EFFECT OF HETEROLOGOUS SEMINAL PLASMA AND SEMEN EXTENDERS ON MOTILITY OF FROZEN-THAWED RAM SPERM

BY

Gracinda André Mataveia

Submitted in partial fulfilment of the requirements for the degree of Master of Science in the Department of Production Animal Studies in the Faculty of Veterinary Science, University of Pretoria

Date submitted: September 2006

Acknowledgements

The author wishes to acknowledge with sincere appreciation Professor S.J. Terblanche, Professor J.O. Nöthling and Professor D. Gerber for their efficient guidance, patience and encouragement during the realization of this thesis.

My sincere gratitude also goes to Ms Annete v Veenhuizen who guided me through the technique of semen freezing, Mrs Anne-Marie Human and Mr. Abram M. Komane who made sure that all necessary materials where available in time, Ms Ilse M. Luther for her patience during the preparation of the randomly numbered cover slips for the motility evaluation of frozen-thawed semen, Mr Samuel Motaung who took good care of the animals and gave valuable assistance during semen collection, Ms Ester Botha with her special contribution on particular aspects of concentration determination.

I am also indebted to Mrs Ellen Nel, Departmental Administrator for the Section of Reproduction in Production Animal Studies who with her motherly skills, took good care of all aspects of daily life in the Section of Reproduction.

For all the friendly people at the Faculty of Veterinary Science, who made me feel at home during my stay in Onderstpoort, a grateful acknowledgement.

To my family for the patience and comprehension during the moments of absence, especially my husband who always found a special word to encourage me throughout this odyssey.

Dedication

To my father who always believed in his young girl but could not enjoy this important moment of his daughter's life.

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Summary

EFFECT OF HETEROLOGOUS SEMINAL PLASMA AND SEMEN EXTENDERS ON MOTILITY OF FROZEN-THAWED RAM SPERM

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Gracinda André Mataveia

Promoter: Prof. S.J. Terblanche

Co-Promoters: Prof. J.O. Nöthling

Prof. D. Gerber

Degree: MSc

Frozen-thawed ram semen crosses the cervix poorly, necessitating laparoscopic insemination. Acceptable fertility can be achieved with frozen-thawed ram semen deposited at the external cervical opening if ram seminal plasma is added. Homologous seminal plasma improves the fertility of frozen-thawed sperm of boars and dogs. Heterologous seminal plasma may have effects as well; the addition of bovine seminal plasma increases the ability of buffalo sperm (*Syncerus caffer*) to fertilize bovine oocytes in vitro.

The aim of the current study was to compare the effects of seminal plasma of rams and bulls, dog prostatic fluid, protein-free TALP, TrilEq (Triladyl with 0.5 ml of Equex STM paste added to each 100 ml) and skim milk upon longevity and percentages of progressively and aberrantly motile frozen-thawed ram sperm.

Three ejaculates from each of 6 rams (2 Dorpers, 2 Döhne merinos, and 2 merinos), aged 2 to 4 years, were extended in TrilEq, pooled and frozen as a single batch per ram at 200 × 10^6/ml in 0.25 ml straws. Seminal plasma of rams was obtained from the same rams, while seminal plasma of five bulls were obtained by centrifugation of their ejaculates and dog prostatic fluid consisted of the post-sperm fractions of the ejaculates of 5 dogs. Within a

species, the seminal plasma or prostatic fluid from different donors was pooled and frozen in aliquots at −18 °C. The 108 straws (6 rams, 6 diluents, 3 replicates) were thawed in random order. Once thawed, a straw was emptied into a tube with 0.85 ml of the appropriate medium at 37 °C and kept at that temperature for 6 h. The percentage of progressively motile sperm was estimated at ×200 magnification immediately (time zero) and 2, 4 and 6 h after thawing. One person thawed the semen and prepared motility specimens, while another performed all motility evaluations. Data were evaluated by means of repeated-measures ANOVA, with rams as subjects and time and medium as fixed effects. Non-significant interactions were removed from the model. Pairwise comparison of means was done by means of Bonferroni's test ($P < 0.05$). The model included Ram, Time, Medium, and Ram × Medium, and Time × Medium interactions, which were all significant ($P < 0.01$).

Mean progressive motility decreased from each time to the next and were 39.0% (0 h), 26.0% (2 h), 19.6% (4 h) and 12.6% (6 h); SEM 1.38%, n = 108. Mean motility was higher for skim milk (39.9%) than for all other media except TrilEq (27.7%), which was better than bull seminal plasma (13.0%), whereas TALP (20.5%) and ram seminal plasma (21.9%) were similar to TrilEq and bull seminal plasma (SEM 2.85%, n = 72). The interactions (Ram × Medium or Time × Medium) were mainly due to dog prostatic fluid, ram seminal plasma, TrilEq, and TALP, while milk resulted in the best and bull seminal plasma in the lowest motility.

This study shows that heat-treated skim milk maintains progressive motility of frozen-thawed ram sperm better than dog prostatic fluid and seminal plasma of bulls and rams, TrilEq and protein-free TALP. In contrast to ram seminal plasma, skim milk is known to result in poor fertility of frozen-thawed ram semen after cervical insemination. It would thus appear that maintenance of progressive motility in vitro may be a poor indicator of fertility after cervical insemination.

Sumário

EFEITO DE PLASMAS SEMINAIS HETEROLOGOS E DILUENTES DO SÉMEN NA MOTILIDADE DOS ESPERMATOZÓIDES DESCONGELADOS DE CARNEIRO

POR

Gracinda André Mataveia

Supervisor: Prof. S.J. Terblanche

Co-Supervisores: Prof. J.O. Nöthling

Prof. D. Gerber

Grau académico: MSc

O semen descongelado de carneiros atravessa com dificuldade o cérvix, necessitando por isso de inseminação por meio da laparoscopia. Pode-se registar uma fertilidade aceitável com o sémen de carneiro se este for depositado na abertura externa do cérvix e quando for adicionado plasma seminal de carneiro ao sémen. Plasmas seminais homólogos melhoram a fertilidade dos espermatozoides descongelado de porcos e de cães. Os plasmas seminais não-homólogos também têm um bom efeito; a adição de plasma seminal de touros aumenta a capacidade do espermatozoides do búfalo (*Syncerus caffer*) fertilizar os oócitos bovinos in vitro.

O objectivo deste estudo era o de comparar os efeitos do plasma seminal de carneiros e bovinos, fluido prostático de cães, TALP sem proteina, TrilEq (Triladyl com 0.5 ml de pasta Equex STM adicionados a cada 100 ml) e leite desnatado na longevidade e percentagens de espermatozoides de carneiro descongelado com motilidade progressiva e aberrante.

Três ejaculados de cada um dos 6 carneiros (2 Dorpers, 2 Döhne Merinos e 2 Merinos), com idades entre os 2 e os 4 anos, foram diluídos em TrilEq, misturados e congelados como uma amostra única por carneiro, em palhetas

de 0.25 ml e com concentrações da ordem dos $200x10^6$ espermatózoides/ml. O plasma seminal de carneiros foi obtido dos mesmos carneiros, enquanto que o plasma seminal do touro foi obtido de 5 touros após centrifugação dos seus ejaculados e o fluído prostático do cão consistiu da última fracção de ejaculados de 5 cães. O plasma seminal ou fluído prostático de cada uma das diferentes espécies foi misturado e congelado por partes à -18 °C.

As 108 palhetas (6 carneiros, 6 diluentes, 3 réplicas) foram descongeladas de forma aleatória. Uma vez descongeladas, o seu contéudo foi vertido para dentro de um tubo com 0.85 ml do meio apropriado a 37 °C e mantido a esta temperatura durante 6 horas. A percentagem de espermatozoides com motilidade progressiva era estimada com uma ampliação de x200 imediatamente (tempo zero), e 2, 4 e 6 horas após o descongelamento. Uma pessoa descongelava o sémen e preparava as amostras, enquanto que uma outra realizava todas as avaliações da motilidade. Os dados foram analizados usando a ANOVA, com os carneiros como sujeitos e o tempo e o meio como efeitos fixos. As interacções não-significantes foram removidas do modelo. A comparação par-a-par das médias foi feita por meio do teste de Bonferroni ($P < 0.05$). O modelo incluiu as interacções entre Carneiro, Tempo, Meio, e Carneiro x Meio e Tempo x Meio, que foram todas significativas ($P < 0.01$).

A motilidade progressiva média decresceu de cada tempo para o seguinte e perfazia 39.0% (0 h), 26.0% (2 h), 19.6% (4 h) e 12.6% (6 h); desvio padrão 1.38%, n = 108. A motilidade média foi mais alta para o leite desnatado (39.9%) do que para os outros meios excepto o TrilEq (27.7%), que foi melhor que o plasma seminal do touro (13.0%), enquanto que o TALP (20.5%) e o plasma seminal do carneiro (21.9%) foram similares ao TrilEq e ao plasma seminal do touro (n = 72, desvio padrão 2.85%). As interacções (carneiro × meio e tempo × meio) foram atríbuidas ao fluído prostático do cão, plasma seminal do carneiro, TrilEq e TALP, enquanto que o leite resultava na melhor e o plasma seminal do touro na mais baixa motilidade.

Este estudo mostra que o leite desnatado mantém a motilidade progressiva do sémen de carneiro descongelado melhor que o fluído prostático do cão e o

plasma seminal do touro e do carneiro, TrilEq e TALP sem proteina. Contrariamente ao plasma seminal de carneiro, o leite desnatado é conhecido por resultar numa baixa fertilidade do sémen de carneiro descongelado quando se usa a inseminação cervical. Deste modo poderia parecer que a manutenção da motilidade progressiva in vitro pode ser um indicador não apropriado da fertilidade após a inseminação cervical.

1 INTRODUCTION

Artificial Insemination (AI) in sheep has been widely used in commercial breeding programs around the world with both, fresh and frozen-thawed semen. According to Salamon and Maxwell (2000) research in the field of artificial insemination in sheep started at the beginning of the twentieth century. It led to the development of methods to use artificial insemination as a routine in sheep farming. After the First World War, a team headed by Milovanov implemented AI programs in sheep breeding operations on a large scale in the former Soviet Union (Salamon and Maxwell, 2000).
The need to use frozen-thawed semen that can be stored over extended periods of time and the demand for semen on farms located far from artificial insemination centres where rams were living stimulated research in semen cryopreservation and storage.

Cryopreservation of semen allows preserving specific genetic traits. However, freezing and thawing sperm has a detrimental effect on some sperm characteristics such as motility and membrane stability (Gillan et al., 2004). Damage of membranes occurs during the freezing and thawing process, but not during storage. In the case of ram spermatozoa, most damage occurs between –10°C and -25°C (Byrne et al., 2000; Salamon and Maxwell, 1995).

Sperm damage occurring during cryopreservation leads to low fertility rates due to inadequate transport and reduced viability of frozen-thawed semen in the reproductive tract of ewes (Byrne et al., 2000; Eppleston et al., 1994; Gillan et al., 2004; McPhie et al., 2000; Salamon and Maxwell, 1995).

The addition of seminal plasma, skim milk, reconstituted milk or synthetic media to frozen-thawed ram sperm increases their motility and ability to move through the reproductive tract of ewes, and resulted in increased fertility (Gillan et al., 2004; McPhie et al., 2000; Maxwell et al., 1999, Salamon and Maxwell, 2000).

The objective of the present study was to compare the effect of different media on the progressive and aberrant motility of frozen-thawed ovine

sperm. Media used in this trial have shown some beneficial effect on sperm progressive motility, *in vitro* viability or fertility in at least one species. Although several studies describe the effect of seminal plasma (Gerber et al., 2003; Günzel-Apel and Ekrod, 1991; Maxwell et al., 1999 and McPhie et al., 2000) and other media (Nöthling et al., 2005 and Sirivaidyapong et al., 2000) on the progressive motility of frozen-thawed spermatozoa, to our knowledge there is no report comparing the effect of different media on frozen-thawed ram spermatozoa.

1.1 Hypotheses

The addition of heat-treated skim milk, protein-free TALP, dog prostatic fluid and seminal plasma from rams and bulls to frozen-thawed ram spermatozoa alter the percentages of progressive and aberrantly motile sperm (Motility) and the rate at which Motility changes over time (Rate), in comparison with frozen-thawed semen to which only freezing extender is added ($P<0.05$).

The effects of heat-treated skim milk, protein-free TALP, dog prostatic fluid and seminal plasma from rams and bulls on Motility and Rate at various times after thawing are statistically different ($P<0.05$).

2 LITERATURE REVIEW

The necessity to use rams over extended periods of time, or at different times of the year, stimulated research on storage of spermatozoa under artificial conditions. This could be achieved by methods that reduced or arrested the metabolism of spermatozoa and thereby prolonged their fertile life (Salamon and Maxwell, 2000).

According to Salamon and Maxwell (2000) there are many reports on the fertility of liquid stored semen after cervical insemination, mainly from the former Soviet Union. After the First World War, under the leadership of Milovanov, artificial insemination with fresh and diluted semen was used on a large scale in sheep breeding programmes in the Soviet Union to fertilise a large number of ewes with semen of outstanding rams. This procedure required transport of the semen from the points or centres of collection to sites of insemination at more distant farms.

Salamon and Maxwell (2000), however, report that fertility declines rapidly when semen stored in a liquid state (at temperatures of 0-5 °C, 10-15 °C or ambient temperature) for more than 24 h was used for cervical insemination. The decrease in fertility was at a rate of 10-35% per day of storage. Thus, while 68 – 75% of ewes lambed from insemination with fresh semen in single cycle, the lambing rates for semen stored for 24, 48 and 72 hours were 45-50%, 25-30% and 15-20% respectively. According to Pérez et al. (1996) and Valcárcel et al. (1996 and 1997), the main changes that occurred during storage included reduction in motility and damage to plasma/acrosomal membranes of the spermatozoa. The effects described above are accompanied by a decline in transport and survival of spermatozoa in the female reproductive tract and reduction in fertility.

A major obstacle to the exploitation of frozen semen is that freezing and thawing of spermatozoa of any species generally leads to decrease in the percentage of motile cells post-thawing. This decrease is described as a result of damage to the membrane structures (Quinn et al., 1969; Nath, 1972, cited by Valcárcel et.al., 1996) or a deficient sperm transport

associated with a decreased respiratory activity (Gillan and Maxwell, 1999 cited by Maxwell et al., 1999) or a disruption of the axonemal elements of the tail, which may not only reduce the number of motile spermatozoa but also affect the type of motility (Watson, 1995 cited by Gillan et.al. 2004). In case of ram spermatozoa, most damage occurs between –10ºC and -25ºC. (Byrne et al., 2000; Salamon and Maxwell, 1995). Valcárcel et al. (1994, cited by Valcárcel et al., 1997) demonstrated that at least 30% of those spermatozoa that remained motile after freezing and thawing had damage to the plasma membrane. According to them those spermatozoa rapidly lose their motility at 37˚C and would therefore be non-functional in vivo.

Unlike cervical artificial insemination with fresh and chilled semen, which has been used successfully in sheep breeding programmes around the world for a number of decades, the use of frozen-thawed semen with cervical artificial insemination has been of limited value in commercial programmes due to low lambing rates, between 25 and 45% (Olesen, 1993; Evans and Maxwell, 1987). Olesen (1993) and Salamon and Maxwell (2000) associate this low fertility with a reduction in number of viable spermatozoa reaching the site of fertilization. In support of this view is the fact that intrauterine insemination by laparoscopy with frozen-thawed semen can be as effective as cervical insemination with fresh semen (Eppleston et al., 1994). It seems that the cervix constitutes the initial barrier to the ascent of frozen -thawed spermatozoa in the ewe (Salamon and Maxwell, 2000; Wulster- Radcliffe and Lewis, 2002; Gil et al., 2002). The cervix of many species, including the ewe, acts not only as reservoir but also as a barrier to spermatozoa, particularly those with impaired function (Maxwell et al., 1999).

The problem of low fertility after cervical insemination with frozen-thawed ram semen could be due to a variety of factors but remains unresolved. In a review paper, Salamon and Maxwell (1995) report that inadequate transport and reduced viability of frozen-thawed spermatozoa in the genital tract of the ewe are the major causes of low fertility after cervical insemination. These authors report a high egg fertilisation rates (85-95%) when thawed semen was deposited into the uterus or oviducts, concluding that the presumably

morphologically normal spermatozoa maintained their fertilising capacity after freeze thawing, provided that cervical transport is not required.

Different techniques and devices have been used to deposit thawed semen deep into cervical canal of the ewe. The cervical traction method (which consisted of pulling the entrance of the cervix into the vagina by forceps) and the cervical traction combined with digital manipulation of the cervix through the rectum (Andersen et al., 1973) allowed semen deposition to depth of 2-5 cm into the cervical canal. Both methods improved the fertility, but were stressful to the animal.

The problem of the cervical barrier in the ewe can be overcome by deposition of frozen-thawed semen into the uterus via the cervix (transcervical insemination) or directly into the uterus by laparoscopy. The transcervical insemination methods are of varying repeatability in penetrating the cervix, are time consuming and rather stressful to the animal. In an experiment conducted in Australia the pregnancy rate at day 70 for ewes inseminated by laparoscopy (48%) was higher than for ewes inseminated by transcervical intrauterine (32%) or cervical (9%) methods (Windsor et al., 1994). Wulster-Radcliffe et al. (2002) described trans-cervical insemination as costly, time consuming, requiring technical proficiency, limiting the number of times a ewe can be used and requiring anesthetic. Laparoscopic intrauterine insemination is the most effective method for the use of frozen-thawed semen in ewes, but requires expertise and is relatively costly (Salamon and Maxwell, 2000). Since it was demonstrated that cervical insemination with frozen-thawed ram sperm was inefficient (Salamon and Lightfoot, 1967, cited by Maxwell et al., 1999), intrauterine insemination has been the only way to obtain consistently high levels of fertility (Salamon and Maxwell, 1995).

A recent study (Maxwell et al., 1999) indicates that frozen-thawed spermatozoa supplemented with ram seminal plasma may yield acceptable fertility after cervical insemination.

In a review paper Katila (2001) summarises the work of Overstreet and Tom (1982) and that of Einarsson and Viring (1973) concluding that seminal plasma has a role in facilitating transport of sperm and/or in protecting it in the genital tract and thereby extending their survival time. Human seminal plasma is, among others, rich in prostaglandins (Harper, 1988; Allsopp et al., 1997) and oxytocin (Katila, 2001). These two hormones are potent stimulators of uterine contractions, which have a positive effect on sperm transport to the ampulla of the uterine tubes where fertilization takes place.

Freezing and thawing brings about changes in ram spermatozoa which reduce their ability to penetrate the cervix, resulting in unacceptably low conception rates when ewes are inseminated into the vagina or the cervix itself. Recent work has shown that freezing and thawing processes bring about capacitation-like changes to spermatozoa (Gillan and Maxwell, 1999), perhaps because seminal plasma, which is believed to contain decapacitation factors, is removed during preparation of spermatozoa for freezing. In the presence of seminal plasma, motility of frozen-thawed spermatozoa was better and there were more uncapacitated and less acrosome-reacted cells in comparison with controls.

Frozen-thawed ram spermatozoa were also better able to penetrate cervical mucus after addition of seminal plasma. Addition of seminal plasma has a beneficial effect on membrane stabilization (Gillan and Maxwell, 1999 cited by McPhie et al., 2000). The frozen-thawed ram spermatozoa in the presence of seminal plasma had increased motility, an increased ability to penetrate the cervical mucus in vitro and increased fertility after cervical AI (Gillan et al., 2004). Plasma addition increased the percentage of ewes pregnant after insemination of frozen-thawed but not fresh spermatozoa (Maxwell et al., 1999). There are indications that seminal plasma of species other than sheep affects fertility in general, and fertility of frozen-thawed sperm deposited into the vagina. Insemination results in swine largely depend on the number of spermatozoa inseminated, sperm transport in the genital tract of the sow and the period of time between insemination and ovulation (Weitze et al., 1990). The fertilization rates and sperm transport can both be increased by

increasing the insemination dose as well as additional seminal plasma pre-treatment (Olar et al., 1989; Weitze et.al., 1990). It was shown that the lower the sperm dose, the more effective was the additional seminal plasma pre-treatment. Concannon and Baptista (1989) reported that vaginal deposition of frozen-thawed dog semen resulted usually in poor fertility. Nöthling and Volkmann (1993) found that the addition of autologous prostatic fluid to frozen thawed dog spermatozoa significantly improved the litter size, the ratio between the number of conceptuses and the number of corpora lutea and pregnancy rate in bitches after intravaginal insemination. Dog prostatic fluid decreases motility and viability of spermatozoa when added to fresh semen prior to incubation, prior to cooling, or prior to extension and freezing (Sirivaidyapong, 2001; England and Allen, 1992; Günzel-Apel and Ekrod, 1991). In their study England and Allen (1992) report an increase in the number of morphologically abnormal spermatozoa after dilution of specimens with prostatic fluid. Sirivaidyapong (2001) recommends a centrifugation of semen before cryopreservation to reduce dog prostatic fluid and its effect on motility and viability.

TALP contains albumin, which stimulates capacitation of sperm in various species (Ellington et al., 1999; Stewart-Savage, 1993; Sirivaidyapong et al., 2000). This effect has been reported for species like for example the hamster (Stewart-Savage, 1993) and the cat (Andrews et al., 1992) and is strongly influenced by the Ca^{2+} ions present in the medium (Sirivaidyapong et al., 2000). On the other hand, albumin induces the acrosome reaction in dogs (Sirivaidyapong et al., 2000) and horses (Ellington et al., 1999). TALP has also been used as Albumin-free TALP (afTALP) with a comparable effect as that of TALP containing albumin (Nöthling et al., 2005). These findings may be used to support the results described by Sirivaidyapong et al. (2000), who associate the induction of the acrosome reaction with the presence of Ca^{2+} ions in the medium.

TALP is normally used in forms which differ in composition. These differences result from differences in the contents of some basic components

or on the presence or absence of some supplements added to the basic composition (Andrews and Bavister, 1989; Ying et al., 1999).

A milk-based extender with 5% egg yolk has traditionally been used for freezing ram semen in Norway and Sweden and protects such spermatozoa well (Gil et al., 2003). Whole, skimmed or reconstituted milk have also been used for many years as extenders for ram semen (Salamon and Maxwell, 2000). The success of these diluents has been attributed to their protein fraction, which may buffer against changes in pH and chelate any heavy metals present and partially protect spermatozoa during cooling for storage.

Before dilution of semen, the whole, skim or reconstituted milk should be heated at 92-95°C for 8-10 minutes to inactivate the lactenins in the protein fraction which is toxic to spermatozoa. Some investigators found that skim milk was better than whole milk for storage of semen at 2-5°C (Salamon and Maxwell, 2000). When combined with antibiotics, skim milk was as effective as egg yolk-glucose-citrate diluent for chilled storage of ram semen. Addition of 5% egg yolk and 15% glucose to the skim milk was claimed to improve the viability of spermatozoa during chilled storage (Feredean et al., 1967, cited by Salamon and Maxwell, 2000).

Gerber et al. (2003) showed that the addition of seminal plasma from domestic bulls enhances the ability of frozen-thawed spermatozoa from African buffalo (Syncerus caffer) to fertilize bovine oocytes in vitro. It therefore seems justified to evaluate the effect of heterologous seminal plasma first on the in vitro viability and later on the fertility of frozen-thawed ram sperm.

The aim of this study was to compare the effects of heat-treated skim milk, protein-free TALP dog prostatic fluid and seminal plasma from rams and bulls on the motility of frozen-thawed ram sperm.

3 MATERIALS AND METHODS

3.1 Experimental animals

Two Dorper, 2 Merino and 2 Dohne Merino rams, 2 to 4 years of age, were used. Their semen quality was evaluated before commencement of the trial and they were trained to serve into an artificial vagina while mounting a non-oestrous ewe held in a neck clamp (Evans and Maxwell, 1987). The rams were moved to Onderstepoort in March 2004 and the trial commenced in April. The last ejaculates were collected in May 2004, which coincides with the optimum breeding season for sheep in South Africa.

3.2 Location and husbandry

The rams were housed in open-roofed pens with cement floors at the Section of Reproduction, Department of Production Animal Studies, Faculty of Veterinary Science.

Animals were clinically healthy, and vaccinated with *Clostriduim perfringens* Type D alum-precipitated vaccine.

Daily care and feeding of the animals was the responsibility of the Onderstepoort Animal Teaching Unit (OATU). Each day the rams each received 0.3 kg of commercial sheep pellets and Lucerne hay and water *ad libitum*.

3.3 Experimental design

A repeated measures block factorial design with the 6 rams as subjects, nested in breed, was used. The 2 within factor variables were Medium (6 levels) and Time after thawing (4 levels). The 6 levels for Medium were TrilEq, heat-treated skim milk, protein-free TALP, ram seminal plasma, bull seminal plasma and dog prostatic fluid. The four levels for Time were 0, 2, 4, and 6 h

(t_0, t_2, t_4 and t_6) after thawing. All interactions between Ram, Medium and Time were evaluated.

For each ram x medium combination 3 straws were thawed and evaluated separately at each level of Time; i.e. 18 straws from each ram were thawed for and each evaluated at t_0, t_2, t_4 and t_6.

The percentages of progressively and aberrantly motile spermatozoa were evaluated as the response variables.

3.4 Collection, preparation and storage of media

3.4.1 TrilEq

TrilEq was used as freezing extender and it was also added as control medium after thawing of the semen. It was prepared in advance and stored at -70 °C until shortly before use. TrilEq consisted of 100 ml of fully constituted Triladyl™ (Triladyl™, Minitüb, Tiefenbach, Germany), plus 0.5 ml Equex STM paste (Nova Chemical Sales, Scituate, MA, USA).

The composition of Triladyl™ is a trade secret; but it is a Tris-based extender. According to the label Triladyl™ contains glycerol, tris, citric acid, fructose, tylosin, gentamicin, lincomycin and spectinomycin. To make up Triladyl™ one volume of the Triladyl™ concentrate was extended with three volumes of deionised water and one volume of egg yolk. Finally, to each 100 ml of extender, 0.5 ml of Equex STM paste was added to give 100.5 ml of TrilEq, which was used in the study.

3.4.2 Seminal plasma from rams and bulls

Ejaculates were collected from rams and bulls using an artificial vagina. Nine ejaculates collected from the rams used for the trial were pooled and centrifuged at 3500 g for 30 minutes. The supernatant was filtered through a 0.22 µm filter. Complete absence of sperm was confirmed under a phase

contrast microscope at a x 200 magnification. Aliquots of 0.85 ml of the filtered seminal plasma were transferred into 20 Cryo-tubes (NUNC, Denmark) and stored at –70 °C.

The seminal plasma from 8 bulls (2 Holsteins, 2 Ayrshires, 2 Tulis and 2 Brahmans) belonging to the Taurus Artificial Insemination Centre was pooled and treated in a similar manner as described for the ram seminal plasma.

3.4.3 Dog prostatic fluid

Ejaculates from 6 dogs were collected by means of digital massage, pooled and treated as describe for the seminal plasma of rams and bulls, except that the centrifugation time was reduced to 15 minutes.

3.4.4 Heat-treated skim milk

Ultra High Temperature (**UHT**) Skim Milk from Clover (Clover S.A. (Pty) Ltd., www.clover.co.za) was used. The analysis per litre of UHT skim milk was as follows: 4.9 g carbohydrates, 3.4 g protein, 0.4 g milk fat and 123 mg calcium.

3.4.5 Protein-free TALP

The composition of Protein-free TALP used was: 113.96mmol/l NaCl, 3.19 mmol/l KCl, 24.88 mmol/l $NaHCO_3$, 0.40 mmol/l NaH_2PO_4 monohydrate, 0.49 mmol/l $MgCl_2$ hexahydrate, 2.00 mmol/l $CaCl_2$ dihydrate, 10.07 mmol/l HEPES, 0.01 mmol/l Phenol red, 0.50 mmol/l sodium pyruvate, 15.78 mmol/l sodium lactate, 0.025 g/l gentamycin (Nöthling et al. 2000 and Ying et al., 1999).

3.5 Collection of ejaculates used for freezing

Rams were allowed to ejaculate once a day over three successive days; these ejaculates were discarded. The rams were then rested for three days before three ejaculates were collected from each ram by means of an artificial vagina with 60-minute intervals. These three ejaculates were extended, pooled after cooling to 5 °C and frozen as one batch, as described in Section 3.9. The sperm concentration was determined using a haemocytometer (compare section 3.8 below).

3.6 Evaluation of fresh ejaculates

Fresh ejaculates were evaluated macroscopically and microscopically immediately after collection (Nöthling, 2000). Macroscopically the following characteristics were evaluated: pH, volume, colour, and consistency. Microscopically the mass motility and individual motility were determined. Individual motility was expressed as the percentages of progressively motile, aberrantly motile and non-motile spermatozoa.

The pH was measured with an indicator paper (pH range 5 to 10). The volume of the ejaculate was measured directly on the graduated tube into which it was collected. Colour and consistency were subjectively assessed.

Determination of the mass motility was based on the wave motion characteristics of semen in a hanging drop placed in the centre of a cover slip. Coverslip and microscope stage were prewarmed at 37 °C (Nöthling, 2000). The mass motility was scored in a 6-point scale ranging from 0 to 5 (Nöthling, 2000). Only specimens with a mass motility score of 4 or higher were used for the trial.

Individual sperm motility was estimated in a specimen on a warm stage (37 °C) under a phase-contrast microscope, using x200 magnification. A drop of semen was placed on a glass slide, which was covered with a coverslip. After placing the slide on the microscope the percentages of

progressively motile, aberrantly motile and immotile sperm in 10 selected fields (starting at the edge and ending at the centre of the coverslip) was estimated (Nöthling, 2000). From the results obtained average values were calculated. The individual sperm motility is expressed in terms of percentages of progressively motile, aberrantly motile and immotile spermatozoa. This method was used for fresh and frozen-thawed specimens as well as during post-thaw incubation.

3.7 Sperm morphology

Morphology of fresh and of frozen-thawed spermatozoa was evaluated on Eosin-Nigrosin smears (Barth and Oko, 1989 and Nöthling, 2000). This comprehensive evaluation was used to determine the percentages of normal and abnormal sperm.

3.8 Determination of sperm concentration

Sperm concentration (defined as the number of spermatozoa per ml) was determined on pooled ejaculates, using a haemocytometer (Chemineau and Cagnié, 1991; Daza, J.F., 1994; Evans and Maxwell, 1987) and adjusted with freezing extender to approximately 200 x 10^6/ml.

3.9 Processing and storage of semen

On the day of semen collection, the mass motility was determined and ejaculates with a mass motility of 4 or higher were extended with two equal volumes of TrilEq, which had been kept at 30 °C, subsequent to which the 14 ml centrifugation tube with the extended semen was placed in a 50 ml tube with water at room temperature that served as a water jacket. This 50 ml tube was then placed in a beaker with a capacity of 250 ml that was filled with water at 15 °C and placed in a cool room at 5 °C. With this

method semen reaches a temperature close to 5 °C after 150 minutes (Figure 3.1). Another 2 ejaculates were collected with 60-minute intervals and processed the same way as the first one. Based on the cooling rate (Figure 3.1) the 3 ejaculates were pooled 2½ h after the third ejaculate had been placed in the cool room.

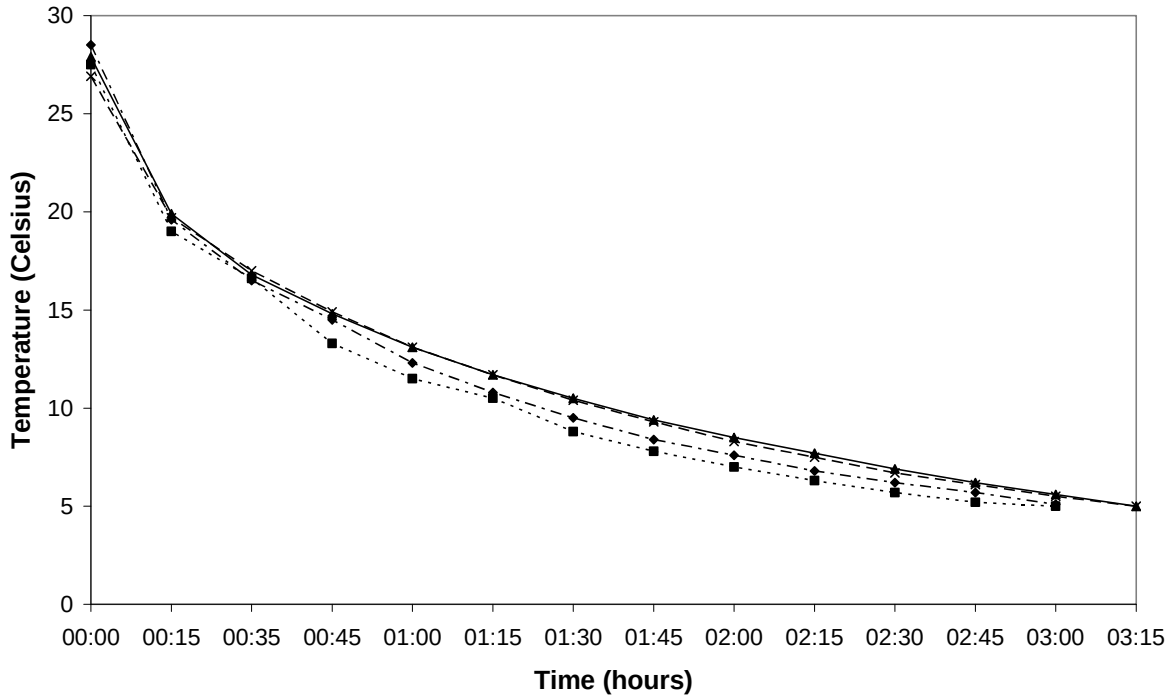

Figure 3.1**:** The cooling rate of four ejaculates in a cool room at 5 °C, showing that their temperatures decreased below 7 °C after 2½ h in a cool room at 5 °C

While the extended ejaculates were being cooled, fifty 0.25 ml French straws were marked with the date and ID of the ram and placed into a cool room at 5 °C. Two hours after the 3 ejaculates were pooled the spermatozoa were mixed and drawn into the marked 0.25 ml French straws that were then sealed with PVC powder. The straws were placed horizontally on a rack and 5 mm apart in stable nitrogen vapour, 4 cm above liquid nitrogen in a Styrofoam box. Twenty minutes later the straws were plunged into the liquid nitrogen and stored in a liquid nitrogen container at -196 °C.

3.10 Post-thaw evaluation of semen

In order to determine whether the frozen semen was suitable for further use, 2 straws from each ram were thawed and the individual motility as well as the percentage of live spermatozoa were determined as described by Nöthling (2000) and in Section 3.6 above.

3.11 Evaluation of frozen-thawed sperm after the addition of different media

In order to determine the sequence in which ejaculates and media had to be thawed a random number was allocated to each ram by medium combination (Table 8.1 in the Appendix). The semen-medium sets were then sorted in ascending order according to the random numbers.

3.11.1 Evaluation of post-thaw motility

One person prepared all slides for the assessment of motility and another performed all the assessments. The person preparing the slides thawed and mixed the contents of a straw of semen with 0.85 ml of the appropriate medium; according to the allocated random numbers. She then placed a drop of the mixed semen onto a cover slip that was then inverted and placed on a glass slide that was labelled with the random number. This person then handed the slide over to the person who evaluated the motility. All glassware was kept a 37 °C.

The second operator evaluated the motility of the specimens and recorded the values against the allocated random number.

The motility of the semen specimen was estimated within 5 minutes after dilution with the medium (t_0), and again two, four and six hours later (t_2, t_4, t_6).

Sets of semen and media were thawed with 10-minute intervals; media were always thawed 10 minutes before the corresponding straw of semen had to be thawed in order to allow the medium to reach 37 °C before thawing the semen.

At t_2, t_4 and t_6 the motility was again evaluated as described above. The 4.5 ml tubes containing the extended semen were capped and placed in a water bath at 37 °C until after the last evaluation at t_6.

All the data generated during this trial are contained in Table 8.1 in the Appendix.

3.11.2 Supravital staining

The percentages of spermatozoa that stained red (had damaged membranes or were dead) and those that remained white (that had intact membranes or were alive) were determined on an Eosin-Nigrosin smear made of the frozen-thawed semen (Nöthling, 2000).

3.12 Statistical analysis

The statistical package NCSS (www.ncss.com, Kaysville, UT USA) was used for all statistical analyses.

The coefficient of correlation between the percentages of progressively motile spermatozoa of frozen-thawed semen immediately after thawing and the percentages of spermatozoa that were non-stained on an eosin-nigrosin smear from the corresponding specimens was determined.

A repeated measure ANOVA was used to test for differences among the post-thaw motility with the rams as subjects nested in breed. Media and Time were within factors.

Where differences among the means existed, the Bonferroni multiple comparison test was used to determine which means differed (Maroco, 2004).

4 RESULTS

4.1 Evaluation of fresh ejaculates

4.1.1 Macroscopic evaluation

Results of the macroscopic evaluation of fresh semen are presented in Table 4.1. Collected ejaculates showed pH values varying between 6.5 and 7.5, while their volumes varied between 0.6 ml and 1.7 ml. With exception of the ram identified as Dorper 3, which produced white semen, the remaining rams produced semen with an ivory colour. Consistency of all collected specimens was thick creamy.

Table 4.1
Results of the macroscopic examination of fresh semen

Breed	Ram	Ejaculate number	pH	Volume (ml)	Colour	Consistency
Dohne Merino	9.15	1	7.5	0.8	Ivory	Thick creamy
		2	7.5	0.6	Ivory	Thick creamy
		3	7.5	0.6	Ivory	Thick creamy
Dohne Merino	9.26	1	6.5	1.8	Ivory	Thick creamy
		2	6.5	1.7	Ivory	Thick creamy
		3	6.5	1.2	Ivory	Thick creamy
Merino	435	1	6.5	1.4	Ivory	Thick creamy
		2	6.5	1.5	Ivory	Thick creamy
		3	6.5	1.4	Ivory	Thick creamy
Merino	436	1	7.5	1.5	Ivory	Thick creamy
		2	7.5	1.3	Ivory	Thick creamy
		3	7.5	1.3	Ivory	Thick creamy
Dorper	2	1	7.5	1.5	Ivory	Thick creamy
		2	7.5	1.0	Ivory	Thick creamy
		3	7.5	0.5	Ivory	Thick creamy
Dorper	3	1	7.0	1.0	White	Thick creamy
		2	7.0	1.0	White	Thick creamy
		3	7.0	1.1	White	Thick creamy

4.1.2 Microscopic evaluation

The results of the microscopic evaluation of the fresh semen are presented in Table 4.2. Collected ejaculates all showed very good mass motility with a score of 5. Within rams, the individual sperm motility was quite similar and the percentages progressively motile spermatozoa all exceeded 70%, whereas the percentages of aberrantly motile spermatozoa were low (1% or lower). There was a large variation in the concentration of spermatozoa among rams.

Table 4.2
Results of the microscopic examination of fresh semen

Breed-ID	**Ejaculate number**	**MM**	**Individual motility**			**Conc**	**Mor**
			Prog	**Aber**	**Immot**		
	1	5	97	0	3		
Dohne Merino 9.15	2	5	100	0	0	1884	98
	3	5	94	0	6		
	1	5	92	0	8		
Dohne Merino 9.26	2	5	90	0	10	5391	96
	3	5	95	0	5		
	1	5	96	1	3		
Merino 435	2	5	90	0	10	2328	99
	3	5	96	0	4		
	1	5	77	1	22		
Merino 436	2	5	89	1	10	4125	94
	3	5	82	1	17		
	1	5	83	1	16		
Dorper 3	2	5	90	0	10	2022	80
	3	5	84	0	16		
	1	5	75	0	25		
Dorper 2	2	5	84	0	16	2787	97
	3	5	84	0	16		
Average		5	88.8	0.3	10.9	3089.5	94

MM Mass Motility; **Prog** percentage of progressive motile spermatozoa; **Aber** percentage of aberrant motile spermatozoa; **Immot** percentage of immotile spermatozoa; **[Conc]** concentration of spermatozoa in million per millilitre; **Mor** percentage of morphologically normal spermatozoa.

4.2 Evaluation of frozen-thawed specimens

Post thaw motility and live/dead staining of frozen-thawed pooled ejaculates are presented in Table 4.3, before media was added. The percentages of the progressive motile spermatozoa and the percentages of the unstained spermatozoa show that the semen used in the experiments was good quality semen.

Table 4.3
Individual motility and supravital staining after thawing

Breed-ID	Straw	Individual motility			Supravital staining	
		Prog	Aber	Immot	Live	Dead
Dohne Merino 9.15	1st	79	0	21	60	40
	2nd	62	0	38	54	46
Dohne Merino 9.26	1st	46	0	54	50	50
	2nd	55	0	45	63	37
Merino 435	1st	47	0	53	53	47
	2nd	62	0	38	60	40
Merino 436	1st	44	0	66	40	60
	2nd	41	1	58	41	59
Dorper 3	1st	68	1	31	68	32
	2nd	40	0	60	60	40
Dorper 2	1st	52	0	48	52	48
	2nd	62	0	38	50	50
Average		54.8	0.2	45.8	54.3	45.8

Prog percentage of progressive motile spermatozoa; **Aber** percentage of aberrant motile spermatozoa; **Immot** percentage of immotile spermatozoa; **Live** percentage of non-stained spermatozoa; **Dead** percentage of red stained spermatozoa.

Figure 4.1 shows that there was a significant positive correlation between the percentage live spermatozoa and the percentage progressively motile spermatozoa in frozen-thawed semen.

Table 4.4

Percentages motile spermatozoa at different times after the semen was thawed and the various types of fluids added (each value represents the mean ± SD of the 18 values derived from 3 straws from each of 6 rams)

	Fluid					
Hours after thawing	**TrilEq**	**Ram**	**Bull**	**Dog**	**Milk**	**AfTALP**
Progressively motile spermatozoa						
0	38.05±2.78	35.36±2.78	42.27±2.78	36.93±2.78	45.09±2.78	36.56±2.78
2	32.66±2.78	28.92±2.78	7.96±2.78	25.30±2.78	41.15±2.78	19.94±2.78
4	26.15±2.78	16.77±2.78	1.94±2.78	19.32±2.78	38.89±2.78	14.49±2.78
6	13.91±2.78	6.62±2.78	4.56±2.78	9.51±2.78	34.49±2.78	11.08±2.78
Aberrantly motile spermatozoa						
0	2.28±0.85	1.65±0.85	1.77±0.85	2.03±0.85	2.07±0.85	3.33±0.85
2	5.88±0.85	1.79±0.85	0.67±0.85	2.83±0.85	1.76±0.85	18.2±0.85
4	1.72±0.85	1.78±0.85	0.14±0.85	1.98±0.85	1.62±0.85	19.4±0.85
6	1.34±0.85	1.61±0.85	0.12±0.85	0.97±0.85	1.53±0.85	13.1±0.85

TrilEq = Triladyl with Equex STM paste, **Ram** = seminal plasma of rams, **Bull** = seminal plasma of bulls, **Dog** = prostatic fluid of dogs, **Milk** = heat-treated skimmed milk, **afTALP** = albumin-free TALP

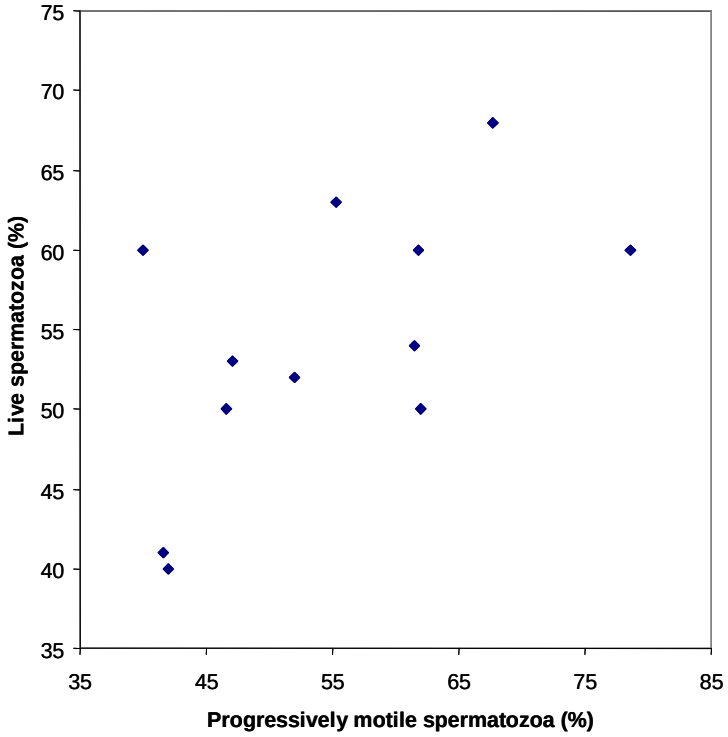

Figure 4.1**:** Percentages of live (non-stained on eosin-nigrosin smears) and progressively motile spermatozoa in 12 straws (2 from each of 6 rams) immediately after thawing (coefficient of correlation = 0.58, $p < 0.05$)

4.3 The effects of different rams, media and time after thawing on motility

The effects of adding the different media to frozen thawed sperm on individual progressive motility (Figure 4.2 to Figure 4.4) and aberrant motility (Figure 4.5 to Figure 4.7) for the six rams and the six media measured at t_0, t_2, t_4 and t_6 are shown below.

The individual progressive motility decreased over the time (Figure 4.2). The individual progressive motility measured for the 6 rams showed a maximum for ram 9.15 and a minimum for 436 (Figure 4.3) while within the media skim milk showed the highest and bull seminal plasma the lowest value of the individual progressive motility (Figure 4.4).

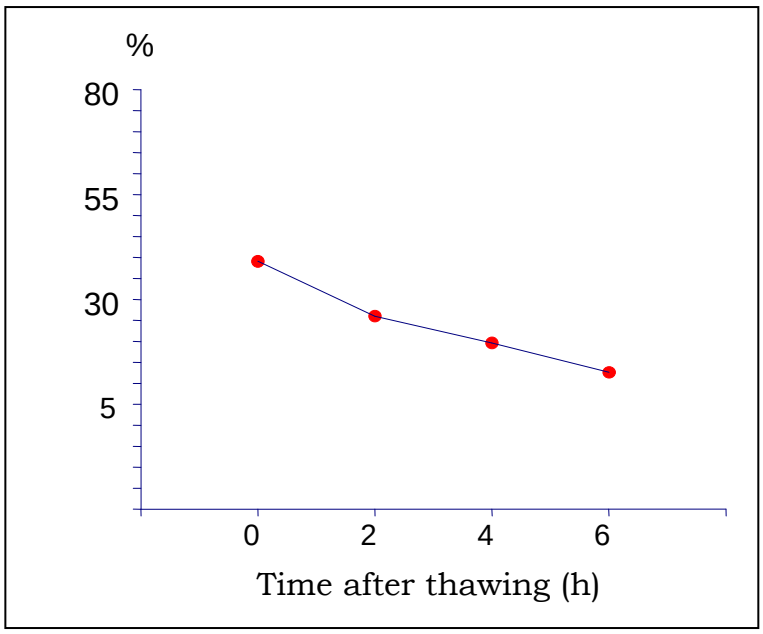

Figure 4.2: Mean post-thaw progressive motility (%) of sperm after various times of post-thaw incubation in water at 37 °C (values represent the pooled means of all rams, media and straws, n = 108)

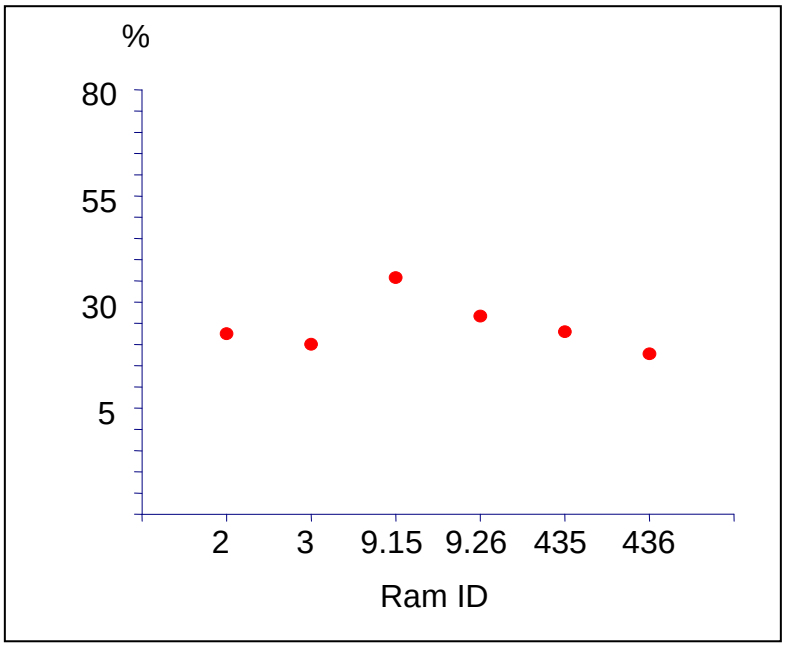

Figure 4.3: Mean post-thaw progressive motility (%) of sperm for the six different rams (values represent the pooled means of all media, times after thawing and straws, n = 72)

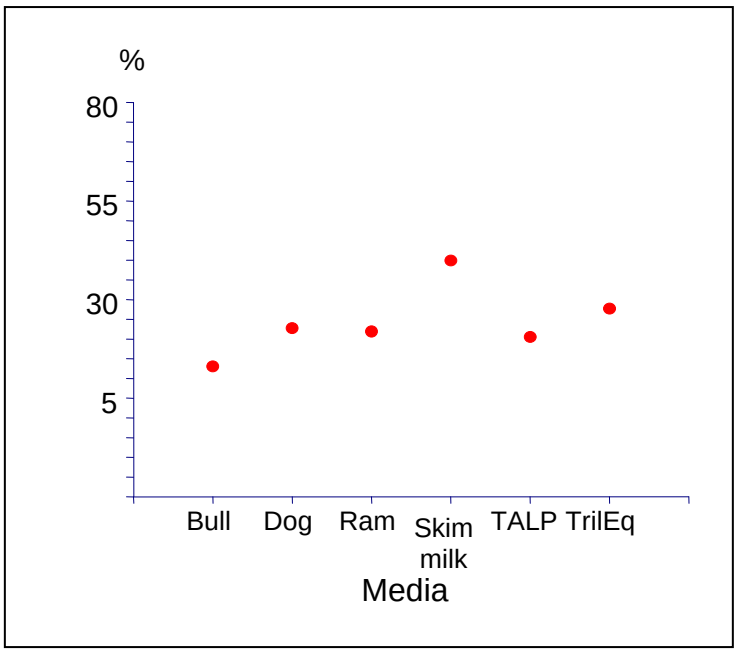

Figure 4.4: Mean post-thaw progressive motility (%) of sperm for the six different media (values represent the pooled means of all rams, times after thawing and straws, n = 72)

The variation of the aberrant motility over the time showed an irregular behaviour. It increased slightly during the first 2 h after thawing and thereafter decreased slightly until 6 h after thawing (Figure 4.5). The aberrant motility measured for the 6 rams showed similar values (Figure 4.6) while within the media TALP showed the highest and the remaining media similar values of the aberrant motility (Figure 4.4).

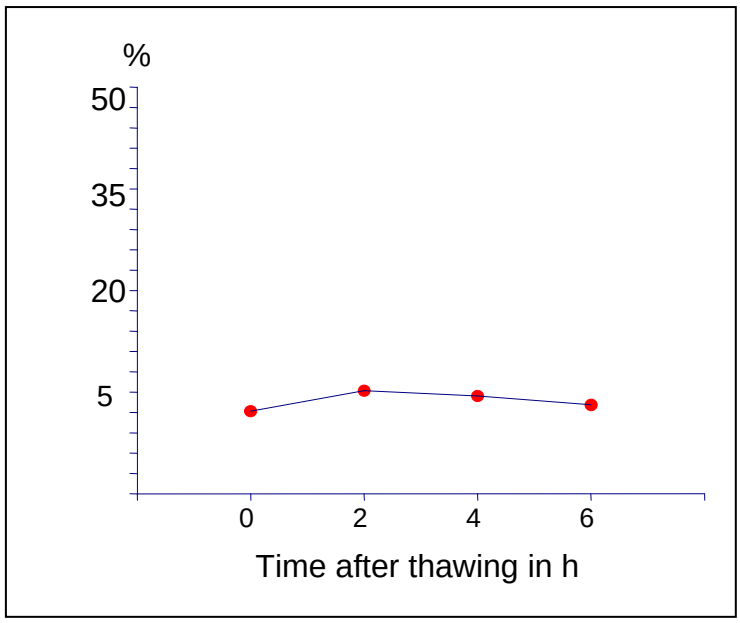

Figure 4.5: Mean post-thaw aberrant motility (%) of sperm at t_0, t_2, t_4 and t_6 (values represent the pooled means of all rams, media and straws, n = 108)

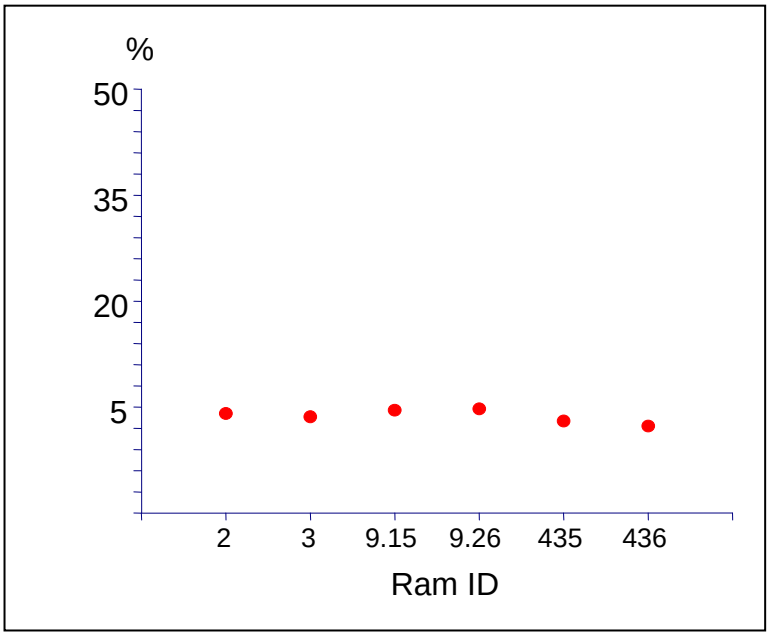

Figure 4.6: Mean post-thaw aberrant motility (%) of sperm for the six different rams (values represent the pooled means of all media, times after thawing and straws, n = 72)

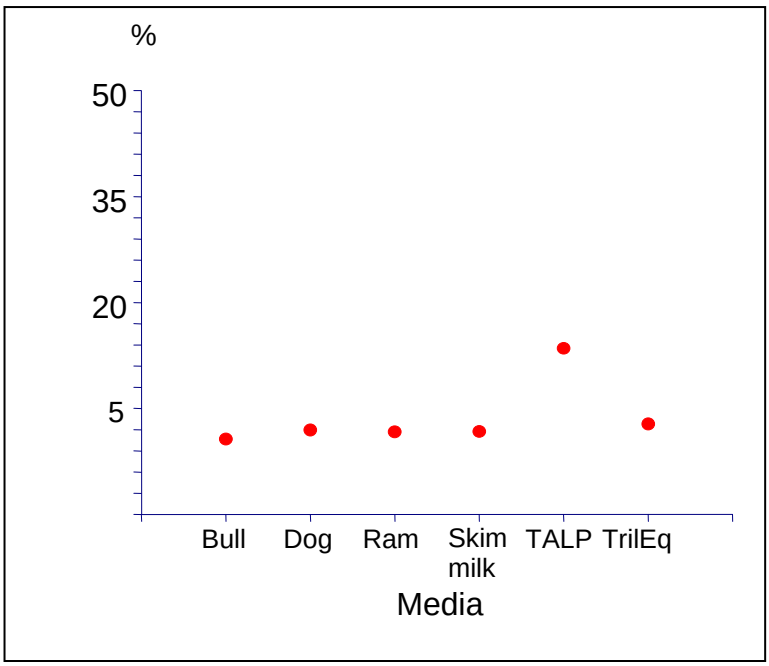

Figure 4.7: Mean post-thaw aberrant motility (%) of sperm for the six different media (values represent the pooled means of all rams, times after thawing and straws, n = 72)

4.3.1 Analysis of variance for motility

Ram, time and media had all a significant effect on the progressive motility. There was also a significant interaction between Ram and Medium and between Time and Medium (Table 4.5).

Ram, time and media had all a significant effect on the aberrant motility. There was also a significant interaction between Ram and Medium and between Time and Medium (Table 4.5).

Table 4.5

Results of the analysis of variance for progressive and aberrant motility

	Probability level	
	Progressive motility	**Aberrant motility**
Ram	<0.001	<0.001
Time	<0.001	<0.001
Medium	<0.001	<0.001
Ram x Time	>0.05	>0.05
Ram x Medium	<0.001	<0.001
Time x Medium	<0.001	<0.001

4.3.2 Difference between various time groups on motility (Bonferroni multiple comparison test)

Pairwise comparisons of the means for progressive motility (Table 4.6) showed that all time groups (t_0, t_2, t_4 and t_6) were significantly different from each other ($p<0.05$).

Table 4.6

Bonferroni Multiple Comparison Test for the time groups

	Different from groups	
Group	**Progressive motility**	**Aberrant motility**
0 hours (t_0)	t_2, t_4, t_6	t_2, t_4
2 hours (t_2)	t_0, t_4, t_6	t_0, t_6
4 hours (t_4)	t_0, t_2, t_6	t_0
6 hours (t_6)	t_0, t_2, t_4	t_2

The mean percentage aberrantly motile spermatozoa at t_0 differs from those at t_2 and t_4; that at t_2 differs from those at t_0 and t_6, whereas the means at t_4 and t_6 differ only from those at t_0 and t_2, respectively (Table 4.6).

4.3.3 Pairwise comparisons of the effects of media on motility

The Bonferroni test shows that progressive motility differed among media (Table 4.7). Bull seminal plasma shows the lowest values while skim milk gave the highest values of the progressive motility. Skim milk shows a significant difference from all remaining media except TrilEq, while TrilEq differs significantly only from bull seminal plasma.

The results on aberrant motility show a much similar behaviour. All fluids except TALP yielded similar aberrant motility.

Table 4.7
Pairwise comparisons of motility of frozen-thawed ram spermatozoa extended in various fluids after thawing (Bonferroni's test, $P<0.05$, n = 72 per group, only different means shown)

	Different from groups	
	Progressive motility	**Aberrant motility**
Bull	TrilEq, skim milk	TALP
TALP	Skim milk	Bull, Ram, Skim milk, Dog, TrilEq
Ram	Skim milk	TALP
Dog	Skim milk	TALP
TrilEq	Bull	TALP
Skim milk	Bull, TALP, Ram, Dog	TALP

4.3.4 The interaction between Ram and the Medium on motility

Results of the interaction among rams on progressive motility, for the various media, are presented in Figure 4.8. For all rams skim milk showed the highest values of the progressive motility whereas, for all but 2 rams, bull seminal plasma resulted in the lowest percentage progressively motile spermatozoa.

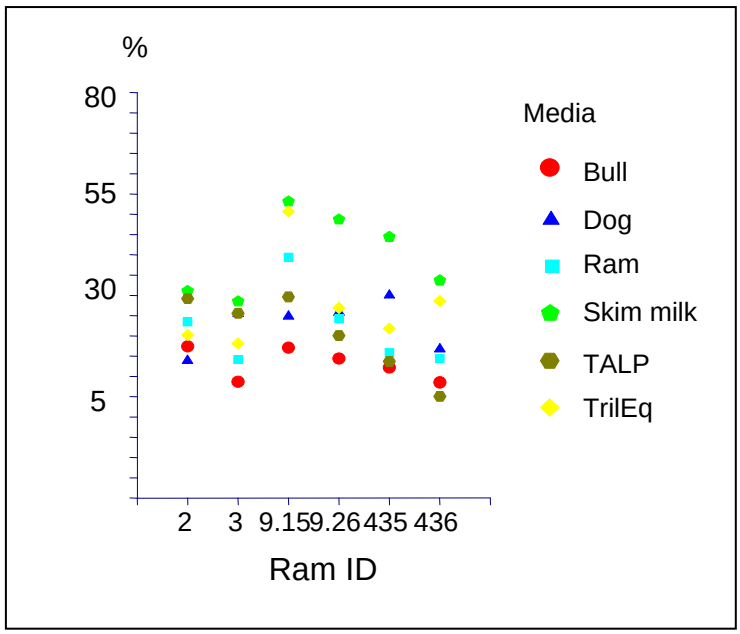

Figure 4.8: The interaction between Ram and Medium on the percentage progressively motile spermatozoa (each value represents the mean of the motility of 3 straws each measured 0, 2, 4 and 6 h after thawing)

The interaction among rams with respect to aberrant motility (Figure 4.9) showed the highest values for TALP whereas the remaining media resulted in lower values that were quite similar within rams. Pairwise comparison of means (Table 4.7) showed that TALP resulted in higher percentages aberrantly motile spermatozoa than all other media, which were similar.

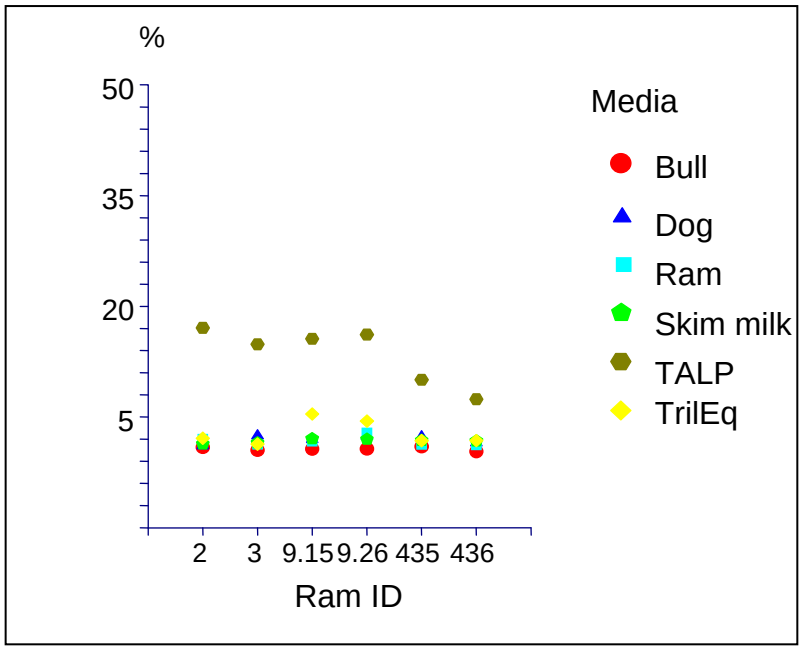

Figure 4.9: The interaction between Ram and Medium on the percentage abberantly motile spermatozoa (each value represents the mean of the motility of 3 straws each measured 0, 2, 4 and 6 h after thawing)

4.3.5 The interaction between Time and Medium on motility

The results presented in Figure 4.10 and Figure 4.11 show the interactions between Time and Medium on progressive and aberrant motility, respectively.

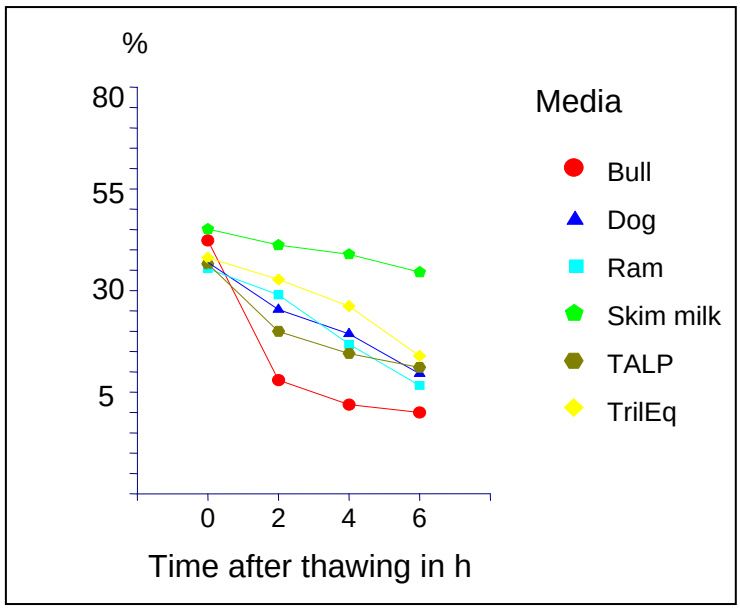

Figure 4.10: Mean post-thaw progressive motility (%) of sperm incubated in different media after thawing in water at 37 °C (values represent the pooled means of the six rams and of all straws, n=12)

Figure 4.10 shows that progressive motility decreased over time for all media. Skim milk resulted in the highest mean percentages of progressive motile spermatozoa, while bull seminal plasma resulted in the lowest, except for time t_0.

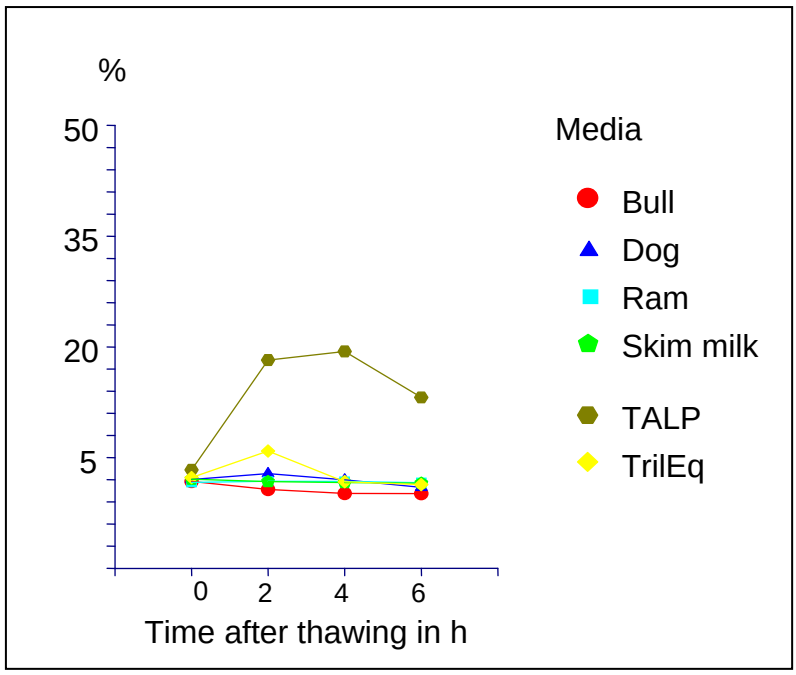

Figure 4.11: Mean post-thaw aberrant motility (%) of sperm incubated in different media after thawing in water at 37 °C (values represent the pooled means of the 6 rams and of all straws, n=12)

Figure 4.11 shows that TALP resulted in the highest values of aberrant motility whereas the remaining media showed similar values, which decreased over time. TALP initially resulted in an increase in the percentages of aberrantly motile spermatozoa from time t_0 to t_2-t_4, followed by a decrease. During estimation of motility for specimens to which TALP was added, spermatozoa showed hyperactive motion, which may be responsible for the different behaviour between TALP and remaining media described above.

5 DISCUSSION

The aim of this study was to compare the effects of media (heat-treated skim milk, protein-free TALP, seminal plasma of rams and bulls, and dog prostatic fluid) and post-thaw incubation (for up to 6 h) on the percentages progressively and aberrantly motile spermatozoa of rams.

5.1 Fresh semen quality

According to Hafez (1993) the average ejaculate volume for rams ranges from 0.8 to 1.2 ml, while Chemineau and Cagnié (1991) give an average value for rams of 1.0 to 1.5 ml, with individual values varying from one collection to another. Evans and Maxwell (1987) report an average ejaculate volume of about 1.0 ml for rams, when using an artificial vagina. The ejaculates from each ram used in the current study had an average volume of 1 ml or higher, except those of Dohne Merino 9.15, which had an average volume of 0.7 ml. The volumes of the ejaculates used in the current study were normal.

Dorper 3 produced white semen, which, according to Evans and Maxwell (1987), is normal. The other rams produced semen with an ivory colour, which, according to Nöthling (2000) is normal for highly concentrated bull semen.

The consistency of semen depends on the concentration of spermatozoa therein. Semen specimens of thick consistency contain a lower concentration of spermatozoa than those with a lower consistency. The consistency of ram semen with a mass motility of 5 is normally thick creamy (Evans and Maxwell, 1987).

The mass motility scores of all ejaculates used in this study were 5, which, according to Evans and Maxwell (1987) is very good. Daza (1994) stated that speciments with a mass motility of 4 or 5 (good or very good mass motility) can be used in artificial insemination experiments, whereas Evans and Maxwell (1987) stated that specimens with a mass motility of 3 or lower may result in decreased fertility.

The fresh semen used in the current study had at least 70% progressively motile spermatozoa which, according to Derivaux (1992) and Daza (1994). is good as they state that good quality semen must have a minimum of 60 % progressively motile spermatozoa and a mass motility of 4 or 5.

Values of the concentration of undiluted specimens used in the current study varied between $1884x10^6$ and $5391x10^6$ spermatozoa per ml. Only 2 rams in the current study (Dohne Merino 9.26 and Merino 436) yielded ejaculates with higher concentrations than the $3500x10^6$ to $6000x10^6$ spermatozoa per ml that, according to Evans and Maxwell (1987), characterise good quality semen. The lower concentration of some fresh ejaculates seems not to affect the percentages of progressive motile spermatozoa. Dohne Merino 9.15 with the lowest concentration show the highest percentages of progressive motile spermatozoa while Dohne Merino 9.26 and Merino 436, with the highest concentrations, have intermediate percentages of progressive motile spermatozoa.

From the above it seems that the quality of the ejaculates used was good, implying that the results of the study are valid for good quality ram semen in general.

5.2 Progressive motility after thawing

The frozen-thawed semen used in this study had good individual sperm motility. According to Daza (1994) good quality frozen-thawed bull semen has at least 50% progressively motile spermatozoa, although satisfactory fertility results in an artificial insemination program may already be achieved with semen that has more than 30% progressively motile spermatozoa. Chemineau and Cagnié (1991) stated that good quality frozen-thawed ram semen must have at least 30% of living cells after thawing. Each of the frozen-thawed ejaculates used in this study had at least 40% living spermatozoa, suggesting that the semen was of good quality. Although the criteria used by Daza (1994) for bulls and by Chemineau and Cagnié (1991) for rams look different, they establish a common minimum limit of 30% as semen with acceptable quality for use in artificial insemination programmes.

There was a significant positive correlation between the percentage of progressive motile spermatozoa and percentage of live spermatozoa of thawed semen. A correlation coefficient of 0.58 indicates a significant relationship between percentage of progressive motile- and the percentage of live spermatozoa. This correlation is easy to understand since live (unstained) spermatozoa include progressively motile and aberrantly motile spermatozoa. The percentage of aberrantly motile spermatozoa for most of the specimens, except where TALP was added, was below 3%, meaning that the percentage live (unstained) spermatozoa in a semen specimen is closely approximated by the percentage progressively motile spermatozoa. The deviations from the ideal correlation coefficient (r=1) is probably caused by the subjectivity of the method used in the estimation of progressive motility, the existence of some aberrant motile spermatozoa and some live but immotile spermatozoa that may exist.

The correlation described above suggests that the minimum limit of 30% used by Daza (1994) for bull semen and by Chemineau and Cagnié (1991) for ram semen are not contradictory.

Immediately after adding any of the media (t_0) the mean progressive motility of all specimens was above 30%, the percentage defined as the minimum value for good quality frozen-thawed semen which on insemination may lead to satisfactory fertility results. (Chemineau and Cagnié, 1991 and Daza, 1994). This good average semen quality was measured with all extenders at time t_0; with skim milk, ram seminal plasma and TrilEq at time 2 hours; and with skim milk and TrilEq at time 4 hours. Skim milk was the only medium that yielded an average percentage of progressively motile spermatozoa above 30% over the 6 hour period that the experiment took place. These results show that skim milk was the best medium used with respect to the maintenance of progressive motility in the study while bull seminal plasma was the worst.

The positive effect of skim milk on maintenance of the individual progressive motility supports its further use as an extender for frozen-thawed ram semen.

Skim milk resulted in a significantly higher mean percentage progressive motility than all other media except TrilEq. The effect of TrilEq is a surprising one since glycerol contained in TrilEq is known to have a spermotoxic effect, especially at relatively high temperatures as the 37°C in our experiments. The Bonferroni test shows that bull seminal plasma is significantly different from skim milk and TrilEq but not from TALP, ram seminal plasma and dog prostatic fluid.

Although skim milk maintains better progressive motility it thus not necessarily results in good fertility after cervical insemination. Contrary to skim milk, ram seminal plasma does not maintain progressive motility as good as skim milk does, but is known to improve the fertility of cervically inseminated frozen-thawed ram sperm. Therefore maintenance of progressive motility can not be used as a good indicator of fertility.

Bull seminal plasma shows one of the worst effects on the progressive motility. Results of the Bonferroni pairwise comparison of means show that bull seminal plasma is only statistically different from TrilEq and skim milk but not from the remaining media. Although bull seminal plasma does not result in good maintenance of progressive motility of frozen-thawed ram sperm, it increases the ability of buffalo sperm to fertilize bovine oocytes in vitro (Gerber et al., 2003). This apparent contradiction shows that a low progressive motility of frozen-thawed ram spermatozoa after addition of bull seminal plasma does not necessarily indicate an inability to fertilize. On the other hand, the decrease of progressive motility of frozen-thawed ram sperm over time may partially explain the contradiction reported. At time zero progressive motility was in average above the minimum limit of 30%. Since during in vitro fertilization trials sperm is added to the oocytes shortly after thawing and addition of media, one should expect a better result in this case

when compared with an addition of sperm after a waiting time of 4 to 6 hours, where the worst effect of bull seminal plasma was registered.

Since the positive effect of some media on maintenance of the progressive motility of frozen-thawed ram sperm can not be used as a reliable predictor of good results in fertility, it would be interesting to compare the results of the progressive motility with results from fertility experiments. This comparison will test the reported correlation between progressive motility and fertility. Since the technique used in artificial insemination seems to have an influence on the fertility results (Maxwell et al. 1999; Gillan et al. 2004; McPhie et al., 2000; Ritar and Ball, 1993), the use of different artificial insemination techniques in these trials will help explain the influence of the technique used on obtained results.

The results of the analysis of variance show that Ram, Time and Medium all had significant influences on the progressive motility. There is an interaction between Ram and Medium. Skim milk maintained progressive motility better than all other mediums for all rams, while bull seminal plasma resulted in the lowest progressive motility for all rams except Dorper 2 and Merino 436. Time also interacted with Medium. Skim milk maintained progressive motility over the time better than all other mediums while bull seminal plasma showed the worst effect, with a rapid decline between 0 and 2 hours. Results presented showed no interaction between Ram and Time.

The results of the Bonferroni pairwise comparison of means for the time groups show a systematic and significant decrease of the progressive motility over time, with the progressive motility of each specimen at a certain time becoming significantly different from the progressive motility of the remaining times. This may be caused by a decrease of mitochondrial activity with time (Maxwell et al., 1999), with the consequent reduction of progressive motility caused by the increasing number of immotile spermatozoa with time. This result supports the practice used in Artificial Insemination of using semen immediately after thawing it, to avoid deterioration of its quality with time and consequently poor fertility results.

5.3 Aberrant motility after thawing

Aberrant motility of ram spermatozoa in TALP shows behaviour different from that observed with the other media. TALP shows the highest percentage of aberrant motility with spermatozoa showing a hyperactivated movement, while the remaining media show nearly similar values, when compared to one another. TALP is known to induce capacitation, enabling spermatozoa to undergo the acrosome reaction and to express hyperactivated motility (Fraser, 1987; Sirivaidyapong et al., 2000). The Bonferroni test shows that addition of TALP gives an aberrant motility different from that of all other media, while the remaining media resulted in similar aberrant motility, when compared with each other except TALP.

TALP results in the highest percentage of aberrant motility, except for time t_0, and a relative large variation over time while the remaining media resulted in similar and nearly constant values over time. This is not supported by the Bonferroni test for the time groups, where no regular tendency can be derived from variation of the aberrant motility when going from time 0 to time 2 hours, from time 2 to 4 hours, and so on. Except for TALP, the other media resulted in a marked decrease in the percentage progressively motile spermatozoa over time, associated with only a small decrease in the percentage aberrantly motile spermatozoa over the same time, suggesting that progressively motile spermatozoa became immotile over time, which may be associated with the inability of the spermatozoa to survive over a long period.

The better capacity of albumin free TALP to maintain the progressive motility of thawed dog sperm than dog prostatic fluid, reported by Sirivaidyapong (2000), can not be extended to rams. In the current trial the post thaw individual progressive motility of semen where TALP was added did not differ from semen where dog prostatic fluid was added. The aberrant motility was, however, significantly higher with TALP than dog prostatic fluid. Aberrant motility in TALP, differently from remaining media, shows first a drastic

increase from t_0 to t_2 and t_4, followed by a decrease at t_6, that was, however, still significantly higher than at t_0.

According to Daza (1994) bull spermatozoa requires about 5 hours to undergo capacitation, subsequent to which they are ready to fertilize the oocyte. The higher values of the aberrant motility in TALP may be associated with the hyperactive movement of spermatozoa in this medium, as was observed in our experiments. According to Bavister and Yanagimachi (1977), this effect may be caused by the pyruvate contained in TALP, which acts as an energy source for sperm motility and acrosome reaction. The effect of pyruvate is supported by other TALP components like glucose and lactose. The effect of TALP lasts for more than the six hours of our experiment, time above the minimum time needed for the spermatozoa to reach the ampulla, where fertilization occurs (Gillan et al., 2004). The fact that TALP maintains motility (although it is aberrant motility) might have a beneficial effect on fertilization, assuming that during this period the capacitation of sperm, stimulated by TALP, takes place (Nöthling et al., 2005).

The effect of TALP is also associated with Ca^{2+} present in the medium (Sirivaidyapong, 2000). This author reports a better induction of capacitation and acrosomic reaction caused by TALP or other media where Ca^{2+} was present. Sirivaidyapong (2000) used TALP that contained Ca^{2+}, with and without bovine serum albumin and, in both cases, obtained results with no significative difference. In some media where Ca^{2+} was inhibited by addition of components like e.g. EDTA, a strong complexing agent for Ca^{2+}, induction of capacitation and acrosomic reaction was strongly reduced.

6 CONCLUSIONS

Heat treated skim milk maintains the progressive motility of ram sperm frozen-thawed with TrilEq better than dog prostatic fluid, seminal plasma of bull and ram and protein-free TALP.

Our study describes the motility of frozen-thawed semen. These results cannot be used to predict the fertilizing capacity without further trials that test the correlation between the motility and the fertility. Such trials would have to include artificial insemination of ewes. Fertility in artificial insemination depends on the technique used to inseminate; suggested studies must also pay attention to the influence of the technique used on the results.

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8 APPENDIX

Table 8.1
Motility of frozen-thawed ejaculates diluted with the different media at times t_0, t_2, t_4 and t_6 (data are sorted in ascending order according to ram, medium, straw and time)

Random No.	Date	Breed	ram	medium	straw	t	Prog	Aber	Immot
0.3967746380	Jun 30	Dorper	2	Bull	a	0	46.67	1.28	52.05
0.3967746380	Jun 30	Dorper	2	Bull	a	2	0.00	0.00	100.00
0.3967746380	Jun 30	Dorper	2	Bull	a	4	0.00	0.00	100.00
0.3967746380	Jun 30	Dorper	2	Bull	a	6	0.00	0.00	100.00
0.2389407698	Jun 25	Dorper	2	Bull	b	0	55.37	1.63	43.00
0.2389407698	Jun 25	Dorper	2	Bull	b	2	36.81	1.48	61.71
0.2389407698	Jun 25	Dorper	2	Bull	b	4	34.92	2.51	62.57
0.2389407698	Jun 25	Dorper	2	Bull	b	6	0.08	2.12	97.80
0.2070021430	Jun 25	Dorper	2	Bull	c	0	34.99	1.77	63.24
0.2070021430	Jun 25	Dorper	2	Bull	c	2	0.00	0.00	100.00
0.2070021430	Jun 25	Dorper	2	Bull	c	4	0.00	0.00	100.00
0.2070021430	Jun 25	Dorper	2	Bull	c	6	0.00	0.00	100.00
0.4504005638	Jun 30	Dorper	2	Dog	a	0	34.40	1.39	64.21
0.4504005638	Jun 30	Dorper	2	Dog	a	2	23.32	1.15	75.52
0.4504005638	Jun 30	Dorper	2	Dog	a	4	18.31	1.02	80.67
0.4504005638	Jun 30	Dorper	2	Dog	a	6	8.43	0.88	90.69
0.6286186089	Jul 01	Dorper	2	Dog	b	0	30.33	1.31	68.36
0.6286186089	Jul 01	Dorper	2	Dog	b	2	21.73	1.06	77.21
0.6286186089	Jul 01	Dorper	2	Dog	b	4	3.54	1.46	95.00
0.6286186089	Jul 01	Dorper	2	Dog	b	6	0.20	0.10	99.70
0.0949471526	Jun 24	Dorper	2	Dog	c	0	23.87	2.78	73.36
0.0949471526	Jun 24	Dorper	2	Dog	c	2	2.63	1.17	96.20
0.0949471526	Jun 24	Dorper	2	Dog	c	4	0.01	0.99	99.00
0.0949471526	Jun 24	Dorper	2	Dog	c	6	0.00	0.00	100.00
0.7403390064	Jul 05	Dorper	2	Ram	a	0	48.08	1.76	50.17
0.7403390064	Jul 05	Dorper	2	Ram	a	2	50.92	1.75	47.33
0.7403390064	Jul 05	Dorper	2	Ram	a	4	13.06	0.87	86.07
0.7403390064	Jul 05	Dorper	2	Ram	a	6	0.21	4.39	95.40
0.2720078915	Jun 29	Dorper	2	Ram	b	0	40.59	2.24	57.17
0.2720078915	Jun 29	Dorper	2	Ram	b	2	35.58	2.59	61.83
0.2720078915	Jun 29	Dorper	2	Ram	b	4	17.73	6.20	76.07
0.2720078915	Jun 29	Dorper	2	Ram	b	6	14.25	1.11	84.64
0.4033905148	Jun 30	Dorper	2	Ram	c	0	30.60	1.09	68.31
0.4033905148	Jun 30	Dorper	2	Ram	c	2	20.88	0.84	78.28
0.4033905148	Jun 30	Dorper	2	Ram	c	4	9.32	0.39	90.29
0.4033905148	Jun 30	Dorper	2	Ram	c	6	0.01	0.99	99.00
0.8801909161	Jul 06	Dorper	2	Skim milk	a	0	20.41	1.18	78.40
0.8801909161	Jul 06	Dorper	2	Skim milk	a	2	15.39	1.61	83.00
0.8801909161	Jul 06	Dorper	2	Skim milk	a	4	5.65	0.51	93.84
0.8801909161	Jul 06	Dorper	2	Skim milk	a	6	0.00	0.00	100.00
0.8836740417	Jul 06	Dorper	2	Skim milk	b	0	43.72	1.92	54.36
0.8836740417	Jul 06	Dorper	2	Skim milk	b	2	48.86	1.56	49.57
0.8836740417	Jul 06	Dorper	2	Skim milk	b	4	33.25	1.54	65.21
0.8836740417	Jul 06	Dorper	2	Skim milk	b	6	0.01	0.79	99.20
0.7031757916	Jul 05	Dorper	2	Skim milk	c	0	49.07	2.24	48.69
0.7031757916	Jul 05	Dorper	2	Skim milk	c	2	53.77	1.83	44.40
0.7031757916	Jul 05	Dorper	2	Skim milk	c	4	52.36	1.62	46.02
0.7031757916	Jul 05	Dorper	2	Skim milk	c	6	50.43	1.50	48.07

Bull: Seminal plasma from bulls; **Dog:** Prostatic fluid from dogs; **Ram:** seminal plasma from rams; **Skim milk:** UHT-milk, **TALP:** sperm talp; **TrilEql™:** commercially available Tris semen extender.

Random No.	Date	Breed	ram	medium	straw	t	Prog	Aber	Immot
0.2872086171	Jun 29	Dorper	2	TALP	a	0	45.12	1.31	53.57
0.2872086171	Jun 29	Dorper	2	TALP	a	2	25.33	20.95	53.71
0.2872086171	Jun 29	Dorper	2	TALP	a	4	20.92	18.51	60.57
0.2872086171	Jun 29	Dorper	2	TALP	a	6	28.78	19.24	51.98
0.3496577935	Jun 29	Dorper	2	TALP	b	0	39.78	4.15	56.07
0.3496577935	Jun 29	Dorper	2	TALP	b	2	22.66	17.01	60.33
0.3496577935	Jun 29	Dorper	2	TALP	b	4	23.93	22.24	53.83
0.3496577935	Jun 29	Dorper	2	TALP	b	6	22.23	20.37	57.40
0.5906284361	Jul 01	Dorper	2	TALP	c	0	56.16	2.56	41.29
0.5906284361	Jul 01	Dorper	2	TALP	c	2	17.62	32.74	49.64
0.5906284361	Jul 01	Dorper	2	TALP	c	4	18.15	22.54	59.31
0.5906284361	Jul 01	Dorper	2	TALP	c	6	29.36	23.31	47.33
0.2367942281	Jun 25	Dorper	2	TrilEq	a	0	21.28	1.84	76.88
0.2367942281	Jun 25	Dorper	2	TrilEq	a	2	14.43	1.64	83.93
0.2367942281	Jun 25	Dorper	2	TrilEq	a	4	1.79	8.64	89.57
0.2367942281	Jun 25	Dorper	2	TrilEq	a	6	0.03	1.37	98.60
0.4882463018	Jun 30	Dorper	2	TrilEq	b	0	53.69	1.90	44.40
0.4882463018	Jun 30	Dorper	2	TrilEq	b	2	39.50	2.64	57.86
0.4882463018	Jun 30	Dorper	2	TrilEq	b	4	34.60	1.33	64.07
0.4882463018	Jun 30	Dorper	2	TrilEq	b	6	16.47	1.39	82.14
0.2556289603	Jun 29	Dorper	2	TrilEq	c	0	32.56	1.27	66.17
0.2556289603	Jun 29	Dorper	2	TrilEq	c	2	14.45	1.60	83.95
0.2556289603	Jun 29	Dorper	2	TrilEq	c	4	13.71	0.79	85.50
0.2556289603	Jun 29	Dorper	2	TrilEq	c	6	0.01	0.89	99.10
0.1940356219	Jun 25	Dorper	3	Bull	a	0	18.79	1.90	79.31
0.1940356219	Jun 25	Dorper	3	Bull	a	2	0.00	0.00	100.00
0.1940356219	Jun 25	Dorper	3	Bull	a	4	0.00	0.00	100.00
0.1940356219	Jun 25	Dorper	3	Bull	a	6	0.00	0.00	100.00
0.6463569657	Jul 01	Dorper	3	Bull	b	0	38.81	1.78	59.40
0.6463569657	Jul 01	Dorper	3	Bull	b	2	0.00	0.00	100.00
0.6463569657	Jul 01	Dorper	3	Bull	b	4	0.00	0.00	100.00
0.6463569657	Jul 01	Dorper	3	Bull	b	6	0.00	0.00	100.00
0.7386003656	Jul 05	Dorper	3	Bull	c	0	46.56	2.27	51.17
0.7386003656	Jul 05	Dorper	3	Bull	c	2	0.00	0.00	100.00
0.7386003656	Jul 05	Dorper	3	Bull	c	4	0.00	0.00	100.00
0.7386003656	Jul 05	Dorper	3	Bull	c	6	0.00	0.00	100.00
0.9527379927	Jul 06	Dorper	3	Dog	a	0	46.32	2.51	51.17
0.9527379927	Jul 06	Dorper	3	Dog	a	2	45.67	2.17	52.17
0.9527379927	Jul 06	Dorper	3	Dog	a	4	39.54	3.06	57.40
0.9527379927	Jul 06	Dorper	3	Dog	a	6	0.03	1.37	98.60
0.1285911393	Jun 24	Dorper	3	Dog	b	0	26.67	2.45	70.88
0.1285911393	Jun 24	Dorper	3	Dog	b	2	4.08	2.92	93.00
0.1285911393	Jun 24	Dorper	3	Dog	b	4	0.18	3.62	96.20
0.1285911393	Jun 24	Dorper	3	Dog	b	6	0.00	0.10	99.90
0.8891066361	Jul 06	Dorper	3	Dog	c	0	53.59	1.77	44.64
0.8891066361	Jul 06	Dorper	3	Dog	c	2	41.49	2.29	56.21
0.8891066361	Jul 06	Dorper	3	Dog	c	4	47.48	2.72	49.81
0.8891066361	Jul 06	Dorper	3	Dog	c	6	0.24	4.36	95.40
0.1992248151	Jun 25	Dorper	3	Ram	a	0	16.90	1.38	81.71
0.1992248151	Jun 25	Dorper	3	Ram	a	2	6.38	2.13	91.50
0.1992248151	Jun 25	Dorper	3	Ram	a	4	0.04	3.46	96.50
0.1992248151	Jun 25	Dorper	3	Ram	a	6	0.00	0.00	100.00

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Random No.	date	Breed	ram	medium	straw	t	Prog	Aber	Immot
0.7068240948	Jul 05	Dorper	3	Ram	b	0	19.47	1.37	79.17
0.7068240948	Jul 05	Dorper	3	Ram	b	2	21.79	0.98	77.23
0.7068240948	Jul 05	Dorper	3	Ram	b	4	0.00	0.00	100.00
0.7068240948	Jul 05	Dorper	3	Ram	b	6	0.00	0.00	100.00
0.6928747155	Jul 05	Dorper	3	Ram	c	0	41.14	1.69	57.17
0.6928747155	Jul 05	Dorper	3	Ram	c	2	30.90	1.98	67.12
0.6928747155	Jul 05	Dorper	3	Ram	c	4	32.95	2.05	65.00
0.6928747155	Jul 05	Dorper	3	Ram	c	6	0.00	0.00	100.00
0.4593175585	Jun 30	Dorper	3	Skim milk	a	0	35.55	1.71	62.74
0.4593175585	Jun 30	Dorper	3	Skim milk	a	2	26.71	1.55	71.74
0.4593175585	Jun 30	Dorper	3	Skim milk	a	4	26.12	1.05	72.83
0.4593175585	Jun 30	Dorper	3	Skim milk	a	6	0.11	0.89	99.00
0.5865827185	Jul 01	Dorper	3	Skim milk	b	0	49.78	2.58	47.64
0.5865827185	Jul 01	Dorper	3	Skim milk	b	2	43.15	1.97	54.88
0.5865827185	Jul 01	Dorper	3	Skim milk	b	4	39.64	2.05	58.31
0.5865827185	Jul 01	Dorper	3	Skim milk	b	6	45.20	1.68	53.12
0.6427184073	Jul 01	Dorper	3	Skim milk	c	0	33.13	1.66	65.21
0.6427184073	Jul 01	Dorper	3	Skim milk	c	2	23.61	1.84	74.55
0.6427184073	Jul 01	Dorper	3	Skim milk	c	4	18.35	0.93	80.71
0.6427184073	Jul 01	Dorper	3	Skim milk	c	6	0.40	0.00	99.60
0.9899636066	Jul 06	Dorper	3	TALP	b	0	38.96	2.31	58.74
0.9899636066	Jul 06	Dorper	3	TALP	b	2	12.24	20.02	67.74
0.9899636066	Jul 06	Dorper	3	TALP	b	4	25.02	17.25	57.74
0.9899636066	Jul 06	Dorper	3	TALP	b	6	0.24	3.76	96.00
0.9394526429	Jul 06	Dorper	3	TALP	c	0	56.50	2.05	41.45
0.9394526429	Jul 06	Dorper	3	TALP	c	2	39.69	22.18	38.13
0.9394526429	Jul 06	Dorper	3	TALP	c	4	20.34	36.78	42.88
0.9394526429	Jul 06	Dorper	3	TALP	c	6	30.55	20.95	48.50
0.9965773997	Jul 06	Dorper	3	TALP	c	0	36.65	2.47	60.88
0.9965773997	Jul 06	Dorper	3	TALP	c	2	23.85	23.32	52.83
0.9965773997	Jul 06	Dorper	3	TALP	c	4	21.75	20.92	57.33
0.9965773997	Jul 06	Dorper	3	TALP	c	6	0.59	6.01	93.40
0.5178988092	Jul 01	Dorper	3	TrilEq	a	0	46.02	2.15	51.83
0.5178988092	Jul 01	Dorper	3	TrilEq	a	2	36.71	1.79	61.50
0.5178988092	Jul 01	Dorper	3	TrilEq	a	4	5.43	0.57	94.00
0.5178988092	Jul 01	Dorper	3	TrilEq	a	6	0.50	0.20	99.30
0.7409140982	Jul 05	Dorper	3	TrilEq	b	0	27.35	1.36	71.29
0.7409140982	Jul 05	Dorper	3	TrilEq	b	2	42.41	2.28	55.31
0.7409140982	Jul 05	Dorper	3	TrilEq	b	4	16.65	0.81	82.54
0.7409140982	Jul 05	Dorper	3	TrilEq	b	6	0.11	0.89	99.00
0.9830214746	Jul 06	Dorper	3	TrilEq	c	0	30.85	2.51	66.64
0.9830214746	Jul 06	Dorper	3	TrilEq	c	2	8.46	2.68	88.86
0.9830214746	Jul 06	Dorper	3	TrilEq	c	4	2.49	0.51	97.00
0.9830214746	Jul 06	Dorper	3	TrilEq	c	6	0.00	0.00	100.00
0.6495286212	Jul 01	Dohne Merino	9.15	Bull	a	0	53.10	2.25	44.64
0.6495286212	Jul 01	Dohne Merino	9.15	Bull	a	2	29.23	0.54	70.24
0.6495286212	Jul 01	Dohne Merino	9.15	Bull	a	4	0.00	0.00	100.00
0.6495286212	Jul 01	Dohne Merino	9.15	Bull	a	6	0.00	0.00	100.00
0.5954310992	Jul 01	Dohne Merino	9.15	Bull	c	0	45.71	2.15	52.14
0.5954310992	Jul 01	Dohne Merino	9.15	Bull	c	2	12.92	0.68	86.40
0.5954310992	Jul 01	Dohne Merino	9.15	Bull	c	4	0.00	0.00	100.00
0.5954310992	Jul 01	Dohne Merino	9.15	Bull	c	6	0.00	0.00	100.00
0.6630989024	Jul 01	Dohne Merino	9.15	Bull	c	0	47.62	1.81	50.57
0.6630989024	Jul 01	Dohne Merino	9.15	Bull	c	2	16.39	0.61	83.00
0.6630989024	Jul 01	Dohne Merino	9.15	Bull	c	4	0.00	0.00	100.00
0.6630989024	Jul 01	Dohne Merino	9.15	Bull	c	6	0.00	0.00	100.00

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Random No.	date	Breed	ram	medium	straw	t	Prog	Aber	Immot
0.1393060992	Jun 25	Dohne Merino	9.15	Dog	a	0	37.38	1.74	60.88
0.1393060992	Jun 25	Dohne Merino	9.15	Dog	a	2	36.34	1.93	61.74
0.1393060992	Jun 25	Dohne Merino	9.15	Dog	a	4	41.39	2.30	56.31
0.1393060992	Jun 25	Dohne Merino	9.15	Dog	a	6	0.00	0.00	100.00
0.3155046290	Jun 29	Dohne Merino	9.15	Dog	b	0	49.56	2.20	48.24
0.3155046290	Jun 29	Dohne Merino	9.15	Dog	b	2	39.68	7.05	53.26
0.3155046290	Jun 29	Dohne Merino	9.15	Dog	b	4	12.42	4.73	82.86
0.3155046290	Jun 29	Dohne Merino	9.15	Dog	b	6	0.00	0.00	100.00
0.6371859675	Jul 01	Dohne Merino	9.15	Dog	c	0	46.42	1.98	51.60
0.6371859675	Jul 01	Dohne Merino	9.15	Dog	c	2	34.29	1.55	64.17
0.6371859675	Jul 01	Dohne Merino	9.15	Dog	c	4	0.00	0.00	100.00
0.6371859675	Jul 01	Dohne Merino	9.15	Dog	c	6	0.00	0.00	100.00
0.4501178702	Jun 30	Dohne Merino	9.15	Ram	a	0	42.98	1.86	55.17
0.4501178702	Jun 30	Dohne Merino	9.15	Ram	a	2	41.84	1.35	56.81
0.4501178702	Jun 30	Dohne Merino	9.15	Ram	a	4	29.41	1.02	69.57
0.4501178702	Jun 30	Dohne Merino	9.15	Ram	a	6	19.33	0.96	79.71
0.3601350993	Jun 29	Dohne Merino	9.15	Ram	b	0	43.67	1.93	54.40
0.3601350993	Jun 29	Dohne Merino	9.15	Ram	b	2	48.08	1.76	50.17
0.3601350993	Jun 29	Dohne Merino	9.15	Ram	b	4	29.39	2.14	68.48
0.3601350993	Jun 29	Dohne Merino	9.15	Ram	b	6	36.10	1.35	62.55
0.3654583245	Jun 29	Dohne Merino	9.15	Ram	c	0	52.15	2.18	45.67
0.3654583245	Jun 29	Dohne Merino	9.15	Ram	c	2	52.93	2.07	45.00
0.3654583245	Jun 29	Dohne Merino	9.15	Ram	c	4	36.93	1.90	61.17
0.3654583245	Jun 29	Dohne Merino	9.15	Ram	c	6	39.88	1.47	58.64
0.4547851224	Jun 30	Dohne Merino	9.15	Skim milk	a	0	55.85	2.08	42.07
0.4547851224	Jun 30	Dohne Merino	9.15	Skim milk	a	2	55.62	1.69	42.69
0.4547851224	Jun 30	Dohne Merino	9.15	Skim milk	a	4	59.52	1.77	38.71
0.4547851224	Jun 30	Dohne Merino	9.15	Skim milk	a	6	55.77	2.26	41.98
0.3015174233	Jun 29	Dohne Merino	9.15	Skim milk	b	0	49.46	2.14	48.40
0.3015174233	Jun 29	Dohne Merino	9.15	Skim milk	b	2	49.62	1.97	48.40
0.3015174233	Jun 29	Dohne Merino	9.15	Skim milk	b	4	51.57	2.59	45.83
0.3015174233	Jun 29	Dohne Merino	9.15	Skim milk	b	6	49.98	1.62	48.40
0.6727283161	Jul 05	Dohne Merino	9.15	Skim milk	c	0	50.31	2.15	47.55
0.6727283161	Jul 05	Dohne Merino	9.15	Skim milk	c	2	50.92	2.49	46.60
0.6727283161	Jul 05	Dohne Merino	9.15	Skim milk	c	4	58.88	1.88	39.24
0.6727283161	Jul 05	Dohne Merino	9.15	Skim milk	c	6	49.87	2.08	48.05
0.2866593593	Jun 29	Dohne Merino	9.15	TALP	a	0	52.53	1.89	45.57
0.2866593593	Jun 29	Dohne Merino	9.15	TALP	a	2	23.75	22.13	54.12
0.2866593593	Jun 29	Dohne Merino	9.15	TALP	a	4	19.39	19.70	60.90
0.2866593593	Jun 29	Dohne Merino	9.15	TALP	a	6	11.87	18.90	69.24
0.8049080326	Jul 06	Dohne Merino	9.15	TALP	b	0	48.49	1.85	49.67
0.8049080326	Jul 06	Dohne Merino	9.15	TALP	b	2	33.85	21.74	44.40
0.8049080326	Jul 06	Dohne Merino	9.15	TALP	b	4	22.46	21.16	56.38
0.8049080326	Jul 06	Dohne Merino	9.15	TALP	b	6	16.06	19.27	64.67
0.4202048980	Jun 30	Dohne Merino	9.15	TALP	c	0	45.02	1.81	53.17
0.4202048980	Jun 30	Dohne Merino	9.15	TALP	c	2	33.05	22.07	44.88
0.4202048980	Jun 30	Dohne Merino	9.15	TALP	c	4	27.69	19.17	53.14
0.4202048980	Jun 30	Dohne Merino	9.15	TALP	c	6	21.10	17.47	61.43
0.7098934168	Jul 05	Dohne Merino	9.15	TrilEq	a	0	60.42	2.39	37.19
0.7098934168	Jul 05	Dohne Merino	9.15	TrilEq	a	2	61.79	3.02	35.19
0.7098934168	Jul 05	Dohne Merino	9.15	TrilEq	a	4	59.08	2.39	38.52
0.7098934168	Jul 05	Dohne Merino	9.15	TrilEq	a	6	55.72	2.44	41.83
0.2822796333	Jun 29	Dohne Merino	9.15	TrilEq	b	0	43.66	2.03	54.31
0.2822796333	Jun 29	Dohne Merino	9.15	TrilEq	b	2	13.05	33.14	53.81
0.2822796333	Jun 29	Dohne Merino	9.15	TrilEq	b	4	53.67	2.59	43.74
0.2822796333	Jun 29	Dohne Merino	9.15	TrilEq	b	6	52.77	1.94	45.29

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0.5975478766	Jul 01	Dohne Merino	9.15	TrilEq	c	0	55.35	2.39	42.26
0.5975478766	Jul 01	Dohne Merino	9.15	TrilEq	c	2	53.97	8.60	37.43
0.5975478766	Jul 01	Dohne Merino	9.15	TrilEq	c	4	46.80	1.94	51.26
0.5975478766	Jul 01	Dohne Merino	9.15	TrilEq	c	6	51.47	1.94	46.60
0.6434382877	Jul 01	Dohne Merino	9.26	Bull	a	0	48.19	2.17	49.64
0.6434382877	Jul 01	Dohne Merino	9.26	Bull	a	2	27.21	0.00	72.79
0.6434382877	Jul 01	Dohne Merino	9.26	Bull	a	4	0.00	0.00	100.00
0.6434382877	Jul 01	Dohne Merino	9.26	Bull	a	6	0.00	0.00	100.00
0.4825381184	Jun 30	Dohne Merino	9.26	Bull	b	0	48.82	2.20	48.98
0.4825381184	Jun 30	Dohne Merino	9.26	Bull	b	2	2.50	2.50	95.00
0.4825381184	Jun 30	Dohne Merino	9.26	Bull	b	4	0.00	0.00	100.00
0.4825381184	Jun 30	Dohne Merino	9.26	Bull	b	6	0.00	0.00	100.00
0.5618393994	Jul 01	Dohne Merino	9.26	Bull	c	0	46.05	1.28	52.67
0.5618393994	Jul 01	Dohne Merino	9.26	Bull	c	2	0.00	0.00	100.00
0.5618393994	Jul 01	Dohne Merino	9.26	Bull	c	4	0.00	0.00	100.00
0.5618393994	Jul 01	Dohne Merino	9.26	Bull	c	6	0.00	0.00	100.00
0.5131679889	Jun 30	Dohne Merino	9.26	Dog	a	0	44.34	2.26	53.40
0.5131679889	Jun 30	Dohne Merino	9.26	Dog	a	2	41.18	3.09	55.74
0.5131679889	Jun 30	Dohne Merino	9.26	Dog	a	4	46.06	1.75	52.19
0.5131679889	Jun 30	Dohne Merino	9.26	Dog	a	6	46.87	1.49	51.64
0.3796380175	Jun 29	Dohne Merino	9.26	Dog	b	0	28.04	1.89	70.07
0.3796380175	Jun 29	Dohne Merino	9.26	Dog	b	2	24.81	2.36	72.83
0.3796380175	Jun 29	Dohne Merino	9.26	Dog	b	4	29.58	2.88	67.55
0.3796380175	Jun 29	Dohne Merino	9.26	Dog	b	6	11.17	0.83	88.00
0.0223920395	Jun 24	Dohne Merino	9.26	Dog	c	0	17.45	2.72	79.83
0.0223920395	Jun 24	Dohne Merino	9.26	Dog	c	2	15.78	5.73	78.50
0.0223920395	Jun 24	Dohne Merino	9.26	Dog	c	4	3.89	1.47	94.64
0.0223920395	Jun 24	Dohne Merino	9.26	Dog	c	6	0.11	2.49	97.40
0.1356091571	Jun 25	Dohne Merino	9.26	Ram	a	0	25.71	1.13	73.17
0.1356091571	Jun 25	Dohne Merino	9.26	Ram	a	2	22.12	2.00	75.88
0.1356091571	Jun 25	Dohne Merino	9.26	Ram	a	4	7.13	2.38	90.50
0.1356091571	Jun 25	Dohne Merino	9.26	Ram	a	6	0.31	5.29	94.40
0.1058803818	Jun 24	Dohne Merino	9.26	Ram	b	0	42.06	2.04	55.90
0.1058803818	Jun 24	Dohne Merino	9.26	Ram	b	2	24.40	3.56	72.05
0.1058803818	Jun 24	Dohne Merino	9.26	Ram	b	4	17.33	1.74	80.93
0.1058803818	Jun 24	Dohne Merino	9.26	Ram	b	6	0.01	0.99	99.00
0.9707492535	Jul 06	Dohne Merino	9.26	Ram	c	0	63.37	1.92	34.71
0.9707492535	Jul 06	Dohne Merino	9.26	Ram	c	2	50.36	1.81	47.83
0.9707492535	Jul 06	Dohne Merino	9.26	Ram	c	4	36.20	1.64	62.17
0.9707492535	Jul 06	Dohne Merino	9.26	Ram	c	6	1.89	10.00	88.11
0.4285105548	Jun 30	Dohne Merino	9.26	Skim milk	a	0	52.87	2.34	44.79
0.4285105548	Jun 30	Dohne Merino	9.26	Skim milk	a	2	47.35	1.82	50.83
0.4285105548	Jun 30	Dohne Merino	9.26	Skim milk	a	4	49.01	1.87	49.12
0.4285105548	Jun 30	Dohne Merino	9.26	Skim milk	a	6	51.12	3.19	45.69
0.6752409194	Jul 05	Dohne Merino	9.26	Skim milk	b	0	52.53	2.59	44.88
0.6752409194	Jul 05	Dohne Merino	9.26	Skim milk	b	2	60.16	1.63	38.21
0.6752409194	Jul 05	Dohne Merino	9.26	Skim milk	b	4	59.55	1.88	38.57
0.6752409194	Jul 05	Dohne Merino	9.26	Skim milk	b	6	52.07	2.00	45.93
0.8324231784	Jul 06	Dohne Merino	9.26	Skim milk	c	0	51.91	1.69	46.40
0.8324231784	Jul 06	Dohne Merino	9.26	Skim milk	c	2	44.38	1.55	54.07
0.8324231784	Jul 06	Dohne Merino	9.26	Skim milk	c	4	36.63	1.63	61.74
0.8324231784	Jul 06	Dohne Merino	9.26	Skim milk	c	6	26.72	1.54	71.74

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0.9190636059	Jul 06	Dohne Merino	9.26	TALP	a	0	40.62	1.57	57.81
0.9190636059	Jul 06	Dohne Merino	9.26	TALP	a	2	18.84	21.42	59.74
0.9190636059	Jul 06	Dohne Merino	9.26	TALP	a	4	16.09	22.24	61.67
0.9190636059	Jul 06	Dohne Merino	9.26	TALP	a	6	14.49	16.77	68.74
0.7208679564	Jul 05	Dohne Merino	9.26	TALP	b	0	48.60	1.81	49.60
0.7208679564	Jul 05	Dohne Merino	9.26	TALP	b	2	34.67	20.69	44.64
0.7208679564	Jul 05	Dohne Merino	9.26	TALP	b	4	21.26	22.07	56.67
0.7208679564	Jul 05	Dohne Merino	9.26	TALP	b	6	4.30	15.70	80.00
0.1692123158	Jun 25	Dohne Merino	9.26	TALP	c	0	30.98	2.78	66.24
0.1692123158	Jun 25	Dohne Merino	9.26	TALP	c	2	5.60	14.90	79.50
0.1692123158	Jun 25	Dohne Merino	9.26	TALP	c	4	2.34	41.59	56.07
0.1692123158	Jun 25	Dohne Merino	9.26	TALP	c	6	3.06	12.44	84.50
0.2654960255	Jun 29	Dohne Merino	9.26	TrilEq	a	0	43.24	2.03	54.74
0.2654960255	Jun 29	Dohne Merino	9.26	TrilEq	a	2	6.08	30.18	63.74
0.2654960255	Jun 29	Dohne Merino	9.26	TrilEq	a	4	44.36	1.52	54.12
0.2654960255	Jun 29	Dohne Merino	9.26	TrilEq	a	6	0.01	0.99	99.00
0.6139724655	Jul 01	Dohne Merino	9.26	TrilEq	b	0	36.09	2.02	61.88
0.6139724655	Jul 01	Dohne Merino	9.26	TrilEq	b	2	46.25	2.29	51.45
0.6139724655	Jul 01	Dohne Merino	9.26	TrilEq	b	4	25.30	0.98	73.71
0.6139724655	Jul 01	Dohne Merino	9.26	TrilEq	b	6	6.53	0.47	93.00
0.2137494418	Jun 25	Dohne Merino	9.26	TrilEq	c	0	28.34	6.85	64.81
0.2137494418	Jun 25	Dohne Merino	9.26	TrilEq	c	2	44.26	1.90	53.83
0.2137494418	Jun 25	Dohne Merino	9.26	TrilEq	c	4	25.26	1.07	73.67
0.2137494418	Jun 25	Dohne Merino	9.26	TrilEq	c	6	16.57	2.86	80.57
0.1244433242	Jun 24	Merino	435	Bull	a	0	27.01	1.94	71.05
0.1244433242	Jun 24	Merino	435	Bull	a	2	0.01	0.59	99.40
0.1244433242	Jun 24	Merino	435	Bull	a	4	0.00	0.00	100.00
0.1244433242	Jun 24	Merino	435	Bull	a	6	0.00	0.00	100.00
0.0849889580	Jun 24	Merino	435	Bull	b	0	46.05	1.95	52.00
0.0849889580	Jun 24	Merino	435	Bull	b	2	16.66	2.91	80.43
0.0849889580	Jun 24	Merino	435	Bull	b	4	0.00	0.00	100.00
0.0849889580	Jun 24	Merino	435	Bull	b	6	0.00	0.00	100.00
0.1206477287	Jun 24	Merino	435	Bull	c	0	54.74	1.73	43.52
0.1206477287	Jun 24	Merino	435	Bull	c	2	1.60	2.80	95.60
0.1206477287	Jun 24	Merino	435	Bull	c	4	0.00	0.00	100.00
0.1206477287	Jun 24	Merino	435	Bull	c	6	0.00	0.00	100.00
0.0805940470	Jun 24	Merino	435	Dog	a	0	48.83	1.67	49.50
0.0805940470	Jun 24	Merino	435	Dog	a	2	29.75	3.37	66.88
0.0805940470	Jun 24	Merino	435	Dog	a	4	35.49	2.29	62.21
0.0805940470	Jun 24	Merino	435	Dog	a	6	35.24	1.62	63.14
0.1637103724	Jun 25	Merino	435	Dog	b	0	30.29	2.37	67.33
0.1637103724	Jun 25	Merino	435	Dog	b	2	12.83	1.88	85.29
0.1637103724	Jun 25	Merino	435	Dog	b	4	37.53	3.74	58.74
0.1637103724	Jun 25	Merino	435	Dog	b	6	31.35	2.43	66.21
0.2022742843	Jun 25	Merino	435	Dog	c	0	32.29	2.06	65.64
0.2022742843	Jun 25	Merino	435	Dog	c	2	16.96	3.36	79.69
0.2022742843	Jun 25	Merino	435	Dog	c	4	11.92	1.11	86.97
0.2022742843	Jun 25	Merino	435	Dog	c	6	37.63	1.71	60.67
0.5162844634	Jul 01	Merino	435	Ram	a	0	46.87	1.49	51.64
0.5162844634	Jul 01	Merino	435	Ram	a	2	34.07	1.27	64.67
0.5162844634	Jul 01	Merino	435	Ram	a	4	32.05	1.21	66.74
0.5162844634	Jul 01	Merino	435	Ram	a	6	7.16	0.37	92.47
0.6612187472	Jul 01	Merino	435	Ram	a	0	19.08	1.42	79.50
0.6612187472	Jul 01	Merino	435	Ram	a	2	14.75	0.40	84.86
0.6612187472	Jul 01	Merino	435	Ram	a	4	3.78	0.42	95.80
0.6612187472	Jul 01	Merino	435	Ram	a	6	0.01	0.99	99.00

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0.2434318211	Jun 25	Merino	435	Ram	c	0	25.46	1.30	73.24
0.2434318211	Jun 25	Merino	435	Ram	c	2	5.74	1.36	92.90
0.2434318211	Jun 25	Merino	435	Ram	c	4	1.49	3.51	95.00
0.2434318211	Jun 25	Merino	435	Ram	c	6	0.01	0.99	99.00
0.6312667599	Jul 01	Merino	435	Skim milk	a	0	51.22	1.85	46.93
0.6312667599	Jul 01	Merino	435	Skim milk	a	2	43.73	1.56	54.71
0.6312667599	Jul 01	Merino	435	Skim milk	a	4	36.44	1.75	61.81
0.6312667599	Jul 01	Merino	435	Skim milk	a	6	50.18	1.74	48.07
0.3357744706	Jun 29	Merino	435	Skim milk	b	0	39.71	2.06	58.24
0.3357744706	Jun 29	Merino	435	Skim milk	b	2	37.37	1.84	60.79
0.3357744706	Jun 29	Merino	435	Skim milk	b	4	38.18	2.06	59.76
0.3357744706	Jun 29	Merino	435	Skim milk	b	6	36.10	1.28	62.62
0.4585732834	Jun 30	Merino	435	Skim milk	c	0	59.26	2.53	38.21
0.4585732834	Jun 30	Merino	435	Skim milk	c	2	39.68	1.77	58.55
0.4585732834	Jun 30	Merino	435	Skim milk	c	4	51.59	1.74	46.67
0.4585732834	Jun 30	Merino	435	Skim milk	c	6	49.25	1.80	48.95
0.6732525711	Jul 05	Merino	435	TALP	a	0	40.44	1.89	57.67
0.6732525711	Jul 05	Merino	435	TALP	a	2	21.04	20.89	58.07
0.6732525711	Jul 05	Merino	435	TALP	a	4	16.14	28.62	55.24
0.6732525711	Jul 05	Merino	435	TALP	a	6	13.78	23.32	62.90
0.7051254306	Jul 05	Merino	435	TALP	b	0	14.67	0.93	84.40
0.7051254306	Jul 05	Merino	435	TALP	b	2	1.90	10.10	88.00
0.7051254306	Jul 05	Merino	435	TALP	b	4	0.58	6.12	93.30
0.7051254306	Jul 05	Merino	435	TALP	b	6	0.01	0.99	99.00
0.2448389374	Jun 25	Merino	435	TALP	c	0	25.08	1.64	73.29
0.2448389374	Jun 25	Merino	435	TALP	c	2	25.75	3.11	71.14
0.2448389374	Jun 25	Merino	435	TALP	c	4	1.84	9.59	88.57
0.2448389374	Jun 25	Merino	435	TALP	c	6	2.87	13.27	83.86
0.4776315571	Jun 30	Merino	435	TrilEq	a	0	27.87	1.32	70.81
0.4776315571	Jun 30	Merino	435	TrilEq	a	2	20.49	2.10	77.40
0.4776315571	Jun 30	Merino	435	TrilEq	a	4	16.27	0.66	83.07
0.4776315571	Jun 30	Merino	435	TrilEq	a	6	0.06	1.74	98.20
0.0105693880	Jun 24	Merino	435	TrilEq	b	0	32.82	2.34	64.83
0.0105693880	Jun 24	Merino	435	TrilEq	b	2	49.51	3.27	47.21
0.0105693880	Jun 24	Merino	435	TrilEq	b	4	43.83	2.24	53.93
0.0105693880	Jun 24	Merino	435	TrilEq	b	6	45.94	1.68	52.38
0.2412168455	Jun 25	Merino	435	TrilEq	c	0	12.27	1.52	86.21
0.2412168455	Jun 25	Merino	435	TrilEq	c	2	8.09	2.70	89.21
0.2412168455	Jun 25	Merino	435	TrilEq	c	4	4.13	0.88	95.00
0.2412168455	Jun 25	Merino	435	TrilEq	c	6	0.01	0.99	99.00
0.8719577754	Jul 06	Merino	436	Bull	a	0	41.21	1.41	57.38
0.8719577754	Jul 06	Merino	436	Bull	a	2	0.00	0.00	100.00
0.8719577754	Jul 06	Merino	436	Bull	a	4	0.00	0.00	100.00
0.8719577754	Jul 06	Merino	436	Bull	a	6	0.00	0.00	100.00
0.7630195352	Jul 06	Merino	436	Bull	b	0	34.16	1.05	64.79
0.7630195352	Jul 06	Merino	436	Bull	b	2	0.00	0.00	100.00
0.7630195352	Jul 06	Merino	436	Bull	b	4	0.00	0.00	100.00
0.7630195352	Jul 06	Merino	436	Bull	b	6	0.00	0.00	100.00
0.2586912699	Jun 29	Merino	436	Bull	c	0	26.98	1.35	71.67
0.2586912699	Jun 29	Merino	436	Bull	c	2	0.00	0.00	100.00
0.2586912699	Jun 29	Merino	436	Bull	c	4	0.00	0.00	100.00
0.2586912699	Jun 29	Merino	436	Bull	c	6	0.00	0.00	100.00
0.2127281383	Jun 25	Merino	436	Dog	a	0	29.31	1.78	68.90
0.2127281383	Jun 25	Merino	436	Dog	a	2	10.71	3.57	85.71
0.2127281383	Jun 25	Merino	436	Dog	a	4	10.68	0.85	88.47
0.2127281383	Jun 25	Merino	436	Dog	a	6	0.00	0.10	99.90

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0.8509040377	Jul 06	Merino	436	Dog	b	0	44.74	1.95	53.31
0.8509040377	Jul 06	Merino	436	Dog	b	2	36.14	2.65	61.21
0.8509040377	Jul 06	Merino	436	Dog	b	4	0.01	0.99	99.00
0.8509040377	Jul 06	Merino	436	Dog	b	6	0.00	0.00	100.00
0.4909131432	Jun 30	Merino	436	Dog	c	0	40.86	1.74	57.40
0.4909131432	Jun 30	Merino	436	Dog	c	2	18.06	3.65	78.29
0.4909131432	Jun 30	Merino	436	Dog	c	4	9.69	0.63	89.69
0.4909131432	Jun 30	Merino	436	Dog	c	6	0.00	0.00	100.00
0.9558328319	Jul 06	Merino	436	Ram	a	0	42.08	1.75	56.17
0.9558328319	Jul 06	Merino	436	Ram	a	2	24.47	2.15	73.38
0.9558328319	Jul 06	Merino	436	Ram	a	4	0.01	0.99	99.00
0.9558328319	Jul 06	Merino	436	Ram	a	6	0.00	0.00	100.00
0.0575301192	Jun 24	Merino	436	Ram	b	0	3.75	1.25	95.00
0.0575301192	Jun 24	Merino	436	Ram	b	2	6.32	2.11	91.57
0.0575301192	Jun 24	Merino	436	Ram	b	4	30.12	2.00	67.88
0.0575301192	Jun 24	Merino	436	Ram	b	6	0.00	0.00	100.00
0.5973495250	Jul 01	Merino	436	Ram	c	0	32.47	1.86	65.67
0.5973495250	Jul 01	Merino	436	Ram	c	2	28.99	2.08	68.93
0.5973495250	Jul 01	Merino	436	Ram	c	4	5.00	0.00	95.00
0.5973495250	Jul 01	Merino	436	Ram	c	6	0.00	0.00	100.00
0.3997399600	Jun 30	Merino	436	Skim milk	a	0	39.56	2.61	57.83
0.3997399600	Jun 30	Merino	436	Skim milk	a	2	36.80	1.82	61.38
0.3997399600	Jun 30	Merino	436	Skim milk	a	4	24.02	1.57	74.40
0.3997399600	Jun 30	Merino	436	Skim milk	a	6	41.44	1.85	56.71
0.4139327226	Jun 30	Merino	436	Skim milk	b	0	50.56	2.56	46.88
0.4139327226	Jun 30	Merino	436	Skim milk	b	2	28.86	1.52	69.62
0.4139327226	Jun 30	Merino	436	Skim milk	b	4	34.92	1.34	63.74
0.4139327226	Jun 30	Merino	436	Skim milk	b	6	43.59	1.91	54.50
0.6707602891	Jul 05	Merino	436	Skim milk	c	0	26.69	1.45	71.86
0.6707602891	Jul 05	Merino	436	Skim milk	c	2	34.66	1.60	63.74
0.6707602891	Jul 05	Merino	436	Skim milk	c	4	24.30	1.41	74.29
0.6707602891	Jul 05	Merino	436	Skim milk	c	6	18.59	1.41	80.00
0.2571571439	Jun 29	Merino	436	TALP	a	0	3.65	22.97	73.38
0.2571571439	Jun 29	Merino	436	TALP	a	2	5.45	14.31	80.24
0.2571571439	Jun 29	Merino	436	TALP	a	4	2.12	12.02	85.86
0.2571571439	Jun 29	Merino	436	TALP	a	6	0.08	1.82	98.10
0.2270172446	Jun 25	Merino	436	TALP	b	0	9.49	3.44	87.07
0.2270172446	Jun 25	Merino	436	TALP	b	2	3.88	1.62	94.50
0.2270172446	Jun 25	Merino	436	TALP	b	4	0.33	2.17	97.50
0.2270172446	Jun 25	Merino	436	TALP	b	6	0.08	1.92	98.00
0.9438951047	Jul 06	Merino	436	TALP	c	0	25.30	2.53	72.17
0.9438951047	Jul 06	Merino	436	TALP	c	2	9.74	18.62	71.64
0.9438951047	Jul 06	Merino	436	TALP	c	4	0.55	6.45	93.00
0.9438951047	Jul 06	Merino	436	TALP	c	6	0.01	0.99	99.00
0.9745989098	Jul 06	Merino	436	TrilEq	a	0	44.70	2.23	53.07
0.9745989098	Jul 06	Merino	436	TrilEq	a	2	43.33	1.89	54.79
0.9745989098	Jul 06	Merino	436	TrilEq	a	4	24.51	1.94	73.55
0.9745989098	Jul 06	Merino	436	TrilEq	a	6	0.24	4.36	95.40
0.6643838488	Jul 05	Merino	436	TrilEq	b	0	42.86	2.99	54.14
0.6643838488	Jul 05	Merino	436	TrilEq	b	2	32.56	2.27	65.17
0.6643838488	Jul 05	Merino	436	TrilEq	b	4	22.27	1.61	76.12
0.6643838488	Jul 05	Merino	436	TrilEq	b	6	3.00	0.00	97.00
0.6740408879	Jul 05	Merino	436	TrilEq	c	0	45.52	1.93	52.55
0.6740408879	Jul 05	Merino	436	TrilEq	c	2	52.60	1.85	45.55
0.6740408879	Jul 05	Merino	436	TrilEq	c	4	30.51	0.54	68.95
0.6740408879	Jul 05	Merino	436	TrilEq	c	6	0.90	0.00	99.10

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