CHAPTER 4

Effect of level of egg yolk and dilution techniques on post-thaw semen quality

4.1 Introduction

Glycerol has been used as a cryoprotectant in extenders for freezing semen since the initial discovery made by *Polge et al. (1949)*. However in some cases, for example, in avian artificial insemination *(Sexton, 1973; Lake & Ravie, 1984)* and in the cervical insemination of ewes *(Abdelhakeam et al., 1991 b)* glycerol has been shown to cause adverse effects on fertility. The study of *Slavik (1987)* of ram semen has shown that glycerol may alter the normal acrosomal reaction with a consequent decrease in fertility rate. This problem can be overcome by using laparoscopic insemination but this technique requires highly trained technicians and aseptic procedures *(Evans & Maxwell, 1987)* which are not always cost effective and are inconvenient compared with cervical insemination.

Nagase et al. (1964 a) and *Gibson & Graham (1969)* have reported the successful freezing of bull semen using glycerol-free extenders. The successful freezing of ram semen in terms of post-thaw motility percentage using glycerol-free extenders has been reported by *Abdelhakeam et al. (1991 a,b)*. The effects of these

extenders on aspects of spermatozoal structure such as acrosomal integrity were not evaluated in these studies and require further investigation.

The experiments in this chapter were designed to determine suitable egg yolk levels and the best method of freezing ram semen with TEST extenders *(Abdelhakeam et al., 1991b)* and also to assess semen samples for acrosomal integrity using an amidase assay *(Edward et al., 1992).* Due to the concentration of normal ram semen samples, the expected maximum concentration of the diluted semen (1:4; semen:extender) used was 1000 x 10^6 sperm/ml. which resulted in a maximum number of sperm available in a 40µl sample (the standard used in the amidase assay, *(Edward et al., 1992)* of 40 x 10^6 sperm cells.

4.2 Materials and methods

4.2.1 The amidase assay determination

This experiment was designed to determine the correlation between optical density (OD) readings and the level of damage to the acrosomes of spermatozoa in the various extenders. Once these relationships were established, they could then be used to predict the integrity of acrosomes in samples from the series of experiments involving mainly frozen/thawed semen which follows.

Semen samples

Semen samples were collected as described in the Section 3.2 and after pooling spermatozoa concentration was measured as described in Section 3.3.1. The samples were then diluted in the extenders (described in the next Section) to

concentrations of 1.0 x 10^9, 0.5 x 10^9, 0.25 x $10^9$and 0.125 x 10^9 spermatozoa / ml. which resulted in 40 x 10^6, 20 x 10^6, 10 x 10^6 and 5 x 10^6 spermatozoa per test when a volume of 40 µl of each sample was used for amidase assay (as described in Section 3.3.3).

Semen extenders

Eleven extenders were used for calibration of the assay (Table 4.1).

Table 4.1 Details of the extenders used throughout Chapter 4 (reference code in parenthesis).

1. Standard extender as described in Section 3.4.1 was to be used as a control extender (C).
2. Modified standard was produced by excluding glycerol and mixing with 10 % of egg yolk (V/V) instead of 15% as in the standard (MS 10).
3. As in # 2 but with 20 % egg yolk (V/V) (MS 20).
4. As in # 2 but with 30 % egg yolk (V/V) (MS 30).
5. As in # 2 but with 40 % egg yolk (V/V) (MS 40).
6. As in # 2 but with 50 % egg yolk (V/V) (MS 50).
7. The TEST base extender as described in Section 3.4.2 with 10% egg yolk (V/V) added (T 10).
8. As in # 7 but with 20% egg yolk (T 20).
9. As in # 7 but with 30% egg yolk (T 30).
10. As in # 7 but with 40% egg yolk (T 40).
11. As in # 7 but with 50% egg yolk (T 50).

All extenders were centrifuged at 10,000 G for 10 min to separate the large protein and lipid particles in egg yolk, the precipitate was discarded and only the supernatant used.

To simulate damage to the acrosome in order to obtain a range of relative OD readings from the given spermatozoa concentrations, triton X100 was added to the diluted samples at 0.05% (V/V) *(Edward et al., 1992)*. The samples were then incubated for 30 min at 37 °C to ensure that acrosome disruption occurred.

Experimental design

The experiments were repeated 5 times with each replicate conducted on a separate day. Thus, there were 11 extenders in each replicate and measurements were made at the levels of 40×10^6, 20×10^6, 10×10^6 and 5×10^6 sperm/test in order to have four means for regression analysis.

Statistical Analysis

The experiment was a 4 concentrations by 11 extenders factorial design with 5 replicates per cell. Regression analyses of the OD readings from the 4 concentrations were conducted for each extender, and the regression lines were constrained to pass through the origin.

4.2.2 Experiment 1 The determination of suitable glycerol-free freezing extenders

Semen samples

Semen samples were obtained and processed in the manner described in Sections 3.2 and 3.3. The extenders used were the same as for the amidase assay determination (Table 4.1) and samples were diluted 1:4 (semen:extender) within one hour of collection.

Freezing and thawing technique

After dilution with the extenders, the semen samples were put into a refrigerator to cool at a constant rate from 37 °C to 5 °C in 1.5 h. They were then held at 5 °C for a further 1 h. as described by *Evans & Maxwell (1987)*. The freezing and thawing method has been described in Section 3.5.

Semen evaluation

Semen evaluation was the same as described in Section 3.3, with the exception that the motility percentage was determined using only two drops of 10 μl of sample and only two areas of examination for each drop (in duplicate) before freezing (after reaching 5 °C). The evaluation after freezing was as described in Section 3.3. The amidase assay was undertaken in duplicate before freezing from only 1 drop of sample, but, after freezing was done on two drops of each pellet (each duplicated).

Statistical analysis

The experiment evaluated 11 extenders used on 6 different days of collection. The data were analysed by analysis of variance (ANOVA), with significant differences between means ($p < 0.05$) being detected using a Duncan's multiple range test *(Snedecor & Cochran, 1967)*.

4.2.3 Experiment 2 The effect of dilution techniques on post-thaw semen quality

Semen samples

The semen samples were obtained as described in Section 3.2. In this experiment the best extender identified previously (Experiment 1), in terms of post-thaw semen performance, TEST with 30% egg yolk V/V (T 30), was used and it was compared to the control extender from Experiment 1.

Dilution techniques, freezing and thawing

The techniques for dilution can be classified into two categories. The "warm dilution" method involved mixing semen with the extenders at 30 - 37 °C, while "cold dilution" involved the mixing of semen and extenders after cooling to 5°C. The "standing time" after mixing was varied in the "warm dilution" method, while "standing time" both before and after mixing was varied in the case of the "cold dilution" method, as summarised in Table 4.2

Table 4.2 Details of the dilution methods used in Experiment 2 (times in hours).

Warm dilution technique before mixing + after mixing standing time	Cold dilution technique before mixing + after mixing standing time
0* + 2	1 + 1
0* + 3	2 + 0#
0* + 4	1 + 2
	2 + 1
	3 + 0#
	1 + 3
	2 + 2
	3 + 1
	4 + 0#

*before mixing 0 h. = diluted immediately after collection
#after mixing 0 h. = sample frozen immediately after dilution at 5°C

The control extender from Experiment 1 was used as an inter-experiment link, and the warm dilution technique 0 + 3 was used as the internal control.

With the exception of some variation due to the effects of dilution method, the details of freezing and thawing were the same as in Experiment 1.

Results from Experiment 1 indicated below-average semen quality after freezing, a situation attributed to the foam containers used. For subsequent work the technique was modified by using metal containers to provide more uniformity of temperature during the cooling phase.

Semen was evaluated in the same manner as described for Experiment 1 and since the same extender was used throughout the experiment, before freezing figures were measured only for the control, 0 + 2, 0 + 3, 0 + 4 and 1 + 1 treatments. This was to ensure good quality of the samples and to minimise fluctuation of the temperature in the refrigerator.

Statistical analysis

The experimental design consisted of 12 dilution techniques, each replicated 6 times (days). Analysis was the same as in Experiment 1. A control extender (as for Experiment 1) was included to monitor variation between the experiments.

4.3 Results

4.3.1 Amidase assay determination

The mean relationships between OD and acrosome damaged spermatozoa at 40 X 10^6, 20 X 10^6, 10 X 10^6 and 5 X 10^6 spermatozoa per test sample are shown in Figure 4.1 for each extender. Regression coefficient (r^2) values ranged from 0.93 - 0.98 and were all significant ($p < 0.05$).

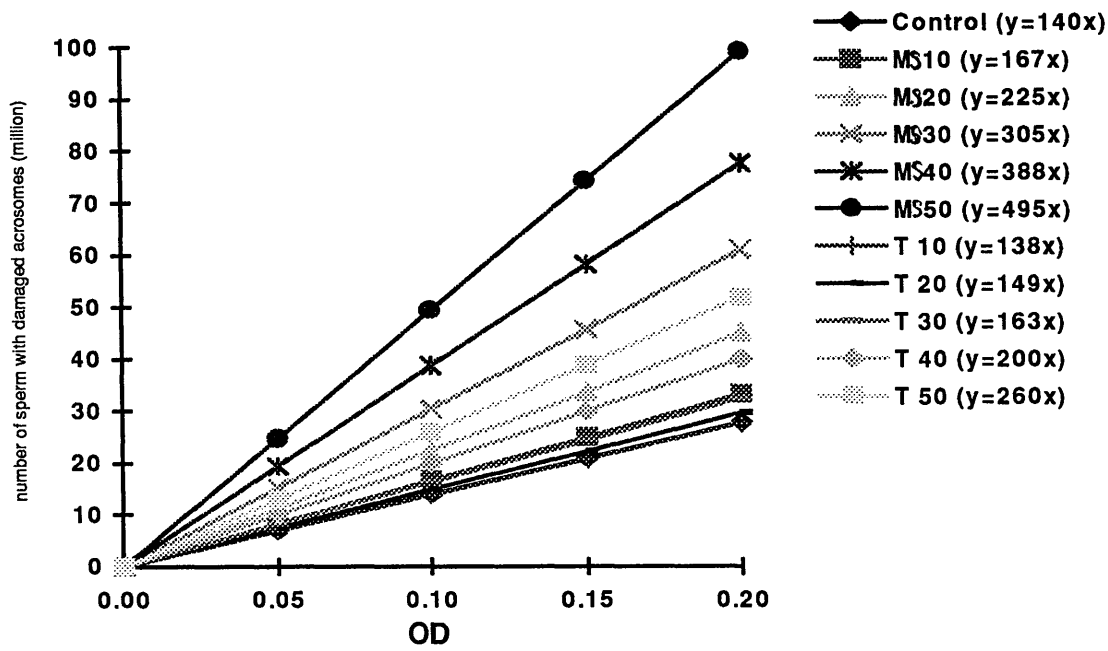


Figure 4.1 The regression analysis and the fitted line plot of amidase assay for each extender.

There were significant differences ($p < 0.05$) between the slopes of all the lines depicted in Figure 4.1 *(Snedecor & Cochran,1967).*

4.3.2 Experiment 1

The relative contributions of semen from individual rams to the pooled sample varied markedly between days (Table 4.3). The lowest pooled semen volume was on day 3 and the highest was on day 4, but the lowest pooled sperm number was on day 1 and the highest was on day 4. While the percent contributions of semen volume from each ram also changed on a day by day basis, it was noticeable that the contribution from M 3 was lowest on all days except day 6 (Table 4.3).

Semen volume was significantly different between rams ($p < 0.05$) but not between days, while semen concentration differed between days ($p < 0.05$) but not rams.

Table 4.3 The percent semen contribution (volume) of each ram on each day in Experiment 1.

Ram	Day 1	Day 2	Day 3	Day 4	Day5	Day 6
M 1	16.5	17.7	26.0	21.7	13.4	21.7
M 2	13.5	17.7	15.6	10.1	18.5	8.7
M 3	9.7	12.7	5.3	7.8	10.2	11.4
C 1	21.4	19.6	16.7	27.1	19.3	16.5
C 2	21.4	19.6	22.9	19.4	23.5	24.3
C 3	17.5	12.7	13.5	13.9	15.1	17.4
Total Volume	10.3	10.2	9.6	12.9	11.9	11.5
Total Concentration(x 10^9 cell/ml.)	3.4	3.9	4.7	4.0	4.1	4.0
Total Sperm(x 10^9 cell)	34.8	40.2	45.6	52.1	49.3	46.2

There were significant differences between both extenders and days ($p < 0.001$) and the interaction between extenders and days was also significant ($p < 0.001$) in terms of motility percentage and percentage of acrosome integrity both before freezing and after freezing.

The mean quality of semen in each extender in terms of post-thawing motility percentage and percentage of intact acrosomes is shown in Figure 4.2. The 'MS' extender group yielded virtually zero motility after freezing and a similar result in terms of after freezing acrosome integrity with the exception of MS 10. The motility after freezing was not significantly different between Control, T 10, T 20 and T 30. The control extender showed that glycerol protected the integrity of acrosomes better than the rest ($p < 0.05$). T 20 and T 30 yielded the second best scores in terms of after freezing acrosome integrity and those scores did not differ significantly from each other ($p > 0.05$).

When adding together the motility and acrosome integrity scores after freezing of 'T' extenders (consider both scores as having equal weighting), it was shown that T 30 had the best overall score of 68.5 (Table 4.4). The worst overall score was from T 50.

Table 4.4 The ranking of total semen quality score after freezing among 'T' extenders.

Rank	Extender	Overall mean of after freezing motility %	Overall mean of after freezing acrosome integrity %	Total score
1	T 30	28.8	39.7	68.5
2	T 20	24.3	40.7	65.0
3	T 10	23.6	26.5	50.1
4	T 40	14.1	23.8	37.9
5	T 50	2.6	7.4	10.0

The variation in semen quality between the days of the experiments (replicates) is shown in Figure 4.3 (motility) and Figure 4.4 (acrosome integrity), before freezing and after thawing. In Figure 4.3, motility before freezing was mostly above the 70% level for 'MS' extenders, with the exceptions of MS 30 on day 4 and both MS 40 and MS 50 on day 5. For T extenders, motility before freezing appeared to be less variable and mostly above the 60% level. Motility after freezing with 'MS' extenders was virtually zero, as it was also for T 50. The semen quality after freezing varied markedly between days. From Figure 4.4, it can be seen that the before freezing acrosome integrity was more variable between days among 'MS' extenders than the 'T' extenders, with T 50 being most variable amongst the latter. After freezing, most of the 'MS' extenders were associated with very low acrosome integrity values (below 10% and close to zero) with the exception of MS 10. 'T' extenders appeared to be better than the 'MS' group with the exception of T 50. Most 'T' extenders yielded integrity scores above 20%, but T 10 and T 40 on day 5 and T 40 on day 6 were exceptions and had lower values.

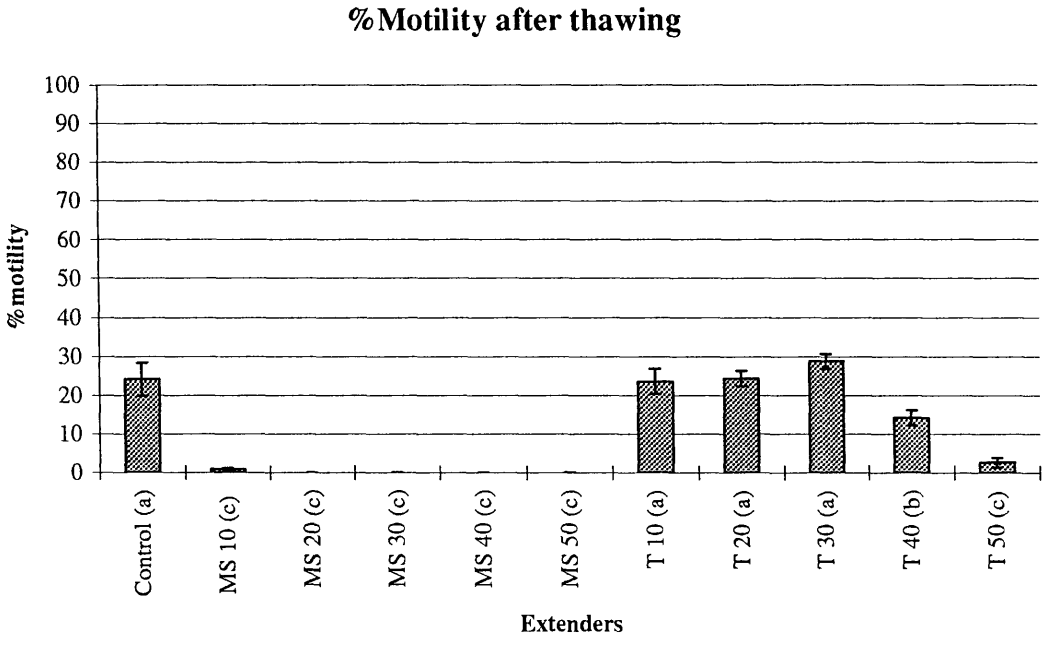
The correlation coefficients (r) from "Spearman" rank correlation analysis are shown in Tables 4.5 and 4.6. The results indicate that although there were marked variations in the after freezing semen quality from each treatment (extenders) on a daily basis, the ranks within the same treatment between days were fairly consistent for both motility and acrosome integrity. All r values in Tables 4.5 and 4.6 were significant ($p < 0.01$).

Table 4.5 "Spearman" rank correlation of motility after freezing between days (n=11).

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Day 1	1.00					
Day 2	0.72	1.00				
Day 3	0.81	0.86	1.00			
Day 4	0.93	0.82	0.93	1.00		
Day 5	0.76	0.90	0.84	0.80	1.00	
Day 6	0.88	0.89	0.84	0.92	0.86	1.00

Table 4.6 "Spearman" rank correlation of acrosome integrity after freezing between days (n=11).

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Day 1	1.00					
Day 2	0.93	1.00				
Day 3	0.89	0.92	1.00			
Day 4	0.90	0.88	0.86	1.00		
Day 5	0.84	0.89	0.94	0.83	1.00	
Day 6	0.85	0.82	0.93	0.78	0.90	1.00



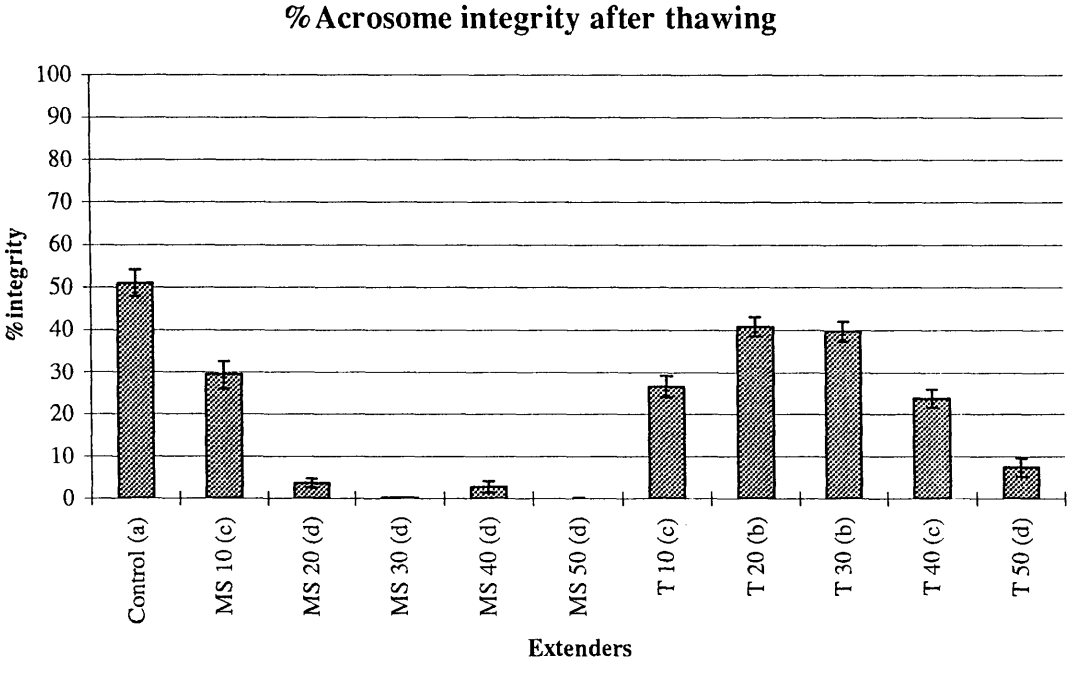
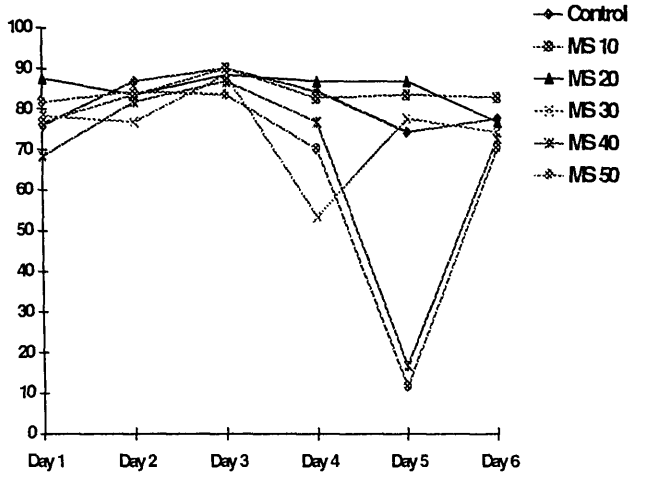
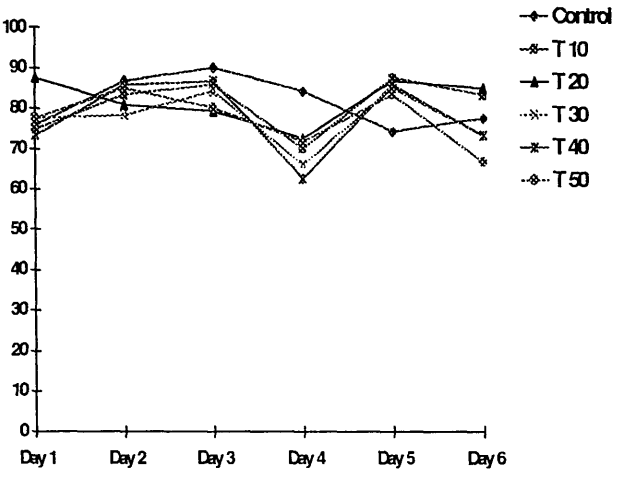


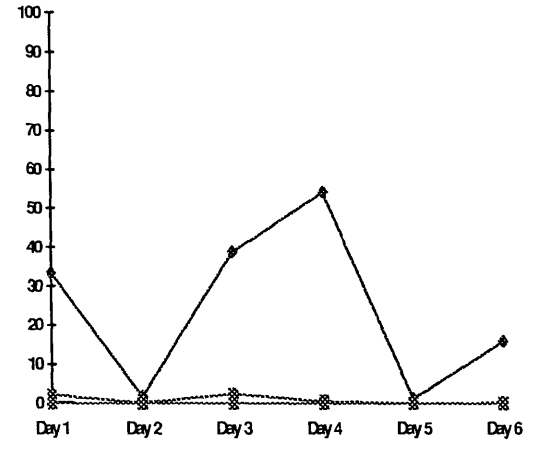
Figure 4.2 The comparison of semen quality after thawing between the extenders. (*extenders followed by the same letter in parenthesis do not differ significantly; p >0.05)



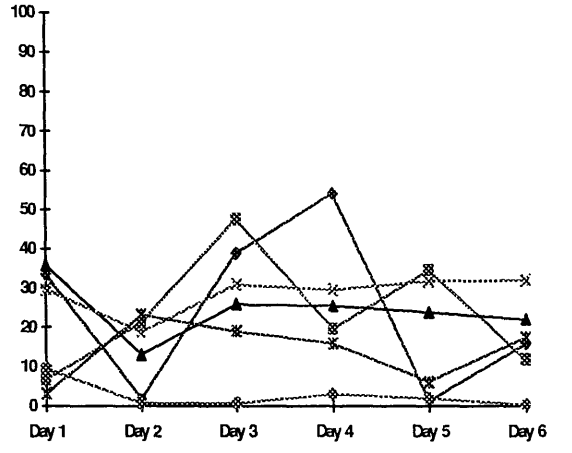
(a) **Before freezing**; Modified standard (MS) extenders



(b) **Before freezing**; TEST (T) extenders

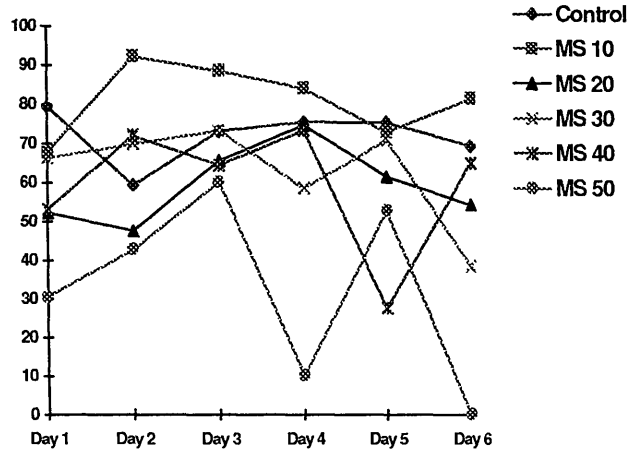


(c) **After freezing**; 'MS' extenders

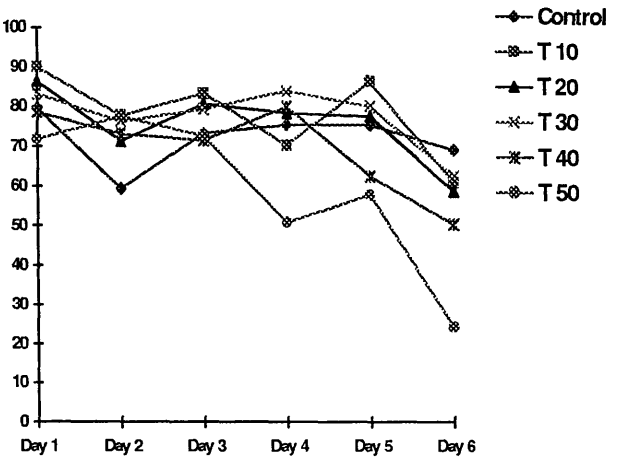


(d) **After freezing**; 'T' extenders

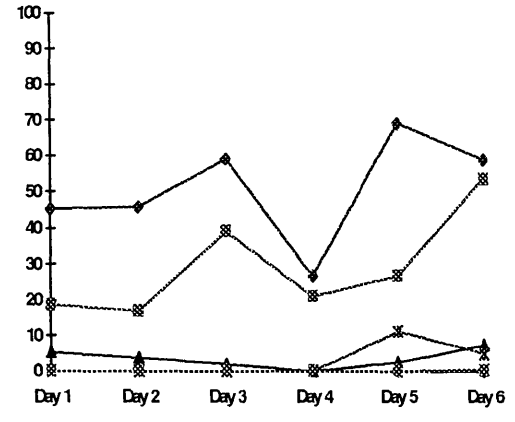
Figure 4.3 The variation of motility percentage before and after freezing between days in the different extenders (see Table 4.1) as compared to the control.



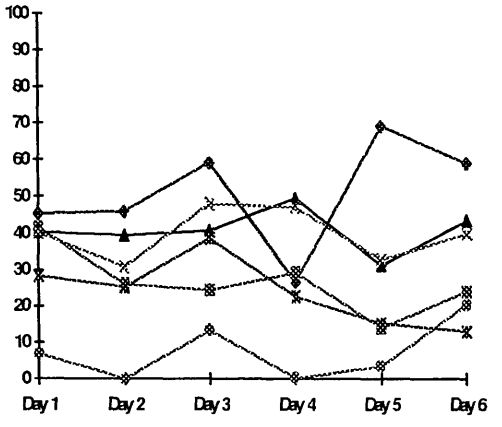
(a) **Before freezing**, 'MS' extenders



(b) **Before freezing**, 'T' extenders



(c) **After freezing**; 'MS' extenders



(d) **After freezing**; 'T' extenders

Figure 4.4 The variation of acrosome integrity percentage between days, before and after freezing, for 'MS' and T extenders as compared to the control.

4.3.3 Experiment 2

The relative contributions of semen from individual rams to the pooled samples varied markedly between days (Table 4.7), as did total volume and concentration of the pooled semen. Days 4 and 6 had lower levels of semen volume than the other days.

Mean semen volume did not differ between rams and days while mean semen concentration was significantly different between days ($p < 0.05$) but not between rams.

Table 4.7 The percent semen contribution (volume) of each ram on each day in Experiment 2.

Ram	Day 1	Day 2	Day 3	Day 4	Day5	Day 6
M 1	18.7	9.7	18.2	11.6	18.1	17.6
M 2	26.0	17.3	14.1	12.7	14.4	14.8
M 3	19.9	11.6	20.2	23.3	18.9	16.2
C 1	10.4	24.0	20.2	23.3	16.2	6.8
C 2	10.4	16.3	9.1	5.8	16.2	20.3
C 3	14.6	21.1	18.2	23.3	16.2	24.3
Total Volume	9.6	10.4	9.9	8.6	11.1	7.4
Total Concentration(x 10^9 cell/ml.)	4.2	4.1	4.1	3.5	4.4	3.9
Total Sperm(x 10^9 cell)	40.6	43.2	40.6	30.6	48.4	29.0

The quality of semen before freezing in terms of motility and percentage of intact acrosomes was well above 80% for both parameters. There were significant differences between methods of dilution and between days ($p < 0.001$) in terms of both post-thawing motility percentage and acrosome integrity percentage. The interaction between method of dilution and days was also significant ($p < 0.001$) (Figure 4.5).

The semen quality arising from each dilution method in terms of post-thawing motility percentage and percentage of intact acrosomes is shown in Figure 4.5. The 1+1 dilution method yielded the best score for motility after freezing; however, this score was still comparable with the 1+2 method ($p > 0.05$). For after freezing acrosome integrity, it was clearly shown that the control dilution method (from Experiment 1) with the glycerolated extender, gave the best protection of acrosomes, while 2+0 had the second best, but was also comparable with the score of 1+2 ($p > 0.05$).

To rank overall means of both motility and acrosome integrity after freezing, the two separate scores were added together, both values being given equal weight (Table 4.8). It can be clearly seen that 1 + 2 dilution method gave the best overall score of 100.1, while the others dilution methods ranged down from 92.9 to 72.8.

Table 4.8 The ranking of total semen quality score after freezing for each of the dilution methods (excluding Control).

Rank	Dilution method	Overall mean of after freezing motility %	Overall mean of after freezing acrosome integrity %	Total score
1	1 + 2	52.1	48.0	100.1
2	1 + 1	53.5	39.4	92.9
3	3 + 1	47.0	43.5	90.5
4	2 + 2	44.6	45.0	89.6
5	2 + 1	43.7	43.7	87.4
6	2 + 0	30.1	54.7	84.8
7	1 + 3	45.3	39.0	84.3
8	0 + 2	45.1	33.2	78.3
9	0 + 3	33.8	43.5	77.3
10	4 + 0	38.9	38.0	76.9
11	3 + 0	30.5	43.4	73.9
12	0 + 4	39.4	33.4	72.8

The variation of semen quality between days of doing the experiments (replicates) in terms of post-thawing motility percentage and the percentage of intact acrosomes is shown in Figure 4.6. Though there was an interaction between days and dilution method ($p < 0.001$), the variation of each dilution method between days appeared to be less than in Experiment 1 (Figure 4.3). In the case of acrosome

integrity after thawing, The control gave the best score on almost every day (replicate).

The results from the "Spearman" rank correlation analysis are shown in Tables 4.9 (motility) and 4.10 (acrosome integrity). The 'r' values from Table 4.9 were moderate and some were significant; namely between Day 1 and Day 3, Days 1 and 5, Day 3 and Days 4, 5 and 6, Day 4 and Day 5 ($p < 0.01$). However, the 'r' values from Table 4.10 were low and inconsistent with the single exception of the 'r' value between Day 3 and Day 6 ($p < 0.01$), which was high and significant.

Table 4.9 "Spearman" rank correlation of motility after freezing between days (n=13).

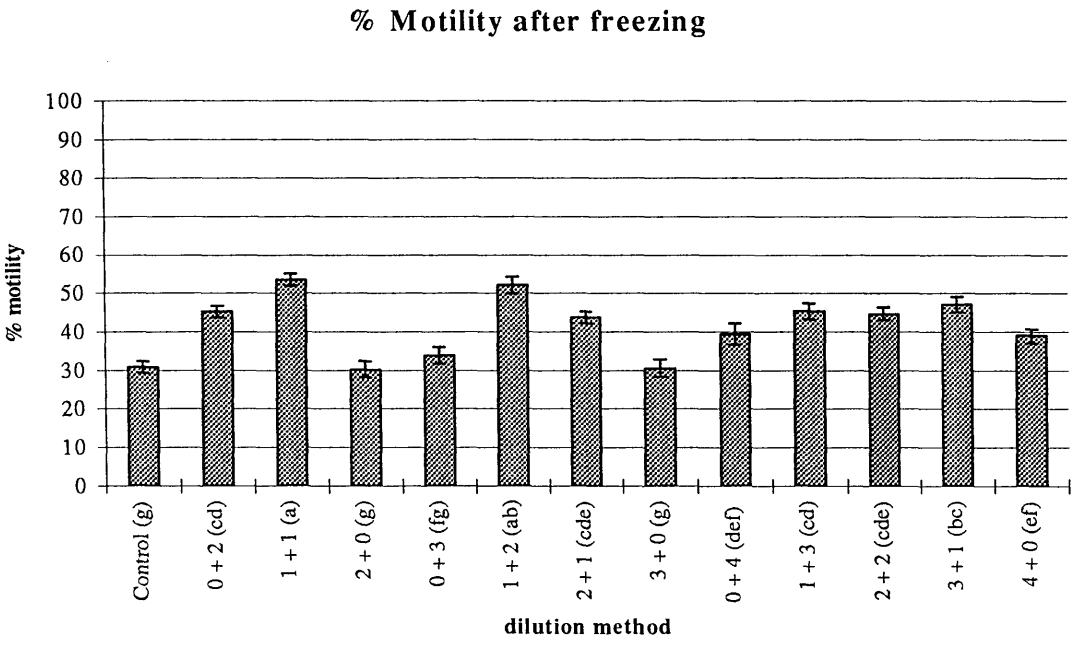
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Day 1	1.00					
Day 2	0.60	1.00				
Day 3	0.71*	0.44	1.00			
Day 4	0.65	0.47	0.74*	1.00		
Day 5	0.68*	0.58	0.78*	0.86*	1.00	
Day 6	0.64	0.65	0.70*	0.63	0.60	1.00

* $p < 0.01$

Table 4.10 "Spearman" rank correlation of acrosome integrity after freezing between days (n=13).

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Day 1	1.00					
Day 2	0.38	1.00				
Day 3	0.56	0.54	1.00			
Day 4	0.46	0.50	0.36	1.00		
Day 5	0.03	0.22	0.58	0.23	1.00	
Day 6	0.29	0.43	0.85*	0.18	0.53	1.00

* $p < 0.01$



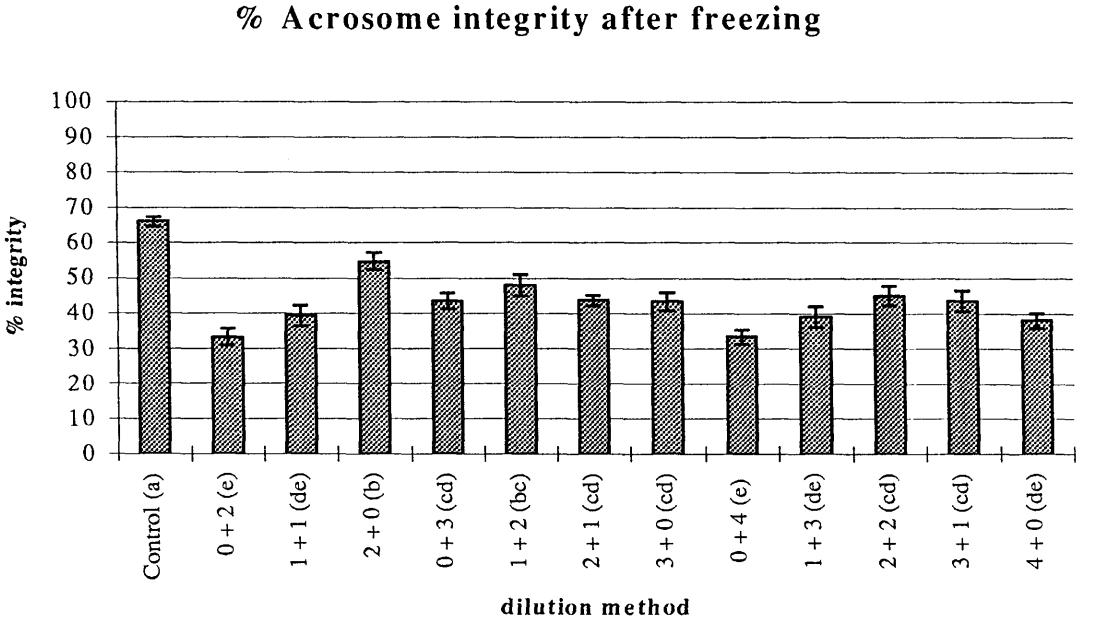
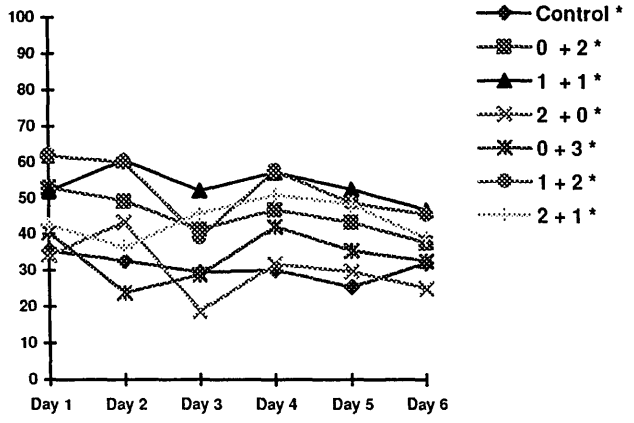
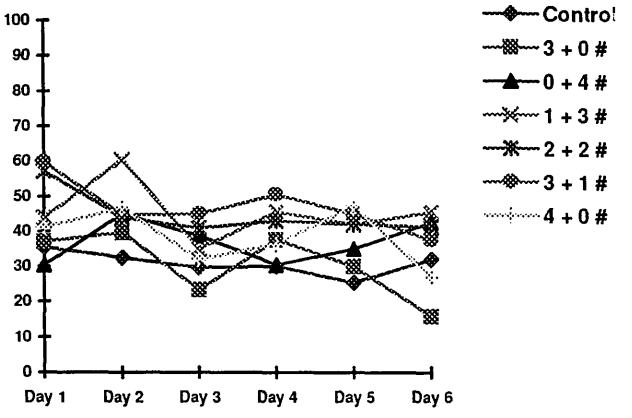


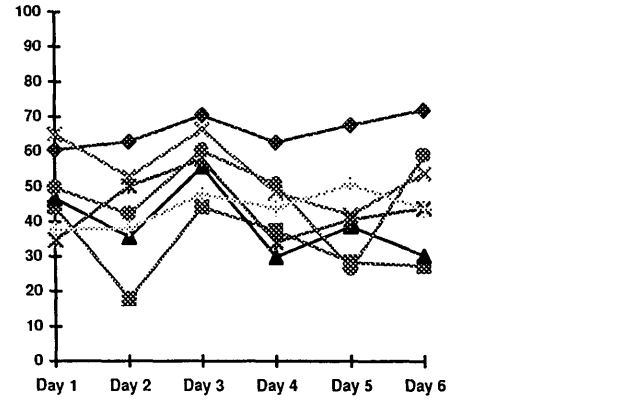
Figure 4.5 The semen quality comparison between method of dilution after thawing *letters in the parenthesis indicate mean differences ($p < 0.05$).



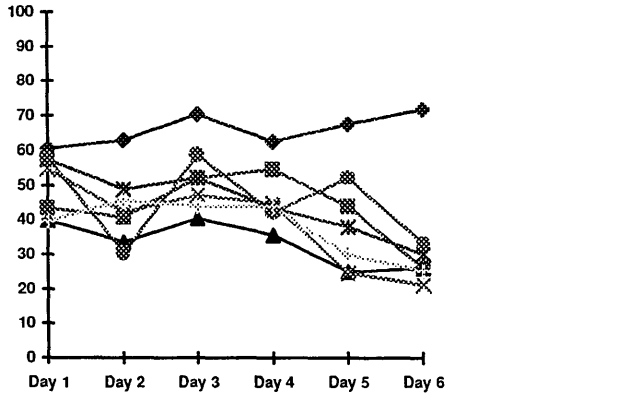
(a) **Motility after freezing**; for indicated* dilution methods



(b) **Motility after freezing**; for indicated# dilution methods



(c) **Acrosome integrity after freezing**; for indicated* dilution methods



(d) **Acrosome integrity after freezing**; for indicated# dilution methods

Figure 4.6 The variation of after freezing semen quality between days, for each dilution methods as compared to the control.

4.4 Discussion and conclusion

4.4.1 Amidase assay determination

To achieve fertilisation, spermatozoa not only have to be alive after thawing but also have to have a functional acrosome. Although some other minor factors are also involved, these two factors are considered to play the major role in determining sperm fertility *(Froman et al, 1987)*. In sheep there have been many reports which indicate that semen treatments ie.; dilution of semen or the freezing process, are able to cause dysfunction of the acrosome *(Healey, 1969; Watson & Martin, 1975; Olson & Winfrey, 1991)*. Thus when assessing the techniques to be used to preserve spermatozoa, both the movement of spermatozoa (motility percentage) and the integrity of the acrosome need to be considered.

The results of the amidase activity measurements were all highly correlated with the number of spermatozoa with damaged acrosomes (r^2 range from 0.93 to 0.98), agreeing with the findings of *Edward et al. (1992)*. However, in the current work there was no consistency between the extenders studied, with the slope (coefficient) of each line being significantly different from all others (Figure 4.1). There were notable differences in each case, with a progressive increase in the slopes with an increasing proportion of egg yolk in the same type of extender (167 to 495 in case of 'MS' extenders and 138 to 260 in case of 'T' extenders; Figure 4.1). The only exception was the control, in which case 15% of egg yolk yielded a coefficient of 140, which was an exceptionally low value, when it is considered that the major components of the control extender were similar to those of the 'MS' extenders.

However, the control extender did include glycerol, which suggests that both glycerol and egg yolk have a significant impact on this assay. A possible reason for this is indicated by *Froman et al. (1987)* who suggested that before conducting this assay spermatozoa should be free from impurities such as protein from the extenders and glycerol, and that washing with Fi coll-400 solution before the amidase assay may thus be necessary. By using a sperm population model that included only different numbers of sperm, *Froman et al.* claimed that the washing process (including centrifugation) did not alter the actual number of damaged acrosomes. However, it could be argued that this would be true only where all acrosomes were damaged, and that in the case of semen samples containing only a proportion of acrosome damaged sperm (as after the freezing process *(Watson & Martin, 1972; Watson, 1975)*), some additional damage due to washing and centrifugation would be expected.. Unfortunately, it is impossible to prove or disprove this from the existing data.

Edward et al. (1992) modified the "micro" amidase assay *(Froman et al., 1987)* by using a "macro" assay. They used the raw sample without washing and showed good correlations between OD readings and the number of acrosome damaged ram spermatozoa. Those results appear to be a valid measurement, and to be practically useful in the determination of semen quality. The results from the present experiment have confirmed these findings, although, they also clearly indicate that the assay must be validated for each type of extender. In addition, it is worthy of mention that a modification to the technique of *Edward et al.* by using filter paper to remove precipitates is a good way to prevent problems associated with the resuspension of the precipitate when the supernatant has to be decanted into a spectrophotometer cuvette.

This filtration step does slow down the process, but has the added advantage of removing any surface layer of lipids present on the sample after centrifugation (observed in the present experiment).

There are alternative methods to determine acrosome integrity such as the Giemsa staining of acrosome membranes as developed by *Watson (1975)*, and examining acrosome structure by the electron microscope *(Healey, 1969)*. Both the above authors claimed that their methods could reliably detect acrosomal integrity, however, both methods are also subjective and depend partly on the skill of the examiners, and in case of electron microscopy require complicated and protracted technical procedures. Due to the limited resources available for the studies in this thesis, together with the objective nature of measurement of amidase assay, the author selected this assay for use. The results of the present experiment confirm that it is a suitable and practical means of measurement of acrosome integrity.

4.4.2 Experiment 1 & 2

The results from these two experiments showed that ram semen can be frozen successfully with glycerol-free extenders and thus confirmed similar findings reported by *Abdelhakeam et al.(1991 b)*. However, there were also very highly significant interactions between extenders and days in Experiment 1 ($p < 0.001$) and also between dilution methods and days in Experiment 2 ($p < 0.001$).

A number of explanations for these interactions are apparent. Firstly, there were quite marked variations in the percent contribution of semen samples from the individual rams (Table 4.3) on different days ($p < 0.05$) in terms of semen

concentration and in terms of semen volume ($p < 0.05$). There were also significant differences in semen concentration between rams (Table 4.7) in Experiment 2 ($p < 0.05$). Such variations between ejaculates seem to be a common trend in most reports *(*eg. *Entwistle & Martin, 1972; Salamon & Visser, 1972; Watson & Martin 1975)*, unfortunately *Abdelhakeam et al. (1991 a,b)*, who reported similar results to those discussed here, did not comment on variations between ejaculates and no direct comparisons are thus possible.

However, it seems likely that differences between ejaculates would have contributed to the current interactions. *Salamon (1964)* found that semen volume, sperm density, seminal plasma composition (including fructose and citric acid concentration) differed significantly from ejaculate to ejaculate within the same ram and between rams and also between days. The only exception was the citric acid concentration, which he found to be consistent in concentration in the ejaculates from the same ram between days *(Salamon, 1964)*. Allowing seminal plasma time to incubate with sperm also has beneficial effects on post-thawing semen quality *(Pavelko & Crabo, 1976; Abdelhakeam et al., 1988, 1991 a)*. The possibility that differences in semen quality before freezing could effect semen quality after freezing will be discussed further in Chapter 5.

An unusual observation recorded in Figure 4.3 (a) is the large decline in semen quality before freezing in the MS 40 and MS 50 treatments on the Day 5, which contributed to the significant interaction. However, when the data was reanalysed using either missing data or the overall means to subtitute for these observations the interaction was still highly significant ($p < 0.001$). The same outcome occurred when

all unusual observations were similarly reanalysed. The causes of these markedly divergent observations appear to be non biological in origin; handling errors during the assays cannot be excluded.

At another level, the interactions between days and treatments could possibly be due to the fact that days varied in order of the treatments. These variations are clearly shown in Figures 4.3, 4.4 and 4.6. Even though treatment ranking varied between the days, the semen quality after freezing, in terms of motility percentage and acrosomal integrity percentage in Experiment 1, was consistent and r values were high between days (Tables 4.5 & 4.6). In Experiment 2 (Tables 4.9 & 4.10), the r values for after freezing acrosome integrity were low and inconsistent. The low r values in this case may be due to the relative small range in values obtained (Figures 4.5 and 4.6).

In Experiment 1, the results for motility during the prefreezing phase showed that increasing the concentration of egg yolk had an adverse effect on motility. This can be explained by the fact that the protein and other components in egg yolk increased the viscosity of the extenders which may thus have "slowed down" the spermatozoa. Another possible reason could be an effect of increased osmotic pressure, which has been shown to increase with greater concentrations of egg yolk and to have an the important effect on sperm "life" *(Salamon & Lightfoot, 1969)*. The results of the current experiment do not agree with *Abdelhakeam et al. (1991 b)*, who found that at 4hr after dilution, the before freezing motility rate increased in a progressive manner with increasing levels of egg yolk, an effect they attributed to the benefit of egg yolk in preventing cold shock. In Experiment 1, the measurements were

done immediately after mixing rather than 4 h. later, and the results were not greatly different between 'T' extenders (average from 77.4% for T 50 to 81.9% for T 20). In terms of acrosomal integrity before freezing, the results paralleled those of motility; the percentage of intact acrosomes was reduced as the concentration of egg yolk increased which suggests the possibility that osmotic pressure may have been increasing and having a negative effect on acrosome integrity, even though other workers such as *Fiser & Fairfull (1989)* have reported that the acrosome of ram spermatozoa has a good tolerance to changing osmotic pressure, as when adding glycerol. However, it can be argued that the changes in osmotic pressure in the study of *Fiser & Fairfull* were relatively small (glycerol 4%; added either immediately at 5 °C or gradually increased to that concentration). Unfortunately, to this author's knowledge, there are no reports on the likely effects of osmotic pressure on the acrosome membrane at such high concentrations of egg yolk as used in the current work. However, the results from Experiment 1 clearly showed the negative effects of high (40-50%) concentrations of egg yolk on acrosome integrity.

After freezing, those extenders lacking glycerol and based on Tris-citrate ('MS') were inferior to those based on TEST (T), at least in terms of spermatozoal motility. The only exception was at the lowest level of egg yolk for 'MS' extenders and at the highest level of egg yolk for T extenders where the MS 10 value of 24.2% was comparable with T 10 - T 30 and the T 50 value of 2.6% was comparable with those for MS 20 - MS 50 at ($p=0.05$) (Figure 4.2). This result is in agreement with *Abdelhakeam et al. (1991 b)* who also found that the best level of egg yolk in 'T' extenders was between 25 and 30%. Nevertheless, at 30% of egg yolk in Experiment

1 the motility level was higher than reported by *Abdelhakeam et al. (1991 b)* using a comparable warm dilution technique (28.8% vs 15.9%, respectively). This difference may have resulted from the fact that there was a period of at least 1 h. separating the first and the last semen collections each day in the current work. The benefit to spermatozoa from holding them in the presence of seminal plasma prior to dilution has been reported by *Pavelko & Crabo (1976)* and *Abdelhakeam et al. (1988, 1991 a)* found that such a delay permits seminal plasma constituents to bind to the spermatozoal membrane and that these may then act to protect the cell from cold shock.

The best result recorded in Experiment 1, when using both motility and acrosome integrity after freezing as the basis for comparison (Table 4.4), was at 30 % of egg yolk, although no significant differences were noted between T 10 - T 30, MS 10 and the control ($p=0.05$). The results of the acrosome integrity assay clearly showed the protection provided by glycerol to acrosome structure as previously reported *(Watson & Martin, 1975)*. The highest integrity percentage was from the control extender (50.8%) which contained 5% (V/V) of glycerol. However, in the case of the T extenders without glycerol, acrosomal integrity was still relatively high in both T 20 and T 30 (40.7 and 39.7%, respectively). On the other hand, the 'MS' extenders had very low acrosomal integrity scores (0.0 to 29.3%). This difference between extenders suggests that although there is a protective effect of egg yolk on the spermatozoa cell as previously reported by *Watson & Martin (1975)* and *Pace & Graham (1974)*, there must be an interaction between buffers and egg yolk or other components of the extender to cause such variation in protection of the acrosome.

After changing to metal containers for chilling semen samples (Section 4.2.3), the overall mean score for controls (inter experimental control) increased from 24.2 to 30.8% in the case of motility after freezing and from 50.8 to 66.0% in the case of intact acrosomes after freezing. In fact, in Experiment 2, in which the control for comparison purposes to Experiment 1 was the 0 + 3 treatment, values also improved (28.8 to 33.8% for motility and 28.8 to 43.5% for acrosomes). From these results it can be concluded that cooling time and constancy of the temperature are very important aspects; as have been mentioned in the reviews of *Memon & Ott (1981)* and *Evans & Maxwell (1987).*

The results from the current experiments agreed with those of *Abdelhakeam et al. (1991a,b)* in terms of the cold dilution method. The most suitable method found in this experiment was to reduce the temperature of both semen and extenders for 1 h. prior to mixing and to then maintain both at 5°C for a further 2 h. (1 + 2) (Table 4.8); *Abdelhakeam et al. (1991 a)* on the other hand recommended the 3 + 1 treatment. This difference in results can be explained as above in terms of the fact that in the current study there was a time of at least 1 h. before finishing the semen collections which may have influenced the results. While the 1 + 2 method of dilution did not yield the best results in terms of semen quality parameters after freezing (motility among the 'T' extenders was best at 1 + 1, and acrosome integrity was best at 2 + 0), it was the second best in both cases and had the maximum overall score of 100.1 from Table 4.8. The most suitable combination was thus concluded to be 1 + 2. However, from the practical point of view the second ranking treatment in Table 4.8, the 1 + 1

dilution method, could be considered as a good alternative because the time required is then only 2 h. compared with 3 h. for the 1 + 2 method.

In conclusion, the best combination of glycerol-free extenders revealed in Experiments 1 and 2 was TEST at 325mOsm with 30% of egg yolk (V/V) and 10% (V/V) of isoosmotic maltose using a cold dilution method of 1 + 2 h. (reduce temperature to 5 °C for 1 h. before mixing and leaving at 5 °C for a further 2 h.). This combination will be used for further detailed evaluation in the rest of this thesis. The variation in quantity and quality of the ejaculates observed in Experiments 1 and 2 appeared to be an important factor influencing the consistent performance of the extenders, and consequently the next chapter (Experiment 3) was designed to investigate these effects.

CHAPTER 5

The repeatability of post-thaw semen quality of individual rams

5.1 Introduction

Most breeds of ram exhibit large individual variations in sexual behaviour and "fresh" semen qualities *(Haynes & Haresign, 1987)*. Semen quantity and quality also vary with age, seasonal and temperature effects, breed and the individual within the breed *(Evans & Maxwell, 1987)*. There have also been some reports indicating that fertility of semen from individual animals differs significantly, both in the bull *(Beatty et al., 1976)* and the ram *(Maxwell, 1980, 1986 b)*. However, there do not appear to be studies reported which have examined the variation in quality of post-thawed semen from individual rams within a breed nor between the breeds. Most experiments have evaluated the freezing quality of pooled semen; for example, *Abdelhakeam et al. (1991 a)* used pooled semen from a group of Finn rams to study the influence of freezing semen in glycerol free extenders.

The experiment reported in this chapter was thus designed to investigate possible variations in the freezing quality of semen, both from individual rams and from rams of two dissimilar breeds (the Merino, a wooled breed; and the Coopworth, a long-wooled meat breed).

5.2 Material and methods

5.2.1 Experiment 3

Semen samples

Semen samples were collected and processed using the procedures described in Section 3.2.The experiment commenced in February 1993 (the end of summer) and dietary intakes were adequate to maintain live weight. Individual semen samples were collected from three Merino (M1, M2, M3) and three Coopworth rams (C1, C2, C3) (two ejaculates from each ram were pooled every day) and were frozen for later evaluation. A portion of each sample was pooled with all others and used as a control. Only 0.5 ml. from each sample was diluted with 2.0 ml. of T 30 extender (1:4 dilution rate). Samples were collected on 6 days at approximately 3 day intervals.

To avoid the potential confounding of the effects of ram with time from collection to freezing, the finishing time for semen collection of each ram was recorded and the standing time at 37 °C was standardised.

Freezing and freezing technique

Semen was frozen using the cold dilution technique of 1+2 (premixing + postmixing standing time) which was described in Section 4.4.2. The prefreezing semen quality was measured immediately after mixing with the extender and consisted of assessments of motility and acrosome integrity. The post-freezing semen quality, including motility percentage and percentage of intact acrosomes, was measured in the same manner as described in Experiments 1 and 2.

Experimental design & analysis

The control (pooled sample) was used as a inter-experimental control and was not used in the ANOVA analysis. Thus the experimental design consisted of 2 breeds, each represented by 3 rams each of which was replicated 6 times. The mean differences between rams and between breeds were analysed by ANOVA.

Two evaluators were used to assess post-freezing motility (4 replicates/evaluator) and the effect of evaluator was included in the model. Overall means are reported as the mean of each ram and breed. "Spearman" rank correlation analysis was used for the ranking of scores between evaluators. Regression analysis was used to analyse the relationships between the two measurements of semen quality (motility and acrosome integrity).

5.3 Results

Semen volume differed significantly ($p < 0.05$) between days but not between rams. There were no significant differences in semen concentration either between rams or days. However, there was a significant difference in mean semen volume between breeds (M=1.7 ± 0.1, C=2.3 ± 0.2 ml.; $p < 0.05$) but not for semen concentration (M=4.2 ± 0.2 x 10^9, C=4.1 ± 0.2 x 10^9 sperm/ml.; $p > 0.05$).

The percentages of progressive motility and of acrosome integrity were significantly different ($p < 0.001$) between rams, and between days, and there was also a significant interaction between rams and days ($p < 0.001$). The mean values of each ram are shown in Figure 5.1.

The mean percent motility after freezing was significantly different ($p < 0.001$) for rams, days and evaluators, but not for the interaction between days and evaluators. However, when using "Spearman" rank correlation for rams between evaluators (overall means 38.7% vs 34.3%), a significant correlation coefficient of 0.89 emerged ($n=7$; $p < 0.01$). The results of post-freezing motility percentage are shown in Figure 5.2.

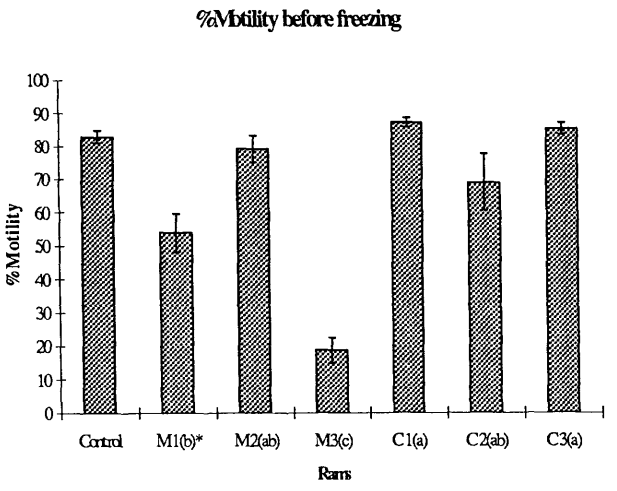
There were significant differences between rams and days and a significant interaction between rams and days($p<0.001$) for post-freezing acrosome integrity and the mean values of each ram are shown in Figure 5.2. The repeatability (intraclass correlation) of semen motility after freezing for different rams was moderate (0.54). Repeatability was low for acrosome integrity after freezing (0.27).

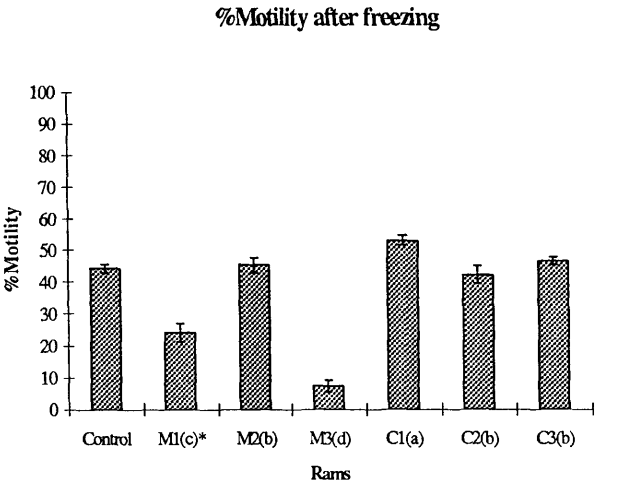
The pre-freezing motility was significantly different ($p < 0.001$) between the breeds (Merino 50.6 ± 4.9% vs Coopworth 80.4 ± 3.2%). However, differences were found to be non significantly different between the breeds in pre-freezing acrosome integrity (Merino 82.2 ± 1.4% vs Coopworth 83.1 ± 1.6%)

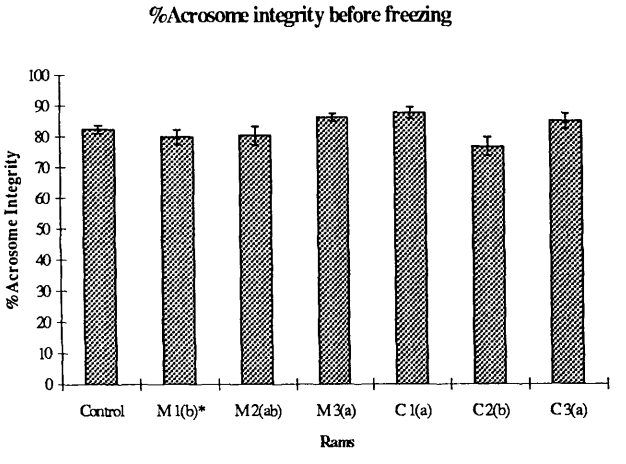
There was a significant difference ($p < 0.001$) between breeds (Merino 25.7 ± 1.9% vs Coopworth 47.3 ± 1.2%) in post-freezing motility but no significant difference in post-freezing acrosome integrity (Merino 36.0 ± 2.1% vs Coopworth 37.9 ± 1.9%)

There was a significant regression relationship ($r^2 = 0.37$; $p < 0.001$) between motility before freezing and after freezing for means recorded on the same day. However, the relationships between motility and acrosome integrity before freezing,

or motility and acrosome integrity after freezing or acrosome integrity before and after freezing were not significant with the r^2 values of 0.00, 0.04 and 0.00 respectively.







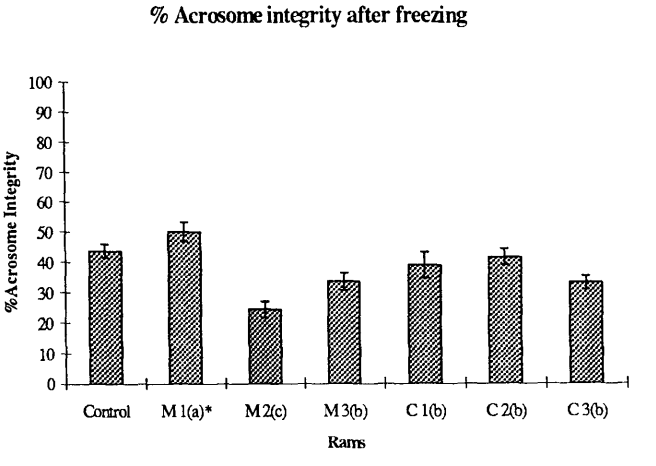
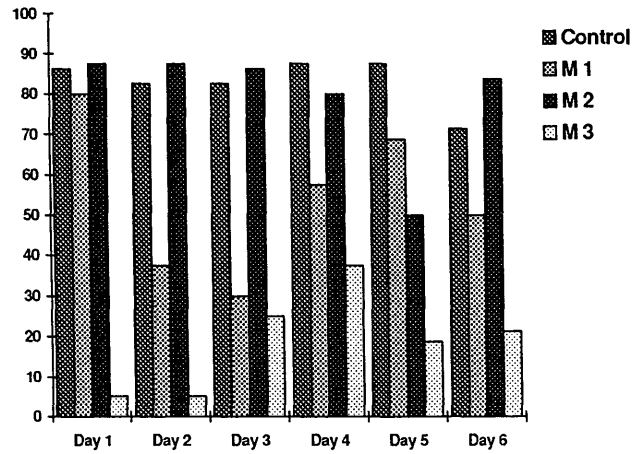
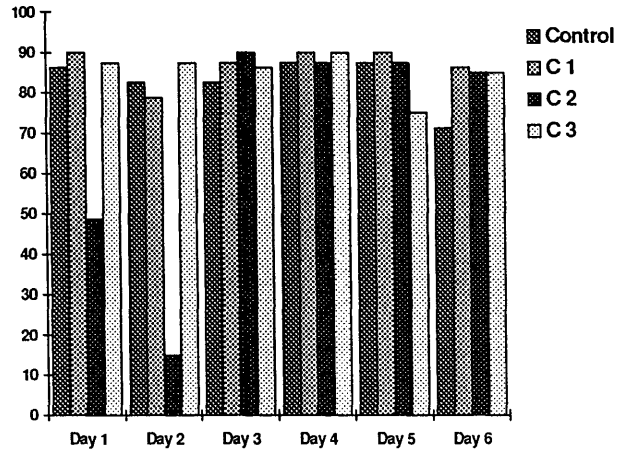


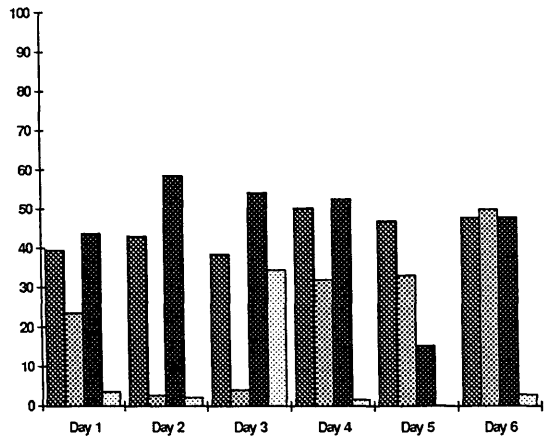
Figure 5.1 The mean ± SE of semen qualities of individual rams. *ram numbers followed by the same letter in parenthesis do not differ significantly ($p > 0.05$).



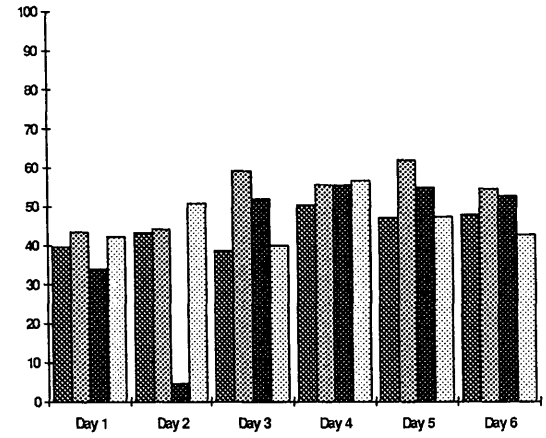
(a) **Motility before freezing** (Control vs Merinos)



(b) **Motility before freezing** (Control vs Coopworths)

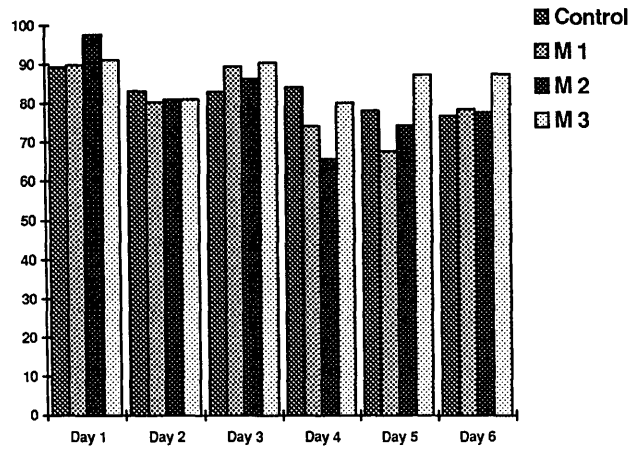


(c) **Motility after freezing** (Control vs Merinos)

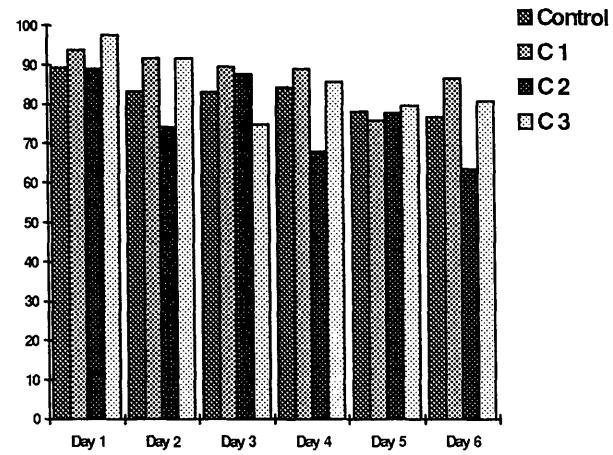


(d) **Motility after freezing** (Control vs Coopworths)

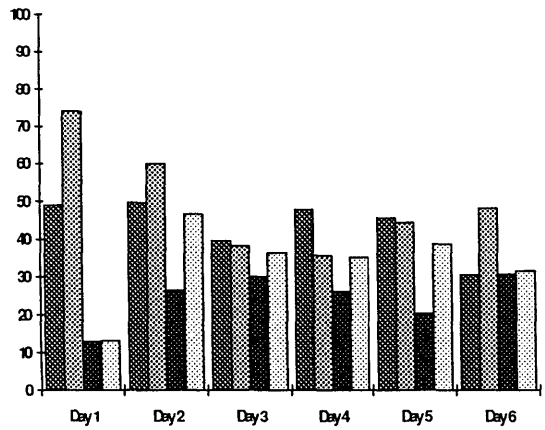
Figure 5.2 The variation in motility between days.



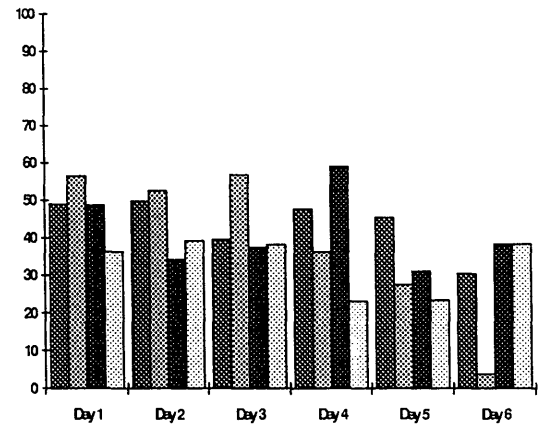
(a) **Acrosome integrity before freezing** (Control vs Merinos)



(b) **Acrosome integrity before freezing** (Control vs Coopworths)



(c) **Acrosome integrity after freezing** (Control vs Merinos)



(d) **Acrosome integrity after freezing** (Control vs Coopworths)

Figure 5.3 The variation in acrosome integrity between days.

5.4 Discussion and conclusion

The results clearly showed that there was large variation between individual rams for both before freezing and after freezing semen quality (Figures 5.1 - 5.3) and the repeatability of semen quality after freezing in terms of motility was moderate and acrosome integrity was low (Section 5.3). *Salamon (1964)* found that the quality of fresh ram semen varied greatly between animals, days, and ejaculates (all $p < 0.001$). Amongst ejaculate volume, semen concentration, fructose concentration and citric acid concentration in seminal plasma, only citric acid concentration values did not differ significantly between days but were still significantly different between rams and ejaculates *(Salamon, 1964)*. *Salamon* suggested that the differences in fructose concentration may have a significant impact on spermatozoa survival rate if semen is incubated with an inadequate energy source. Furthermore, *Quinn & White (1968)* and *Quinn et al. (1968)* also found that incubating ram semen in an extender with low pH (6.0 and 6.8 vs 7.6) resulted in an increase in the cold shock tolerance of spermatozoa. Due to the fact that ram spermatozoa utilise fructose as an energy source for activity and produce lactic acid, which can decrease the pH in semen, cold shock tolerance can be expected to increase with time after collection. If the concentration of fructose varies as in the finding of *Salamon (1964)*, the utilisation of fructose may not be equal in all semen samples and as a consequence unequal semen qualities may occur after cold shock.

Holding sperm with seminal plasma also has been reported to have a positive effect on semen quality. *Rajamannan et al. (1971)* found that holding bull sperm with

seminal plasma for a 15 - 20 min period before dilution could improve the fertility of poor quality semen. Seminal plasma proteins in pig semen have also been shown to play an important role in fertility. Thus *Pavelko & Crabo (1976)* found that the seminal plasma proteins in pig semen coated the spermatozoal membrane during incubation and the attachment occurred primarily during the first 2 h. of incubation (determined by ^{125}Iodine). They also reported that spermatozoa from 'fertile' semen has a high level of this coating of seminal plasma proteins and that in it the coating will remain intact after the freezing and thawing processes, whereas in poor fertility semen this coating is lost during freezing. The benefit associated with seminal plasma proteins which coat ram semen has not yet been investigated, although, *Abdelhakeam et al. (1988, 1991 b)* has shown that holding ram sperm with seminal plasma also resulted in a better recovery rate after freezing with glycerol-free extenders. From these findings, we can assume that seminal plasma proteins may be present in ram semen in unequal quantities in different semen samples, and that as a result, differences in semen quality after freezing may occur. These may be reasons for the significant differences in the performance of semen samples between rams and days in the current experiment. When we consider that the freezing process and extender used in the present experiment were fixed, it seems highly likely that the differences observed can be attributed to the seminal plasma. Future research is needed to investigate exactly which factors in the seminal plasma are actually involved.

The breed difference in semen quality in terms of progressive motility percentage and percentage of acrosome integrity may not be attributed to the fact that Merinos produced less semen volume than Coopworth rams (1.7 vs 2.2 ml., $p < 0.05$);

however, the semen concentrations were similar for both breeds. Thus the chances of spermatozoa to have beneficial from seminal plasma could be similar. The breed differences were significant both before and after freezing for motility but not for acrosome integrity, and it would seem that whatever the cause of the differences between breeds the protective characteristics were evident for motility but not for integrity of the acrosome. Future studies with larger numbers of rams are necessary before the differences observed here could be attributed to genetic characteristics. At the observed percentage difference (approximately 30% from Merinos to Coopworths) and with coefficients of variation of the order of 20% (approximately 21% from the current experiment), the work of *Berndtson (1991)* indicates that the number of rams needed would be at least 37 per breed at $p < 0.05$ and an 80% confidence level. It is also worthy to note that, in bulls, there have been several reports on breed difference in average ejaculate volume *(reviewed by Graves, 1978)*; however, *Graves* commented that because those reports were from small groups of bulls of the various breeds, the results which could be attributed partly to variation between individual bulls. Thus, he concluded that it is doubtful that real breed differences in semen volume have yet been established.

Considering the fact that the control for the present experiment was pooled from all rams, we would expect the overall mean of values such as those in Figure 5.1 to approximately the midpoint of the range. In fact, this did not occur, possibly because Coopworth semen contributed more to the pooled sample (see Section 5.3) through its greater volume and better motility (pre- and post-freezing), and exhibited less variation between rams than the Merino samples (Figure 5.1).

The significant interactions observed between rams and days (see Section 5.3) may have resulted from changes in the rank order of semen quality between days from the same ram as shown in Figures 5.2 and 5.3. There was no significant interactions between days and evaluators. The "Spearman" rank correlation (Section 5.3) analysis suggest that although there was a significant difference between the evaluators in overall means (38.7% vs 34.32%), the rank scores appeared to be similar, with both evaluators in basic agreement on the ranking of rams on any one day. This suggests that the overall means of post-freezing motility percentage can be used with some confidence.

There is agreement in the literature that most breeds of ram can produce semen throughout the year but that there is a period, concurrent with the breeding season of the ewes, when semen production is highest *(*eg. *Dutt, 1960; Lees, 1965: Papelko & Clegg, 1965).* The dietary intakes of protein and energy have also been shown to affect the production of spermatozoa in Australian Merino rams *(Lindsay et al., 1979; Masters & Fels, 1984; Cameron et al., 1988)* and they may override the influence of photoperiod *(Masters & Fels, 1984).* The present experiment commenced at the beginning of autumn, at the peak of breeding season *(Evans & Maxwell, 1987)*, and feed intake was adequate throughout. Therefore, seasonal and nutrition effects are unlikely to have influenced the current results.

The significant results of the regression analysis between means of motility before and after freezing implied that post-freezing motility can be predicted from motility before freezing. In fact, the low r^2 value (0.37; Section 5.3) suggests that many other factors contribute to the post-freezing motility as well as the

characteristics of semen which determine pre-freezing motility, and the predictive ability is in fact so low as to be impractical.

There were no significant relationships between motility and acrosome integrity either before or after freezing (r^2= 0.00 and 0.04 respectively). *Watson (1975)* also found no significant correlations (actual values not reported) between motility and acrosome integrity and more recently *Berndtson et al. (1981)* and *Tamuli & Watson (1994)* have confirmed the relative independence of the two measurements in bull and pig spermatozoa respectively. In bulls, motility and acrosome integrity after freezing were in fact significantly correlated in the work of *Berndtson et al. (1981)*, but the correlations were low (r^2= 0.33 or less), and as a consequence only about 5% of the variations in either parameters could be account for by variation in the other. *Tamuli & Watson (1994)* did not study the motility of boar and ram semen, but did record that in both species the fact of a spermatozoan staining as either 'live' or 'dead' was unrelated to acrosome integrity. These reports all indicate that motility and acrosome integrity are poorly related, and as much are in agreement with the findings from Experiment 3.

Taken together, the results discussed in the preceding paragraph all indicate that to best predict the likely fertility of a semen sample, separate estimates of both motility and acrosome integrity are required. This is the current author's recommendation. Ideally, these two parameters should be assessed concurrently in the same sperm cells, but at present this is not technically possible. With estimates based on whole semen samples difficulties in interpretation can occur because we do not know, for example, whether motile cells are the ones with intact acrosomes or not.

'Microtechniques' will need to be developed for concurrent assessments in individual spermatozoa before this limitation can be overcome.

In conclusion, there appear to be various factors (as yet unidentified) in the seminal plasma of ram semen which influence the performance of the frozen-thawed semen. These variations appear to be important in limiting the evaluation of semen freezing techniques. Because the outcome of the evaluation can depend of the group of animals studied, or the breed (for instance, the semen from Coopworth rams in the present study was of better quality after freezing than from Merinos), the technique might be valid only in specified groups of animals.

CHAPTER 6

The components of egg yolk protecting spermatozoa from freezing damage

6.1 Introduction

The low density lipoprotein extract (LDF) from egg yolk was shown by *Watson (1976)* to be one of the factors involved in protecting bull and ram spermatozoa from freezing damage. It is also known that LDF exerts its protective effect by binding to the cell membrane in the same manner as egg yolk *(Watson, 1975). Vishavanath et al. (1992)* extracted three portions from LDF, namely water soluble amaranth (WTM), saline soluble amaranth (SLM), and citrate soluble amaranth (CLM). These LDF portions were found to each protect bull spermatozoa from cold shock to some extent *(Vishavanath et al., 1992)*; of them, the WTM fraction was also found to keep incubated spermatozoa alive longer than raw egg yolk, and CLM gave the least protection. However, information in this area is limited since *Watson (1976)* evaluated frozen semen with extenders incorporating glycerol, while *Vishavanath et al. (1992)* investigated only chilled semen. Although ram spermatozoa can be successfully frozen in egg yolk based extenders without glycerol *(Abdelhakeam et al., 1991 a,b)*, there have been no reports of which fractions of the egg yolk exert the freezing protection.

The experiments in this chapter were thus designed to investigate the relative protection afforded by the various LDF components of egg yolk to ram spermatozoa during freezing and to determine if sperm frozen in glycerol-free extenders have the capacity to fertilise ova.

6.2 Materials and methods

6.2.1 The amidase assay determination

This experiment was designed to determine the correlation between the optical density (OD) reading and the percentage of damage to the spermatozoal acrosome in various extenders. These values were to be used to determine the integrity of acrosomes in samples from subsequent experiments.

Semen samples & extenders

Semen samples were taken from the pooled semen of six rams; the processing methods were as described in Sections 3.2 and 3.3.

This experiment used TEST buffers as described in Section 3.4.2, and raw egg yolk was replaced by the low density lipoproteins extracted from egg yolk as detailed by *Shannon et al. (1991) (cited by Vishwanath et al., 1992).* The details of the extraction procedure are as follows:

A 20% egg yolk suspension was prepared in 1.0 litre of distilled water containing 1.25 x 10^6 units of penicillin and 20 mg of streptomycin (Sigma # P-3539). The suspension was left at 4 °C for 2 h. and then centrifuged at 10,000 G at 4 °C for

30 min. The supernatant was taken as the water soluble fraction. Next the precipitate was resuspended in 1.0 litre of 0.9% NaCL containing antibiotic as before, chilled and centrifuged as in the previous step to yield a supernatant which comprised the saline soluble fraction.

Amaranth (Acid red #27, Sigma # A-1016) was added to both the above fractions to a final concentration of 0.1% and the mixtures were shaken until the amaranth was completely dissolved. The suspensions were then centrifuged at 4°C at 10,000 G for 1 h. The two low density precipitates from the individual fractions were collected by vacuum pump and the supernatants discarded. The precipitates were then resuspended in two volumes of 5.8% sodium citrate solution (Univar # 467) and the amaranth was removed by adding DEAE Sephacel® cellulose anion exchanger (Pharmacia # 17-0500-01). The solution was filtered through a Whatman B grade glass filter and more DEAE was added until the pink colour of amaranth was removed from the filtrate. The filtrate was dialysed against three changes of 50 volumes of TEST buffer. The fractions thus obtained were the water soluble amaranth (WTM) and saline soluble amaranth (SLM) fractions respectively. A summary of the overall procedure is outlined in Figure 6.1. Protein concentrations were measured by the Biuret method (COBAS® machine using Roche® Unimate 7 TP # 07 3678 3).

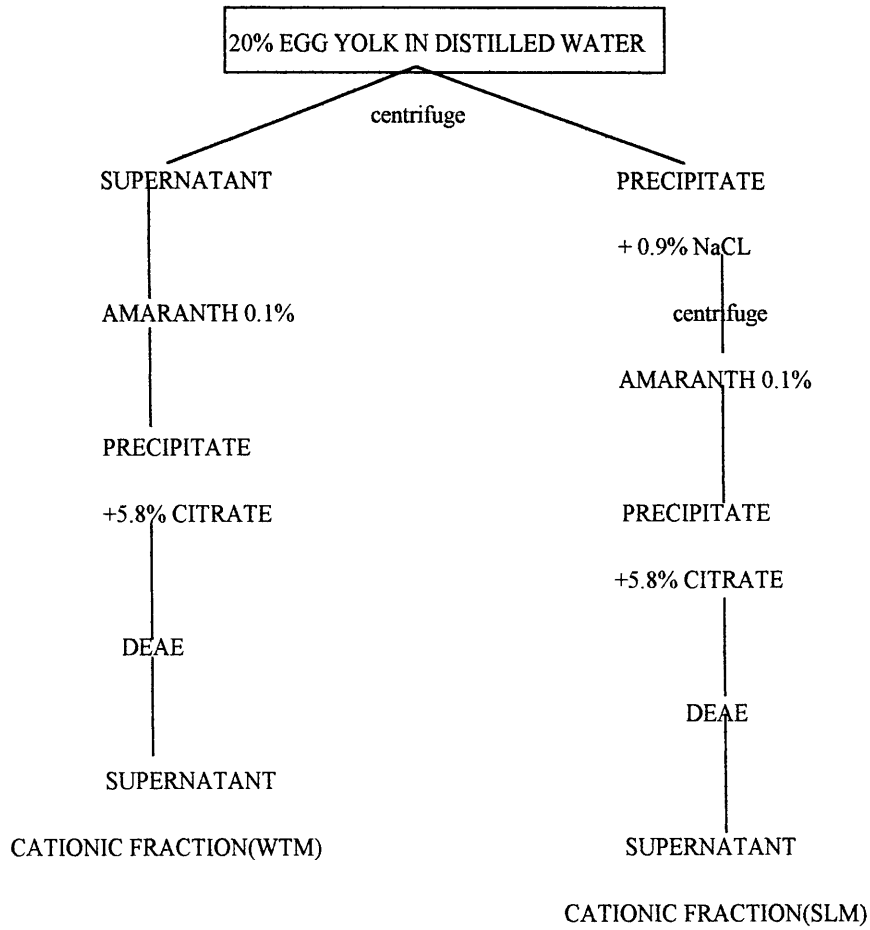


Figure 6.1 Flow chart of the extraction of cationic lipoproteins of egg yolk in water and saline solution.

The concentration of proteins in the extenders was calculated using the total protein in T 30 (Chapters 4 and 5) as a standard. However, a comparable concentration of protein was unable tobe used. *Vishwanath et al. (1992)* found that from the starting protein material (raw egg yolk), 74% was WTM, 14.3% was SLM and 11.7% was citrate soluble fraction (not used in the present study). In the present study, the total protein in T 30 was 25.6 mg%, and by using the proportions

Semen samples & extenders

The semen used was a pooled sample from collections from six rams and the semen processing methods were as described in Sections 3.2 and 3.3.

There were three extenders. T 30 was as described in Section 3.4.2, while WTM and SLM were as described in Section 6.2.1.

Freezing and thawing technique

Freezing was conducted using the "cold dilution" technique of 1+2 (hours premixing + postmixing standing time) which has been described in Section 4.4.2. The prefreezing semen quality was measured 1 h. after mixing with the extenders and consisted of motility percentage and acrosome integrity percentage. The post-thawing semen quality assessment included motility percentage and percentage of intact acrosomes measured in the same manner as that described in Experiments 1 and 2.

Experimental design

T 30 extender was used as a control. The experimental design consisting of 3 extenders (T30, WTM, SLM) replicated 6 times. The mean differences between extenders were analysed by ANOVA.

Two evaluators were used to assess post-thawing motility (4 replicates/evaluator) and the effect of evaluator was included in the model. Overall means are reported as the mean for each extender.

6.2.3 Experiment 5 The fertility test

This experiment was designed to determine whether semen frozen using "glycerol-free" extenders had the capacity to fertilise ova when inseminated into ewes by either the intrauterine or cervical routes.

Semen samples & extenders

The best "day performance" in terms of post-thawing motility and acrosome integrity percentage when the scores from these two parameters were added together (the same calculation as used for Tables 4.4 and 4.8 in Chapter 4) of semen frozen in Experiment 4 were used for this study. However, the results from Experiment 4 indicated that the SLM extender gave better post-thawing semen quality (overall score from both motility and acrosome integrity) than WTM and thus the T 30 (from Day 2; Figure 6.4) and SLM (from Day 1; Figure 6.4) extenders were used in this experiment.

The ewes

Fifty-nine cycling Merino ewes were available for use. Oestrus cycles were synchronised in all ewes by treatment for 9 days with intravaginal progestagen pessaries (Repromap®, Upjohn). Superovulation was induced by an i.m. injection of PMSG (350 i.u./ewe, Folligon®, Intervet) on the day of sponge withdrawal. Forty-eight to fifty two hours after sponge withdrawal, ewes were artificially inseminated.

Oestrus was detected by vasectomised rams fitted with "Sire Sine" harnesses and marking crayons.

The insemination methods

Two insemination techniques; intrauterine (IU) *(Killen & Caffery, 1982)* and cervical *(Evans & Maxwell, 1987)* were used.

For the IU technique, 0.1 ml. of frozen thawed semen was used while the dosage for cervical insemination was 0.4 ml.. These dosages were equivalent to 50 x 10^6 and 200 x 10^6 of active spermatozoa respectively and were above the recommended doses of 20 x 10^6 and 180 x 10^6 for IU and cervical insemination respectively *(Evans & Maxwell, 1987)*. One week after insemination, the ewes were joined with fertile rams fitted with "Sire Sine' harnesses and marking crayons and returns to oestrus were recorded over the next two weeks.

Experimental design & analysis

Two extenders (T 30 and SLM) and two insemination techniques were used. The results were analysed by the Chi-squared technique *(Snedecor & Cochran, 1967)*.

6.3 Results

6.3.1 The amidase assay determination

The mean relationships between the number of sperm with damaged acrosomes (at 40, 20, 10 and 5 x 10^6 spermatozoa per test) and optical density readings in the two extenders are shown in Figure 6.2.

The regression equations were constrained to pass through the origin (blank = 0), and for WTM, $y = 358x$ ($r^2 = 0.94$, $p < 0.005$) and for SLM, $y = 209x$ ($r^2 = 0.99$, $p < 0.001$).

A comparison of the slopes of the lines *(Snedecor & Cochran, 1967)* revealed a significant difference between the extenders ($p < 0.05$).

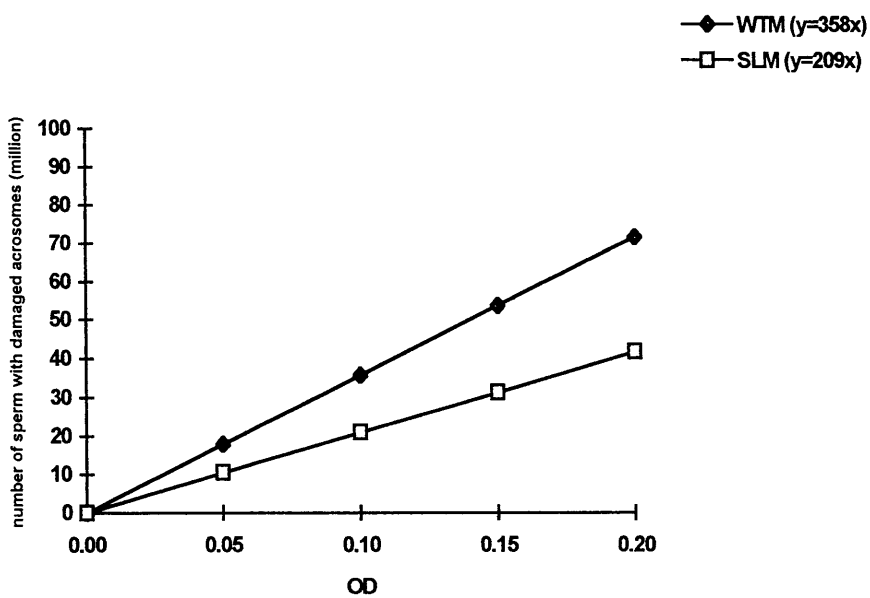


Figure 6.2 The relationships between the number of sperm with damaged acrosomes and OD for the two egg yolk extract extenders used in amidase assay determination.

6.3.2 Experiment 4

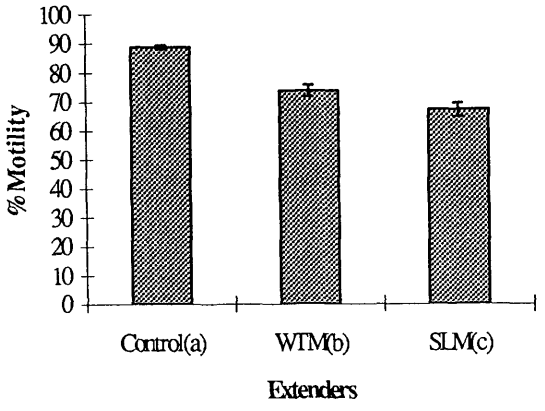
There were significant differences between the means for pre-freezing semen quality in terms of motility and acrosome integrity percentage ($p < 0.001$). There were also significant differences in the means of those parameters between days of collection ($p < 0.001$), and their interaction was also significant ($p < 0.001$).

There were also significant differences between the extenders ($p < 0.005$) and days ($p < 0.001$) in post-thawing motility percentage. The interaction between extenders and days was also significant (p 0.001). However, there were no significant differences between the post-thawing motility scores of the evaluators ($p > 0.8$) nor were there any significant interactions involving evaluators ($p > 0.5$).

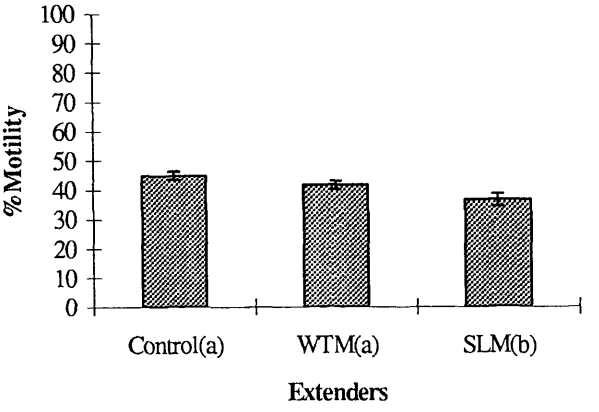
The means for post-thawing acrosome integrity percentage were also significantly different between the extenders ($p < 0.001$) and days ($p < 0.001$).

A summary of the overall quality assessment for each extender is shown in Figure 6.3. The best motility before freezing was with the Control extender; the worst was with SLM extender. For acrosome integrity before freezing the best score was again the Control but in this case WTM extender had the worst score. Only the SLM extender yielded a significantly reduced motility score after freezing ($p < 0.05$); however, WTM gave no protective effect on acrosome integrity after freezing.

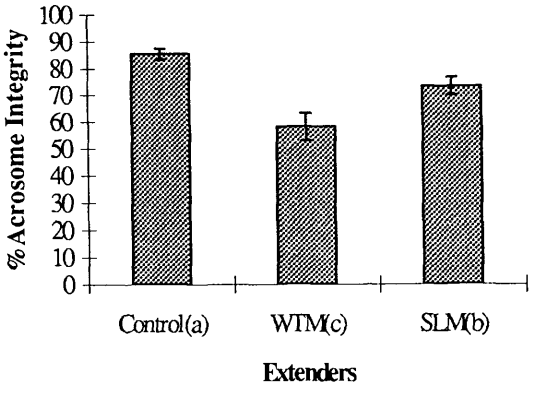
The variations of these measurements by day is shown in Figure 6.4. The only noticeable decline was in the protective effect of SLM on acrosome integrity after freezing. which fell virtually to zero percent on day 6. There was no protection of acrosome at all from the WTM extender.



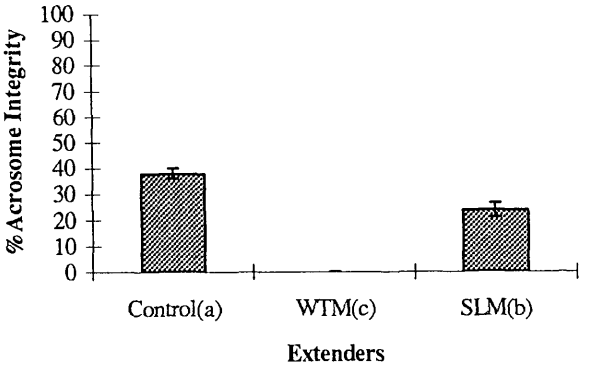
(a) **Motility before freezing** (n=2)



(b) **Motility after freezing** (n=8)



(c) **Acrosome integrity before freezing** (n=2)

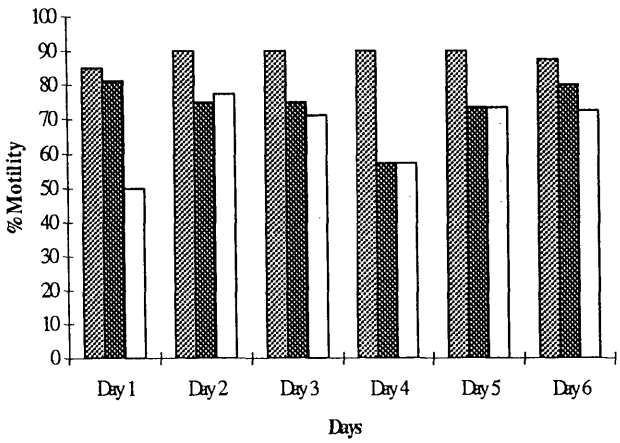


(d) **Acrosome integrity after freezing** *(n=4)

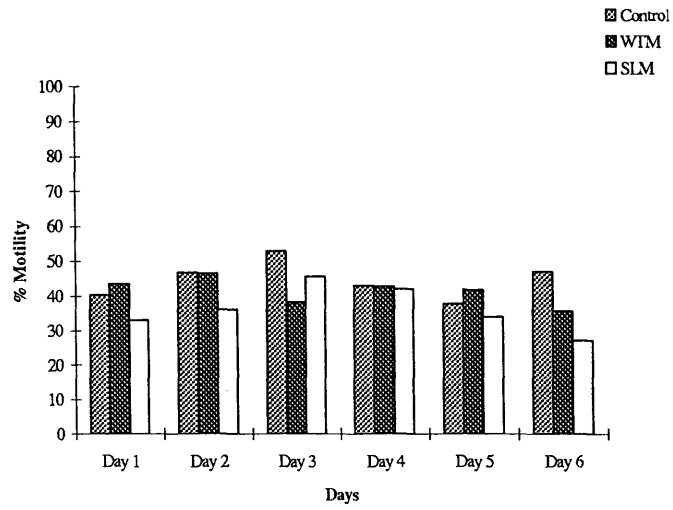
Figure 6.3 Means ± SE of motility and acrosome integrity for each extender.

(extenders followed by the same letters in the parenthesis do not differ significantly; $p > 0.05$).

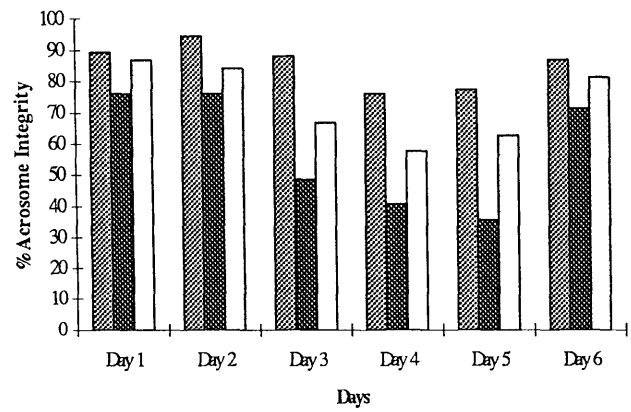
*WTM overall mean = 0.



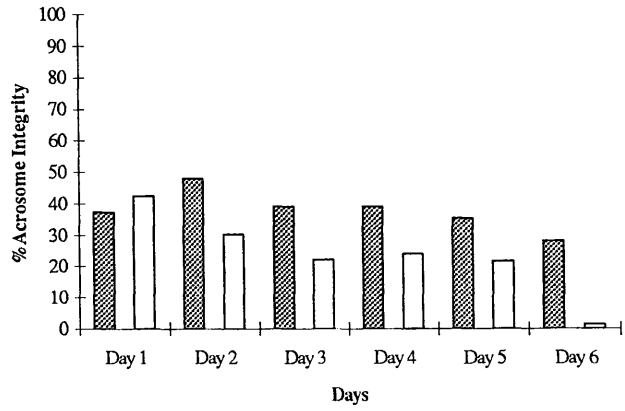
(a) **Motility before freezing** (n=2)



(b) **Motility after freezing** (n=8)



(c) **Acrosome integrity before freezing** (n=2)



(d) **Acrosome integrity after freezing** *(n=4)

Figure 6.4 Daily variations in sperm motility and acrosome integrity before and after freezing.

*WTM means = 0.

6.3.3 Experiment 5

The results of the fertility trial are shown in Table 6.1.

Table 6.1 The results of the fertility trial (with low conception rate for all treatment group).

Extenders	Insemination Method*	No. of ewes	Number of pregnant ewes	Conception rate (%)
T 30	IU	15	3	20.0
T 30	Cervical	14	1	7.1
SLM	IU	14	2	14.2
SLM	Cervical	16	3	18.7

* IU = intrauterine method (laparoscopic technique), Cervical = Cervical insemination technique

6.4 Discussion and conclusions

6.4.1 Amidase assay determination

The results of this experiment confirmed the results from the amidase assay determination in Chapter 4, with the amidase activity measurements being highly correlated with the number of spermatozoa with damaged acrosomes (WTM: r^2 = 0.94 and SLM: r^2 = 0.98). The results also confirmed that this method has to be calibrated for each type of extender as the slopes were again significantly different. Even though the amidase reaction has been claimed to be specific to the acrosin enzyme from the acrosome *(Froman et al., 1987; Edward et al., 1992)*, the current results clearly indicate that the ingredients in the extender (ie: proteins in egg yolk) have a significant effect on the measurement reading with the WTM fraction in this

case having the greatest influence. The evidence from the current experiment (amidase assay determination) confirm those in Chapter 4, which showed that the greater the concentration of protein, the greater the increase in the slope of the response line. However, the concentrations of each of the lipoprotein fractions in the current experiment were not at the same level, and a future study is thus needed to compare extenders at similar concentrations of these lipoproteins. From the present studies it can be concluded that regardless of the type of protein, increasing the concentration resulted in an incremental increase in the slope of the regression equation relating amidase activity to the number of sperm present with damaged acrosomes.

For the process of egg yolk extraction, it is worthy of mention that the concentrations of the final products, namely WTM and SLM, were very much lower (7.6 ± 0.8 and 2.7 ± 0.2 mg/ml.) than the concentrations reported by *Vishwanath et al (1992)* of 59.7 ± 5.3 and 35.4 ± 5.7 mg/ml. respectively. This difference is almost 10 times that found in the present study. The techniques used were almost identical with the exception of differences in the anion exchangers used and in the use of '14GC' containing 2.2 mM $MgSO_4$, 2.5 mM $CaCl_2$ and 3.5 mM K_2HPO_4, rather than the 'TEST' buffer used in current experiment in the final dialysis step (Section 6.2.1). The difference could thus represent a "true" extender effect, or there may have been an error in the concentrations quoted by *Vishwanath et al. (1992).*

In conclusion, the amidase assay was again shown to be a reliable, repeatable and practical means of measurement for acrosome integrity. The assay needs to be

calibrated for each different extender used, but within extenders an increase in protein content is associated with an increase in the slope of the response line.

6.4.2 Experiment 4

The results from this experiment showed quite conclusively that the lipoproteins extracted from egg yolk can protect ram spermatozoa during the freezing process. The pre-freezing semen quality, in terms of motility percentage, showed that WTM alone was able to keep spermatozoa alive to a level comparable with egg yolk (41.6% vs 44.8 % respectively after cold shock during the temperature reduction phase). These results confirm and extend (to ram semen) the finding of *Vishwanath et al. (1992)* that WTM had a protective effect against cold shock comparable with egg yolk when used with bull semen. However, in terms of pre-freezing acrosome integrity, WTM was inferior to both egg yolk and SLM. In addition, the post-thawing acrosome scores showed that WTM gave no protection at all (0% integrity). While SLM was inferior to both egg yolk and WTM in terms of motility percentage after freezing (36.5%, 44.8% and 41.6% respectively, $p < 0.05$), it still maintained the integrity of acrosomes after freezing. The fact that unfractionated egg yolk was far superior to the separate lipoprotein fractions suggests that there were some beneficial factors in raw egg yolk that were screened out in the extraction process or damaged by the freeze drying process. *Watson (1973)* reported that LDF was able to be freeze dried, but the same author *(1976)* subsequently found that the freeze drying process caused some degree of reduction in freezing protection. He also suggested that this decline could be compensated for by using a higher concentration of the LDF fraction. Whether this would be true for the present protocol remains to be determined.

However, it has been clearly shown that ram spermatozoa can be protected from freezing damage by LDF of egg yolk.

Watson (1976) found that egg yolk protects spermatozoa against the loss of motility during the chilling and freezing process by its attachment to the spermatozoal plasma membrane. This attachment is associated with the negatively charged membrane and the cationic characteristic of the egg yolk lipoproteins and the attachment could be broken by washing twice with yolk-free extender. *Vishavanath et al. (1992)* found that all three LDF fractions from egg yolk containing mainly cationic proteins. In Experiment 4, the maintenance of motility during the post-freezing phase was similar to the result of *Vishwanath et al. (1992)*, with WTM being of more benefit to the spermatozoa than SLM. However, if we consider acrosome integrity, SLM was superior to WTM in both cold shock protection (before freezing) and freezing injury protection. It is thus possible to conclude that at least these two fractions provide protection to spermatozoa and help to maintain both motility and acrosome integrity when they are combined in raw egg yolk.

There were no significant differences between evaluators, nor in the interactions of evaluators and days, and evaluators and extenders. These results mean that the evaluators were in agreement not only about the quality of semen (motility) after freezing but also in the day-to-day variation in post-freezing motility. Thus, the motility scores were able to be used with a high level of confidence.

The significant interaction between extenders and days for pre- and post-freezing semen qualities could have resulted from the apparently random

inconsistencies of semen quality observed between days (Figure 6.4) which were nevertheless found to be significant ($p < 0.001$) in all cases. These problems have already been discussed in Chapter 5.

In conclusion, the protective function of egg yolk when freezing ram semen appears to depend on the whole of the egg yolk and is not fully manifest in any of the lipoprotein extracts studied here.

6.4.3 Experiment 5

The fertility levels in this trial were very disappointing and initially could be taken to suggest that the semen was of poor fertility. However, when using the same T 30 extender with fresh semen from the same group of rams used in the current experiment, at a dilution rate of 1: 10 (semen : extender) and laparoscopy intrauterine insemination, fertilisation rates of 68.4% (372/574) were recorded in another trial conducted at the same time as the present experiment *(King, unpublished data; pers. comm.)*. With the limited number of ewes available to the current author, the limited aim of Experiment 5 was to establish whether non-glycerolated extenders were able to support the ability of spermatozoa to fertilise. The answer to this question was a clear 'yes'. For the future, the main intention will be in increasing conception rate to AI in the field, preferably within the breeding season, using this type of extender. With coefficients of variation of the order of 20 to 25% for ewe fertility (non-return rate), the work of *Berndtson (1991)* indicates that a group of 29-45 ewes per treatment would be needed in any such trial to detect a 15% difference in fertility at $p < 0.05$ and 80% confidence. Quite large animal resources will clearly be needed for such work.

More detailed examination of the pattern of onset of oestrus in the present study suggests that synchronisation was poor. Eight out of fifty-nine ewes did not show oestrus at all (13.5% of ewes used) while another 18 ewes only showed oestrus more than 60 h. after sponge withdrawal. *Evans & Robinson (1980)* observed that the onset of oestrus after removal of sponges (progesterone pessaries) was at a mean 43.2 h., while *Jabbour & Evans (1991)* found the best possible time for intrauterine insemination was around 44 h. after sponge removal. These results suggest that the time of intrauterine insemination should be around the same time as the onset of oestrus. Although the mean was similar in this study, the range was considerable, perhaps associated with the fact that this experiment was conducted at the very end of breeding season. *Maxwell (1986 b)* also found that the onset of oestrus varied greatly (from less than 12 to 72 h.) after sponge removal late in anoestrus (in December in Australia). There are a number of reasons why variation in the onset of oestrus could result in poor fertility. For example, if sperm is deposited into the uterus relatively early (ie 24 and 36 h.) after sponge removal, or in other words relatively early compared to ovulation time, there is evidence that increased embryonic losses can occur, possibly due to ageing of sperm in vivo *(Maxwell et al., 1984)*. The estimated fertilisable life of sheep ova is in the range 12 - 24 h. after ovulation *(Dzuik, 1965)*. These data suggest that if spermatozoa enter the oviduct within this period of time (12 - 24 h. after ovulation), successful fertilisation is most likely. This has been confirmed by the finding of *Maxwell (1986 b)* that the best fertilisation rate (57.4%) following intrauterine insemination of frozen-thawed semen occurred at approximately 16 h. after expected ovulation time, and that fertilisation rate decreased dramatically to 39.3% when insemination occurred 22 h. after expected ovulation time. From this

combined evidence, it can be concluded that if insemination is conducted at an inappropriate time then poor fertility can result, and that could have been the case in this trial.

The current fertility results, although poor in terms of their ability to compare the extenders (T 30 and SLM) and insemination techniques (IU and cervical) studied, do provide conclusive evidence that ram semen diluted with T 30 or SLM can be frozen and retain its fertility when used by either the cervical or IU routes. *Abdelhakeam et al. (1991, a)* reported a fertilisation rate of 52% (46 ewes) following double cervical insemination, however, they used a TEST extender with 15% egg yolk and with 10% (V/V) of isoosmotic glucose in a straw preservation method. It is thus difficult to compare the results from experiment 5 with those of *Abdelhakeam et al. (1991, a)* because of the many differences in technique and/or timing used.

In conclusion, the current results clearly indicate that the lipoprotein fractions of egg yolk have the ability to support motility and acrosome integrity in ram spermatozoa during freezing and thawing, but that these abilities are less than of entire raw egg yolk. Spermatozoa frozen in non-glycerolated extenders were also clearly shown to retain the ability to fertilise ova; the continuing problem which requires future attention in research is to improve the conception rate when such semen is used in artificial breeding programs under field conditions.

General Discussion

The results from the present studies indicate quite clearly that ram spermatozoa can be frozen with glycerol-free extenders. The results from Experiments 1 and 2 suggested that including glycerol may in fact provide better protection of acrosome integrity after thawing, but they were not directly comparable because the Control extender was based on Tris-citrate and the 'T' extenders on TEST buffer and also because of difference in the sugar components (glucose vs maltose, respectively).

The current findings also suggest that egg yolk can be used as a major cryoprotectant and that the addition of 10% V/V maltose may act additively in terms of cryoprotection *(Nagase et al., 1964 a,b; 1968; Abdelhakeam et al., 1991 b)*. In more recent work, *Molinia et al. (1994)* found that with comparable extenders (Tris based), extenders including glycerol protect ram spermatozoa in terms of post-freezing motility better than glycerol-free ones and that the disaccharides sugars; trehalose and sucrose protect sperm in terms of motility after freezing better than glucose. However, when glycerol was added either at 2.5 or 5.0% V/V the cryoprotective effect of sugar was masked, with the result that a non-significant difference between those three sugars was recorded. Including glycerol at 2.5 or 5.0% V/V with the sugars provided a better protective effect in terms of post-freezing motility than the sugars alone ($p < 0.001$). The results of *Molinia et al. (1994)* indicate

that glycerol is still needed to provide better motility after freezing. Thus, further investigation of the effects of including glycerol in the TEST extender used in the present studies is warranted.

All the current results indicated that the post-freezing quality of ram semen was very much influenced by the individual rams from which it came and by random variation across days of experiment (ie. replicates). In contrast to those findings, it is very interesting to note that only a very few researches have investigated this variation since *Salamon (1964)*, some thirty years ago, first reported significant differences in the fresh ejaculates of different rams. Since the current work began, two additional reports have been published of differences between individual rams in post-freezing semen quality. Thus *Garcia-Artiga et al. (1993)* found a significant difference between individual rams in the ability of sperm to resist the process of freezing in the absence of glycerol but unfortunately no details are provided in their abstract and direct communication with them has not been possible. More recently, *Molinia et al., (1994)* studied the freezing of ram semen with glycerol-free extenders and found significant differences between ejaculates ($p < 0.001$) in both progressive motility, progressive velocity after freezing and mean linear index (by Hamilton Thorn motility analyser). *Molinia et al.* studied mostly pooled semen samples from 10 mature rams from mixed breed and age; however, the difference in semen qualities after freezing were recorded from 3 to 9 separate ejaculates from groups of only 3 rams. While they did report variation between ejaculates, there were no interactions between rams, ejaculates and/or sugar treatments. Thus, they attributed these variations to the inherent variability in semen quality between animals. However, it could be agued

that if variation in semen quality from each ejaculate is present, why did the variation occur in a pattern that did not cause any significant interactions ? With the limited details provided in their paper, it is very difficult to understand the reported variations between rams.

The different semen qualities observed after freezing (in terms of motility and acrosome integrity) samples from individual rams in Chapter 5 appeared to create important problems in the assessment of freezing protocols, including the difficulty of achieving consistent outcomes when using these techniques under practical conditions in the field. It is very likely that semen from groups of rams used in commercial breeding programs (ie. at an AI center), which have already passed checks for breeding soundness, may respond to dilution/freezing treatments (ie. as in the present study) differently from that from ordinary rams which have not been proven. If this be true then any particular study would appear to be valid only in its particular circumstances and not necessarily when applied to others (ie. when a different group of rams is used). Possibly the most suitable place to conduct further research into new freezing protocols would be in an AI center, because one the major benefit of AI is to produce a considerably greater number of offspring than is possible by natural mating. Thus, those selected males at an AI center could produce more consistent semen quality and this would result in less variation than was apparent in the results from the present study. The number of animals actually used should be based on the work of *Berndtson (1991)* as discussed in the Chapters 5 and 6.

Examination of the interactions between treatments and days indicated that differences in semen quality before freezing each day had a non consistent effect on

semen quality after thawing when compared among treatments and between days. For example, in Experiment 1 (Figure 4.3, d) on day 3, the Control extender had lower motility than T 10, but on day 4 this ranking had changed such that T 10 had much lower motility than the Control. Similar results can be seen on days 4 and 5 between T 10 and T 20, and are also present for acrosome integrity after thawing (Figure 4.4 d). A possibly reason for this, which has already been discussed in Chapters 4 and 5, is that individual rams differed in their percent contribution to pooled semen on different days. Unfortunately, to this author's knowledge, there are no other reports available on such a variation, perhaps because most previous workers have tended to discuss only main effects, and have ignored this variation or considered it as a "normal" factor.

With regard to this, it is worth mentioning that the normal practice in the AI center in Thailand where the author works is to select bulls as semen donors on the basis of breeding soundness, but even so it is not possible to satisfactorily freeze semen from all selected bulls by using the same standard freezing protocol. *Berndtson et al. (1981)* report the same problem, and conclude that on this basis some bulls have to be culled. Furthermore, even with bulls that are generally acceptable, not all ejaculates can always pass the freezing process with a good motility score, and these samples have to be discarded. These results and experiences indicate that there is much variation in semen quality between individual bulls, and between ejaculates within the same bull. As discussed in Chapter 4, variable factors affecting freezing performance may be present in the seminal plasma of rams, and future research is needed to identify these factors and determine if they can be controlled. Success in

this work would be of considerable benefit in the use of genetically superior animals whose semen is currently classed as 'not-suitable for freezing'.

Experiment 4 in Chapter 5 also needs to be "followed-up" with further investigation on the protection promoted to spermatozoa during freezing by the lipoprotein extracts from egg yolk. Specifically, various levels of these extracts need to be assessed in order to determine the most suitable concentration for routine freezing. It could be concluded from a comparison of the current results with those of *Vishwanath et al. (1992)* that CLM (citrate soluble lipoprotein extraction of egg yolk) which was left out of the investigations in this thesis may have an important role to play in protecting sperm during freezing. This conclusion is based on the fact that the results from Experiment 4 indicated that ram spermatozoa were not provided with full freezing protection by either WTM or SLM. This investigation should be done as thoroughly as possible not only to identify the actual protective fraction(s) but also to isolate these important fraction(s) in order for them to be used directly as cryoprotectants without having to use raw egg yolk. Unfortunately, due to limited resources, the author was unable to undertaken those investigations in the present studies.

The two parameters used to evaluate the freezing protocol in this thesis need to be further investigated for their correlation with fertility; in the current work their effects appeared to be independent. The motility percentage was found by *Hulet & Ercanbrack (1962)*, to have a correlation coefficient (r) of 0.67 with the fertility of ram semen, whereas the 'r' values reported in the review of *Berndtson et al. (1981)* ranged from 0.21 - 0.84 in the bull. *Berndtson et al.* attributed much of this variation

in the results of different researches to differences in the number of bulls per group and to the different methods used to assess fertility (ie. overall nonreturn rates, laboratory tests of semen quality, etc.)

The correlation with fertility of acrosome integrity as assessed by 'sperm bound amidase assay' in ram semen has not yet been investigated, but in the bull the same correlation has been reported to range from 0.60 - 0.87 *(reviewed by Berndtson et al., 1981)*, the variations were claimed to be due to variation among the groups of bulls studied *(Berndtson et al., 1981)*; *Berndtson et al.* also reported that the r value between motility and the proportion of intact acrosomes was 0.23 which they considered was low. They concluded that these two parameters were in fact independent and that the use of both methods to determine the viability of sperm which have undergone experimental treatments will give a greater assurance than only a single test. Thus, the correlations with fertility of the two parameters used in the current thesis also need to be investigated further in order to make as robust and valid as possible the parameter(s) of semen 'quality' used to predict fertility.

Overall it can be concluded that ram semen can be successfully pellet frozen (in terms of motility and acrosome integrity after freezing) by the cold dilution method (1+2) in a TEST extender containing 30% V/V of egg yolk and 10% V/V of isoosmotic maltose, and that such spermatozoa still maintain some fertility. However, the variation between individual rams in semen quality after freezing has posed some difficulties in the evaluation of freezing protocols, and further work in this area offers promise for improving the fertility of semen after freezing in non-glycerolated extenders.

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