Relationship between laboratory measures of ram sperm competence and field fertility

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ABSTRACT

Previous work has show poor relationships between the maintenance of sperm motility and fertility after AI of ram semen. New laboratory tests of sperm competence have been developed but their relationship to fertility needs to be established. Two large scale trials involving the laboratory evaluation of straws of frozen semen from different rams and the field AI of ewes with the same batches of semen were conducted. In Trial A, 1671 ewes were AI'd with 52 batches of semen from 26 rams on 6 farms. In Trial B 6872 ewes were AI'd with 137 batches of semen from 34 rams on 25 farms. In Trial A, there was no linkage between farms while in Trial B the same batch was used on more than one farm and most farms had more than 1 batch from more than 1 ram. Laboratory evaluation involved: visual motility assessment post thaw and time taken to drop to 5% motility when incubated at 38oC, 8 parameters of velocity and direction of motility using CASA; % live and % acrosome intact and sperm concentration. These parameters were measured immediately post-thaw and in Trial B a repeat measurement was made after 6h at 38°C. In addition, in Trial A the relative IVF value was determined. In Trial A there were significant ($P<0.05$) between farm and ram within farm effects on fertility (% pregnant) but no ejaculate within ram effect. Significant ($P<0.001$) differences were seen in all semen parameters except initial motility, time to 5% and concentration. Parameters correlated with fertility were IVF and % live and together these accounted for 44 % of the variation. In Trial B, there were effects of farm on fertility ($P<0.001$) and breed of ram ($P<0.05$), residual ram variation was non-zero but not significant. There were significant differences between rams and between ejaculates within rams in concentration, visual motility, % live, velocity and directional parameters. The number of live and motile sperm inseminated was correlated ($P<0.001$) with fertility. Significant ($P<0.05$) correlations were also obtained between two CASA parameters; the % live and % visual motility, after 6h of incubation. Multiple regression equations were derived that accounted for between 62 and 75 % of the variation between rams.

Key words: Ram semen; sperm viability measures; fertility.

INTRODUCTION

Determination of the relationship between laboratory measures of sperm function and field fertility of semen is an essential step in the development of protocols for the laboratory evaluation of sperm competence. In the past the duration of the maintenance of sperm motility under incubation has been used as the standard method of semen evaluation for a number of species. Previous work on ram semen diluents (Smith *et al*., 1993) has shown a very poor relationship between maintenance of motility and fertility of fresh stored ram semen. A suite of new laboratory tests that measure a number of sperm functions have been developed (Smith and Murray, 1997) and this publication reports on the relationship between these parameters and field fertility after AI with frozen ram semen.

METHODS

Field Trials: Two large scale field trials involving the laparoscopic insemination of frozen/thawed ram semen were performed. **Trial A:** Involved the AI of 1671 ewes with 52 batches of semen from 26 rams and was conducted on 6 farms. The semen used on each farm was different from that used on the others. Pregnancy was determined by real-time ultrasonic scanning at 50-70 days post insemination. **Trial B:** Involved the AI of 6,872 ewes with 137 batches of semen from 34 rams and was conducted on 25 farms. Linkage between farms was included in this trial with some batches of semen being used on more than one farm and most farms using more than one batch from any ram and more than one ram (see Tables 3 and 4). Inseminations were conducted as part of the commercial operation on these farms and were performed by 11 groups of professional inseminators.

In both trials inseminations were performed laparoscopically using one 0.25ml straw of frozen/thawed semen.

Pregnancy data was determined by either non-returns to service, pregnancy scanning, or lambing records.

Laboratory evaluation: Straws from each batch of semen used in the AI trials was subjected to the following measurements: concentration (by haemocytometer); visual motility - an aliquot of semen was diluted with RSD-1 (Upreti, *et al*., 1995) at 38°C and assessed for percentage motile at 10x magnification immediately after thawing (0h) and at 8-12 h intervals until less than 5% were motile (time to 5%). Eight parameters of velocity and direction of motility were obtained using the 'Hobson Sperm Tracker' (Briggs *et al*., 1996). The parameters measured were: curvilinear velocity (VSL); average path velocity (VAP); straight line velocity (VSL); mean angular displacement (MAD); beat cross frequency (BCF); amplitude of lateral head displacement (ALH); linearity of track (LIN) and percentage of motile sperm (%MOT). The percentage of live or membrane intact sperm (% live) was determined

using SYBR-14 an PI staining and the percentage of acrosomal intact (%PSA) sperm using PSA lectin (Smith and Murray, 1997). In trial A, the relative level of *in vitro* fertility was determined using a heterologous IVF test (Smith and Murray, 1996). In Trial B, the laboratory measures were performed immediately post-thaw and were repeated after straws had been incubated for 6 hours at 38oC, with a further visual motility estimate after 24h. Because the laboratory evaluations were spread over a considerable time period, straws from a control (pooled) semen batch were run on each day of the evaluations to account for any variation over time.

Statistical Analysis: Trial A: All data were analysed using the REML procedures in the Genstat 5.3 statistical package. Percentage of ewes pregnant (% preg.) was analysed as binomial data fitting a nested model (Rams within Farms). For the laboratory data batch within ram was added to the model. The % live was analysed as binomial data and all other parameters as continuous variates with appropriate transformations to achieve homogeneity of variance and normal error distribution.

Analysis of co-variance was performed with (% pregnant) and the other 15 parameters. Correlation of semen viability parameters with field fertility was performed on ram within farm co-variation. A correlation matrix was derived and multi-variate regression analyses were then fitted.

Trial B. Pregnancy data were analysed as binomial data using GLMM procedure, with farm, breed type, ram and batch as random effects to provide individual ram

TABLE 1: Farm and ram summary of fertility results for Trial A.

Farm	Ram	No. Batches	No. Ewes AI'd	Ewes Pregnant No.	Ewes Pregnant %
1	476/89	1	71	26	36.6
	305/89	3	99	42	42.4
	145/90	3	121	49	40.5
	111/90	2	98	46	46.9
	1265/91	2	62	29	46.8
	1070/91	2	54	26	48.1
	1058/91	2	54	26	48.1
			575	**252**	**43.8**
2	115/90	1	38	31	81.6
	180/91	2	59	44	74.6
	59/92	2	61	40	65.6
	1602/92	2	58	43	74.1
			216	**158**	**73.2**
3	2101/91	4	147	108	73.5
	2102/91	4	148	105	70.9
			295	**213**	**72.2**
4	D5/89	2	35	20	57.1
	A60/93	3	35	18	51.4
	A3/93	1	34	17	50.0
	A4/91	2	35	18	51.4
	A21/93	1	37	13	35.1
	XL89/90	1	38	30	78.9
			250	**134**	**53.6**
5	250/87	7	**134**	**56**	**41.8**
6	D65/92	3	99	49	49.5
	D86/89	1	33	17	51.5
	227/90	1	35	22	62.9
	A8/92	1	34	21	61.8
			201	**109**	**54.2**

TABLE 2: Farm summary of Laboratory measures of sperm in Trial A

Parameter		Farm 1	2	3	4	5	6
Relative IVF		105	148	148	122	133	138
Visual Motility	(%)	35	26	34	35	43	41
Live	(%)	28	15	21	16	21	18
PSA intact	(%)	79	70	74	77	75	58
Hrs to 5% motile	(h)	23	20	23	21	27	20
Concentration	($x10^6$/ml)	382	526	429	470	498	380
VCL	(µm/sec)	161	145	172	161	167	162
VAP	(µm/sec)	121	85	126	104	89	103
VSL	(µm/sec)	90	59	90	73	61	75
MAD	(°)	40	60	46	52	61	49
BCF	(Hz)	6.3	13.5	7.3	13.1	16.7	17.8
ALH	(µm)	6.3	7.1	7.1	7.3	7.6	6.3
LIN	(%)	52	40	47	43	36	45
%-MOTILE	(%)	69	20	55	43	38	46

TABLE 3: Fertility values for individual Farms in Trial B

FARM ID	No. of Rams	No. of Batches	Insemi-nator	No. Ewes AI'd	No. Ewes Lambed^	% Lambed	Adjusted% Lambed
1	6	7	A	170	114	67.1	75.0
2	9	11	B	258	177	68.6	74.6
3	6	8	C	143	102	71.3	77.9
4	7	10	D	197	131	66.5	74.6
5	7	14	C	315	213	67.6	69.8
6	22	69	E	1542	682	44.2	54.3
7	5	5	A	150	61	40.7	52.5
8	8	16	E	344	202	58.7	63.6
9	7	9	F	250	138	55.2	65.7
10	7	15	A	492	73	14.8	22.9
11	6	11	A	274	121	44.2	48.4
12	11	17	A	503	214	42.5	46.9
13	4	13	A	359	193	53.8	64.2
14	5	10	G	178	91	51.1	61.8
15	3	3	E	40	23	57.5	66.9
16	9	9	H	242	88	36.4	46.9
17	4	8	E	192	127	66.5	64.2
18	6	9	D	212	138	65.1	73.3
19	4	4	A	120	69	57.5	66.9
20	9	14	I	282	183	64.9	73.7
21	6	6	J	147	60	40.8	45.6
22	5	5	K	225	120	53.3	53.2
23	5	6	K	170	118	69.4	71.2
24	6	7	J	98	39	39.8	52.8
25	3	5	J	49	25	51.2	57.0
Total				**6952**	**3502**	**50.4**	**60.0**

^No. Ewes Lambed = the number of ewes recorded as either (Non Return, Scan Pregnant or Lambed)

@Adjusted % Lambed = This adjusted value is based on a weighting that assumes that on each farm a proportion of the ewes were AI'd with semen of the different Ram Breeds in the ratio of numbers in the overall trial and weighted for the mean values for those breeds. The values are also adjusted for the stage of pregnancy report effect.

fertility estimates. Laboratory data were analysed using REML procedure taking account of the day to day variation using the values for the control semen. The % live was analysed as binomial data and all other parameters as continuous variates with appropriate transformations to achieve homogeneity of variance and normal error distribution. The model included day, breed type ,ram within breed and batch within ram as random effects plus control vs remainder as a fixed effect. Fertility and laboratory estimates for each ram were correlated.

RESULTS

Trial A: The pregnancy data is summarised in Table 1. The mean pregnancy rates between farms ranged from 41.8% (Farm 5) to 73.2% (Farm 2) (P<0.001) while the spread in pregnancy rates between rams within a farm ranged from 53.8% (35.1 to 78.9% on Farm 4) to 2.6% (70.9 to 73.5% on Farm 3). The differences between batches within ram were not significant while those for between rams within farms was marginal (0.05 < P <0.1).

The mean values for the laboratory tests are presented in Table 2. There were significant (P<0.001) between farm effects for all parameters except %PSA, Hrs to 5% motility and concentration. There were no significant between batch within ram effects.

Correlations of sperm evaluation and field fertility: Significant correlations were seen between fertility and IVF (+ 0.521, P<0.003), and % live (- 0.506, P<0.007) which together accounted for 44.3% of the variance.

Trial B: The pregnancy data is summarised in Tables 3 and 4 and is presented both as the raw and the predicted values based on the model used in the analysis. There was a significant (P< 0.001) difference between farms, which ranged from (14.8% on Farm 10 to 71.3% on Farm 2) and a significant difference between breed of ram (P<0.05) but there were no significant differences between rams nor between batches within rams, although the between ram

TABLE 4: Fertility values for individual Rams used in Trial B

Ram ID	Breed	No. of Farms	No.Ewes AI'd	No.Ewes Lambed[#]	% Lambed	Weighted % Lambed[$]	Adjusted % Lambed[@]
1346/81	Romney	1	30	26	86.7	60.0	67.6
278/87	Romney	1	30	25	83.3	64.0	67.5
2665/87	Romney	1	29	20	69.0	83.9	67.2
5012/9	Romney	1	24	18	75.0	61.9	67.2
5121/9	Romney	1	24	19	79.2	67.3	67.3
112/92	Coopworth	2	100	53	53.0	58.0	58.4
115/90	Coopworth	2	98	54	55.1	60.0	58.5
461/93	Coopworth	3	128	64	50.0	57.7	58.3
A12/93	Texel -	1	27	15	55.6	66.5	61.7
A21/93	Texel +	1	24	3	12.5	18.5	57.0
A227/90	Texel +	1	26	13	50.0	61.4	57.8
A6/92	Texel -	1	22	8	36.4	47.6	61.3
D65/92	Dorset	2	61	28	45.9	54.7	67.1
D86/89	Dorset	2	66	36	54.6	63.0	67.5
B08	East Friesian	11	358	159	44.4	45.9	60.5
B09	East Friesian	9	397	179	45.1	45.5	60.3
B12	East Friesian	1	20	7	35.0	42.2	61.3
B16	East Friesian	7	224	102	45.5	53.4	61.8
B19	East Friesian	1	75	39	52.0	59.0	62.0
B21	East Friesian	18	872	433	49.7	50.0	61.0
B23	East Friesian	3	94	45	47.9	52.7	61.6
B26	East Friesian	17	737	359	48.7	49.4	60.9
B27	East Friesian	2	92	38	41.3	48.4	61.3
B32	East Friesian	1	85	24	28.2	34.3	60.4
B37	East Friesian	1	15	8	53.3	53.2	61.5
B40	East Friesian	22	1266	674	53.2	53.5	62.6
B51	East Friesian	4	131	48	36.6	41.7	60.6
B56	East Friesian	15	583	292	50.1	53.0	62.2
B60	East Friesian	11	370	199	53.9	50.7	61.5
B68	East Friesian	7	307	165	53.8	56.9	62.6
B75	East Friesian	1	10	3	30.0	17.6	61.2
B84	East Friesian	1	25	10	40.0	55.9	61.5
B87	East Friesian	11	378	196	51.9	52.9	61.9
B88	East Friesian	2	89	46	51.7	57.5	62.0
B17 fresh	East Friesian	1	134	94	70.2	69.6	66.1
Total		**25**	**6952**	**3502**	**50.4**	**57.4**	**62.3**

[#] No. Ewes Lambed = the number of ewes recorded as either (Non Return, Scan Pregnant or Lambed)

[$] Weighted % Lambed = Adjusted for the between Farm effects but using a Fixed Ram model effect.

[@] Adjusted % Lambed = This is an adjusted value based on a weighting that assumes that on each farm a proportion of the ewes were AI'd with semen from the different Breeds in the ratio of numbers as in the overall trial and adjusted for the between farm effects and weighted for the mean values of those breeds and weighted for an uniform concentration of 400 million sperm per ml.

effect was significant (P<0.05) if a 'fixed ram effect' model was used for the analysis. The majority of the ram breed effect was due to differences in the concentration of the semen used but there was still a significant residual effect of breed following adjustment for concentration. There was no significant difference between the 11 groups of inseminators involved in the trial. The number of farms that individual rams were used on ranged from 1 to 22 (Table 4) while the number of rams used on any one farm ranged from 3 to 22 and batches of semen from 3 to 69 (Table 3). The reliability of the pregnancy data was influenced by the stage of collection with the non-return rate representing 85.3% of ewes lambing and pregnancy scanning 96.0%. There were significant (P<0.001) between farm effects on these parameters.

Laboratory analyses: The data for the laboratory analysis (Table 5) was restricted to 65 batches of semen from 25 rams as samples of the other batches were either unavailable or were lost in transit.

There were significant (P<0.001) between ram effects on sperm concentration and this was predominantly an effect of breed type (or processing centre), with the straws from the East Friesian breed being packaged at a much lower concentration than those for the other breeds.

There were significant (P<0.001) effects of ram on the % live sperm and the % visually motile. There were significant differences between rams in VCL,VSL BCF, ALH, and %MOT. All parameters significantly (P<0.001) changed over the 6 hrs of incubation.

TABLE 5. Mean Ram Breed type values for Laboratory sperm parameters in Trial B.

Parameter		Ram Breed					
		East Freisian	Coop-worth	Dorset	Romney	Texel	Mean
		(13)[a]	(3)	(2)	(2)	(4)	(25)
Live 0h	(%)	24.7	12.5	30.1	21.1	23.2	23.0
Live 6h	(%)	22.9	8.4	15.1	14.7	17.3	18.7
Motile 0h	(%)	42.4	20.4	38.0	29.4	36.7	36.9
Motile 6h	(%)	37.4	12.6	20.9	23.1	25.6	29.5
VCL 0h	(µm/sec)	148	153	154	141	139	147
VCL 6h	(µm/sec)	129	123	139	133	124	129
VAP 0h	(µm/sec)	108	112	114	96	104	107
VAP 6h	(µm/sec)	92	92	102	92	93	93
VSL 0h	(µm/sec)	80	84	85	75	77	80
VSL 6h	(µm/sec)	67	68	66	69	63	67
MAD 0h	(°)	37	31	32	37	33	35
MAD 6h	(°)	38	33	36	41	38	38
BCF 0h	(Hz)	17	18	18	18	16	17
BCF 6h	(Hz)	16	16	16	17	14	16
ALH 0h	(µm)	4.3	4.9	4.0	4.9	4.3	4.4
ALH 6h	(µm)	4.0	3.8	4.1	4.5	4.0	4.0
LIN 0h	(%)	48	53	53	51	52	50
LIN 6h	(%)	47	51	41	47	45	47
MOT 0h	(%)	27	45	28	43	42	33
MOT 6h	(%)	19	24	25	31	31	23
Concentration (x10^6/ml)		133	426	227	376	378	229

[a] = number of rams on which data was obtained.

Relationship of laboratory measures to fertility. The correlation between sperm concentration and fertility (P<0.01) was such that at a mean fertility level of 50% an additional 100 x10^6 sperm / ml would result in an increase of 3.6 ± 1.2 %. A number of laboratory parameters showed significant correlations with the fertility of rams adjusted for farm effects (Table 6).A significant correlation (r=0.424; P<0.05) was found between the change in CASA %MOT over the 6h incubation and fertility adjusted for farm and concentration effects.

TABLE 6: Correlation of Laboratory parameters with fertility in Trial B.

Variate		Correlation coefficient
Adjusted Ram fertility[a]		
Number of live sperm	(0h)	+ 0.538**
Number of live sperm	(change over 6h)	- 0.681***
Number of motile sperm	(0h)	+ 0.798***
Number of motile sperm	(6h)	+ 0.797***
Number of motile sperm	(24h)	+ 0.736***
Number of motile sperm	(change over 6h)	- 0.736***
Number of motile sperm	(change over 24h)	- 0.782***
%MOT	(0h)	+ 0.555**
%MOT	(6h)	+ 0.602**
VAP	(change over 6h)	+ 0.391*
% live	(6h)	- 0.500**
% live	(change over 6h)	- 0.684***
Visual motility	(0h)	- 0.535**
Visual motility	(6h)	- 0.616**

[a] Adjusted Ram fertility = adjusted for Farm but not for concentration.
* = P<0.05; ** = P< 0.01; *** = P<0.001.

Multiple regression analysis showed that for the ram fertility estimate adjusted for farm only the number of motile sperm at 0h was included in the equation and this accounted for 62% of the variance. A similar analysis with the ram fertility estimate adjusted for farm and concentration resulted in the following variables (% MOT-change over 6h; % live -change over 6h; BCF at 6h; MAD at 6h; visual % motile at 24h; number of live sperm at 6h and LIN at 6h) being fitted in that order to an equation that accounted for 75% of the variance.

DISCUSSION

The results of these two trials highlight some important points that need to be carefully considered in any evaluation of fertility of frozen ram semen used in AI.

The first is the major effect of different farms and thus the need to have each batch of semen evaluated on as many farms as possible. This is restricted by the current freezing technology and the high insemination dose rate used which results in low numbers of inseminations per ejaculate. This factor also compromises the accuracy of the pregnancy data due to the small number of ewes that comprise a specific sub-set of data. In trial B, the variation in the pregnancy rates for the same batch of semen on different farms was very large and this resulted in the lack of batch

within ram and also the low between ram effects seen. The lack of any inseminator effect was also due to the large between farm differences in pregnancy rates. Thus factors other than semen quality and inseminator competence are having profound effects on AI pregnancy rates obtained on specific farms.

In Trial A, as there was no linkage of the semen used between farms the analysis was on a between ram within farm model. This showed that the use of a 'relative IVF' test was the laboratory measure that gave the best prediction of field fertility. This however, is a time consuming and relatively difficult procedure and is expensive. In this trial the only other parameter that was significantly related to the fertility was the percentage of live sperm but as this correlation was negative it is difficult to interpret. Interestingly, a negative relationship for this parameter and fertility was also found in Trial B. The significant positive correlations between concentration; the numbers of live and motile sperm inseminated and fertility in Trial B but not Trial A may reflect the overall lower values and wider range of concentrations found in the second trial as well as the increased sensitivity of the fertility test due to higher numbers of ewes. In this case the relationships were positive and the -negative effects of the % live and % visually motile sperm seen in both trials may reflect the tendency of processor's to selectively package sperm of lower initial viability at higher concentrations.

The lack of relationships between the CASA parameters (except %MOT) immediately post-thaw and fertility is supported by the findings of (Eppleston and Maxwell, 1995) in sheep and (den Daas,1997) in cattle. However, when a 6 h incubation was performed and the changes in values were determined then a significant correlation of velocity (VAP) with field fertility was found and velocity and directional parameters were incorporated into the multiple regression equations. This is supported by the similar findings with pig semen after incubation (Holt *et al*., 1997).

In conclusion, laboratory parameters such as: 'relative IVF' , the number of live sperm , %MOT, BCF, MAD as well as changes in these over the 6h of incubation have been shown to be good predictors of the fertility estimates of different rams, once site effects have been accounted for. These parameters will be very useful measures for the development of new semen diluents and handling techniques.

However, the over-riding influence of farm factors other than semen quality or inseminator ability make the use of semen competency measures as a generalised predictor of field fertility with AI very questionable.

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