

## **INSEMINATION RELATED FACTORS AFFECTING FERTILIZATION IN ESTRUS-SYNCHRONIZED CATTLE**

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### **Introduction**

In addition to the requirements for healthy well-managed cattle and the sound application of synchronizing drugs, many other factors can also play a role in determining the success of an AI-estrous synchronization program. Considering the economic investment in semen and drugs, the success of such a program must be judged on the basis of pregnancy rate to the first artificial insemination service. Also, a good first service pregnancy rate response usually signifies conditions are good for second service and the breeding season in general. Additional key factors to be considered as impacting pregnancy rate to first service are semen quality (primarily dependent on choice of bull), the timing of insemination and the competence of the inseminators in handling and placement of semen. In most breeding strategies, whether estrous synchronization is employed or not, the semen quality, placement, and timing of insemination are critical to a successful pregnancy. The nature of subfertility due to the male/inseminate is proving as complex as that due to the female. Recent research in our laboratory utilizing accessory sperm (measure of sperm available for fertilization) and embryo quality (measure of fertilizing sperm and egg competence) have given us some insights to the problems associated with attempts to optimize pregnancy rate to AI. In this presentation I would like to address some of these insights particularly those associated with the semen/bull and the timing of insemination.

### **Compensable and Uncompensable Seminal Deficiencies**

We now know success or failure of an AI dose due to the male or inseminate resides in whether or not the egg was fertilized (fertilization rate) or whether or not the embryo developed normally and hatched in time to signal pregnancy to the dam (embryonic death). Both scenarios are embraced by semen quality and quantity and they must be considered together to address "pregnancy rate". Salisbury and VanDemark (1961) were the first to suggest the nature of the relationship between sperm quality and quantity. They proposed fertility increases with increasing numbers of viable sperm delivered to the cow up to a threshold, after which limiting factors in the female population become important and further increases in sperm are without effect on fertility. From the standpoint of semen quality, Pace et al. (1981) found this relationship to hold true for numbers of structurally intact and motile sperm in the inseminate. Sullivan and Elliott (1968) showed the minimum number of motile sperm required for maximum fertility (threshold) differed among bulls and bulls also differed in the maximum fertility at any

dosage (Figure 1). They also observed low fertility bulls required more sperm be inseminated than high fertility bulls in order to reach their respective maximum fertility. They postulated the

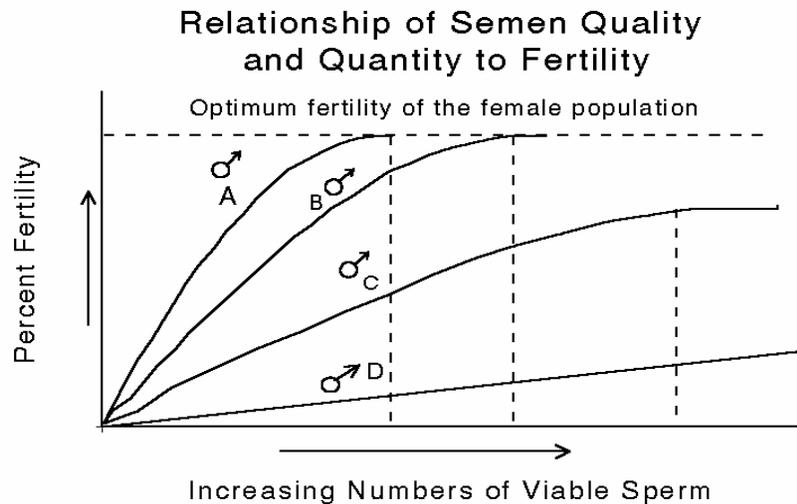


Figure 1. Relationship between pregnancy rate and the number of spermatozoa inseminated. The semen of different bulls varies in the maximum non-return rate and in the rate at which the maximum fertility is achieved with increasing sperm dosage (modified from Sullivan and Elliott (1968)).

requirement of more sperm by the subfertile bulls was due to the presence of abnormal sperm unable to negotiate barriers in the female tract precluding their access to the site of fertilization. This was shown to be true in a later study (Saacke et al., 1998) where sperm with classically misshapen heads did appear as accessory sperm following artificial insemination. From AI data in the Netherlands, den Daas et al. (1992) found the minimum number of sperm required to reach maximum fertility for a given bull (threshold) was independent of the maximum fertility achievable by that bull. Collectively, these studies, cited above, indicate it is now critical to recognize that seminal deficiencies fall into two major categories (compensable and uncompensable). Seminal deficiencies that are **compensable** would be those impacting pregnancy rates when numbers of sperm in the dosage are below threshold levels; i.e. pregnancy rate differences among bulls due to compensable seminal deficiencies would be minimized or eliminated simply by raising sperm numbers per AI dose. Such adjustments in the AI dose are made by reliable AI organizations when such deficiencies are known. However, where semen handling techniques or AI placement of semen is not adequate, impairment of pregnancy rate can be expected simply because lower than threshold numbers of viable competent sperm may be delivered to the cow. Seminal deficiencies that are **uncompensable** would be those that result in subfertility to AI or natural service regardless of sperm dosage and are represented by incompetent sperm that can fertilize, but not sustain an embryo. Such a deficiency is not compensable because incompetent sperm can preempt fertilization by a competent sperm equal to their frequency of occurrence in the semen dose. These deficiencies are intrinsic to the bull and can therefore only be minimized by bull selection. Bulls providing semen with unacceptable

levels of abnormal sperm appear to be the main source of uncompensable traits and should not be offered for semen preservation and use in AI.

### **Accessory Sperm and Their Implication to Pregnancy Rate**

Accessory sperm are those sperm trapped in the zona pellucida (outer covering of the egg), one of the important egg vestments sperm must penetrate in order to fertilize. Although there is only one fertilizing sperm, a range in number of sperm may be simultaneously competing for this honor. Once the fertilizing sperm enters the egg proper, a reaction occurs stopping progress of these competing sperm as well as the binding of additional sperm to the surface of the zona pellucida. Thus, accessory sperm are thought to represent, in number and quality, those sperm competing for fertilization in the oviduct of the cow during that short window in time provided by the ovulated fertilizable egg. Through several years of experimentation in our lab we have now recovered nearly 1000 eggs/embryos from single-ovulating cows 6 days post artificial insemination (nearly 30 different bulls were represented in these studies). Figure 2 shows the distribution of accessory sperm found in the zona pellucida of embryos and eggs from these cows as being very skewed, having an average, median and mode of 12.0, 2.4 and 0 sperm per ovum/embryo, respectively. Of reproductive interest is the association of accessory sperm number per egg/embryo to the fertilization status and embryo quality. This is best described by the median number (50 percentile of cows) of accessory sperm per egg/embryo (Table 1). Clearly, unfertilized eggs are simply sperm hungry, having a median accessory sperm number of 0. These data also show embryo quality tends to be positively related to median accessory sperm number. Good to excellent embryos have more accessory sperm than do degenerate or fair to poor embryos. This rather small difference has been interpreted to suggest the larger accessory sperm numbers are most likely associated with higher embryo quality because they represent greater competition among potential fertilizing sperm at the time of fertilization. There is evidence this competition favors a more competent sperm (i.e., there is sperm selection at the zona pellucida of the egg, Howard et. al., 1993) as well as at other locations in the female tract (previously reviewed, Saacke et al., 2000). On this basis, we ascribed a score to the embryos within categories of increasing accessory sperm number to determine the approximate number of accessory sperm (competing sperm) required to maximize embryo quality in artificially inseminated cows. These data are presented in Figure 3 and were based upon 804 embryos recovered from the 927 ova/embryos represented in Figure 2 and Table 1. It is apparent from Figure 3 that nearly 10 sperm per embryo were necessary to reach the maximum embryo quality index, after which increasing accessory sperm numbers had no influence on embryo quality. Regardless of embryo quality, the mode in accessory sperm number remained 0 suggesting that nature intended few sperm to approach the egg. On the otherhand, this exercise stresses the importance of semen handling and placement in the cow if we are to achieve threshold or above threshold numbers of sperm to the egg (i.e., approach 10 sperm/egg) necessary to maximize both fertilization rate and embryo quality for a general population of bulls. It should also be clear that the large variation in accessory sperm within and across fertilization/embryo status categories (Table 1) would preclude any use of accessory sperm numbers in predicting male fertility. However, increasing accessory sperm numbers could indicate

directions to be taken in adopting reproductive practices and strategies favoring improved pregnancy rates. Improving accessory sperm number would not be expected to bring inseminates or bulls harboring uncompensable traits into a normal reproductive range, thus, use of such bulls should continue to be discouraged.

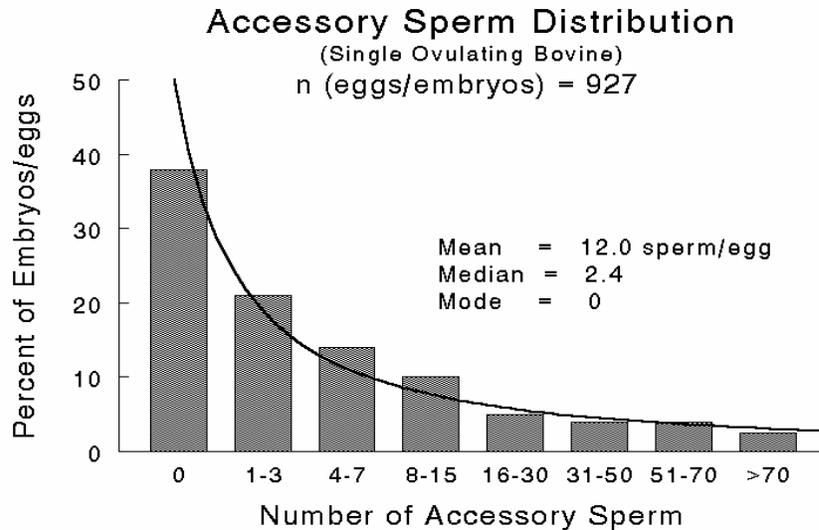


Figure 2. Frequency distribution of accessory sperm per embryo or ovum in artificially inseminated single-ovulating cows. Quality and quantity of semen used varied, but was within acceptable standards for commercial artificial insemination. Similar distributions have been reported for individual experiments utilizing both frozen and fresh semen (Saacke et al., 2000).

Table 1. Relationship of accessory sperm per embryo/ovum to fertilization status and embryo quality (n=927)

Fertilization status/ Embryo quality	n	Mean ± SD	Median
<b>Excellent/good</b>	449	24.5 ± 44.1	<b>7</b>
<b>Fair/poor</b>	213	17.2 ± 32.2	<b>5</b>
<b>Degenerate</b>	80	13.5 ± 38.1	<b>1</b>
<b>Deg/UFO</b>	12	2.7 ± 5.7	<b>0.5</b>
<b>Unfertilized</b>	173	1.6 ± 16.5	<b>0</b>

Embryo quality based upon Lindner and Wright, 1983 as modified for degenerate embryos by DeJarnette, et al., 1992

It is important one understands how embryo quality affects pregnancy rate. The best data on this point is Lindner and Wright (1983), who developed the embryo scoring system we used in the data presented above. They showed embryos classified as excellent to good produce twice as many pregnancies upon transfer to recipients as those classified fair to poor. One would expect much of this difference in embryo performance to carry over to embryos permitted to remain in utero. Of course degenerate embryos and unfertilized eggs produce no pregnancies under any circumstance. Based upon the median number of 2.4 accessory sperm per egg/embryo (Figure 2) and the threshold need nearly 10 sperm per ovum/embryo to optimize embryo quality (Figure 3), it is clear breeding practices favoring sperm access to the egg be adopted where possible. The effort to raise accessory sperm number per egg/embryo using several different strategies in artificially inseminated cows has been a central focus of our research program for the past several years. The outcome of our efforts have been reviewed previously (Saacke et al., 1994 and 2000) and thus, will not be repeated here except to emphasize two of the major positive factors impacting accessory sperm numbers per egg/embryo important to estrous synchronization and timed insemination, i.e., choice of bull and time of insemination relative to ovulation.

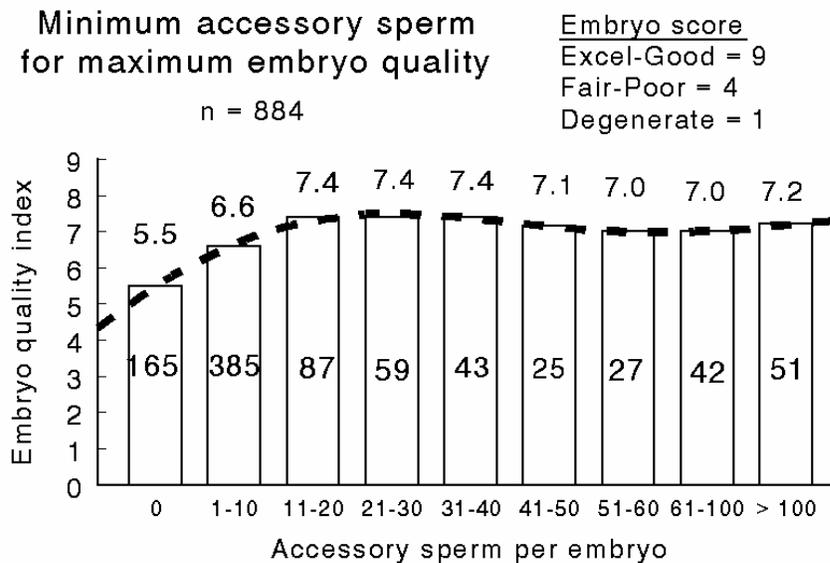


Figure 3. Histogram showing the numbers of accessory sperm required to maximize embryo quality index for 6 day-old embryos (morulae) derived from artificial insemination of single-ovulating cows. Embryo grading was according to Lindner and Wright (1983) as modified by DeJarnette et al., (1992). Embryo quality index was the average embryo quality based on the numerical score listed above. As may be noted, a minimum of 11–20 accessory sperm per embryo was required to maximize embryo quality index. The number within each bar is the number of embryos recovered in that accessory sperm category.

## The Effect of Bulls and Time of Insemination on Sperm Access to the Egg and Embryo Quality

Even when cows are bred at the conventional time following onset of heat (approximately 6-16 hours following onset), there is considerable variation among bulls with respect to numbers of sperm accessing the egg (Nadir et al., 1993). Data from this study comparing four bulls is presented in Table 2. Clearly, Bull A in this comparison has high egg access as denoted by the high accessory sperm number (median of 40 sperm per egg) compared to the other three bulls. It would be expected that such a bull as A would perform as well at low sperm dosages as at normal dosage and/or this bull would be less vulnerable to inseminator error in semen placement and handling than would other bulls.

Table 2. Accessory sperm differences per embryo/egg among bulls used at the same insemination dosage

Bull	n	Median	Mean $\pm$ SD
A	25	40	53 $\pm$ 61
B	37	8	15 $\pm$ 23
C	16	13	36 $\pm$ 65
D	20	2	11 $\pm$ 16

Nadir et al., 1993

Such a bull would be considered to have little to no compensable deficiencies and easily meet threshold numbers of sperm to the cow by AI. Under the same premise, bulls B and C would also match the fertility and embryo quality of bull A, but one would expect that while sperm dosage is appropriate, there is less room for inseminator or semen handling error with these two bulls. For bulls B and C, pregnancy rates could be expected to depend more heavily on dilution rates, inseminator competence and timing of insemination. Based on a median of two sperm per egg, bull D might be more marginal in optimizing fertilization rate and embryo quality under current use in AI. The seminal differences we are addressing across these four bulls would be considered compensable differences. Some of the semen traits involved in these differences are known and used by AI organizations in processing semen and determining sperm dosage rate. However, there are compensable differences among bulls that we still do not understand and can only determine by fertility data from the artificial insemination of adequate numbers of cattle.

With respect to differences among bulls important to embryo quality, i.e., the competence of a bull's fertilizing sperm or the uncompensable deficiency in his semen; our best judge of this is the occurrence of abnormal sperm in the semen. Abnormal sperm in the semen reflect the health of the spermatogenic process in the testes of the bull and in particular, the health of the DNA contributed to the embryo by the male (for review see Saacke et al. 2000). DeJarnette et al., (1992) examined the 6-day-old embryos from cows bred to semen of AI bulls having average and below average quality (within the AI center) based upon counts of abnormal sperm. Their data is shown in Figure 4. Clearly, the below average semen produced fewer excellent to good embryos and greater numbers

of degenerate embryos and unfertilized eggs when compared to semen of average quality. Bulls in AI are generally screened for significant numbers of abnormal sperm prior to acceptance into AI. In addition, in reliable AI organizations, routine examination of semen for abnormal sperm is practiced to check for changes in a bull's spermatogenic status. Sperm morphology evaluation is also one of the main components of the BSE (breeding soundness exam) of bulls practiced by veterinarians in approving breeding bulls for service. Availing oneself of a reliable semen service and/or BSE for bulls will minimize risk of using semen with significant uncompensable deficiencies. Of interest is that abnormal sperm in semen (the best indicator of uncompensable deficiencies), rarely get to the egg in vivo (Saacke et al., 1998). However, these abnormal sperm represent a deficiency in DNA quality that extends to the normal appearing sperm in the same samples (Acevedo et al. 2001) rendering them incompetent in sustaining the embryo after fertilization. Posing a particular problem in uncompensable semen deficiencies among beef breeds in particular, are fat bulls and a percentage of those coming off "hot rations" from test stations, where testicular thermoregulation has been impaired by inguinal fat (Kastelic et al. 1996).

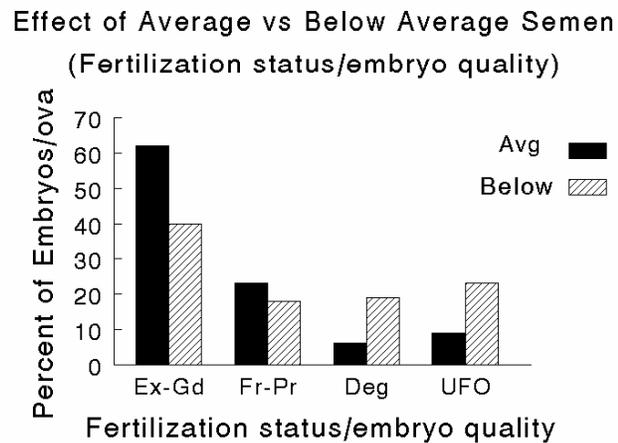


Figure 4. Effect of average and below average semen (based upon content of abnormal sperm) on fertilization status/embryo quality in single ovulating cattle. Both, fertility and embryo quality were influenced by the semen as noted in the shift in distribution across categories (n = 21 and 22 for the average and below average semen, respectively). (DeJarnette et al. 1992).

More recently we have examined the effect of insemination time on numbers of accessory sperm, fertilization status and embryo quality (Dalton et al., 2001). In this experiment, the HeatWatch® system was used to dictate time of artificial insemination for each cow. In this heat detection system, an electronic device is placed on the rump of the cow and a signal is transmitted via antennas to a computer when the device is activated for 2 seconds by the pressure of a mounting cow. On this basis, first mount, duration of mounting and number of mounts were permanently recorded along with the identification of the standing cow. In lactating Holsteins, ovulation occurs  $27.6 \pm 5.4$

hours following the first mount for either natural estrous cycles or prostaglandin synchronized cycles (Walker et al., 1996). Our experimental artificial insemination time was either 0 hour, (heat onset indicated by first mount), 12 or 24 following first mount. However, due to logistics associated with monitoring the computer every three hours followed by retrieving the cow for insemination, actual times of insemination were:  $2.0 \pm 0.9$  hours,  $12.1 \pm 0.6$  and  $24.2 \pm 0.7$  hours following the first mount, respectively. Six days following insemination, the embryo was recovered non-surgically and examined for fertilization status/embryo quality and numbers of accessory sperm according to previously published methods (DeJarnette et al. 1992). Artificial insemination was to one of three bulls used at random and balanced in number of resulting eggs/embryos recovered for each time of insemination. Accessory sperm data are presented in Table 3. Clearly, accessory sperm number per embryo/egg was favored by breeding later, rather than earlier. Fertilization rate and embryo quality are presented in Figure 5 for each insemination interval (0, 12, or 24 hours post estrus onset). From Figure 5, increasing fertilization rate can be observed to follow increasing accessory sperm number (Table 3), as expected. Fertilization rate is favored by breeding late (24 hours post heat onset) and poorest by breeding early, near onset of heat. However, examination of embryo quality in relation to time of insemination shows a shift from high quality embryos achieved by inseminations at/near onset of heat to low quality embryos from insemination at 24 hours following heat onset. On the basis of these data it appears optimum reproductive efficiency (pregnancy rate) is a compromise using our current techniques and recommendations in AI. If we inseminate too early, we suffer from lower fertilization rates (but embryo quality is good) and if we breed too late, we suffer from lower embryo quality (but our fertilization rate is good). Thus, the intermediate time of 12 hours post heat onset would prove optimal when using a precise method for determining heat onset (Figure 6). This optimum was verified in field studies using "HeatWatch®" (Dransfield et al. 1998) where 6-16 hours post onset of heat provided the best pregnancy rates. The basis for pregnancy rate failure by breeding late (24 hour post onset) could reside in the fact that we would often have an aging egg waiting for sperm if we assume ovulation occurs  $27.6 \pm 5.4$  hours post heat onset as detected by HeatWatch®. Sustained sperm transport to the site of fertilization in the oviduct requires a minimum of 4-6 hours following insemination in the cow (Hunter and Wilmut, 1984). Thus, sperm arrival in the oviduct following a 24-hour insemination would be 28 to 30 hours post heat onset, after many eggs were already ovulated. In the current study, this would indicate that a rather large portion of eggs would be aging awaiting sperm arrival. This probably attributes most of the degenerate embryos to late insemination rather than a male-related uncompensable trait. On the other hand, the high embryo quality associated with early insemination suggests duration of sperm residence in the female tract may result in exertion of additional selection pressure favoring fertilization by a more competent sperm, particularly where there are uncompensable sperm deficiencies in the semen (Figure 6). The correct explanation is probably a combination of the two but must await further research.

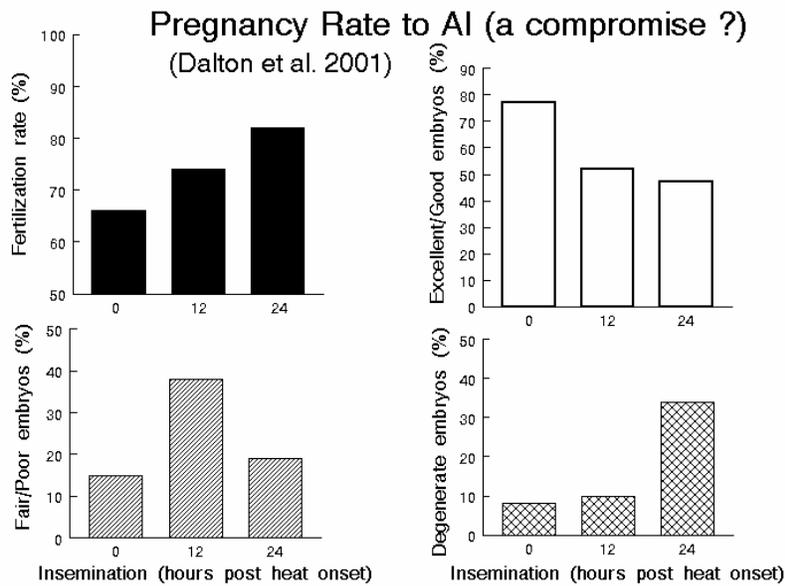


Figure 5. Effect of time of artificial insemination following onset of standing heat (Heat Watch System®) on fertilization status and embryo quality judged 6 days following artificial insemination (n = 117). (Dalton et al. 2001)

**Table 3. Effect of artificial insemination time on accessory sperm per embryo or egg**  
(breeding time post onset of estrus based on HeatWatch System®)

Treatment	n	Mean ± SD	Median	% Fert
0 hour	39	9 ± 23	1	66
12 hour	39	21 ± 46	2	74
24 hour	39	33 ± 53	4	82

•Ovulation 27.6 ± 5.4 hours  
•25 x 10<sup>6</sup> sperm/dose  
(Dalton et al., 2001)

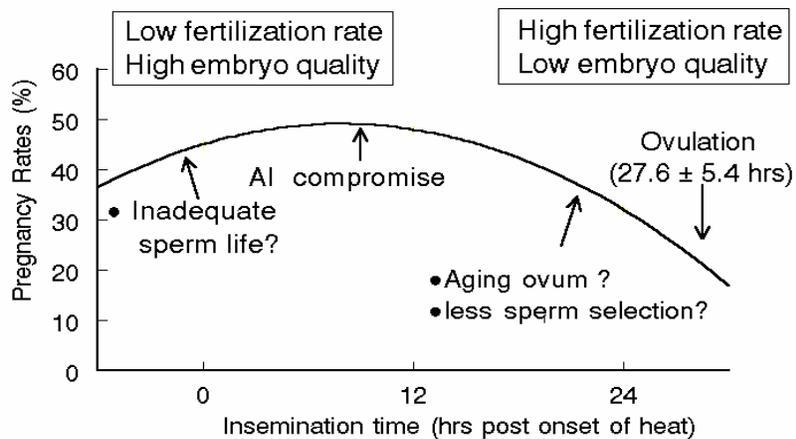


Figure 6. Calculated pregnancy rate from data presented in Figure 5 and based upon the ability of embryos classified excellent to degenerate to constitute a pregnancy (according to Lindner and Wright, 1983). AI as a compromise is based upon early inseminations being inadequate due to high levels of unfertilized ova, and late inseminations characterized by poor embryo quality, most likely due to an aging egg. However, high embryo quality appears to be associated with early insemination and high fertilization rates are associated with late insemination (Saacke et al., 2000).

### **Closing Comments**

Important to the insemination strategies employed with the new burgeoning regimes of estrous synchronization is knowing the time of ovulation and the variation in time over which ovulation can be expected in a group of treated animals. Only by such information can we make the correct decision on when to inseminate in relation to injection events or behavioral clues. The data presented here would indicate insemination must be late enough to maximize sperm access to the egg, but not so late to risk the possibility of an aging egg awaiting sperm arrival in the cow's oviduct. Thus, if a synchronization regime were to postpone ovulation until 30 or 35 hours following heat onset, the 24-hour insemination could be the best in optimizing pregnancy rate (both fertilization rate and embryo quality). Clearly, the CL and follicular control of the estrous cycle in cattle, currently under intensive research, offers tremendous advantages in synchronizing ovulation and tightening the variation in time of ovulation.

Finally, I would end this discussion by again recognizing the magnitude of bull differences that can greatly influence results to a synchronization program. Differences we have seen among bulls in response to time of insemination for one of our studies are shown in Figure 7. Although the trends were similar, the magnitude of differences in performance of bulls at different insemination times is quite great. In a timed insemination program, Bull A would be considered to perform well over a broad time span relative to ovulation, whereas bulls B and C really required later breeding to optimize their efficiency in sperm access to the egg. Unfortunately, and as you might expect, this is difficult, time consuming, expensive data to acquire and therefore not available on commercial bulls. The best protection one can have is to be aware of bull differences and know the expected time and variation in ovulation in order to choose an insemination time maximizing results to most bulls. Lastly, subscribing to a reputable semen source is the best protection against the use of poor quality semen.

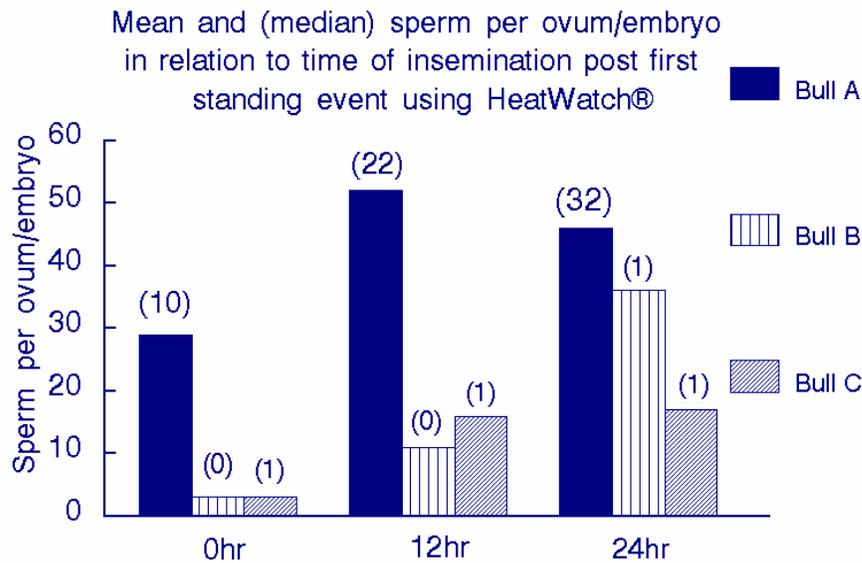


Figure 7. Variation among bulls in sperm access to the egg relative to time of insemination post heat onset. Mean sperm per egg/embryo is shown by the bars and the median number in brackets. Bull A has adequate numbers of sperm accessing the egg at all breeding times while bulls B and C require insemination closer to ovulation (Dalton et al., 2001).

### Acknowledgements

I am very appreciative of the support of the following agencies relative to our male research regarding accessory sperm, fertilization and embryo quality in artificially inseminated cattle: Select Sires Inc., National Association of Animal Breeders and the Virginia Ag Council.

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