ADVANCES IN SEMEN DILUTION

P. SHANNON
New Zealand Dairy Board, Newstead, Hamilton

SUMMARY

Experiments have shown that using Caprogen (a nitrogen-saturated diluent) the number of sperm per insemination can be reduced far below the optimum level for egg yolk citrate without adversely affecting conception rate.

This has had far-reaching effects on bull coverage. In 1960, the average number of inseminations per bull was 4,000; in 1967, the figure will be considerably more than 20,000, and the six top-rated Jersey bulls will average in excess of 40,000 inseminations each. Apart from the increase in ratings which has resulted from this increase, there has also been a substantial saving in operating costs. This is estimated to be some $200,000 annually.

Further reductions in dose rate appear possible on the basis of recent experiments. A reduction to 1.5 million sperm per insemination would increase the coverage of top-rated sires to between 80 and 90,000 inseminations. Use of rediluted deep-frozen semen would further increase the coverage and obviate the need for further bulls.

In 1966, three bulls in the Artificial Breeding Scheme reached a lifetime total of 100,000 inseminations. By the early 1970s, it can reasonably be expected that this will be the average annual coverage per bull.

THE SUCCESS of any commercial A.I. organization depends on two main factors, first, conception rates, and, secondly, the quality of the bulls used. Without good conception rates, no viable A.I. scheme could exist, and without bulls of high genetic merit there would be no justification for its existence.

This paper discusses briefly measures taken to improve fertility, and, in rather more detail, methods used to increase the coverage of highly-rated sires.

METHODS OF IMPROVING CONCEPTION RATES

The conception rate by A.I., although dependent on the inherent fertility of the bulls used, is profoundly affected also by the diluent with which the semen is diluted.

Until 1960, the diluent used in New Zealand was the egg yolk citrate diluent developed by Salisbury *et al.*, 1941. This diluent gave satisfactory results with semen

used on the same day as collection, but only about a third of the bulls used gave satisfactory results with semen used on the day after collection. Satisfactory results had been achieved from 1956 to 1960 by using about two thirds of the semen collected on the day of collection and restricting for use on the day after collection semen which exhibited good storage characteristics.

In 1960, a series of trials was initiated to develop, and test in the field, new diluents which would maintain fertility for a longer period. It was found that the addition of glycine and glycerol markedly improved the storage life of diluted semen. Subsequently, it was found that caproic acid, and nitrogen saturation of the diluent, also improved conception rates. Surprisingly too, this diluent (Caprogen) gave better results when stored at ambient temperatures rather than at 5°C. With this diluent satisfactory fertility can be maintained with all bulls for a period of three days.

INCREASING THE COVERAGE OF BULLS

The seasonal output of semen from Newstead Artificial Breeding Centre in 1967–8, shown in Fig. 1, illustrates the difficulties that have to be overcome in increasing the coverage of proven bulls.

The spring mating season lasted for only 17 weeks during which some 660,000 cows were serviced. Even within

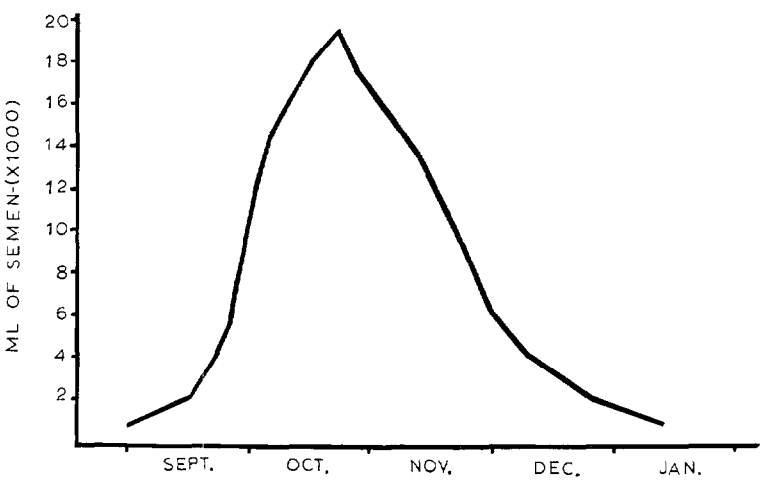

Fig. 1: Seasonal supply of semen from Newstead Artificial Breeding Centre (1967–8).

this period, however, the daily requirements of semen varied widely rising from 600 ml at the beginning of September to 20,000 ml in October and declining to 1,000 ml in mid-December.

Because of such fluctuations in demand for semen, it appears that deep-frozen semen would be ideal for New Zealand conditions. Semen could be collected all the year round and used over the peak period. Initially, the change to a deep-frozen programme was rejected for three major reasons: (1) Conception rates obtained with deep-frozen semen were well below those obtained with liquid semen; (2) Not all semen could be deep frozen; (3) The freezing and storing of semen at low temperatures would have increased costs considerably.

Work was therefore concentrated on improving the coverage of bulls using liquid semen.

DILUTION RATE TRIALS WITH LIQUID SEMEN

Extensive dilution rate trials in America, using the egg yolk citrate diluent, showed that fertility progressively declined with increasing dilution, and that this rate of decline was gradual until a level of 10 million motile sperms (15 million total sperms) was reached below which the decline was accelerated (Salisbury and Van Demark, 1961).

In New Zealand until 1960, the average dilution rate used was 18 million sperm per ml, the dilution rate varying from 25 million for bulls of lower-than-average fertility to 12.5 million for bulls of high fertility. Reduction of sperm numbers from this level resulted in a reduction of fertility especially with semen used on the day after collection.

One of the problems associated with high dilution rates is that, in addition to sperm numbers being reduced, sperm die more quickly than at low dilutions. Because of this, it was felt that reduction in sperm number per insemination, by reducing the volume used per insemination from 1 ml to 0.5 ml, would be more successful than increasing dilution rates.

Two trials were conducted in 1960 and 1961 to test this contention. In 1960, a comparison of conception rates was made when doses of 1 ml and ½ ml per insemination were used. For 9,964 control inseminations, the conception rate was 63.3%, and for 3,513 experimental inseminations, 62.3%. Because the reduction in fertility was so small, the insemination dose was reduced to 0.75 ml

per insemination in the 1961 season and a further large-scale trial was conducted to compare fertility doses of 0.75 ml and 0.5 ml per insemination. The results were: 0.75 ml dose, 407,827 inseminations, 64.3% conception rate; 0.5 ml dose, 69,853 inseminations, 63.3% conception rate.

The slight fall of 1% in conception rate when 0.5 ml dose was used appeared acceptable in view of the reduction in operating costs and the greater utilization of highly-rated proven sires that resulted from its use.

Although this reduction in volume per insemination resulted in a worthwhile increase in bull coverage, further increase could be obtained only by increasing the dilution rate.

Brief mention has already been made of the "dilution effect", namely, that, with increasing dilution the livability of sperm is reduced. Although this has been extensively reported as fact, no completely satisfactory explanation of the phenomenon has been given. Moreover, although it has been observed that the dilution rate effect occurs in diluents containing egg yolk, no critical work on the reasons for the dilution effect in such diluents had been reported.

It was decided, therefore, to investigate two theories given for the dilution effect in diluents containing egg yolk. The diluents used were 14G and 14GC. These were basically egg yolk citrate diluents, supplemented in the case of 14G by the addition of glycine and glycerol, and by glycine glycerol and caproic acid in the case of 14GC. These additions to the citrate diluent have been shown to improve significantly the storage life of sperm.

The two theories tested were (1) That the dilution effect was due to dilution of seminal plasma; and (2) That dilution effects are caused by leakage of essential intracellular substances.

The first theory was tested by adding 10% seminal plasma to semen samples diluted to contain 12.5 million sperm per ml.

If the second theory were correct, the addition of dead sperm should, to some extent, reduce the dilution effect. To test this, 6.25 million dead sperm (killed by cold shock) were added to samples diluted to contain 6.25 million sperm per ml. This was compared with semen diluted to contain 6.25 and 12.5 million sperm per ml.

Neither the addition of seminal plasma nor of dead sperm improved the livability of stored sperm. In fact,

the contrary was the case, livability being markedly reduced by both treatments.

As neither the addition of seminal plasma nor dead sperm reduced the dilution effect, it was concluded that the detrimental effect on livability was due to dilution of live sperm and must result from some function of metabolism.

One possibilty was that when sperm are stored at high concentration they may reduce oxygen tension in the surrounding media. To test the effect of oxygen level on livability of sperm, split ejaculates from 20 bulls were stored at 5° C at concentrations of 12.5 and 200 million sperm per ml in 14G, 14GC, 14GN (14G saturated with nitrogen) and Caprogen (14GC saturated with nitrogen).

At days 2, 6, and 8 of storage, samples were incubated at 37° C. Samples which contained 200 million sperm and were stored at 5° C were rediluted in the appropriate diluent to contain 12.5 million sperm per ml, immediately before incubation.

The results of this trial are shown in Table 1.

The three significant features of this trial were: (1) A significant difference in livability between dilution rates; (2) A significant interaction between nitrogen saturation and dilution effect; (3) A significant interaction between nitrogen saturation, days of storage and dilution rate.

The effect of nitrogen saturation, therefore, was to halt the decline in incubation life associated with aging semen, and this effect was much more marked in semen stored in a highly-diluted form than in a concentrated form. It seems unlikely that this effect is due to nitrogen as such, but rather to a reduction of oxygen tension in the media.

TABLE 1: INCUBATED LIFE (HR) AT 37°C OF SPERM STORED AT 12.5 MILLION AND 200 MILLION SPERM/ML
All Samples Incubated at 12.5 Million Sperm/ml

	Unsaturated Diluents (14G and 14GC)		*N-saturated Diluents (14GN and Caprogen)*	
	Storage Concentration			
Days of Storage	*12.5*	*200*	*12.5*	*200*
2	39.9	45.0	47.6	51.2
6	31.9	42.1	48.3	50.0
8	23.0	37.3	47.8	50.3
Average	31.6	41.5	47.9	50.5

To further test the effect of storage in concentrated or diluted form in nitrogen-saturated diluents, split ejaculates from 20 bulls were diluted in Caprogen to contain either 12.5 or 200 million sperm per ml. Samples were incubated at 12.5 million sperm per ml. After seven days of storage, the incubated life for semen stored at 200 million sperm per ml was 50 hr, and for sperm stored at 12.5 million, 52.1 hr.

The effect of storage concentration on fertility was also tested. Split ejaculates from 7 bulls were diluted to contain either 200 million or 25 million sperm per ml in Caprogen. After 7 or 8 days' storage, material stored at 200 million was rediluted to contain 25 million sperm per ml. Both samples were used on the day the concentrated stored material was rediluted. A 0.5 ml insemination dose was used so that the number of sperm per insemination was 12.5 million. The results of the trial were: storage at 200 million, 1,207 inseminations, 40.8% conception rate; storage at 25 million, 1,179 inseminations, 52.5% conception rate. The difference in fertility was significant.

As there was a beneficial effect of nitrogen saturation on livability of highly-diluted samples, a number of dilution rate trials using the Caprogen diluent have been conducted. A small scale trial in 1963 showed no significant effect on fertility by reducing sperm numbers per insemination from 6.25 million to 3.125 million sperm per ml. Two further trials, conducted in 1964 and 1965, involved semen diluted in Caprogen but stored at ambient temperatures rather than 5° C. The results of these trials are shown in Table 2.

TABLE 2: CONCEPTION RATES IN CATTLE RELATIVE TO NUMBERS OF SPERM (MILLION) INSEMINATED

	Sperm/Insemination		
	5.0	*3.75*	*2.5*
1964 Trial			
No inseminations	15,997	8,574	8,922
% non-returns	68.0	69.3	67.0
	Sperm/Insemination		
	3.75	*2.5*	*1.875*
1965 Trial			
No inseminations	14,110	6,130	6,124
% non-returns	63.6	63.3	61.2

TABLE 3: FERTILITY FOLLOWING INSEMINATION WITH VARIABLE NUMBERS OF SPERM (MILLION) IN CAPROGEN-BASED DILUENTS

	Sperm/Insemination					
	2.5		*1.875*		*1.5*	
Diluent	*No. Insem.*	*%N.R.*	*No. Insem.*	*% N.R.*	*No. Insem.*	*% N.R.*
Caprogen (20% egg yolk)	5,614	63.5	2,107	65.8	1,933	65.2
Caprogen and catalase (20% egg yolk)	8,154	66.6	1,575	67.2	1,601	65.6
Caprogen and catalase (5% egg yolk)	7,160	67.4	1,515	67.5	1,699	68.4

Summarizing these trials, it appears that reducing the numbers of sperm inseminated from 5 million to 2.5 million sperm per insemination does not result in any significant decline in fertility. However, there did appear to be a decline in fertility when sperm numbers were reduced below 2.5 million.

This reduction in fertility with sperm numbers below 2.5 million may be due to the fact that nitrogen saturation does not completely remove oxygen from the diluent. Oxygen levels may therefore again be critical at these very high dilutions. One of the products of aerobic metabolism in bull sperm is peroxide, which has detrimental effects on livability, but can be effectively eliminated by the addition of the enzyme catalase to the diluent. Certainly, catalase improves livability in even nitrogen-saturated diluents and a combination of nitrogen saturation and catalase addition is a most effective protection against dilution effects at very high dilution rates.

To test the effect of catalase additions, a trial was conducted in 1967 to compare the effects of different dilutions with and without catalase and at two different levels of egg yolk. Results of the trial are shown in Table 3. Two features in this table are of interest. First, the decline in conception rate reported in 1965 with inseminations of 1.875 million sperm has not been repeated. Secondly, although the addition of catalase appears to have improved conception rates, it does not appear to have affected the dilution rate response.

FROZEN SEMEN

The possible future role of deep-frozen semen in New Zealand is of interest. One of the reasons it has not been used extensively is the cost of freezing and storing the semen. One way in which these costs could be reduced is by freezing the sperm in concentrated form, then thawing and rediluting it in diluent before sending it into the field. This method would have the further advantage of obviating the need for low temperature equipment in the field.

This is not a new idea. James and Fyvie (1955) very early in the history of deep-frozen semen recognized this technique as a potential money saver. However, the results they obtained were not very promising. With material inseminated within two hours of thawing the conception rate was 59%; used between 4 and 8 hours after thawing, 34% and from 12 to 20 hours after thawing, 6%.

Although these early results looked distinctly unpromising, it was decided to investigate this possibility further.

Work on the reasons for dilution effect suggested one reason for the poor livability of rediluted deep-frozen semen. The addition of dead sperm to a diluted semen sample significantly reduced livability. In the freezing process, a considerable number of sperm is killed so it is distinctly probable that these dead sperm are the reason for the poor survival of thawed frozen sperm. Indeed, the addition of thawed frozen sperm to normally-diluted semen reduces livability in much the same way as the addition of dead sperm.

Laboratory trials have shown that the detrimental effect of dead sperm can be mitigated by reducing the level of egg yolk. Further protection can be afforded by the addition of catalase. In fact, the livability of deep-frozen semen rediluted in Caprogen with 5% egg yolk plus catalase is quite good.

Because of good laboratory results, a small-scale trial was conducted in the 1967 spring mating season. Single

TABLE 4: FERTILITY FOLLOWING INSEMINATION WITH REDILUTED SEMEN AFTER DEEP-FREEZE STORAGE

Semen Used	*No. Insem.*	*% Non-returns*
Day of dilution	 353	63.2
Day after dilution	 654	64.1
2 days after dilution	 80	55.0

ejaculates from 5 bulls were diluted to contain 200 million sperm per ml and deep frozen. After 4 months' storage at −79° C, they were thawed and rediluted in Caprogen with 5% egg yolk plus catalase to contain 50 million sperm per ml. Semen was used on the day of, day after, and two days after dilution, with 0.5 ml for each insemination (25 million sperm). Results from the trial are given in Table 4.

There was no decline in conception rate with semen from the "day of" to the "day after" dilution and the overall results for the five bulls were the same as those obtained with normal liquid semen from Newstead.

The results are highly promising and this work will be greatly extended in the current year when it is hoped to incorporate dilution rate trials in the experiment.

REFERENCES

James, J. P.; Fyvie, A. A., 1955: *Proc. N.Z. Soc. Anim. Prod., 15*: 126.

Salisbury, G. W.; Fuller, H. K.; Willett, E. L., 1941: *J. Dairy Sci., 24*: 905.

Salisbury, G. W.; Van Demark, N. L., 1961: *Physiology of Reproduction and Artificial Insemination of Cattle.* Freeman and Co., San Francisco and London. 639 pp.