# Effect of diluent, dilution rate and storage temperature on longevity and functional integrity of liquid stored alpaca (*Vicugna pacos*) semen.

Morton K. M. [1*], Gibb Z., Bertoldo M. and Chis Maxwell W. M.

Centre for Advanced Technologies in Animal Genetics and Reproduction (ReproGen), Faculty of Veterinary Science, University of Sydney, NSW 2006, Australia.
[1]Present Address: Camel Reproduction Centre, Dubai, UAE.

## Abstract

Liquid storage of alpaca semen was investigated by assessing the motility and acrosome integrity of sperm at 24, 48 and 72 h after i) dilution 1:4 (v/v) with Androhep®, Biladyl®, Lactose, Salamon's and Triladyl® diluents and liquid storage at 4 or 15°C (Experiment 1), ii) dilution 1:1, 1:2, or 1:4 (v/v) with Biladyl® and storage at 4 or 15°C (Experiment 2) and iii) dilution of epididymal sperm with Biladyl® and storage at 4°C for 24 h. In Experiment 1, sperm motility was higher after storage in Biladyl® compared with other diluents ($P<0.05$), and sperm motility was similar for Lactose and Triladyl® diluents but lower for Androhep® and Salamon's diluents ($P<0.05$). Sperm motility was similar at 24 and 48 h after storage at 4 and 15°C, but higher at 72 h after storage at 4°C ($P<0.05$). Acrosome integrity did not decline until 72 h of liquid storage. In Experiment 2, sperm motility was higher after dilution 1:4 than 1:1 or 1:2 with Biladyl® ($P<0.05$). Acrosome integrity was not reduced until 72 h of liquid storage and was not influenced by dilution rate or storage temperature. Epididymal sperm motility and acrosome integrity were similar immediately after harvest (53.0±2.1% and 92.3±2.0% respectively, $P>0.05$) and after 24 h of liquid storage at 4°C (47.0±2.0% and 90.6±0.9%, respectively $P>0.05$). Ejaculated semen and epididymal alpaca sperm can be liquid stored for 72 h and 24 h respectively. Dilution of ejaculated semen 1:4 v/v in Biladyl® results in higher motility and longevity compared with the other diluents. Storage at 4°C is recommended beyond 48 h but 4 and 15°C are equally efficacious for <48 h storage periods. Improvements to longevity and a determination of its fertilizing capacity are required before liquid stored alpaca semen could be used for AI.

Keywords: Camelidae; semen; liquid storage

*Corresponding author: Katherine M. Morton, Ph.D., Camel Reproduction Centre, P.O. Box 79914, Dubai, United Arab Emirates, E-mail: katherine.morton@yahoo.com

## 1. Introduction

Artificial insemination (AI) facilitates the widespread use of genetics from superior sires thereby increasing the rate of genetic gain and is an important tool for animal breeding programs. There is considerable interest in the use of AI in camelid breeding programs. At present the lack of efficient methods to preserve the fertilising lifespan of semen in liquid or frozen form prevents the widespread use of AI in camelids. Successful semen storage is dependant on the reversible reduction in metabolic activity and motility, which prolongs the fertilising lifespan of sperm (Evans and Maxwell 1987). This can be achieved by storing sperm in a liquid or frozen state. Cryopreservation of sperm in camelids is inefficient, primarily owing to a lack of knowledge regarding camelid sperm physiology and the viscous nature of the seminal plasma (Bravo et al. 2000b).

As camelid sperm are generally not tolerant to freezing and thawing

procedures (Morton et al. 2008), liquid storage of sperm may facilitate the development of AI technology. There have been few reports on the liquid storage of camelid semen and the optimal conditions remain to be elucidated. The few studies on liquid storage have generally reported poor results (Dromedary camel: Hassan et al. 1995, Deen et al. 2005; llama: Huanca and Gauly 2001, Giuliano et al. 2006b). While pregnancies have been reported recently after AI with liquid stored semen in both Old and New World camelids, the pregnancy rates were disappointing. Skidmore (2005) reported pregnancy rates of 25–30% after AI with liquid stored sperm (24 h, 4°C) in Dromedary camels, and Giuliano et al. (2006a) reported a 13 % conception rate when llamas were AI with liquid stored semen. These results are encouraging; however, the efficiency is still below commercially acceptable levels.

During liquid storage, the metabolic activity of sperm is reduced by preservation at a reduced temperature, in a slightly acidic environment, gassing diluents with $CO_2$ or under anaerobic ($N_2$) conditions (Vishwanath and Shannon 2000). However, optimal storage conditions are species-specific, reflecting differences in sperm physiology and preferences for pH buffers, diluent tonicity, energy sources, tolerance to cold shock and susceptibility to oxidative damage (Morton et al 2008).

These parameters have yet to be thoroughly investigated for alpacas and development of a suitable liquid storage method will depend on first determining the appropriate basic parameters, such as diluent, storage temperature and dilution rate. The aims of the present study were to i) investigate the effect on sperm longevity and functional integrity of diluent (Androhep®, Biladyl®, Lactose, Salamon's and Triladyl®) and storage temperatures (4 and 15°C), as well as interactions between diluents and storage temperatures, ii) examine the effect of dilution rate (1:1, 1:2, and 1:4 v/v) and any possible interactions between dilution rate and storage temperature, and iii) determine if the methods elucidated from Experiments 1 and 2 could be applied to epididymal alpaca sperm.

## 2. Materials and Methods

Procedures described herein were approved by The University of Sydney's Animal Ethics Committee and performed in accordance with the Australian Code of Practice for Care and Use of Animals for Scientific Purposes.

### 2.1. Animals

Male alpacas (n=16; 7.03±0.6 years old) were maintained under field conditions at The University of Sydney's Animal Reproduction Unit, Cobbitty, NSW, Australia for the duration of the experimental period (June to November 2006). Alpacas were maintained on native pastures, exposed to natural day length and ambient temperatures and supplemented with Lucerne hay (*Medicago sativa*) and commercially formulated alpaca pellets as required.

### 2.2. Reagents and media

Chemicals used were of analytical grade and, where possible, cell culture tested by the manufacturer. All media components were purchased from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated. Androhep®, Biladyl® (fraction A only) and Triladyl® (Minitüb, Tiefenbach, Germany) were reconstituted according to the manufacturers instructions.

Lactose diluent consisted of 11% (w:v) lactose as described by Morton et al. (2007). Salamon's diluent was modified from that described by Evans and Maxwell (1987) and consisted of 300 mM Tris (hydroxymethyl) aminomethane, 27.8 mM fructose, 94.7 mM citric acid. All diluents were prepared using tissue-culture grade filtered water (0.22 μm, Millipore). Diluents were supplemented with 20% (v/v) hens egg yolk, ultracentrifuged (10,000 *g*; 30 min) and then aliquoted (10 mL) for frozen storage at –20°C until use (within 4 weeks).

2.3 Semen collection

Semen was collected as previously described by Morton et al. (2008a,b, 2009). Briefly, semen was collected using a rubber sheep AV (IMV Technologies, L'Aigle, France) with a silicone liner and a camel collecting glass (IMV Technologies). The artificial vagina (AV) was then wrapped in an electric heating pad (Bodyzone™ Heat Pad HP700; Breville, Botany, NSW, Australia), mounted inside the wooden mannequin and placed in the semen collection pen. A male alpaca was allowed to enter the pen and mate with the mannequin. After mating ceased, the AV was removed from the mannequin. Viscous semen contained within the AV was forced into the collecting glass by repeated, sharp downwards thrusts. Collecting glasses containing semen were stored in a 30°C waterbath until evaluation, dilution and processing (which occurred within 10 min of collection).

2.4. Semen evaluation and assessment of sperm characteristics

Ejaculate volume, semen viscosity, sperm concentration, sperm motility, acrosomal status and morphology were determined on neat semen samples. Semen evaluation and sperm assessments were performed as described by Morton et al. (2007, 2009). Graduations on the collecting glass were used to determine ejaculate volume and the thread formation technique (Morton et al. 2008a, 2009) was used to evaluate semen viscosity. Briefly, 50 μL neat semen was removed from the ejaculate using a micropipette and approximately 25 μL was pipetted on to a glass slide. A thread of semen was formed by slowly raising the pipette. The distance between the pipette and the slide at the time the thread broke was measured using a ruler and recorded as the viscosity.

Sperm motility patterns were classified subjectively as either oscillatory or progressive. Sperm traveling at greater than one body length per second were considered to display forward progressive motility, and sperm which did not display forward motility were considered oscillatory. Sperm motility was estimated subjectively to the nearest 5% by examining five fields of the neat or diluted semen sample (10 μL) placed on a pre-warmed slide using phase contrast microscopy (X 100, Olympus, Tokyo, Japan; Evans and Maxwell, 1987). Sperm concentration was determined using a haemocytometer as described by Evans and Maxwell (1987).

Acrosome integrity was assessed by fluorescent isothiocyanate-conjugated peanut agglutin (FITC-PNA) as described by Morton et al. (2007, 2008a). Briefly, an aliquot of sperm was smeared on a slide, air dried and fixed for 30 sec in 96% (v:v) ethanol. Slides were then stored at 4°C for 2-4 wk. Immediately before assessment slides were stained with 100 μg/mL FITC-PNA (Sigma-Aldrich) in phosphate buffered saline (Sigma-Aldrich) for 30 min in a humid 37°C atmosphere. Slides were then rinsed

with PBS to remove excess stain and placed in the dark at 37°C to dry. Fade retardant consisting of 90% (v:v) glycerol (Sigma-Aldrich), 10% (v:v) PBS (Sigma-Aldrich) and 0.1% (w:v) *p*-phenylenediamine (Sigma-Aldrich) was then placed on the stained area of the slide and covered with a cover slip. Sperm were considered acrosome intact if the acrosome stained green while those with no staining or a single band of green staining at the equatorial segment were considered as having non-intact acrosomes.

2.5. Experimental design

**Experiment 1:** Effect of diluent (Androhep®, Biladyl®, Lactose, Salamon's or Triladyl®) and storage temperature (4 or 15°C) on the longevity and functional integrity of liquid stored alpaca sperm

Semen was collected from alpaca males (n=6; 4 ejaculates per male) twice weekly, and assessed for quality parameters. Semen was then divided into five aliquots and diluted 1:4 (v/v) with Androhep®, Biladyl®, Lactose, Salamon's and Triladyl® diluents. Dilution was performed dropwise, and after 1:1 (v/v) dilution with the appropriate diluent, parts of the viscous semen were visible as opaque masses within the diluted semen. The extended semen was then gently pipetted with a 1.0 mL pipette to break up the viscous semen and facilitate proper mixing of the diluent. Pipetting ceased when the opaque mass was no longer visible which coincided with a reduction in semen viscosity (Morton et al. 2008). Aliquots of diluted semen were divided into two parts for storage at 4 and 15°C. Tubes containing diluted semen were placed water jackets (consisting of foil covered plastic boar semen AI bottles (Minitüb, Tiefenbach, Germany) filled with 200 mL of 30°C water and placed inside either a 4 or 15°C refrigerator and cooled to 4 or 15°C over 2 h. Sperm motility and acrosome integrity were assessed immediately after collection, dilution (0 h) and 24, 48, and 72 h after liquid storage.

**Experiment 2:** Effect of dilution rate (1:1, 1:2, or 1:4 v/v) and storage temperature (4 or 15°C) on the longevity and functional integrity of liquid stored alpaca sperm

Semen was collected from alpaca males (n=4; 3 ejaculates per male) and assessed for quality parameters. Semen was then divided into three aliquots and diluted as described above 1:1, 1:2 or 1:4 (v/v) with Biladyl®. Diluted semen was then divided into two parts for at 4 and 15°C. Sperm motility and acrosome integrity were assessed immediately after collection, dilution (0 h) and 24, 48, and 72 h after liquid storage.

**Experiment 3 (pilot study):** liquid storage of epididymal alpaca sperm

Based on the results of Experiments 1 and 2, a pilot study was conducted to investigate the liquid storage of epididymal alpaca sperm. Testes and epididymides from castrated alpaca males (n=10, age: 32.5 ± 1.8 mo) were transported overnight to the laboratory using the method described by Morton et al (2007). Briefly, testes and epididymides were wrapped in gauze, which had previously been soaked in phosphate buffered saline (Sigma, St Louis, MO, USA), and placed in a foam esky on top of a cold pack for transport to the laboratory overnight.

Upon arrival at the laboratory, the epididymides were cleaned, excess tissue was removed, the surface blood vessels were punctured and the blood wiped away. Epididymides were then minced with a scalpel and the sperm allowed to swim out for 30 min into 4.0 mL warm Androhep without egg yolk (AH; Minitüb, Tiefenbach, Germany) in

60 mm petri dishes (Bacto Laboratories, Liverpool, Australia) placed on heated (37°C) stages. Sperm suspensions were centrifuged (300 *g*; 10 min) and the supernatant removed. The resultant pellet was resuspended to a final volume of 1 mL with Biladyl® (as described in Experiment 1 and 2) and cooled to 4°C over 2 h (-0.14°C/min). Cooled resuspended sperm were then stored at 4°C for 24 h. Sperm motility and acrosome integrity were assessed immediately after collection (post harvest) and 24 h after the onset of liquid storage.

2.6. Statistical analysis

Statistical differences were determined after logit transformation of sperm motility and acrosome integrity data. Data were analysed using a linear mixed model (REML) procedure with fixed effects specified for the factor of interest as well as day of collection and a random effect for male to account for repeated observations. Significance of the treatment effects was assessed by Wald chi-square tests, and pairs of means compared using least significant differences (LSD) with $P<0.05$ considered significant. GenStat (Release 10, Lawes Agricultural Trust, Rothamsted, UK), has been used for the analysis.

## 3. Results

3.1. Characteristics of neat semen

For Experiments 1 and 2, a total of 36 ejaculates were collected. The volume (mean±SEM) was 1.48±0.14 mL (range: 1.0-3.2 mL), sperm motility was 48.0± 2.2% (range: 40 – 60%), the total number of sperm in the ejaculate was 455.1±69.7 $x10^6$ (range: 178-1454.4 $x10^6$), semen viscosity was 6.30±0.9 mm (range: 1-15 mm) and acrosome integrity was 91.5±1.9% (range: 83-96%).

3.2. Experiment 1

Data on the motility of sperm are presented in Table 1. Interactions between diluent and length of storage ($P<0.001$) and storage temperature and length of storage ($P<0.001$) on sperm motility were observed but there was no interaction between diluent and storage temperature ($P=0.13$). Motility was similar for sperm stored at 4 and 15°C at 24 and 48 h but was higher at 72 h for 4 than 15°C ($P<0.05$).

Table 1. Motility of ejaculated alpaca sperm after dilution (1:4, v/v) with Androhep®, Biladyl®, Lactose, Salamon's or Triladyl® diluents and liquid storage at 4 or 15°C. Data are means ± SEM. Values within a row (A,B) or column (a,b) with a different superscript are significantly different ($P<0.05$).

| Diluent | Temperature (°C) | Storage time (h) | | | |
|---|---|---|---|---|---|
| | | 0 | 24 | 48 | 72 |
| Androhep® | 4 | $48.6\pm2.7^{A,a}$ | $8.1\pm1.6^{B,a}$ | $5.3\pm0.2^{B,a}$ | $2.1\pm1.1^{C,a}$ |
| | 15 | | $16.9\pm2.9^{B,b}$ | $8.6\pm1.8^{B,a,b}$ | $0.0^{C,b}$ |
| Biladyl® | 4 | $53.3\pm2.2^{A,a}$ | $35.7\pm3.3^{A,c}$ | $16.2\pm2.9^{B,b}$ | $6.5\pm2.4^{C,c}$ |
| | 15 | | $33.7\pm3.9^{A,b,c}$ | $14.8\pm2.6^{B,b}$ | $6.1\pm3.0^{C,c}$ |
| Lactose | 4 | $39.5\pm2.2^{A,a}$ | $17.4\pm2.6^{B,b}$ | $10.0\pm2.1^{B,a,b}$ | $2.9\pm1.6^{C,a,c}$ |
| | 15 | | $20.7\pm2.9^{B,b,c}$ | $9.3\pm2.2^{C,a,b}$ | $1.4\pm1.4^{D,b}$ |
| Salamon's | 4 | $48.1\pm2.2^{A,a}$ | $6.4\pm1.2^{B,a}$ | $4.9\pm0.1^{B,a}$ | $0.0^{C,b}$ |
| | 15 | | $14.1\pm2.8^{B,a,b}$ | $6.7\pm1.2^{B,a,b}$ | $0.4\pm0.4^{C,b}$ |
| Triladyl® | 4 | $52.6\pm1.7^{A,a}$ | $33.3\pm3.9^{A,b,c}$ | $12.9\pm2.8^{B,a,b}$ | $2.1\pm1.2^{C,a,c}$ |
| | 15 | | $30.9\pm3.9^{B,b,c}$ | $11.2\pm2.8^{C,a,b}$ | $0.4\pm0.4^{D,b}$ |

**Table 2.** Acrosome integrity of ejaculated alpaca sperm diluted in Androhep®, Biladyl®, Lactose, Salamon's or Triladyl® diluents and liquid stored at 4 or 15°C. Data are means ± SEM. Values within a row (A,B) or column (a,b) with differing superscripts are significantly different (P<0.05).

| Diluent | Temperature (°C) | Storage time (h) | | | |
|---|---|---|---|---|---|
| | | 0 | 24 | 48 | 72 |
| Androhep® | 4 | 88.4±1.4$^{A,a}$ | 92.8±1.3$^{A,a}$ | 87.5±3.1$^{A,a}$ | 59.3±5.9$^{B,a}$ |
| | 15 | | 92.7±0.9$^{A,a}$ | 88.0±2.3$^{A,a}$ | 57.0±19.8$^{B,a}$ |
| Biladyl® | 4 | 90.8±1.4$^{A,a}$ | 90.3±1.7$^{A,a}$ | 78.4±3.0$^{A,a}$ | 74.0±15.0$^{A,b}$ |
| | 15 | | 90.8±1.8$^{A,a}$ | 80.1±3.5$^{A,a}$ | 55.5±19.1$^{B,a,b}$ |
| Lactose | 4 | 85.3±3.4$^{A,a}$ | 94.2±1.4$^{A,a}$ | 85.0±2.7$^{A,a}$ | 50.0±0.0$^{B,a}$ |
| | 15 | | 86.7±4.3$^{A,a}$ | 79.8±3.7$^{A,a}$ | 56.3±8.6$^{B,a,b}$ |
| Salamon's | 4 | 87.5±1.4$^{A,a}$ | 90.3±2.3$^{A,a}$ | 85.0±2.2$^{A,a}$ | 9.0±0.0$^{B,c}$ |
| | 15 | | 84.4±5.8$^{A,a}$ | 82.5±3.2$^{A,a}$ | 5.0±0.0$^{B,c}$ |
| Triladyl® | 4 | 91.9±1.3$^{A,a}$ | 90.7±3.5$^{A,a}$ | 84.6±2.9$^{A,a}$ | 47.0±23.7$^{B,a}$ |
| | 15 | | 93.4±1.1$^{A,a}$ | 81.4±3.5$^{A,a}$ | 25.0±24.0$^{B,a}$ |

**Table 3.** Motility of ejaculated alpaca sperm diluted 1:1, 1:2 or 1:4 (v/v) with Biladyl® and liquid stored at 4 or 15°C. Data are means ± SEM. Values within a row (A,B) or column (a,b) with differing superscripts are significantly different (P<0.05).

| Dilution rate | Temperature (°C) | Storage time (h) | | | |
|---|---|---|---|---|---|
| | | 0 | 24 | 48 | 72 |
| 1:1 | 4 | 47.8±4.2$^{A,a}$ | 19.6±3.2$^{B,a}$ | 11.8±3.8$^{B,a,b}$ | 2.6±2.1$^{C,a}$ |
| | 15 | | 28.3±3.9$^{B,b}$ | 16.4±4.1$^{B,a}$ | 3.1±1.8$^{C,a,b}$ |
| 1:2 | 4 | 52.3±3.3$^{A,a}$ | 34.5±4.0$^{B,b,c}$ | 19.7±4.7$^{C,a}$ | 6.6±3.1$^{D,a,b}$ |
| | 15 | | 31.3±4.2$^{B,b}$ | 21.2±5.0$^{C,a}$ | 6.3±2.8$^{D,a,b}$ |
| 1:4 | 4 | 56.8±3.1$^{A,a}$ | 44.0±3.5$^{B,c}$ | 31.1±5.4$^{C,b}$ | 9.2±3.9$^{D,b}$ |
| | 15 | | 39.8±3.4$^{B,c}$ | 25.4±5.2$^{B,a,b}$ | 7.8±3.5$^{C,a,b}$ |

Motility of sperm was higher after storage in Biladyl® compared with all other diluents (P<0.05). Motility was similar for Triladyl® and Lactose, but was reduced for Androhep® and Salamon's diluents (P<0.05). Motility had declined for sperm stored at 4 and 15°C in Androhep®, Lactose, Salamon's and sperm stored at 15°C in Triladyl by 24 h after the onset of liquid storage (P<0.05). By contrast, sperm motility did not decline until 48 h after the onset of liquid-storage for sperm stored at 4 and 15°C in Biladyl®, and 4°C in Triladyl (P<0.05). The majority of the loss in sperm motility occurred in the first 24 h after the onset of liquid storage.

**Table 4.** Acrosome integrity of ejaculated alpaca sperm diluted 1:1, 1:2 or 1:4 (v/v) with Biladyl® and liquid stored at 4 or 15°C. Data are means ± SEM. Values within a row (A,B) or column (a,b) with differing superscripts are significantly different (P<0.05).

| Dilution rate | Temperature (°C) | Storage time (h) | | | |
|---|---|---|---|---|---|
| | | 0 | 24 | 48 | 72 |
| 1:1 | 4 | 86.7±2.7$^{A,a}$ | 87.3±1.7$^{A,a}$ | 80.2±2.0$^{A,a}$ | 60.5±2.4$^{A,a}$ |
| | 15 | | 88.7±1.8$^{A,a}$ | 78.7±2.2$^{A,a}$ | 56.2±2.2$^{B,a}$ |
| 1:2 | 4 | 89.3±1.4$^{A,a}$ | 89.6±1.8$^{A,a}$ | 79.4±2.0$^{A,a}$ | 52.0±7.8$^{B,a}$ |
| | 15 | | 86.2±2.7$^{A,a}$ | 76.9±3.2$^{A,a}$ | 58.8±2.2$^{A,a}$ |
| 1:4 | 4 | 89.9±2.1$^{A,a}$ | 90.4±1.1$^{A,a}$ | 78.7±2.4$^{A,a}$ | 68.2±1.9$^{A,a}$ |
| | 15 | | 88.9±1.6$^{A,a}$ | 77.4±3.0$^{A,a}$ | 54.5±7.8$^{A,a}$ |

Data on the acrosome integrity of sperm are presented in Table 2. Overall, there was no effect of diluent or temperature on acrosome integrity although interactions were observed between diluent and length of storage (P=0.005), and between storage temperature and length of storage (P<0.001). Overall, a decline in acrosome integrity of sperm was not observed until 72 h after the onset of liquid storage (P=0.05) and the decline in acrosome integrity was similar for sperm stored at 4 and 15°C.

3.3. Experiment 2

Data on the motility of sperm stored at 4 and 15°C after 1:1, 1:2, and 1:4 (v/v) dilution in Biladyl® are presented in Table 3. Sperm motility was affected by dilution rate and length of storage (P<0.001) but not storage temperature (P=0.79), and no interactions were observed (P>0.05). Motility was higher after 1:4 than 1:2 or 1:1 dilution, and was better 1:2 than 1:1 dilution (P<0.05). For all dilution rates and temperatures, sperm motility declined (P<0.05) by 24 h after the onset of liquid storage, with the majority of the loss in motility occurring during the first 24 h after the onset of liquid storage.

The acrosome integrity data are presented in Table 4. There was no influence of dilution rate or storage temperature on acrosome integrity of sperm and no interactions were observed (P>0.05). Acrosome integrity declined after 72 h of storage (P=0.005).

3.4. Experiment 3

Motility and acrosome integrity of epididymal alpaca sperm were similar immediately after harvest and 24 h after liquid storage at 4°C (P>0.05; Table 5).

**Table 5.** Motility and acrosome integrity of epididymal alpaca sperm immediately after harvesting (post harvest) and storage at 4°C for 24 h (liquid stored). Data means ± SEM. Values within a column (a,b) with differing superscripts are significantly different (P<0.05).

| Sperm type | Motility | Acrosome integrity |
|---|---|---|
| Post | 53.0±2.1$^{a}$ | 92.3±2.0$^{a}$ |
| Liquid | 47.0±2.0$^{a}$ | 90.6±0.9$^{a}$ |

## 4. Discussion

The present study investigated the effects of diluent and storage temperature (Experiment 1) and dilution rate and storage temperature (Experiment 2) on the longevity and functional integrity of liquid stored alpaca sperm. The results demonstrate that alpaca semen can be liquid stored at 4 or 15°C. Sperm retained acceptable

rates of motility for 48 h and some samples remained motile for 96 h (data not presented). Biladyl® was the most suitable of the diluents tested, and dilution at a rate of 1:4 with this medium produced superior results compared with lower dilution rates. Furthermore, alpaca epididymal sperm were liquid stored at 4°C for 24 h without loss of motility or acrosome integrity.

Variation in the effectiveness of diluents in preserving the longevity and fertilising capacity of sperm during liquid storage can be attributed to their different constituents. Three of the diluents examined in this study are Tris based (Biladyl®, Triladyl®, and Salamon's), while Androhep® and Lactose media are based on Hepes (a zwitterionic buffer) and the sugar lactose, respectively. Tris based diluents are commonly used for the liquid storage of bull (Vishwanath and Shannon 2000), buck (Leboeuf et al. 2000) and ram semen (Evans and Maxwell 1987), and have been used for liquid storage of alpaca (Vaughan et al. 2003), camel (Vyas et al. 1998, Deen et al. 2004, Wani et al. 2005, Niasari-Naslaji et al. 2006) and llama sperm (Ratto et al. 1999, Giuliano et al. 2006b).

Sperm motility was higher after storage in Biladyl® compared with Androhep®, Lactose, Salamon's or Triladyl®. Interestingly the survival of sperm stored in Biladyl®, Triladyl® and Salamon's diluents differed markedly, despite the similarities in their constituents. Salamon's diluent comprises 300 mm Tris (hydroxymethyl)aminomethane, 94.7 mM citric acid and 27.8 mm fructose while Biladyl® is a two-step extender based on Triladyl®, comprising 100 mM Tris, 33 mM citric acid, 28 mM fructose solution with antibiotics. This suggests that the reduced Tris and citric acid in Biladyl® and Triladyl® may be responsible for the improvement in longevity for sperm stored in these media compared to Salamon's diluent.

In contrast to the results of the present study, Vaughan et al. (2003) concluded that Triladyl® was superior to Biladyl® for liquid storage of ejaculated alpaca semen. However, full details of the experiment and analyses were not presented. Vyas et al. (1998) concluded that Tris was superior to Lactose diluent for the liquid storage of Dromedary camel semen at 5°C, and Deen et al. (2004) concluded that Tris was superior to Biociphos, as sperm remained motile for up to 96 h in the former compared with only 24 h for the latter medium. Survival of Dromedary camel sperm in Tris-tes egg yolk and Tris-lactose egg yolk was reported to be superior to that in citrate egg yolk, sucrose egg yolk and Tris-fructose egg yolk diluents (Wani et al. 2005). Moreover, Niasari-Naslaji et al. (2006) observed that Tris and Camel Green Buffer (zwitterionic buffered, IMV USA) resulted in superior motility of liquid stored Bactrian camel sperm compared with lactose and sucrose diluents. Liquid storage of llama semen at 5°C was superior after dilution in Lactose diluent compared with Tris, PBS (supplemented with 40% llama serum) and skim-milk diluents (Giuliano et al. 2006b). Taken together, these results suggest that the survival of camelid semen is generally better after storage in Tris diluents than diluents based on other organic buffers (such as citrate), sugars (glucose, lactose or sucrose) and milk proteins. However, as mentioned previously, the amount of Tris in the Tris based diluents confounds comparison, particularly as the amount is often not specified. Further studies are clearly required to determine the optimal levels of Tris, and other constituents in diluents for the liquid storage of alpaca semen.

Until the present study, the longevity of camelid semen liquid-stored at different temperatures had not been investigated. Previous studies have stored the semen at 5°C only (alpaca: Vaughan et al. 2003; Bactrian camel: Niasari-Naslaji et al. 2006; Dromedary camel: Deen et al. 2005; llama: Ratto et al. 1999, Huanca and Gauly 2001, Giuliano et al. 2006a) or failed to comment on sperm survival after Dromedary camel epididymal sperm were liquid stored at 4 and 23°C (Wani et al. 2005). The results of the present study demonstrate successful liquid storage of alpaca semen at 15°C. Furthermore, storage of alpaca sperm for up to 48 h was equally efficient at 4 and 15°C. A temperature of 4°C is recommended for storage periods exceeding 48 h, although the survival of liquid stored alpaca sperm beyond 48 h was poor in the present study.

Survival of llama sperm after 72 h of liquid storage has been demonstrated previously by Ratto et al. (1999) and Huanca and Gauly 2001 who utilised epididymal and ejaculated samples, respectively. Moreover, liquid stored Dromedary camel epididymal sperm retained motility for 8 days during liquid storage at 4 and 23°C (Wani et al. 2005). In the present study, alpaca sperm were still motile after 96 h of liquid storage. While the mean motility was low (< 5 %), the motility of individual samples ranged between 0 and 20 % demonstrating high levels of variation between males. Motilities of 50 % or more were observed in individual samples after 72 h of liquid storage which is encouraging. However, improvements to the longevity of liquid stored semen are required and its fertilizing capacity is yet to be determined.

Various dilution rates have been utilised in the liquid storage of camelid semen. Llama semen is most frequently diluted 1:1 (Huanca and Gauly 2001, Giuliano et al. 2006a,b) whilst Bactrian and Dromedary camel semen have been diluted 1:10 (Niasari-Naslaji et al. 2006) and 1:3 (Vyas et al. 1998, Deen et al. 2004). However, no direct comparisons of dilution rate have been made. The results from the present study demonstrate that survival of alpaca semen after 1:4 dilution rate is superior to a 1:2 and 1:1 dilution rates. These results are in agreement with the observations of Vaughan et al. (2003) who suggest a 1:4 dilution for alpaca semen, and the observations of Rigby et al. (2001) who concluded higher dilution rates were beneficial for stallion semen. Increased dilution rates, above 1:4, may be beneficial although further research is required to confirm this.

In a series of experiments, Niasari-Naslaji et al. (2006) demonstrated the impact of diluent osmolarity and pH of the survival of Bactrian camel sperm stored at 4°C. Tris diluents with an osmolarity of 300-360 mOsm/kg provided acceptable sperm survival whilst those with osmolarities of 270 or 390 mOsm/kg yielded poor survival rates. Furthermore, sperm liquid stored in Tris diluent with a pH of 6.9 were motile 24 h after the onset of storage, whereas a pH of 5.5, 6, 7.5, 7.9 or 8.9 resulted in a loss of sperm motility between 4 and 12 h after the onset of liquid storage. The increased survival of Bactrian camel sperm under slightly acidic conditions (pH 6.9 at the onset of liquid storage) would be expected, given the relationship between sperm metabolism and pH identified by Koellikker (1856; cited by Vishwanath and Shannon 2000). Interestingly storage of alpaca semen at slightly acidic conditions in Androhep® or Lactose diluents did not improve sperm survival in the present study. However, adjustment of the pH of the optimum diluent from the present study (Biladyl®) was not investigated, and

this medium might be more beneficial for alpaca sperm longevity during liquid storage if the pH were made slightly acidic.

Sperm motility declined throughout liquid storage in the present study with the most prominent reduction in motility occurring during the first 24 h, agreeing with Giuliano et al. (2006b) who observed a significant reduction to llama sperm motility during 24 h of liquid storage. Vaughan et al. (2003) observed high sperm motility (45–50%) for sperm liquid stored for 24 h in Biladyl® and Triladyl® but the authors did not disclose the initial motility of the samples thereby preventing a comparison of the loss in motility during the first 24 h of storage. Vaughan et al (2003) also reported 10 and 5% reductions in sperm motility between 24 and 48 h of liquid storage in Biladyl® and Triladyl®, respectively. This is similar to the results of the present study, where a 15% reduction in sperm motility was observed between 24 and 48 h of liquid storage. Interestingly, the loss in motility between harvesting and 24 h of liquid storage was minimal for epididymal sperm in the present study.

Previously, epididymal alpaca sperm have been stored in Lactose diluent at 4°C for 24 h with less than a 5% reduction in sperm motility (Morton et al. 2008a). Ratto et al. (1999) also reported motilities of 20-50% for epididymal llama sperm stored for 72 h at 5°C. These results demonstrate that epididymal alpaca and llama sperm, unlike ejaculated sperm, tolerate liquid storage procedures, probably owing to the exposure of the latter to the viscous seminal plasma. Epididymal alpaca sperm also tolerate freezing and thawing better than ejaculated sperm, retaining approximately 30–40% of the original motility after thawing (Morton et al. 2007, 2008a). Furthermore, epididymal alpaca sperm which were transported overnight to the laboratory, liquid stored at 4°C for 24 and then frozen and thawed, retained 35–40% of their original motility (Morton et al. 2008a) demonstrating their robustness. The recent use of epididymal sperm models to develop semen preservation techniques in alpacas has challenged perceptions about alpaca sperm and further highlight the possible role of the viscous seminal plasma as a major hindrance to the development of assisted reproductive technologies in alpacas and other camelids.

In summary, the research presented in the present study demonstrated successful liquid storage of ejaculated alpaca semen at both 4 and 15°C, and epididymal sperm at 4°C. Dilution of semen at 1:4 in Biladyl® for liquid storage is advantageous whilst storage temperatures of 4 or 15°C for periods up to 48 h yielded similar results. However, a temperature of 4°C is recommended for storage periods greater than 48 h. Despite these advances, further research is required to improve the longevity and determine the fertilizing capacity of liquid stored ejaculated alpaca semen.

## 5. Acknowledgements

This research was supported by the Rural Industries Research and Development Corporation (RIRDC) and the Australian Alpaca Association. The authors wish to thank Ms. K.J. Bailey for technical assistance and Mr. B. Biffin and K. Tribe for on-farm assistance and animal management.

## References

Bravo PW, Ccallo M, Garnica J, 2000a. The effect of enzymes on semen viscosity in llamas and alpacas. Small Ruminant Res 38, 91-95.

Bravo PW, Skidmore JA, Zhao XX, 2000b. Reproductive aspects and storage of semen

in Camelidae. Anim Reprod Sci 62, 173-193.

Deen A, Sumant V, Mamta J, Sahani MS, 2004. Refrigeratory preservation of camel semen. J Camel Prac Res 11, 137-139.

Evans G, Maxwell WMC, 1987. Salamon's artificial insemination of sheep and goats. Butterworths, Sydney, Australia.

Giuliano S, Chaves MG, Director A, Trasorras V, Miragaya M, 2006a. Inseminación artificial con semen refrigerado en llamas. Resultados preliminares. Proc. IV Congreso Mundial sobre Camelidos. Santa Maria, Argentina. p. 24 [Spanish]

Giuliano S, Director A, Gambarotta M, Trasorras V, Miragaya M, 2006b. Influencia de diluyentes en semen refrigerado a 5ºC durante 24 hs. en la especie Lama glama. Proc. IV Congreso Mundial sobre Camelidos. Santa Maria, Argentina. p. 26 [Spanish]

Hassan MM, Saeed M, Rizwan M, 1995. Semen collection by artificial vagina and cryopreservation of camel (Camelus dromedarius) spermatozoa. Pakistan Vet J 15, 105-108.

Huanca W, Gauly M, 2001. Conservacion de semen refrigerado de llamas. Rev Inv Vet Peru **1**, 460-461 [Spanish].

Jasko DJ, Moran DM, Farlin ME, Squires EL, 1991. Effects of seminal plasma dilution or removal on spermatozoal motion characteristics of cooled stallion semen. Theriogenology 35, 1059-1067.

Leboeuf B, Restall B, Salamon S, 2000. Production and storage of goat semen for artificial insemination Anim Reprod Sci 68, 113-141.

Morton KM, Bathgate R, Evans G, Maxwell WMC, 2007. Cryopreservation of epididymal alpaca (Vicugna pacos) sperm: a comparison of Citrate, Tris and Lactose based diluents, and pellets and straws. Reprod Fertil Dev 19, 792-96.

Morton KM, Vaughan JL, Maxwell WMC, 2008a. The continued development of artificial insemination technology in Alpacas. Rural Industries Research and Development Corporation, Kingston, ACT, Australia.

Morton KM, Ruckholdt M, Evans G, Maxwell WMC, 2008b. Quantification of the DNA difference, and separation of X- and Y-bearing sperm in alpacas (*Vicugna pacos*). Reprod Domest Anim 43 638-642.

Morton KM, Thomson PC, Bailey KJ, Evans G, Maxwell WMC, 2009. Quality parameters for alpaca (*Vicugna pacos*) are affected by semen collection procedure. Reprod Domest Anim in press.

Niasari-Naslaji A, Mosaferi S, Bahmani N, Gerami A, Gharahdaghi AA, Abarghani A, Ghanbari A, 2007. Semen cryopreservation in Bactrian camel (Camelus bactrianus) using SHOTOR diluent: Effects of cooling rates and glycerol concentrations. Theriogenology 68, 618-625.

Niasari-Naslaji A, Mosaferi S, Bahmani N, Gharahdaghi AA, Abarghani A, Ghanbari A, Gerami A, 2006. Effectiveness of a tris-based extender (SHOTOR diluent) for the preservation of Bactrian camel (*Camelus bactrianus*) semen. Cryobiology 53, 12-21.

Ratto MH, Wolter M, Berland M, 1999. Refrigeration of epididymal sperm from lama with three different extenders. Proceedings of the 2nd Congreso Mundial sobre Camelidos. Cusco, Peru p. 79.

Rigby SL, Brinsko SP, Cochran M, Blanchard TL, Love CC, Varner DD, 2001. Advances in cooled semen technologies: seminal plasma and semen extender. Anim Reprod Sci **68**, 171-180.

Skidmore JA, 2005. Reproduction in dromedary camels: an update. Anim Reprod **2**, 161-171.

Vaughan JL, Galloway D, Hopkins D, 2003. Artificial insemination in alpacas (*Lama pacos*). Rural Industries Research and Development Corporation, Kingston, ACT, Australia.

Vishwanath R, Shannon P, 2000. Storage of bovine semen in liquid and frozen state. Anim Reprod Sci 62, 23-53.

Vyas S, Goswami P, Rai AK, Khanna ND, 1998. Use of tris and lactose extenders in preservation of camel semen at refrigerated temperature. Indian Vet J 75, 810-812.

Wani N, Nowshari M, Wernery U, 2005. Storage of camel (*Camelus dromedarius*) epididymal spermatozoa in different extenders and at different temperatures. Biol Reprod Suppl 73, 693.