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FACTORS AFFECTING FERTILIZATION IN ESTROUS 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 that 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. Current 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 that 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 that 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 that the minimum number of motile sperm required for maximum fertility (threshold) differed among bulls and that bulls also differed in the maximum fertility at any dosage (Figure 1). They also observed that low fertility bulls required that more sperm be inseminated than for high fertility bulls in order to reach

their maximum fertility. They postulated that the 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 be true in a later study (Saacke et al., 1998). From AI data in the Netherlands, den Daas et al. (1992) found that 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 that 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 could 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 sperm may be delivered to the cow. Seminal deficiencies that are **uncompensable** would be those that result in subfertility to AI 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 the incompetent sperm have a chance of preempting 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.

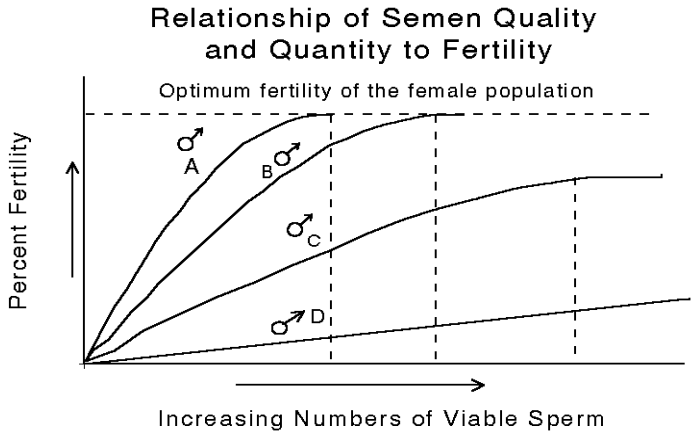

Figure 1. Relationship between pregnancy rate and the number of spermatozoa inseminated. The semen of different bulls varies in the maximum non-return rate and in the rate at which the maximum fertility is achieved with increasing sperm dosage (modified from Sullivan and Elliott (1968).

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 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

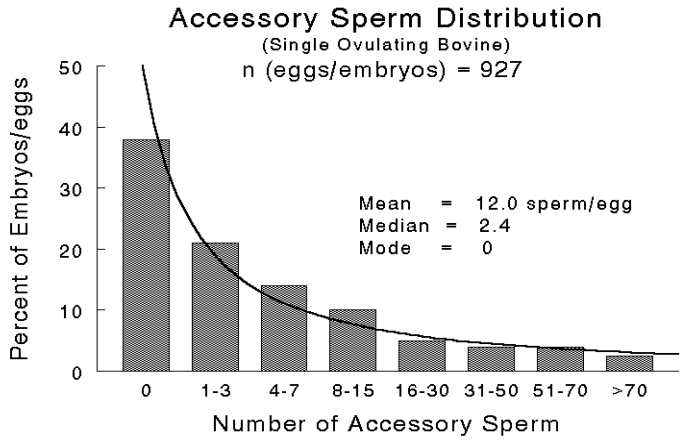

Figure 2. Frequency distribution of accessory sperm per embryo or ovum in artificially inseminated single-ovulating cows. Quality and quantity of semen used varied, but was within acceptable standards for commercial artificial insemination. Similar distributions have been reported for individual experiments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

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 importance is the association of accessory sperm number per egg/embryo to the fertilization status and embryo quality. This is best described by the median (50 percentile of cows) number 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 that embryo quality is positively related to median accessory sperm number. Good to excellent embryos have more accessory sperm than do degenerate

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
Excellent/good	449	24.5 ± 44.1	**7**
Fair/poor	213	17.2 ± 32.2	**5**
Degenerate	80	13.5 ± 38.1	**1**
Deg/UFO	12	2.7 ± 5.7	**0.5**
Unfertilized	173	1.6 ± 16.5	**0**

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

or fair to poor embryos. This has been interpreted to suggest that 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 and that this competition favors a more competent sperm (i.e., there is sperm selection in the zona pellucida of the egg, Howard et. al., 1993). 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 884 embryos recovered from the 927 ova/embryos represented in Figure 2 and Table 1. It is apparent from Figure 3 that approximately 11 to 20 sperm per embryo were necessary to reach the maximum embryo quality index, after which increasing accessory sperm numbers had no further relationship to embryo quality. This 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., greater than 11 sperm/egg) necessary to maximize both fertilization rate and embryo quality.

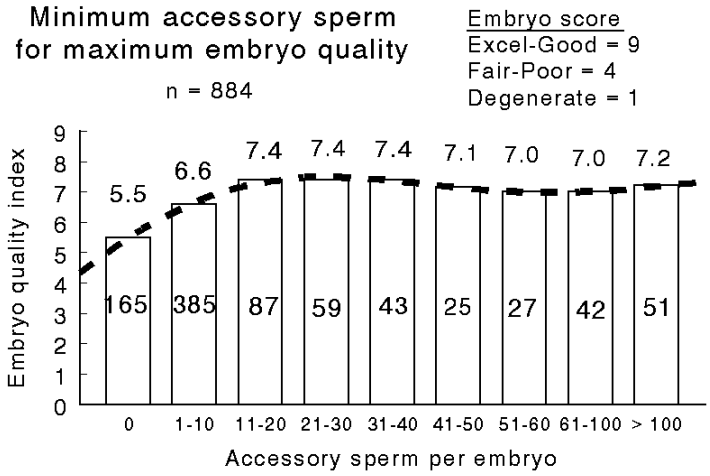

Figure 3. Histogram showing the numbers of accessory sperm required to maximize embryo quality index for 6 day-old embryos (morulae) derived from artificial insemination of single-ovulating cows. Embryo grading was according to Lindner and Wright (1983) as modified by DeJarnette et al., (1992). Embryo 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.

Important to this discussion is that one understands how embryo quality affects pregnancy rate. The best data on this point is that of Lindner and Wright (1983), who developed the embryo scoring system we used in the data presented above. They showed that embryos classified as excellent to good produce twice as many pregnancies upon transfer to recipients as those that are 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 for more than 11 sperm per ovum/embryo to optimize embryo quality (Figure 3), it is clear that 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.

The effect of bulls and time of insemination on sperm access to the egg and embryo quality

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 4 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 3 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 that this bull would be less vulnerable to inseminator error than the other bulls when maximizing fertility and embryo quality. 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

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

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

Nadir et al., 1993

appropriate, there is less room for inseminator or semen handling error with these two bulls. For bulls B and C, pregnancy rates will depend more heavily on inseminator competence and timing of insemination. Based on a median of 2 sperm/egg, bull D would be expected to be inferior in optimizing fertilization rate and embryo quality under current use in AI. The seminal differences that we address 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 yet understand and can only be determined 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 are 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 bulls 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. Posing a particular problem in uncompensable semen deficiencies are fat bulls and a percentage of those coming off "hot rations" from test stations, where testicular thermoregulation has been impaired.

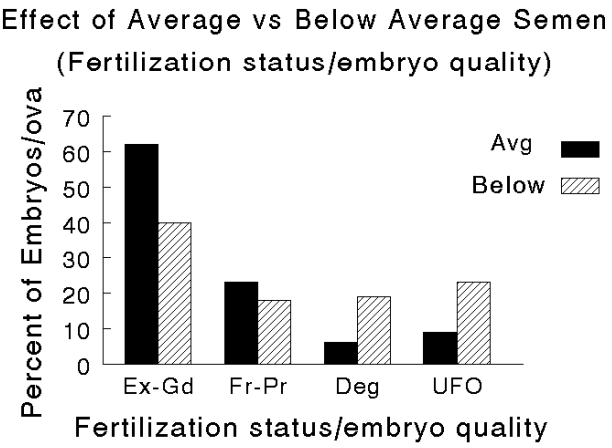

Figure 4. Effect of average and below average semen (based upon content of abnormal sperm) on fertilization status/embryo quality in single ovulating cattle. Both, fertility and embryo quality were influenced by the semen as noted in the shift in distribution across categories (n = 21 and 22 for the 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 ± 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 ± 0.9 hours, 12.1 ± 0.6 and 24.2 ± 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).

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 10^6 sperm/dose

(Dalton et al., 2001)

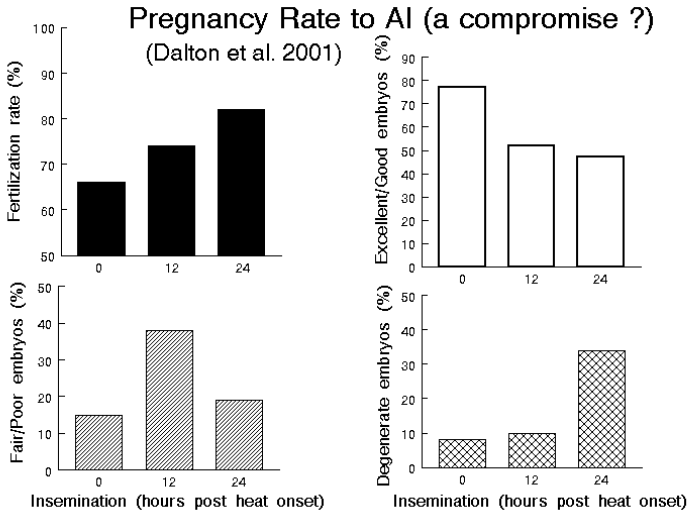

Figure 5. Effect of time of artificial insemination following onset of standing heat (HeatWatch System®) on fertilization status and embryo quality judged 6 days following artificial insemination (n = 117). (Dalton et al. 2001).

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 that 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.

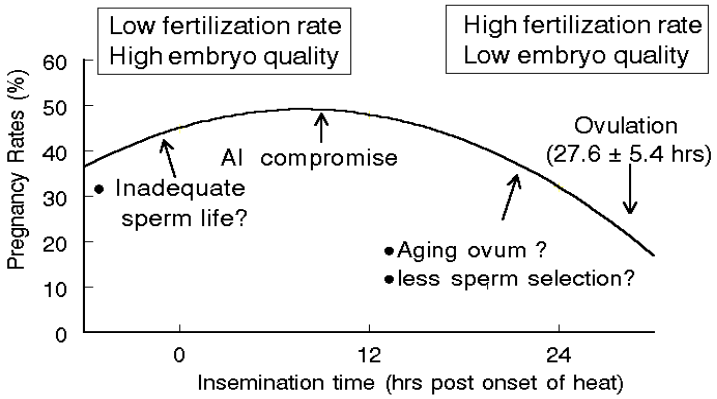

Figure 6. Calculated pregnancy rate from data presented in Figure 5 and based upon the ability of embryos classified excellent to degenerate to constitute a pregnancy (according to Lindner and Wright, 1983). AI as a compromise is based upon early inseminations being inadequate due to high levels of unfertilized 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).

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 that ovulation occurs 27.6 ± 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. This would indicate that in the current study, a rather large portion of eggs would be aging awaiting sperm arrival. This probably accounts for most of the degenerate embryos from this late insemination. On the other hand, the high embryo quality associated with early insemination suggests that

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.

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. 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 that 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 as well as tightening the variation in the ovulatory event.

Finally, I would end this discussion by again recognizing the magnitude of bull differences that can greatly influence results to a synchronization program. Differences that we have seen among bulls in response to time of insemination for one of our studies is 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 and therefore 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 to avoid a problem bull, subscribe to a reliable source of quality semen.

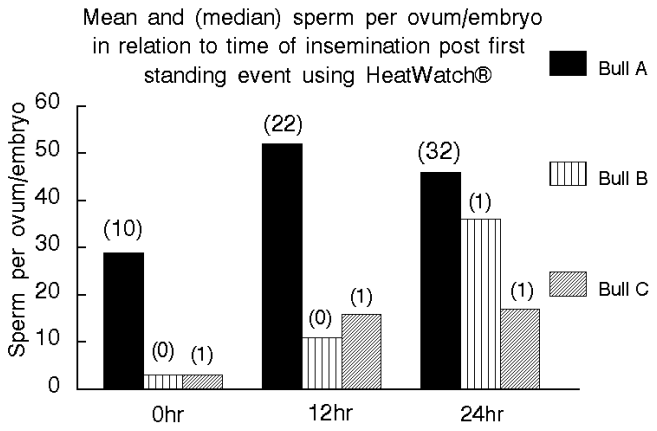

Figure 7. Variation among bulls in sperm access to the egg relative to time of insemination post heat onset using the HeatWatch® system of recording mounts. Mean sperm per egg/embryo is shown by the bars and the median number in brackets. Bull A has adequate numbers of sperm accessing the egg at all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

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