

Measuring the Concentration of Spermatozoa from Honey Bees¹ with Spectrophotometry^{2,3}

JOHN R. HARBO

Bee Breeding and Stock Center Laboratory, Agric. Res. Serv., USDA, Baton Rouge, LA 70808

ABSTRACT

A technique was derived for measuring the concentration of sperm in honey bees, *Apis mellifera* L., by spectrophotometric absorbance. A positive linear correlation ($r = 0.957$) existed between absorbance at 230 nm and

sperm concentration in 0.5 M NaCl. With our spectrophotometer, the formula correlating millions of sperm per 5 ml (x) to absorbance (y) was $y = -0.0096 + 0.1224x$.

Mackensen and Roberts (1948) first described a method of counting spermatozoa from honey bees, *Apis mellifera* L. It involved dispersing spermatozoa in a known volume of water, transferring a tiny subsample onto a hemacytometer slide, and counting the spermatozoa within the defined volume of a counting chamber. However, this procedure was tedious and time consuming, and, because of the small (0.1–0.9 μ l) samples, it yielded variable results.

My objective was to devise a technique by which a spectrophotometer could be used to measure the concentration of honey bee sperm. Cattle breeders routinely use a similar method to estimate the concentration of spermatozoa when storing sperm for later use in artificial insemination programs (Salisbury et al. 1943, Foote 1968 and 1972).

Throughout the paper I refer to "absorbance" readings because the absorbance (logarithmic) scale of the spectrophotometer was used. However, the phenomenon that I measured was probably light scattering or turbidity rather than true absorbance.

METHODS AND MATERIALS

Preparing Sperm Suspensions.—Spermatozoa were counted from both the male ejaculate and the female spermatheca. The intact spermatheca taken from the honey bee queen was immersed in 5 ml of 0.5 M NaCl, the spermathecal membrane was broken, the spermatozoa were dispersed, and the empty membrane was removed. Semen from the male was collected in an insemination tip and injected into a known volume of 0.5 M NaCl (usually > 5 ml). In both cases, the spermatozoa were dispersed by agitating with the sucking and expelling action of a pipet.

Hemacytometer Counts.—Sperm concentration was estimated by counting a known volume, 0.1 μ l, in a hemacytometer grid and multiplying by 50,000 to obtain the number of spermatozoa per 5 ml. However, to reduce sample error, I sometimes counted all 9 grids in a hemacytometer, a volume of 0.9 μ l.

All but 23 of the observations in Fig. 1 represent the mean of eight 0.1- μ l counts. Of the remaining, 8 are from more than 8 such counts, 7 are from fewer than 8, and 8 are from larger (0.9 μ l samples).

Absorbance Readings.—I used a Beckmann DU-2

spectrophotometer with a slit width of about 0.14 mm and the wavelength set at 230 nm; absorbance decreased with longer wavelengths and increased with shorter wavelengths. Two hundred thirty nm was selected because it absorbed well and because it was as close as I wanted to approach the minimum wavelength limit (220 nm) of the silica cells.

Three to 4 ml of the sperm suspension were put into a spectrophotometric cell, and 1 of the other cells served as a blank (0.5 M NaCl only). Because absorbance gradually decreased with time (ca. 5%/h during the 1st hour, but more slowly thereafter), absorbance readings were made less than 15 min after the sperm suspension was prepared.

Excessive agitation of the sperm suspension with a pipet and shaking when in the spectrophotometric cell caused an increase in the absorbance reading. When in the cell, the spermatozoa can be mixed, if desired, by inversion.

Sampling Accuracy.—To estimate the repeatability or coefficient of variation for spectrophotometric measurements, I prepared 8 sperm suspensions with 4 μ l of semen and 40 ml of 0.5 M NaCl in each. From each preparation, 3–7 four- μ l samples were drawn, and the coefficient of variation was derived. To measure the coefficient of variation on the hemacytometer counts, I randomly selected the counting data from 18 of the 104 observations in Fig. 1.

RESULTS

Hemacytometer counts and absorbance readings from 104 sperm suspensions are plotted in Fig. 1. The observations represent counts and absorbance readings made from 1972 to 1974. Each circle in Fig. 1 represents a sample of sperm taken from the spermatheca of a queen in 1972, ca. 2/3 of which were samples of spermatozoa from a single inbred line. Each square represents a sample taken from a spermatheca in 1973 or 1974, and each triangle represents a sample taken from a male ejaculate. Spermatozoa represented by squares and triangles were of miscellaneous genetic origin. The distribution in Fig. 1 indicates that genetic type or environmental condition of sperm before dilution had no apparent effect on absorbance. For example, sperm from a male ejaculate, sperm from the queen's spermatheca, and sperm from different genetic stock all exhibited similar absorbancy.

With simple regression analysis, I found a positive

¹ Hymenoptera: Apidae.
² In cooperation with Louisiana Agric. Exp. Stn.
³ Mention of a commercial or proprietary product in this paper does not constitute an endorsement of this product by the USDA. Received for publication Apr. 22, 1975.

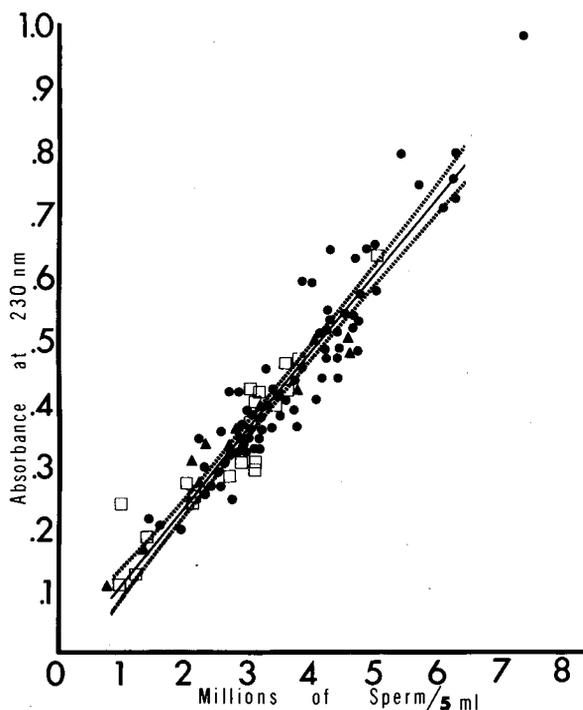


FIG. 1.—The relationship between spectrophotometric absorbance and hemacytometer counts when 0.5 M NaCl is used to dilute the sperm. The squares and circles represent samples of spermatozoa taken from a spermatheca, and the triangles represent samples of semen. The solid line is the regression line ($y = -0.0096 + 0.1224x$), and the dotted lines are the 95% confidence limits for the regression line. See text for further explanation.

linear correlation between sperm counts and absorbance readings ($r = 0.957$). The formula for the regression line is $y = -0.0096 + 0.1224x$. The regression line and 95% confidence limits for the expected value of y are graphed in Fig. 1.

The final step was to estimate sperm concentration from absorbance. Because absorbance became the independent variable and sperm concentration was the dependent variable, the axes were reversed. The formula, then, became $y = 0.08 + 8.17x$; y = millions of spermatozoa per 5 ml and x is absorbance units.

DISCUSSION

Spectrophotometric measurement has 2 limiting factors. First, the counts must be made soon after the spermatozoa are suspended in the salt solution because absorbance drops ca. 5% during the 1st hour. Foote (1972) observed a similar drop with time when measuring the concentration of bull spermatozoa. Secondly, the conversion formula is only valid for spermatozoa that are suspended in 0.5 M NaCl; perhaps the osmolar concentration of the solution affects the conformation of the sperm cells, and this, in turn, affects absorbance or light scattering.

On the other hand, spectrophotometric measurement is faster, easier, and less variable than the manual counting technique. The coefficient of variation for hemacytometer counts was 13.8%; spectrophotometric absorbance had a coefficient of variation = 1.0% when the samples were taken within a 15-min interval.

Since the spectrophotometric measurements were less variable than the counts made in this study, I apparently calibrated a less variable method with a more variable method. Therefore, the error in Fig. 1 may be a reflection of nonrepeatability of the counts rather than error in the spectrophotometric technique. Assuming that the errors in the hemacytometer counts were high as often as they were low, then the spectrophotometric measurements are probably more accurate than the error in Fig. 1 indicates.

As visualized in Fig. 1, error increases as absorbance values extend away from the midpoint, either higher or lower. When absorbance is < 0.1 or > 0.8 , there is very little experimental data to support the validity of the formula. Progressive dilutions of concentrated samples indicated, however, that the relationship between absorbance and sperm concentration continued to be linear when the absorbance reading reached 1.7.

The constant in the regression formula (-0.0096) can be explained, perhaps, by faulty preparation of samples. When preparing some of the first samples, I was not aware of the drop in absorbance with time. Therefore, when samples were too concentrated to count with a hemacytometer, I diluted them a second time. This caused a delay of perhaps 15 min, while samples not requiring a second dilution were usually counted within 5 min of preparation.

When samples were diluted a second time, only the second dilution was included in Fig. 1. The points representing delayed readings (second dilutions) were many of the circles in the 3.0–4.5 million range in Fig. 1. Because most of these sample points were near and slightly below the mean, their effect was probably a slight increase in the slope and a general lowering of the regression line. Both of these effects tend to reduce the zero intercept and are probably responsible for the constant (-0.0096). Although I hesitate to make subjective changes in the formula, I suggest removing the constant. This changes the regression formula to simply $y = 0.1224x$ and the conversion formula to $y = 8.17x$.

ACKNOWLEDGMENT

I am grateful to Ms. Mary Toaston, Biological Technician, and to Mr. John D. Tallant, Statistician, USDA, ARS, Southern Regional Research Center, New Orleans, LA, for their valuable assistance.

REFERENCES CITED

- Foote, R. H. 1968. Standards of sperm concentration, polystyrene latex particles as an aid in quality control. Proc. 2nd Tech. Conf. on Artificial Insemination and Reprod. Natl. Assn. of Animal Breeders 95–7.

1972. How to measure sperm cell concentration by turbidity (optical density). Proc. 4th Tech. Conf. on Artificial Insemination and Reprod. Natl. Assn. of Animal Breeders 57-61.
- Mackensen, O., and W. C. Roberts. 1948. A manual for the artificial insemination of queen bees. U.S. Bur. Entomol. Plant Quarantine ET-250. 33 pp.
- Salisbury, G. W., G. H. Beck, I. Elliot, and E. L. Willett. 1943. Rapid methods for estimating the number of spermatozoa in bull semen. J. Dairy Sci. 26: 69-77.
-