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RECENT ADVANCES IN THE DEEP-FREEZE PRESERVATION OF RAM SEMEN

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Research on the frozen storage of ram semen has been going on for more than 20 years and in general followed the same lines as with bull semen. In an excellent review by Lightfoot (1969) most aspects of the freezing procedures were discussed fully, and detailed assessments were presented on the effects of diluent composition and methods of dilution, cooling, freezing and thawing. A brief discussion of some of these procedures is presented here as an introduction to the review of fertility results with frozen ram spermatozoa.

The diluents commonly used are isotonic to slightly hypertonic media and the inclusion of sugars, notably those of higher molecular weight, was found to have beneficial effects. The commonly used cryoprotective agent, as for bull semen, is glycerol. Dilution of the semen at 30°C with the glycerol-containing diluent (one-step dilution) can be done successfully and this has considerable practical advantages over the method of addition of the glycerol-containing fraction at 5°C (two-step dilution). Lower rates of dilution (1:2 to 1:4) of semen are generally reported to be more suitable, but this can be influenced by factors such as the composition of the diluent and the method of freezing. Results on cooling rates indicated that slow cooling to 5°C over one to two hours is required and that relatively short periods of equilibration at 5°C gave satisfactory results with ram semen.

Ram semen can be frozen successfully in ampóules, straws ("paillettes") or in pellet form, and choice of freezing method seemed dependent largely on individual preferences and needs. It is noteworthy though that the straws and even more so pellets, offer substantially greater utilization of storage space in the liquid nitrogen containers than do ampoules. Two important considerations should further be borne in mind when the semen is frozen in pellet form. First, through lack of a sealed "container", bacterial contamination in liquid nitrogen is possible and the probable event of migration of spermatozoa between semen-pellets of different sires (Merkt, Weitze & Lorrmann, 1967) may raise doubt on the parenthood of the progeny obtained. In the second place, pelleted semen makes possible the use of a thawing solution which can have a substantial effect on the recovery rate on thawing, as well as on the survival of cells during post-thawing incubation. It further follows that the use of a thawing solution implies further dilution of the semen and reconcentration (by centrifugation) before insemination is necessary to obtain satisfactory fertility. The findings of the reports by Lightfoot (1969) were further substantiated by results of Lightfoot & Salamon (1969a, b), Salamon & Lightfoot (1969) and Salamon & Brandon (1971). It was, nevertheless, indicated by these authors that numerous factors of diluent composition interacted with each other and with methods of dilution, cooling, freezing and thawing. It is therefore unwise to recommend a certain diluting media when other freeze-thawing procedures are not specified. This complex influence of various factors therefore explains to a great extent the wide variation and often total contradiction found in published results.

The early research on the fertility of frozen-thawed ram spermatozoa has been reviewed by Emmens (1961), Emmens & Robinson (1962), Sadleir (1966), Leidl (1968) and Lightfoot (1969). In the report by Lightfoot a full summary of the literature up to that time is presented and the author also referred to the problem of evaluating some results where the authors used unsatisfactory experimental designs and/or provided insufficient details on the conduct of experiments. Subsequently, Lopyrin (1969) also criticised the methods used by some Soviet workers who claimed high fertility. When these claims were tested under strict supervision "none of the authors could confirm their own results and obtain a fertility rate higher than 14% after one insemination. Evidently, this was due to the replacement of fertile teaser rams by vasectomised rams. Thus, the possibility of occasional fertilization of ewes by natural mating has been excluded". In the review presented here, which covers research since 1968, lack of essential details in reports was still encountered and this is evident in the summary of the literature presented in Table 1. Nevertheless, the fertility results following insemination with frozen-thawed ram semen improved markedly in the past five years. They are still lower than those obtained with fresh-diluted semen, but the important point is that these results are at least repeatable. In the discussion that follows, these results will be examined under several headings relating to the most relevant procedures of freeze-thawing and insemination. Thus, diluent composition is dealt with first, followed by dilution rate, freezing and thawing methods, insemination techniques, finally concluding with the effect of long-term storage on fertility.

1. Diluent composition

Diluents used successfully for frozen storage of ram spermatozoa can be divided broadly into sugar- and Tris*-based media. The former group includes mono- (glucose and fructose), di- (lactose) and trisaccharides (raffinose) which were usually combined with sodium citrate. Media containing Tris, alone or in combination with different sugars (e.g. fructose, glucose), have also been used successfully for the frozen storage of ram spermatozoa.

The high fertility results, obtained with semen frozen in glucose-citrate-yolk, claimed by earlier workers (Mackepladze, Gugušvili, Bregadze & Haratišvili, 1960; Lopatko, 1963) could not be confirmed by Branny, Pilch & Wierzbowski (1966) and Salamon (1967). Similarly, only low to modest results were obtained following cervical in-

* Tris: tris (hydroxymethyl)-aminomethane.

Table 1

Summary of literature on the fertility of frozen-thawed ram spermatozoa

Reference	Diluent	Dilution rate	Freezing method	No. cells/ inseminate ($x10^6$)*	No. of insemi- nations	Fertility		Comments
						No. of ewes	% Pregnant	
Aamdal & Andersen (1968)	75,3% of 11% lactose – 20% yolk – 4,7% glycerol.	1:4 –	Straw	–	2	18	61,0	Non-return.
		1:6	Straw	–	2	8	62,5	Pregnant at slaughter.
Fraser (1968)	71,5% of 11% lactose – 25% yolk – 3,5% glycerol.	–	Pellet	–	1	–	31,0	Non-return.
		–	Pellet	–	2	–	56,0	Non-return.
		–	Pellet	–	3	–	80,0	Non-return.
Loginova & Zeltobrjuh (1968)	12% lactose – 2% sodium citrate – 20% yolk and	1:5 – 1:6	Ampoule, straw & pellet	–	1	12	68,8	Fertilized eggs; tubal insemination.
	4,8% glucose – 2% sodium citrate – 20% yolk (4,2 – 9,6% glycerol).			–	1	33	5 –15	CR. Cervical insemination.
	As above, and also 9% lactose – 20% yolk – 4,2% glycerol.	"	"	–	1	17	88,0	CR. Tubal insemination.
		"	"	–	1	16	12,0	CR. Cervical insemination.
		"	"	–	2 + 3	125	12,5–32	Lambing. Cervical insemina- tion.
Lunca (1968)	1% fructose – 2,8% citrate – 25% yolk – 7% glycerol.	1:4 –	Straw	50*	–	45	45,0	Conception rate.
		1:14	Straw	20–25*	–	55	44,0	Conception rate.
	Milk-yolk (9:1) – 7% glycerol.	"	Straw	50*	–	48	44,0	Conception rate.
		"	Straw	20–25*	–	52	43,0	Conception rate.
Platov (1968)	Lactose-yolk – 3,5% glycerol.	1:3	Pellet	–	1 + 2	45	22,0	Lambing.
	Do., 3,5% ethylene glycol.	1:3	Pellet	–	1 + 2	60	22,0	Lambing.
	Do., 1,75% ethylene glycol.	1:1	Pellet	–	1 + 2	54	5,5	Lambing.
	Lactose-yolk-albumin – 3.5% glycerol.	–	Pellet	–	1	135	24,0	Lambing.

Table 1 (contd.)

Reference	Diluent	Dilution rate	Freezing method	No. cells/ inseminate $(x10^6)$*	No. of insemi- nations	Fertility		Comments
						No. of ewes	% Pregnant	
Volkov (1968)	Lactose-yolk	–	Pellet	–	2	59	11,8	Fertilized eggs.
		–	Pellet	–	2	39	12,8	Pregnant at laparotomy.
		–	Pellet	–	2	95	15,8	Lambing.
Kalev *et al.* (1969)	Glucose-sodium citrate-yolk	–	–	50–60	–	537	32,0	Conception rate.
Mattner *et al.* (1969)	247 mM glucose – 49 mM NaCl – 5 mM Na-phosphate – 17 mM fructose	1:19	Ampoule	30	1	6	83,3	Ewes with fertilized eggs: Uterine insemination.
		"	Ampoule	3	1	7	14,3	
		"	Ampoule	80–120	1	8	87,5	
	– 6% yolk – 7,5% glycerol	"	Ampoule	"	1	9	33,3	Ewes with live embryos: Uterine insemination.
Colas & Brice (1970)	75% of 11% lactose – 20% yolk – 5% glycerol	1:2	Straw	190	2	. 50	60,0	Lambing.
		"	Straw	"	2	63	44,4	Pregnant at laparotomy.
Kalev *et al.* (1970)	75% of 11% lactose-20% yolk – 5% glycerol	–	Pellet	70–80*	2	340	24,1	Lambing.
	3% glucose – 2,5% citrate – 7% glycerol.	–	Pellet	70–80*	2	560	24,08	Lambing.
Lightfoot & Salamon (1970a)	166,5 mM raffinose – 68 mM citrate – 15% yolk – 6% glycerol.	1:1	Pellet	120	1	53	12,5	Ewes with fertilized eggs.
	Do. 5% glycerol, re-concentrated semen.	1:3	Pellet	80	1	14	22,2	Eggs with sperm on zona.
		"	Pellet	160	1	14	37,5	
		"	Pellet	20	1	8	–	) No. of sperm in cervix
		"	Pellet	60	1	8	–	) increased as dose increased.
		"	Pellet	180	1	8	–	)
		"	Pellet	160	1	9	66,6	Ewes with fertilized eggs.

Table 1 (contd.)

Reference	Diluent	Dilution rate	Freezing method	No. cells/ inseminate ($x10^6$)*	No. of insemi- nations	Fertility		Comments
						No. of ewes	% Pregnant	
(1970b)	Same as 1970a. Re- concentrated semen.	1:3	Pellet	160	2	40	50,0	Lambing. Cervical insemi- nation.
		"	Pellet	"	2	37	29,7	Lambing. Cervical traction insemination.
		"	Pellet	"	1	30	40,0	Lambing. Uterine insemi- nation.
Salamon & Lightfoot (1970)	166,5 mM raffinose – 68 mM citrate – 15% yolk – 5% glycerol. Reconcentrated semen (except for 1st treat- ment).	1:1	Pellet	120	1	269	5,6	Non-return (6% glycerol).
		1:3	Pellet	150	1	46	41,3	Lambing.
		"	Pellet	"	2	43	46,5	Lambing.
		"	Pellet	50	1	45	17,8	Lambing.
		"	Pellet	"	2	43	25,6	Lambing.
		"	Pellet	150	1	46	8,7	Lambing.
		"	Pellet	"	2	43	44,2	Lambing.
		"	Pellet	80	1+2	116	44,8	Lambing. Cervical insemi- nation.
		"	Pellet	240	"	115	47,8	
		"	Pellet	150	2	17	64,7	Lambing. No relaxin.
		"	Pellet	"	2	52	44,2	Lambing. Relaxin.
		"	Pellet	"	2	99	24,2	Lambing. No oxytocin.
		"	Pellet	"	2	96	37,5	Lambing. 0,5 Units oxytocin.
		"	Pellet	"	2	96	20,8	Lambing, 5,0 Units oxytocin.
Samouilidis (1970)	250 mM Tris – 1,25% fructose – 25% yolk – 8% glycerol	1:2	Straw	–	1	45	11,1	Lambing.
		"	Straw	–	2	92	52,2	Lambing.
		"	Straw	–	2+3	298	51,7	30–60 day non return.
		"	Straw	–	1	307	36,3	

Table 1 (contd.)

Reference	Diluent	Dilution rate	Freezing method	No. cells/ inseminate (x10^6)*	No. of insemi- nations	Fertility		Comments
						No. of ewes	% Pregnant	
Bureaunu & Negoită (1971)	Glucose-citrate – 7% glycerol.	–	Ampoule	–	–	–	49,8	Conception rate.
Colas *et al.* (1971)	75% of 11% lactose – 20% yolk – 5% glycerol.	1:2	Straw	190	2	133	20,3	Lambing and non-return. No PMSG.
		”	Straw	”	2	139	49,0	Lambing and non-return. 400 Units PMSG.
		”	Straw	”	2	89	37,1	NR. Insemination at onset of oestrus.
		”	Straw	”	2	98	53,0	NR. Insemination 12 hr from onset of oestrus.
Kareta *et al.* (1971)	Fructose-citrate-yolk – 8% glycerol.	1:2 – 1:8	Ampoule	28–72	2+3	204	48,0	Lambing.
Pexton & Botkin (1971)	9% skimmilk – 0,25% fructose – 7% glycerol.	1:4 & 1:19	–	–	2	16	0	
	2% glycine – 0,5% fructose – 2% citrate – 20% yolk – 0 glycerol.	”	–	–	2	16	6,3	Lambing. Double inseminations done in two successvie cycles.
	1,5% ribose – 2,2% phosphate – 25% yolk – 7,5% glycerol.	”	–	–	2	16	6,3	
	2,5% fructose – 2,94% citrate – 20% yolk – 7,5% glycerol.	”	–	–	2	16	18,8	

Table 1 (contd.)

Reference	Diluent	Dilution rate	Freezing method	No. cells/ inseminate ($x10^6$)*	No. of insemi- nations	Fertility		Comments
						No. of ewes	% Pregnant	
Salamon (1971)	166,5 mM raffinose – 68 mM citrate – 15% yolk – 5% glycerol. Reconcentrated semen.	1:4	Pellet, –79°C	155	1	157	47,1	Lambing.
		"		"	2	161	59,0	Lambing.
		"	"	"	1	93	40,9	Lambing.
		"	"	"	2	98	55,1	Lambing.
		"	Pellet, –140°C	"	1	92	39,1	Lambing.
		"		"	2	95	50,5	Lambing.
Andersen & Aamdal (1972)	11% lactose-yolk – glycerol (see Aamdal & Andersen 1968).	1:4 – 1:6	Straw	60–75*	2	283	46,0	Lambing.
		"	Straw	"	2	40	70,0	Conception rate.
		"	Straw	120–150*	2	40	70,0	Conception rate.
Colas (1972)	75% of 11% lactose – 20% yolk – 5% glycerol	–	Straw	180	2	133	20,8	Lambing. No PMSG.
		–	Straw	"	2	130	45,2	Lambing, 400 Units PMSG.
		–	Straw	"	2	82	57,3	Lambing.
		–	Straw	"	2	76	38,4	Lambing.
		–	Straw	"	2	40	52,5	Lambing.
		–	Straw	"	2	133	26,3	Lambing.
Dzuik *et al.* (1972)	325 mOsm Tes-Tris-0,25% fructose – 20% yolk – 5% glycerol.	1:3	Pellet	–	1	56	13,0	Lambing.
Kareta *et al.* (1972)	Fructose-citrate-yolk – 8% glycerol.	1:1 – 1:8	Ampoule	<50	2+3	119	49,6	Lambing.
			Ampoule	50–60	"	75	57,3	Lambing.
		"	Ampoule	>60	"	66	43,9	Lambing.
	Fructose-citrate-yolk – 8% glycerol + bull seminal plasma	"	Ampoule	<50	"	25	48,0	Lambing.
		"	Ampoule	50–60	"	20	60,0	Lambing.
		"	Ampoule	> 60	"	37	70,3	Lambing.

Table 1 (contd.)

Reference	Diluent	Dilution rate	Freezing method	No. cells/ inseminate ($x10^6$)*	No. of insemi- nations	Fertility		Comments
						No. of ewes	% Pregnant	
Linge (1972)	Tris-yolk.	1:2	Straw	–	–	87	54,0	Lambing. Thaw at 75°C.
		”	Straw	–	–	31	55,0	Lambing. Thaw at 72°C.
		”	Straw	–	–	15	20,0	Lambing. Thaw at 35°C.
Loginova & Zeltobrjuh (1972a)	Glucose-citrate-yolk – 8% glycerol	1:5	Straw	–	1	40	12,5	Ewes with fertilized eggs: Cervical insemination.
		”	Straw	–	2+3	27	18,5	
		”	Straw	–	1	3	100	Ewes with fertilized eggs; Uterine insemination, 9, 13 or 15 hr before ovulation respectively.
		”	Straw	–	1	11	90,9	
		”	Straw	–	1	12	33,3	
(1972b)	4,8% glucose – 2% citrate – 20% yolk – 2,4% glycerol.	1:6	Ampoule	–	2+3	31	32,5	Lambing. Cervical insemination.
	12% lactose – 2% citrate – 20% yolk – 2,4% glycerol.	”	Ampoule	–	”	32	12,5	Lambing. Cervical insemination.
	Do.	”	Pellet	–	”	31	25,8	Lambing. Cervical insemination.
	9% lactose – 20% yolk – 2,4% glycerol.	”	Pellet	–	”	31	29,0	
	4,8% glucose – 2% citrate – 20% yolk.	1:6	–	–	–	20	10,0	CR. Cervical insemination.
	5% glucose – 2,16% phosphate – 20% yolk.	1:6	–	–	1	17	88,2	CR. Uterine insemination.
Nauk (1972)	Raffinose-inositol-K-Ca-Mg-20% yolk – 5% glycerol.	1:2	–	–	2	) 102	30,0	Lambing.
	Lactose	1:2	–	–	2	)	22,0	Lambing.

Table 1 (contd.)

Reference	Diluent	Dilution rate	Freezing method	No. cells/ inseminate ($x10^6$)*	No. of insemi-nations	Fertility		Comments
						No. of ewes	% Pregnant	
Remes (1972)	75% of 11% lactose – 20% yolk – 5% glycerol.	1:2	Pellet	30–50	2	14	64,3	Lambing.
Salamon (1972)	166,5 mM raffinose – 68 mM citrate – 15% yolk – 5% glycerol. Frozen for 3 years.	1:4	Pellet	150	1	75	49,3	Lambing.
		”	Pellet	”	2	97	55,7	Lambing.
Andersen *et al.* (1973)	250 mM Tris-1% fructose – 20% yolk – 8% glycerol.	1:5 –	Straw	150*	1	53	54,0	CR. Intra-uterine insemination. Synchronised ewes.
		1:7	Straw	”	1	24	29,0	Do. Deep cervical insemination.
		”	Straw	”	1	83	89,0	CR. Intra-uterine insemination. Non-synchronised ewes.
		”	Straw	”	1	60	45,0	Do. Deep cervical insemination.
		”	Straw	”	2	21	57,0	CR. Cervical insemination.
Visser & Salamon (1973)	300 mM Tris–94,7 mM citric acid – 27,75 mM glucose – 15% yolk – 5% glycerol.	1:4	Pellet	180	1	35	22,9	Lambing.
		”	Pellet	”	2	35	57,1	Lambing.
	166,5 mM raffinose – 68 mM citrate – 15% yolk – 5% glycerol.	1:4	Pellet	180	1	34	35,3	Lambing.
		”	Pellet	”	2	33	54,5	Lambing.
	Tris-glucose-yolk-glycerol, as above. Reconcentrated semen.	1:4	Pellet	90	1	46	37,0	Lambing.
		”	Pellet	”	2	50	38,0	Lambing.
	Do. Unconcentrated semen.	”	Pellet	”	1	58	37,9	Lambing.
		”	Pellet	”	2	68	44,1	Lambing.

Table 1 (contd.)

Reference	Diluent	Dilution rate	Freezing method	No. cells/ inseminate $(x10^6)$*	No. of insemi- nations	Fertility		Comments
						No. of ewes	% Pregnant	
Salamon & Visser (1974)	166,5 mM raffinose – 68 mM citrate – 15% yolk – 5% glycerol.	1:4	Pellet	180	1	34	55,9	Lambing. Semen frozen for 2 weeks.
		"	Pellet	"	2	34	52,9	
		"	Pellet	"	1	35	42,9	Lambing. Semen frozen for 5 years.
		"	Pellet	"	2	35	62,9	
Visser & Salamon (1974)	300 mM Tris-94,7 mM citric acid – 27,75 glucose – 15% yolk – 5% glycerol. Reconcentrated semen.	1:4	Pellet	90	1	36	19,4	Lambing.
		"	Pellet	"	2	34	35,3	Lambing.
		"	Pellet	180	1	37	29,7	Lambing.
		"	Pellet	"	2	33	57,6	Lambing.
	360 mM Tris – 113,7 mM citric acid – 33,30 mM glucose – 18% yolk – 6% glycerol. Unconcentrated semen.	1:2	Pellet	90	1	39	30,8	Lambing.
		"	Pellet	"	2	35	37,1	Lambing.
		"	Pellet	180	1	38	34,2	Lambing.
		"	Pellet	"	2	33	54,5	Lambing.

*Motile spermatozoa; total number where indicated with an asterisk (*).

NR: Non-return.

CR: Conception rate.

Note: In many papers essential details concerning the conduct of the experiments were not clearly stated or were not available in translation.

semination when the diluent contained glucose at a concentration of 3% (24% lambing: Kalev, Marinov, Zagorski, Kitchev, Bak'rdzhiev & Zhekov, 1970) or 4,8 to 5% (12,5 to 32% lambing, 12,5 to 18,5% ewes with fertilized eggs, 10 to 32,5% lambing: Loginova & Zeltobrjuh, 1968, 1972a, b), while a number of workers reported high egg fertilization and conception rate following surgical insemination into the oviduct (69 to 88%: Loginova & Zeltobrjuh, 1968) or uterus of ewes (14 to 88%: Mattner, Entwistle & Martin, 1969: 33 to 100%: Loginova & Zeltobrjuh, 1972a; 88%: Loginova & Zeltobrjuh, 1972b). Kalev, Zagorski, Zahriev, Kitchev & Georgiev (1969) and Bureanu & Negoiţă (1971) further claimed conception rates of 23 to 73% and 50% respectively after cervical insemination, but unfortunately details of the diluent composition were lacking. Varying degrees of success have also been reported with the use of a fructose-citrate medium (44 to 45% conception: Lunca, 1968; 48% lambing: Kareta, Pilch & Wierzbowski, 1971; 44 to 50% lambing: Kareta *et al.*, 1972; 19% lambing: Pexton & Botkin, 1971).

Following the development of a lactose (8,25%)-sodium citrate (2%)-yolk diluent for bull semen by Nagase & Graham (1964), many workers used this medium, or slight variations of it, for frozen storage of ram semen. Some workers reported satisfactory fertility with this diluent in 1968 [Aamdal & Andersen (61 to 62,5% pregnancy), Fraser (31 to 80% non-return), Platov (6 to 24% lambing) and Volkov (12 to 16% fertility and lambing)]. Loginova & Zeltobrjuh (1968) obtained only 5 to 15% non-returns, but it is not clear from their report which results belong to the glucose or the lactose diluents. Subsequently lactose-citrate was used with success for frozen storage of ram spermatozoa and the fertility results of some French workers were particularly encouraging. Colas & Brice (1970), Colas, Brice, Courot & Cottier (1971) and Colas (1972) reported lambing and pregnancy rates of 10 to 68%. Lambing results were generally low (10 to 21%) in ewes inseminated at the first oestrus after cessation of the progestagen treatment, but improved markedly (to 45 to 68%) when the ewes were injected with Pregnant Mare Serum Gonadotrophin (PMSG, 400 I.U.) prior to insemination. By using this combined progestagen-PMSG treatment, Colas *et al.* (1971) obtained higher fertility when the first of two inseminations was performed 12 hours after the onset of oestrus (53% non-return) than immediately after oestrus detection (37% non-return). Colas & Brice (1970) and Colas (1972) used two inseminations, 50 and 60 hours after sponge withdrawal. Their reports showed that insemination at appointed times can result in satisfactory fertility (45 to 68% lambing), with the additional benefit of eliminating the need for detection of oestrous animals by teaser rams. Remes (1972) reported similar high fertility (64% lambing), but only 14 ewes were inseminated. In a trial with 340 animals, Kalev *et al* (1970) could only obtain 24% lambing, following a non-return rate of 44%. Loginova & Zeltobrjuh (1972b) likewise obtained only 12,5% lambing.

Following extensive laboratory investigations, Lightfoot & Salamon (1970a, b) and Salamon & Lightfoot (1970) reported on the fertility of ram spermatozoa frozen in 166,5 mM raffinose-68 mM sodium citrate-yolk diluent. After initial low fertility (5,6 to 26% lambing) the authors subsequently improved and modified the procedures of freeze-thawing and insemination and lambing rates of 45 to 65% were consistently obtained in later trials. These results were later confirmed by Salamon (1971, 1972).

The use of Tris in diluents for freezing ram semen was investigated and resulted in satisfactory fertility. Thus, Samouilidis (1970) reported non-return rates of up to 52% and Linge (1972) obtained 55% lambing with Tris-fructose-yolk and Tris-yolk respectively. Andersen, Aamdal & Fougner (1973) obtained 57% conception in 21 ewes, but the authors also claimed 89% pregnancy when the semen was deposited into the uterus by non-surgical means (see *Insemination techniques*, p. 11). After detailed studies by Salamon & Visser (1972), a Tris-glucose-yolk diluent was selected for fertility trials. Lambing rates of 19,4 to 37,9% after single and 53,3 to 57,6% after double insemination were subsequently reported by these authors (Visser & Salamon 1973, 1974).

2. Dilution rate

An important effect of varying the prefreezing dilution rate on recovery and survival of ram spermatozoa was illustrated by Lightfoot & Salamon (1969b). Best results were obtained when the semen was diluted 4- to 6-fold, with lower recovery for both lower and higher rates. This effect was, however, dependent on whether the diluent components were adjusted to have the same concentration in the diluted semen, regardless of dilution rate. Furthermore, with pellet-frozen semen, prefreezing and thawing dilution rate interacted. Recovery was poor with low prefreezing (1:1) or high thawing dilution (1:7), while thawing dilution of 1:1 was suitable for 4- to 16-fold prefreeze dilution. Thawing in a dry tube was only successful with 16-fold prefreeze dilution and lower rates became increasingly deleterious.

In most of the reports on the fertility of frozen ram spermatozoa, relatively low prefreezing rates were employed (1:2 to 1:6). Lunca (1968), Kalev *et al.* (1970), Kareta, Pilch & Wierzbowski (1971, 1972) and Andersen, Aamdal & Fougner (1973) adjusted dilution rates according to ejaculate-density or to obtain a certain cell number in the inseminate. It is therefore difficult to determine whether the effect on fertility was one of dilution rate *per se* or of variation in diluent composition. Platov (1968) obtained substantially higher fertility (22 cf. 6% lambing) when semen was diluted 1:3 as against 1:1 with lactose-yolk, whereas Pexton & Botkin (1971) reported similar results for rates of 1:4 to 1:19. It is not indicated whether diluent adjustments were made for these dilution rates and the mentioned criticism therefore still applies. Subsequent to their laboratory investigations, Lightfoot & Salamon (1970a, b), Salamon & Lightfoot (1970) and Salamon (1971, 1972) obtained satisfactory lambing results with pellet-frozen semen diluted 4- to 6-fold prior to freezing, followed by 3- to 4-fold dilution at thawing.

3. Method of freezing

Few investigations were carried out to determine the suitability of different freezing "containers" for ram semen.

Results of Salamon (1967) and Loginova & Zeltobrjuh (1968) showed no difference for ampoules, straws or pellets. Fertility was generally low for all methods. With improved freezing procedures that followed these reports, satisfactory fertility results have been obtained with semen frozen in ampoules, straws and in pellet form. No direct comparisons were, however, made and choice of method seems to depend on personal preferences and circumstances.

4. Method of thawing

Recovery and survival of spermatozoa after freezing is influenced substantially by the thawing process, where additional injury to the cells can occur with sub-optimal techniques. In the research reviewed here most of the attention was directed towards thawing temperature and more recently also to thawing solutions for semen frozen by the pellet method.

Lightfoot (1969) indicated in his review that bull and ram semen can be thawed successfully at 37 to 40°C or at 0 to 5°C, with only marginally better results at the higher temperatures. In the reports since 1968 on the fertility of frozen ram spermatozoa most workers used thawing temperatures of 37 to 45°C. Aamdal & Andersen (1968) examined different thawing temperatures for semen frozen in straws and found 75°C superior to 35°C as judged by differential staining of the spermatozoa. They subsequently used semen thawed at 75°C in fertility trials and obtained 61 to 62,5% pregnancy in 26 ewes. These results were later confirmed by Andersen *et al.* (1973). Linge (1972) compared 72 to 75°C and 35°C and obtained substantially higher lambing results with the semen thawed at the higher temperatures (54 and 55% cf. 20%). Nauk (1972) reported rather poor lambing results when thawing the semen at 70°C.

The thawing of pellet-frozen semen presents an additional problem to optimal temperature. Several reports indicated that thawing of the pellets in a solution was beneficial in the case of ram (Salamon 1968), bull (Essich 1966; Idris 1971; Heydorn & Paufler 1973) and jackass and stallion spermatozoa (Krause & Grove 1967), whereas Salamon (1973) found better recovery and survival of boar spermatozoa when the pellets were thawed in dry, warmed tubes. The beneficial effect of a thawing solution for pellet-frozen ram spermatozoa has been described in detail by Lightfoot & Salamon (1969b) and Salamon & Brandon (1971). They examined several solutions, and important interactions with diluent composition and dilution rate were described. It was further pointed out that "some kind of repairing action provided by the thawing solution to the sperm cells not lethally injured during freezing may also be possible". Furthermore, "that the solution acting beneficially on the recovery of cells will not necessarily be an optimal milieu for the maintenance of viability of spermatozoa as the time elapses from thawing". The authors subsequently used semen thawed in a solution in fertility trials and obtained 45 to 65% lambing following reconcentration of the thawed semen (Lightfoot & Salamon 1970a, b; Salamon & Lightfoot 1970; Salamon 1971, 1972). In the latter two reports inositol-citrate thawing solution was used in preference to sodium citrate or glucose-citrate used previously for semen frozen in raffinose-citrate diluent. Visser & Salamon (1973, 1974) illustrated, however, that semen frozen in Tris-based media can be thawed successfully without using a thawing solution ("dry" thawing). Lambing results following the use of semen thawed in dry tubes or in a solution were indistinguishable (41,3 cf. 37,5% and 38,6 cf. 35,0% respectively in the two trials).

Thawing of the pellets in the cervix of the female ("cold insemination") has also been reported for the cow (Stoye, Mahler & Dieckmann, 1966; Merkt, Weitze & Brunkhorst, 1966), while Salamon & Lightfoot (1970) reported one lambing from 7 ewes inseminated in this way.

5. Insemination techniques

Two lines of approach are available in the insemination of ewes: Surgical insemination into the oviducts or uterus and normal insemination into the cervix. Bypassing of the cervix by surgery has been seen as a way of improving fertility with frozen ram semen and Salamon and Lightfoot (1967) argued that the principal failure of transport and survival of the spermatozoa occurred at this part of the female tract. Reports of high egg fertilization and lambing rates following surgical insemination into the oviducts (Salamon & Lightfoot, 1967: 57% ewes with fertilized eggs; Loginova & Zeltobrjuh, 1968: 69% fertilized eggs and 88% conception) or uterus (Salamon & Lightfoot 1967:88 to 93% ewes with fertilized eggs; Mattner, Entwistle & Martin, 1969: 88% ewes with fertilized eggs; Lightfoot & Salamon, 1970a: 67% ewes with fertilized eggs; 1970b: 40% lambing; Loginova & Zeltobrjuh, 1972a: 33 to 100% ewes with fertilized eggs; 1972b: 88% conception) should, however, be judged with care. First, the technique is not a practical one for large scale application and seems to offer little economy in the amount of semen required for insemination. Secondly, egg fertilization does not necessarily reflect lambing rate and high embryonic mortality has been reported by Salamon & Lightfoot (1967) and Mattner *et al.* (1969).

Cervical insemination in the ewe presents the problem of depth of insemination into the posterior part of the cervix. Several workers have attempted to perform insemination in the ewe as deep as possible into the cervix. Results were however contradictory. Lightfoot & Salamon (1970b) used "cervical traction" and found that deep cervical insemination (1 to 3 cm) reduced lambing to 30% as against 50% with normal cervical insemination. Similar results were also obtained by Salamon & Lightfoot (1970) and Andersen *et al.* (1973, depth of insemination 2,5 to 5,0 cm). Contrary to this, Ten & Bon (1965) and Kareta, Pilch & Wierzbowski (1972) found that lambing improved with deep cervical insemination (2 to 5 and >1,5 cm deep respectively).

It should be pointed out that part of the discrepancy in results can probably be explained by the fact that different degrees of depth have been used by the various workers. Differences in numbers of cervical papillae and consistency and abundance of cervical mucus may also have been contributing factors (Salamon & Lightfoot, 1970). Salamon

& Lightfoot (1970) further tested relaxin and oxytocin in an attempt to perform deeper insemination and to improve transport of the spermatozoa in the female tract. Results with both hormone treatments were negative and in fact decreased fertility.

Although no definite conclusions can be drawn on the minimum number of motile cells required in the inseminate, the literature in Table 1 indicates that satisfactory fertility can be obtained with 50 million up to 190 million motile spermatozoa. These numbers of cells were usually contained in small volumes (0,05 to 0,20 ml) and indeed Salamon & Lightfoot (1970) found a decrease in lambing when the inseminate volume was increased from 0,1 to 0,3 ml, both volumes containing 150 million motile cells. Salamon (1971) further stressed that a high concentration of motile cells together with careful insemination are necessary prerequisites for high fertility. Careful handling of ewes to reduce stress to a minimum, can also contribute to the establishment and retention of a sufficient cervical sperm population to ensure maximum chance of fertilization. Replenishment of this population by double insemination improved fertility results, as is indicated in a number of reports (Fraser, 1968; Salamon & Lightfoot, 1970; Samouilidis, 1970; Salamon, 1971). It was shown, however, by Salamon (1971, 1972) and Salamon and Lightfoot (1970) that the time of single and double inseminations in relation to the detection of oestrus had an effect on the lambing results. They found that the second insemination (usually 12 hours after the first) was most beneficial when the first was performed early in oestrus (1 to 3 hours after detection) and that little advantage can be gained if the first was performed at about the middle of oestrus (12 to 17 hours after detection). Visser & Salamon (1974) showed that the fertility improved with the increase of the total number of motile spermatozoa (90 to 360 x 10^6) deposited by either one or two inseminations 12 to 25 hours after the detection of oestrus. The question therefore arises as to whether a single insemination with a large number of motile cells (e.g. 360 x 10^6) will produce fertility results equivalent to two inseminations (each with 180 x 10^6 motile cells).

Finally, the high fertility obtained by Andersen *et al.* (1973) following "uterine" insemination by a non-surgical method, merits attention. The authors claimed that in 62% of 220 ewes it was possible to deposit the semen through the cervix into the uterus by means of traction of the cervix with a special "sponge holder" and manipulation of the inseminating pipette via the rectum of the ewe. Of 53 synchronised ewes 54% conceived, while conception rate in 83 naturally cycling ewes was 89%. Even though these results may be considered as very satisfactory, the practical application of this method remains to be proven, and more information is needed on the amount of time and labour involved with this technique.

6. Effect of long-term storage on fertility

Bull semen frozen-stored for several years maintains its fertilizing capacity, and satisfactory fertility has been obtained after storage for 3 years (Lindström, Holmström & Jokinen, 1972), 4 (Melrose), 1962) 5 to 6 (Cassou, 1972), 8 (Mixner & Wiggin, 1964) and 12 years (Mixner, 1968). Although a number of workers used ram semen after frozen storage for several weeks (e.g. Aamdal & Andersen, 1968; Volkov, 1968; Lightfoot & Salamon, 1970a, b; Salamon & Lightfoot, 1970; Salamon, 1971; Kareta, Pilch & Wierzbowski, 1972; Andersen, Aamdal & Fougner, 1973) the only report on semen stored for several years seems to be by Salamon (1972). Lambing after insemination with semen stored for three years was satisfactory (53%) and similar to results for semen of the same bank used after five to eight weeks of storage. Semen from this bank has subsequently been used by Salamon & Visser (1974) and lambing after five years storage was again 53%. It can therefore safely be assumed that the fertilizing capacity of ram semen stored in liquid nitrogen will not appreciably decrease with age, similar to results with frozen-stored bull semen.

Conclusions

From this review on the fertility of frozen ram semen, two important conclusions can be drawn. First, several combinations of freezing and thawing methods can result in moderate to satisfactory fertility. In any endeavour to determine suitable procedures it is, however, essential that interactions between these factors are recognised and the experiments should be planned in such a way as to be able to determine these or any other effects. Secondly, it is evident that apart from the freeze-thawing techniques and media used, the time and method of insemination have a significant effect on the fertility. It is most important to establish a sufficient cervical population of motile cells. Efficient restraint and the careful handling of ewes during and after insemination will help to retain this population and enhance the successful transport of spermatozoa to the site of fertilization.

References

AAMDAL, J. & ANDERSEN, K., 1968. Freezing of ram semen in straws. *Proc. VIth Int. Congr. Anim. Reprod.* 2, 977.

ANDERSEN, K. & AAMDAL, J., 1972. Artificial insemination with frozen semen in sheep in Norway. *Wld Rev. Anim. Prod.* 8, 77.

ANDERSEN, K., AAMDAL, J. & FOUGNER, J.A., 1973. Intra-uterine and deep cervical insemination with frozen semen in sheep. *Zuchthyg.* 8, 113.

BRANNY, J., PILCH, J. & WIERZBOWSKI, S., 1966. Low temperature freezing of ram semen. II. Diluent trials. *Med. Wet.* 22, 290.

BUREAUNU, A. & NEGOIȚA, V., 1971. Freezing ram semen in ampules to –196°C. *Anim. Breed. Abstr.* 40, 605.

CASSOU, R., 1972. Development of fertility rates in terms of age of semen. *Proc. VIIth int. Congr. Anim. Reprod.* 2, 1421.

COLAS, G., 1972. Ewe fertility following insemination with liquid or deep frozen semen in the course of oestrusses induced by progestins during the breeding season. *Proc. VIIth int. Congr. Anim. Reprod.* 2, 925.

COLAS, G. & BRICE, G., 1970. Fertility of fluorogestone acetate-treated ewes inseminated with frozen semen: Preliminary results. *Ann. Zootech.* 19, 353.

COLAS, G., BRICE, G., COUROT, M. & COTTIER, M., 1971. Artificial insemination in the plan of intensification of ovine production: Present state and prospectives. *Bull. Tech. Inform.* 257, 1.

DZUIK, P.J., LEWIS, J.M., GRAHAM, E.F. & MOYER, R.H., 1972. Comparison between natural service and artificial insemination with fresh or frozen semen at an appointed time in the ewe. *J. Anim. Sci.* 35, 572.

EMMENS, C.W., 1961. Dilution, transport and storage of ram semen. *Proc. Conf. School of Wool Tech., Univ. N.S.W., Austr.* p. 118.

EMMENS, C.W. & ROBINSON, T.J., 1962. Artificial insemination in the sheep. In: *The semen of animals and artificial insemination*, ed. J.P. Maule, Edinburgh: Comm. Bur. Anim. Breed. Gen.

ESSICH, G., 1966. *Zur Tiefkühlkonservierung von Bullensperma in Pillenform und kleine Kunststoffröhrchen.* Vet.-med. Diss. Tierärztl. Fak., Ludwig-Maximillians – Univ., München. (*Anim. Breed. Abstr.* 36, 341).

FRASER, A.F., 1968. Progress in the artificial insemination of sheep with frozen semen. *Proc. VIth int. Congr. Anim. Reprod.* 2, 1033.

HEYDORN, K.P. & PAUFLER, S., 1973. Untersuchungen über die Tiefgefrierung von Bullensperma in Pellets bei Verwendung verschiedenen Verdünnern und verschiedenen Auftaulösungen. *Dt. tierärztl. Wschr.* 80, 424.

IDRIS, O.E., 1971. Hannover Thawing Solution (HTS) – eine neue Auftaulösung, die eine mehrtägige Erhaltung der Befruchtungsfähigkeit von aufgetauten, pelletierten Bullensperma ermöglicht. Vorläufige Mitteilung. *Dt. tierärztl. Wschr.* 78, 413.

KALEV, G., ZAGORSKI, D., ZAHARIEV, Z., KITCHEV, G. & GEORGIEV., G., 1969. Insemination of ewes with deep frozen semen. *Veterinarnomed. Nauk.* 6, 13. (*Anim. Breed. Abstr.* 38, 1506).

KALEV, G., MARINOV, P., ZAGORSKI, D., KITCHEV, G., BAK'RDZHIEV, K. & ZHEKOV, G., 1970. Effect of some diluents on viability of deep-frozen ram spermatozoa. *Veterinarnomed. Nauk.* 7, 31. (*Anim. Breed. Abstr.* 40, 608).

KARETA, W., PILCH, J. & WIERZBOWSKI, S., 1971. The freezing of ram semen at low temperatures. III. The fertility of ewes after insemination with frozen semen. *Med. Wet.* 27, 734.

KARETA, W., PILCH, J. & WIERZBOWSKI, S., 1972. Fertility of frozen ram semen diluted in citrate added bull seminal plasma or not. *Proc. VIIth int. Congr. Anim. Reprod.* 2, 1479.

KRAUSE, D. & GROVE, D., 1967. Deep-freezing of jackass and stallion semen in concentrated pellet form. *J. Reprod. Fert.* 14, 139.

LEIDL, W., 1968. Neue Verfahren in der Künstlichen Besamung der Wirbeltiere. *Proc. VIth int. Congr. Anim. Reprod.* 2, 951.

LIGHTFOOT, R.J., 1969. *Studies of factors affecting the survival, physiological transport and fertility of ram spermatozoa after freezing by the pellet method.* Ph. D. (Agric.) thesis, University of Sydney.

LIGHTFOOT, R.J. & SALAMON, S., 1969a. Freezing ram spermatozoa by the pellet method. II. The effects of method of dilution, dilution rate, glycerol concentration, and duration of storage at 5°C prior to freezing on survival of spermatozoa. *Aust. J. biol. Sci.* 22, 1547.

LIGHTFOOT, R.J. & SALAMON, S., 1969b. Freezing ram spermatozoa by the pellet method. III. The effects of pellet volume, composition of the thawing solution, and reconcentration of the thawed semen on survival of spermatozoa. *Aust. J. biol. Sci.* 22, 1561.

LIGHTFOOT, R.J. & SALAMON, S., 1970a. Fertility of ram spermatozoa frozen by the pellet method. I. Transport and viability of spermatozoa within the genital tract of the ewe. *J. Reprod. Fert.* 22, 385.

LIGHTFOOT, R.J. & SALAMON, S., 1970b. Fertility of ram spermatozoa frozen by the pellet method. II. The effects of method of insemination on fertilization and embryonic mortality. *J. Reprod. Fert.* 22, 399.

LINDSTRÖM, U., HOLMSTRÖM, B.-G. & JOKINEN, L., 1972. Pellets: Experiences and results of storage over three years. *Proc. VIIth int. Congr. Anim. Reprod.* 2, 1427.

LINGE, F., 1972. Field trials with frozen semen. *Fårskötsel* 52, 12. (*Anim. Breed. Abstr.* 41, 1679).

LOGINOVA, N.V., & ZELTOBRJUH, N.A., 1968. Evaluation of various methods of freezing semen. *Ovtsevodstvo (Mosk.)* 14, 22. (*Anim. Breed. Abstr.* 37, 582).

LOGINOVA, N.V. & ZELTOBRJUH, N.A., 1972a. Viability of frozen ram semen in different parts of the genital tract. *Proc. VIIth int. Congr. Anim. Reprod.* 2, 1507.

LOGINOVA, N.V. & ZELTOBRJUH, N.A., 1972b. Survival rate and fertilizing ability of frozen ram semen. *Anim. Breed. Abstr.* 41, 1680.

LOPATKO, M.I., 1963. Laboratory and field tests on methods of freezing ram semen to –196°C. In: *Artificial insemination of farm animals.* Forest Steppe Inst. Anim. Breed., Ukrainian S.S.R. Kharkov Book Publisher.

LOPYRIN, A.I., 1969. Recurrent problems of artificial insemination of sheep. *Zhivotnovodstvo (Mosk.)* 31, 75.

LUNCA, N., 1968. Some aspects of freezing ram semen. *Proc. VIth int. Congr. Anim. Reprod.* 2, 1615.

MACKEPLADZE, I.B., GUGUŠVILI, K.F., BREGADZE, M.A. & HARATIŠVILI, G., 1960. Storage and use of frozen bull and ram semen. *Zhivotnovodstvo (Mosk.)* 22, 77.

MATTNER, P.E., ENTWISTLE, K.W. & MARTIN, I.C.A., 1969. Passage, survival, and fertility of deep-frozen ram semen in the genital tract of the ewe. *Aust. J. biol. Sci.* 22, 181.

MELROSE, D.R., 1962. Artificial insemination in cattle – Ultra-low temperature storage of semen. In: *The semen of animals and artificial insemination,* ed. J.P. Maule. Edinburgh: Comm. Bur. Anim. Breed. Gen.

MERKT, H., WEITZE, K.-F. & BRUNKHORST, F., 1966. Die Kaltbesamung mit Kapselpellets praxisreif. *Dt. tierärztl. Wschr.* 74, 505.

MERKT, H., WEITZE, K.-F. & LORRMANN, W., 1967. Semen pellets sealed in a capsule, a method of improving the pellet method in the deep-frozen storage of bull semen. *Dt. tierärzt. Wschr.* 73, 603.

MIXNER, J.P., 1968. Fertility of bull semen frozen for twelve years. *Proc. VIth int. Congr. Anim. Reprod.* 2, 1095.

MIXNER, J.P. & WIGGIN, S.H., 1964. The effects of aging on the motility and fertility of frozen bull semen. *Proc. Vth int. Congr. Anim. Reprod.* 4, 264.

NAGASE, H., & GRAHAM, E.F., 1964. Pelleted semen: Comparison of different extenders and processes on fertility of bovine spermatozoa. *Proc. Vth int. Congr. Anim. Reprod.* 4, 387.

NAUK, V.A., 1972. Experimental freezing of ram semen at low temperatures. *Nauk V.I. Lenina,* 1972 (1), 40, 47. (*Anim. Breed. Abstr.* 40, 3295).

PEXTON, J.E. & BOTKIN, M.P., 1971. Fertility of frozen and fresh extended ram semen. *J. Anim. Sci.* 33, 264.

PLATOV, E.M., 1968. The effect of freezing on the content of adenosine-polyphosphates in semen. *Ovtsevodstvo (Mosk.)* 14, 14.

REMES, E., 1972. The artificial insemination of sheep in Finland. *Wld Rev. Anim. Prod.* 8, 73.

SADLER, R.M.F.S., 1966. The preservation of mammalian spermatozôa by freezing. *Lab. Pract.* 15, 413.

SALAMON, S., 1967. Observations on fertility of ram semen frozen by different methods. *Aust. J. exp. Agric. Anim. Husb.* 7, 559.

SALAMON, S., 1968. Deep freezing of ram semen: Recovery of spermatozoa after pelleting and comparison with other methods. *Aust. J. biol. Sci.* 21, 351.

SALAMON, S., 1971. Fertility of ram spermatozoa following pellet freezing at –79 and –140°C. *Aust. J. biol. Sci.* 24, 183.

SALAMON, S., 1972. Fertility of ram spermatozoa frozen-stored for three years. *Proc. VIIth int. Congr. Anim. Reprod.* 2, 1493.

SALAMON, S., 1973. Deep freezing of boar semen. III. Effects of centrifugation, diluent and dilution rate, pellet volume and method of thawing on survival of spermatozoa. *Aust. J. biol. Sci.* 26, 239.

SALAMON, S. & BRANDON, M.R., 1971. Effect of composition of thawing solution on survival of ram spermatozoa frozen by the pellet method. *Aust. J. biol. Sci.* 24, 355.

SALAMON, S. & LIGHTFOOT, R.J., 1967. Fertilization and embryonic loss in sheep after insemination with deep frozen semen. *Nature, London,* 216, 194.

SALAMON, S. & LIGHTFOOT, R.J., 1969. Freezing ram spermatozoa by the pellet method. I. The effect of diluent composition on survival of spermatozoa. *Aust. J. biol. Sci.* 22, 1527.

SALAMON, S. & LIGHTFOOT, R.J., 1970. Fertility of ram spermatozoa frozen by the pellet method. III. The effects of insemination technique, oxytocin and relaxin on lambing. *J. Reprod. Fert.* 22, 409.

SALAMON, S. & VISSER, D., 1972. Effect of composition of Tris-based diluent and of thawing solution on survival of ram spermatozoa frozen by the pellet method. *Aust. J. biol. Sci.* 25, 605.

SALAMON, S. & VISSER, D., 1974. Fertility of ram spermatozoa frozen-stored for 5 years. *J. Reprod. Fert.* 37, 433.

SAMOUILIDIS, S., 1970. *Ein Beitrag zur Tiefkühlkonservierung von Schaf- und Ziegenbocksamen mit Hilfe des Pellet- bzw. Paillette-verfahrens.* Vet.-med. Diss., Tierärztl. Fak., Ludwig-Maximillians-Univ., München. (*Anim. Breed. Abstr.* 39, 1408).

STOYE, H., MAHLER, R. & DIECKMANN, W., 1966. Versuche zur Verlegung des Auftauvorganges von Tiefgefriersperma ins weibliche Genitale beim Rind. *Zuchthyg.* 1, 2.

TEN EN BON., 1965. Physiological and biochemical characters of semen should be considered in insemination with frozen semen. *Ovtsevodstvo (Mosk.)* 11, 11.

VISSER, D. & SALAMON, S., 1973. Fertility of ram spermatozoa frozen in a Tris-based diluent. *Aust. J. biol. Sci.* 26, 513.

VISSER, D. & SALAMON, S., 1974. Fertility following insemination with frozen-thawed reconcentrated and unconcentrated ram semen. *Aust. J. biol. Sci.* In press.

VOLKOV, A.S., 1968. Conception in ewes inseminated with deep frozen semen. *Anim. Breed. Abstr.* 37, 593.