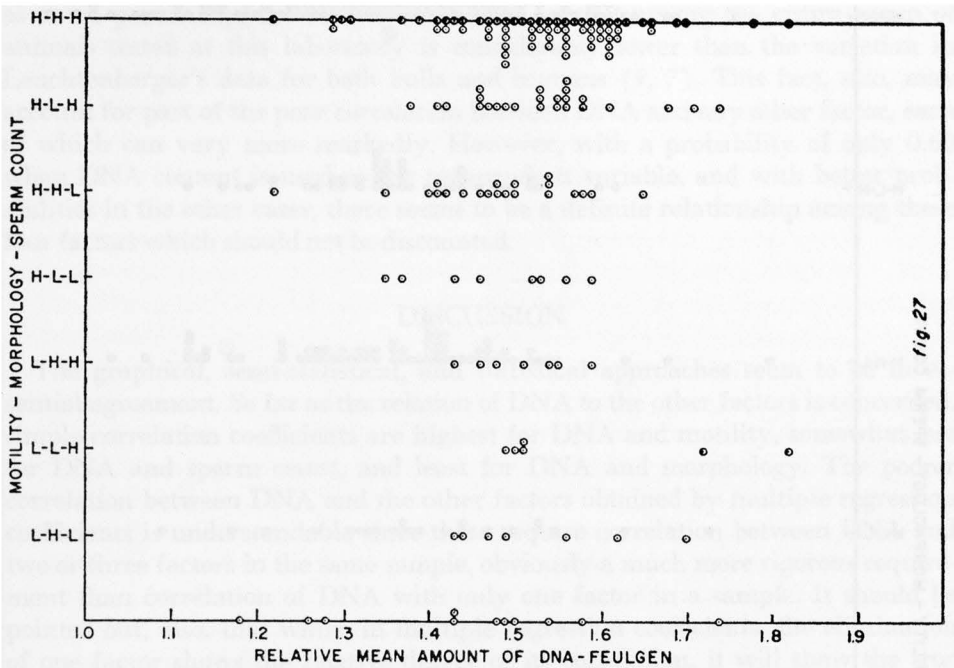
FIG. 27. Relationship between relative DNA means and combinations of 'low' and 'high' motility, morphology and sperm count in 155 samples of spermatozoa from 135 Group B bulls of unknown fertility under two years of age except 20 samples from 17 bulls between two and three years of age.

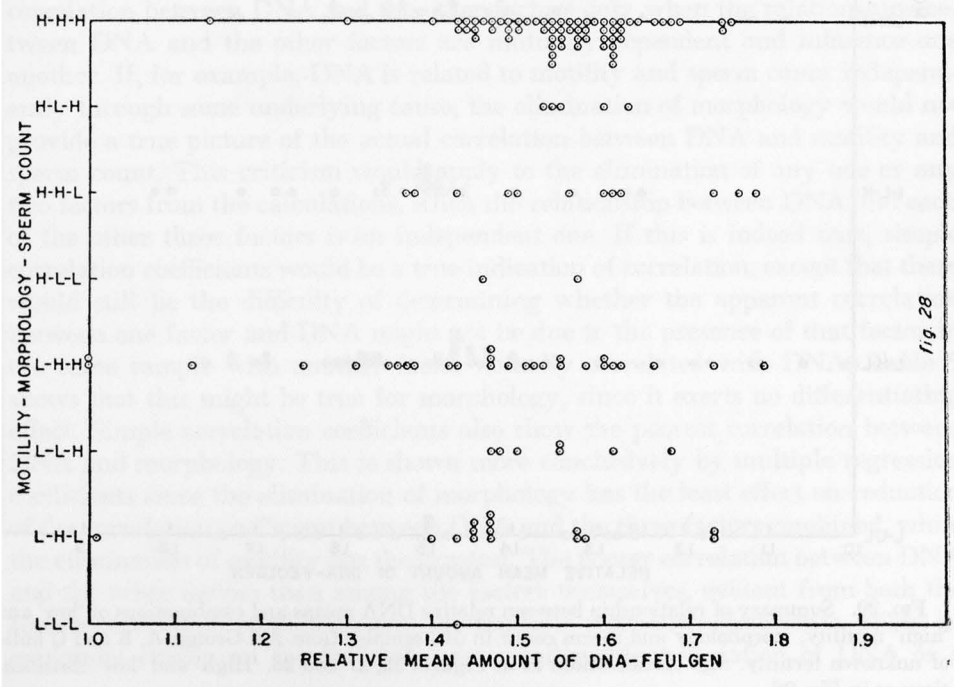
'H' and 'L' equivalents as in Fig. 26.

FIG. 28. Relationship between relative DNA means and combinations of 'low' and 'high' motility, morphology and sperm count in 135 samples of spermatozoa from 21 Group C bulls of unknown fertility. Bulls from which samples were taken were originally 12 to 18 months of age; and samples were taken over a period of a year to a year and a half.

'H' and 'L' equivalents as in Fig. 26.

contents would weight the DNA factor heavier than the coding of the other three variables. This method, however, does not assume that DNA values greater than 1.60 will not increase fertility. The fact that higher DNA contents were not coded higher accordingly might be partial explanation of their poorer correlation and regression coefficients. It should be further noted that the coding system used in





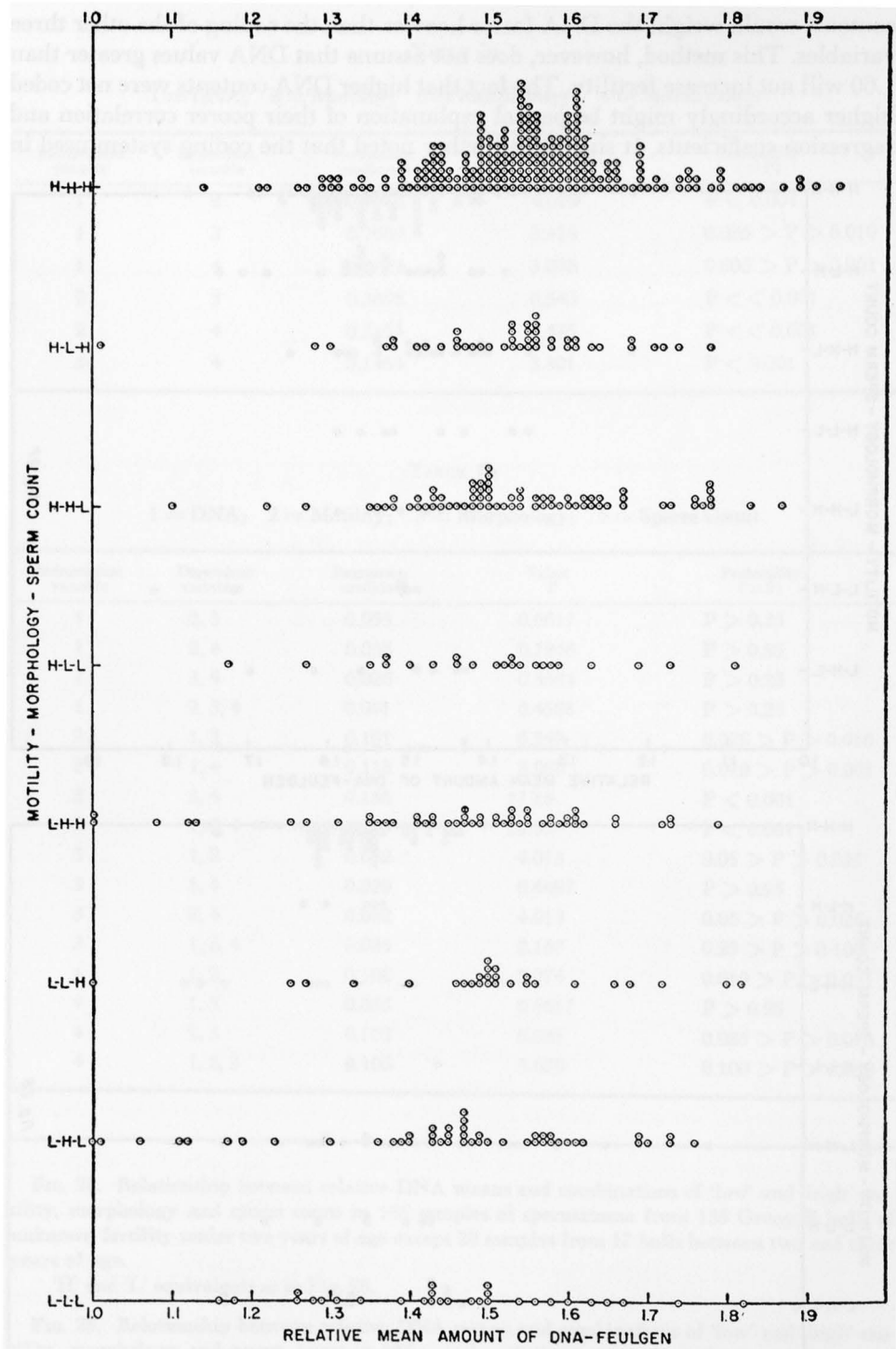


FIG. 29. Summary of relationship between relative DNA means and combinations of 'low' and 'high' motility, morphology and sperm count in 518 samples from 256 Groups A, B and C bulls of unknown fertility. Age classifications as in Figures 26, 27 and 28. 'High' and 'low' classifications as in Fig. 26.

these statistical analyses is different from that used in the non-statistical approach which is presented in the main body of this article. However, similar results were obtained with both methods, justifying the use of different codes.

From these data it cannot be said that DNA content will predict the other three variables, either individually or collectively, with any great accuracy. However, as noted previously, the variation of DNA content among the entire group of animals tested at this laboratory is considerably lower than the variation in Leuchtenberger's data for both bulls and humans (*9*, 7). This fact, also, may account for part of the poor correlation between DNA and any other factor, each of which can vary more markedly. However, with a probability of only 0.60 when DNA content is used as the independent variable, and with better probabilities in the other cases, there seems to be a definite relationship among these four factors which should not be discounted.

DISCUSSION

The graphical, semi-statistical, and statistical approaches seem to be in essential agreement. So far as the relation of DNA to the other factors is concerned, simple correlation coefficients are highest for DNA and motility, somewhat less for DNA and sperm count, and least for DNA and morphology. The poorer correlation between DNA and the other factors obtained by multiple regression coefficients is understandable since these require correlation between DNA and two or three factors in the same sample, obviously a much more rigorous requirement than correlation of DNA with only one factor in a sample. It should be pointed out, also, that while, in multiple regression coefficients, the elimination of one factor shows the relative degree of its correlation, it will show the true correlation between DNA and the other factors only when the relationships between DNA and the other factors are mutually dependent and influence one another. If, for example, DNA is related to motility and sperm count independently through some underlying cause, the elimination of morphology would not provide a true picture of the actual correlation between DNA and motility and sperm count. This criticism would apply to the elimination of any one or any two factors from the calculations, when the relationship between DNA and each of the other three factors is an independent one. If this is indeed true, simple correlation coefficients would be a true indication of correlation, except that there would still be the difficulty of determining whether the apparent correlation between one factor and DNA might not be due to the presence of that factor in the same sample with another factor actually correlated with DNA. Table 5 shows that this might be true for morphology, since it exerts no differentiating effect. Simple correlation coefficients also show the poorest correlation between DNA and morphology. This is shown more conclusively by multiple regression coefficients since the elimination of morphology has the least effect on reduction of the correlation coefficient between DNA and the three factors combined, while the elimination of motility has the greatest. The poorer correlation between DNA and the other factors than among the factors themselves, evident from both the simple correlation coefficients and the multiple regression coefficients, might be explained chiefly on the basis of greater independent variation of DNA or a

blurring by experimental error of actual correlation over a narrow range of variation. The latter might be one aspect of the general reduction of genetic variation in Santa Gertrudis bulls.

Thus, all lines of evidence point to a relationship between DNA and the other factors, particularly motility and sperm count. The logical consequence of this relationship has been shown to be true, that those groups and bulls poorest in the factors of motility, sperm count, and morphology are, in general, poorest in DNA. The relationship may be a complex one and may depend on some underlying factor. At the present time, the analysis seems to have been extended as far as the data justify.

We are confronted with a relationship too striking to dismiss as chance and at the same time with numerous exceptions that demand explanation. Yet, there are explanations that can be offered. It is not too difficult to understand why the correlation between morphology and DNA should be weak. Inasmuch as morphologically abnormal sperm are excluded from measurement, a causative effect between DNA and morphology in the individual sperm, even if it exists, would not show up as it would between DNA and motility, since no distinction is made, of course, in DNA analysis between motile and non-motile sperm. As for the percentage of abnormality in the whole sample, there is less reason to believe that this would affect or be affected by the DNA to the same extent that the sperm count might. There is greater complexity to accounting for the exceptions to the correlation between motility and DNA and sperm count and DNA. First of all, it is necessary to allow for experimental error, which can reach a maximum of 15% in a single sample. This could easily account for a number of instances when low DNA is found with high factors and vice-versa. Then, it is quite possible that the motility and/or sperm count might be affected by causes extraneous to basic physiological capacity, such as long retention of sperm before ejaculation, lowering the motility, or, conceivably, ejaculation shortly before the sample was taken, lowering the sperm count. These circumstances could explain some instances of low factor-high DNA combinations. To explain the few instances when low DNA is associated with high factors and vice-versa in all three samples, it is necessary to go a little further. It is more than possible, it is likely that motility and sperm count are affected by physiologically basic causes that might have nothing to do with DNA. Conversely, one cannot reject the idea that DNA might be affected in a fraction unconcerned with motility and sperm count. After one has given due consideration to all these possibilities, it becomes unreasonable to expect a black and white correlation. In view of the many opportunties for non-correlation, the statistical correlation actually found becomes all the more impressive.

The probability that has emerged is further vindication of the time and effort involved. If the number of samples had been smaller, the individual inconsistencies might have been so numerous that one would have been justified in discarding the possibility of any relationship; or, if the consistency had slightly outweighed the inconsistency, possibly because of the restriction of samples of poor quality, the difference might have been too slight to be convincing or even to warrant a follow-up. The validity of the conclusions reached rests upon exhaustive analysis of a large amount of unselected data, both of which conditions are prerequisite to the statistical argument.

Evidence for the relationship of DNA to motility and sperm count has a direct bearing on the problem of elimination of the DNA-doubtful animals prior to breeding, for sperm count and motility examination are easily and rapidly carried out, while DNA analysis, like all quantitative techniques, is comparatively difficult and time-consuming. If a rigorous screening program had been applied to the 57 animals charted in Fig. 30, to the extent of rejecting all bulls that did not produce high motility, morphology, and sperm count levels in at least two out of the three tests, 15 bulls with at least two of the three tests in the low DNA range would have been eliminated; but, on the other hand, four would have escaped. Seventy-five per cent of the animals of doubtful DNA would have been eliminated without the necessity of DNA analysis, but about seven per cent of the total of fifty-seven bulls would have escaped even this rigorous degree of screening.

Important as is the application of this information to animal breeding, its theoretical significance has even wider horizons. Although the quantitative constancy of DNA, its correlation with degree of ploidy of the nucleus is, in general, well established, opinion is not sufficiently unanimous to reject the possibility of

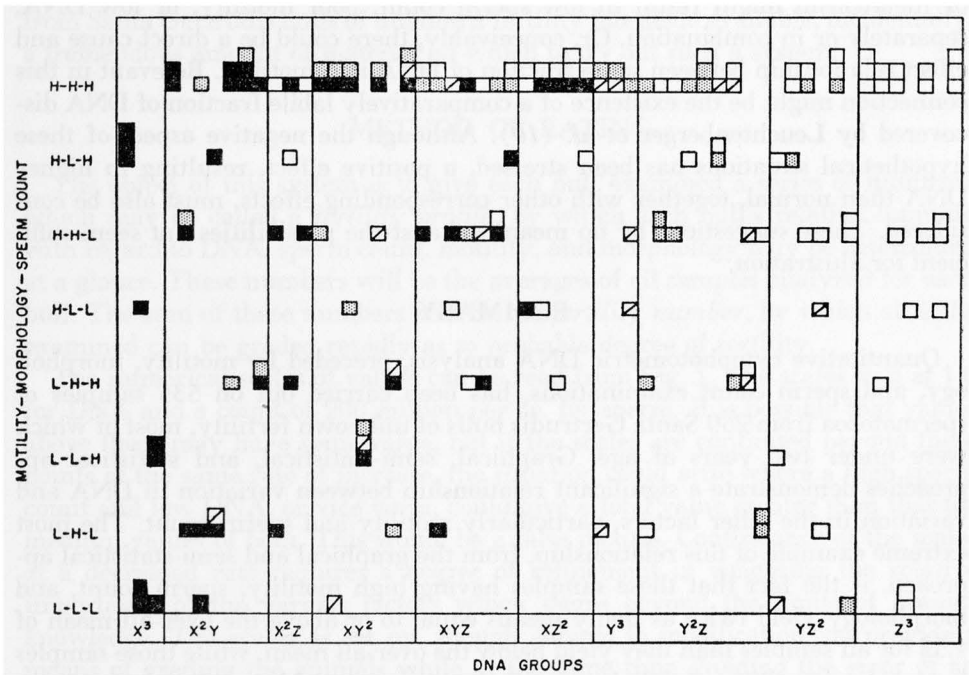


FIG. 30. Relationship between levels of relative DNA means and combinations of 'high' and 'low' motility, morphology and sperm count in samples of spermatozoa from 57 bulls, considered individually, of unknown fertility under two years of age. 'H' is equivalent to motility better than fair, morphology less than 25% abnormality, sperm count 100 million per cc or higher. 'L' is equivalent to motility fair or worse, morphology greater than 20% abnormality, sperm count less than 100 million per cc.

$X < 1.45$ in relative DNA units and is represented by the solid square symbol.

$Y = 1.45 - 1.59$ in relative DNA units and is represented by the square symbol containing a diagonal line for the interval 1.45 — 1.51 inclusive, and by the stippled square symbol for the interval 1.52 — 1.59 inclusive.

$Z > 1.59$ in relative DNA units and is represented by the open square sysmbol.

any variation. Perhaps the most extreme statement of the constancy theory in its relation to cytophotometric technique has been made by Patau and Swift (*19*). A more liberal view is expressed by Leuchtenberger (*12*). Brachet (*2*) has reviewed evidence for variation. Further suggestive evidence has recently been obtained by the technique of autoradiography (*4, 21*), and Schultz, in a recent theoretical paper (*24*), has considered some of the wider implications of possible quantitative variation in DNA. In the absence of direct evidence, one can only speculate on how the apparent relationship between DNA and motility and sperm count could be expressed in physiological terms. In general, it could be an association rather than a correlation, each being a separate and fundamentally equal aspect of some underlying deficiency with multiple, radiating effects. Or some degree of pleiotropism could be involved. Or there could be a cause and effect relationship between DNA and the other factors, which might also include elements of pleiotropism. Specifically, there might be some impaired capacity for DNA synthesis in the testis, of genetic or environmental origin, resulting in fewer germ cells or more sluggish spermatogenesis, together with a smaller amount of DNA in the individual sperm. Some basic defect in testis development or metabolism might result in low sperm count, poor motility, or low DNA, separately or in combination. Or, conceivably, there could be a direct cause and effect relationship between some fraction of DNA and motility. Relevant in this connection might be the existence of a comparatively labile fraction of DNA discovered by Leuchtenberger *et al.* (*10*). Although the negative aspect of these hypothetical situations has been stressed, a positive effect, resulting in higher DNA than normal, together with other corresponding effects, must also be considered. These suggestions by no means exhaust the possibilities but seem sufficient for illustration.

SUMMARY

Quantitative cytophotometric DNA analysis, preceded by motility, morphology, and sperm count examinations, has been carried out on 535 samples of spermatozoa from 259 Santa Gertrudis bulls of unknown fertility, most of which were under two years of age. Graphical, semi-statistical, and statistical approaches demonstrate a significant relationship between variation in DNA and variation in the other factors, particularly motility and sperm count. The most extreme example of this relationship, from the graphical and semi-statistical approach, is the fact that those samples having high motility, sperm count, and morphology yield twice as many means equal to or above the over-all mean of 1.52 for all samples than they yield below the over-all mean, while those samples having low motility, morphology, and sperm count yield a ratio that is almost the reciprocal. Correlation coefficients and multiple regression coefficients show the highest correlation among the factors themselves exclusive of DNA but also show a significant correlation between DNA and the other factors. This is highest for DNA and motility, intermediate for DNA and sperm count, and lowest for DNA and morphology. There are many exceptions, for which plausible explanations have been offered. The practical and theoretical significance of the data is discussed.

VI. A Rating System for Bulls of Unknown Fertility

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The classification of a large number of young, untested bulls according to breeding potential becomes a difficult juggling feat if one attempts to rate the individual bulls by comparison of their respective scores on three or more factors of fertility, and perhaps on several samples of semen. If, on the other hand, all the results of the separate examinations and analyses could be summed up in one over-all fertility rating, the task would be greatly simplified. And it would be most convenient to be able to express this rating as a number. Then, all those of a certain numerical level could be retained for breeding, or a sales valuation could be placed on a bull according to the level of his fertility number. In the following paper an attempt has been made to do this. The extensive investigation carried out on the deoxyribonucleic acid (DNA) content of semen spermatozoa from Santa Gertrudis bulls of unknown fertility has made available, incidentally, a tremendous amount of material on which to try out such a system.

METHOD OF RATING

The object of this system is to give each bull examined a series of numbers, which may be called a *fertility profile*, by which each bull's relative standing with regard to DNA, sperm count, motility, and morphology may be determined at a glance. These numbers will be the averages of all samples analyzed for each bull. The sum of these numbers will be the *fertility number*, by which all bulls examined can be graded rapidly as to probable degree of fertility.

The numerical scales of values can be read from Fig. 1. The scales stop at 1.6 for DNA and a count of 200 million per cc. for sperm. It is possible that values above these may have some value, but if the scales are continued beyond these points at the same rate of increase, the situation could arise where a high sperm count and low DNA, or vice versa, could give a total value greater than that for medium values of both. This would be a false picture. Continuance of the scales at a decreased rate requires a degree of refinement in weighting the relative importance of the various factors which seems beyond the limits of present knowledge. The scales as set up, though subject to modification, do provide a means of grading the animals while at the same time avoiding the error of an artificial precision.

In the light of present knowledge at least, sperm count, motility, and morphology can be expressed in values relative to each other more easily than they can be related to DNA. The scales have been arranged so that an increase in motility from *fair* to *excellent* increases the final value the same amount as an increase in sperm count from 10 to 100 million.* The morphology scale has been

* The sperm count could also be modified according to the percentage of non-motile sperm, when this information is available.

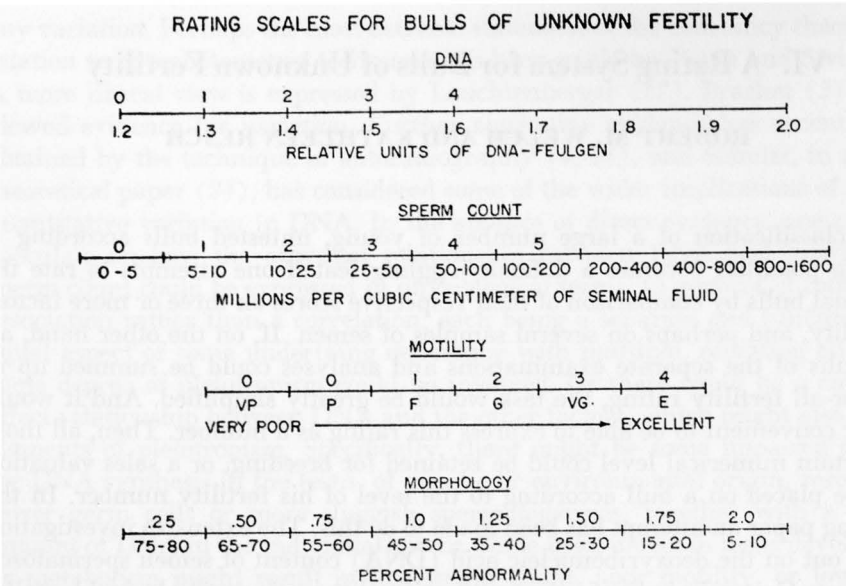


FIG. 1. Rating scales for bulls of unknown fertility.

arranged so that increasing abnormality and increasing sperm count are balanced more or less against each other.

DNA is probably an all or none factor, but there are degrees of statistical probability. For instance, it is statistically more probable that a value of 1.5 or 1.6 is satisfactory as to DNA than one of 1.4 or 1.3. The scale has been arranged so that an increment of one-tenth in the DNA value increases the probability of satisfying the DNA requirement to the same extent as doubling the sperm count improves the animals fertility in this respect.

APPLICATION OF RATING SYSTEM

Individual sample fertility profiles and both individual and cumulative fertility numbers for the bulls of known fertility and infertility and the Groups A, B, and C bulls of unknown fertility are listed in Tables 1 through 7. Group A animals were prospective herd sires tested during the first year; Group B, 135 prospective herd sires tested during the second year; and Group C, 21 reject animals tested over an extended period. These tables serve two main purposes: they furnish a means of determining the value of multiple tests for fertility number or for any individual factor; and they offer a quick and convenient method of evaluating quantitatively the over-all quality of a group of bulls in the same or different years.

For example, one might ask the questions: What is the advantage of two samples over one sample in determining the most reliable fertility number? Are three tests better than two? Is there any advantage of taking more than three samples and spreading these over a considerable period of time? In answering these questions, one can look at the tables from a considerable number of angles. If one assumes that one point in the fertility number is an acceptable margin of devi-

ation, there are still 23 bulls of the 57 in Table 2 that have a cumulative fertility number for two samples differing by more than one point from the individual fertility number for the first sample. Presumably, for these 23 bulls, one sample would not have provided a reliable fertility number within the limit set. There

TABLE 1

Fertility ratings of Santa Gertrudis bulls of known fertility and infertility

		Fertility profile				Fertility no.	
	No. of bull	DNA	Sperm count	Motility	Morphology	Individual	Cumulative
Group 1—Fertile	1 (C $\frac{1}{1}$ 34)	0.0	3	1	2.0	6.0	..
		1.2	3	3	2.0	9.2	..
		3.3	5	4	2.0	14.2	9.8(11.8)
		4.0(1.61)	5	4	2.0	15.0	..
		3.6	4	4	2.0	13.6	..
		4.0(1.63)	5	4	2.0	15.0	12.2(14.5)
	9 (K pt)	2.1	3	2	2.0	9.1	..
		2.7	4	4	2.0	12.7	..
		3.4	5	3	2.0	13.4	11.7(13.1)
		3.7	5	2	2.0	12.7	..
		4.0(1.66)	4	3	2.0	13.0	12.2(13.7)
	2 (= L^{R} 3)	3.8	5	2	2.0	12.8	..
		2.4	5	4	2.0	13.4	..
		3.8	5	4	2.0	14.8	13.7(14.8)
		3.0	4	3	2.0	12.0	..
		2.3	3	0	2.0	7.3	12.1(14.2)
	4 (L 707)	4.0(1.61)	4	4	2.0	14.0	..
		4.0(1.73)	4	4	2.0	14.0	..
		2.4	3	2	2.0	9.4	12.5(14.0)
		1.6	4	4	2.0	11.6	..
		2.2	3	1	2.0	8.2	11.4(13.5)
	3 (W 956)	3.7	5	4	2.0	14.7	..
		0.9	3	3	2.0	8.9	..
		1.2	3	2	2.0	8.2	10.6(12.0)
		3.7	4	3	2.0	12.7	..
		4.0(1.61)	3	2	2.0	11.0	11.1(13.1)
Group 10—Fertile	28	3.1	5(410)	4	2.0	14.1	..
		4.0(1.90)	5	4	1.75	14.75	..
		3.2	5(265)	4	1.50	13.7	14.2(14.5)
	22	4.0(1.86)	4	3	1.75	12.75	..
		3.3	5	1	1.75	11.05	..
		4.0(1.67)	5	4	1.75	14.75	12.9(14.3)
	993	3.7	5(300)	3	1.75	13.45	..
		3.4	5	2	2.0	12.4	..
		1.9	5(630)	4	2.0	12.9	12.9(14.0)
	734	3.8	5	4	2.0	14.8	..
		4.0(1.71)	5	2	2.0	13.0	..
		3.1	4	2	1.50	10.6	12.8(13.9)
	2	2.1	4	4	1.50	11.6	..
		4.0(1.61)	2	3	1.75	10.75	..
		4.0(1.78)	4	4	2.00	14.0	12.1(13.9)

TABLE 1—Continued

Fertility ratings of Santa Gertrudis bulls of known fertility and infertility

		Fertility profile				Fertility no.	
	No. of bull	DNA	Sperm count	Motility	Morphology	Individual	Cumulative
Group 1—Infertile	10 (KMD)	2.5	4	0	2.0	8.5	. .
		2.0	5	4	2.0	13.0	. .
		3.7	5	4	2.0	14.7	12.1(14.1)
		3.2	4	4	2.0	13.2	. .
		2.9	3	2	2.0	9.9	11.9(13.9)
	11 (LMD)	2.7	4	2	2.0	10.7	10.7
	8 (L 727)	2.5	4	2	2.0	10.5	. .
		3.2	4	1	2.0	10.2	. .
		1.6	4	1	2.0	8.6	9.8(10.4)
		3.6	4	0	2.0	9.6	. .
		3.5	5	2	2.0	12.5	10.3(11.4)
	5 (K–51)	3.5	4	0	2.0	9.5	. .
		4.0(1.72)	4	0	2.0	10.0	. .
		3.3	3	0	2.0	8.3	9.3(9.8)
	7 (C–15)	1.4	3	0	2.0	6.4	. .
		1.9	3	0	2.0	6.9	. .
		2.1	3	2	2.0	9.1	7.5(8.0)
		2.2	3	0	2.0	7.2	. .
		4.0(1.65)	4	2	2.0	12.0	8.3(9.4)
	6 (C–322)	2.8	4	1	2.0	9.8	. .
		1.0	3	0	2.0	6.0	. .
		2.0	3	0	2.0	7.0	7.6(8.4)
		2.1	3	0	2.0	7.1	7.5

No morphology examination was made of Group 1 bulls, but, in order to complete profile, rating of "2" has been arbitrarily assigned to each bull.

Sperm count of Group 1 bulls was recorded as "low", "medium" or "high" from ranch and laboratory examination and amount of centrifuged sediment present. Profile ratings of 3, 4 and 5 were assigned respectively to low, medium and high sperm count.

Numbers in parenthesis in extreme right column is for each bull: 1st—fertility number for best 2 out of 3 samples; 2nd—fertility number for best 3 out of 5 or 4 out of 6 samples. Each factor considered independently.

TABLE 2

Fertility rating of Group A bulls of unknown fertility derived from three samples of spermatozoa each

	Fertility profile				Fertility no.	
No. of bull	DNA	Sperm count	Motility	Morphology	Individual	Cumulative
10–108	4.0(1.90)	5(220)	4	2.0	15.0	. .
	4.0(1.69)	5(290)	4	2.0	15.0	15.0
	3.8	5(236)	4	2.0	14.8	14.9(15.0)
9–78	4.0(1.64)	3	4	2.0	13.0	. .
	4.0(1.63)	5(845)	4	2.0	15.0	14.0
	4.0	5(1470)	4	2.0	15.0	14.3(15.0)
10–48	4.0(1.61)	5(1110)	4	2.0	15.0	. .
	4.0(1.71)	5(252)	2	2.0	13.0	14.0
	4.0(1.65)	5(350)	4	1.75	14.75	14.2(15.0)
3–1	3.0	5	4	2.0	14.0	. .
	3.4	5(522)	4	1.75*	14.15	14.1
	3.2	5(255)	4	1.75	13.95	14.0(14.2)
3–8	3.7	4	4	2.0	13.7	. .

	2.6	5(288)	4	1.75*	13.35	13.5
	4.0(1.69)	5(1680)	4	1.75	14.75	13.9(14.7)
8–74	4.0(1.69)	5(310)	4	1.75	14.75	. .
	4.0(1.87)	5(300)	2	1.75	12.75	13.8
	3.6	5(790)	4	1.50	14.10	13.9(14.8)
10–111	3.6	5(400)	4	2.0	14.6	. .
	3.0	5(306)	4	2.0	14.0	14.3
	1.9	5(840)	4	2.0	12.9	13.8(14.3)
9–84	3.8	5	4	2.0	14.8	. .
	4.0(1.78)	2	4	1.75	11.75	13.3
	4.0(1.63)	5(490)	4	1.75	14.75	13.8(14.9)
10–110	2.1	5(200)	2	2.0	11.1	. .
	4.0(1.65)	5(480)	4	2.0	15.0	13.0
	4.0(1.76)	5(420)	4	2.0	15.0	13.7(15.0)
8–72	4.0(1.89)	5(420)	4	1.75	14.75	. .
	4.0(1.83)	5(320)	2	2.0	13.0	13.9
	4.0(1.75)	5(1000)	2	2.0	13.0	13.6(14.0)
3–2	2.3	5	4	2.0	13.3	. .
	3.3	5(720)	4	2.0*	14.3	13.8
	2.3	5(540)	4	2.0	13.3	13.6(13.8)
8–76	2.9	5(580)	4	2.0	13.9	. .
	3.5	5(980)	4	1.5	14.0	14.0
	4.0(1.78)	5(410)	2	1.5	12.5	13.5(14.5)
8–92	4.0(1.61)	5	1	2.0	12.0	. .
	4.0(1.75)	5	2	2.0	13.0	12.5
	4.0(1.94)	5(600)	4	2.0	15.0	13.3(14.0)
8–67	4.0(1.71)	5(570)	4	2.0	15.0	. .
	3.7	5(240)	2	2.0	12.7	13.9
	3.5	5(520)	2	1.75	12.25	13.3(13.8)
9–100	4.0(1.72)	5(270)	4	2.0	15.0	. .
	4.0(1.77)	3	2	1.75	10.75	12.9
	4.0(1.65)	5(350)	3	2.0	14.0	13.3(14.5)
10–113	3.9	5(410)	3	1.75	13.65	. .
	4.0(1.84)	5(400)	3	1.75	13.75	13.7
	1.7	5(380)	4	1.50	12.20	13.2(14.2)
9–95	4.0(1.63)	3	4	2.0	13.0	. .
	3.2	5	4	2.0	14.2	13.6
	2.3	4	4	2.0	12.3	13.2(14.1)
3–9	3.4	5(352)	4	2.0	14.4	. .
	1.6	4	3	2.0	10.6	12.5
	4.0(1.69)	5(238)	3	2.0	14.0	13.0(14.2)
10–03	2.5	4	3	2.0	11.5	. .
	3.6	5(225)	4	2.0	11.6	13.0
	1.9	5(220)	4	1.75	12.65	12.9(14.0)
7–58	3.5	5	4	1.75	14.25	. .
	2.9	4	4	2.0	12.9	13.6
	2.8	4	3	1.5	11.3	12.8(13.6)
8–103	2.8	4	4	2.0	12.8	. .
	2.1	5(1000)	1	2.0	10.1	11.4
	4.0(1.79)	5(250)	4	2.0	15.0	12.6(14.4)
9–102	4.0(1.76)	3	4	1.75	12.75	. .
	4.0(1.81)	4	4	1.25	13.25	13.0
	4.0(1.67)	2	4	1.75	11.75	12.6(13.2)
9–97	4.0(1.60)	5(290)	4	2.00	15.00	. .
	4.0(1.61)	5(380)	1	1.75	11.75	13.4
	1.9	5(620)	2	2.00	10.90	12.6(14.0)

TABLE 2—Continued

Fertility rating of Group A bulls of unknown fertility derived from three samples of spermatozoa each

No. of bull	Fertility profile				Fertility no.	
	DNA	Sperm count	Motility	Morphology	Individual	Cumulative
3–11	3.2	3	4	2.00	12.2	. .
	3.3	5(244)	4	2.00	14.3	13.2
	1.4	5(640)	3	1.75	11.15	12.6(14.2)
4–50	3.3	4	4	1.5	12.8	. .
	3.6	5(1480)	3	2.0	13.6	13.2
	1.5	5(380)	3	2.0	11.5	12.6(14.0)
8–88	3.7	5(210)	0	2.00	10.7	. .
	4.0(1.79)	5	2	1.75	12.75	11.7
	2.8	5(340)	4	2.00	13.8	12.4(13.8)
3–14	3.7	2	2	1.5*	9.2*	. .
	3.2	5(240)	4	2.0	14.2	11.7
	4.0	5(835)	3	1.5	13.5	12.3(14.1)
6–N–1	2.2	5	2	2.0	11.2	. .
	2.2	5(915)	4	2.0	13.2	12.2
	3.5	5(295)	2	2.0	12.5	12.3(12.8)
4–55	1.7	4	3	1.5	10.2	. .
	4.0(1.64)	5	3	1.75	13.75	12.0
	4.0(1.61)	5	2	2.0	13.0	12.3(13.9)
10–114	4.0(1.61)	5(1120)	2	1.5	12.5	. .
	4.0(1.81)	5(460)	2	1.75	12.75	12.6
	0.6	5	4	1.75	11.35	12.2(13.8)
9–96	4.0(1.76)	5(450)	3	1.75	13.75	. .
	4.0(1.76)	2	2	1.25	9.25	11.5
	2.4	5(440)	4	1.75	13.15	12.1(14.2)
2–75	3.9	4*	2	2.0*	11.9	. .
	3.5	4*	2	1.75*	11.25	11.6
	4.0	5(310)	2	1.75	12.75	12.0(12.3)
2–71	2.5	3*	1	1.75*	8.25	. .
	3.6	5*	3	2.0*	13.6	10.9
	3.1	5*	3	2.0*	13.1	11.7(13.4)
8–101	3.0	4	3	2.0	12.0	. .
	2.9	5	1	2.0	10.9	11.4
	4.0(1.79)	5	1	1.75	11.75	11.6(12.5)
3–10	0.3	3	1	2.0	6.3	. .
	4.0(1.63)	5(644)	3	2.0	14.0	10.2
	3.4	5(910)	4	2.0	14.4	11.6(14.2)
7–49	1.7	5(255)	2	2.0	10.7	. .
	3.8	4	2	2.0	11.8	11.2
	4.0(1.73)	5(230)	1	2.0	12.0	11.5(12.9)
9–99	4.0(1.76)	3	1	2.0	10.0	. .
	3.1	4	3	1.25	11.35	10.7
	4.0(1.72)	3	4	1.75	12.75	11.4(12.9)
8–68	0.7	5(870)	4	2.0	11.7	. .
	3.3	5(620)	1	2.0	11.3	11.5
	0.1	5(480)	4	2.0	11.1	11.4(13.0)
6–N–2	2.1	5(540)	1	2.0	10.1	. .
	2.4	5(980)	3	2.0	12.4	11.2
	3.8	5(740)	1	1.75	11.55	11.4(12.1)
3–7	1.0	5	4	1.50	11.5	. .

	1.8	5	4	1.50	12.3	11.9
	0.0	5	3	1.50	9.5	11.1(11.9)
2–70	4.0(1.69)	3*	1	1.75*	9.75	. .
	3.5	5*	3	1.75*	13.25	11.5
	3.3	5	0	1.75	10.05	11.0(12.5)
8–71	2.3	5	2	2.0	11.3	. .
	4.0(1.72)	5	2	1.5	12.5	11.9
	0.7	5	1	2.0	8.7	10.8(12.2)
4–49	3.0	4	0	1.5	8.5	. .
	3.9	5	2	1.75	12.65	10.6
	1.4	5(455)	3	1.75	11.15	10.8(12.7)
8–70	1.7	4	4	2.0	11.7	. .
	1.0	2	1	2.0	6.0	8.9
	4.0(1.89)	5(640)	3	2.0	14.0	10.6(12.8)
7–61	3.0	4	1	1.0	9.0	. .
	4.0(1.68)	5(910)	2	1.25	12.25	10.6
	4.0(1.66)	5(217)	0	1.0	10.00	10.4(11.6)
4–54	1.8	1	2	1.75	6.55	. .
	4.0(1.73)	4	3	1.50	12.50	9.5
	3.0	5	2	2.00	12.00	10.4(12.4)
6–N–3	3.6	4	0	1.0	8.6	. .
	4.0	3	2	1.75	10.75	9.7
	4.0(1.63)	3	2	1.75	10.75	10.1(11.2)
9–81	3.3	5(300)	1	1.0	10.3	. .
	2.6	5(250)	1	0.75	9.35	9.8
	2.0	5(290)	1	1.0	9.00	9.6(10.0)
10–49	4.0(1.66)	5(910)	4	2.0	15.0	. .
	2.1	0	0	2.0*	4.1	9.6
	2.3	2	3	2.0	9.3	9.5(12.2)
10–109	2.5	5(670)	0	1.75	9.25	. .
	1.5	5(370)	0	1.75	8.25	8.8
	3.4	4	0	2.00	9.40	9.0(9.8)
9–90	3.9	0	1	1.75	6.65	. .
	3.5	3	1	1.75	9.25	8.0
	4.0(1.78)	2	2	2.0	10.00	8.6(9.8)
3–12	3.5	5	2	1.75	12.25	. .
	0.9	4	1	1.25	7.15	9.7
	0.0	3	1	2.0	6.00	8.5(10.1)
9–80	4.0(1.67)	1	1	1.5	7.5	. .
	4.0(1.82)	2	1	1.25	8.25	7.9
	4.0(1.63)	2	2	1.5	9.5	8.4(9.0)
3–4	3.6	2	2	2.0	9.6	. .
	2.4	4	2	2.0	10.4	10.0
	0	3	0	2.0	5.0	8.3(10.5)
8–69	0.7	5	1	0.5	7.2	. .
	2.2	4	1	1.5	8.7	8.0
	1.3	5	1	1.0	8.3	8.1(9.0)
4–56	2.9	3	1	1.75	8.65	. .
	0.8	5	3	1.25	10.05	9.4
	0.0	2	1	2.0	5.0	7.9(9.7)
4–52	2.0	1	2	1.25	6.25	. .
	0.0	3	1	1.25	5.25	5.8
	1.1	3	1	1.50	6.60	6.1(7.4)

* Estimated from available information.

Numbers in parenthesis in the extreme right column are fertility numbers for the best two out of three combinations in each sample group.

TABLE 3

Fertility rating of Group A bulls of unknown fertility derived from two samples of spermatozoa each

No. of bull	Fertility profile				Fertility no.	
	DNA	Sperm count	Motility	Morphology	Individual	Cumulative
8–73	3.0	5	3	2.0	13.0	..
	4.0(1.67)	4	4	2.0	14.0	13.5
9–105	4.0(1.79)	5(240)	2	1.75	12.75	..
	3.2	5(200)	4	1.50	13.7	13.2
9–98	4.0(1.64)	4	4	1.75	13.75	..
	4.0(1.67)	4	2	2.0	12.0	12.9
10–50	2.8	4	4	2.0	12.8	..
	4.0(1.78)	3	4	2.0	13.0	12.9
9–91	3.8	4	4	1.50	13.5	..
	3.1	3	2	2.0	10.1	11.8
10–02	3.3	3	2	2.0	10.3	..
	3.4	5	3	2.0	13.4	11.8
9–87	4.0(1.87)	4	2	1.75	11.75	..
	3.1	5	2	1.75	11.85	11.8
7–46	3.1	5	4	2.0	14.1	..
	4.0	2	1	2.0	9.0	11.6
8–65	3.3	3	2	1.75	10.05	..
	3.1	5(940)	2	2.0	12.1	11.1
9–89	4.0(1.83)	3	2	2.0	11.0	..
	3.4	4	2	1.50	10.9	11.0
8–77	0.0	3	4	2.0	9.0	..
	1.4	5	4	2.0	12.4	10.7
7–48	3.8	3	1	2.0	9.8	..
	4.0(1.66)	5(265)	0	2.0	11.0	10.4
4–61	2.4	2	1	1.25	6.65	..
	4.0(1.64)	5	3	0.75	12.75	9.7
7–60	2.5	4	1	1.25	8.75	..
	3.6	5(217)	0	1.25	9.85	9.3
4–48	1.9	3	1	1.0	6.9	..
	2.4	5	3	0.75	11.15	9.0
7–56	3.0	3	2	2.0	10.0	..
	0.0	5(360)	1	2.0	8.0	9.0
7–59	0.7	4	2	0.75	7.45	..
	3.0	5(260)	1	1.0	10.0	8.7
7–57	4.0(1.63)	3	1	1.0	9.0	..
	2.9	5(280)	0	0.5	8.4	8.7
8–94	0.0	5	1	1.0	7.0	..
	4.0(1.80)	5	0	0.75	9.75	8.4
7–55	2.8	4	1	2.0	9.8	..
	0.0	4	1	1.75	6.75	8.3
7–47	3.0	0	2	1.75	6.75	..
	4.0(1.61)	3	0	1.75	8.75	7.8
8–79	0.6	2	1	1.0	4.6	..
	3.0	5	1	1.25	10.25	7.4
4–60	0.0	3	2	1.0	6.0	..
	4.0	3	0	1.5	8.5	7.2
7–50	3.0	0	1	1.75	5.75	..
	4.0(1.65)	2	1	1.50	8.50	7.1

9–82	1.8	0	1	2.0	4.8	..
	4.0(1.69)	1	1	1.75	7.75	6.3
10–1003	0.5	5(210)	0	1.50	7.0	..
	3.6	0	0	2.0	5.6	6.3
7–62	3.2	1	1	1.75	6.95	..
	1.9	1	0	2.0	4.9	5.9

TABLE 4

Fertility rating of Group A bulls of unknown fertility derived from one sample of spermatozoa each

	Fertility profile				
No. of bull	DNA	Sperm count	Motility	Morphology	Fertility number
8–85	3.6	5	2	2.0	12.6
5–67	3.5	5	2	2.0	12.5
10–19	2.9	3	4	2.0	11.9
3–5	3.1	5	0	1.25	9.35
7–51	4.0(1.62)	1	2	1.75	8.75
7–54	0.0	5(315)	1	2.0	8.0
9–93	4.0(1.69)	2	0	1.75	7.75
4–57	0.0	3	1	1.75	5.75
7–45	1.4	0	1	1.0	3.4
7–52	0.0	1	0	2.0	3.0

are 36 bulls out of the 57, the first sample of which differs by more than one point in the fertility number from the second. If the acceptable margin of deviation is increased to two, the number of exceptions decreases considerably but would still include about 10% of the bulls. It seems fairly obvious that a single test would not provide a reliable fertility number for as high as 95% of the bulls. If one compares the cumulative mean of two samples with the cumulative mean of three samples, there are only seven bulls in which the difference amounts to over one point in the fertility numbers and none in which the difference is over two. Of these seven, five could be detected by the fact that the first and second samples deviate by more than two points. According to this, three samples would have little advantage over two, especially when a third sample is taken if the deviation between first and second is over two points. This does not hold, however, if one considers the mean of the best two samples out of three, each factor considered independently, a fairer number than the mean of all three. According to this, there are 25 bulls in which the difference between the mean of two samples and the mean of the best two out of three exceeds one point in the fertility numbers, and, in some cases, the difference reaches as high as three to four points. Of these 25, the first sample differs from the second by more than two points in 16 bulls, by more than one point in three bulls, and by one point or less in 6 bulls. Thus, there would be nine bulls of the 57 in which the first sample would not differ enough from the second to indicate the desirability of taking an additional sample. If, therefore, one uses the mean of best two out of three samples as the most reliable fertility number, three samples would be desirable if one wants to rate 95% or more of the bulls within the limit set of one point permissible devi-

TABLE 5

Fertility rating of Group B bulls of unknown fertility derived from three samples of spermatozoa each

No. of bull	Fertility profile				Fertility no.	
	DNA	Sperm count	Motility	Morphology	Individual	Cumulative
13–21	2.3	3	1	1.50	7.8	..
	4.0(1.70)	5(350)	1	1.50	11.5	9.6
	A. 4.0(1.73)					
	2.6	5(455)	1	1.75	10.35	9.85(10.92)
13–20	3.8	3	1	1.25	9.05	..
	2.8	4	1	0.25	8.05	8.55
	3.1	5	1	0.25	9.35	8.82(9.7)
12–176	2.6	2	2	0.50	7.1	..
	0.3	3	0	0.25	3.85	5.48
	A. 0.9					
	0.4	5(240)	1	1.75	9.95	6.96(9.03)
	A. 4.0(1.62)					

Fertility rating of Group B bulls of unknown fertility derived from two samples of spermatozoa each

No. of bull	Fertility profile				Fertility no.	
	DNA	Sperm count	Motility	Morphology	Individual	Cumulative
13–7	4.0(1.63)	5(380)	4	2.00	15.0	..
	3.4	5(470)	2	2.00	12.4	13.7
12–169	4.0(1.68)	5(310)	3	2.00	13.7	..
	A. 3.4					
	3.6	5	3	2.00	13.6	13.6
12–171	4.0(1.86)	5	4	2.00	15.0	..
	A. 4.0(1.77)					
	1.7	5(580)	3	2.00	10.95	13.0
	A. 0.2					
13–9	4.0(1.69)	5	2	1.75	12.75	..
	3.8	5(880)	2	1.50	12.3	12.52
12–167	4.0(1.71)	4	1	2.00	11.0	..
	A. 4.0(1.70)					
	4.0(1.61)	5(280)	3	2.00	14.0	12.50
13–10	4.0(1.70)	5	4	2.00	15.0	..
	3.4	2	2	2.00	9.4	12.20
14–8	2.8	5	0	1.75	9.55	..
	3.6	5(260)	4	2.00	14.6	12.08
13–16	2.3	4	1	1.75	9.05	..
	3.1	5	4	2.00	14.1	11.6
13–8	3.1	5	1	1.75	10.85	..
	2.0	5	2	1.25	10.05	10.45
	A. 1.6					
13–25	3.4	2	1	1.00	7.4	..
	3.8	5(500)	2	1.75	12.55	10.0
12–164	2.4	4	0	2.00	8.4	..
	4.0(1.83)	5	1	1.25	11.25	9.8
	A. 4.0(1.81)					
14–14	3.1	5	0	1.0	9.1	..
	3.0	5(280)	0	1.0	9.0	9.0
14–10	2.9	5(940)	0	1.25	9.15	..
	2.3	3	0	1.50	6.8	7.98

12–173	2.9	3	0	0.25	6.15	. .
	4.0(1.73)	2	1	0.50	7.5	6.83
	A. 4.0(1.74)					

A.—Additional determination on duplicate slide.

TABLE 6

Fertility ratings of Group B bulls of unknown fertility derived from one sample of spermatozoa each

No. of bull	Fertility profile				Fertility number
	DNA	Sperm count	Motility	Morphology	
13–28	4.0(1.62)	5	4	2.00	15.0
19–69	4.0(1.61)	5(680)	4	2.00	15.0
12–165	4.0(1.61)	5(490)	4	2.00	15.0
19–72	4.0	5(300)	4	2.00	15.0
18–49	4.0(1.72)	5(330)	4	2.00	14.9
	A. 3.8				
17–31	3.9	5(200)	4	2.00	14.9
14–2	4.0(1.68)	5	4	1.75	14.75
	A. 4.0(1.88)				
12–B1	3.7	5	4	2.00	14.7
12–166	4.0(1.65)	5(500)	4	1.75	14.65
	A. 3.8				
18–51	3.9	5(750)	4	1.75	14.65
19–71	3.9	5(560)	4	1.75	14.65
14–19	4.0(1.61)	5	4	1.50	14.5
19–74	3.2	5(430)	4	2.00	14.2
14–9	3.2	5(870)	4	2.00	14.2
13–17	3.6	5(300)	4	1.50	14.1
16–22	4.0(1.65)	5(510)	3	2.00	14.0
	A. 4.0(1.67)				
18–53	4.0(1.80)	5(525)	4	1.00	14.0
	A. 4.0(1.62)				
19–63	4.0	5	3	2.00	14.0
14–13	3.5	5(210)	4	1.50	14.0
15–15	2.9	5(1070)	4	2.00	13.9
12–F	2.9	5(720)	4	2.00	13.9
15–9	2.9	5	4	2.00	13.9
12–P	2.8	5(810)	4	2.00	13.8
16–23	4.0(1.62)	5(640)	3	1.75	13.75
	A. 4.0(1.63)				
13–27	2.7	5(220)	4	2.00	13.7
12–$\frac{W}{1}$ 14	3.9	4	4	1.75	13.65
18–52	3.6	5(660)	3	2.00	13.6
13–6	3.6	5	3	2.00	13.6
14–7	3.3	5	4	1.25	13.55
15–8	3.0	5	4	1.50	13.5
13–15	2.7	5	4	1.75	13.45
17–42	2.0	5(250)	4	1.75	13.45
	A. 3.4				
16–24	4.0(1.70)	5	3	2.00	13.35
	A. 2.7				
16–27	3.3	5(710)	3	2.00	13.3

TABLE 6—Continued

Fertility ratings of Group B bulls of unknown fertility derived from one sample of spermatozoa each

No. of bull	Fertility profile				Fertility number
	DNA	Sperm count	Motility	Morphology	
16–30	3.3	5(760)	3	2.00	13.3
18–48	2.7	5	4	1.50	13.2
18–54	3.6	5(640)	3	1.50	13.1
15–2	2.3	5(270)	4	1.75	13.05
12–82	4.0(1.62)	5(560)	2	2.00	13.00
12–J	4.0(1.60)	5(610)	2	2.00	13.0
18–58	4.0(1.68)	5(590)	4	0.50	12.9
	A. 2.8				
17–33	3.9	5	2	2.00	12.9
17–39	2.9	5(400)	3	2.00	12.9
18–55	2.9	5	3	2.00	12.9
17–43	2.9	5	3	2.00	12.9
17–35	3.6	5(510)	3	1.25	12.85
12–168	4.0(1.74)	5	3	2.00	12.8
	A. 1.6				
19–64	3.3	5	3	1.50	12.8
13–3	4.0(1.66)	5	2	1.75	12.75
13–13	1.7	5(560)	4	2.00	12.7
16–18	2.6	5(950)	3	2.00	12.6
14–1	3.4	5(690)	2	2.00	12.4
13–5	4.0(1.65)	5	2	1.25	12.25
	A. 4.0(1.72)				
16–29A	4.0(1.65)	5(245)	2	2.00	12.25
	A. 2.5				
19–65	2.2	5	3	1.50	12.25
	A. 3.3				
14–4	3.4	5(315)	2	1.75	12.15
14–6	1.9	4	4	1.75	12.15
	A. 2.9				
16–20	3.4	5	2	1.75	12.15
18–60	1.0	5	3	1.75	12.15
	A. 3.8				
12–P_1	1.9	5	4	2.00	12.10
	A. 0.3				
16–25	3.6	5(450)	2	1.50	12.10
18–46	2.6	5	3	1.50	12.10
13–14	4.0(1.65)	4	2	2.00	12.00
17–45	3.0	4	3	2.00	12.00
12–$\frac{1}{10}$–6	4.0(1.66)	4	4	2.00	12.00
	A. 0.0				
14–12	3.2	5	2	1.75	11.95
14–16	3.2	5(270)	2	1.75	11.95
18–47	2.9	5	3	1.00	11.90
18–50	2.6	5(230)	3	1.25	11.85
15–11	2.1	4	4	1.75	11.85
15–5	2.8	3	4	2.00	11.8
13–(10–6)	3.0	5(310)	2	1.75	11.75

12–$\frac{R}{1}$3	3.7	4	2	2.00	11.70
12=137	0	5	4	1.75	11.70
	A. 1.9				
14–15	4.0(1.75)	5	2	0.75	11.68
	A. 4.0(1.88)				
	A. 3.8				
17–38	1.9	5	3	1.75	11.65
	A. 1.9				
15–4	0.9	5(250)	4	1.75	11.65
15–3	0.9	5(230)	4	1.75	11.65
17–37	3.6	5(280)	2	1.00	11.60
17–36	2.8	4	3	1.75	11.55
12–X	2.0	5	2	1.50	11.50
	A. 4.0(1.70)				
15–10	2.7	3	4	1.75	11.45
13–22	3.4	4	2	2.00	11.40
14–5	3.3	5	2	1.00	11.30
18–56	0.6	5	2	2.00	11.25
	A. 3.9				
13–23	2.2	5	2	2.00	11.20
13–2	3.4	5(415)	1	1.75	11.15
15–7	2.4	5(220)	2	1.75	11.15
16–19	3.4	4	2	1.75	11.15
13–24	2.1	5	2	2.00	11.10
15–13	2.1	5(1370)	2	2.00	11.10
19–68A	2.3	5(270)	3	0.75	11.00
	A. 2.2				
13–19	3.0	4	2	1.75	10.75
15–1	1.5	4	4	1.25	10.75
19–70	3.9	4	2	0.75	10.65
16–29B	3.9	5(425)	0	1.75	10.65
17–32	1.6	5(360)	2	1.50	10.60
	A. 2.6				
19–68B	3.8	2	3	1.75	10.55
18–57	4.0(1.62)	4	2	2.00	10.45
	A. 0.9				
16–21	3.5	5(330)	0	1.75	10.25
17–40	0.4	4	3	1.75	10.25
	A. 2.6				
13–11	2.2	4	2	2.00	10.20
16–26	4.0(1.64)	4	0	2.00	10.00
	A. 4.0(1.61)				
18–59	3.2	4	2	0.75	9.95
12–175	0.9	5	2	1.00	9.73
	A. 4.0(1.75)				
	A. 1.3				
19–62	3.3	3	2	1.00	9.30
19–73	1.5	4	2	1.50	9.20
	A. 1.9				
13–1	3.6	2	2	1.50	9.10
12–$\frac{1}{12}$2	2.9	2	2	2.00	8.90
13–18	2.7	3	1	2.00	8.70

TABLE 6—Continued

Fertility ratings of Group B bulls of unknown fertility derived from one sample of spermatozoa each

No. of bull	Fertility profile				Fertility number
	DNA	Sperm count	Motility	Morphology	
14–3	3.6	3	0	2.00	8.60
17–44	1.8	2	2	2.00	8.10
	A. 2.4				
19–66	2.1	3	2	0.75	8.05
	A. 2.5				
12–$\frac{K}{4}$8	0.00	2	2	1.75	6.30
	A. 1.1				
16–17	2.9	1	0	2.00	5.90
14–20	2.1	3	0	1.25	5.30
	A. 0.0				
12–174	3.0	1	0	0.25	4.25
12–177	0.0	2	0	0.25	2.50
	A. 0.5				

A.—Additional determination on duplicate slide.

TABLE 7

Fertility ratings of Group C bulls of unknown fertility derived from several samples of spermatozoa each

No. of bull	Fertility profile				Fertility no.	
	DNA	Sperm count	Motility	Morphology	Individual	Cumulative
O5	3.5	5(560)	4	2.00	14.5	. . .
	4.0(1.62)	5(530)	4	2.00	15.0	. . .
	3.5	5(550)	4	2.00	14.5	14.7(14.8)
	4.0(1.66)	5	4	2.00	15.0	14.8
	. .	5(780)	4	2.00	. . .	. . .
U11	2.4	5(1140)	4	2.00	13.4	. . .
	3.4	5(580)	4	2.00	14.4	. . .
	4.0	5(1010)	4	2.00	15.0	14.3(14.7)
	4.0	5(760)	4	2.00	15.0	. . .
	3.4	5(2030)	4	1.50	13.9	. . .
	3.2	5	1	2.00	11.2	13.8(14.7)
	2.7	5(670)	4	2.00	13.7	13.8
O9	3.6	5(490)	2	2.00	12.6	. . .
	2.9	5(200)	4	2.00	13.9	. . .
	3.4	5(210)	4	2.00	14.4	13.6(14.5)
	4.0(1.61)	5(300)	4	2.00	15.0	. . .
	2.9	5(710)	4	2.00	13.9	. . .
	2.7	5(290)	0	1.50	9.2	13.2(14.5)
	3.4	5	4	1.75	14.15	13.3
U9	2.3	5(250)	4	2.00	13.3	. . .
	2.7	5(320)	4	2.00	13.7	. . .
	2.4	5(210)	4	2.00	13.4	13.5(13.6)
	4.0(1.63)	5(430)	4	2.00	15.0	. . .
	3.5	5(430)	1	2.00	11.5	. . .
	3.4	5	4	2.00	14.4	13.6(14.4)

	3.5	5	4	2.00	14.5	...
	4.0	5(840)	4	2.00	15.0	...
	3.7	3	1	2.00	9.7	13.4(14.7)
	2.9	4	2	2.00	10.9	13.1
O8	3.3	5(270)	4	2.00	14.3	...
	4.0(1.74)	5(580)	4	2.00	15.0	...
	3.8	5(930)	4	2.00	14.8	14.7(14.9)
	3.4	5(510)	4	2.00	14.4	...
	0.5	5(430)	1	2.00	8.5	...
	2.0	5(560)	2	1.50	10.5	12.9(14.6)
	..	5(480)	4	1.75	...	...
O6	2.9	5(630)	4	1.75	13.65	...
	1.0	5	4	2.00	12.0	...
	2.6	5	4	2.00	13.6	13.1(13.8)
	3.4	5	2	2.00	12.4	12.9
	..	3	2	1.75	...	...
U2	3.7	5	3	2	13.7	...
	2.0	4	0	2	8.0	...
	4.0(1.75)	5(200)	4	2	15.0	12.2(14.4)
	3.6	5(260)	4	2	14.6	...
	3.7	5	2	2	12.7	12.8
	..	0	.	.	...	...
U7	4.0(1.61)	5(310)	4	2.00	15.0	...
	..	3	4	2.00	...	...
	4.0(1.67)	5(410)	4	2.00	15.0	...(15.0)
	4.0(1.74)	5(1010)	4	2.00	15.0	15.0
	4.0(1.66)	5(290)	.	1.75	...	...
	2.5	4	0	2.00	8.5	...(15.0)
	2.5	5(855)	2	1.75	11.25	...
	2.5	5(405)	4	1.75	13.25	13.0
	3.1	5(1150)	3	2.00	13.1	...(14.4)
	4.0(1.61)	5	4	2.00	15.0	...
	2.7	4	0	2.00	8.7	12.8
	2.3	5	0	2.00	9.3	12.4(14.2)
U6	3.3	5(480)	4	1.50	13.8	...
	2.9	5(570)	1	1.75	10.65	12.2
U15	1.1	5(2190)	1	2.00	9.1	...
	3.7	5(630)	2	2.00	12.7	...
	4.0(1.69)	5(400)	4	2.00	15.0	12.3(13.8)
	3.0	5(660)	4	2.00	14.0	...
	2.7	5(450)	0	2.00	9.7	...
	..	5(740)	2	2.00	...	12.1(13.4)
U1	2.7	5	1	2.00	10.7	...
	4.0(1.68)	5(260)	4	2.00	15.0	...
	3.5	5	1	2.00	11.5	12.4(13.3)
	4.0	5(245)	1	2.00	12.0	...
	4.0(1.61)	2	2	1.75	9.75	11.8
O7	4.0(1.62)	3	3	2.00	12.0	...
	4.0	4	3	2.00	13.0	...
	4.0(1.73)	5(385)	1	2.00	12.0	12.3(13.5)
	3.0	5(245)	0	1.50	9.5	...
	1.7	4	4	2.00	11.7	11.6
U5	0.0	4	0	2.00	6.0	...
	4.0	5	4	2.00	15.0	...
	3.3	5(440)	2	2.00	12.3	11.1(13.6)
	3.9	5	4	2.00	14.9	...

TABLE 7—Continued

Fertility ratings of Group C bulls of unknown fertility derived from several samples of spermatozoa each

No. of bull	Fertility profile				Fertility no.	
	DNA	Sperm count	Motility	Morphology	Individual	Cumulative
	4.0(1.79)	5(320)	1	2.00	12.0	...
	1.7	5(240)	0	2.00	8.7	11.5(13.6)
	..	5	4	1.75	...	...
U4	3.5	5(230)	2	1.50	12.00	...
	3.2	5(680)	4	1.75	13.95	...
	4.0(1.61)	5(330)	0	1.75	10.75	12.2(13.5)
	4.0(1.78)	3	2	2.00	11.00	...
	4.0	5(355)	0	1.75	10.75	...
	3.5	5(253)	0	1.50	10.00	11.4(12.7)
	4.0(1.69)	5(295)	2	1.50	12.50	...
	2.8	5(415)	0	1.00	8.80	...
	2.9	5(630)	0	1.75	9.65	11.0(12.3)
	4.0	5(249)	2	1.00	12.00	...
	4.0(1.68)	5(840)	0	1.50	10.50	...
	4.0(1.62)	5(815)	2	1.75	12.75	11.2(13.8)
U8	4.0(1.62)	5	2	2.00	13.00	...
	4.0(1.64)	5(310)	4	2.00	15.00	...
	4.0(1.63)	5(250)	3	1.50	13.50	13.8(14.5)
	3.8	5(250)	2	1.75	12.55	...
	2.5	3	0	1.75	7.25	...
	3.5	5(310)	0	1.25	9.75	11.8(13.6)
	4.0(1.61)	5	2	1.75	12.75	...
	2.6	4	2	1.25	9.85	...
	2.3	2	0	1.50	5.80	11.0(12.6)
	4.0(1.76)	4	2	1.75	11.75	...
	3.7	4	0	1.75	9.45	...
	4.0(1.66)	5	0	1.75	10.75	11.0(12.7)
U14	0.0	5(640)	0	2.00	7.00	...
	2.7	4	0	2.00	8.70	...
	3.3	5(500)	4	2.00	14.30	10.0(12.0)
	4.0(1.62)	5(250)	1	2.00	12.00	...
	2.3	5(940)	2	2.00	11.30	...
	3.1	5(600)	0	1.75	9.85	10.5(12.0)
	3.0	5(655)	4	2.00	14.00	11.0
U10	2.5	3	1	2.00	8.50	...
	2.3	3	2	2.00	9.30	...
	4.0(1.61)	5	2	2.00	13.00	10.3(11.3)
	2.5	5	2	2.00	11.50	...
	1.8	5(410)	1	2.00	9.80	...
	3.8	3	1	2.00	9.80	10.3(11.5)
	3.8	5(730)	4	1.75	14.55	10.9
U12	2.7	4	1	2.00	9.70	...
	2.3	4	1	2.00	9.30	...
	4.0(1.61)	5(800)	4	2.00	15.00	11.3(12.4)
	1.5	5(380)	1	2.00	9.50	...
	4.0(1.61)	5(550)	0	1.25	10.25	...
	3.7	4	2	1.25	10.95	10.8(12.4)
U13	3.5	5(630)	2	2.00	12.50	...
	0.0	5(444)	1	2.00	8.00	...

	2.8	5(350)	3	2.00	12.80	11.1(12.7)
	0.0	5(240)	2	2.00	9.00	...
	3.3	5(460)	1	1.75	11.05	...
	3.0	3	2	1.75	9.75	10.5(12.4)
	2.2	5(580)	1	1.75	9.95	...
	2.7	5(320)	0	2.00	9.70	...
	..	1	1	...	...	...
	2.7	4	0	2.00	8.70	10.2(11.8)
	3.8	5	1	2.00	11.80	10.3
U3	3.8	5	2	2.00	12.80	...
	4.0(1.68)	5(560)	1	1.75	11.75	...
	1.8	5(320)	0	2.00	8.80	11.12(12.4)
	0.0	5	1	2.00	8.00	10.3
	..	5	2	2.00	...	...
19	4.0(1.73)	2	0	2.00	8.00	...
	1.6	5	0	2.00	8.60	8.3

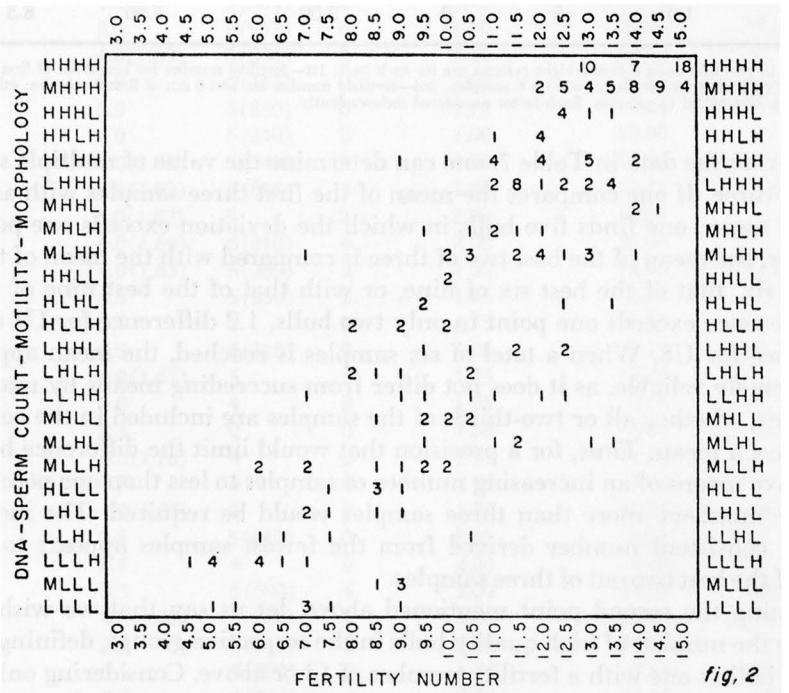
Numbers in parenthesis in extreme right column are for each bull: 1st—fertility number for best 2 out of first 3 samples; 2nd—fertility number for best 4 out of first 6 samples; 3rd—fertility number for best 6 out of first 9 samples; 4th—fertility number for 8 best out of 12 samples. Each factor considered independently.

ation. From the data in Table 7, one can determine the value of multiple samples beyond three. If one compares the mean of the first three samples with any succeeding mean, one finds five bulls in which the deviation exceeds one point. If, however, the mean of the best two of three is compared with the mean of the best four of six, that of the best six of nine, or with that of the best nine of twelve, the difference exceeds one point in only two bulls, 1.2 difference for U4 and 1.9 difference for U8. When a total of six samples is reached, the mean appears to be extremely reliable, as it does not differ from succeeding means by more than one point, whether all or two-thirds of the samples are included in the computation of each mean. Thus, for a precision that would limit the difference between successive means of an increasing number of samples to less than one point in the fertility numbers, more than three samples would be required. The most adequately consistent number derived from the fewest samples appears to be the mean of the best two out of three samples.

Pursuing the second point mentioned above, let us say that we wish to determine the number of high quality bulls in the respective groups, defining a high quality bull as one with a fertility number of 13 or above. Considering only those bulls given at least three tests and taking the best two out of the first three, we find eight out of ten bulls of known fertility in the high quality group and only one out of five bulls of known infertility in the high quality group. Thirty-three of 57 Group A bulls are in the high quality group; none of the three bulls in Group B given three tests are in the high quality group; and 14 of 21 Group C bulls are in the high quality group. If we wish a more complete comparison of the Groups A and B bulls, it will be necessary to make it on the basis of one sample, as most of the Group B bulls received only one test. Using only the first sample when there are more than one, we find that in Group A 24 bulls out of 94 fall into the high quality group, while in Group B, 45 out of 135 fall into the high quality group. This would indicate that the Group B bulls are, as a group, slightly superior to the Group A bulls, but an estimate based on the results of a single sample per bull might be misleading. It will be recalled that, when the fertility number

was based on the best two out of three samples, 33 of 57 Group A bulls tested three times fell into the high quality group. This kind of comparison could be carried out from a number of different points of view. An over-all mean could be computed for any number of bulls over as long a period as records are kept. Any individual bull could be compared with the highest rated bulls or with the group average. The group average could be computed from year to year to determine improvement or decline. The point seems clear that the assignment of a fertility number to each bull makes it possible to put breeding potential on a simple quantitative basis, susceptible to mathematical manipulation.

It should be taken into consideration, however, that even a comparatively high fertility number may mask one or more comparatively low factors. A fertility number of 13, for instance, might contain a sperm count profile number of 3 or a



FIGURES 2 through 6. The composition of fertility numbers in terms of individual factor profiles. Each plotted number represents the total fertility numbers, derived as explained in each figure, of a given level falling under the respective profile classification. All profile letters are derived from the individual sample profile numbers or from the means, as the case may be, and are assigned according to the following scale:

DNA—H, 4; M, 2.5–3.9; L, less than 2.5
Sperm count—H, 4.5 and above
Motility—H, 2 and above
Morphology—H, 1.62 and above.

FIG. 2. Individual samples of Group A.
FIG. 3. Individual samples of Group B.
FIG. 4. Mean of first two samples of two and three sample bulls of Group A.
FIG. 5. Mean of all samples of Group A bulls with three samples.
FIG. 6. Mean of best two out of three samples of Group A bulls with three samples, each factor considered separately.

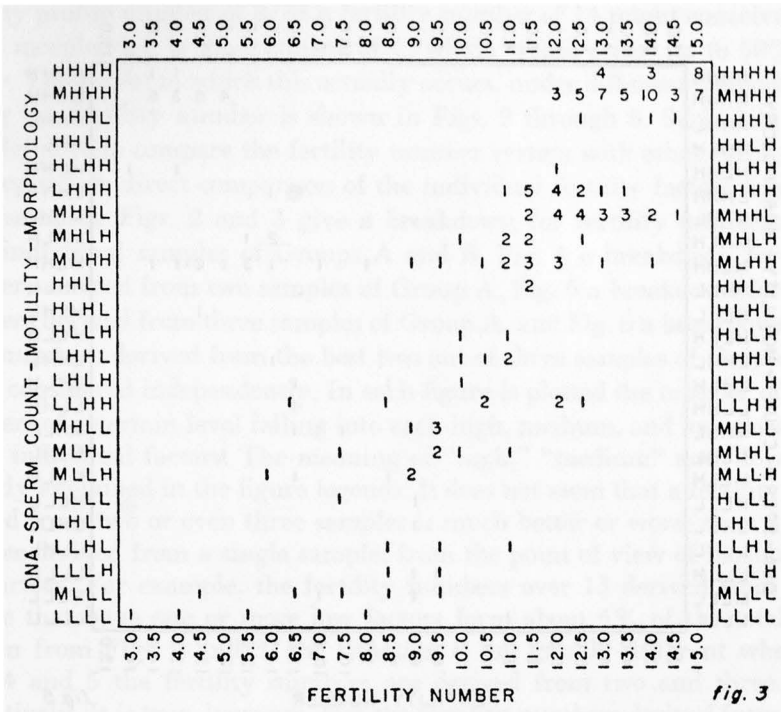

DNA-SPERM COUNT-MOTILITY-MORPHOLOGY
HHHH MHHH HHHL HHLH HLHH LHHH MHHL MHLH MLHH HHLL HLHL HLLH LHHL LHLH LLHH MHLL MLHL MLLH HLLL LHLL LLHL LLLH MLLL LLLL
3.0 3.5 4.0 4.5 5.0 5.5 6.0 6.5 7.0 7.5 8.0 8.5 9.0 9.5 10.0 10.5 11.0 11.5 12.0 12.5 13.0 13.5 14.0 14.5 15.0
FERTILITY NUMBER

fig. 3

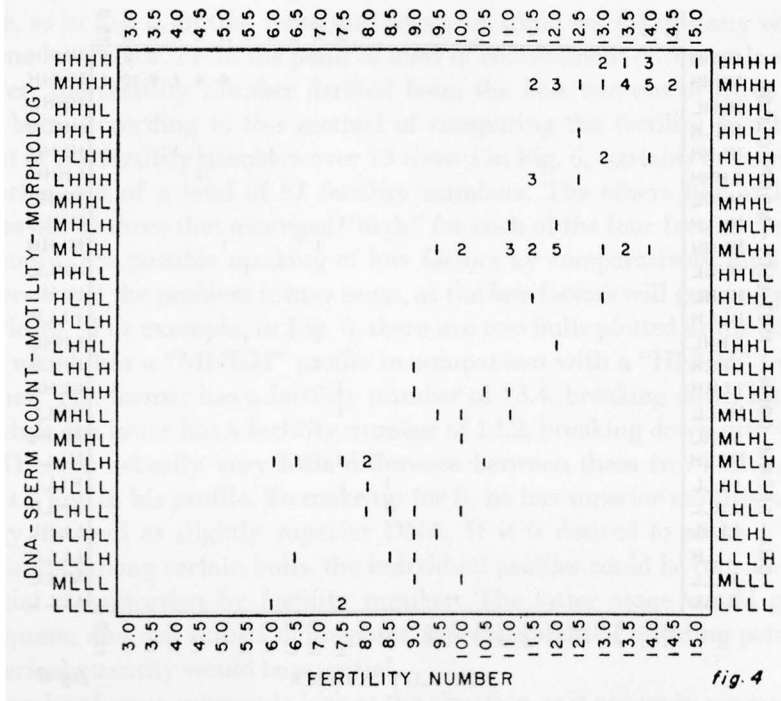

DNA-SPERM COUNT-MOTILITY-MORPHOLOGY
HHHH MHHH HHHL HHLH HLHH LHHH MHHL MHLH MLHH HHLL HLHL HLLH LHHL LHLH LLHH MHLL MLHL MLLH HLLL LHLL LLHL LLLH MLLL LLLL
3.0 3.5 4.0 4.5 5.0 5.5 6.0 6.5 7.0 7.5 8.0 8.5 9.0 9.5 10.0 10.5 11.0 11.5 12.0 12.5 13.0 13.5 14.0 14.5 15.0
FERTILITY NUMBER

fig. 4

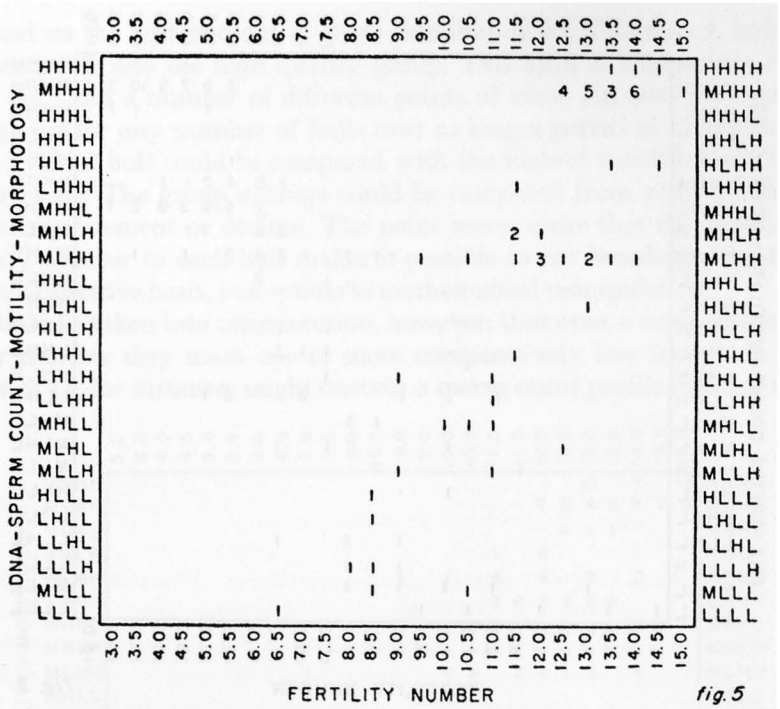

DNA-SPERM COUNT-MOTILITY-MORPHOLOGY
HHHH
MHHH
HHHL
HHLH
HLHH
LHHH
MHHL
MHLH
MLHH
HHLL
HLHL
HLLH
LHHL
LHLH
LLHH
MHLL
MLHL
MLLH
HLLL
LHLL
LLHL
LLLH
MLLL
LLLL
3.0 3.5 4.0 4.5 5.0 5.5 6.0 6.5 7.0 7.5 8.0 8.5 9.0 9.5 10.0 10.5 11.0 11.5 12.0 12.5 13.0 13.5 14.0 14.5 15.0
FERTILITY NUMBER

fig. 5

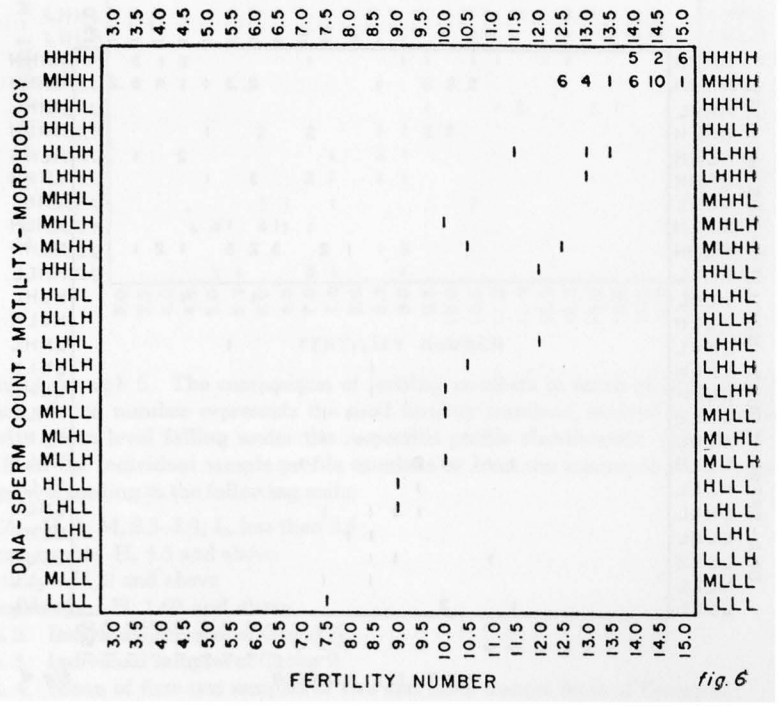

DNA-SPERM COUNT-MOTILITY-MORPHOLOGY
HHHH
MHHH
HHHL
HHLH
HLHH
LHHH
MHHL
MHLH
MLHH
HHLL
HLHL
HLLH
LHHL
LHLH
LLHH
MHLL
MLHL
MLLH
HLLL
LHLL
LLHL
LLLH
MLLL
LLLL
3.0 3.5 4.0 4.5 5.0 5.5 6.0 6.5 7.0 7.5 8.0 8.5 9.0 9.5 10.0 10.5 11.0 11.5 12.0 12.5 13.0 13.5 14.0 14.5 15.0
FERTILITY NUMBER

fig. 6

motility profile number of 2; or a fertility number of 14 might conceivably contain a morphology profile number of 1, which would mean up to 50% abnormality. The extent to which this actually occurs, under different methods of computing the fertility number is shown in Figs. 2 through 6. Such data make it possible, also, to compare the fertility number system with other rating systems that depend on direct comparison of the individual fertility factors from one or more samples. Figs. 2 and 3 give a breakdown for fertility numbers derived from individual samples of Groups A and B, Fig. 4 a breakdown for fertility numbers derived from two samples of Group A, Fig. 5 a breakdown for fertility numbers derived from three samples of Group A, and Fig. 6 a breakdown for fertility numbers derived from the best two out of three samples of Group A, each factor considered independently. In each figure is plotted the number of fertility numbers of a certain level falling into each high, medium, and low classification of the individual factors. The meaning of "high," "medium" and "low" is sufficiently explained in the figure legends. It does not seem that a fertility number derived from two or even three samples is much better or worse than a fertility number derived from a single sample, from the point of view of masking fewer low factors. For example, the fertility numbers over 13 derived from a single sample that have one or more low factors form about 5% of the total, as can be seen from Figs. 2 and 3; the situation is not greatly different when, as in Figs. 4 and 5 the fertility numbers are derived from two and three samples respectively. It is true, however, that the fertility numbers derived from two and three samples seem to be a slight improvement over those derived from a single sample, as in Fig. 2, in that those numbers over 13 do not include any with more than one low factor. From the point of view of concealment of fewest low factors, however, the fertility number derived from the best two out of three samples seems best. According to this method of computing the fertility number, only one out of the fertility numbers over 13 shown in Fig. 6, contains as many as one low factor, out of a total of 57 fertility numbers. The others had at least two samples out of three that averaged "high" for each of the four factors considered.

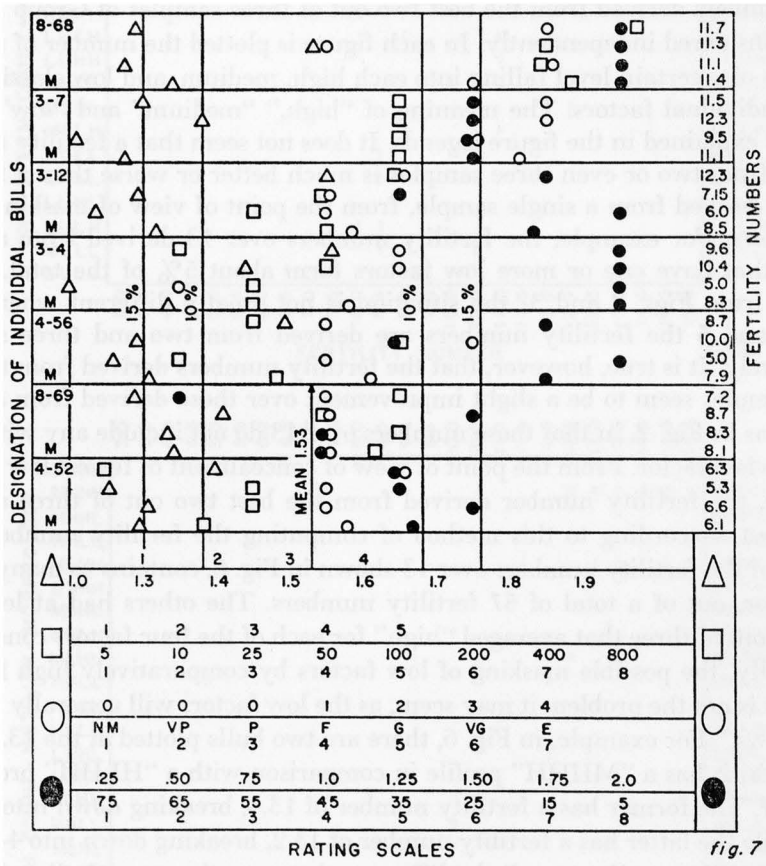
Actually, the possible masking of low factors by comparatively high fertility numbers is not the problem it may seem, as the low factors will generally be high level "lows." For example, in Fig. 6, there are two bulls plotted at the 13.5 level, one of which has a "MHHH" profile in comparison with a "HLHH" profile for the other. The former has a fertility number of 13.4, breaking down into 3.4–5–3–2; while the latter has a fertility number of 13.2, breaking down into 4–3.5–4–1.75. There is actually very little difference between these two bulls, although one has a low in his profile. To make up for it, he has superior motility and morphology, as well as slightly superior DNA. If it is desired to make a fine discrimination among certain bulls, the individual profiles could be considered after an initial classification by fertility number. The latter alone would generally be adequate; and, for statistical purposes, representation of breeding potential by a numerical quantity would be essential.

It may be of some interest to look at the situation as it occurs in representative, individual bulls from the standpoint of DNA variation. For this purpose, repre-

sentative bulls of various DNA classifications, drawn from Group A bulls with three samples each and from Group C bulls are illustrated in Figs. 7 through 12.

DISCUSSION AND SUMMARY

The fertility factors on which the fertility number is based are the conventional indices of motility, morphology, and sperm count plus the newer factor of the DNA content of the semen spermatozoa. Other factors could, of course, be added whenever there is sufficient data to justify including them. Motility, morphology, and sperm count examinations are quickly and easily determined and should be carried out for all bulls rated. DNA analysis is time-consuming, particularly



FIGURES 7 through 11. Profile graphs of representative bulls of Group A with three samples from the standpoint of DNA variation in the mean of three samples. The lowest line of numbers in each individual scale is used in positioning the various factor symbols and in computing the "M" or mean line. The top line, as in Fig. 1, is used in computing the individual fertility numbers, the mean fertility number being the mean of the individual numbers.

FIG. 7. Lowest DNA.

FIG. 8. Medium DNA.

FIG. 9. Highest DNA.

FIG. 10. Most fluctuating in DNA.

FIG. 11. Least fluctuating in DNA.

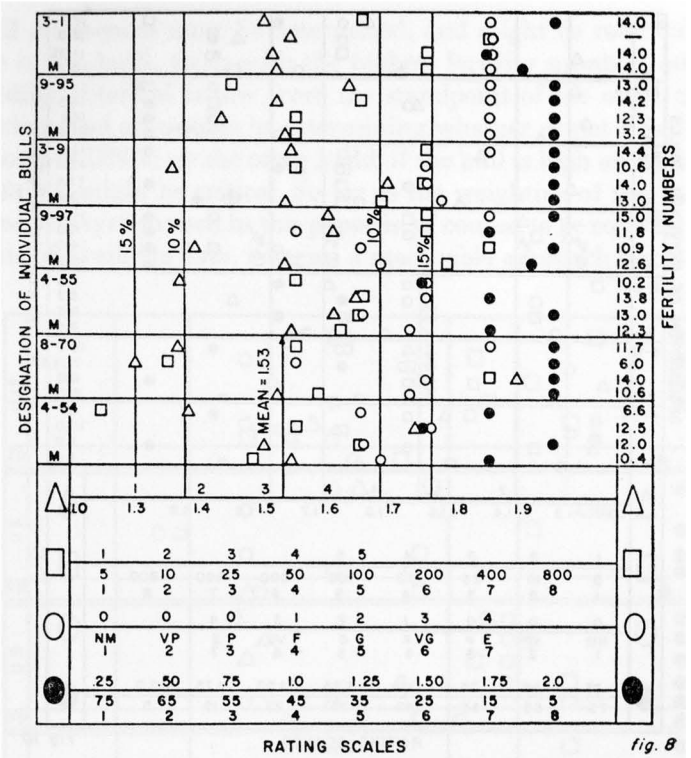

DESIGNATION OF INDIVIDUAL BULLS
FERTILITY NUMBERS
3-1
M
14.0
—
14.0
14.0
9-95
M
13.0
14.2
12.3
13.2
3-9
M
14.4
10.6
14.0
13.0
9-97
M
15.0
11.8
10.9
12.6
4-55
M
10.2
13.8
13.0
12.3
8-70
M
11.7
6.0
14.0
10.6
4-54
M
6.6
12.5
12.0
10.4
15 %
10 %
10 %
15%
MEAN = 1.53
1 2 3 4
1.0 1.3 1.4 1.5 1.6 1.7 1.8 1.9
1 2 3 4 5
5 10 25 50 100 200 400 800
1 2 3 4 5 6 7 8
0 0 0 1 2 3 4
NM VP P F G VG E
1 2 3 4 5 6 7
.25 .50 .75 1.0 1.25 1.50 1.75 2.0
75 65 55 45 35 25 15 5
1 2 3 4 5 6 7 8
RATING SCALES

fig. 8

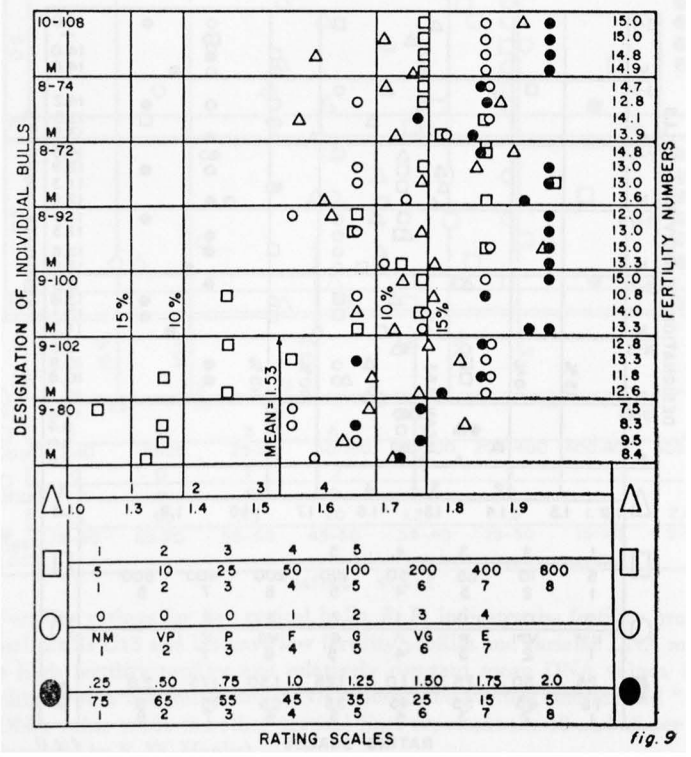

DESIGNATION OF INDIVIDUAL BULLS
FERTILITY NUMBERS
10-108
M
15.0
15.0
14.8
14.9
8-74
M
14.7
12.8
14.1
13.9
8-72
M
14.8
13.0
13.0
13.6
8-92
M
12.0
13.0
15.0
13.3
9-100
M
15.0
10.8
14.0
13.3
9-102
M
12.8
13.3
11.8
12.6
9-80
M
7.5
8.3
9.5
8.4
15 %
10 %
10 %
15%
MEAN = 1.53
1 2 3 4
1.0 1.3 1.4 1.5 1.6 1.7 1.8 1.9
1 2 3 4 5
5 10 25 50 100 200 400 800
1 2 3 4 5 6 7 8
0 0 0 1 2 3 4
NM VP P F G VG E
1 2 3 4 5 6 7
.25 .50 .75 1.0 1.25 1.50 1.75 2.0
75 65 55 45 35 25 15 5
1 2 3 4 5 6 7 8
RATING SCALES

fig. 9

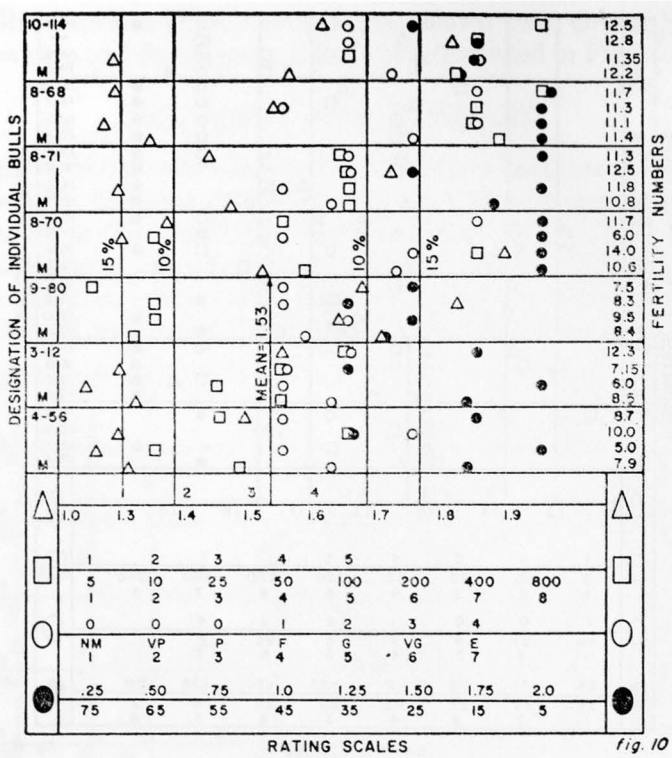

DESIGNATION OF INDIVIDUAL BULLS
FERTILITY NUMBERS
10-114
8-68
8-71
8-70
9-80
3-12
4-56
M
12.5 12.8 11.35 12.2
11.7 11.3 11.1 11.4
11.3 12.5 11.8 10.8
11.7 6.0 14.0 10.6
7.5 8.3 9.5 8.4
12.3 7.15 6.0 8.5
9.7 10.0 5.0 7.9
15%
10%
10%
15%
MEAN=1.53
1 2 3 4
1.0 1.3 1.4 1.5 1.6 1.7 1.8 1.9
1 2 3 4 5
5 10 25 50 100 200 400 800
1 2 3 4 5 6 7 8
0 0 0 1 2 3 4
NM VP P F G VG E
1 2 3 4 5 6 7
.25 .50 .75 1.0 1.25 1.50 1.75 2.0
75 65 55 45 35 25 15 5
RATING SCALES

fig. 10

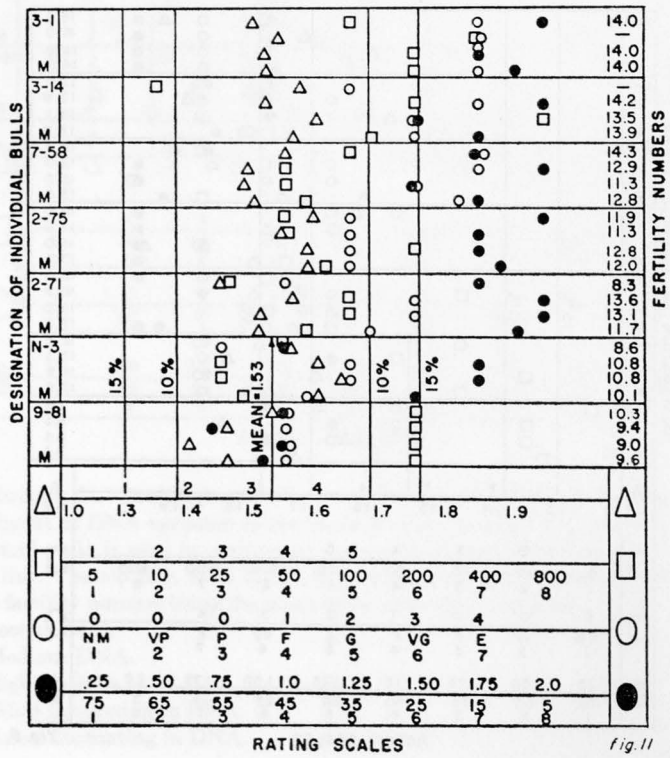

DESIGNATION OF INDIVIDUAL BULLS
FERTILITY NUMBERS
3-1
3-14
7-58
2-75
2-71
N-3
9-81
M
14.0 — 14.0 14.0
— 14.2 13.5 13.9
14.3 12.9 11.3 12.8
11.9 11.3 12.8 12.0
8.3 13.6 13.1 11.7
8.6 10.8 10.8 10.1
10.3 9.4 9.0 9.6
15%
10%
MEAN=1.53
10%
15%
1 2 3 4
1.0 1.3 1.4 1.5 1.6 1.7 1.8 1.9
1 2 3 4 5
5 10 25 50 100 200 400 800
1 2 3 4 5 6 7 8
0 0 0 1 2 3 4
NM VP P F G VG E
1 2 3 4 5 6 7
.25 .50 .75 1.0 1.25 1.50 1.75 2.0
75 65 55 45 35 25 15 5
1 2 3 4 5 6 7 8
RATING SCALES

fig. 11

when small differences must be determined, and might be reserved for a small percentage of the bulls, those with the highest fertility numbers otherwise. If a bull's breeding potential is low from the standpoint of the other three factors, there is little added advantage in determining whether or not it is also low from the standpoint of DNA; on the other hand, if the bull is high on the other factors, the DNA factor might be critical. So far as the weighting of the various factors is concerned, the system used in this paper is, of course, to be considered tentative. On the basis of available data, it seems a reasonable approach but is always sub-

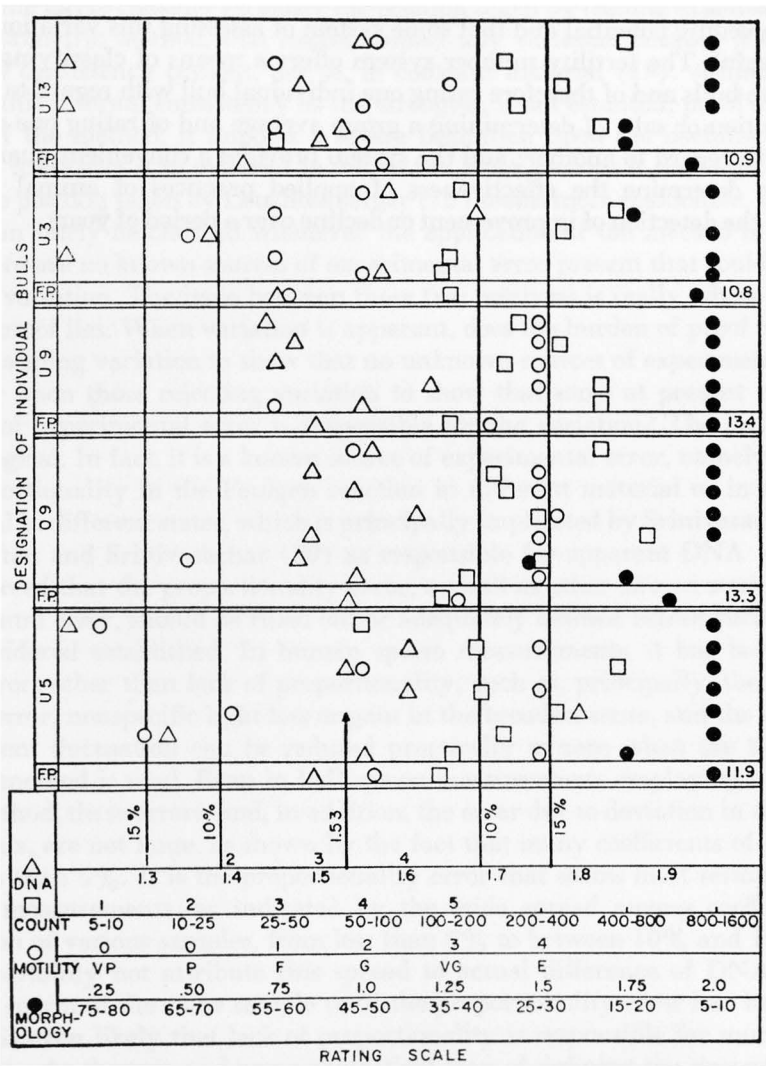


FIG. 12. Fertility ratings for five typical bulls. F. P. indicates the fertility profile for the respective animal. Bulls U13 and U3 have low fertility profiles and variable DNA means. Bulls U9 and O9 have high fertility profiles and relatively constant mean DNA values. Bull U5 has a medium fertility profile but fluctuating DNA values. The vertical line marked "1.53" indicates the normal DNA value while the other vertical lines represent the 10 and 15 per cent deviation from 1.53. (Prepared by E. W. Hanly).

ject to revision. It should be emphasized that the amount of testing required to determine the fertility number is dependent upon the degree of precision desired. If, for example, one is satisfied to know that a fertility number of 13 represents a breeding potential of somewhere between 11 and 15, it would obviously require fewer tests than determining the number with a precision of plus or minus one.

Whatever modifications or applications of the system might be made, it is clear from the most cursory examination of the spread of the fertility numbers that, in a large group of bulls, there is a tremendous variation in quality or, more precisely, breeding potential and that some system of assessing this variation should prove useful. The fertility number system offers a means of classifying young, unproven bulls and of therefore rating one individual bull with regard to another for retention or sale, of determining a group average and of rating one group of bulls with regard to another; and the system provides a convenient quantitative index to determine the effectiveness of applied practices of animal genetics through the detection of improvement or decline over a period of years.

VII. General Discussion and Summary

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No one familiar with quantitative DNA research is inclined to suggest lightly, on the basis of cytophotometric data, that DNA variation exists in certain material. One has to consider seriously the position taken by leading exponents of the cytophotometric method, that makes suspect any variation because it lacks the internal consistency present, per se, in constant material (*19*). Although they regard this internal consistency as the strongest, if not essential, proof of the validity of the method, it imposes a severe limitation upon the usefulness of the method to restrict it to material in which the DNA content is constant. The less extreme position taken by Leuchtenberger (*12*) seems more reasonable, that variation can fairly be claimed whenever the application of the method is uniform and there are no known sources of experimental error present that could account for the variation. The issue between these two positions is really where the burden of proof lies. When variation is apparent, does the burden of proof rest upon those claiming variation to show that no unknown sources of experimental error exist or upon those rejecting variation to show that some at present unknown source of experimental error is responsible for the variation? The latter seems more logical. In fact, it is a known source of experimental error, namely the lack of proportionality in the Feulgen reaction in different material or in the same material in different states, which is principally implicated by Srinivasachar (*25*) and Patau and Srinivasachar (*20*) as responsible for apparent DNA variation. It is agreed that the proportionality error, as well as other known sources of experimental error, should be ruled out or adequately defined before variation can be considered established. In human sperm measurements, it has been shown that errors other than lack of proportionality, such as, principally, the distributional error, nonspecific light loss or gain in the broadest sense, and the error due to current fluctuation can be reduced practically to zero when the two-wavelength method is used. Even in bull sperm measurements employing a less precise method, these errors, and, in addition, the error due to deviation in size measurements, are not large, as shown by the fact that many coefficients of variation are less than 5%. It is the proportionality error that seems most serious in bull sperm measurements, as indicated by the wide spread among coefficients of variation of various samples, from less than 5% to between 10% and 15%. One could certainly not attribute this spread to actual difference of DNA content among sperm of the same sample until the proportionality error had been ruled out. It is even likely that lack of proportionality is responsible for much of this variation. As there is no known theoretical way of defining the proportionality error, the best approach towards a definition of this, together with other sources of error, seems an empirical one. It was for this reason that a large scale experiment was specially designed in which the conditions under which the bulk of the sample analyses was carried out were duplicated, except that different smears of the same sample were used to determine the deviation between smears on the

same slide, on different slides stained in the same container, and on different slides stained in different containers. On the basis of 184 separate corrections, the maximum error was set at 15%; in 80% of the corrections, however, the error did not exceed 10%.

It has been described in detail how small but consistent deviations in excess of those to be expected from the established limits of error were found in both Group A and Group B bulls. Whether the samples were considered individually, or an over-all mean for a number of samples was computed, or whether additional determinations were made on duplicate slides, there were always a few more than there should have been that exceeded the calculated experimental error. In the group first tested, one of the bulls of known infertility, C-322, had an over-all mean that was lower than that to be expected from experimental error, and another, C-15, had a similar over-all mean on the basis of four out of five tests. Both animals were definitely lower in DNA than any of the bulls of known fertility tested. There was less variation in the Group C animals than in any other group. Only one of the 21 bulls tested, U13, gave an over-all mean that was lower than that to be expected from experimental error and this by only a few points, too close to be convincing in view of the number of samples normal in DNA yielded by this bull. From the standpoint of the individual tests, there were a number that deviated by more than the maximum 15% error, but the fluctuation was far less than that encountered in some of the Group A bulls on the basis of three tests. The comparative lack of variation in the Group C animals can be considered an indirect confirmation of the variation found in the bulls of other groups, for, if experimental error were responsible for the variation, one might expect the deviation to be much the same in all groups tested. As will be seen below there is a reason that can be advanced to explain the reduced variation in Group C.

The statistical evidence for variation is further supported by the distribution of the DNA means of the Group A bulls given three tests. As shown in Fig. 4, Article IV, there are more bulls having all low means or all high means and fewer having mixed means than one would expect from chance. The statistical significance of this distribution is not on such a high level of confidence that it can be advanced as conclusive proof, but it does fit into a consistent picture of variation.

One of the strongest evidences for variation is the statistically significant correlation found between low DNA and low sperm count and/or poor motility and, to a lesser extent, with morphology. If the deviating DNA means were due to experimental error, one would expect them to be randomly distributed with regard to all other factors and to show no correlation with any of them. It follows that, if one group shows less variation than another, such as Group C, it should also be of higher quality with regard to the factors of motility, morphology and sperm count. This has been found to be true by a comparative tabulation of the number of low factors present in Groups A, B and C collectively for each group, and in Groups A and C for the individual animals on the basis of three tests. If a low factor is defined as motility worse than good, sperm count less than 100 million per cubic centimeter, and morphology consisting of more than 20% abnormal forms, there were 96 low factors in a total of 143 samples in Group C, or 67%; there were 131 low factors in a total of 155 samples in Group B, 85%; and there

were 245 low factors in a total of 246 samples in Group A, practically 100%. The superiority, from this standpoint, of the Group C bulls is even more impressive when we examine the individual tabulation. On the basis of the first three tests given the Group C bulls, there was only one bull out of the 19 given three or more tests that had as many as four low factors in the three samples. In marked contrast, there were 16 bulls of the 57 Group A bulls given three tests that had four or more low factors in the three samples. It is of added significance that five of the seven bulls having the lowest over-all means of the 57 in this group had four or more low factors in the three samples, while not a single bull in the seven bulls having medium over-all DNA means had as many as four low factors in the three samples. To complete the breakdown for the bulls having four or more low factors in the three samples, two of the seven bulls with the highest over-all DNA means, two of the seven least fluctuating, three of the most fluctuating, and four of those not classified were in this category. The significance of the differences is not immediately apparent except between the bulls with the lowest over-all means and the other groups.

The correlation of DNA means with other factors furnishes an internal proof of the actual existence of deviating DNA means comparable to the consistency of results in constant material advanced as the strongest, and, by some, the only adequate, proof of the validity of the method. In the presence of correlation, constancy is no longer indispensable, for it is just as unlikely that experimental error would result in correlation as that experimental error would result in constant results.

Most of the data on correlation have been derived from bulls of unknown fertility. The results for bulls of known fertility and infertility, presented in the separate correlation paper, confirm the conclusions reached on the basis of data from bulls of unknown fertility and suggest a new possibility, that correlation between the DNA content of the semen sperm and other factors occurs in both fertile and infertile animals. More evidence is needed on this point. It should be emphasized, however, that the correlation argument does not exclude the existence of DNA deviation independent of the other factors. As detailed in the correlation paper, some cases of deviation cannot be explained on the basis of correlation.

The results are similar to those obtained by the Leuchtenberger group in that DNA variation has been found, but they differ in a number of particulars. As the work of the Leuchtenberger group was done on men and bulls of known fertility and infertility and ours mostly on bulls of unknown fertility, it is impossible to make a comparison of the incidence of DNA deviation found, but the results can be compared from the standpoint of degree, type, and relation to other factors. As the work of the Leuchtenberger group progressed, the trend was towards finding a predominantly fluctuating type of DNA abnormality with many extremely deviating means. The DNA abnormality found here, on the other hand, appears to be mostly of the constantly low or high variety just beyond the limits of experimental error, with few means departing markedly from the normal. In the group of known infertility, the two apparently deviating bulls gave all means except one at the low end of the scale. In Groups A and B there were both low and high single and over-all means, while the instances of fluctuation that ap-

peared in the bulls of Group A given three tests were, as shown by Fig. 4, Article IV, fewer than would be expected from a chance distribution of the means among the 57 bulls involved. In Group C there were no low or high over-all means in excess of experimental error, and the bull most fluctuating in DNA had only about one-third of his total samples beyond the normal range in contrast with 50 to 75% of the total means beyond the normal range in cases of deviation in men of known infertility. The Leuchtenberger group found deviation from the normal in all the germinal cell stages of the infertile testes examined. The only testis work done here was on five bulls of known infertility. No deviation was found in the primary spermatocytes from values obtained for the corresponding stage in the testes of bulls normal in DNA content of their semen sperm. This does not necessarily contradict the low values for semen sperm obtained for two of the bulls of known infertility, as the experimental error of the testis measurements was considerably greater than that for the semen sperm measurements, and, conceivably, there is the possibility of loss of DNA after sperm maturation from sperm normal in DNA to begin with, or failure to synthesize DNA by sperm inadequate in DNA. The latter two possibilities would involve a metabolic type of DNA.

The research of the Leuchtenberger group on bulls (*9*), though less extensive, was similar to the results obtained in human material. There was a clear separation between the means of 15 fertile and 15 infertile bulls, although some of the means of 55 other fertile bulls fell into the infertile range. The over-all mean of the 15 infertile bulls was 1.08, far lower than the over-all mean would be for bulls examined here whose DNA means exceeded the limits of experimental error. The latter had means, individual or cumulative for two or more samples, that fell into the upper range of infertile bull means published by the Leuchtenberger group.

It is in regard to the correlation of DNA with the other factors of motility, sperm count, and morphology that the results here differ mainly from those of the Leuchtenberger group, which found no evidence of correlation. Since the correlation found here is not an invariable one but rather statistically significant and, furthermore, does not exclude the existence of DNA abnormality without correlation, the difference between the results of the two groups may be more apparent than real and may simply be due to a more exhaustive follow-up of the possibility here in a greater quantity of material.*

Although it was not practical to retain the bulls showing deviation and to follow up the DNA analyses with breeding tests, the data accumulated have considerable practical application. (The correlation of most of the DNA deviation found with the other factors made breeding tests less essential.) The comparatively limited incidence and slight degree of DNA abnormality found, requiring considerable refinement of analysis for its detection, lead to the conclusion that it is not the kind of test to apply routinely to a large number of relatively ordinary bulls; and, indeed, the correlation of most of the cases of DNA abnormality

* Another possible explanation might be that the material was unselected here, while there appears to have been some restriction of poor samples in the Leuchtenberger work on human material. On the other hand, it may be that the non-correlated type of DNA abnormality is more prevalent in human material than in bulls.

found with poor motility and/or sperm count and/or morphology indicates that it is not an essential test when the escape from detection of a few bulls out of a hundred would be of no serious consequence. It is in the high quality bulls, normal in all other respects, that the presence of DNA abnormality causing infertility would be of most economic detriment. If the test is restricted to these, it would not be impractical to carry out the refinements of method developed during the course of the work for the determination of DNA, in semen sperm analyses made at different times, with a high degree of accuray and precision. The data accumulated have also made it possible to make a large scale application of a tentative rating system for bulls of unknown fertility designed to put breeding potential on a quantitative, numerical basis, with all the facility of manipulation that such a quantity implies. The actual rating of the bulls examined during the course of the work has made it clear in the most concrete terms what a wide range of variation in breeding potential exists in bulls in which it might not be otherwise possible to make a discrimination; and, therefore, how desirable some such system of rating would be in order to make proper disposition of individual bulls and assess the results of breeding practices over a period of years.

The theoretical significance of the results depends largely upon the cause of the deviation. If it is simply a question of irregular distribution of chromosomes, there would be little need for reflection. It is highly unlikely, however, that aneuploidy could be so widespread and so consistent as to produce, not individual, aberrant values, but deviating means. Furthermore, the work of the Leuchtenberger group has shown that, in the testis material examined, the deviation is not peculiar to any particular stage of spermatogenesis but is present in all, thus ruling out the possibility of loss during spermatogenesis (*8*, *11*). It seems a likely possibility that some variation in DNA synthesis is responsible for the deviation. In those instances in which no correlation with the other factors was found, the variation in synthesis would seem to involve the genetic fraction or one closely related, the exact amount of which, as Leuchtenberger suggests, would be critically necessary for sperm function. In those instances in which correlation was present, the variation in synthesis would seem to involve a metabolic fraction, and significantly, to implicate DNA quantitatively in cell physiology. If, indeed, there is some variation in DNA synthesis involved, there is no evidence to indicate that other organs of the body besides the testis are affected. The fundamental question arises: Is the variation in DNA synthesis generalized and quantitative, rising above the threshold in the testis because of the greater need for synthesis of DNA in that organ, or is the variation a qualitative one, peculiar to the testis? Along this line, an experiment was planned, using *Drosophila virilis*, to determine whether sterility could be produced by a general interference in nucleic acid synthesis brought about by the use of aminopterin, without affecting the viability of the fly. At the same time, it was planned to investigate the biosynthesis of DNA in the testis and other organs of *D. virilis*, by determining what compounds, if any, could be utilized by the fly for DNA synthesis when the normal pathway of synthesis was blocked. If a variation in DNA synthesis exists and affects only the testis, qualitatively or quantitatively, it would be an example of differential DNA synthesis. This suggests a parallel from basic research, the marked reduction of DNA in the salivary gland of the lethal mutant 1(2)gl, *D. melanogaster*, strik-

ingly disproportionate to the effect in other organs (*31*). The parallel seemed close enough to justify extension of the inquiry in this direction, and, accordingly, a cytochemical and biochemical investigation was planned. The two problems in basic research, complementary to the main subject under investigation, will be taken up in separate papers.

GENERAL SUMMARY

1. Cytophotometric DNA analysis, accompanied, in all except a few instances, by morphology, motility and sperm count examination, has been carried out on 658 samples of semen spermatozoa from 275 Santa Gertrudis bulls of the King Ranch in the following classifications: 64 samples from 10 bulls of known fertility and 6 of known infertility; 246 samples from 98 Group A bulls of unknown fertility under two years of age, 57 of which received three tests each; 155 samples from 135 Group B bulls of unknown fertility under two years of age including additional determinations on duplicate slides, whenever possible, for deviating DNA means; 180 samples from 21 Group C bulls of unknown fertility originally under two years of age, tested over a period of approximately 18 months followed by check-up tests on doubtful animals; and 13 samples from 5 miscellaneous bulls. While these bulls were adequate for the purpose of the research, they were classified by the ranch as comparatively less desirable Santa Gertrudis stock; and the bulls of Group C would not have been used for breeding.

2. DNA content of testis and liver nuclei from bulls of known infertility has been compared with that from bulls established as relatively normal in the DNA content of their semen sperm.

3. A method has been formulated for comparative DNA analysis of semen sperm over an extended period of time that limits the error to 15% and to 10% in 80% of cases, and refinements have been described for further reduction of the error.

4. When staining was controlled to the Leuchtenberger normal mean of 1.52, for comparison of results, an over-all mean of 1.53 ± 0.01 was obtained for all the samples of Group A and an over-all mean of 1.52 ± 0.008 for all the samples of both Groups A and B.

5. Two of the six bulls of known infertility gave DNA means for their semen sperm lower than those obtained for any of the 10 animals of known fertility. No difference in DNA content beyond the possible limits of experimental error was discovered between the primary spermatocytes and liver nuclei of the infertile bulls and similar material from bulls established as relatively normal in the DNA content of their semen spermatozoa.

6. In Group A, 14% of the total samples and 19% of the bulls given three tests showed a deviation from the over-all mean of 1.53 in excess of the calculated experimental error. In the group of bulls given three tests, there were more with all excessively low or high over-all means and fewer with fluctuating means than would be expected from a chance distribution of the means among the 57 bulls.

7. In Group B, on a basis of cumulative means for the 135 bulls involved, 10% deviated from the fiduciary mean of 1.52 by more than would be expected from

experimental error; on a basis of confirmation of deviation by additional measurements on a duplicate slide, 3% of the 101 figuring in the calculation deviated in excess of experimental error. These were taken to represent those bulls deviating to such a degree that the maximum experimental error would not be sufficient to bring either the original mean or the duplicate slide mean within normal limits.

8. In Group C, no bull had a cumulative mean that differed significantly from the over-all mean. In the bull displaying the greatest fluctuation, four means out of a total of eleven fell beyond the limits of the five most constant of the bulls tested.

9. In the bulls of Groups A, B and C a significant correlation was discovered between the DNA content and the motility and sperm count, and, to a lesser extent, the morphology of their semen sperm. In the most extreme form, those samples having high motility, morphology, and sperm count yielded twice as many means equal to or above the over-all mean of 1.52 for all samples than they yielded below the over-all mean, while those samples having low motility, morphology, and sperm count yielded a ratio that was almost the reciprocal. The bulls showing the least variation of DNA, those of Group C, had fewest low motility, morphology and sperm count factors in their samples, while the bulls showing the greatest variation in DNA, those of Group A, had the highest number of low factors. Limited data indicated that correlation was present in animals of known fertility as well as in those of known infertility and unknown fertility. The conclusions reached from graphical and semi-statistical approaches are, in general, confirmed by correlation coefficients and multiple regression coefficients. These show that, while the strongest correlation exists among the factors of motility, morphology, and sperm count, excluding DNA, there is a significant correlation between DNA and the other factors. This is highest for DNA and motility, intermediate for DNA and sperm count, and lowest for DNA and morphology.

10. On a basis of the four factors of DNA content of semen sperm, motility, morphology and sperm count, a rating system was devised, and all the bulls, with a few exceptions for various reasons, were given fertility profiles and fertility numbers. Practical application of such a system was discussed.

11. Four types of evidence supporting the DNA variation found were discussed.

12. Compared to the DNA deviation found in human material, and to a limited extent in bull material, by the Leuchtenberger group, the deviation found in Santa Gertrudis bulls is slight. The fact that most of this is correlated with the other factors reduces the economic significance of DNA deviation in the class of bulls tested and decreases the probability of its occurrence in the higher quality Santa Gertrudis bulls.

13. The practical application and theoretical significance of the results were discussed and complementary, basic research outlined.

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