Storage of bovine semen in liquid and frozen state

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Abstract

This review describes the historical and current methods used for storage of bovine semen. The essential physiological differences between liquid and frozen semen, their relative advantages and disadvantages are addressed, and the current state of technology, the procedures used, their merits and future possibilities are also discussed. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Liquid semen; Spermatozoa numbers; Frozen bovine semen

1. Introduction

Genetic advancement in the cattle population, particularly in the dairy sector has relied on two processes, namely, the use of bulls of high genetic merit and the selective rearing of calves of high breeding merit as replacements. Artificial insemination has remained the main vehicle for the rapid dissemination of valuable genes and the method of choice for dairy farmers worldwide to improve the genetic quality of their stock. This steady level of genetic progress in dairy cattle is primarily due to advances in semen technology. For the purposes of this review, the reference will be mostly with the dairy industry, for this is where technical advancement in semen technology has been captured most successfully (Chupin and Schuh, 1993; Chupin and Thibier, 1995; Cunningham, 1998; Foote, 1998).

The potential genetic contribution of a sire is described by Foote (1998) as:

\[
\text{Contribution of a sire to genetic improvement} = \text{Number of progeny per sire} \times \text{Genetic superiority of the sire.}
\]

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The number of progeny per sire will be determined by: (i) total sperm output of the bull; (ii) the number of sperm used per insemination; and (iii) the percentage of cows calving to a single insemination. This can be represented as:

\[
\text{Number of progeny per sire} = \left( \frac{\text{Number of sperm per sire}}{\text{number of sperm inseminated per cow}} \right) \times \text{Proportion of cows calving to a single insemination.}
\]

These principles effectively determine the number of bulls required for a dairy cow population. The equation becomes particularly attractive when very few bulls are needed to service a large population of dairy cows thereby significantly raising the selection intensity (Shannon, 1978).

The requirement of semen from bulls of high genetic merit has been the main impetus for developing and refining storage technologies for cattle semen. In a 1980 survey, it was believed that the total number of inseminations worldwide exceeded 130 million (Bonadonna and Succi, 1980). A more recent survey (Chupin and Thibier, 1995) showed that the total number of doses of semen produced exceeded 200 million, with more than 95% of this processed as frozen product. About 4 million doses were used as liquid semen, and this was restricted primarily to New Zealand with smaller amounts in France, The Netherlands, Australia and Eastern Europe. In this review, the main focus will be on the well-known storage technologies, which include liquid and frozen stored semen along with a brief discussion on emerging technologies for semen storage in cattle.

2. Liquid storage of semen

There have been many reports since the turn of the century on diluting media for semen of livestock with much of this work originating in the former Soviet Union (Anderson, 1945). Each diluent, ranging from a simple salt solution to the more complex buffered medium had its own merits. Perhaps the accepted principle of semen dilution technology was that survival of spermatozoa for extended periods was inversely related to their metabolic activity. To be useful for artificial insemination, diluted semen had to have a minimum shelf-life of between 2 and 4 days to provide for easy transport and use in distant locations. It was this guiding principle that led to the initial storage temperature of 5°C (Salisbury and Van Demark, 1961). The predominant effect of storage at 5°C was a lowering of metabolic rate of spermatozoa, which contributed to extended survival. The discovery that egg yolk was a useful additive in increasing the preserving properties of the various media, added further impetus to this work (Phillips, 1939). Many extenders have been developed for liquid storage of semen, and this chapter only provides a brief description of some of the major developments. Detailed discussions on some of the early diluents are available in a comprehensive review by Foote (1978).

2.1. Diluents for storage of semen at refrigerated temperatures

The early diluents for storage at refrigerated temperatures were significantly influenced by the discovery of egg yolk as a useful additive, and the primary buffering
Table 1
Comparison of 60- to 90-day non-returns (% NRR) to inseminations with bovine semen diluted to 8 million spermatozoa/ml in CUE and Tris media. Data from Foote (1978). Diluent compositions are shown in Table 2.

<table>
<thead>
<tr>
<th>Diluent</th>
<th>No. 1st inseminations</th>
<th>% NRR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CUE</td>
<td>5981</td>
<td>73.0</td>
</tr>
<tr>
<td>Tris</td>
<td>5673</td>
<td>73.3</td>
</tr>
</tbody>
</table>

component in these diluents was phosphate (Phillips, 1939). Subsequently, citrate was found to have adequate buffering capacity with an enhanced period of survival of spermatozoa stored at 5°C (Willett and Salisbury, 1942). Citrate then became the salt of choice, as its chelating properties improved the solubility of protein fractions in egg yolk. Egg yolk has been a more common additive, but homogenised whole milk, fresh or reconstituted skim milk and coconut milk have also been used to preserve fertility of bovine spermatozoa (Melrose, 1962; Norman et al., 1962).

Many of the zwitterionic buffers (Good et al., 1966) provided good buffering capacity over a wide pH range. Tris, TES, MES, HEPES, PIPES, MOPS and BES titrated with hydrochloric acid were tested at various pH levels as semen diluents and all of them were similar in suitability (Foote, 1972) cited by Foote (1978). However, the Tris-based diluent has become more universal, and this buffering medium combined with egg yolk and glycerol has been tested extensively (Davis et al., 1963a,b). Motility of spermatozoa was slightly lower in Tris medium buffered to lower pH (6.25 vs. 6.75) than in Cornell University Extender (CUE), but good results were obtained in a fertility trial with both media (Table 1).

A further field trial demonstrated that a lower pH (6.5) and the presence of glycerol in Tris medium improved fertility, compared with higher pH and absence of glycerol (Foote, 1970). The results were comparable with semen diluted with CUE. The conclusion from these series of studies was that the Tris diluent was suitable for storage of semen at refrigerated or ambient temperature and also in frozen state. The diluents shown in Table 2, and some subsequent modifications to these have been used in field trials with varying success (Bartlett and Van Demark, 1962; Van Demark and Bartlett, 1962; Shannon, 1965, 1978; Foote, 1978). In the case of the Illini Variable Temperature (IVT) diluent, the main changes have been in the ratio of bicarbonate to citrate and the concentration of glucose increasing from 0.3% to 1.2%.

2.1.1. Milk-based diluents

The historical origin of milk as a diluent for bull semen has been described by Salisbury and Van Demark (1961). The first report on the use of milk originated in Germany (Koelliker, 1856) cited by Salisbury and Van Demark (1961). This observation went unnoticed until Underbjerg et al. (1942) compared fertility of bull semen stored in egg yolk–phosphate and autoclaved milk diluents. The results were similar for both media. The methods of preparation of milk diluents and the various combinations have been reviewed by Melrose (1962) and Foote (1978). The important aspect of storage of semen in milk-based extenders was the necessity to inactivate the lactenin (a toxic
Table 2
Composition of diluents for storage of bovine semen at low (5°C) and ambient temperatures. Ingredients are in g/100 ml of medium

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Egg yolk–phosphate (Phillips, 1939)</th>
<th>Egg yolk–citrate (Salisbury et al., 1941)</th>
<th>Original IVT diluent (Van Demark et al., 1957)</th>
<th>CUE (Foote et al., 1960)</th>
<th>Tris medium for ambient storage (Foote, 1970)</th>
<th>CAPROGEN® (Shannon, 1965)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature of storage</td>
<td>5°C</td>
<td>5°C</td>
<td>5°C</td>
<td>5°C and ambient</td>
<td>5°C and ambient</td>
<td>Ambient</td>
</tr>
<tr>
<td>Tris</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potassium hydrogen phosphate</td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium hydrogen phosphate</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>3.6</td>
<td>2</td>
<td>1.45</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>0.21</td>
<td>0.21</td>
<td>0.21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.04</td>
<td>0.04</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose</td>
<td>0.3</td>
<td>0.3</td>
<td>1.25</td>
<td>0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citric acid</td>
<td>0.087</td>
<td>1.675</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Catalase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caproic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg yolk (%)</td>
<td>50</td>
<td>50</td>
<td>10</td>
<td>20</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Gas phase</td>
<td>CO₂</td>
<td>self carbonating</td>
<td></td>
<td></td>
<td></td>
<td>N₂</td>
</tr>
</tbody>
</table>
factor) by heating. Different preparations of milk needed different heating requirements to selectively inactivate the lactenin, but still maintain the integrity of the protein and sugar moieties in milk. Glycerol added to the milk-based diluents was advantageous and maintained fertility of bull spermatozoa for 4 days (O’Connor and Smith, 1959; Almquist, 1962). In general, milk-based extenders have been successful in maintaining fertility and gave comparable results to egg yolk–citrate diluents (Foote, 1978). Egg yolk (5–10%) added to milk-based extenders seemed to inactivate the toxic factor in milk. This process has overcome the problem of heating the skim or fortified milk before addition to semen, and also adequately maintained the fertility of diluted bovine semen stored at 5°C.

2.2. Storage of semen at ambient temperature

It has been the maxim of semen dilution technology that survival of spermatozoa in diluted state is inversely related to their metabolic activity. The pursuit of this principle led to the development of egg yolk diluents for storage of semen at 5°C (Phillips, 1939) and deep freeze techniques for total immobilisation of spermatozoa at very low temperatures (Polge et al., 1949; Polge and Rowson, 1952). In order to store semen at temperatures above 5°C and achieve satisfactory results, alternative methods of metabolic inhibition were attempted. Thus, Van Demark and Sharma (1957), proposed CO₂ narcosis, Norman et al. (1958) suggested lowering the pH and Shannon (1965) proposed N₂ gassing as methods to inhibit metabolic activity of spermatozoa. Storage at 5°C reduces metabolic activity, but not all changes associated with lower temperatures are beneficial to spermatozoa. For example, the activity of the Na⁺/K⁺ pump decreases with reduced temperatures such as 5°C, but is unable to cope with diffusion of ions across the cell membrane (Quinn and White, 1966, 1967; Sweadner and Goldin, 1980). A consequent increase in the intracellular concentration of Na⁺ is detrimental to survival of spermatozoa (Makler et al., 1981). It was then postulated that storage at ambient temperature may be superior to storage at 5°C, provided the medium in which spermatozoa are suspended inhibits those pathways that are detrimental to their survival at higher temperatures (Shannon and Curson, 1972b, 1982a, 1984). The temperature of storage is an important consideration. The optimum temperature range is considered to be 18°C to 24°C (Shannon and Curson, 1984). Storage at temperatures above this results in lower fertility, compared to storage at 5°C (Foote and Bratton, 1960; Bartlett and Van Demark, 1962).

Another significant development in ambient temperature diluent technology has been the change in the level of egg yolk in the diluent. The ratio of egg yolk to buffer became particularly important at higher storage temperatures than it was with storage at 5°C (Foote and Bratton, 1960; Shannon and Curson, 1983). Historically, semen diluents have included anywhere from 12.5% to 50% egg yolk in the medium. Efforts to decrease the egg yolk concentration in the simple phosphate and citrate buffers gave no advantage in survival of spermatozoa or fertility (Foote and Bratton, 1960). Egg yolk does protect the sperm cells from the toxic effects of seminal plasma; however, it also provides substrates (aromatic amino acids such as L-phenylalanine) for H₂O₂ production by an aromatic amino acid oxidase (AAA0) released from dead cells to the detriment of live
Table 3
Percentage of NRRs (49 day) for semen diluted in CAPROGEN® media containing differing levels of egg yolk. Data from Shannon and Curson (1983)

<table>
<thead>
<tr>
<th>Trial</th>
<th>Egg yolk (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20</td>
</tr>
<tr>
<td>1</td>
<td>66.5 ± 0.4</td>
</tr>
<tr>
<td>2</td>
<td>67.4 ± 0.3</td>
</tr>
<tr>
<td>3</td>
<td>67.6 ± 0.7</td>
</tr>
</tbody>
</table>

spermatozoa (Shannon and Curson, 1972b, 1982b). The amount of egg yolk required in semen diluents to provide protection against seminal plasma toxins is proportional to the amount of seminal plasma in diluted semen (Shannon and Curson, 1972a). Thus, when semen is diluted to a high rate, and the seminal plasma concentration is consequently reduced, there would be some advantage in reducing the egg yolk concentration. This was borne out in a large fertility trial, where a decrease in egg yolk concentration from 20% to 5% had no detrimental effect on fertility. Further decrease of egg yolk concentration affected fertility of some sires, suggesting that the level of egg yolk was insufficient in some cases to be completely protective (Table 3).

2.2.1. Coconut milk extender
Coconut milk extenders have been equivalent to skim milk–glycerol, CUE or CAPROGEN® in maintaining motility and survival of spermatozoa (Norman and Rao, 1972; Foote, 1978). This extender is quite simple and contains 15% decanted coconut milk boiled for 10 min, 2.2% sodium citrate, antibiotics and 5% egg yolk. The presence of egg yolk was essential to provide a lipid component to the medium. In some cases, the medium has been supplemented with 0.1% calcium carbonate (Norman et al., 1958). A recent report on the use of Coconut extract and Coconut milk on ram semen showed dramatic maintenance of motility over a 48 h period of 30°C but no fertility trials have been reported (Chairussuyuhur et al., 1993). There have been no recent reports on significant advances in this front in the literature.

2.2.2. Immobilisation of spermatozoa by low pH
An early observation in 1924 by Krshyshkovsky and Pavlov, cited by Norman et al. (1958), showed that spermatozoa were immobilised when placed in sealed tubes at room temperature, but subsequent exposure to air produced a resumption of activity. Inhibition of sperm motility was due to the decrease of pH by the accumulation of lactic acid in the medium. Further studies by Norman et al. (1958) confirmed the finding and suggested the decrease of pH as an effective method to inhibit metabolic activity of spermatozoa. In this study, conclusive evidence was obtained that lowering the pH substantially reduced metabolic activity measured by O₂ consumption, lactate production, and motility of live spermatozoa. This effect was reversible, as activity resumed when the old diluting medium was replaced after 150 h incubation with fresh medium at neutral pH. By merely altering the pH from 5.76 to 7.45, motile activity of spermatozoa could
be altered from relative senescence to rapid movement (Norman et al., 1958). The effect of acidic conditions on sperm metabolism was identified by Koelliker (1856), cited by Norman et al. (1958) and Guenther (1907), who concluded that activity of spermatozoa is a function of hydrogen ion concentration. A decrease in pH down to 5.5 was well tolerated by spermatozoa, and the effect could be reversed by alkaline conditions, but a pH below 5.5 was spermicidal and caused irreversible enzyme denaturation.

2.2.3. Immobilisation of spermatozoa in diluents gassed with carbon dioxide

Carbon dioxide was found to be a very effective inhibitor of sperm motility (Shettles, 1940). Early experiments showed that sperm motility was reversibly inhibited when exposed to CO₂ for short periods, but prolonged exposure to this gas proved toxic to sperm cells. Van Demark and Sharma, 1957 proposed CO₂ narcosis as an effective means to maintain viability and fertilising ability of bovine spermatozoa for 6 to 7 days at room temperatures. The first diluent designed on the basis of CO₂ immobilisation of spermatozoa was the IVT diluent (Van Demark et al., 1957). The IVT diluent contained a mixture of salts, sugar and antibacterial agents, and was saturated with CO₂ until the pH decreased to 6.35. The final mixture contained 10% egg yolk. Bovine semen extended with this diluent and stored at room temperature maintained fertility for over 3 days (Bartlett and Van Demark, 1962). Several diluents were then formulated using this concept to optimise sperm survival and to extend the shelf-life of diluted semen (Table 2). By optimising the concentration of bicarbonate in the medium (0.1 M), and at higher concentrations of glucose (0.067 M) in the IVT diluent, sperm motility was maintained at high levels (> 45%) for over 90 days at 5°C. While motility was maintained for extended periods, fertility decreased sharply after 2 days of storage at 5°C, (Bartlett and Van Demark, 1962). This inconsistency in fertility result was evident also in other studies, where CO₂ narcosis was used to maintain motility and fertility of spermatozoa for extended periods (Dunn and Foote, 1958; McFee et al., 1958; Foote et al., 1960). The main difference between the IVT diluent and the CUE was that CUE was self carbonating and relied on the action of citric acid on bicarbonate to release CO₂ with no major effect on the pH of the medium (Table 2). The CUE was a substantial improvement on the IVT diluent in this regard. Numerous field trials have confirmed that CUE along with CAPROGEN® are the most successful liquid semen diluents to date (Table 4; Shannon, 1964; Foote, 1978).

Table 4
Fertility of bull spermatozoa stored in CUE and CAPROGEN® diluents. Data from Shannon (1964) and expressed as % NRR at 49 days

<table>
<thead>
<tr>
<th>Diluent</th>
<th>Day of collection</th>
<th>Day after collection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of inseminations</td>
<td>% NRR</td>
</tr>
<tr>
<td>CUE</td>
<td>59,091</td>
<td>62.8</td>
</tr>
<tr>
<td>CUE + glycerol</td>
<td>30,058</td>
<td>63.1</td>
</tr>
<tr>
<td>CAPROGEN®</td>
<td>73,993</td>
<td>62.8</td>
</tr>
<tr>
<td>CAPROGEN® + glycerol</td>
<td>35,351</td>
<td>62.7</td>
</tr>
</tbody>
</table>
Fig. 1. Incubated life of spermatozoa (h) stored at 12.5 million/ml and 200 million/ml at 5°C. The samples were incubated at 37°C at 12.5 million sperm/ml. Data from Shannon (1965).

2.2.4. Metabolic inhibition by nitrogen gassing

Shannon (1964, 1965) developed the CAPROGEN diluent for bovine semen, which replaced the self-carbonating concept with an ingenious method to reduce the dissolved O₂ levels in the medium with N₂ gas. This had no effect on the pH, but substantially reduced the metabolic activity of spermatozoa. An additional effect of N₂ gassing was that it nullified the adverse effect of high dilution on sperm survival. Nitrogen saturation halted the decline in incubation life of stored spermatozoa. This effect was more noticeable when spermatozoa were stored in a dilute form (12.5 million/ml), compared to storage in a concentrated state (over 200 million/ml, Fig. 1). At the same time, inclusion of volatile saturated fatty acids (Shannon, 1962) and catalase (Shannon, 1972, 1973; Macmillan et al., 1978) significantly improved the length of time that diluted bovine semen could be stored. The diluent was originally developed for use at 5°C, but proved superior for semen stored at temperatures between 15°C and 27°C (Shannon and Curson, 1984). The conception rates with semen stored in CAPROGEN® were 0.9% better at the elevated temperatures on the day of collection and 2.3% higher on the following day compared to semen stored at 5°C (Table 5; Shannon, 1971).

2.3. Requirements for storage of bull semen at ambient temperatures

Storage of semen at 5°C was not always easy or convenient, therefore, methods to store semen at ambient or room temperatures were actively investigated. The require-

<table>
<thead>
<tr>
<th>Storage times</th>
<th>Storage at 5°C</th>
<th>Ambient temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>6–12 h</td>
<td>268,201</td>
<td>44,354</td>
</tr>
<tr>
<td>12–24 h</td>
<td>172,663</td>
<td>29,772</td>
</tr>
</tbody>
</table>

* Significantly different to storage at 5°C (p < 0.05).
ments regarding diluting media that could accommodate the decrease in metabolic activity of spermatozoa when stored at ambient temperature are the following.

(i) An energy source in the form of simple sugars (glucose, fructose). Bovine spermatozoa function adequately under both aerobic and anaerobic situations and the rate of respiration can be controlled by the level of substrates (Hammerstedt et al., 1988; Krzyzosiak et al., 1999) and temperature of storage (Hammerstedt and Hay, 1980; Inskeep and Hammerstedt, 1985).

(ii) A buffered medium to accommodate pH changes, usually in the form of citrate, Tris, HEPES, MOPS, or bicarbonate buffers.

(iii) Glycerol, a dual purpose additive, to provide osmolality to the medium and as an invasive thermal protectant. In liquid semen diluents, glycerol has been shown to reduce the decline in fertility associated with the aging of spermatozoa (Shannon, 1964). In some cases, however, it did decrease fertility with semen used shortly after collection (O’Connor and Smith, 1959; Almquist, 1962). The effect of glycerol may depend on the basic diluent to which it has been added. The addition of 1% glycerol to an egg yolk–citrate–glycine diluent increased the conception rate by $1.4 \pm 0.7\%$ for semen stored for 24 h at ambient temperature (Shannon, 1964). The beneficial effect of glycerol was also noticed when added to milk and Tris diluents with an increase in conception rates of 1.4% and 2.5%, respectively (Foote, 1970; Bratton and Foote, 1973, cited by Foote, 1978 and Stewart 1964).

(iv) Egg yolk or other macromolecular substances such as skim milk, whole milk, or coconut milk provide thermal protection to spermatozoa. The inclusion of macromolecular substances, particularly egg yolk has benefit even when sperm cells are not subjected to cold shock. Perhaps the most important effect of egg yolk is its ability to bind seminal plasma fractions that have a detrimental effect on survival of spermatozoa (Shannon and Curson, 1972a; Al-Somai et al., 1994; Prendergast et al., 1995).

(v) A combination of antibiotics to provide both bacteriostatic and bactericidal protection.

Other additives to diluents for storage of semen at ambient temperature have also influenced the keeping quality of bovine semen with some positive effects on conception rates. Some of these additives are listed below.

2.3.1. Glycine

The reason why glycine has been used as an additive to semen diluents is still not clear. Many plants, unicellular organisms and marine elasmobranchs, accumulate compatible solutes, which provide protection during stress situations (Yancey and Somero, 1979; Aspinall and Paleg, 1981). Some of these compounds such as free amino acids and quaternary nitrogen containing compounds retard thermal denaturation of enzymes (Paleg et al., 1981), provide thermal protection (Heber et al., 1971) and maintain enzyme structure and function (Bowlus and Somero, 1979). Conflicting fertility results have been reported with diluents containing glycine (Strom, 1956; Stower and Bud Hasaim, 1957; Foote et al., 1958; Salisbury and Van Demark, 1961; Shannon, 1964). A fertility trial with 20% egg yolk–citrate–glucose diluent containing 1% glycine gave a fertility advantage of 2.1% on the day of collection and 2.7% with semen stored for 24 h, compared to a simple 20% egg yolk–citrate diluent (Shannon, 1964). The advantage
appears to be directly attributable to the glycine–glucose moiety. Presence of glycine in diluents for frozen semen has been detrimental to post-thaw survival of spermatozoa (Martin, 1965).

2.3.2. Caproic acid
Volatile fatty acids included in semen diluents is presumed to aid in maintaining membrane integrity and fluidity (Shannon, 1962). While this feature has not been investigated in detail, it is well known that lower-order fatty acids will prolong the respiratory activity of spermatozoa for extended periods (Mann and Lutwak-Mann, 1981). Caproic acid enhances cell survival and fertility of diluted bovine semen. In a large-scale fertility trial, addition of caproic acid had no significant effect on fertility with semen used on the day of collection, but a significant increase (+1.3%) was seen on the day after collection (Table 6; Shannon, 1962).

2.3.3. Catalase
The medium for dilution and storage of semen has significant effects on the integrity of spermatozoa. Within an aerobic or even a partially aerobic system, the production of reactive oxygen species is inevitable (Mann and Lutwak-Mann, 1975; Fridovich, 1978, 1981; Mann et al., 1980). The most damaging are the superoxide anion (O$_2^-$), peroxide (H$_2$O$_2$) and the hydroxyl free radical (OH). The reactions producing these free radicals are more active when semen is stored at ambient temperatures than in frozen state. Peroxide is the more pernicious of the pro-oxidants formed. The main source of peroxide during storage of bovine semen at ambient temperature is the oxidative de-amination of aromatic amino acids by a membrane bound AAAO as shown in the Eq. (1).

\[ \text{R} \cdot \text{CH}_2\text{CH(NH}_3\text{)} \cdot \text{COOH} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{AAAO}} \text{R} \cdot \text{CH}_2\text{COCOOH} + \text{H}_2\text{O}_2 + \text{NH}_3 \]

(1)

The reaction was first described for bovine spermatozoa by Tosic and Walton (1946, 1950). Subsequently, Shannon and Curson (1972b) demonstrated that AAAO activity was restricted to the dead sperm population. The properties of AAAO are well established. The enzyme is heat and acid labile: it is inactive in live spermatozoa and is

Table 6
Conception rates of semen diluted in 14G* diluent containing caproic acid (0.025%). Data from Shannon (1962) and expressed as % NRR at 49 days

<table>
<thead>
<tr>
<th>Age of semen</th>
<th>14G (control)</th>
<th>14G + caproic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of inseminations</td>
<td>% NRR</td>
</tr>
<tr>
<td>6±18 h</td>
<td>224,275</td>
<td>64.1</td>
</tr>
<tr>
<td>24±36 h</td>
<td>95,587</td>
<td>64.3</td>
</tr>
</tbody>
</table>

*The 14G diluent is the precursor to the current CAPROGEN® medium containing all the basic ingredients except caproic acid.
* Significantly different to control (p < 0.05).
Table 7
Effect of catalase in CAPROGEN diluted on fertility of bovine semen stored at ambient temperature and used on day of collection (6–18 h) and day after collection (24–48 h). Data from Shannon (1968) and fertility was measured as % NRR at 49 days

<table>
<thead>
<tr>
<th>Age of semen</th>
<th>CAPROGEN</th>
<th>CAPROGEN + catalase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of inseminations</td>
<td>% NRR</td>
</tr>
<tr>
<td>Trial 1</td>
<td>6–18 h</td>
<td>94,671</td>
</tr>
<tr>
<td></td>
<td>24–48 h</td>
<td>148,286</td>
</tr>
<tr>
<td>Trial 2</td>
<td>6–18 h</td>
<td>30,233</td>
</tr>
<tr>
<td></td>
<td>24–48 h</td>
<td>44,126</td>
</tr>
</tbody>
</table>

* Significantly different to CAPROGEN (p < 0.05).

released from dead cell membranes in the presence of citrate. It is confined to the tail region of bull spermatozoa and the activity of AAAO is also affected by the oxygen tension in the diluent. It was established from kinetic studies that the response of this enzyme increased with increasing temperature and duration of storage (Shannon and Curson, 1972b, 1982a,b). The enzyme is specific for L- aromatic amino acids and in particular, L-phenylalanine was significantly more toxic than L-tyrosine or L-tryptophan (Macmillan et al., 1972). Egg yolk is an excellent source of aromatic amino acids, particularly L-phenylalanine.

The levels of peroxide generated in the storage medium can be minimised by the presence of catalase (Shannon, 1968). The significance of this reaction is that in the breakdown of peroxide, half the amount of oxygen is returned back to the system (Eq. 2)

\[ 2\text{H}_2\text{O}_2 \xrightarrow{\text{Catalase}} 2\text{H}_2\text{O} + \text{O}_2 \]  

(2)

Two separate field trials substantiated the effect of catalase and the results are shown in Table 7. With extended storage times, the beneficial effect of added catalase became evident and the field trial clearly emphasises the need to either reduce or abolish endogenous peroxide production during liquid storage of semen.

2.4. Factors influencing fertility after liquid storage

2.4.1. The effect of duration of storage on fertility of spermatozoa

Bovine spermatozoa stored at ambient temperature in CAPROGEN diluted show a slow decrease in motility over an extended period (about 3–4 weeks), but the concomitant decrease in fertility is significantly higher (Fig. 2). Fertility, as measured by non-return rate (NRR), is maintained for the first 3 to 5 days after dilution (69.9 ± 1.2%), followed by an accelerating decrease in NRR until day 10 of storage (41.5 ± 3.7%) (Vishwanath and Shannon, 1997). Bartlett and Van Demark (1962) showed a similar dichotomy in response to storage at 5°C. Semen stored in a medium containing bicarbonate, catalase and glucose and gassed with CO₂ had 56% motile spermatozoa after 40 days of storage. However, fertility decreased in the first few days of storage and was down on average by 8–14% by day 3 (Bartlett and Van Demark, 1962).
The physiological reasons for this rapid decline in fertility of spermatozoa stored at ambient temperature is presumed to be due to three factors: extracellular oxidative stress, effects of seminal plasma and endogenous free radical production. The combined effects of these three factors could lead to significant damage to spermatozoa. This is perhaps the main reason why semen stored at ambient temperature is restricted in shelf-life with a declining fertilising potential. (Vishwanath and Shannon, 1997).

2.4.2. Effect of number of spermatozoa inseminated on fertility of liquid stored semen

An important factor that contributes to the fertility of diluted semen is the dose rate or absolute sperm numbers in a given inseminate. Salisbury and Van Demark (1961) have described the relationship between semen quantity (sperm numbers) and semen quality (fertilising potential). The central point in this concept is that up to a threshold level,
fertility is under the influence of sperm numbers. This threshold varies significantly between bulls. Once this level is attained, fertility is unaffected by further increases in sperm concentration. This effect has been adequately demonstrated in field trials where some of the inherent differences in the fertility of different bulls can be compensated for by altering the absolute numbers of spermatozoa in the inseminate (den Daas, 1992; Shannon and Vishwanath, 1995). The effect of sperm numbers being able to influence fertility is a defined compensable element and increasing sperm concentration does alter the probability of fertilisation (Saacke et al., 1994; Shannon and Vishwanath, 1995). However, increasing sperm numbers does not alter the rate of decrease in fertility after 5 to 6 days of storage at ambient temperature (10–21°C, Vishwanath, unpublished information). The change in NRR for different sperm concentrations in liquid semen is shown in Fig. 3. The probability of fertilisation is quite high on the first day of use at all three sperm concentrations (4, 6 and 8 million/ml), and they are not significantly different from each other. On the contrary, with storage, the NRR is lower on days 2 and 3 at the lowest sperm concentration of 4 million/ml (p < 0.05). The reasons could be:

- probability of fertilisation decreased due to lower sperm numbers;
- effect of dilution compounding the sperm aging effect; and
- bull × dose rate interaction.

3. Frozen storage of semen

Effective storage of semen in frozen state implies a complete arrest in the developmental process of sperm cells that began in the testis and continued through the epididymis and after ejaculation. The logical conclusion for this process is the presence of spermatozoa in the female tract preparing for fertilisation. Watson (1995) described the frozen preservation as “a hiatus in the process that demarcates this continuum of sperm development with a period of suspended animation that eventually leads to successful fertilisation”. There is an argument that spermatozoa stored at −79°C (in dry ice) or at −196°C (in liquid nitrogen) retain their fertilising potential indefinitely. Mazur (1980) proposed a time period of between 3000 and 10,000 years before genome destruction, while insemination trials with frozen stored semen (Salisbury and Hart, 1970) suggest a far shorter time period for survival at the above-mentioned low temperatures with no change in fertilising potential. However, other evidence suggests that this could have been due to inadequate maintenance of temperatures. With more advanced cryogenic vessels, it is possible to maintain spermatozoa in a frozen state for a very long period.

The fortuitous discovery of glycerol as an effective cryoprotective agent (Polge et al., 1949; Polge and Rowson, 1952) introduced a completely new system of semen storage, a method which is widely in practice today. An axiom of semen freezing is that even with the best preservation techniques and all the developments that have occurred over the years, the best cell recovery after thawing is just over 50%.
3.1. Effects of freezing

The stresses associated with freezing are primarily the temperature changes that spermatozoa are subjected to during the process of cooling, the injurious effects of the media components and cryoprotectants themselves during the process, and finally the effects of thawing. The basic principles of freezing were established quite early and several authors have reviewed the physical effects of each of the processes mentioned above (Mazur, 1963, 1965; Watson, 1979, 1995). The physical effects of cooling and then freezing a cell suspension results in a number of changes in the external environment in terms of water and solute movement. The rate at which water moves out of the cell during this process plays an important role in determining cooling rates, which have to be optimised for cell survival after thawing (Mazur, 1963, 1977). The curves for survival vs. cooling rate show a biphasic response. The optimum cooling rate is between 80°C/min and 120°C/min with faster and slower cooling rates resulting in lower survival (Watson, 1979, 1995; Woelders, 1997). This also has bearing on a period of withholding times at different temperatures to allow for equilibration before freezing. The purpose of this equilibration time at certain temperatures is to allow for the translocation of water and thereby reduce the injurious effect of ice nucleation during the freezing and thawing process.

3.2. Diluents and cryoprotectants for freezing of semen

The basic components of the diluents for freezing of semen are essentially the same as those used for ambient temperature storage. The general requirements are:

- ionic or non-ionic substances to maintain the osmolarity and to buffer the medium;
- a source of lipoprotein or high molecular weight material to prevent cold shock, such as egg yolk or milk;
- glycerol, propane-diol or DMSO to offer cryoprotection;
- glucose or fructose as an energy source; and
- other additives such as enzymes and antibiotics.

Phillips (1939) made a crucial discovery when he found that egg yolk added to the semen diluent had a beneficial effect on fertility. Phillips and Lardy (1940) recommended the use of a diluent containing equal parts of phosphate buffer and egg yolk as a protective agent against cold shock. Salisbury et al. (1941) presented conception results from field trials with bovine semen diluted in an egg yolk–sodium citrate diluent (EYC). Since then, egg yolk in combination with Tris and citrate has been the most common constituent in most freezing diluents for bovine semen. The reason why citrate is a better ionic substitute than phosphate is because phosphate inhibits the oxidation of lactic acid causing its accumulation (White, 1956). Davis et al. (1963a,b) developed a 0.2 M Tris-buffered 20% egg yolk extender (TRIS-EY). When compared with EYC and skim milk, TRIS-EY was found to be significantly better at maintaining post-thaw motility. Steinbach and Foote (1964) confirmed this observation. Field trials with cooled but unfrozen semen at sperm concentrations of 4 to 8 million/ml failed to show evidence of improved fertility with TRIS-EY compared with EYC (Foote, 1970). Compositions of these diluents and their modifications are shown in Table 8.
Table 8
Composition of Tris- and citrate-based diluents for freezing bovine semen. All diluents contain antibiotics at approximately 100,000 units of penicillin and dihydrostreptomycin/100 ml of the diluent. All values are given in g/100 ml of the medium, unless otherwise mentioned.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluent</td>
<td>(Davis et al., 1963a)</td>
<td>(Steinbach and Foote, 1964)²</td>
<td>(Steinbach and Foote, 1964)²</td>
<td>(De Leeuw et al., 1993)</td>
</tr>
<tr>
<td>Tris</td>
<td>3.03</td>
<td>2.4</td>
<td></td>
<td>3.15c</td>
</tr>
<tr>
<td>Glycerol</td>
<td>14 ml</td>
<td>8.8 ml</td>
<td>7.2 ml</td>
<td>6 ml</td>
</tr>
<tr>
<td>Fructose</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Citric acid</td>
<td>1.69</td>
<td>1.354</td>
<td></td>
<td>1.23</td>
</tr>
<tr>
<td>monohydrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium citrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dihydrate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egg yolk</td>
<td>20 ml</td>
<td>20 ml</td>
<td>20 ml</td>
<td>20 ml</td>
</tr>
</tbody>
</table>

¹Original diluent modified by Foote (1970) to include fructose.
²Tris (hydroxymethyl) aminomethane.
³Tris (hydroxymethyl) aminomethane hydrochloride.

3.2.1. Egg yolk

There have been many attempts to find the protective component in egg yolk with the aim to prepare a ‘chemically defined diluent’, and thus reduce the possibility of transmission of harmful pathogens, the likely inconsistencies between different batches of egg yolk and the yolk globules which interfere with microscopic examination of diluted semen. Early experiments on the protective action of egg yolk soon led to research in the specific components of egg yolk that might be responsible for the cryoprotective action, namely, phosphatidylcholine (lecithin), phospholipids, lipid extracts, lipoprotein fractions and specific lipoproteins (Mayer and Lesley, 1945; Blackshaw, 1954; Blackshaw and Salisbury, 1957; Martin, 1963; Lanz et al., 1965; Gebauer et al., 1970). There was a consensus among these studies that lecithin provides some protection to spermatozoa during cold shock and freezing. One of the major problems with lecithin is that it is insoluble in aqueous solutions, forming an unstable suspension. It partly precipitates in the absence of vigorous stirring and can break down to form lysolecithin which is toxic to spermatozoa (Jones, 1976). Synthetic liposomes made up of dioleoyl phosphatidyl choline, phosphatidyl choline, phosphatidyl serine and combinations thereof with cholesterol were effective in preventing cooling damage to spermatozoa, but were a poor substitute compared to egg yolk in preventing freeze–thaw damage (De Leeuw et al., 1993).

The major component of egg yolk offering protection is a phospholipid moiety of a low density lipoprotein fraction (Pace and Graham, 1974; Foulkes, 1977; Watson, 1981). Further analysis of the type of lipoprotein extracted from whole egg yolk by water, saline and citrate revealed that the major extract is a cationic lipoprotein fraction that preferentially bind to the sperm membrane and afford far greater protection. This protection is assumed to result from the lipoprotein binding to the sperm membrane via the charged protein moiety, which aids in stabilising the plasma membrane through the
Table 9
Recovery of spermatozoa and survival at 37°C after cold shock in CAPROGEN® containing 5% whole egg yolk or an equivalent amount of water-based lipoprotein extract (WTM) of egg yolk. Data from Vishwanath et al. (1992)

<table>
<thead>
<tr>
<th>Diluent</th>
<th>Recovery (%)</th>
<th>Survival (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAPROGEN® (no egg yolk)</td>
<td>&lt;5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CAPROGEN® (5% egg yolk)</td>
<td>80&lt;sup&gt;b&lt;/sup&gt;</td>
<td>104&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Water extract (WTM)</td>
<td>80&lt;sup&gt;c&lt;/sup&gt;</td>
<td>116&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values with different superscripts differ, <i>p</i> < 0.05.

critical temperature zones, with the lipid portion acting as thermal insulation (Vishwanath et al., 1992). This water-based fraction was significantly better than egg yolk in maintaining survival of spermatozoa recovered after cold shock (Table 9; Vishwanath et al., 1992). Field fertility with the same fraction in ambient temperature stored semen where it was used as a thermal protectant also revealed a significant advantage (Table 10).

Evidence suggests that the whole lipoprotein molecule is required to protect spermatozoa during cold shock. Selective digestion of either the protein or the lipid fractions did not offer the same protection against cold shock (Watson, 1981; Prendergast et al., 1994). It is possible that the protein or the lipid fractions are unable to bind to the membrane and offer thermal protection on their own.

The amount of egg yolk commonly used in freezing media is between 15% and 30% on a volume to volume basis. This range in egg yolk concentration is based on results obtained by Van Demark et al. (1957) where the percentage of motile spermatozoa after freezing and thawing was similar for 15% and 30%, but was significantly lower at egg yolk concentrations above and below this range.

3.2.2. Ions

The ions in the storage medium are not mainly for ionic strength, but more for maintaining osmolarity (Watson, 1979). This purpose is adequately served by zwitterionic buffers, amino acids, α-keto acids and a combination of salts and carbohydrates (Miller and Van Demark, 1954).

3.2.3. Cryoprotectants

3.2.3.1. Glycerol. The discovery of the protective action of glycerol (Polge et al., 1949) was an important technological breakthrough, as it reduced the mechanical damage to

Table 10
Fertility of semen from five sires diluted in CAPROGEN® containing 5% egg yolk or an equivalent amount of water-based egg yolk extract (WTM). Data from Vishwanath et al. (1992). Ejaculates were split between the two treatments and fertility measured as % NRR at 24 days

<table>
<thead>
<tr>
<th>Diluent</th>
<th>No. of inseminations</th>
<th>% NRR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAPROGEN® (5% egg yolk)</td>
<td>88,49</td>
<td>66.6</td>
</tr>
<tr>
<td>Water extract (WTM)</td>
<td>61,95</td>
<td>68.5°</td>
</tr>
</tbody>
</table>

° Indicates significantly different to CAPROGEN® (<i>p</i> < 0.05).
spermatozoa during the freezing process. Glycerol and other penetrating cryoprotectants such as DMSO reduce cell damage by preventing the concentration effects of extracellular media. Colloids such as sucrose and dextran on the other hand, are non-penetrating cryoprotectants inducing the formation of ice crystal lattices, which are thought to form external shields around sperm membranes (Karow and Webb, 1965; Schmehl et al., 1986; Nicolajsen and Hvidt, 1994). The role of cryoprotectants is a matter of substantial debate and the current thinking on the nature of the protective effects of penetrating and non-penetrating cryoprotectants are discussed by Watson (1995).

Glycerol is the most widely used cryopreservative for bull spermatozoa. Conventional methods of freezing in 0.25- or 0.5-ml straws have used approximately 7% glycerol as the optimum concentration for citrate–yolk and Tris–egg yolk extenders (Pickett and Berndtson, 1974, 1978; Rodriguez et al., 1975; Watson, 1979, 1990; De Leeuw et al., 1993). The optimum concentration of glycerol for freezing bovine semen is also influenced by other components in the diluent (Woelders et al., 1997). Suffice to say, glycerol concentrations for freezing have been between 0.25 M (2.25%) and 1 M (9%) with many studies demonstrating toxicity beyond this concentration (Fahy, 1986).

3.2.3.2. Sugars and polyols. Nagase et al. (1964) found that xylose, fructose, glucose, galactose, maltose, sucrose and raffinose were all effective for rapid freezing of bull semen. Sugars perform several functions in the diluent. They add osmotic pressure to the medium and act as cryoprotectants (Watson, 1979). Polyols, like glycerol and several sugars, form hydrogen bonds with the polar head groups of membrane lipids (Crowe and Crowe, 1984; Crowe et al., 1985). The main effect of sugars and polyols is their ability to replace the water molecule in the normally hydrated polar groups and this property helps to stabilise the membrane during transition through the critical temperature zones (Woelders, 1997). Sugars, like glycerol, alter the mechanical properties of the diluent by increasing its viscosity. This prevents the eutectic crystallisation of solutes and increases the glass-forming tendency of the medium, a property used increasingly in vitrification media (Nicolajsen and Hvidt, 1994). A recent study (Woelders et al., 1997), using trehalose and sucrose, demonstrated a significant interaction between cooling rates and the presence of sugars. In this study, an isotonic sugar medium where the Tris–citrate components were substituted with sucrose and trehalose was significantly superior to a Tris–citrate–egg yolk medium in preserving acrosome integrity. The suggestion was that by using a cooling rate in the optimum range of 76–140°C/min a further improvement in viability after thawing could be achieved. Trehalose is a common substitute for water molecules in the dehydration process and is the sugar found in many organisms that survive anhydrobiosis (Woelders et al., 1997).

3.2.4. Milk diluents
Milk proved to be a suitable diluent for storage of bull semen both in the liquid and frozen state (Salisbury and Van Demark, 1961; Melrose, 1962). A 10% whole milk or skim milk diluent in combination with 7% glycerol and antibiotics has been very successfully used as a diluent for freezing semen for many years. Along with the Tris–egg yolk–citrate diluent developed by Davis et al. (1963a), milk diluents have been routinely used in many AI centres to freeze semen (Almquist et al., 1954; Ahmad and
Foote, 1985). Various studies have attempted to improve the whole milk or skim milk extenders, but have generally been unsuccessful (Foote et al., 1993). The main disadvantage with milk extenders is the poor visibility of spermatozoa under a microscope, which makes post-thaw evaluations difficult. It is for this reason that egg yolk diluents in combination with Tris and citrate are preferred.

3.3. Freezing protocols: processing, cooling rates, freezing and thawing

3.3.1. Initial processing and temperature equilibration

Methods of processing of semen immediately after collection vary considerably. It is mostly guided by the methods of use that have suited the organisation, requirements for disease control and semen export protocols. Based on these requirements, procedures vary between organisations that are actively engaged in the international trade of bovine semen. Semen is usually diluted at 30°C to 37°C with an equilibrating medium containing all the basic constituents of the freezing diluent. In most cases, glycerol is omitted from this medium. The ratio of dilution is approximately 1:5 semen/diluent. This procedure is necessary to provide a buffering medium for spermatozoa, some antibiotic protection and to provide thermal insulation during the subsequent cooling. The Certified Semen Services (CSS, USA) procedure for entry of all imported semen into the USA requires undiluted semen to be treated with antibiotics before initial dilution (Lorton et al., 1988). It is generally considered that the slow cooling of diluted semen to 5°C before freezing has beneficial effects (Ennen et al., 1976; Gilbert and Almquist, 1978). This period, during which glycerol is usually added is sometimes erroneously referred to as the glycerol equilibration period. Glycerol equilibrates quite rapidly across the cell membrane at 5°C and can be added at any time during the cooling period (Martin, 1965; De Leeuw et al., 1993). Once the diluted semen attains the holding temperature of 5°C, the mixture is extended to the final sperm concentration with the same medium containing glycerol. All subsequent manipulations such as filling and sealing into straws are usually conducted at 5°C. The final sperm concentration per milliliter of diluted semen differs between organisations. It also depends on stipulations by importing countries. The relationship between the total number of spermatozoa per breeding unit and threshold fertility is discussed in a later section. The average sperm concentration is between 10 and 20 million per breeding unit, depending on the demand of semen from individual sires.

3.3.2. Effect of cooling rates

A two factor theory accounts for the effects of cooling rates on spermatozoa (Mazur, 1965; Mazur et al., 1972). A slow cooling rate exposes spermatozoa to the ‘solution effects’ such as increasing salt concentration, increasing osmolality and changing pH. Fast cooling may not allow intracellular water to pass out, leading to intracellular ice nucleation (Mazur, 1963, 1977; Mazur et al., 1972). In practice, the cooling rate at which freezing damage occurs in spermatozoa is several orders lower than the calculated rate at which ice nucleation should occur. A recent report (Woelders, 1997) shows a clear relationship between survival of bull spermatozoa and cooling rate (Fig. 4). The
Fig. 4. Relation between % motile bull spermatozoa post-thaw and the cooling rate. Values are means ± se for five bulls. Data from Woelders (1997).

optimum cooling rate was 100°C/min, and this is greater than the optimum of 26–52°C/min suggested earlier for bovine spermatozoa (Robbins et al., 1976). A further detailed study by Woelders et al. (1997) suggests a significant interaction between the osmolality of the medium and cooling rate. Supra-optimum cooling rates are tolerated at higher osmolalities where the medium contains sucrose or trehalose.

3.3.3. Methods of freezing

The technique of freezing semen in 0.25- or 0.5-ml straws in liquid N₂ is now universal (Cassou, 1964). This technology is well established and all the equipment required for filling semen in straws and racking them up for freezing is readily available. The appliances used for freezing of semen range from static freezers to controlled programmable freezers. Although there has been considerable research in determining optimum cooling rates and adapting cooling curves for individual bulls, there has been very little progress in the practical sense. The programmable freezers currently available cannot reliably alter or allow customisation of cooling rates for a large batch of straws in a production environment (Wagtendonk, personal communication). Many semen processing organisations continue to use static vapour freezers where the straws containing semen are subjected to uncontrolled freezing conditions, depending on the distance of the straw chamber from the liquid N₂ layer. In these freezers, the rate of decrease in temperature is between 150°C/min and 300°C/min. The advantage of the static vapour freezer is that all the straws in any given freezing cycle are subjected to the same cooling rates, because the straws are placed in one layer above the liquid N₂ level. In programmable freezers, the straws are placed in more than one layer, and this contributes to considerable variation in cooling rates in individual layers within a freezing cycle (Wagtendonk, personal communication).

3.4. Thawing of semen

The general theory is that rapid thawing of semen is advantageous to prevent injury during rewarming. Mazur (1984) observed that rapid thawing prevents the possibility of re-crystallisation of water molecules, which could be injurious to cell membranes. A
potential problem in slow thawing is the osmotic change due to ingress of water during the process, as this is considered to be more damaging than water egress during freezing (Curry and Watson, 1994). Studies by Holt and North (1994) have also confirmed this observation with ram spermatozoa. There are some practical considerations regarding the temperature at which semen used in field conditions should be thawed to obtain maximum fertility. This is a question often asked with many anecdotal references to higher fertility with semen thawed at higher temperatures ($>25^\circ\text{C}$). There are some earlier studies evaluating fertility of semen thawed at temperatures of $30^\circ\text{C}$, $15^\circ\text{C}$ and $4^\circ\text{C}$ (Arnott, 1961). The 60–90 day NRR followed the trend of $4^\circ\text{C} > 15^\circ\text{C} > 30^\circ\text{C}$ in thawing temperature. Other studies have also suggested a lower temperature of around $4^\circ\text{C}$ as favourable for thawing, compared to higher temperatures of around $15^\circ\text{C}$ (Pickett et al., 1965; Pickett and Berndtson, 1978). A recent field trial to determine the effect of thawing semen at $35^\circ\text{C}$ compared with ambient temperature ($14 \pm 3^\circ\text{C}$) showed no difference in NRR between the two thawing temperatures (Table 11; Vishwanath, 1998).

### 3.5. Re-dilution of bulk frozen semen

The concept of re-dilution of frozen–thawed semen was first explored by James and Fyvie (1955). The idea was to develop it as a convenient method to harness the out-of-season production potential of a bull. The initial trials were not very successful and the fertilising ability of re-diluted semen could be retained for only a few hours. The technique was later successfully modified for field use (Shannon, 1972; Macmillan et al., 1978). In this system, the semen was frozen in straws at high sperm concentrations in an egg yolk–citrate–glycerol diluent and re-diluted with CAPROGEN® upon thawing (Shannon, 1965) and before using as liquid semen. This technology was used very efficiently for a number of years in many isolated areas in New Zealand. The AI technician diluted the contents of a 0.25-ml straw in a test tube containing 5 ml of CAPROGEN® and completed the day’s inseminations. Inseminations of 0.5 ml volumes were made by using a syringe pipette. At the end of the day, the remaining diluted semen was discarded, as earlier trials had shown a significant decrease in fertility of the re-diluted semen stored overnight (Shannon, 1978). The process has been improved so that large volumes of concentrated semen (5–25 ml) can be bulk frozen (patent pending) and re-diluted in the production laboratory on the days required for use. This is then dispatched as standard liquid semen and used within 60 h of re-dilution. The sperm concentration of the re-diluted material range between 6 and 10 million spermatozoa/in-

### Table 11

<table>
<thead>
<tr>
<th>Temperature of thawing</th>
<th>Number of inseminations</th>
<th>% NRR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ambient (14 ± 3°C)</td>
<td>1182</td>
<td>72.7 ± 1.3</td>
</tr>
<tr>
<td>35 ± 1°C</td>
<td>1105</td>
<td>71.4 ± 1.4</td>
</tr>
</tbody>
</table>

Fertility of frozen semen from nine bulls thawed at $35^\circ\text{C}$ or at ambient temperature. Data from Vishwanath (1998) and fertility expressed as % NRR at 24 days.
The results with re-diluted frozen semen are shown in Fig. 5. The percentage NRR for the first 2 days of use was not significantly different for the two types of semen, but was significantly lower on day 3 of use with re-diluted semen ($p < 0.05$). Two aspects that could potentially affect this system are the significant bull × sperm concentration interaction, which determines the final sperm concentration in the inseminate and, secondly, the bull × age of semen interaction, which limits the number of days semen can be used in a diluted state in the field.

### 3.6. Effect of sperm numbers on fertility of semen stored in the liquid or frozen state

Bulls differ in their inherent fertility and follow similar fertility trends whether their semen is diluted and stored in liquid or frozen state. This effect is shown in Fig. 6. The percentage NRR of liquid and frozen semen for the same 11 bulls at optimum sperm concentration (liquid 2.5 million/inseminate, frozen 20 million/inseminate) range between 62% and 72% (Shannon and Vishwanath, 1995). When sperm concentrations were decreased to sub-optimum levels (liquid 0.5 million/inseminate, frozen 5 million/inseminate), fertility declined by an average of 7% for liquid semen and by 7.9% for frozen semen. There was a significant bull × dose rate interaction in the frozen semen system, which was not seen, in the liquid system ($p < 0.01$). This clearly highlights the fact that semen ejaculates from different bulls inherently differ in their susceptibility to freezing as measured by their NRR. Further evidence suggests that this could be due to a reduced probability of fertilisation or an altered pattern of survival of frozen–thawed spermatozoa in the female reproductive tract (Shannon and Vishwanath, 1995). A similar bull × dose rate interaction for frozen semen was observed by den Daas (1992). Much greater differences between bulls regarding NRR was seen at lower than at higher sperm concentrations.

The true relationship between the number of spermatozoa inseminated and fertility was first proposed by Salisbury and Van Demark (1961). This was further explained by a model proposed by Schwartz et al. (1981), which relates the number of spermatozoa to the probability of conception, based on a Poisson distribution. The validity of the model.
was reaffirmed by the results of a series of field trials (den Daas, 1992). Bulls differ in their maximum NRR value, and this is reached as the number of spermatozoa per inseminates increases. The rate at which this maximum value is approached also differs between bulls. The added variation to this is the inherent fertility difference between sires that occur depending on time of insemination. There is a significant interaction between insemination time, sperm concentration, stage of oestrus of the cow (pre- or post-oestrus) and fertility of individual sires (Macmillan and Curnow, 1977).

The differences in sire fertility have been illustrated in two separate studies by Macmillan and Watson (1975), and Macmillan and Curnow (1977). The stage of oestrus at insemination did not significantly influence the results for sires of above average fertility, but below average sires had reduced conception rates with mid and early oestrus inseminations. The observation suggests that there could be an in vivo decline in fertilising ability of spermatozoa, which differs between sires.
The reasons for the differences in the response to dilution rate are not known. It is possible that individual bulls react differently to a single freezing protocol that is normally used by semen processing companies. Perhaps, customising freezing protocols for individual bulls could help in reducing the dose rates of frozen semen (Parkinson and Whitfield, 1987; den Daas, 1992). The fact that higher numbers of spermatozoa are required for insemination with frozen semen due to losses in the freezing process is not unexpected. The number of live spermatozoa required for insemination is far lower when using liquid than frozen semen; at the same dose rate of 2 million motile sperm, NRRs for liquid and frozen semen were 68.1% and 59.7%, respectively.

3.7. Advantages and disadvantages of liquid stored and frozen–thawed semen

The relative advantages and disadvantages in the field usage of liquid stored and frozen–thawed semen are listed in Table 12. The most obvious advantage of liquid stored semen is one of sperm numbers, and this has been the main impetus in further refining liquid semen technology for extensive use in New Zealand and other countries. An average ejaculate of 5 ml containing 1.5 billion spermatozoa/ml will yield between 350 and 500 frozen semen straws at a dose rate of 15 to 20 million spermatozoa/inseminate. On the other hand, the same ejaculate as liquid semen will yield 7500 straws at a dose rate of 1 million/inseminate and 3750 straws at the higher sperm concentration. The storage costs of liquid semen are very low. If semen utilisation is high within the shelf-life period after dilution, the economic advantages are quite significant (Shannon, 1978; Curson et al., 1991). The obvious disadvantage with liquid stored semen is the limited shelf-life. By contrast, the most important advantage of frozen–thawed semen is the possibility of long-term storage capability. This allows for extensive testing of the processed semen, and is a reliable method for genetic insurance of valuable bulls. The biggest disadvantage in a high demand situation is the high sperm numbers required for insemination to maintain the same level of fertility as for liquid stored semen. In New Zealand, a unique system of seasonal breeding operates. More than 2.5 million inseminations are performed in a 16- to 20-week period during the Spring months of September to Summer (Fig. 7). More than 85% of these inseminations

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Frozen–thawed semen</th>
</tr>
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<tbody>
<tr>
<td>Low sperm numbers</td>
<td>Long term storage.</td>
</tr>
<tr>
<td>High sire utilisation</td>
<td>Flexibility of use.</td>
</tr>
<tr>
<td>Inexpensive storage</td>
<td></td>
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<tr>
<td>Ease of use in the field</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Disadvantages</th>
<th></th>
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<tbody>
<tr>
<td>Limited shelf life</td>
<td>High sperm numbers.</td>
</tr>
<tr>
<td></td>
<td>Expensive to store.</td>
</tr>
</tbody>
</table>
Fig. 7. The distribution of inseminations in New Zealand during the spring mating period (1st September–31st December). Data from Vishwanath et al. (1996).

are with liquid stored semen, which has a 4-day shelf-life (Curson et al., 1991; Vishwanath et al., 1996).

3.8. Semen packaging

The current methods for semen packaging are largely based on the French mini-straw, the 0.25-ml pailette (Cassou, 1964). The procedure has been in operation for many years and has generally worked well for packaging frozen and liquid semen in many countries (Chupin and Schuh, 1993). The straw allows essential details of the sire to be recorded and this has become mandatory for semen traded internationally. The packaging method for liquid semen in New Zealand is the minitub straw sealed at both ends with glass beads (Minitub, Germany).

Freezing of semen in pellets was first described by Japanese workers (Nagase and Niwa, 1964; Nagase et al., 1964) and is still used in some countries for storing semen from young sires and for local use. This procedure is not widely practised because details of the sire cannot be easily recorded on a pellet.

4. Commercially available diluents

Most AI companies prepare their own semen diluents with minor modifications to suit their requirements. In the last few years, a few proprietary brands have been available and they are listed below. The move to eliminate egg yolk from the system has been quite strong because of a perceived health risk associated with a biological material being included in the diluting media. However, suitable alternatives have not been as successful as either milk or egg yolk (van Wagendonk-de Leeuw et al., 2000). Some of the diluents listed below are dual purpose and recommended for both liquid and frozen storage of semen. The list is by no means exhaustive; nor has a detailed market survey
been conducted on the relative merits of all the media available. The common ones cited in literature are the following.

1. Biladyl — supplied by Minitüb Germany and used with frozen semen. It consists of two fractions A and B for step-wise dilution of semen with addition of egg yolk, water and antibiotics as prescribed by CSS.

2. Triladyl — supplied by Minitüb Germany and used with frozen semen. This is similar to Biladyl and is used as a one-step dilution medium.

3. Biociphos — supplied by IMV, L’Aigle, France. It is a one-step dilution medium containing soy bean extract, replacing the egg yolk fraction.

4. Laciphos — supplied by IMV. It is a skim milk-based powder medium requiring addition of water. Both Biociphos and Laciphos are supplied with antibiotic fractions that satisfy either the USA (CSS-certified antibiotic) or the EEC antibiotic formulae.

5. Tris concentrate — Gibco BRL, Manufactured by Holland Genetics, The Netherlands. This is a 5× concentrate of the popular Tris-based medium (Davis et al., 1963a) and modified by De Leeuw et al. (1993). It is the most common diluent for freezing semen. The Tris diluent has been used also for liquid semen (Foote, 1978).

6. CAPROGEN® concentrate — concentrated liquid semen diluent for storage at ambient temperatures (18°C to 24°C) supplied by Livestock Improvement, New Zealand. It is a diluent extensively used for liquid storage of semen with no drop in fertility over a 4-day period (Curson et al., 1991).

5. Future directions

5.1. Liquid stored semen

Major gains can be made with liquid semen technology if the decline in fertility upon storage at ambient temperature is halted or reduced. To this end, the physiological processes that contribute to aging of spermatozoa upon storage in vitro need to be understood. Reactive oxygen species, which are more likely to be generated in an ambient temperature system have to be contained in the media in which the spermatozoa are suspended. Intracellular activity could also contribute to the production of these free radicals and the slowing down of respiratory activity could be beneficial.

5.2. Frozen semen

The future direction of research on freezing semen should be directed towards improvement of freezing protocols to allow a lower number of spermatozoa to be included per breeding unit. The current freezing protocols require from 40 to 100 million spermatozoa/ml of diluting medium and each inseminate contains between 10 and 25 million spermatozoa. For any substantial gain to be made in this direction, future work needs to concentrate on diluent composition and repeatable cooling rates to protect the sperm cells during the freezing process.

5.3. Alternative storage technologies

Storage of semen in frozen state is expensive, as liquid N₂ and well-insulated containers are essential. The on-going cost of storing semen in liquid N₂ can be
prohibitive. There has always been an interest in developing alternative strategies for long-term storage of bovine spermatozoa, and experiments on desiccation, vitrification and freeze drying have been attempted in the past with limited success (Larson and Graham, 1976; Jeyendran et al., 1981, 1983). A recent review by Holt (1997) discusses the future requirements for preservation of germplasm and how these strategies need to be re-visited in the light of modern knowledge on the response of sperm membranes to severe manipulations. The next breakthrough in semen storage technology will almost certainly involve some of these strategies.

6. Concluding remarks

The use of liquid stored semen allows high utilisation of individual sires. This is possible because of the low sperm numbers required to maintain fertility and the extended shelf-life of up to 4 days of use in the field. If the effects of semen age can be overcome, the efficiency and utilisation of liquid stored semen could be increased quite significantly. The essential difference between liquid and frozen–thawed semen lies in the response of bulls to sub-optimum dose rates. While all bulls respond similarly to sub-optimum dose rates with liquid stored semen, the response varies greatly when frozen–thawed semen is used. This means that higher than required dose rates are recommended for frozen–thawed semen because of the possibility of a bull × dose rate interaction. The use of bulk-frozen semen re-diluted and used as liquid semen is an option to combine the convenience of frozen semen technology with the efficiency of liquid semen.

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