Flow cytometric sex-sorting and cryopreservation of dairy buck spermatozoa

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The dairy goat industry in Australia is expanding due to an increase in consumer demand for alternative dairy products as a result of demographic and social changes in society. Pre-determination of sex through sperm sorting coupled with AI has the potential to improve the efficiency of production of Australian dairy goat herds by selecting for the most productive sex; in this instance, replacement does for breeding and milk production. Sex pre-selection can only be achieved efficiently by separation based on the difference in DNA content of X- and Y-chromosome-bearing spermatozoa.

Artificial insemination of non-sexed fresh and frozen-thawed sperm is commercially available to dairy goat producers, yet unfortunately not widely adopted. To date no efforts have been made to pre-select the sex of goat kids using sex-sorted sperm. Offspring of pre-determined sex have been successfully produced using fresh (non-frozen) and frozen-thawed spermatozoa in pigs, cattle and sheep. Regardless, substantial losses are still evident before, during and after sorting and freezing as well as a general reduction in the survival rate, membrane integrity and fertilising ability of sexed and frozen sperm compared with non-sexed and non-frozen counterparts.

Keywords: Artificial insemination, Dairy goat, Goat, Sex-sorting, Sex pre-selection, Spermatozoa

Introduction

The ability to pre-determine the sex of offspring prior to conception is a highly desired technology for incorporation into assisted breeding programs for both production animals and wildlife. Selection of sex has important implications for populations in which one sex has more intrinsic value, for instance; stud operations and female dairy replacements. The efficiency of production would be improved by reducing animal wastage and allowing for the dissemination, manipulation and storage of superior genetic stock (Parrilla *et al*. 2004).

To date, sex pre-selection can only be achieved efficiently by separation of sperm based on the difference in DNA content of X- and Y- chromosome-bearing spermatozoa coupled with assisted reproductive technologies (ARTs), namely artificial insemination (AI), in-vitro fertilisation (IVF) and embryo transfer (ET) (Maxwell *et al*. 2004). Advances in flow cytometry have led to the development of the high speed Beltsville sperm sexing technology, allowing for faster sort rates whilst maintaining a sort purity of 85-95% (Johnson *et al*. 1999; Johnson and Welch 1999; Maxwell *et al*. 2004).

Offspring of pre-determined sex using flow cytometry have been successfully produced using fresh (non-frozen) and frozen-thawed spermatozoa in several mammalian species; pigs (Grossfeld *et al*. 2005), cattle (Schenk *et al*. 1999; Seidel *et al*. 1999), sheep (Hollinshead *et al*. 2003; de Graaf *et al*. 2006; de Graaf *et al*. 2007b) bottlenose dolphins (O'Brien and Robeck 2006) and humans (Fugger 1999). Dairy cattle are the only production species to commercially adopt sex-sorted sperm, as the value of the end product justifies the premium paid for sorting (Hohenboken 1999; Seidel 2003a). The value and demand for goat milk as an alternative dairy product is rising and with it the potential for commercially adopting sex-sorted buck sperm to advance the growth of the industry. To date no efforts have been made to pre-select the sex of goat kids using sex-sorted sperm.

Artificial insemination of non-sexed fresh and frozen-thawed sperm is commercially available to dairy goat producers, yet not widely adopted. The cost-benefit analysis reveals the cost efficiency of natural matings with bucks outweighs the added benefits of improved genetic selection and year-round production of milk, hair and meat associated with AI (Leboeuf *et al.* 2000). Standard protocols have been established specifically for the cryopreservation of goat sperm (Evans and Maxwell 1987). Interestingly the presence of egg yolk; a standard cryoprotectant in the sperm freezing of many species, has been

found to interact with the seminal plasma proteins in a goat ejaculate with detrimental effects incurred on sperm motility and viability during cryopreservation (Leboeuf *et al.* 2000; Dorado *et al.* 2007).

Overview of the dairy goat industry

The dairy goat industry in Australia is expanding due to an increase in consumer demand for alternative dairy products as a result of demographic and social changes in society (Stubbs and Abud 2002). At present the Australian dairy goat industry lacks the supply capabilities necessary for developing new export markets. The commercial adoption of assisted reproductive technologies to increase the efficiency of production is likely to provide opportunities to establish new export markets, particularly in Asia (Stoney and Francis 2001).

Domestic markets for goat milk rely on the consumer perception of added 'health' and 'nutritional' benefits compared to cow milk (Stoney and Francis 2001). Despite its current expansion, the small size of the dairy goat niche market is a barrier against genetic and productive gains in the industry. There is a real need for commercialisation in the industry with a move away from traditional 'cottage farms' to large scale operations incorporating compelling marketing strategies to capture larger market shares. Artificial insemination protocols have been established (Purdy 2006; Dorado *et al*. 2007; Mara *et al.* 2007) and offered on a commercial level to improve the rate of genetic gain, yet the incorporation of this technology into breeding programs has been slow. The costs, in terms of dollars and reduced viability is seen to outweigh the benefits to breeding programs of extending breeding and lactation beyond the breeding season to consistently supply the demand for products throughout the year (Leboeuf *et al*. 2000).

Pre-determination of sex through sperm sorting coupled with AI has the potential to improve the efficiency of production of Australian dairy goat herds by selecting for the most productive sex; in this instance, replacement does for breeding and milk production. There is also the additional benefit associated with AI of faster rates of genetic gain as genes from superior males are able to be transported across the country in a liquid or frozen state, negating the need for and costs associated with live animal transport.

Sperm sexing

Over the years, numerous efforts have been made to separate sperm into two distinctive sex-chromosome populations for sex selection based on presumed differences in weight, density, size, motility and surface charge (Johnson 1994; van Munster *et al.* 1999). No study based on physical attributes has been successful in demonstrating a skewing of the sex ratio in the resultant offspring (Seidel 2003b). To date the only effective method for pre-determining sex with separated sperm is based on flow cytometry and relies on a difference in the DNA content of X- and Y-chromosome-bearing spermatozoa after staining sperm with the fluorescing DNA-binding dye, Hoechst 33342 (Maxwell *et al*. 2004). This section will focus on the applications of sexing technology, the advances and constraints of the technology and how sperm sexing can be incorporated into the Australian dairy goat industry.

Practical applications

The costs and complexities involved with sex-sorting of mammalian sperm have limited the commercialisation and adoption of this technology to niche applications in several species with all applications requiring AI or IVF (Seidel and Garner 2002). The largest beneficiary of sex-sorting technology lies with production agriculture with practical applications revolving around situations in which one sex intrinsically provides more value to production. This is true for female herd replacements in the dairy industry for breeding and lactation, male steers for meat production due to their efficiency with growth rates and also in stud male operations (Lu *et al*. 1999; Seidel 2003b).

Pre-determination of sex has various other uses in terms of research in biotechnology namely by advancing progress and limiting the number of animals required for studies, in companion, show and sporting animals; to reproduce superior genetics, but in particular for managing captive breeding and repopulation of wildlife (O'Brien *et al*. 2003; Seidel 2003a). In species with single sex dominated social structures, selecting for female offspring provides a means of accelerating the repopulation of

endangered species, which is especially true for those with slow growth rates and those unable to breed in captivity (Maxwell *et al.* 2004). Sex pre-selection also has important implications for human medicine by negating the risk of inheritance of sex-linked genetic diseases by selecting for female offspring (Seidel 2003b).

Current status of sex-sorting

Countless numbers of offspring have been produced using the Beltsville sperm-sexing technology since the first reported case of successful pre-determination of sex in rabbit pups in 1989 (Johnson *et al.* 1989). At this time, standard speed orthogonal sperm-sorters had been developed and were capable of sorting 350, 000 sperm per hour, after modifications were made to account for the asymmetry of sperm head morphology and to reduce the random orientation of sperm relative to the fluorescence detector. This led to the incorporation of a second detector measuring forward fluorescence and a beveled needle that altered the fluid stream from cylindrical to flat (Johnson and Welch 1999) and correctly orientated 20-40% of intact sperm to the laser beam (Johnson and Pinkel 1986).

The paddled shape and general asymmetry of sperm head morphology makes orientation relative to the detector crucial to maintain high accuracy during the sorting process. If not correctly orientated the asymmetry of the sperm head was shown to cause differential fluorescence after staining with DNA-binding dye, Hoechst 33342, concealing the relatively small differences in DNA content that flow cytometry operates on. Without a clear distinction of the DNA content of a sperm head, individual spermatozoa may be sorted into incorrect tubes or flow directly through the system as waste (Johnson 1999). Generally speaking, a greater difference in DNA content between X and Y sperm populations allows for a more accurate and efficient separation of the two populations (Maxwell *et al*. 2004). The inherent difference in DNA content has been characterised for most domestic livestock and recently this has extended to include wild primates and ungulates (O'Brien *et al*. 2003; Maxwell *et al*. 2004).

Further refinements to standard flow cytometric technology included replacement of the beveled needle by an orientating nozzle with a ceramic tip giving sperm less time to lose orientation, which improved correct orientation of sperm to 70% (Johnson and Welch 1999). Conversion to high speed modified flow cytometers operating under increased pressure (40-50psi) improved the accuracy of sperm sorting - the purity of sorted populations, and the efficiency - the time taken to sort a sample (Maxwell *et al*. 2004).

It is due to the advancements in flow cytometric technology that it is currently possible to operate at high sorting speeds in the order of 10-15 million sperm per hour with purities in excess of 95% (Evans *et al.* 2004; Maxwell *et al.* 2004). Johnson and Welch (1999) reported that sorting speeds of up to 20 million sperm per hour are achievable, although these speeds are generally associated with sexed populations of lower purities (75-80%). Although faster sort rates are theoretically possible and achievable, at this point where advances are still being made to reduce the high levels of sperm loss from handling, a compromise of lowered purity renders this approach less desirable.

Considerable between-species differences in sperm characteristics necessitate the need for establishing species-specific protocols for the handling and processing of sperm during sorting. De Graaf *et al.* (2007c) described the standard procedure for the preparation, processing and flow cytometric sorting of ram spermatozoa. Ejaculates are collected, diluted to a concentration of 200×10^6 sperm/ml and stained with the bisbenzimide fluorescent DNA-binding-dye Hoechst 33342 in a 34°C waterbath for one hour. As Hoechst has been deemed detrimental to sperm in large amounts (Durand and Olive 1982), it is best to minimise the concentration of Hoechst prior to sorting to reduce sperm damage whilst adding enough to ensure adequate resolution for sorting.

X-chromosomes intrinsically have a higher DNA content than Y-chromosomes, thus spermatozoa bearing X-chromosomes will absorb more dye and fluoresce more brightly compared with spermatozoa bearing Y-chromosomes. Food dye is added, and acts by permeating through the membranes of dead and membrane compromised spermatozoa, quenching their fluorescence. The modified flow cytometer then acts to remove the compromised population through the manual addition of 'gates' to select for the fluorescing population; thus the collected population should be composed of sperm with higher viability.

Orientation of sperm relative to the fluorescent detectors and the break of fluid stream into small, uniform droplets are crucial for sorting with high accuracies (Johnson 1995). Theoretically, each droplet should contain a single live spermatozoon, under ideal working conditions (Suh *et al.* 2005). Droplets

that are empty, contain multiple spermatozoa or are incorrectly oriented pass directly through the laser and collect as waste (Johnson 1995). The brightest fluorescing population, displayed by the 90° histograms, are manually 'gated' and selected as the viable, correctly orientated sperm populations for sorting to simulate high purity populations (Johnson 1999; de Graaf *et al*. 2007c). Sperm that are not showing fluorescence are considered non-viable and not included in sorted populations. Orientated, fluorescing sperm are seen by the 0° detector and charged either positively or negatively depending on their classification as either an X- or Y-chromosome-bearing sperm, depending on the degree of fluorescence. As the droplets pass through the electrostatic field they are deflected by oppositely charged brass plates and collected in separate tubes (Johnson 1999).

Limitations

Since the successful inception of sexed sperm to produce offspring in 1989 (Johnson *et al*. 1989), the techniques and equipment associated with sorting have undergone continual refinement to reduce the inefficiencies inherent in the process. Despite this, substantial losses are still evident before, during and after sorting alongside a general reduction in the survival, membrane integrity and fertilising ability of sexed sperm compared with non-sexed counterparts (Suh *et al*. 2005). The poor physiological condition of sperm post-sorting combined with the lowered fertility, high costs inherent in the process and low availability of sexing services limit the adoption of this technology for commercial purposes (Seidel 2003).

Despite improvements in the sorting technology, the relative inefficiencies still result in a limited number of sperm able to be sorted (Johnson 1999; Maxwell *et al*. 2004). A major loss of 30% of sperm occurs due to incorrect orientation during sorting even with integration of the ceramic tipped orienting nozzle (Maxwell *et al.* 2004). Seidel and Garner (2002) reiterate the concept and state that with an accuracy of 90%, recovery of sperm is limited to 22%, and of this number half belong to each chromosomal population. Johnson (1999) attempts to quantify this phenomenon by attributing the cause to compromised flow integrity; the orientation of sperm relative to the detector, aggregates of sperm in each droplet, non-uniform staining, poor resolution and coiled tails of sperm. Regardless of the cause, until further refinements are made to reduce the losses associated with sorting, optimisation of methodologies for collecting, preparing and handling sperm prior to and post sorting are necessary.

Several authors (Maxwell *et al*. 2004; Suh *et al*. 2005; Garner *et al.* 2006) report damages to sperm due to the effects of high dilution rates, staining with Hoechst 33342, mechanical stress, changing media, exposure to a UV laser and high pressures. Furthermore, they clarify that damage can be minimised by ensuring constant temperatures (37°C), optimal osmolarities, species specific collection and cryopreservation media and appropriate laser intensities. Due to the high pressures necessary for sufficient speed of sorting, sperm are expelled from the nozzle into a tube containing collection media at a rate approaching 90km/hr (Suh *et al.* 2005). Not surprisingly, this has been found to cause considerable reductions in viability and motility, not only from the speed, but also as a result of the rapid immersion of sperm into new medium (Johnson 1999).

A study by Suh *et al.* (2005) aimed to quantify the damage to sperm as a result of high pressure cytometric sorting and found that reducing the operating pressure significantly improved post-sort viability of sperm. A recommended reduction in pressure from 50psi to 40psi is warranted following this study, as although reducing pressure to 30psi showed most improvement in viability, the difference between 30-40 psi was not significant enough to justify the reduction in sorting resolution (Suh *et al.* 2005).

The composition of the catch medium- the medium that sperm are expelled into post-sorting- also has important implications in the maintenance of viability and motility of sex-sorted spermatozoa. A good catch medium will buffer the fall of sorted sperm from the flow cytometer, provide a nutrient base for sorted sperm while sorting is underway and be compatible with other media used throughout the sorting process (Bathgate 2008 pers. comm).

The use of Hoechst staining has met with much controversy, with suspicions that the DNA-binding stain has mutagenic properties likely to increase the probability of abnormalities in animals produced using sexed sperm (Durand and Olive 1982). Hoechst is a bisbenzimide stain that binds to the grooves of the DNA helix and is subsequently excited by a UV laser light, resulting in fluorescence. Despite reports of damage to DNA integrity and potential for mutation, numerous studies performed since this time have

found no evidence to support these claims. Catt *et al*. (1997) found no evidence of increased DNA 'nicks' after exposure to high levels of Hoechst stain. Seidel and Garner (2002) also report no difference in motility or DNA integrity after analysis with a chromatin stability assay. Although no effects on sperm DNA are currently apparent, further investigations into resultant embryos from sorted spermatozoa are warranted. Offspring derived from sex-sorted sperm are physiologically normal with no reports to date of obvious genetic abnormalities (Johnson 1989; Johnson and Welch 1999; Seidel and Garner 2002; de Graaf *et al.* 2007c).

The aforementioned speed of sorting is one of the main limitations to commercialisation of the sex-sorting process. As mentioned previously, cattle are the only production species with commercially available sex-sorted sperm and this is in part due to the low dose of insemination required. Seidel and Garner (2002) report the process of sorting bull spermatozoa requires 8 minutes to achieve sufficient numbers of sexed sperm for a commercial cattle AI dose; 2×10^6 spermatozoa per insemination dose. In comparison, sorting of ram spermatozoa generally requires 30 minutes to achieve sufficient numbers of sperm per dose; 8×10^6 spermatozoa (Maxwell *et al*. 2004; de Graaf *et al*. 2007a).

Overall the main constraint to sexing technology is the depressed viability and fertility of sperm post sorting. Numerous studies have documented lowered pregnancy rates compared with unsexed controls (Johnson *et al*. 1989; Seidel *et al.* 1999; Hollinshead *et al*. 2002) due in part to embryo mortalities (Morton *et al*. 2005), reduced insemination dose, timing of insemination relative to ovulation and reduced viability in the female reproductive tract (Maxwell *et al.* 2004). Sperm physiology has been characterised in part, particularly in relation to the reactions necessary for fertilisation. Sorted spermatozoa have been found to exhibit altered membranes and partial capacitation in response to the sorting process (Maxwell *et al.* 2004); such a response is a function of acrosomal reactions as the sperm matures. Thus, sorting is causing premature ageing of the sperm populations, similar to that experienced during transit in the female tract, freeze thawing and long term chilling of sperm (Seidel 2003b; Maxwell *et al.* 2004). The reduction in the in-vitro and in-vivo fertilising lifespan of spermatozoa necessitates the use of higher doses of sperm for AI whilst also timing AI closer to ovulation and inseminating close to the ovaries (Seidel and Garner 2002).

Upon consideration of the constraints involved with sex-sorting sperm it comes as no surprise that there is still progress to be made before the technology will be ready for extensive industry incorporation. At this point, sexed sperm are considered very valuable due to the cost and time involved in their preparation, and as a result low numbers should be used in conjunction with ARTs such as IVF, laparoscopic intrauterine insemination, intra cytoplasmic sperm injection and low dose insemination (Maxwell *et al*. 2004).

Incorporation of sex-sorted sperm into the dairy goat industry

At the time of writing, only one published paper has attempted the flow cytometric sorting of goat sperm based on the difference in DNA content between X- and Y- bearing populations. Parrilla *et al* (2004) demonstrated that goat sperm could be separated into high purity X- and Y-chromosome-bearing populations with high sort rates and good accuracy. However, this study did not further assess the post-sort quality and viability of sperm or the potential for sorted sperm to retain fertilising ability in-vivo and in-vitro.

The relative merit of the study lies in the characterisation of the average difference in DNA content between X- and Y-chromosome-bearing goat spermatozoa; determined to be 4.4% (Parilla *et al.* 2004). In comparison with other production species, the 'split' of goat sperm into two populations is more resolved, and the larger difference in DNA would increase the accuracy and efficiency of sorting goat sperm. The DNA difference is similar to values established for ram spermatozoa; 4.2% (Figure 2) (Johnson and Welch 1999) providing further support to arguments of considerable between-species similarities. Between-species similarities in other ARTs, namely; cryopreservation media and chilled storage IVF hint at the potential for initial use of ram protocols for the sexing of goat sperm (Bathgate 2008 pers. comm.).

Further investigations are warranted to characterise the fertilising ability of sex sorted goat sperm in-vivo and in-vitro. Initial research (Parrilla *et al.* 2004) suggests that the difference in DNA between chromosomal sperm populations will contribute to the accuracy and efficiency of this process.

Cryopreservation

Goat sperm was first frozen at -79°C in 1950 (Smith and Polge) with the resulting fertility of thawed sperm deemed to be too low for any practical application (Barker 1957). Since then, alterations and refinements to the process of freezing; addition of cryoprotectants and the rate of cooling, has led to improvements in the post-thaw survival, membrane integrity and fertilising ability of frozen goat spermatozoa (Leboeuf *et al*. 2000). Artificial insemination of sexed, frozen sperm will likely drive the commercialisation of sex-sorting technologies so improvements in determining optimal freezing methods are warranted. Based on protocols established for rams it is likely that sex-sorting goat sperm will take approximately 30 minutes per sample, due to the considerable between-species similarities. Coupled with the fact that inseminate doses should also be doubled to counteract the inherent losses involved with sorting and freezing, the need arises for a 'bank' of sorted sperm in preparation for heat onset of the doe.

Practical applications

The need to store and transport sperm are the drivers behind the development of cryopreservation protocols. Limitations to sexing technologies include the distance between males, females and the flow cytometer. At present, reliable sorting relies on the use of fresh or chilled (4-5°C) ejaculates, necessitating the need for males to be located close to the flow cytometer (Maxwell *et al.* 2004). More importantly, sorted sperm may need to be transported considerable distances for use in the artificial insemination of females. Seidel (2003b) reports that in well-managed herds, fertility of frozen, sexed sperm is 70-80% of non-sorted sperm.

In relation to the Australian dairy goat industry, both artificial insemination and cryopreservation have potential benefits in improving the progress of industry commercialisation. Artificial insemination may serve to improve the production of milk, meat and hair by controlling reproduction, in particular synchronising the time of kidding, selecting for improved genotypes, storing genetic material and increasing the number of offspring produced per sire (Leboeuf *et al.* 2000). Controlling reproduction can achieve acceptable fertility in the non-breeding season thereby improving the productivity of an enterprise by supplying end-products year round. Artificial insemination also allows for the efficient spread of superior genetics on a spatial and temporal basis without transmitting disease, improving genetic gains on an industry basis (Leboeuf *et al.* 2000).

Recently Mara *et al* (2007) characterised the potential for long term viability of chilled sperm as an alternative to freezing. No compromise to fertility was reported with semen chilled at 4°C over 24hr, warranting the investigation of long term chilled preservation of sperm to overcome the inherent losses involved with current cryopreservation protocols. This study has arisen due to consumer pressure for organic dairy milk products, free from the hormones used to routinely synchronise oestrus in AI protocols including those involving frozen sperm.

Cryopreservation of goat sperm

Protocols for the cryopreservation of goat sperm have long been established (Evans and Maxwell 1987) with nuances specific to goats characterised and circumvented to maintain viability and fertility post-thaw. The main hindrance of goat ejaculates relative to other domestic species is the deleterious effects of egg yolk on sperm survival through an interaction with seminal plasma components (Chauhan and Anand 1990; Ritar and Ball 1993; Leboeuf *et al*. 2000). This concept will be further evaluated in the proceeding section.

Leboeuf *et al*. (2000) describes the conventional method for freezing goat sperm. Initial dilution using a glycerol-based cryoprotectant at 30°C is followed by cooling to 4°C over 1-1.5 hours by placing the tube containing sperm in a volume of water and cooling the body of water. Sperm can then be frozen as pellets or straws and immersed in liquid nitrogen. The method for freezing in straws involves a two-step process; straws are loaded, capped and suspended horizontally in liquid nitrogen vapour 4-5cm above liquid nitrogen and after 4-5 minutes straws are immersed in the liquid nitrogen. The procedure for freezing on dry ice is comparably simpler, involving the freezing of a pellet of sperm on -79°C dry ice until solid and then immersing in -196°C liquid nitrogen.

The velocity of freezing is able to be regulated by the size of the pellet. Maxwell *et al.* (1994) report that the rates of cooling, freezing and thawing ram sperm are dependent on the surface-volume ratio of the package in which the sperm is frozen. Furthermore, Salamon and Maxwell (1995) describe that ram spermatozoa frozen in pellets are able to tolerate a wide range of freezing temperatures, ranging from -79°C to -160°C, rendering them the most versatile method for freezing. Holding straws in liquid nitrogen vapour prior to immersion attempts to create a freezing temperature close to that of dry ice (-60°C to 80°C) (Maxwell *et al.* 1994)

In terms of time, post-thaw motility and survival, freezing in pellets imparts better viability (Ritar *et al*. 1990; Ritar and Ball 1991; Maxwell *et al*. 1995) compared with straw freezing, but with the advent of computerised freezing machines this difference may be inconsequential upon consideration of an industry requirement for labeling of biomaterials (Purdy 2006). Identification of the date and dose of semen is possible with polyvinyl chloride (PVC) straws and this is particularly useful during banking for long-term storage of cryopreserved sperm. The drawback of freezing in pellets on dry ice not only relates to the limitations of labeling but also extends to cover the threat of contamination throughout the process. Sperm come into contact with the surface of the dry ice and are plunged directly into liquid nitrogen, neither of which can be assumed sterile.

With regards to the in-vivo fertility of cryopreserved sperm, numerous studies have documented the pregnancy rates following AI of frozen sperm in various breeds of goats; 51% in Angoras (Ritar and Salamon 1983), 39% in Cashmeres (Ritar *et al.* 1990), 47% in Floridas (Dorado *et al.* 2007), 50% in Beetals and Bengals (Singh *et al.* 1995) and 57% in Murciano-Granadinas (Salvador *et al.* 2005). Studies have also reported fairly high kidding rates of goats inseminated with sperm stored for long periods. Fougner (1979) observed kidding rates of 63% after intrauterine insemination of sperm stored for 1-3 years. Similarly Waide *et al.* (1977) witnessed kidding rates of 63%, 79% and 77% with sperm stored for 1-30 days, 31-102 days and 210-1022 days, respectively. These studies demonstrate that goat sperm can be successfully cryopreserved whilst maintaining reasonably high fertility in-vivo.

Current status and limitations

Inherent to any cryopreservation protocol are losses in sperm numbers and viability due to the extra handling involved with the actual process of freezing. This has been attributed to the cooling rate, cold shock associated with temperature changes and the introduction of egg yolk and glycerol as cryoprotectants. Species differences and male to male variation in sperm numbers and viability post-thaw have also been observed (Leboeuf *et al.* 2000; Purdy 2006).

Freezing and thawing compromises the structural integrity, biochemical processes and functionality of sperm; motility is reduced, membrane integrity is compromised, fertilising ability is suppressed and transport through cervical mucus is impaired (Maxwell *et al*. 2004; Purdy 2006; Dorado *et al*. 2007). Interestingly, Cox *et al*. (2002) observed a direct correlation between the ability of spermatozoa to migrate efficiently in cervical mucus in goats with the ability to colonise the oviducts and fertilise mature oocytes in vivo.

A study by Dorado *et al*. (2007) found that freezing Florida goat sperm decreased motility substantially, yet the in-vivo fertility results obtained in the study remained in the average range of values determined by other authors for other breeds of goat to be representative of successful freezing. The group also identified that the structural integrity of the plasma and acrosome membranes of goat spermatozoa were more vulnerable to freezing (50% of acrosomes remained intact one hour post thawing) than the locomotor apparatus, as motility was not suppressed to the same extent as damage to membranes (Dorado *et al*. 2007). The general conclusions of the study included the use of a greater number of sperm per insemination, inseminating close to the site and time of ovulation and by using goat sperm populations with highly rapid forward progressive motility to overcome the defects inherent in cryopreservation of goat spermatozoa.

The addition of egg yolk to cryopreservation media as a cryoprotectant is routine in formulating freezing protocols for most domestic species. Egg yolk protects the plasma and acrosome membranes of sperm against temperature related injury such as cold shock, as during freezing the rate of cooling goat sperm is quite variable and at times rapid (from 4°C to -79°C in one step, and -79°C to -196°C in another; Dorado *et al.* 2007). During preservation, sperm cells also lose anti-oxidant enzymes and proteins, increasing the vulnerability to peri-oxidative damage and compromising survival and fertility (Mara *et al.* 2007).

Remarkably the seminal plasma components, namely the enzyme originating from the bulbourethral gland secretion of a goat ejaculate causes specific problems in relation to preserving semen in egg yolk based diluents (Ritar and Salamon 1982; Mara *et al*. 2007). An egg yolk coagulating enzyme (EYCE) present in the seminal plasma of buck sperm exhibits a lipase activity on lecithin, a compound found in egg yolk and subsequently employed in many semen extenders, resulting in fatty acid and lysolecithin production (Dorado *et al.* 2007). Lysolecithin in particular is quite toxic to sperm cells, resulting in acrosomal damage and reduction in motility and survival (Leboeuf *et al*. 2000).

The problems with EYCE can largely be circumvented by one of two methods; washing sperm immediately after collection to remove excess seminal plasma, or by using a lower percentage of egg yolk in the cryodiluent (in the range of 1.5-5%) (Leboeuf *et al*. 2000). Washing to remove seminal plasma coupled with the addition of 1.5-6% egg yolk was found to improve survival of sperm kept at 37°C for six hours (Ritar and Salamon 1982). The relative downfall in this method lies in its complexity and the time it takes to wash and equilibrate sperm samples and despite all these efforts sperm losses still ensue. A study by Corteel (1975) observed that washing for removal of seminal plasma in dairy goat breeds caused no improvement in fertility. Similarly Leboeuf *et al*. (2000) documented that the detrimental interactions involving seminal plasma proteins vary in intensity between the breeding and non-breeding season, as well as observing considerable male to male variation within breeds.

Concluding remarks

Domestic and global drivers for commercialisation of the dairy goat industry in Australia are demanding high quality goat milk products at low costs with a consistent supply year-round. Realistically these goals may only be achieved through adoption of assisted reproductive technologies; namely sex-sorting, cryopreservation and artificial insemination of buck spermatozoa. Artificial insemination and cryopreservation protocols have already been established for dairy goats in Australia to improve the efficiency of production and dissemination of superior genetics, yet are not widely practiced. It is likely that artificial insemination of sexed, frozen sperm will drive the commercialisation of sex-sorting technologies in the dairy goat industry.

It has been shown that goat sperm can be sex sorted (Parrilla *et al.* 2004) and frozen (Leboeuf *et al.* 2000; Dorado *et al*. 2007). It is not known whether sex-sorted goat sperm retain their fertilising ability post-sort, and furthermore, the viability of goat sperm after coupling sex-sorting with cryopreservation. As documented in other species, further refinements are needed in relation to the collection, preparation and handling of sperm prior to and post-sorting to reduce the loss of sperm numbers and reductions in viability inherent in flow cytometric sex-sorting. In relation to dairy goats, a protocol to circumvent the problems of egg yolk as a cryoprotectant is warranted.

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