FLOW-CYTOMETRIC SORTING OF RAM SPERMATOZOA: PRODUCTION OF LAMBS OF A PRE-DETERMINED SEX USING *IN VIVO* AND *IN VITRO* FERTILISATION

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Declaration

Apart from the assistance mentioned in the Acknowledgements, the studies contained within this thesis were planned and executed by the author, and have not been previously submitted for any degree to a University.

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Summary

Birth of offspring of a pre-determined sex using flow cytometrically sorted fresh spermatozoa was first achieved in rabbits by Johnson *et al*. (1989). Since then offspring have been produced using sex-sorted spermatozoa from several different species (reviewed by Johnson, 2000). Initially, efficiency of the sex-sorting technology was poor with only low numbers of spermatozoa sorted per hour. Thus, the offspring derived from flow cytometrically sorted spermatozoa were produced with the use of artificial reproductive technologies (ART) such as *in vitro* fertilisation (IVF) and culture (IVC), intracytoplasmic sperm injection (ICSI) and deep artificial insemination (AI) which facilitated low dose insemination of potentially compromised spermatozoa. More recently, the development of high-speed sorters (Johnson and Welch, 1999) has facilitated the production of offspring using conventional AI techniques with low dose inseminates (Seidel *et al*., 1999) and successful cryopreservation of sorted spermatozoa (Schenk et al., 1999; Johnson *et al*., 2000; Lindsey *et al*., 2002; Schenk and DeGrofft, 2003). Increased efficiency of sorting bull spermatozoa has evolved through significant instrumentation and biological developments which have enabled the commercialization of the sperm sexing technology in the dairy industry, although conception rates in cows after low dose AI with sexed frozen-thawed spermatozoa are still lower than after standard frozen semen AI (Seidel *et al*., 1999). Subsequently, over 20 000 calves of pre-determined sex have been produced from commercially available sex-sorted frozen-thawed bull spermatozoa (Seidel, 2003). However, similar developments have not been made in the sheep industry and were examined in this thesis.

In this study, successful cryopreservation of sex-sorted ram spermatozoa and production of offspring of the pre-determined sex (X: 94.4 %; Y: 100 %) was achieved after low dose (2-4 x 10^6 total) insemination using conventional laparoscopic intrauterine (IU) AI. However, the overall pregnancy rate for ewes inseminated with sex-sorted frozen-thawed spermatozoa was low (25 %) compared to ewes inseminated with a commercial dose (140 x 10^6 total) of non-sorted frozen-thawed spermatozoa (54 %).

Cryopreservation has been found to not only reduce the proportion of motile spermatozoa, but cause the remaining spermatozoa to undergo changes that advance membrane maturation thereby shortening their lifespan, especially after *in vivo* fertilisation (Gillan and Maxwell, 1999). It was found that sorting prior to cryopreservation accelerated the maturation of sperm membranes and after co-incubation with oviducal cells *in vitro*, sorted frozen-thawed spermatozoa were released more rapidly than non-sorted (control) frozen-thawed spermatozoa. The potentially reduced lifespan of sorted frozen-thawed spermatozoa, and practical constraints on the number of spermatozoa that can be sorted for an insemination dose, makes insemination close to the site of fertilisation and time of ovulation critical for successful fertilisation. After treatment of ewes with GnRH to increase the precision of insemination in respect to the time of ovulation, there was no difference in pregnancy rate between ewes inseminated before, during or after the assumed time of ovulation. Furthermore, there was no difference in pregnancy rate after IU AI with similar doses of sorted frozen-thawed and non-sorted frozen-thawed spermatozoa in GnRH-treated ewes. The minimum dose of sorted frozen-thawed spermatozoa required for

commercially acceptable pregnancy rates determined after IU AI was high (20 x 10^6 motile).

Consequently, alternative methods for efficiently producing large numbers of offspring of a pre-determined sex using flow cytometrically sorted ram spermatozoa were investigated. Ram spermatozoa can be stored for short periods of time in a chilled state (liquid storage) or for an indefinite period of time in a frozen state (frozen storage; Salamon and Maxwell, 2000). The fixed location of the sperm sorter requires the need for transport of semen from the point of collection to the site of sorting and processing, but also from the sperm sorter site to the recipient females under artificial conditions. In this study, ram spermatozoa liquid stored for 24 h prior to sorting were efficiently sorted, frozen, thawed and after *in vitro* fertilisation and culture produced a high proportion of grade 1 blastocysts. Similarly, spermatozoa stored at reduced temperatures after sorting maintained high sperm quality for up to 6 days.

Furthermore, frozen-thawed spermatozoa from rams and some non-human primates were successfully prepared for sorting and efficiently sorted producing spermatozoa with high quality *in vitro* parameters. The quality of frozen-thawed ram spermatozoa after sorting was such that successful re-cryopreservation after sorting was possible. Low numbers of frozen-thawed sorted and re-frozen and thawed spermatozoa were optimal for IVF and a high proportion of grade 1 *in vitro* embryos of a pre-determined sex were produced. These embryos were either transferred immediately or vitrified prior to transfer, extending the application of the sperm sexing technology further. The birth of lambs of pre-determined

sex after transfer of both fresh and vitrified embryos derived from frozen-thawed sorted spermatozoa was achieved.

The findings in this thesis suggest that sorted frozen-thawed ram spermatozoa may have more advanced membrane maturation state than non-sorted frozen-thawed spermatozoa, resulting in a decreased fertilizing lifespan in the female reproductive tract. Despite this, the use of sexed ram spermatozoa in a number of physiological states (fresh, liquid, frozen) with several different ARTs is possible in producing significant numbers of offspring of a pre-determined sex. Improved efficiency in both sperm sexing and associated reproductive technologies is required for commercialization to be achieved in the sheep industry.

List of Abbreviations

AI	artificial insemination
ANOVA	analysis of variance
BSA	bovine serum albumin
CO_2	carbon dioxide
COC	cumulus oocyte complex
CTC	chlortetracycline
DNA	deoxyribonucleic acid
ET	embryo transfer
FBS	fetal bovine serum
FISH	fluorescence *in situ* hybridisation
FITC	fluorescein isothiocyanate
FSH	follicle stimulating hormone
g	gravity, acceleration due to
G	gauge
GnRH	gonadotrophin releasing hormone
H199	HEPES-buffered tissue culture medium 199
H_2O	water
HEPES	N-(2-hydroxyethyl) piperizine-N'-(2-ethanesulphonic acid)
HSA	human serum albumin
H33342	Hoechst 33342
IU	international units
IVC	*in vitro* culture/cultured
IVF	*in vitro* fertilisation/fertilised
IVM	*in vitro* maturation/matured
IVP	*in vitro* production/produced
LH	lutenising hormone
mg	milligram
ml	milliliters

mOsmol	milli-osmole
M	molar
N_20	nitrogen dioxide
no.	number
O_2	oxygen
OECM	oviductal epithelial cell monolayer
pers. com.	Personal communication
PBS	phosphate –buffered saline
PMSG	pregnant mare serum gonadotrophin
pH	hydrogen ion concentration,-log 10 of
PI	propidium iodide
s.e.m./SEM	standard error of the mean
SP	seminal plasma
Sperm	spermatozoa
SOF	synthetic oviduct fluid
TCM-199	tissue culture medium-199
UTJ	utero-tubal junction
UV	ultraviolet
v/v	volume:volume ratio
w/v	weight:volume ratio
X sperm	X-chromosome bearing spermatozoa
Y sperm	Y-chromosome bearing spermatozoa
μ (prefix)	micro ($x10^{-6}$)
m (prefix)	milli ($x10^{-3}$)
°C	degrees centigrade

Table of Contents

2. Papers: This thesis is based on the following papers:

Literature Review

1.1 Introduction

The efficiency and accuracy at which spermatozoa can be separated according to sex has developed a long way over the past 15 years. The first report of live offspring of pre-determined sex following surgical insemination of flow cytometrically sorted spermatozoa was in rabbits (Johnson et al., 1989). Since this success many offspring have been produced using the sperm sexing technology in humans (Levinson *et al*., 1995; Fugger *et al*., 1998) and livestock (cattle: Cran *et al*., 1993; pigs: Johnson *et al*., 1991; sheep: Catt *et al*., 1996; Cran *et al*., 1997) and wildlife (elk: Schenk and DeGrofft, 2003) species using a number of assisted reproductive technologies including surgical and deep artificial insemination, *in vitro* fertilisation, culture and embryo transfer.

This thesis examines the production of lambs of pre-determined sex using flow cytometrically sexed ram spermatozoa combined with assisted reproductive technologies. With particular reference to sheep, the literature review examines primarily the sperm sexing technology and more briefly, the artificial reproductive technologies used in combination with sexed spermatozoa to produce offspring. Greater detail of the assisted reproductive technologies used in this study and the biological effects sorting and processing has on spermatozoa are discussed in the relevant papers.

1.2 Sex-Predetermination

Pre-determination of sex prior to fertilisation allows the production of offspring of the desired sex from a particular mating. The benefits of determining the sex prior to conception for livestock producers, wildlife conservation programs and companion animal breeders have been reviewed previously (Morrell, 1991; Cran, 1992; Windsor *et al*., 1993; Seidel and Johnson, 1999; Seidel, 2003).

The use of sex pre-selection technology has enormous potential for increasing production efficiency in the domestic livestock industry through reduced costs of progeny testing, increased selection of breeding stock, rapid genetic gain, reduced wastage and increased utilization of facilities (Hohenboken, 1999). The Australian sheep industry earns approximately $ 4-5 AUD billion in exports of sheep products, which represents 15 % of total agricultural export earnings. Approximately one million ewes are laparoscopically inseminated nationally each year, of which half are stud ewes, and approximately 500 000 rams are sold nationally each year (W. M. C. Maxwell, pers. com.). With the use of frozen-thawed sexed sperm and artificial insemination (AI), real gains for the sheep industry would be applied to genetic improvement through increased selection intensity of sires and the widespread use of superior rams. This potential gain in genetic improvement and production through incorporation of the sexing technology has been estimated to be worth between $ 80 and $ 100 AUD million per year (W. M. C. Maxwell, pers. com.).

The pre-determination of the sex of offspring prior to conception is also an important management tool in conservation programs involving captive endangered species.

Manipulation of the sex ratio plays an important role in the population management of certain wildlife species in captivity such as the gorilla which requires a social organization containing only one mature male (O'Brien *et al*., 2003). Demand for sexed semen in companion animals is also increasing, often simply for sex preference or, in the case of polo ponies, for the selection of females.

Methods described to aid sex selection in humans have been reported as far back as Hippocrates in 460-377 BC (reviewed by Zarutskie *et al*., 1989; Windsor *et al*., 1993) but are all purely folklore. Since the identification of the sex chromosomes (Guyer, 1910) and the revelation that genetic material carried by the X and Y chromosomes determines the sex of offspring after fertilisation of an oocyte with an X- or Y- chromosome bearing spermatozoa (Painter, 1923) many scientific techniques have been devised to separate spermatozoa into X- and Y- enriched populations. A number of physiochemical differences of spermatozoa have been proposed including motility, swimming velocity, density, spermatozoa head volume, surface charge, cell surface antigens and the presence of an F-body in the Y-chromosome (Table 1: modified from Johnson, 1995). These differences have formed the basis of many sperm separation techniques which have been extensively reviewed by Gledhill *et al*. (1988), Amann (1989), Windsor *et al*. (1993), Johnson (1992, 1995), Flaherty and Matthews (1996) and Seidel and Garner (2002). However, the efficacy of these techniques has been disproved or shown to have low reproducibility because there is either no difference in the physiochemical property between X- and Y- chromosome bearing spermatozoa or the difference is so small that it is of little use (Windsor *et al*., 1993).

The exception is the separation of X- and Y- chromosome bearing spermatozoa based on a measurable difference in DNA content (Gledhill *et al*., 1976; Moruzzi, 1979; Pinkel *et al*., 1982; Garner *et al*., 1983; Johnson *et al*., 1987) by flow cytometric sorting (Johnson and Pinkel, 1986). This is the only validated (Welch and Johnson, 1999; Johnson, 2000) and reproducible method of separating spermatozoa into X- and Y- populations enriched at greater than 90% (Seidel *et al*., 1999; Welch and Johnson, 1999; Johnson, 2000; Garner, 2001; Seidel and Garner, 2002).

To date, attempts to establish the presence of an X- or Y- specific antigen on the cell surface of spermatozoa have been unsuccessful, despite extensive investigation over the past twenty years (Pinkel *et al*., 1985; Hancock, 1987; Hendriksen *et al*., 1993 and 1996; Howes *et al*., 1997). However, if a surface antigen were identified, significant improvements in sorting efficiency could be achieved through employment of either an immunological separation technique (Blecher *et al*., 1999) or the incorporation of flow cytometry and fluorescent labelling. The development of an immunological method is dependent on the presence of active gene expression by the X and / or Y chromosome within the haploid X- and Y- bearing spermatids (Hendriksen, 1999). However, during spermatogenesis, large numbers of developing spermatocytes and spermatids are connected by intercellular bridges and therefore may contain proteins derived from either sex chromosome in their cytoplasm or on their cell membrane (Koo *et al*., 1979). The economic implications of the existence of sex specific proteins probably warrants further investigation despite the recent failure, using protein analysis of flow cytometrically sorted

porcine (Hendriksen *et al*., 1993, 1996) and bovine (Howes *et al*., 1997) spermatozoa, to detect any differences between X- and Y-chromosome bearing spermatozoa and the failure to produce offspring of pre-determined sex using the immunological spermatozoa separation technique developed by Blecher *et al*. (1999).

Another recent report investigating an alternate spermatozoa sexing method is the separation of spermatozoa based on sperm head volume. Van Munster *et al*. (1999) demonstrated using differential interference contrast microscopy that the difference in volume of X- and Y-chromosome bearing bovine spermatozoa matched the difference in DNA content of spermatozoa. However, spermatozoa samples were only able to be sorted using a flow cytometer with incorporated interference optics at 300 spermatozoa per second and at 60-66 % purity (Van Munster, 2002).

Further developments of both of these sorting techniques may result in an efficient and thus alternate sex separation method that does not rely on the use of potentially cytotoxic and mutagenic agents such as Hoechst 33342 stain and ultra-violet (UV) light (see section 1.2.5.1), which are currently required for the separation of spermatozoa based on DNA content by flow cytometry (see below).

Sexing of embryos via embryo biopsy and PCR and sexing of foetuses via selective abortion after ultrasound diagnosis of sex are also ways of predetermining the sex of potential offspring after fertilisation, but this thesis will focus on the sexing of spermatozoa as a means of sex pre-selection. Despite greater accuracy in the pre-determination of sex

using the embryo biopsy technique (100 %) compared to the flow cytometric sorting of spermatozoa (> 90%), the latter has a major advantage over embryo sexing in that there need be few or no wasted embryos.

Table 1: Summary of potential differences between X- and Y- chromosome bearing spermatozoa (modified from Johnson, 1995).

Parameter	Proposed X and Y difference	Reference
DNA	Less DNA content in Y spermatozoa	Pinkel *et al*. (1982); Moruzzi (1979)
Size	X spermatozoa larger	Cui and Matthew (1993)
Density	X spermatozoa heavier	Harvey (1949)
Swimming velocity	Y spermatozoa swim faster	Ericsson *et al*. (1973)
Motility	Y spermatozoa more motile	Sarkar (1984)
pH	pH sensitivity	Roberts (1940)
Surface charge	X migrate to cathode	Kaneko *et al*. (1984)
Sperm head volume	Lower volume in Y	Van Munster et al. (1999a)
Spermatozoa surface	H-Y antigen present	Hendriksen *et al*. (1993)
Spermatozoa surface	Specific protein	Blecher *et al.* (1999); Howes *et al.* (1997)
F-body	Long arm on Y chromosome	Barlow and Vosa (1970)

1.2.1 Principles of flow cytometric sorting

The X chromosome of mammals is larger than the Y chromosome (Schmitz *et al*., 1992) and therefore has a higher DNA content (Moruzzi, 1979). It is this difference in DNA content that is the basis for sperm separation by flow cytometry. The greater the difference in DNA content between X- and Y- chromosome bearing spermatozoa, the more efficiently the two populations can be resolved and separated. This quantitative difference varies among species (Moruzzi, 1979; Garner *et al*., 1983) with most domestic livestock having a

difference in DNA content ranging from 3.6-4.2 % (Johnson, 1992; Johnson and Welch, 1999). Ram and bull spermatozoa have a difference of 4.2 % and 3.8 % respectively.

Sperm morphology also plays a key role in the ability to effectively separate spermatozoa by sex by flow cytometry (Rens *et al*., 1998; 1999). Spermatozoa have a flat, paddle-shaped head which contains densely packed chromatin. For accurate interpretation of the difference in DNA content between X- and Y- chromosome bearing spermatozoa by flow cytometry, each spermatozoon has to be correctly oriented to the excitation source (see below). These features are not only the basis of sex separation by flow cytometry but they determine the level of efficiency at which spermatozoa can be separated into X and Y populations.

Separating spermatozoa according to sex by flow cytometric sorting involves the staining of a suspension of spermatozoa with a vital, non-intercalating, fluorescent, bisbenzimide dye which binds to the minor groove of the DNA helix (Hoechst 33342; Johnson *et al*., 1987). Spermatozoa are also stained with a non-toxic food dye prior to sorting. The food dye penetrates the membranes of non-viable spermatozoa reducing the intensity of Hoechst 33342 fluorescence facilitating the selection of only viable (plasma membrane intact) spermatozoa for sorting and the gating of non-viable (plasma membrane non-intact) from the population to be sorted (Fig 2 (i), R2; Johnson and Welch, 1999).

After staining and incubation (34°C), spermatozoa are then passed in single file under hydrodynamic pressure (40-50 psi; see section 1.2.5.2) through a focused 200 mW argon-

laser beam with an ultraviolet (UV) wavelength of 333-363 nm (Fig 1(i and ii)). Ultraviolet light causes the DNA stain bound to the spermatozoa to fluoresce. The fluorescence intensity from each spermatozoon is collected through two optical lenses located at 90° and 0° to the laser beam (Fig 1(ii)). The fluorescence is then transmitted through a Longpass filter (LP400) in order to block UV scatter and stray light before reaching the photomultiplier tubes (PMT).

Correctly oriented spermatozoa have their side or edge facing the 90° fluorescence detector and their flat surface or front facing the 0° fluorescence detector. The hydrodynamic forces exerted on each spermatozoon facilitates their alignment with the longest axis parallel to the direction of the flow (Sharpe *et al*., 1997; Fig 1 (ii and iii)). The 'orientating nozzle' assists in the alignment or orientation of the sperm head of each spermatozoon about the flow axis (Rens *et al.*, 1999; Fig 1 (ii)). Fluorescent light is preferentially emitted along the plane of the sperm head because of its flat shape and the high refractive index of the densely packed chromatin. Thus, the role of the PMT located at 90° to the laser is to determine the 'correctly oriented' spermatozoa by detecting the spermatozoa that are demonstrating maximal fluorescence emission or 'side angle fluorescence'. Based on fluorescence data from the 90° detector, the correctly oriented and viable spermatozoa are selected for high purity sorting (Fig 2 (i), spermatozoa in R1), thus excluding the non-oriented, agglutinated and non-viable spermatozoa (Fig 2 (i), R2) from the population to be sex-sorted.

Only correctly oriented spermatozoa have their flat surface or 'forward angle' fluorescence signal then detected by the PMT located at 0° to the laser. Flat orientation allows the most accurate determination of DNA content. Fluorescence signals from the 0° detector are plotted into a two-dimensional zoom plot for sort gate placement around the X- and Y-chromosome bearing sperm populations (Fig 2 (ii)). The gated populations are then depicted in histogram format (Fig 2 (iii)). X-chromosome bearing spermatozoa have a greater DNA content than Y-chromosome bearing spermatozoa and therefore emit a stronger fluorescence signal. The spermatozoa population or peak at the lower fluorescence level represent the Y-chromosome bearing spermatozoa population (Fig 2 (ii): Y-sort region and (iii): Y peak) and the spermatozoa population or peak at the higher fluorescence level represent the X-chromosome bearing spermatozoa population (Fig 2 (ii): X-sort region and (iii): X peak).

Even within the spermatozoa population selected for sorting there is some variation in orientation which results in overlap in the bimodial distribution of the X- and Y-chromosome bearing spermatozoa. Heterogeneity in chromatin composition of spermatozoa, polymorphism, instrument alignment and resolution also play a role in the degree of overlap between the two sperm populations. The greater the difference in DNA content (species dependent) and the greater the staining uniformity (ejaculate and preparation dependent), the greater the resolution between the two sperm populations which is reflected in the degree of separation between the two peaks of the histogram (Fig 2 (iii)). However, sort gates are placed so that the population of spermatozoa in the area of overlap and also the extreme edges (aneuploid spermatozoa which occur at low levels in

most livestock species) are discarded and thus allows high purities (that is, 90 % or greater) to be achieved. The higher the required purity for a sperm sample, the smaller the sort region gates are set (Fig 2 (ii)). However, sorting at higher purity reduces the number of spermatozoa available for sorting, thus reducing sort rates. There is therefore an inverse relationship between purity and sorting rates.

Fluorescent signals from both detectors are produced at a rate of 180 000 measurements per second (Seidel and Garner, 2002). The signals are digitized and the information analysed by a computer and relayed back in time for each correctly oriented spermatozoon to be encased in a droplet formed by vibrations set up by a piezoelectric mechanism in the column (Fig 1 (iii)). Each droplet is given either a positive or negative charge depending on the amount of DNA that was measured and the positioning of the sort gates. The charged droplets are then passed between positively and negatively charged deflection plates which separate the spermatozoa into separate collection tubes (Fig 1 (iv)). Droplets containing more than one spermatozoon, a non-oriented spermatozoon or a non-viable spermatozoon are not electrostatically charged and remain in the central stream of sheath fluid which flows to the waste tank (Fig 1 (iv)).

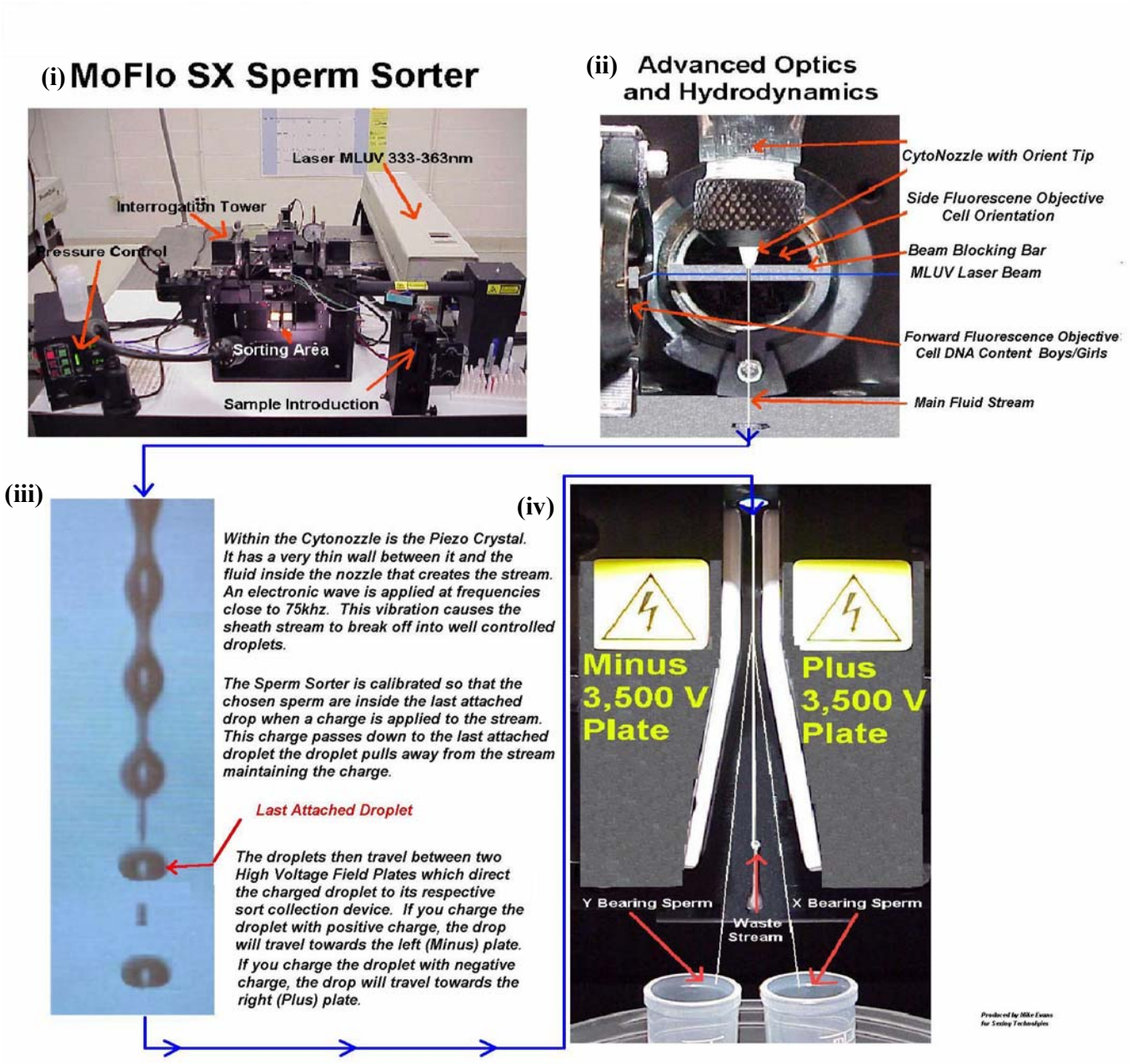

Fig 1 contributed by K. M. Evans, 2004

Fig 1: Photographs illustrating (i) the components of the MoFloSX® sperm sorter used at the The University of Sydney, Australia; (ii) the orientating CytoNozzle with a stream of spermatozoa flowing in single file past the laser beam with the side and forward angle detectors collecting fluorescence data from each passing spermatozoon; (iii) formation of spermatozoon encased droplets and (iv) deflection of charged droplets containing either a X- or a Y-chromosome bearing spermatozoon into either the left or right collection tube and non-charged droplets containing incorrectly oriented, aneuploid, non-viable or agglutinated spermatozoa passing into the waste stream.

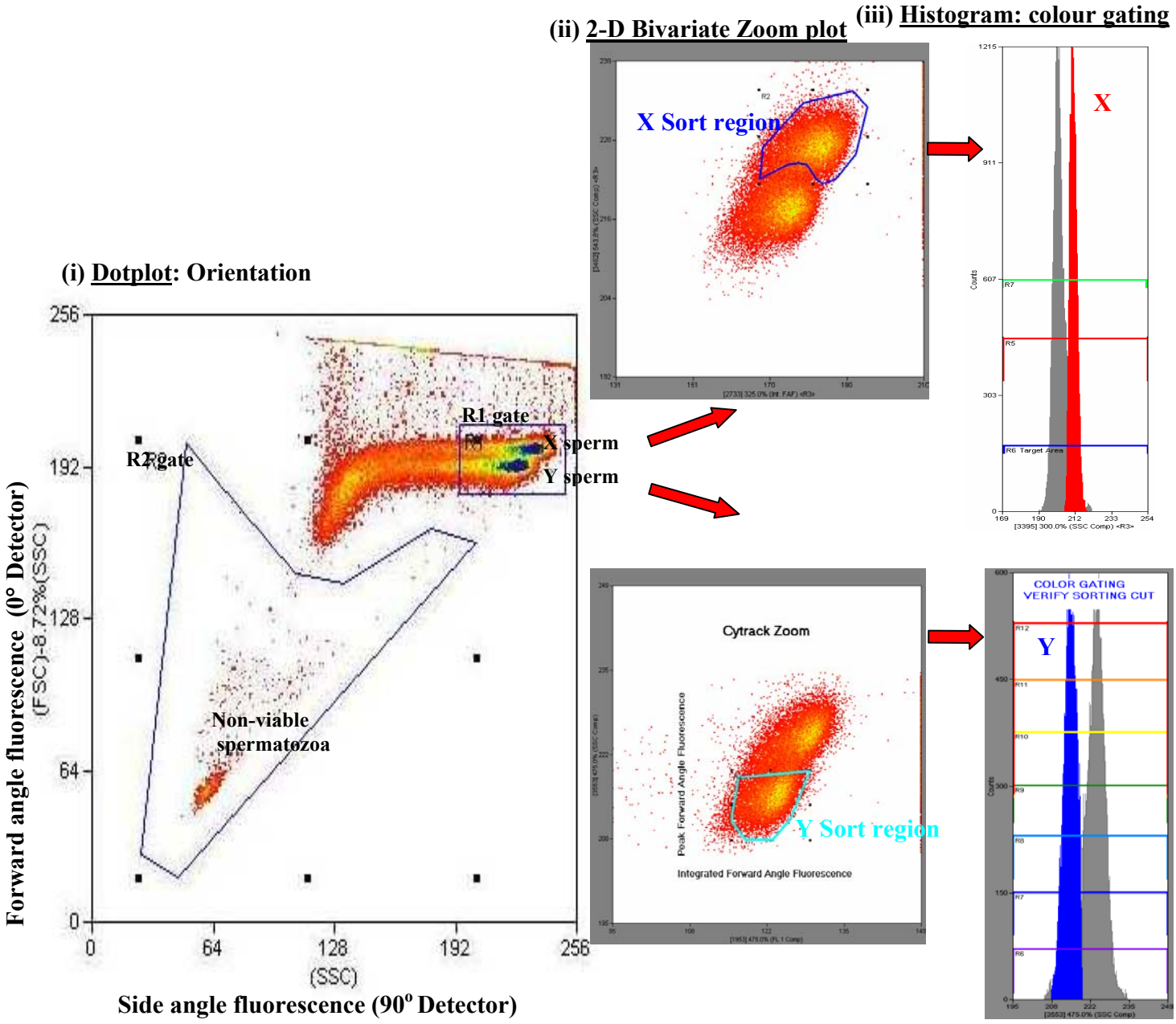

Fig 2: Computer generated output during the sorting of a fresh sample of ram spermatozoa on a MoFlo SX®.

The X and Y axis on the dotplot (i), two-dimensional (2-D) zoom plot (ii) and histogram (iii) represent increasing levels of fluorescence.

Each dot in the dot plot (i) and zoom plot (ii) represents a spermatozoon. Information from the 90° fluorescence detector is illustrated in the dotplot (i). A gate (R1) is placed around the viable spermatozoa emitting maximum side angle fluorescence. The forward angle (front) fluorescence emitted by these correctly oriented spermatozoa in R1 is captured by the 0° detector and analysed for DNA content. The population of sperm at the higher fluorescence (Y axis of the dot plot (i); X and Y axis of the zoom plot (ii) and X axis of the histogram (iii)) represents the X-chromosome bearing spermatozoa and the population at the lower fluorescence represents Y-chromosome bearing spermatozoa. Spermatozoa emitting low levels of fluorescence have damaged membranes and are considered non-viable. Their H33342 fluoresence is quenched by food dye so that they can be excluded from the viable population (R2 (i)). The spermatozoa in between sort gates R1and R2 are viable but incorrectly oriented and are therefore not included for high purity sorting. These spermatozoa and the non-viable spermatozoa are not given a charge and are passed into the waste line. Spermatozoa in between the two populations on the zoom plot (ii) and in between the two peaks on the histogram (iii) represent incorrectly oriented spermatozoa. Spermatozoa at the edges of the peaks represent aneuploid spermatozoa (low levels in livestock species). Sort gates are placed so that these spermatozoa are excluded from the sex-sorted population (Y and X Sort regions (ii)). Using colour gating technology, shifts in the proportion of correctly oriented spermatozoa are easily identified during sorting (iii) and in combination with the Cytrack technology, the continual adjustment of the sorting gates is possible thus allowing high X and Y purity to be maintained.

1.2.2 Confirmation of the purity of sex-sorted spermatozoa

A simple, quick, cost-effective and accurate method for determining the purity of sex-separated spermatozoa is essential to ensure the purity of the sample and to predict the sex ratio of the offspring that will result from its use. This is economically important as there are significant costs involved in the production of offspring of the undesirable sex. There are several accurate methods for differentiating X from Y chromosome-bearing spermatozoa, or for determining the sex of embryos after fertilisation, which have also been utilized to validate the effectiveness of sex separation by flow cytometry (Welch and Johnson, 1999). These methods include polmerase chain reaction (PCR; sperm: Welch *et al*., 1995; embryo: Cran *et al*., 1993), fluorescence in situ hybridization (FISH; Johnson *et al*., 1993; Kawarasaki *et al*., 1998), karyotyping following interspecific *in vitro* fusion (Yanagimachi *et al*., 1976) and the sex ratio of offspring.

However, these techniques are labour intensive, time consuming, expensive and restricted by availability of probes (FISH) or hamster oocytes (karyotyping). These factors limit their use for routine determination of the purity of sex-separated sperm samples. Flow cytometric DNA analysis or 'sort re-analysis' is currently the most effective, rapid and most importantly, validated method of routinely determining the purity of flow cytometrically sorted sperm samples from species with greater than 3 % difference in DNA content (Welch and Johnson, 1999). After sorting, a sample of spermatozoa is removed and sonificated to remove the tails and midpieces in order to improve orientation. They are then re-stained and incubated to maintain staining uniformity during re-analysis. The spermatozoa heads are passed back through the flow cytometer, but not sorted, and the

number of spermatozoa for each sex is counted. Using a curve-fitting math model to fit a double-gaussian-peak curve, the purity of X- and Y- chromosome bearing spermatozoa enrichment of a sample is determined within 30 min (Welch and Johnson, 1999).

1.2.3 Development of sperm sorting technology

There have been many developments in the sperm sexing technology since the evolution of the first flow cytometers modified for sperm sorting by Johnson and Pinkel (1986). Improvements in instrumentation and sperm handling during sorting have enabled the combination of this technology with other reproductive technologies, such as cryopreservation, in the production of offspring.

1.2.3.1 Standard speed sperm sorters

The first modification to the standard orthogonal flow cytometer / cell sorter was the replacement of the side scatter detector with a detector that measured forward fluorescence (0° to the laser beam). This allowed resolution of X- and Y-chromosome bearing spermatozoa populations with species which had greater than 3.5 % difference in DNA content (Johnson, 2000). The second addition was a wedge shaped injection tube or bevelled needle which increased the proportion of correctly oriented spermatozoa to the laser beam from approximately 10 % to 20-40 % (Johnson and Pinkel, 1986). However, sperm samples were only able to be sorted at approximately 350 000 spermatozoa/ h under 0.84 kg/cm^2 (Johnson *et al*., 1989). Consequently, due to the low numbers of available sperm, the first offspring produced from sex-sorted spermatozoa were achieved with the

combination of reproductive technologies such as surgical insemination, deep-intrauterine insemination, *in vitro* fertilisation (IVF) and embryo transfer (ET; Table 2).

1.2.3.2 High speed sperm sorters

Replacement of the bevelled needle with an orientating nozzle (Rens *et al*., 1998; 1999) increased the proportion of oriented spermatozoa to 70 % (Johnson and Welch, 1999). The orientating nozzle has an interior tapering ellipse that is able to exert hydrodynamic orientating forces on spermatozoa right up until they are released in front of the laser (Fig 1(ii); Cytonozzle with orient tip). This gives less time for the spermatozoa to lose orientation compared to the beveled needle and thus greater numbers of spermatozoa are available for high purity sorting. The development of the high speed sorter which operates under higher pressures (40-50 psi) and therefore faster flow rates (optimum output: 20-25 000 spermatozoa or events /sec) than the standard speed sorter (40 times faster sorting speed) in combination with the orientating nozzle has allowed significant increases in sorting rates (ram and bull: 4000 spermatozoa /sec) to be achieved while maintaining greater than 85-90 % purity of sorted sperm samples.

Instrumentation developments such as the integration of the forward angle fluorescence (FAF) signals has facilitated a 2 - 4 % increase in X and Y purity (K. M. Evans, unpublished data). Plotting peak versus the integrated signal (Fig 2, ii) allows the elimination of agglutinated spermatozoa from the sorted sample, thereby not only improving purity but increasing the proportion of live spermatozoa as it is usually non-viable (dead) spermatozoa that undergo agglutination. Furthermore, integrated signals are

presented in a two dimensional zoomed plot with FAF data (Fig 2 ii). This not only facilitates greater precision in sort gate placement but allows spermatozoa to be selected for sorting based on two parameters (bivariate sorting; Fig 2 (ii)) rather than one (FAF; univarite sorting) as previously done (Johnson, 1995). This allows higher X and Y purities to be achieved at faster sorting rates.

Improvements in software programs for sperm sorting such as the development of the 'Cytrack' program have resulted in significant economic gains, particularly for large commercial sorting centers that run a number of sperm sorters at one time. Prior to Cytrack, an operator was required to continually monitor the unit to keep data in the exact position in relation to the region being sorted to maintain high sort purity. Cytrack uses a digital signal processor (DSP) to track the sorting data and hold it still regardless of instrument instability which means that an operator is no longer required once the system is set up and the sorting is initiated. Therefore, a number of sperm sorters can now be managed by only one operator.

These recent developments have all contributed to the commercialisation of sexed bull semen in the United Kingdom (Cogent®, U.K) and United States of America (XY Inc®, Colorado, USA).

1.2.3.3 Biological developments

Alongside the instrumentation developments, many biological developments have occurred that have resulted in greater numbers of viable and fertile spermatozoa being produced per hour.

The discovery that the vital, non-intercalating stain H33342 gave improved separation compared to other dyes without the need for decondensation was an important step towards the production of fertile sorted spermatozoa (Keeler *et al*., 1983; Johnson *et al*., 1987; Johnson and Clarke, 1988). Spermatozoa were stained with not only H33342 but with propridium iodide to identify the non-viable spermatozoa (Johnson *et al*., 1994). This stain, which has potential mutagenic effects, has now been replaced by red food dye in order to quench the H33342 fluorescence of spermatozoa with damaged membranes, allowing them to be excluded from the viable population during sorting (Fig 2(i): R2 gate; Johnson and Welch, 1999; Schenk *et al*., 1999).

The development of the high-speed sperm sexing has resulted in a reduction in the dilution of spermatozoa during staining, sorting and time spent diluted in the collection tubes (Maxwell and Johnson, 1999). However, spermatozoa still undergo significant dilution after high speed sorting and may benefit from the addition of seminal plasma at stages during the sorting process (Catt *et al*., 1997b).

The methods of preparing and handling of sperm samples before, during and after sorting play an important role in the viability and fertility of sorted spermatozoa. Constant

temperature, osmolarity of buffers, pH and sterility of staining, sheath, collection and cryopreservation media are essential to maintaining and improving the viability and fertility of spermatozoa that have gone through many potentially damaging processes. Diluent requirements are not only specific to sorted spermatozoa but are often species-specific as well, and many optimization studies have been carried out (bull: Schenk *et al*., 1999; ram: Catt *et al*., 1997a; boar: Maxwell *et al*., 1997; stallion: Buchanan *et al*., 2000).

The supplementation of 20 % egg yolk (v/v) was an important addition to the collection media as it acts to protect the sorted spermatozoa against the effects of dilution after they are injected into the collection tube at 90 km/h (Johnson *et al*., 1989; Johnson, 1991). Optimisation of centrifugation speed and temperature after sorting and cryopreservation methods for sorted spermatozoa (Schenk *et al*., 1999) have also played an important role in achieving viable, fertile spermatozoa not only post-sort but post-thaw.

These instrumentation and biological developments have allowed not only the production of offspring using conventional and low dose AI in many species, but have enabled the successful cryopreservation of sorted spermatozoa in cattle, sheep, horses and elk to be achieved, bringing the sex sorting technology closer to commercialization in several of the livestock species (Table 2).

Table 2: Production of the first offspring of pre-determined sex derived from flow-cytometrically sorted spermatozoa on standard speed and high-speed flow cytometers in combination with other reproductive technologies.

Insemination	Type of sorted spermatozoa	Dose (x 10^6 total spermatozoa)	Offspring species	Reference
Standard speed sperm sorter				
Surgical AI (uterine)	Fresh	0.3	rabbit	Johnson *et al*., 1989
Surgical AI (oviduct)	Fresh	0.3	swine	Johnson *et al*., 1991
IVM, IVF, IVC, ET fresh embryos	Fresh	1.0 /ml	cattle	Cran *et al.,* 1993
IVM, IVF, IVC, ET of fresh and frozen-thawed embryos	Fresh	0.02 /oocyte	cattle	Cran *et al*., 1994
IVM, ICSI, IVC, ET	Fresh	-	sheep	Catt *et al*., 1996
In *vivo* matured oocytes, IVF, ET	Fresh	-	swine	Rath *et al.,* 1996
IVF, IVC, ET	Fresh	0.0025 / 0.81µl droplet	human	Levinson *et al*., 1995
Conventional laparoscopic AI (uterine body)	Fresh	0.1	sheep	Cran *et al.*, 1997
Deep AI (uterine horns)	Liquid stored fresh;	0.1-2.5	cattle	Seidel *et al*., 1997
(i)Conventional AI (uterine) (ii)IVF,IVC,ET; (iii) ICSI,IVC,ET	Fresh and Frozen-thawed	0.13 motile (AI)	human	Fugger *et al.*, 1998
High speed sperm sorter				
Conventional AI (uterine body) and Deep AI (uterine horns)	Liquid stored and frozen-thawed;	1.5-3	cattle	Seidel *et al*., 1999; Doyle *et al*., 1999
Surgical AI (oviduct)	Frozen-thawed	0.4	swine	Johnson *et al*., 2000
Conventional laparoscopic AI (uterine body) and Deep AI (oviducal)	Frozen-thawed	2-4	sheep	Hollinshead *et al*., 2001
Deep AI (tip of uterine horns)	Fresh	25	horse	Buchanan *et al*., 2000
Deep AI (Hysteroscopic AI-utero-tubal junction)	Fresh and Frozen-thawed	5 (motile)	horse	Lindsey *et al*., 2002

Deep AI (non surgical-anterior uterine horn)	Fresh	70-140	swine	Vazquez *et al.*, 2003
Conventional AI (uterine body)	Frozen-thawed	5	elk	Schenk and DeGrofft, 2003

(For brevity, only offspring derived from the first combination of flow cytometrically sorted spermatozoa (greater than 85 % purity) with an ART is reported. Over 20 000 offspring have been produced using sex-sorted semen.)

1.2.4 Efficiency of sperm sorting

Despite these modifications that have increased the number of viable sex-sorted spermatozoa, there are significant losses of spermatozoa during the sorting process. Depending on a number of factors such as male, ejaculate and operator, approximately only 30 % of a sperm sample prepared for sorting is sex-sorted. The remainder of the sample is lost at various stages before, during and after sorting (Garner *et al*., 2001; Seidel and Garner, 2002). The most significant loss (30 %) is attributed to incorrectly oriented spermatozoa. Future research into improving orientation will have a significant impact on sorting efficiency and commercialisation of flow cytometric sperm sorting. However, even with perfect orientation, the sperm sorters today have a sorting upper limit of 10 000 live spermatozoa of each sex / second (Seidel and Garner, 2002).

Alternatively, the development of a sorting system which is less dependent on spermatozoa orientation for high purity sorting would have a significant impact on sorting efficiency. The 360° radially symmetric optical configuration which combines orientation-independent illumination and fluorescence collection with high speed sorters (Sharpe *et al*., 1997) and the separation of spermatozoa based on sperm head volume through the incorporation of interferometry with flow cytometry (Van Munster, 2002) are examples of recently reported techniques that may eventually achieve this goal.

Obviously, the greatest improvement in sorting efficiency would be achieved through elimination of the need for spermatozoa orientation in high purity sorting. The presence and detection of a surface sex-linked antigen or membrane protein would enable the combination of immunological and flow cytometry technologies that would fulfill this objective. Not only would this method of sex sorting eliminate the sperm orientation requirement but also the potentially damaging effects of H 33342 and UV light on spermatozoa.

1.2.5 Effect of flow cytometric sorting and processing on spermatozoa fertility

The sperm sorting process comprises many different stages which may have adverse effects on the viability, fertility and genotype of spermatozoa. These include nuclear staining and incubation, exposure to UV light, dilution during staining and sorting, exposure to mechanical forces while passing through the flow cytometer and projection into the collection tube. After sorting, spermatozoa are centrifuged to remove the sheath fluid and concentrate the sorted spermatozoa. They are then processed for either immediate use, liquid storage or cryopreservation. These post-sorting processes compound damage to sperm quality that may have already occurred during sorting.

1.2.5.1 Potential damage to DNA integrity

There have been previous reports of mutations in actively mitotic cells after exposure to H33342 (Durand and Olive, 1982). However, Catt *et al*. (1997b) found no increases in endogenous DNA nicks after exposure of spermatozoa to high levels of H33342, and Libbus *et al*. (1987) reported no effect of H33342 itself on spermatozoa DNA but

suggested that the entire sorting process may lead to chromosome damage. More recently, there was no reported difference in motility or DNA integrity (as determined by chromatin stability assay) between spermatozoa that had been passed through the sorter with and without staining (Garner *et al*., 2001; Seidel and Garner, 2002). Similarly, after staining bull spermatozoa with either 149 μM or 224 μM there was no difference in post-sort and thaw motility (Schenk *et al*., 1999) and there was no reported difference in cleavage rates (Merton *et al*., 1997) or blastocyst development rates (Zhang *et al*., 2003) after IVF with stained or non-stained spermatozoa.

The effect of the UV light that spermatozoa are exposed to during the sorting process has also been reported as potentially damaging to DNA integrity. Libbus *et al*. (1987) concluded that either the direct action of UV light or the laser-excited fluorescence of H33342 induced DNA damage. On the other hand, Catt *et al*. (1997b) found no evidence of endogenous DNA nicks in spermatozoa that had been exposed to high levels of UV light and more recently it was reported that after IVF with boar spermatozoa sorted on a high speed MoFlo® cell sorter and exposed to up to 125 mW laser power had no detrimental effect on embryo development (Guthrie *et al*., 2002). It was hypothesized that this latter result was most likely attributed to the short duration spermatozoa were exposed to UV light (1 μsec) which was facilitated by the high speed sorting equipment. Furthermore, all high-speed sperm sorters are fitted with an argon laser so that spermatozoa are not exposed to damaging low UV wavelengths that are absorbed by nucleic acids and proteins (Seidel and Garner, 2002). The condensed packaging of the chromatin in spermatozoa DNA may

make it more stable to the potentially mutagenic effects of UV light and H33342 than the DNA found in somatic cells.

Despite these potentially mutagenic factors that spermatozoa are exposed to during sorting, births of over 20 000 calves after insemination with sex-sorted, frozen-thawed bull spermatozoa (Seidel, 2003) and offspring produced from seven mammalian species including humans, have been reported without any apparent alteration to phenotype (Amann, 1989; Seidel and Johnson, 1999; Garner, 2001; Johnson, 2000; Seidel and Garner, 2002; Table 2).

1.2.5.2 Potential damage to spermatozoa viability and fertility

Although the advent of the high speed sperm sorter has significantly reduced the rate at which spermatozoa are diluted during staining and sorting, ram spermatozoa still undergo a 10-fold dilution prior to staining, a further 2-fold dilution after staining and incubation and a 100-fold dilution by sheath fluid during sorting. This extensive dilution results in the removal of many beneficial seminal plasma components such as anti-oxidants and proteins that maintain membrane stability. Capacitation-like changes have been observed after the sorting of both ram and boar spermatozoa (Catt *et al*., 1997a; Maxwell and Johnson, 1997, 1999; Maxwell *et al*., 1997, 1998). These membrane changes, which have also been observed after freezing and thawing of ram spermatozoa (Gillan *et al*., 1997), reduce the fertilising lifespan of spermatozoa after *in vivo* insemination (Watson, 1995). However, these studies also demonstrated that the addition of 10 % seminal plasma to the staining medium helped prevent capacitation-like changes and when added to the collection

medium helped restore the decapacitated state of both ram and boar spermatozoa after flow-cytometric sorting.

However, the acrosome integrity of sorted spermatozoa is high (Hollinshead *et al*., 2002a). Prior to sorting spermatozoa are stained not only with H33342 but with a food dye that penetrates the non-viable spermatozoa (plasma membrane non-intact) and quenches the intensity of the fluorescence emitted from these spermatozoa, thus allowing them to be gated out from the viable population available for sorting.

The effect sorting pressure has on spermatozoa viability and fertility has recently been investigated. Suh and Schenk (2003) found higher motility after thawing, and Campos-Chillon and De La Torre (2003) reported higher cleavage and blastocyst rates after IVF with bull spermatozoa sorted at 40 psi compared to 50 psi. Furthermore, reducing the sorting pressure to 40 psi did not lower sort rate or purity when compared to 50 psi (J. L. Schenk pers. comm.). The effect of sorting under lower pressure on pregnancy rates after AI is yet to be seen.

Lower pregnancy rates after fertilisation with sex-sorted spermatozoa compared to non-sorted spermatozoa have been reported in a number of studies (Johnson *et al*., 1989; Johnson, 1991; Johnson, 1995; Cran *et al*., 1993; Seidel *et al*., 1999; Hollinshead *et al*., 2002b; Seidel and Garner, 2002). However, whether this loss is due to DNA damage and increased early embryonic loss or other factors such as dose rate, timing of insemination relative to ovulation, manipulation of the female reproductive tract or reduced viability in

the female reproductive tract of sorted spermatozoa after liquid transport or cryopreservation prior to insemination is yet to be determined.

1.3 Storage of spermatozoa

The preservation of semen has interested livestock breeders since AI was first considered (Salamon and Maxwell, 1995). Since the development of large scale AI programs in the twentieth century, the need to inseminate large numbers of females with semen from genetically superior males required transport of semen from the collection point or centre to the site of the female (Salamon and Maxwell, 2000). With the development of the sperm sexing technology, methods of transporting semen to the site for sex-sorting and further processing are required due to the fixed location of the sperm sorter. The aim of storing spermatozoa is to prolong their fertilising capacity. The preservation of semen for short-term (liquid) storage has been achieved by reducing the metabolism of spermatozoa through reductions in storage temperatures, and for long-term (frozen) storage by arresting the metabolism by storage at sub-zero temperatures (Salamon and Maxwell, 2000). Although the fertilising capacity of spermatozoa may be prolonged by storage in a liquid or frozen state, the storage processes inevitably reduce the proportion of motile spermatozoa and cause degenerative changes to spermatozoal membrane integrity, which ultimately reduces fertilising capacity after AI (Maxwell and Watson, 1996). These changes may have an even greater impact on the viability and fertility of sex-sorted spermatozoa which have been exposed to many potentially membrane damaging processes prior to preservation, especially after low dose AI. However, development of successful preservation protocols for sex-sorted spermatozoa will facilitate the widespread distribution and commercialization of the sperm-sexing technology (Schenk *et al*., 1999; Seidel, 2003).

The first reports of semen preservation were made by Spallanzani (1776) when he discovered that frog, stallion and human spermatozoa were immobilized when cooled in snow for 30 min, but were motile again after re-warming. Since this discovery, significant work has been carried out over the last century to determine the optimum cooling rates, temperatures and diluent compositions at which to preserve spermatozoa of domestic, wildlife and human spermatozoa.

1.3.1 Liquid storage of spermatozoa

The storage of semen in a 'chilled' state was extensively investigated by workers in the early nineteenth century (Maxwell and Salamon, 1993). Studies particularly concentrated on the effect of decreased temperatures on the physiology of spermatozoa. It was first observed by Milovanov in 1934 that if spermatozoa were rapidly cooled to temperatures close to 0°C a high proportion of spermatozoa became irreversibly immotile. This phenomenon was termed 'temperature shock', and is now commonly known as the 'cold shock' effect. Early research focused on ways to avoid cold shock. Optimum cooling rates for ram and bull spermatozoa were determined (10°C / h; Birrillo and Pulhaljskii, 1936;sited in Maxwell and Salamon, 1993) and the addition of lipids such as egg yolk (Lardy and Phillips, 1939; Willet and Sailsbury, 1942), lipoproteins, milk and phospholipids (Blackshaw, 1953) to storage diluents helped protect spermatozoa against the irreversible effects of cold shock. These findings laid the basis for the development of diluents for short-term storage of ram spermatozoa at reduced temperatures (reviewed by Maxwell and Salamon, 1993).

1.3.1.1 Temperatures used for short-term liquid storage of spermatozoa

The objective of storing spermatozoa below ambient (21°C) temperatures is to decrease their metabolism thereby prolonging their viability. However, a wide range of storage temperatures (0-15°C) have been reported to maintain viability and fertility of ram spermatozoa. Initially investigators reported 10-15°C to be the optimum temperature (Chang and Walton, 1940), but many Soviet workers found 0-5°C maintained greater spermatozoal viability. There have been many studies, mainly from the Soviet Union during the 1950s and 1960s, that examined the fertility of liquid stored ram spermatozoa (reviewed by Maxwell and Salamon, 1993). Based on these studies it is recommended that ram semen stored at 5°C be used within 24 h of storage for cervical AI (Evans and Maxwell, 1987) and within 6 d for intrauterine AI (Salamon *et al*., 1979). For use in an IVF system, ram spermatozoa stored for 7 d at 5°C resulted in high fertilisation rates, but even after 14 d storage *in vitro* fertilisation still occurred (Stojvanov *et al*., 1994). Since ram semen stored at 15°C has a shorter viable lifespan than semen stored at 5°C therefore it is recommended that it be used for AI within 6-12 h of storage (Evans and Maxwell, 1987).

1.3.1.2 Diluents used for short-term liquid storage of spermatozoa

The most commonly used diluents for storage of ram spermatozoa are classified as natural (whole cow's milk, milk powder, skim milk and ultra-heat-treated (UHT) milk) or synthetic. The synthetic diluents comprised a buffer (Tris, citrate, phosphate, Tes, Hepes, Mops, Mes or Pipes), an energy source (fructose, glucose or mannose), lipid for protection of the cell membrane against cold shock (egg yolk) and antibiotics to prevent growth of contaminating organisms (Maxwell and Salamon, 1993; Salamon and Maxwell, 2000).

All liquid storage diluents are designed to preserve the fertilising capacity of spermatozoa during *in vitro* storage at low temperatures. However, this is carried out in different ways depending on the diluent composition. Milk acts as a buffer against changes in pH and as a heavy metal chelating agent (Jones, 1969). All milk diluents need to be heated at 92-95°C for 10 min to destroy the toxic component in the protein fraction called lactenin (Flipse *et al*., 1954) except for UHT milk which is a commercially available preparation that has already been heat-treated and sterilised. An organic buffered diluent commonly used for storage of ram spermatozoa is the Tris-based diluent (Evans and Maxwell, 1987). The commercial Hepes-buffered diluent AndroHep (Minitüb, GmbH, Landshut, Germany) is widely used for the liquid storage of boar semen (Weitze, 1991). These diluents have buffering capacity superior to phosphate and citrate (Watson, 1979) and function to maintain the intracellular pH of spermatozoa.

However, irrespective of diluent, the motility and membrane integrity of spermatozoa deteriorate during cooling and liquid storage at low temperatures. These degenerative changes may be the result of lipid peroxidation and the production of excessive amounts of reactive oxygen species (ROS; Hammerstedt, 1983) which impair motility and the capacity for fertilisation after liquid storage (Maxwell and Watson, 1996). There have been several methods described to protect liquid-stored spermatozoa from the damaging effects of ROS, including storage in a low oxygen atmosphere (Salisbury *et al*., 1976) and the addition of anti-oxidants to storage diluents (Hammerstedt, 1983). The addition of the anti-oxidants may delay the membrane destabilization associated with sperm ageing and increase the

lifespan of liquid stored spermatozoa and in particular of flow-cytometrically sorted liquid stored spermatozoa. After the addition of the antioxidants superoxide dismutase and catalase to ram spermatozoa stored in a Tris-based diluent overall motility, acrosome integrity and fertility improved (Stojanov *et al*., 1994; Maxwell and Stojanov, 1996).

1.3.2 Frozen storage of spermatozoa

Due to the limited period of time that liquid stored spermatozoa can maintain fertility, further investigation into the storage of semen below 0°C was carried out. The first report of successful frozen storage of spermatozoa was in 1937 by Bernstein and Petropavlovsky (cited in Salamon and Maxwell, 2000), when rabbit, guinea pig, ram, boar, bull, fowl, duck and stallion spermatozoa were frozen with 9.2 % glycerol at -21°C. However, the cryoprotective property of glycerol was not established until 1949 when Polge *et al*. (1949) inadvertently used a solution of glycerol during the freezing of fowl spermatozoa at -79°C. Soon after this discovery the first offspring from frozen-thawed spermatozoa were produced (cattle: Stewart, 1951; human: Bunge *et al*., 1954 and sheep: Salamon and Lightfoot, 1967). Hence, successful cryopreservation and production of offspring in sheep did not occur until sixteen years after the first born calf from frozen-thawed bull semen. Cryopreservation methods and diluents used to freeze bull spermatozoa were initially used to freeze ram spermatozoa, unsuccessfully. This prompted extensive studies into different components of cryopreservation diluents (buffers and cryoprotectants) and processing techniques (dilution, cooling, freezing and thawing rates) that best protected ram spermatozoa against the dramatic changes in temperature that occur during the freeze-thawing processes (reviewed by Salamon and Maxwell, 1995a, 2000). It was not until the

development of the pelleting technique using dry ice to freeze (-80 to -95°C) ram spermatozoa (Lightfoot and Salamon, 1969b; Visser and Salamon, 1974a) and plastic straws, using liquid nitrogen vapour to freeze (-75 to -125°C) ram spermatozoa that acceptable post-thaw motility and fertility rates were achieved (Salamon, 1970; 1971).

1.3.2.1 Diluents used for long-term frozen storage of spermatozoa

The function of a cryopreservation diluent is to inhibit metabolism and further membrane maturation and to provide an environment to conserve membrane integrity and function (Watson, 1995). A range of diluents have been used to freeze ram spermatozoa, and the diluents most commonly used today are based on either Tris tris(hydroxymethyl)aminomethane) or zwitterions (Tes, hepes and Pipes) buffers. Salamon and Visser (1972) carried out the initial studies on the Tris-glucose diluent after pellet freezing of ram spermatozoa, and it is still a widely recommended diluent for the frozen storage of ram spermatozoa (Triladyl, Minitüb). However, Tris has poor buffering capacity below pH 7.5. This prompted further investigation into ram freezing diluents that contain zwitterion buffers as they can be used in the pH range of pH 6.0 to pH 8.0. A recent study reported that ram spermatozoa frozen and thawed in a zwitterion buffered diluent had higher post-thaw motility and acrosome integrity than ram spermatozoa cryopreserved in the traditional Tris-glucose freezing diluent (Molinia *et al*., 1994). It was suggested that the zwitterion buffers may have greater capacity to pick up hydrogen ions in the surrounding medium, thereby aiding the dehydration process (Prins and Weidel, 1986).

1.3.2.2 Frozen storage of sex-sorted spermatozoa

There are no published reports on the cryopreservation of flow cytometrically sorted ram spermatozoa. However, sex-sorted bull spermatozoa have been successfully cryopreserved and offspring produced (Seidel *et al*., 1999). Spermatozoa are exposed to many potentially damaging events during the sorting process (see above) which may be further compounded by cryopreservation. To maintain sorted spermatozoa viability and fertilising capacity after thawing, different cryopreservation procedures from those used by the commercial AI industry are required. Optimization studies on the equilibration time, freezing diluent, egg yolk preparation and general handling of sorted spermatozoa during the freeze-thawing processes have been reported for bull spermatozoa (Schenk *et al*., 1999). Similar studies will need to be carried out for the successful cryopreservation of sorted ram spermatozoa.

1.3.3 Events during cooling, freezing and thawing for liquid and frozen storage of spermatozoa

Observations on the events that occur in spermatozoa undergoing cooling, freezing and thawing processes have been extensively reviewed by Watson (1979); Amann and Pickett (1987); Watson (1995) and Woelders (1997). During the processes of cooling, freezing and thawing, spermatozoa are exposed to a series of changes in their physical and chemical environment. During cooling, the lipids in the membranes of spermatozoa undergo a series of phase transitions which ultimately impair the function of the membrane proteins. As spermatozoa are cooled below freezing point, the water in the cryopreservation diluent freezes, leaving the spermatozoa suspended in a highly concentrated solution. This creates an osmotic gradient between the inside and outside of the spermatozoon, whereby water

flow out of the cell, dehydrating it. If the spermatozoa are cooled too quickly, intracellular water cannot flow into the extracellular environment quickly enough, resulting in intracellular freezing and crystal formation. Intracellular crystalisation has a detrimental effect on the post-thaw viability of the spermatozoon. However, if the spermatozoa are cooled slowly, water continues to move out of the cell until it is dehydrated. Extracellular freezing and crystals form, which are less detrimental to spermatozoa viability. The optimal cooling rate for spermatozoa is between 10-80°C/min (Watson, 1995). With the addition of the cryoprotective substance, glycerol (0.5 M), the optimum cooling rate for ram spermatozoa is 50-60°C/min (Duncan and Watson, 1992). During thawing spermatozoa are exposed to the same processes as during freezing, but in reverse order and with less control (Hammerstedt *et al*., 1990).

There are a number of processes involved in the cryopreservation and thawing of spermatozoa which are potentially damaging to spermatozoa. Cryogenic injury of spermatozoa can occur at any stage during the freeze-thawing process but it is the cooling to sub-zero temperatures and re-warming to ambient temperatures, not the storage at -179°C, that causes the most damage (Watson, 1995). Furthermore, certain organelles and structures within the spermatozoon may respond differently to different aspects of the freezing process. Therefore, the optimal cryopreservation procedures must represent a compromise aiming to preserve the integrity of different structures with different cryobiological requirements in the largest proportion of cells (Watson, 1995). Spermatozoa undergo ultrastructural (plasma, mitochondrial, acrosomal membranes), biochemical and functional damage during the cryopreservation process. Details on the

specific nature of this biological damage have been extensively reviewed by Salamon and Maxwell (1995b) and Watson (1995). After freeze-thawing, only 40-60 % of ram spermatozoa preserve their motility (Salamon and Maxwell, 2000). However, of the remaining motile population, a proportion have (i) undergone changes that advance the maturation of sperm membranes and have (ii) sustained mitochondrial injuries which impair sperm transport in the female reproductive tract (Gillan and Maxwell, 1999). These changes contribute to poorer fertility after AI with an equivalent dose of motile frozen-thawed spermatozoa compared to fresh, motile spermatozoa (Watson, 1995).

1.3.4 Viability and fertility of liquid stored and frozen-thawed spermatozoa

The reduction in fertility of stored (liquid or frozen) ram spermatozoa compared to fresh has been extensively reviewed (Maxwell and Salamon, 1993; Salamon and Maxwell, 1995a, 1995b; Gillan and Maxwell, 1999; Watson, 1995; Maxwell and Watson, 1996; Curry 2000). In 1951, Austin and Chang identified the need for ejaculated spermatozoa to undergo a process of capacitation before they were able to participate in fertilisation. Capacitation is the first stage of a series of membrane destabilization events and a prerequisite for fertilisation that is characterized by an influx of calcium ions, an efflux of cholesterol and redistribution of intrinsic membrane proteins and lipids (Yangimachi, 1994; Harrison, 1996). Further membrane destabilization ultimately results in the acrosome reaction which is an irreversible morphological and biochemical change that results in a loss of spermatozoal viability (Acosta *et al*., 1988). The processes of cooling, freezing and re-warming involved in the storage of spermatozoa has been reported to advance the maturation of sperm membranes, increasing the proportion of capacitated and acrosome-

reacted spermatozoa and thus reducing their lifespan (Watson, 1995). The changes that occur to liquid and frozen stored spermatozoa during cooling, freezing, thawing and re-warming render them immediately able to partake in fertilisation, unlike fresh spermatozoa which still have to undergo the processes of membrane maturation described above. However, stored spermatozoa rapidly decline in fertilising ability after incubation *in vitro* or *in vivo* (Gillan and Maxwell, 1999).

In *vitro* studies have demonstrated that frozen-thawed spermatozoa are released earlier from oviducal epithelial cell monocultures (Gillan *et al*., 2000) and penetrate oocytes earlier, thus reaching a more advanced stage of development after co-incubation for 20-22 h than fresh spermatozoa (Maxwell *et al*., 1996; Gillan *et al*., 1997). These studies reflect the physiological readiness of frozen-thawed spermatozoa to participate in fertilisation. Studies with zona-free hamster oocytes also found that fresh spermatozoa required incubation before penetration of the oocyte (cattle: Wheeler and Seidel, 1986; human: Crister *et al*., 1987; ram: Garde *et al*., 1993). Similarly, liquid stored (5°C) goat spermatozoa had higher *in vitro* fertilisation rates compared to fresh spermatozoa (Pomares *et al*., 1995-unpublished; cited in Maxwell and Watson, 1996).

High fertilisation rates have been achieved after intrauterine or oviducal insemination with low numbers of frozen-thawed ram and bull spermatozoa (sheep: Maxwell *et al*., 1993; cattle: Seidel *et al*., 1995) and after intrauterine insemination with liquid stored (5°C) ram spermatozoa (Salamon *et al*., 1979). However, fertility of liquid stored and frozen-thawed ram spermatozoa was lower than fresh spermatozoa after cervical insemination (Liquid

stored: Salamon *et al.*, 1979; Frozen-thawed: Lightfoot and Salamon, 1970; Armstrong and Evans, 1984; Maxwell and Hewitt, 1986). This difference between fresh and stored spermatozoa is most likely attributable to the site of deposition. Intrauterine and oviducal insemination places spermatozoa in closer proximity to the oocytes and overcomes the cervical barrier and problem of impaired sperm transport resulting from cryoinjury (Salamon and Maxwell, 1995b). Alternatively, it may further support the theory that the process of preservation (liquid or frozen) advances the maturation of sperm membrane. Capacitated spermatozoa placed low in the female reproductive tract may undergo premature acrosome reactions and subsequently die before fertilisation or if the aged spermatozoa participate in fertilisation embryonic mortality may be increased (Gillan and Maxwell, 1999). Ultimately, fertility is lower for stored spermatozoa than fresh.

Chlortetracycline hydrochloride (CTC) is a fluorescent antibiotic that traverses the cell membrane of spermatozoa and enters intracellular compartments that have a high concentration of calcium ions (Tsien, 1989). It has been demonstrated in many species that the membrane status of spermatozoa (intact, capacitated and acrosome-reacted) can be assessed by CTC staining (mouse: Saling and Storey, 1979; human: Lee *et al*., 1987; stallion: Varner *et al*., 1987; boar: Wang *et al*., 1995; Maxwell and Johnson, 1997; bull: Fraser *et al*., 1995; Cormier *et al*., 1997 and ram: Perez *et al*., 1996; Gillan *et al*., 1997). The processes of cooling, freezing and thawing has been observed to advance the capacitation-like changes of spermatozoa in many species (ram: Perez *et al.,* 1996; Gillan *et al*., 1997; boar: Maxwell and Johnson, 1997; Green and Watson, 2001; bull: Cormier *et*

al., 1997), and further incubation of previously capacitated spermatozoa caused continued membrane destabilization, acrosome reaction and ultimately cell death (Gillan *et al*., 1997). Given that sex-sorted spermatozoa are subjected to known physical changes such as extreme dilution, nuclear staining, UV exposure and pressure, maintaining viability through the storage process is highly critical. To date there have been no thorough examinations of optimum storage conditions for ram spermatozoa before and after sex-sorting.

1.4 Use of processed semen

The functional changes that stored spermatozoa have undergone need to be taken into consideration when performing AI (Curry, 2000) as the processes of semen preservation reduce the number of motile spermatozoa, and the remaining motile population have undergone membrane changes that reduce their fertilising lifespan (Maxwell and Watson, 1996). This may have greater implications for the fertility of spermatozoa that have undergone flow cytometric sorting prior to liquid storage or cryopreservation. Insemination of liquid stored and frozen-thawed spermatozoa close to the site of fertilisation and time of ovulation are therefore critical for successful *in vivo* fertilisation. The numbers of spermatozoa inseminated also play a role in the rate of fertilisation but, in the case of sexed semen, this is a limiting factor. However, these limitations are overcome with *in vitro* fertilisation (IVF). Due to the proximity of the oocytes to spermatozoa, and due to the physiological state that stored spermatozoa are in (capacitated), relatively low numbers of stored spermatozoa are required for successful IVF.

1.4.1 Artificial Insemination

AI allows the rapid dissemination within a flock or herd of genetic and economically desirable traits. With the development of successful cryopreservation protocols for ram spermatozoa and of an intrauterine insemination technique (Killen and Caffery, 1982) significant growth of utilization of AI with frozen-thawed spermatozoa has taken place in the Australian sheep industry (Evans, 1988; 1991). Commercialisation of sexed semen in the cattle industry has recently become possible with improvements in sorting efficiency and the development of successful cryopreservation protocols that have enabled the use of

standard commercial AI techniques (Schenk *et al*., 1999). These developments are yet to take place in the sheep industry. Limitations that will need to be overcome for the successful commercialisation of sexed ram semen include the cervical barrier and low dose insemination of compromised (liquid stored or frozen-thawed) spermatozoa. There have been many developments in several aspects of AI in sheep which have helped overcome these limitations, including intrauterine insemination, control of ovulation and definition of the optimum time and minimum dose of spermatozoa for insemination (Evans, 1988).

Spallanzani (1776) not only reported the successful preservation of semen, but also demonstrated that the use of AI could result in the birth of live young.. By the turn of the century the use of AI in other mammals was reported (Ivanov, 1907). AI in sheep was extensively used in the former USSR for over 50 years and many developments occurred during this time (Maxwell and Salamon, 1993). Commercial AI with fresh diluted ram semen has been practised in many countries including France, Ireland, New Zealand and Australia for several decades (Evans, 1988). Initially, intrauterine insemination was performed via laparotomy (Salamon and Lightfoot, 1967). However, more recently, commercial AI with frozen-thawed ram spermatozoa has been made possible with the development of a laparoscopic intrauterine insemination technique (Killen and Caffery, 1982; Evans and Maxwell, 1987). There has been a steady increase in the use of AI in the Australian sheep industry since this development with 20 000 ewes inseminated in 1983 (Maxwell, 1984), 250 000 in 1988 (Maxwell and Wilson, 1990), 500 000 in 1994/5 (Maxwell and Watson, 1996) and approximately 1 million ewes are inseminated annually in Australia (W.M.C. Maxwell pers. com.). This is only a small proportion of the entire

sheep population in Australia, but importantly it represents the genetically influential stud ewe population (Maxwell and Watson, 1996). This section of the sheep industry would benefit economically from the use of sexed semen in their AI programs.

Use of AI is limited in the sheep industry mainly because of the costs involved with laparoscopic AI. This may also have a negative impact on the commercialization of sex-sorted ram spermatozoa in Australia, particularly with extensive wool and meat producers. Alternative semen deposition techniques have been developed that require minimum technical skills and have few associated costs including vaginal, cervical and transcervical AI. To date, these techniques result in poor fertility after insemination with liquid stored (Salamon *et al*., 1979; Maxwell and Salamon, 1993) or frozen-thawed (Lightfoot and Salamon, 1970a; Armstrong and Evans, 1984; Maxwell and Hewitt, 1986; Windsor *et al*., 1994) spermatozoa due to the effectiveness of the cervix to act as a barrier to sperm transport, preventing the establishment of an adequate cervical population of functionally intact spermatozoa (Salamon and Maxwell, 2000). This problem would be further exacerbated by low dose AI, a constraint associated with sexed semen.

Laparoscopic insemination into the uterus is the only commercially available AI technique in sheep which could be used in conjunction with sexed ram semen. It not only effectively circumvents the cervix but deposits semen close to the site of fertilisation, which is important after insemination with liquid or frozen stored spermatozoa. High fertilisation rates were achieved with fresh and frozen-thawed spermatozoa (Maxwell and Butler, 1984; Maxwell *et al*., 1984; Maxwell and Hewitt, 1986; Salamon and Maxwell, 2000) and lower

numbers of spermatozoa per inseminate could be used (Walker *et al*., 1984; Maxwell, 1986b; Eppleston *et al*., 1986; Jabbour *et al*., 1988; Maxwell *et al*., 1993).

High fertility after laparoscopic AI is achieved if used in conjunction with a synchronized oestrus. The most commonly used method for synchronizing ewes today is insertion of an intravaginal sponge impregnated with fluorogestone acetate (FGA; 30 mg Chronogest) for 12 days (Robinson, 1965) in conjunction with an intramuscular injection of 400 I.U. of pregnant mare serum gonadotrophin (PMSG) at sponge removal (Evans and Robinson, 1980). The optimum time of insemination after sponge removal has been reported to be between 48 and 65 h. In one experiment the median time of ovulation has been determined to be between 55.8 and 59.7 h after sponge removal (Maxwell, 1986a). Insemination both before (Davies *et al*., 1984; McKelvey *et al*., 1985) and after (Maxwell 1986a; Eppleston *et al*., 1986) the known time of ovulation has resulted in high pregnancy rates. Intrauterine insemination at the time of ovulation can interfere with ovum pick up and result in low fertility. Similarly, early and late insemination in respect to ovulation, with frozen-thawed spermatozoa can result in aged spermatozoa or oocytes participating in fertilisation and thus increased embryonic mortality (Maxwell, 1986a).

Increased synchrony of ovulation can be gained through the use of gonadotrophin releasing hormone (GnRH). It was reported that when GnRH was incorporated into the synchronization regime the period over which ovulation occurred was reduced to 12 h (Eppleston *et al*., 1991). Despite the increase in synchrony of ovulation there have been few reports of increased fertility after intrauterine insemination with commercial doses of

frozen-thawed ram spermatozoa (Maxwell, 1986a; Walker *et al*., 1989a; Eppleston *et al*., 1991). However, importantly, Smith *et al*. (1986) found significant improvement in fertility in ewes treated with GnRH after low dose insemination of frozen-thawed spermatozoa.

Intrauterine insemination allows the use of much lower doses of spermatozoa than other forms of insemination. Fertility data after intrauterine insemination with a range of doses of fresh and frozen-thawed spermatozoa have been reviewed by Evans (1988) and Salamon and Maxwell (1995; 2000). Overall, low dose insemination results are variable. This variability may be related to time of insemination and ovulation or ewe factors (Evans, 1988). Acceptable fertility has been achieved with doses as low as 1×10^6 total frozen-thawed spermatozoa (Walker *et al*., 1984; Maxwell *et al*., 1993). However, the recommended minimum effective dose for intrauterine insemination with frozen-thawed or fresh ram semen is 20×10^6 motile spermatozoa and pregnancy rates of 70 % and greater are expected (Evans and Maxwell, 1987).

The site of insemination may also play a role in fertility. Maxwell (1986b) found that insemination into the tip of the uterus resulted in lower lambing rates than insemination into the middle or base of the uterine horns. However, others have reported no difference in fertility after insemination at the tip, middle or bottom of the uterine horns (Reinhold *et al*., 1990). Similarly there are varying reports after insemination into only one or both horns (Salamon and Maxwell, 2000). Recently, in order to inseminate closer to the site of fertilisation, a technique for embryo transfer using a mini-laparotomy was developed (Maxwell *et al*., 1993). Time of insemination relative to ovulation is important using this

technique as the manipulation involved can disrupt ovum pick up and inhibit sperm transport.

At present, intrauterine insemination is the only commercial AI technique for sheep that allows acceptable and repeatable pregnancy rates with frozen-thawed semen, but also allows low dose insemination (Salamon and Maxwell, 2000), which will have important implications in the commercialization of sexed ram spermatozoa. So far approximately 20 000 calves have been produced using sexed frozen-thawed bull spermatozoa and commercial AI techniques (Seidel, 2003). Pregnancy rates after AI of heifers with spermatozoa that were sorted by sex prior to freeze-thawing were only 70-90 % of those achieved with non-sorted (control) frozen-thawed spermatozoa (Seidel *et al*., 1999). However, the same number of motile spermatozoa were not used for the sorted (1.5-3.0 x 10^6 total spermatozoa) and control (20 x 10^6 total spermatozoa) treatments in this study. Improved sorting and cryopreservation techniques as well as increased sorting efficiency may overcome this discrepancy in pregnancy rates. However while the numbers of sexed spermatozoa able to be sorted is still a restricting factor the use of *in vitro* fertilisation and culture systems to produce large numbers of pre-sexed embryos may be a more economical and practical option for stud producers and owners of valuable animals, particularly in the Australian sheep industry.

1.4.2 *In vitro* production of embryos and subsequent transfer

In *vitro* production (IVP) of embryos is an important tool in the production of offspring derived from sex-sorted spermatozoa (cattle: Cran *et al.,* 1993, 1994, 1995; pigs: Rath *et*

al., 1997, 1999; Abeydeera *et al*., 1998; sheep: Catt *et al*., 1996; humans: Levinson *et al*., 1995; Fugger *et al*., 1998). Despite the improved efficiency in flow cytometric sorting of spermatozoa, only low numbers of spermatozoa are able to be produced per hour, which limits commercial viability. IVF provides an alternate and effective method for the production of offspring of the desired sex. There has been considerable improvement in the efficiency of IVP, particularly in cattle, making possible the production of large numbers of viable embryos at relatively low costs for commercial use (Lu and Polge, 1992; R. Fry pers. com). The production of embryos of a pre-selected sex has potential commercial application in the sheep industry and is also a potentially valuable tool for management of certain captive wildlife species and valuable animals.

The first offspring produced by embryo transfer (ET) was in 1891 by Heape after he transplanted embryos from one rabbit to another (Heape, 1891). Since then successful transfer of *in vivo* derived embryos has occurred in many species (reviewed by Betteridge, 1981). A much longer period of time elapsed before the successful transfer and production of offspring derived from *in vitro* produced embryos was reported. The first report of successful culture of pre-implantation embryos (8-cell) was in the mouse in 1949 by Hammond (1949). Continued improvements in culture media (Whitten, 1956; 1957) eventually resulted in the birth of the first offspring derived from cultured mouse embryos (Biggers and McLaren, 1958). It wasn't until the early 1980's that the first offspring were born after *in vitro* fertilisation of *in vivo* matured oocytes (cattle: Brackett *et al*., 1982; sheep: Hanada, 1985). Further improvements in the *in vitro* culture system of many species eventually enabled *in vitro* maturation and fertilisation of oocytes to develop to the

blastocyst stage. Following these developments, the first births after transfer of *in vitro* produced embryos in cattle (Lu *et al*., 1988; Czlonkowska *et al*., 1991) and sheep (Pugh *et al*., 1991) were reported. The viability of *in vitro* derived blastocysts has since been demonstrated in many species (cattle: Goto *et al*., 1988; Polge *et al*., 1991; Sinclair *et al*., 1995; sheep: Shorgan *et al*., 1990; Walker *et al*., 1992; Thompson *et al*., 1995; O'Brien *et al*., 1996; Brown and Radziewic, 1998).

In *vitro* derived embryos result in lower pregnancy rates after transfer compared to *in vivo* derived embryos (Tervitt and Rowson, 1974; Holm *et al*., 1996). *In vitro* embryo culture is also associated with prolonged gestation periods, increased birth weights, and an increased incidence of dystocia and perinatal death (sheep: Walker *et al*., 1992; Holm *et al*., 1994, 1996; Brown and Radziewic, 1998; cattle: Sinclair *et al*., 1995; Farin and Farin, 1995; Hasler *et al*., 1995) compared to *in vivo* produced embryos. Furthermore, Brown and Radziewic (1998) reported that the growth rates and testis weights of males derived from *in vitro* produced embryos were significantly greater than in lambs born from natural matings. This 'large calf / lamb' syndrome reported after transfer of *in vitro* derived embryos has been associated with asynchronous recipients (Rowson and Moore, 1966; Wilmut *et al*., 1981; Young *et al*., 1995; Naitana *et al*., 1995), embryo manipulation (Walker *et al*., 1996), progesterone supplementation early in gestation (Kleeman *et al*., 1994), and the presence of sera in the culture media, as these effects were not seen if bovine serum albumin (BSA) was used as the source of protein in the culture media instead of human serum (Thompson *et al*., 1995). Improvements in the techniques of *in vitro* maturation and culture are still

required, especially if embryos are to be cryopreserved prior to transfer as IVP embryos have increased sensitivity to cryoinjury (Leibo and Luskutoff, 1993; Leibo *et al*., 1995).

Production of *in vitro*, pre-sexed embryos followed by cryopreservation and subsequent transfer would further facilitate the commercial application of the sperm sexing technology. The first offspring produced after cryopreservation of *in vivo* produced embryos were cooled at a rate of 0.2°C / min and dimethylsulfoxide (DMSO) was used as a cryoprotectant (mouse: Whittingham *et al*., 1972; cattle: Wilmut and Rowson, 1973; sheep: Willadsen *et al*., 1974). Since these first reports, offspring from many different species have been produced following transfer of both *in vivo* and *in vitro* produced embryos cryopreserved using a variety of cryoprotectants and slow or fast cooling and warming rates (reviewed by Niemann, 1991; Palasz and Mapletoft, 1996). However, pregnancy rates are lower and embryonic loss higher after the transfer of *in vitro* produced embryos compared to *in vivo* derived embryos after cryopreservation (Greve *et al*., 1993; Dobrinsky, 1996). The most important factor affecting freezability is embryo quality (Leibo and Loskutoff, 1993) for which unfortunately there is no objective measurement yet. Despite the higher freezing sensibility of *in vitro* produced embryos (Greve *et al*., 1993; Pollard and Leibo, 1994), significant improvements in culture systems and cryopreservation techniques have enabled pregnancy rates between 35 to 50 % in sheep and cattle to be achieved after the transfer of two or multiple embryos (Lane *et al*., 1998; Walmsley *et al.*2000*;* Dattena *et al*., 2000; Peura *et al*., 1999; 2002).

In 1985, Rall and Fahy introduced vitrification as a new method to cryopreserve mammalian embryos without the formation of ice crystals. In contrast to conventional equilibrium or slow-rate freezing, vitrification involves the use of rapid cooling and warming rates and high concentrations of cryoprotectants in order to totally eliminate ice crystal formation and reduce chilling injury (Vajta *et al*., 1998). However, high concentrations of cryoprotectants may lead to toxic and osmotic injuries requiring minimal periods of exposure during the vitrification process. Methods that have allowed direct contact between the cryodiluent solution and liquid nitrogen have further reduced the detrimental effects of chilling injury and the time that the embryo is exposed to potentially toxic concentrations of cryoprotectant (Vajta *et al*., 1998). Such methods include the dropping of embryos into liquid nitrogen (Riha *et al*., 1991) or using electron microscope grids during the cooling and warming process (Martino *et al*., 1996). However, the development of the Open Pulled Straw (OPS) technology has allowed high speed freezing, thawing, storage and in-straw dilution resulting in improved survival for *in vitro* produced embryos and high pregnancy and birth rates after transfer (Vajta *et al*., 1998). The vitrification method also offers the advantage compared to slow rate conventional freezing of being simple, quick and cost-effective, which is important in field conditions (Vajta, 2000). Development of vessels in which embryos can be cooled at even higher rates (Lane *et al*., 1999) and improvements in culture media through addition of antioxidant (Lane *et al*., 2002) and hyaluronan (Lane *et al*., 2003) is likely to eventually close the gap in pregnancy rates between fresh and cryopreserved embryos after transfer.

Further improvement in *in vitro* embryo production systems will allow the production of higher quality embryos and combined with further improvements in cryopreservation techniques will make cryopreserved, *in vitro* produced pre-sexed embryos a commercial option for both livestock producers and wildlife conservation programs in the near future.

1.5 Concluding remarks and aims

The pre-determination of the sex of offspring using spermatozoa separated by fluorescence differential sorting has been achieved in a number of species using a variety of reproductive technologies (Table 2). The commercialisation of sexed bull spermatozoa has been a result of an interaction between several factors including the development of successful cryopreservation protocols (Schenk *et al*., 1999), increased sorting efficiency (Rens *et al*., 1998, 1999; Johnson and Welch, 1999), cost saving software improvements such as the development of Cytrack, and instrumentation advancements including the zoomed integrated plot system for gating spermatozoa populations for sex-sorting (K.M. Evans, unpublished data). Such developments with ram spermatozoa would have significant benefits for wool and meat producers in not only Australia but in other countries such as the United Kingdom and Europe where each animal has a higher economic value and is more intensely managed. However, for these developments to take place an understanding of the effect sex-sorting, cooling, freezing, thawing and storage have on the viability and fertility of ram spermatozoa, especially after *in vivo* fertilisation is required. It has been previously demonstrated that preserving spermatozoa in either a chilled (liquid) or frozen state causes changes to spermatozoa membranes that reduce their lifespan and hence fertility compared to fresh spermatozoa (Maxwell and Watson, 1996). However, the fertility of frozen-thawed spermatozoa can be improved after *in vivo* insemination close to the site of fertilisation and time of ovulation (Maxwell *et al*., 1993) or after *in vitro* fertilisation, culture and embryo transfer (O'Brien *et al*., 1996). The latter is particularly

advantageous for sorted sperm samples as low numbers of spermatozoa are required for successful fertilisation.

The overall aim of the work reported in this thesis is to develop ways in which sex-sorted ram spermatozoa can be effectively used in conjunction with other available reproductive technologies and ultimately commercialized for use in the wool and sheep industry or as a management tool for certain non-human primate captive species. This aim comprises the following objectives:

(i) Determine and optimise of the processes and diluents required for the successful sorting and cryopreservation of ram spermatozoa in order to produce offspring from sex-sorted frozen-thawed ram spermatozoa using deep and conventional AI techniques.

(ii) Determine the minimum number of sorted frozen-thawed ram spermatozoa and the optimum time to inseminate relative to ovulation to achieve commercially acceptable pregnancy rates.

(iii) Investigate the effects of sorting and cryopreservation on the quality and functional status of ram spermatozoa using *in vitro* sperm assessments.

(iv) Development of short-term storage (liquid or chilled) protocols for transport of ram spermatozoa before and after flow cytometric sorting using sorting parameters and *in vitro* sperm characteristic assessments before and after sorting.

(v) Determine the most effective preparation method for ram and non-human primate species transported to the sorting site in a frozen state using sorting parameters and *in vitro* sperm quality testing before and after sorting.

(vi) Produce offspring of the desired sex after the transfer of fresh and vitrified *in vitro* produced embryos derived from ram spermatozoa transported to and from the sorting site in a frozen state.

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