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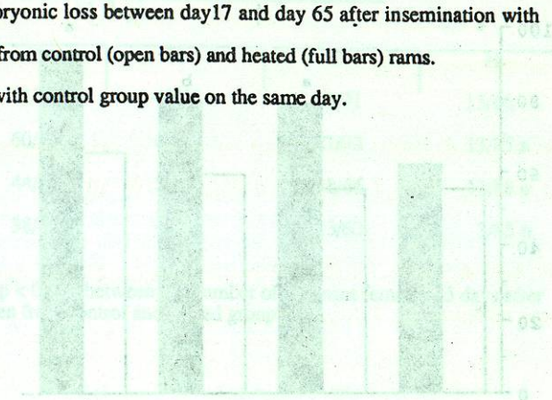
Legend.

Figure 1. Percentage of embryonic loss between day17 and day 65 after insemination with frozen-thawed spermatozoa from control (open bars) and heated (full bars) rams.
a = $p<0.01$ and b = $p<0.05$ with control group value on the same day.



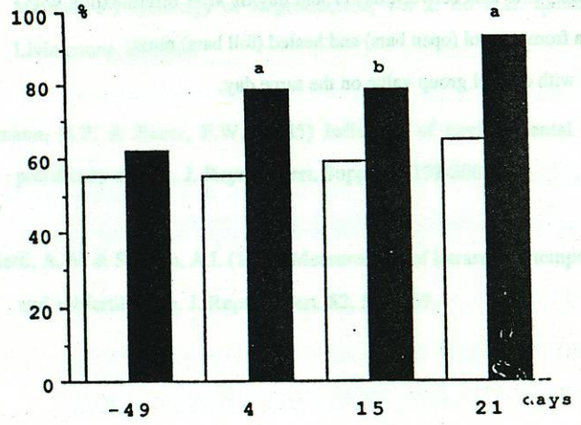


Table 2. Pregnancies at 17 and 65 days after insemination with frozen-thawed spermatozoa from control (C) or heated (H) rams.

	Nb of ewes pregnant/nb of ewes inseminated			
	17 days		65 days	
days	C	H	C	H
-49	51/91	40/66	23/91	15/66
4	60/92	61/85	27/92	13/85 a
15	44/66	56/88	18/66	12/88 a
21	38/63	41/85	13/63	3/85 b

a = $p < 0.04$ and b = $p < 0.001$ between the number of pregnant females 65 days after insemination with semen from control and heated groups.

Table 1. Fresh semen characteristics (mean±sem) in control (C) and heated (H) group.
Total sperm count (10^9 spermatozoa/ejaculate) = sperm count X volume.
Dead = percentage of dead spermatozoa assessed on 200 cells.
Motility (%) was the proportion of total cells for which path velocity >10 µ/sec.
Rapid (%) was the proportion of all cells moving with a path velocity > 95 µ/sec.

	Total Sperm Count		Dead		Motility		Rapid	
Days	C	H	C	H	C	H	C	H
-49	3.1 (0.7)	4.5 (0.3)	12.4 (2.7)	17.3 (3.2)	63.2 (10.1)	79.1 (10.5)	28.9 (6.0)	39.1 (8.5)
4	2.3 (0.4)	2.7 (0.4)	16.9 (8.8)	28.5 (10.3)	56.8 (10.6)	47.7 (11.5)	24.0 (3.9)	12.5 **b** (6.1)
15	2.5 (0.2)	2.9 (0.8)	12.7 (3.0)	38.3**ab** (1.8)	77.0 (2.7)	55.2 (8.2)	36.9 (2.7)	18.8 (7.2)
21	3.6 (0.7)	2.6 **b** (0.5)	8.5 (1.6)	56.9 **a** (7.2)	74.6 (0.9)	17**abc** (6.4)	38.7 (6.9)	5.5 **ab** (2.0)

a = $p < 0.01$ with control group value on the same day;
b = $p < 0.05$ and c = $p < 0.01$ with values at day -49 and day 15 respectively in the heated group .

THE REPEATABILITY OF COMPUTERISED IMAGE ESTIMATIONS OF FRESH, STRAW AND PELLET-FROZEN RAM SEMEN CHARACTERISTICS AND THEIR CORRELATION WITH FERTILITY.

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The characteristics of frozen-thawed ram semen vary between rams and between samples within ram (1). Computerised image analysis of semen has been used extensively in species other than sheep (2, 3). However, little is known about fresh and frozen-thawed ram semen characteristics measured by these systems, or the variability in estimations of these semen characteristics between rams and between samples within ram. There is also minimal information on the relationships between these semen characteristics and fertility, and their use for fertility prediction. This paper presents some results of computerised image analysis of ram semen and some procedures used to analyze this kind of data. These analyses involved estimation of the variance components and repeatabilities of semen characteristics in fresh and frozen-thawed ram semen. The correlation between some semen characteristics and fertility were estimated, an equation of fertility prediction for one particular experiment was formulated, and the most important semen characteristics for fertility prediction were determined.

MATERIALS AND METHODS

1. Experimental Procedure.

Experiment 1. Semen was collected by artificial vagina from 4 adult Merino rams, diluted in a tris-based medium (4) to a concentration of 200 million spermatozoa per ml, drawn into straws (0.5 ml; IMV, L'Aigle, France) and frozen stored in liquid nitrogen (-196°C). Three weeks later, semen was thawed (37°C) and evaluated using computerised image analysis (Hamilton Thorn Motility Analyzer, Daintree Industries, Victoria).

Experiment 2. Semen was collected from 65 Merino rams by electro-ejaculation. Only one ejaculate per ram was obtained. Half the semen was assessed within 10 minutes of collection by computerised image analysis and the other half was processed with a tris-based diluent (4) and frozen in pellet form. Three weeks later the pellets were thawed (37°C) and evaluated using computerised image analysis.

Experiment 3. Semen was collected from Merino rams by artificial vagina and diluted with a tris-based diluent (4) to 400 million spermatozoa/ml, then split in half. The first half was processed in 0.5 ml straws and the second half diluted to 200 million spermatozoa/ml and packed in the same type of straws. The straws were frozen in liquid nitrogen. Semen was thawed (37°C) and assessed for the number of motile spermatozoa/ml (MC), progressive motility, motility, progressive velocity and straighteness using computerised image analysis. With the results of MC and by varying the volume of diluted semen, six treatments were determined.

Treatment 1: Intrauterine insemination with < 35 x 10^6 motile spermatozoa/ml.
Treatment 2: Intrauterine insemination with 35 to 50 x 10^6 motile spermatozoa/ml.
Treatment 3: Intrauterine insemination with 50 to 65 x 10^6 motile spermatozoa/ml.
Treatment 4: Intrauterine insemination with 65 to 85 x 10^6 motile spermatozoa/ml.
Treatment 5: Intrauterine insemination with > 85 x 10^6 motile spermatozoa/ml.
Treatment 6: Cervical insemination with 244 to 280 x 10^6 motile spermatozoa/ml.

A group of 373 ewes were treated with progestagen sponges (30 mg Chronogest, Intervet) for 12 days and received 400 i. u. PMSG (Folligon, Intervet) at sponge removal. The ewes were randomly allocated to treatments 1 to 6 for insemination 50 hours after sponge removal. Pregnancy was determined by ultrasound 50 days after insemination (5).

2. Semen characteristics.

Table.1. Abbreviations and definitions of the semen characteristics assessed by the Hamilton Thorn image analyzer.

Semen Characteristic	Definition
Velocity VAP (VAP)	The five-point running average path velocity, averaged over all cells for which VAP > LVV see below.
Medium VAP velocity (MVV)	Medium value of VAP velocity pre-set as a threshold by the user.
Low VAP velocity (LVV)	Low value of VAP velocity pre-set as a threshold by the user.
Mean track speed (VCL)	Average value of the track speed over all cells for which VAP > LVV. This is computed by taking the total distance covered by a cell in its track, taking straight lines for each cell between the successive 5 to 20 points acquired, summing the distances and dividing by the total elapsed time.
Mean straightness (mean STR)	Ratio of VCL/VAP. Measures departure of the cell path from straight line.
Threshold of STR (So)	The straightness threshold value pre-set by the user.
Total sperm (TOTAL)	The sum of all motile and non-motile spermatozoa.
Motile sperm (MOTILE)	The total number of motile cells.
Progressively motile sperm (PROG)	The progressive cell population. This is composed of all cells with VAP greater than the set for medium velocity (MVV) and the mean straightness (STR) greater than a set threshold value (So). Both MVV and So are set by he user on the main gates screen.
Motility (MOT)	The proportion of total cells for which path velocity VAP > 0
Rapid (RAPID, RAPID%)	The fraction of all cells moving with velocity VAP > MVV.
Medium (MEDIUM, MEDIUM %)	The fraction of all cells moving with velocity between LVV and MVV: i. e. MVV > VAP > LVV.
Slow (SLOW, SLOW %)	The fraction of all cells moving with velocity < LVV; i.e. LVV > VAP > 0.
Static (STATIC, STATIC %)	The fraction of all cells which are not moving at all; i.e. VAP= 0
Mean progressive velocity (mean VSL)	The velocity measured in a straight line from beginning to end of track, averaged over all cells for which VAP > LVV.
Mean linearity (mean LIN)	Ratio of VSL/VCL. Measures departure of the cell track from a straight line.
Mean lateral head displacement (mean ALH)	displacement measured in the cell track, averaged over all cells for which the straightness exceeds the threshold straightness (STR > So), and for which VAP > LVV.

Modified from the Hamilton Thorn Research user manual, (6). For some of the traits, standard deviation is calculated as well, and an ending ST is added to the abbreviation.

3.Statistical analyses.

Experiment 1. The data had a hierarchical structure. Variance components were estimated by fitting a model which included the effects of rams, batches within ram, and straws within batch. All effects were treated as random and the last mentioned term was the error. Repeatabilities were estimated from the intra-class correlation, combining the appropriate variance components.

Experiment 2. Variance components were estimated by analysis of variance. The fresh semen and frozen semen data were analyzed separately, fitting a model which included the effects of rams and samples within ram. Repeatability was calculated from the intra-class correlation, combining the appropriate variance components.

Experiment 3. The data were first analysed by analysis of contrasts (chi-square) between treatments. From the raw data the correlations between frozen-thawed semen characteristics and fertility were estimated by simple correlation analysis (7). The coefficient regression and intercept were estimated using the equations [b = (r) x STD Y/ STD X] and [a = Y - b * X.]. These two values were replaced in the general equation of regression Y = a + bX, and then with the following estimation of X: X = Y - a/ b, the increment in correlated semen characteristics (X_1, X_2...X_n) which increased the conception rate by 5, 10 and 15% and their confidence limits were estimated. The confidence limits, together with the variance components within rams from experiment 1, were used to determine the minimum number of straws which need to be assessed to detect the required increments in correlated semen characteristics.

The regression (or prediction) equation of fertility was also estimated. For this all semen characteristics were used in the multiple regression analysis. The statistical program used considered 0.5 as a limit of significance for the values of the semen characteristics to be included in the regression model (8).

RESULTS AND DISCUSSION

Experiment 1. Table 2 shows the values for the different frozen-thawed ram semen characteristics assessed by computerised image analysis, and Table 3 their variance components and repeatabilities.

Table 2. Straw frozen-thawed ram semen characteristics assessed by computerised image analysis.

Semen characteristics	Mean	Standard Deviation	Range Minimum	Maximum
Total sperm/ml	263	44.9	128	397
Motile sperm/ml	120	47.1	12	281
Progressive motility	31	12.3	5	64
Motility	45	13.8	14	7
Progressive velocity	118	16.7	52	159
Lateral head displacement	5	0.8	2	7
Straightness	82	4.3	67	9
Path velocity	127	16.5	58	170
% Rapid cells	32	13.0	5	67
% Medium cells	13	5.1	4	37
% Slow cells	2	9.8	0	108
% Static cells	53	14.1	17	86

Table 3 Variance components and repeatabilities for straw frozen-thawed semen characteristics assessed by computerised image analysis.

Semen characteristic	Variance component (%) Ram	Batch	Straws	Repeatability Straws	Batches
Total sperm/ml	2	10	88	0.12	0.02
Motile sperm/ml	9	13	78	0.22	0.09
Progressive motility	16	16	69	0.31	0.16
Motility	3	2	75	0.25	0.03
Progressive velocity	5	25	70	0.30	0.05
Lateral head displacement	0	57	43	0.48	0.00
Straightness	29	10	62	0.38	0.29
Cell path velocity	5	24	71	0.29	0.05
% Rapid cells	14	15	70	0.30	0.14
% Medium cells	15	12	73	0.27	0.15
% Slow cells	0	2	98	0.02	0.00
% Static cells	2	22	76	0.24	0.02

The number of rams was too low for a reliable estimation of the variance component between rams. Nevertheless, the model was fitted to show how this kind of information could be analyzed and interpreted. Another limitation of the data is that between ram variation may have been reduced due to selection of rams on the basis of semen attributes.There was a significant difference between batches in all semen characteristics ($p < 0.001$) except slow cells, and between rams for straightness ($p < 0.01$) and percentage of medium cells ($p < 0.05$). The largest variance component was between straws (Table 3). The repeatability for all semen characteristics between straws within batches was low, and even lower between batches within ram. Such low repeatabilities would indicate that more than one straw must be assessed to obtain reliable information from a batch of frozen semen and that several batches must be assessed from each ram. Further studies are required using larger numbers of unselected rams.

Experiment 2. Tables 4 and 5 show the different values for fresh and frozen-thawed ram semen characteristics collected from 65 rams by eletro-ejaculation and assessed by computerised image analysis. The variance components and repeatabilities are presented in Table 6.

Table 4. Fresh ram semen characteristics assessed by computerised image analysis.

Semen Characteristics	Mean	Standard Deviation	Range Minimum	Maximum
Total sperm/ml	2517	1708.6	99	9926
Motile sperm/ml	1552	1308.6	22	8306
Progressive motility	40	20.2	0	80
Motility	59	22.4	6	94
Progressive velocity	115	32.8	41	197
Lateral head displacement	6	1.2	3	10
Straightness	76	10.7	45	93
Path velocity	128	32.4	48	211
% Rapid cells	40	21.5	0	83
% Medium cells	19	15.0	0	73
% Slow cells	0	0.5	0	5
% Static cells	40	22.3	6	93

Table 5. Pellet frozen-thawed ram semen characteristics assessed by computerised image analysis.

Semen Characteristics	Mean	Standard Deviation	Range Minimum	Maximum
Total sperm/ml	478	504.0	36	3322
Motile sperm/ml	117	251.3	0	2053
Progressive motility	10	9.8	0	43
Motility	18	13.6	0	70
Progressive velocity	99	48.0	0	254
Lateral head displacement	4	2.0	0	12
Straightness	76	21.7	0	100
Path velocity	105	48.5	0	257
% Rapid cells	10	10.0	0	45
% Medium cells	7	6.4	0	38
% Slow cells	1	2.1	0	22
% Static cells	82	13.8	27	100

Table 6. Variance components and repeatabilities of fresh and frozen-thawed semen characteristics assessed by computerised image analysis.

Semen Characteristic	Variance component (%) Between Rams		Within ram		Repeatability	
	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen
Total sperm/ml	78	80	22	20	0.79	0.80
Motile sperm/ml	78	76	22	24	0.78	0.76
Progressive motility	69	72	31	28	0.69	0.72
Motility	71	70	29	30	0.71	0.70
Progressive velocity	79	42	21	58	0.79	0.42
Mean lateral head displacement	67	34	33	66	0.61	0.34
Straightness	74	32	26	68	0.74	0.32
Path cell velocity	79	40	21	60	0.79	0.40
% Rapid cells	68	72	32	28	0.68	0.72
% Med.cells	79	51	21	49	0.79	0.51
% Slow cells	19	25	81	75	0.19	0.25
% Static cells	70	74	30	26	0.70	0.74

Variance components and repeatability estimates from fresh and frozen semen were, in general of similar magnitude (Table 6). The repeatability estimates were higher than those for straw-frozen semen in experiment 1. Such repeatabilities would suggest that reliable estimates of semen characteristics could be obtained by computerised image analysis of fresh and pellet-frozen semen. A major limitation of this study is that results are based on one ejaculate collected by electrical stimulation from each ram. Further studies are required using a larger number of ejaculates per ram, preferably collected by the more usual artificial vagina method.

Experiment 3.

1. Semen characteristics and fertility.

Freezing concentration of spermatozoa had no effect on any of the objectively assessed semen characteristics or fertility. So data were pooled for the 6 insemination treatments and are presented in Table 7.

Table 7. Pregnancy following artificial insemination with frozen thawed semen.

Treatment	Mean MC inseminated	No. ewes pregnant/ inseminated (%	
1	23.3	15/37	(40.5)*
2	39.5	33/63	(52.4)
3	58.6	44/77	(57.1)
4	77.1	39/68	(57.4)
5	104.5	18/31	(58.1)
6	189.7	14/97	(14.4)**

* Treatment 1 < treatment 4 ($p < 0.05$), ** treatment 6 < all other treatments ($p < 0.001$).

The pregnancy rate was lower after cervical insemination than intrauterine insemination, even with the lowest dose of spermatozoa. There appeared to be no advantage in increasing the inseminate dose for intrauterine insemination above 40 million motile spermatozoa.

2. Correlation between semen characteristics and fertility and minimum number of straws to be assessed.

The raw data from the experiment was used for the correlation analysis presented in Table 8.

Table 8. Correlation between post-thawing semen characteristics and fertility.

	Motile sperm/ml	Progressive motility	Motility	Progressive velocity	Straightness	Fertility 65 days
Motile sperm/ml	1.00					
Progressive motility	0.31	1.00				
Motility	0.34	0.98 **	1.00			
Progressive velocity	-0.07	-0.20	-0.27	1.00		
Straightness	-0.57	0.08	-0.04	0.28	1.00	
Fertility 65 days	0.21	0.51 *	0.52 *	0.09	0.10	1.00

* $p < 0.01$, ** $p < 0.001$

In this experiment, fertility was significantly correlated with progressive motility (Figure 1) and motility percentage (Figure 2). The correlations between the rest of the semen characteristics and fertility were not significant.

Figure 1. Relationship between progressive motility and fertility.