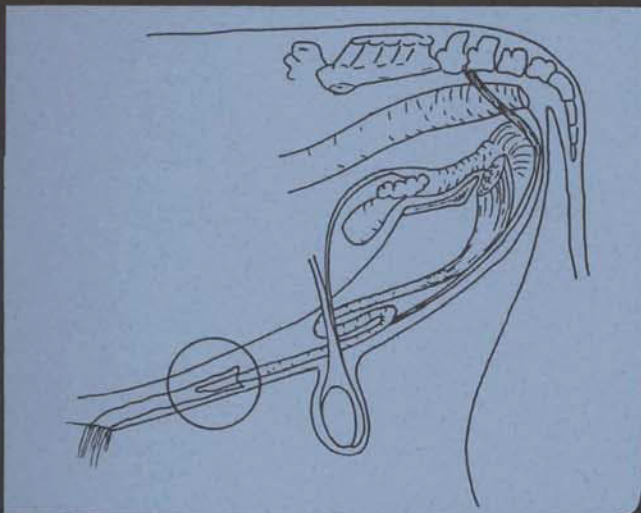


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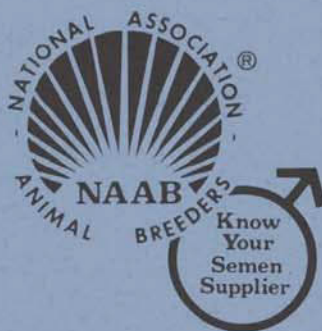
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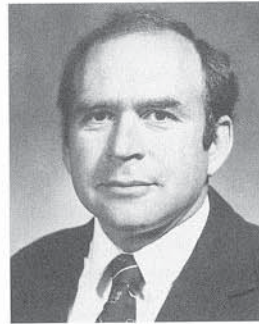


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FERTILITY OF BULLS ON THE EDGE OF THE DOSE-RESPONSE CURVE FOR NUMBERS OF SPERM PER INSEMINATE

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SEIDEL

George E. Seidel, Jr. was raised on a dairy farm in Pennsylvania. He received a B.S. degree from Penn State in Dairy Science in 1965, and M.S. (1968) and Ph.D. (1970) degrees from Cornell. For the past 28 years, he has been at the Animal Reproduction and Biotechnology Laboratory, College of Veterinary Medicine and Biomedical Sciences, at Colorado State University in Fort Collins. His current title is University Distinguished Professor. Current research projects include in vitro oocyte maturation and fertilization, use of antioxidants for culturing embryos, genetic control of early embryonic development, and sexing semen. The latter work resulted in foundation of a company, XY, Inc., which is owned in part by the Colorado State University Foundation. Dr. Seidel currently serves as Director of Science for this company.

Introduction

Reproductive physiologists frequently are interested in measuring fertility. This is a major and difficult undertaking in cattle. Even so, analogous to Winston Churchill's description of democracy, the infrastructure for evaluating fertility in cattle, while terrible, is in many respects the best available for any mammal (1). Ideally, conclusions should be applicable to a sample of ejaculates from a defined population of bulls bred to a defined population of females, inseminated by a defined population of inseminators breeding cows detected in estrus using defined

procedures, and on and on defining ad nauseam. In practice, we have a complex situation in which many of these factors cannot be controlled. Most researchers resort to doing the best possible with the resources available. We are forced to be opportunists, because the costs of doing everything "right" are prohibitive.

I will start by briefly describing problems of measuring fertility in cattle, because these must be kept in mind in interpreting experimental findings, and then review research concerning insemination with low numbers of sperm, including our own recent findings with low numbers of sexed sperm.

Realities of Measuring Fertility in Cattle

1. Binomial variance. Except for very special cases, researchers are stuck with binomial variance when measuring pregnancy rates¹ or non-return rates in cattle. The bottom line is that huge numbers of observations are required for reasonably precise estimates of means. This is because standard deviations due to binomial variance for binomial observations such as percent pregnant are roughly 50 percent for most relevant situations. The standard deviation of the mean, or standard error due to binomial variance, thus is:

$$\sim 50\% \div \sqrt{N}, \text{ or}$$

10% for N of 25

5% for N of 100

2% for N of 625

1% for N of 10,000

Recall that a 95 percent confidence interval of a mean is $\sim \pm 2$ standard errors. When it is necessary to evaluate differences of less than five percentage points between treatments or animals, thousands of observations per group are required to obtain meaningful results. Such numbers are sometimes available for experiments with non-return rates, but are seldom available for actual pregnancy rates.

2. Small differences are important. For most situations in the bovine AI industry, a difference of two or three percentage points in fertility is of great interest and importance. While differences of 10 or more percentage points in fertility are relatively easy to detect, such large effects usually occur only in the context of poor treatments or bulls of low fertility. Treatments that improve fertility 10 or more percentage points over controls will be relatively rare in this industry. Thus, if increasing fertility is the goal, one almost always is considering two to three percentage points effects, and therefore thousands of observations per treatment are required for statistical significance. One means of addressing this conundrum is to lower fertility deliberately by decreasing sperm numbers per insemination dose so that differences between treatments are amplified. Another approach is to use competitive

fertilization procedures (8) to obtain fertility information with fewer animals than with conventional procedures.

3. Laboratory measurements of spermatozoa have limitations in predicting fertility. Predicting fertility of a single semen sample with laboratory procedures is a difficult proposition under the best of circumstances (1). However, laboratory assays are invaluable as a quality control screen, and thousands of doses of semen are discarded daily that do not meet minimum quality control standards. Because there are different reasons why semen from a particular bull or treatment may have superior or inferior fertility in a given situation (7), it is unrealistic to expect that one or two laboratory tests will have high precision in predicting fertility when used alone. This is especially demanding if the range of values under consideration is only six or eight percentage points in pregnancy or non-return rates.

4. Non-return rates are an imperfect surrogate for pregnancy rates. Those in the bovine AI industry are acutely aware of limitations in non-return data, and the need to adjust these data in various ways for valid interpretations. Nevertheless, properly interpreted, non-return data are exceedingly valuable surrogates for pregnancy rates. Unfortunately, useable non-return rate information is becoming scarce due to direct semen sales. Obtaining actual pregnancy data from large herds is likely to become more important than in the past, and increasingly will be worth paying for.

5. Even pregnancy rates are imperfect measures of fertility. Problems with pregnancy determination include accuracy of diagnosis (is she really pregnant), accuracy of attribution (is she pregnant to the insemination under consideration), and whether embryonic or fetal death will occur. Under certain conditions, these errors are small, e.g. skilled personnel determining pregnancy between 40-70 days after insemination. It appears that ultrasonography is more accurate for timing pregnancy than palpation per rectum. Although it is expensive to obtain reliable pregnancy information from a large population of farms, this may be feasible from a small number of selected large farms.

For the special case of comparing fertility among AI sires, the Estimated Relative Conception Rate (ERCR) data calculated by Dairy Records Management Systems in Raleigh, NC are very useful. These data also have limitations, but repeatabilities are provided, and repeatabilities are quite high for bulls with more than 1,000 services. These data can be accessed at:

<http://www2.acs.ncsu.edu/drpc>.

¹ Note that fertilization rates, conception rates, pregnancy rates, calving rates, abortion rates, etc. can be defined in various ways. Functional criteria of success include: 1) Whether the cow lactates at the end of the pregnancy, 2) Whether the conceptus ends up being a successful herd replacement, 3) Whether there is a marketable pregnancy or offspring after birth, and/or 4) Considerations of cost, lack of dystocia, etc. I will define pregnancy as being pregnant with no evidence of impending abortion.

6. There are many populations of cows and heifers. Fertility of females varies according to age and parity, days post-partum, body condition, whether animals are cycling normally, etc. Treatments that are optimum for one population of cows may not be optimum for another. A relatively new finding is that pregnancy rates in dairy cows vary enormously whether ovulation occurs on the ovary ipsilateral or contralateral to the previously gravid uterine horn (5). When semen was placed into the uterine horn ipsilateral to ovulation, the pregnancy rate was 39.6 percent (191/482) in the previously gravid horn vs 51.4 percent (225/438) [$P < .01$] if by chance ovulation was contralateral to the previously gravid horn.

7. Reproductive management is critical. Pregnancy rates are profoundly influenced by accuracy of estrus detection, nutrition, season, estrus synchronization treatment, timing of insemination, site of insemination, semen handling procedures, skill and training of inseminators, fertility of semen, etc. These factors plus differences between populations of females mean that a 35 percent pregnancy rate per insemination may be quite acceptable in one situation, whereas a 70 percent pregnancy rate might be disappointing in another.

8. Bull to bull differences in fertility are enormous. With natural service, many of the differences in fertility among bulls are due to differences in sexual behavior; mating must occur for pregnancy. With AI, the propensity of sperm to survive after cryopreservation varies considerably from bull to bull, particularly in populations that have not been through multiple generations of conception with frozen semen. Another huge effect is an interaction between individual bulls and numbers of sperm per insemination dose (2). In most cases, this effect is masked by inseminating excessive numbers of sperm per insemination dose.

9. Insemination of overwhelming sperm numbers is a common practice. In an attempt to compensate for suboptimal reproductive management, less fertile populations of cattle, suboptimal handling of semen, bull to bull differences, etc., a great excess of sperm usually are packaged per insemination dose. This is a sensible policy, particularly when more sperm can be harvested than can be sold, the situation for most bulls. Lower sperm numbers frequently are used after information is available on fertility, generally from non-return rates. Most bull studs lower sperm numbers for "high demand" bulls, provided fertility does not decline noticeably.

10. Experimental treatments. For testing new treatments that may improve fertility, the most valid results usually will be obtained by using the populations of animals, personnel, and environments in which results will be applied. Unfortunately, the problem that it is difficult to detect treatments that may improve pregnancy rates only one or two percentage points is exacerbated by the fact that farmers' animals are being used, sometimes without their informed consent. Thus, there is a reluctance to use any treatment that may compromise fertility to any extent. Using critical sperm numbers becomes an important issue, because use of excessive sperm numbers per inseminate makes it virtually impossible to demonstrate beneficial treatment effects.

11. Designing and interpreting experiments with low sperm numbers per insemination dose. For applications, ideal sperm numbers are somewhat above the inflection point of the typical sigmoid-shaped dose response curve; to increase sensitivity for experiments, sperm numbers should be somewhat below this point (1). There usually will be a differently-shaped curve for each particular bull-population of cows-environment subgroup, which exacerbates the noise in experiments, increasing the variation above the background binomial variation. There are similar problems with any experimentation, which is why statistical procedures have been developed to design trials and interpret findings.

As an example, in our own studies on lowered sperm numbers per insemination dose, we have had very limited resources, and results apply to the populations and procedures used: well managed Holstein heifers; estrus synchronization with prostaglandin $F_{2\alpha}$; bulls of above average fertility; and well trained inseminators using specific procedures such as uterine horn insemination (9). Any experiment must be interpreted considering similar information.

The Dose-Response Curve for Numbers of Sperm vs Fertility

Early work on fertility vs number of sperm per inseminate has been reviewed by Salisbury and Vandemark (10) and Jondet (4). Their general conclusions were that non-return rates begin to drop asymptotically starting around 10 million total sperm/dose and then drop off more sharply by two million total sperm/dose. These curves are fairly similar for frozen vs cooled, liquid semen if one adjusts numbers for sperm death due to cryopreservation procedures. Amann (1) concluded that critical sperm numbers for cattle were about seven million total cryopreserved sperm per inseminate.

Jondet (4) presented data for four individual bulls at three doses of semen, 0.4, 3, and 13 x 10⁶ motile sperm per inseminate post-thaw (Figure 1). These data illustrate two recurring themes: there are considerable bull differences in fertility, and the rate of decline in fertility as sperm numbers decrease usually is much less for higher than lower fertility bulls. Jondet also found that inseminating more than 24 hours post-estrus resulted in markedly lower non-return rates with 0.4 x 10⁶ motile sperm/dose (30 percent) compared with 57 and 60 percent with 3 and 13 million sperm/dose. In a different study, he also noted that non-return rates at low sperm doses were 10 percentage points less for inseminators achieving low non-return rates than for those achieving high non-return rates.

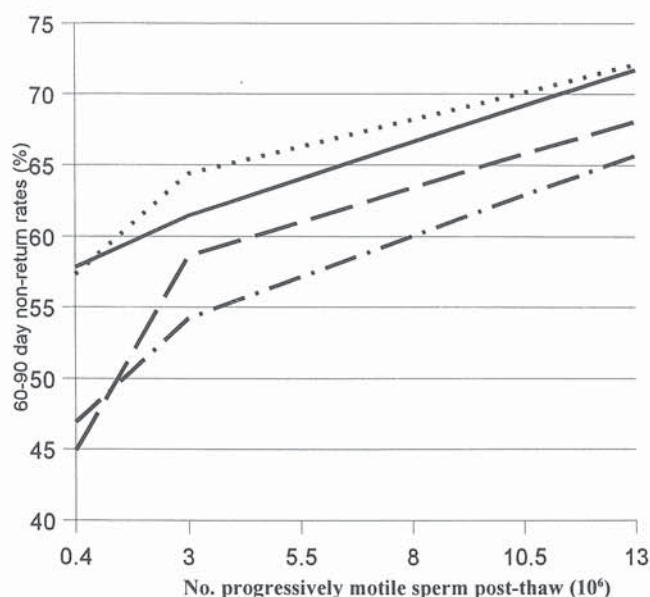


Figure 1. Non-return rates from four bulls (4) [mean 249 services/data point].

A number of other studies concerning the number of sperm/dose have appeared since Jondet (4), nearly all reporting non-return rates. A particularly interesting study by Macmillan and Curnow (6) showed that non-return rates with 0.5 and 2.5 million total sperm (liquid) were 60.5 and 65.9 percent when cows were inseminated late in estrus and 52.3 and 62.0 percent when insemination occurred early in estrus. One of the most thorough studies on numbers of sperm per inseminate was published this year (2). They demonstrated major differences among the 20 bulls studied, in the asymptotic shape of adjusted non-return rates vs sperm numbers per inseminate. Remarkably, several bulls had little or no decline using conventional AI procedures in non-return rates in extrapolations to less than 1 million total sperm/dose. A recent study by Shannon and Vishwanath (15) also is instructive. Non-

return rates with 0.5 and 2.5 million total unfrozen sperm/dose were 61 and 68 percent; with 5 and 20 million frozen sperm/dose, nonreturn rates were 60 and 68 percent.

Our own recent work in this area began with a liquid semen study using insemination doses of 100,000, 250,000, and 2,500,000 total sperm (12). Sperm were deposited into the uterine horn ipsilateral to the ovary with the largest follicle as determined ultrasonographically half a day prior to insemination. Detailed procedures have been described (12); the more important details are that bulls (n=3) were selected from the most fertile half of those available; the female population was Holstein heifers with estrus synchronized by two prostaglandin F_{2α} injections at a 12-day interval; semen was cooled to 5°C and shipped 2,400 km by a commercial, overnight service; inseminators (n=3) were trained for uterine horn insemination using conventional embryo transfer equipment; and five percent v/v seminal plasma was added back to the semen diluted in Cornell Universal extender, and packaged at 0.1 ml/0.25 ml straw.

Results are summarized in Table 1; 92 percent of ovulations (side determined by ultrasonography of the corpus luteum 7-9 days post-insemination) occurred ipsilateral to the ovary with the largest follicle. Pregnancy rates determined using ultrasonography 42-45 days after insemination were 41 percent, 50 percent, and 62 percent for doses of 100,000, 250,000, and 2,500,000 total sperm in heifers ovulating on the same side as insemination. Surprisingly, pregnancy rates were virtually identical with contralateral inseminations, although numbers of observations are low for these groups. Hawk and Tanabe (3) obtained similar results using much higher sperm numbers. Lopez-Gatius (5), however, found that pregnancy rates were significantly lower with insemination contralateral to ovulation in a study designed to compare ipsilateral and contralateral semen deposition. We found no significant difference among bulls (range 41-51 percent pregnant) or inseminators (range 46-52 percent pregnant).

Table 1. Pregnancy rates (main effects means) 42-45 days post insemination with nonfrozen semen.

Total sperm/inseminate	Side ^a	n	% Pregnant
1 x 10 ⁵	Ipsilateral	104	41 ^a
	Contralateral	14	36
2.5 x 10 ⁵	Ipsilateral	103	50 ^{b,c}
	Contralateral	8	50
2.5 x 10 ⁶ (control)	Ipsilateral	55	62 ^c
	Contralateral	2	50

^a Side of insemination relative to ovulation.

^{b,c} Values without a common superscript differ (P<.05).

A second study using frozen semen (11) was done similarly to the one just described with these important differences: 1) total sperm numbers were 200,000, 500,000, or 10,000,000 per 0.25-ml straw; 2) half of the semen was placed into each uterine horn with no attempt to determine ovulation side; and 3) heifers were inseminated once daily, evenings only, one-half or one day after estrus was first detected. Pregnancy rates are presented in Table 2.

Table 2. Pregnancy rates (main effects) 2 months post insemination with frozen semen.

Total sperm/inseminate (10 ⁶)	N	% Pregnant (2 months)	
		Actual mean	Least squares mean
0.2	152	54	51**
0.5	170	68	66
10.0	153	67	64

**Differs from other values, $P < .01$.

There was no significant difference between 500,000 and 10,000,000 total sperm frozen/insemination dose (Table 2), but the pregnancy rate was significantly lower for 200,000 sperm/insemination. There were no significant differences among bulls nor were there differences in pregnancy rates between AI 12 (65%; $n=239$) or 24 (63%; $n=236$) hr post-estrus detection. However, the pregnancy rate differed between the two inseminators (55 vs 65%, $P < .05$), but there was no inseminator X sperm number interaction.

There are some caveats associated with the somewhat surprising results of 68 percent pregnant with 0.5 million total sperm vs 67 percent pregnant with 10 million total sperm. One concern is the limited numbers of observations; the standard errors due to binomial variance alone are approximately four percentage points, so an 80 percent confidence interval on means would be $\sim \pm 6$ percent. Thus, the higher sperm dose might have resulted in a higher pregnancy rate by several percentage points than the lower dose if larger numbers of inseminations had been performed. If we assume that there really is no difference in pregnancy rates between these two doses, the next question is why, since other studies (2,4,15) show declines in non-return rates for most bulls in this range of sperm numbers.

The main differences between our study with frozen semen from studies showing lowered fertility with sperm numbers under one million/dose include:

- 1) Bulls were selected from the top half of the bull battery for fertility.
- 2) Only heifers were inseminated.
- 3) Estrus was induced by prostaglandin $F_{2\alpha}$.

- 4) Site of semen deposition was uterine horns, not the uterine body.
- 5) Smooth, side-opening sheaths were used for semen deposition.
- 6) There was more control of estrus detection, inseminator training, etc.

Any or all of these differences could contribute to explaining why 0.5 million sperm are sufficient for maximal or near maximal fertility under the specified conditions.

Pregnancy Rates with Low Doses of Sexed Semen

Our first efforts at combining sorting of X- and Y-chromosome-bearing sperm with low sperm dose insemination were logistically demanding and yielded variable pregnancy rates (12). Semen was collected early in the morning at Atlantic Breeders Cooperative in Lancaster, PA, transported to the USDA-ARS laboratory in Beltsville, MD, sorted into X- and Y-chromosome-enriched fractions, transported to Colorado State University, Fort Collins, CO, loaded into straws, and inseminated into heifers at various locations just after midnight or on the following day. Approximately 100,000 to 200,000 sexed sperm were inseminated into the uterine horns of these heifers. Pregnancy rates (two-months) ranged from zero to 45 percent (9/20), depending on the replicate; 82 percent (14/17) of the calves were of the designated sex. Pregnancy rates with this unfrozen, sexed semen were higher if inseminations were done between nine and 13 hours post-sorting (22.4%) than 17 to 29 hours post-sorting (2.6%). Also, it was unclear how applicable procedures would be across a population of bulls because pregnancy rates were much higher for one bull than several others. However, there was much confounding in this preliminary work, so the only definitive conclusion was that reasonable pregnancy rates could be achieved with AI of sexed semen under some conditions.

Procedures for flow cytometry/cell sorting were next developed locally in the XY, Inc. laboratory using the principles developed at USDA-ARS and a MoFlo® high-speed flow cytometer/cell sorter manufactured by Cytomation, Inc. This enabled logistically simpler experiments and much faster sorting rates.

For the next experiment (14), the MoFlo® system was operated at 500-600 live sperm/s of each sex, the cattle were only 200 km distant from the instrument, and the bulls were close at hand. The pregnancy rate to term after insemination of sperm selected to have X-chromosomes was 42 percent (Table 3); for the liquid semen control the pregnancy rate was 54 percent; and for the frozen semen control of 15.6×10^6 motile sperm/dose post-thaw, it was 52 percent. These data were balanced across three bulls and two inseminators. Eighteen of 19 calves born from

sexed semen were females. Only one sexed and one control pregnancy detected by ultrasound at 31-34 days post-insemination did not develop to term, indicating pregnancy wastage was minimal after one month of gestation. Fertility of this sexed semen was ~80 percent of controls. In a recent experiment (13), we found that pregnancy rates after artificial insemination of heifers with sexed, frozen-thawed semen were similar to pregnancy rates using sexed, unfrozen semen.

Table 3. Pregnancy rates with unfrozen, sexed semen.

Treatment	No. sperm/ inseminate	Term pregnancy (%)	No. females
Frozen control	15,600,000 motile	15/29 (52%)	11 (73%)
Liquid control	300,000 total	15/28 (54%)	8 (53%)
Liquid sexed	300,000 total	19/45 (42%)	18 (95%)

Future Directions

It is quite clear that under some circumstances it is possible to obtain near maximum fertility, as high as ~70 percent term pregnancy in heifers, with fewer than 10^6 sperm by using relatively standard AI procedures. It similarly is clear that 10^7 sperm are insufficient for maximum fertility under other circumstances, particularly with some bulls. There is great difficulty in determining when it is appropriate to lower sperm numbers and to what extent without lowering fertility. This is a special problem with "high demand" bulls, and also will be an issue during the first years of commercialization of sexed semen. While some progress is likely via changes in procedures such as uterine horn insemination, new methods of testing fertility would be desirable. Perhaps some competitive fertilization system will evolve that provides usable information from a few dozen inseminations using limited sperm numbers.

Nearly 150 bovine pregnancies have been produced with artificial insemination of sexed semen over the course of several experiments. We can conclude the following from these experiments:

- 1) There is no observable increase in abnormalities of calves.
- 2) Semen can be sexed routinely at 90 percent accuracy.
- 3) Pregnancy rates with 300,000 to 1,000,000 sexed sperm/insemination are 70-80 percent of controls (inseminated with many more sperm) with heifers, but possibly lower with cows.
- 4) As expected, there appear to be considerable differences from bull to bull in fertility with low numbers of sexed sperm per inseminate.
- 5) Pregnancy rates with sexed, frozen semen may be similar to those with sexed, liquid semen.

- 6) We can sort in excess of 1,000 live sperm of each sex chromosome composition at 90 percent accuracy per second, and are striving to triple that rate in the near future.
- 7) Sexed bovine semen may be available for retail sales within several years.

Acknowledgments

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