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Storage of poultry semen

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Abstract

Methods of semen collection and artificial insemination (AI) in poultry, requirement for diluents, methods of liquid and frozen storage of avian semen and evaluation of spermatozoa after storage for fertilizing ability are reviewed. Frozen storage of semen from non-domestic birds is also briefly discussed. Published by Elsevier Science B.V.

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1. Introduction

Artificial insemination (AI) has been a critical component of reproduction in turkeys since the 1960s and is used almost exclusively for commercial flock production. The contrast in the size of the toms (large white strains can exceed 33 kg) and hens (approximately 9 kg at the onset of lay) and consequent low fertility of the heavy broad-breasted strains after natural mating has resulted in the complete integration of AI in commercial production. At present, AI in other domestic avian species is not used extensively. But this may change. For example, as fertility in the broiler breeds continues to decline as males are selected for growth, AI may become cost effective in broiler breeder management (Reddy, 1995). The AI procedure is not as simple in ducks and geese because unlike chickens and turkey hens, the oviduct cannot be everted and the commercial demand for AI in these species is limited (Cooper, 1977). AI techniques have also been successfully adapted to cranes and other non-domestic species with special attention to size differences and stress levels in these birds (Gee, 1995).

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Significant progress was made in developing semen diluents and preservation procedures for poultry semen in the 1970s and 1980s. However, the basic procedures for semen collection and AI were established in the 1930s (see review, Lake, 1995). Liquid storage of semen at refrigerated temperatures for up to 6 h in turkeys and 24 h in chickens can result in fertility levels comparable to freshly inseminated semen. Although it was the serendipitous discovery of the freezing qualities of glycerol in freezing chicken semen that advanced semen freezing technology for all species (Polge, 1951), freezing procedures for poultry sperm are not cost effective for commercial flock production.

Compared to the other species outlined in this book, avian species, specifically Galliforms, have some unique physiological characteristics which might influence semen preservation. Unlike bull, ram, or boar spermatozoa, the avian sperm head is cylindrical and not much wider in diameter than the tail (approximately 0.5 μm, Thurston and Hess, 1987). Less cytoplasmic volume and therefore less ability to move cryoprotectants inside the sperm head may be one of the reasons avian spermatozoa do not survive the freezing process well. In addition, the sperm tail is quite long (Thurston and Hess, 1987), at 90 to 100 μm it is approximately eight times the length of the head [compared to bull spermatozoa where tail length is approximately 50 μm (Salisbury and Vandemark, 1961)], making poultry spermatozoa potentially more susceptible to freezing damage. A significant feature of the reproductive physiology of the hen is her ability to store fertile spermatozoa for long periods of time. Sperm storage tubules (SST), which are structures found in the distal half of a the oviduct of all avian species studied to date, sequester and store spermatozoa which are slowly released over time to insure an adequate population of spermatozoa at the site of fertilization (Bakst, 1993). Turkey hens inseminated before the onset of egg production can produce fertilized eggs up to 16 weeks after insemination (Christensen and Bagley, 1989). The precise mechanisms supporting prolonged sperm storage in the SST are unknown but are thought to include reversible suppression of respiration and motility of spermatozoa as well as stabilization of the plasma membrane and maintenance of the acrosome (Bakst, 1993). Although it has yet to be achieved, mimicking the environment for storage of spermatozoa within the hen would profoundly alter current systems for storing semen for extended periods of time in vitro.

This section reviews semen collection and insemination techniques in poultry, diluent requirements and methods for liquid and frozen storage of avian semen and highlights how storage of semen in vitro alters spermatozoa and subsequent fertility.

2. Collection of semen

In 1937 Burrows and Quinn described a non-invasive method, the ‘‘abdominal massage method’’, for collection of semen from roosters. The technique involves restraining the male and gently stroking the back of the bird from behind the wings towards the tail with firm rapid strokes. The male responds with tumescence (erection) of the phallus, at which time the handler gently squeezes the cloaca expressing semen through the external papillae of the ducti deferentis collecting the semen into a

container. For turkeys, the technique is adapted by massaging the area around the cloaca before milking the semen (Lake and Stewart, 1978a,b). Adaptations are also made for species such as waterfowl which have penis-like copulatory appendages (Cooper, 1977) and non-domestic species which require additional restraint (Gee, 1995). The proximity of the cloaca increases the likelihood of obtaining semen contaminated with feces, urates, and bacteria that are detrimental to semen quality.

3. Artificial insemination

The technique currently used for AI in poultry was developed in the 1930s and involves applying pressure to the hen's abdomen and everting the vaginal orifice through the cloaca (Quinn and Burrows, 1936). This procedure is also referred to as cracking, venting or everting the hen. Semen is deposited 2–4 cm into the vaginal orifice concurrently with the release of pressure on the hen's abdomen. Insemination is accomplished with straws, syringes or plastic tubes. In large scale commercial operations, automated semen dispensers using individual straws loaded with a set AI dose are commonly used.

Several factors influence fertility after AI. Semen quality can be affected by age of the tom or rooster, lighting schedule, season, body weight, and diet (Sexton, 1986, 1987), as well as the semen collector. Timing of AI is important and is usually performed in the late afternoon to minimize the number of hens with hard-shelled eggs in the shell gland. In turkeys, Brillard and Bakst (1990) demonstrated that sperm numbers in the SST of hens inseminated before the onset of lay was twice that of hens inseminated at the beginning of egg production. Therefore, turkey hens are generally inseminated before they begin to lay, usually 14 to 17 days after increased lighting for stimulation of egg production. In chickens, AI is usually initiated when 15% to 20% of the hens are in egg production (see Bakst and Brillard, 1995).

4. Liquid storage of semen

4.1. Semen diluents

Diluents are buffered salt solutions used to extend semen, maintain the viability of spermatozoa in vitro, and maximize the number of hens that can be inseminated. Semen extension is important since poultry semen is viscous and highly concentrated, containing 6 (roosters) to 12 (toms) billion spermatozoa/ml. Semen diluents are based on the biochemical composition of chicken and turkey semen (see review, Lake, 1995). Glutamic acid, the most prominent anionic constituent of avian seminal plasma, became a standard component of diluents (Lake and Mc Indoe, 1959). There are many diluents available for poultry semen, both published recipes and commercially available products. Several investigators have compared the composition of various diluents and summarized fertility data across studies (Howarth, 1983; Bakst, 1990; Bootswala and

Miles, 1992). What is evident from these reviews is that there is no standard diluent for poultry semen and that studies are so variable in experimental design including time of insemination, vaginal depth and frequency of AI, number of spermatozoa inseminated and duration of fertility analyzed that discerning the benefits of different diluents is difficult. There are basic characteristics common to nearly all diluents: factors to maintain pH, osmolarity and provide an energy source for spermatozoa (see review, Christensen, 1995). The pH of a diluent can effect the metabolic rate and motility of spermatozoa. Buffering agents, consisting of a mixture of an acid and its conjugate base are formulated into a diluent to limit changes in pH. These usually include the mixture of phosphates, citrates and/or organic zwitterionic molecules such as *N,N*-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid (BES) and *N*-Tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid (TES) in poultry diluents. Zwitterionic molecules are important in diluents for chicken semen because of lactic acid buildup with increased storage time which can lower the diluent pH (Christensen, 1995). Chicken and turkey spermatozoa can tolerate a pH range of 6.0–8.0 (Bogdonoff and Schaffner, 1954; Van Wambeke, 1967). The motility and metabolic rate of spermatozoa can be altered by diluent pH. For example, a low pH reduces motility, lactic acid production, oxygen uptake in chicken spermatozoa, whereas high pH increases metabolic rates in vitro (Bogdonoff and Schaffner, 1954). For 24 h storage of chicken semen, Lake and Ravie (1979) demonstrated that pH of 6.8 and 7.1 improved survival over diluents with pH of 5.8 and 7.4. For turkey semen stored 6 h in vitro, higher fertility resulted when diluent pH was 6.5 compared to 7.5 (Giesen and Sexton, 1982).

Poultry spermatozoa can maintain fertilizing ability in diluents with osmolarities ranging from 250 to 460 mosM/kg H_20. When spermatozoa are placed in a solution of low osmolarity, the net movement of water into the sperm cells causes them to swell. In a hyperosmotic solution, spermatozoa lose water and shrink (Bakst, 1980). This physical property is the basis of commonly used spermatozoa stress tests discussed in subsequent sections. In hypoosmotic conditions spermatozoa display increased incidence of bent necks (Bajpai and Brown, 1964; Clark et al., 1984), a defect frequently found in diluted chicken and turkey semen, which is negatively correlated with fertility. Giesen and Sexton (1982) observed a disappearance of turkey spermatozoa over an 18-h storage period suggesting that spermatozoa are swelling and bursting in vitro. These investigators hypothesized that hypertonic diluents could reverse the swelling and improve survival in vitro.

Important to the development of diluents and storage systems for poultry semen is the physiological differences and metabolic requirements of spermatozoa from different species. Chicken spermatozoa are metabolically competent in aerobic and anaerobic environments in vitro. In contrast, turkey spermatozoa require high levels of oxygen to survive (Sexton, 1974; Wishart, 1981). Aerobic metabolism necessitates the aeration of semen which is generally accomplished by placing diluted semen in a flask to maximize surface to volume ratio and placing the flask on a rotary shaker. More recently, other methods to maximize and control oxygen supply to semen have been investigated. These include the addition of perfluorochemicals, compounds that chelate oxygen (Thurston et al., 1993a; 1994) and the development of environmentally controlled capsules that regulate temperature and oxygen concentration (Hammerstedt and Shultz, 1995).

4.2. Alteration in the function of spermatozoa after liquid storage of semen

Similar to the description of semen diluents, it is difficult to compare results reported in the literature involving the long-term liquid storage of poultry semen because of the variation in experimental design. Such variation includes differences in AI schedules, semen dose, storage interval (which can range from 6 to 48 h), and analysis of fertility results reported. One phenomenon that appears to be consistent in results with liquid semen storage is the decline in fertility with semen stored longer than 6 h after the first 5 to 8 weeks of egg production. Over the course of egg production the efficiency of the SST decreases, therefore, even late season declines in fertility and hatch are not uncommon with fresh inseminations. When semen is stored 24 h or longer in vitro, fertility problems are magnified.

Recently, several assays have been developed which provide information on spermatozoa and oviduct function, and the duration fertility. These methods rely on the laid egg that traditionally has been evaluated as either fertile or infertile. Yet, the freshly laid egg is a tremendous source of information for sperm function in vivo. Sperm numbers and egg penetration at the germinal disc region can be estimated by isolating the investment that surrounds the ovum (yolk). This investment is referred to as the inner perivitelline layer (IPL). At fertilization, spermatozoa penetrate the IPL utilizing acrosomal enzymes to digest holes through the protein fibers of this layer (Bakst and Howarth, 1977; Wishart, 1995a). These holes are clearly discernable utilizing a quick and simple preparation and staining procedure. A second population of spermatozoa that failed to penetrate the IPL but are at the site of fertilization are trapped in the fibrous mesh of the outer perivitelline layer (OPL). This OPL is secreted by the upper oviduct laid and down over the IPL (Bakst and Howarth, 1977). Both the holes in the IPL and the spermatozoa trapped within the OPL can be visualized and quantified in freshly laid eggs. Wishart (1987) provided an estimate of the duration of fertility of individual hens by determining the unit density of spermatozoa embedded in the OPL of eggs and using a previously established mathematical relationship. Likewise, there is a significant positive correlation between the numbers of spermatozoa trapped in the OPL and the holes in the IPL with the number of spermatozoa residing in the SST at the time the egg is laid in chickens (Brillard and Antoine, 1990; Wishart et al., 1993; Wishart, 1995a) and in turkeys (Brillard and Bakst, 1990; Wishart, 1995a). In addition, estimation of individual egg, hen and flock fertility can be performed by evaluating hole numbers in the IPL of freshly laid eggs (Bramwell et al., 1995; Wishart et al., 1995). Although both assays can be used to give similar information on in vivo sperm function, the evaluation of holes is a simpler assay (Wishart, 1995a). Preparation time is shorter and the holes in the IPL are easy to detect, therefore more eggs can be evaluated. When these assays were used to estimate the impact of 24-h storage of semen at 5°C versus fresh semen (Donoghue et al., 1995a, 1996) observed both significantly fewer spermatozoa trapped in the OPL and fewer holes in the IPL of turkey eggs after insemination with in vitro stored semen. Over an 11 week study, almost five-fold fewer spermatozoa were trapped in the OPL of eggs from turkey hens inseminated with stored semen compared to hens inseminated with fresh semen (Donoghue et al., 1995a). It was suggested that fewer spermatozoa reached and entered the SST, consequently, fewer spermatozoa were present at the site of

fertilization around the time of ovulation. When estimating IPL holes, eggs evaluated from the stored semen group averaged 40% fewer holes than eggs evaluated after inseminations with fresh semen (Donoghue, 1996). These studies suggest that using current methods for storing turkey semen for 24 h compromise the intrinsic ability of spermatozoa to reach and/or be stored in the SST.

4.3. Semen evaluation after liquid storage

Determination of the viability of spermatozoa after semen storage is important for several reasons. First, it provides an estimate of semen quality. If inseminated, poor quality semen reduces fertility, increases embryo mortality and forces the hen to rely on spermatozoa from previous inseminations (Thurston, 1995). More traditional semen evaluation procedures include determination of semen volume, color, concentration, motility, viability and morphology of spermatozoa. Many of these assessments correlate with the fertilizing capacity of spermatozoa when fresh semen is evaluated (Wishart, 1995a). Histological and fluorescent stains have been used to determine live–dead sperm ratios (Lake and Stewart, 1978a,b; Bilgili and Renden, 1984; Bayyari et al., 1990; Donoghue et al., 1995b) and metabolic activity (Chaudhuri et al., 1988; Wishart, 1989). However, classical semen evaluation techniques do not correlate highly with fertility results when turkey semen is used after storage for more than 6 h in vitro (Sexton, 1988a; Lake, 1989; Wishart, 1989; Bakst, 1990). Reduced fecundity of stored spermatozoa may be due to the detrimental effect of damaged or non-viable sperm cells on viable cells (Sexton, 1988b). Sexton (1988b) reported that greater than 30% of spermatozoa considered viable after 24-h storage is ''unfit'' to survive in the oviduct. The true magnitude of this unfit sperm population, which is not detectable by conventional viability tests, was first elucidated by Bakst et al. (1991) using a hypoosmotic stress test. No difference was observed in viable spermatozoa when fresh and 24 h stored semen was stained with ethidium bromide in the iso-osmotic range. However, when subjected to low osmolarity, the 24 h stored semen population had fewer viable spermatozoa. Adapting this technique to include live–dead fluorescent stains coupled with flow cytometry resulted in similar observations (Donoghue et al., 1996). Bakst et al. (1991) did not observe any difference in fertility when the sperm numbers were increased to compensate for the non-viable spermatozoa based on the hypo-osmotic stress test. Nevertheless, the determination of which sperm characteristics are altered during liquid storage, and thereby render the spermatozoa ''unfit'', could lead to ways of improving in vitro semen storage.

In vitro sperm–egg interaction assays have been introduced which assess the ability of spermatozoa to hydrolyze holes in fragments of the IPL (Bramwell and Howarth, 1992; Steele et al., 1994) or to bind to solubilized IPL proteins attached to a microtitre plate (Barbato et al., 1994; Hammerstedt et al., 1997). The former assay has been adapted and characterized for routine use with pieces of IPL separated from laid eggs (Robertson and Wishart, 1997; Robertson et al., 1997). The assay — which simply involves counting the numbers of holes per unit area of unstained IPL — can demonstrate 50% to 90% loss of function in chicken spermatozoa stored for 24 h at 5°C, under conditions in which eosin permeability and tetrazolium dye reduction are un-

changed, or reduced by only 20%, respectively (Robertson et al., 1997). With turkey spermatozoa stored at 5°C for 24 h, the assay has shown a 70% reduction in function for aerobic storage and greater than 95% reduction for anaerobic storage. Under the same conditions, assays of eosin permeability and dye reduction could not distinguish between samples stored under these two conditions. It is notable that the changes in sperm function shown in this assay are similar to the changes in the ability of spermatozoa to reach the OPL of the egg in vivo (Donoghue et al., 1995a).

Within both the male and female reproductive tract, factors important to normal sperm function are kept in check but may become harmful to spermatozoa in an in vitro storage system. For example, Van Voorst and Leenstra (1995) recently observed improved fertilization with chicken semen when it was dialyzed before 24 h of storage. These and other investigators (Blesbois and de Reviers, 1992) found that seminal plasma, particularly their content of trace minerals and organic compounds, was deleterious to spermatozoa during in vitro storage. Other possible culprits for damage to spermatozoa during in vitro storage include factors in the seminal plasma like spermiophages, or components of the spermatozoa themselves such as active seminal proteases or lipid peroxides.

Spermiophages, which are immunoresponsive cells found in semen, are more highly concentrated in ejaculates from toms exhibiting the yellow semen syndrome (Thurston et al., 1975). In semen that is pooled from different males, spermiophages derived from one tom may be activated against spermatozoa from different toms due to differences in histocompatability antigens (Barnes et al., 1996). Monoclonal antibodies to spermiophages cross-react with blood monocytes and peritoneal macrophages. Therefore, spermiophages like macrophages could target cells for destruction that are recognized as foreign (Perez et al., 1994). Recently it was shown that the addition of spermiophages to normal turkey semen had no effect on fertilizing capacity, hatchability or embryo mortality of unstored or semen stored for 6 h at 5°C (Barnes et al., 1996). However, the effect of spermiophages during storage for 24 h or longer are unknown.

Enzymes which may be activated and affect the viability of spermatozoa during 24 h storage as a result of damage to the acrosome include acrosin (Richardson et al., 1987; Froman, 1990) and active seminal plasma proteases (Thurston et al., 1993b). This activity could possibly cascade into the hydrolysis of sperm plasma membrane-associated glycoproteins. Several studies provide evidence that surface glycoproteins of chicken spermatozoa may be important determinants of their fertilizing capacity. Fertilization was reduced when neuramidase was used to remove neuraminic acid (sialic acid) residues from plasma membrane glycoproteins (Froman and Thurston, 1984). Additionally, hypertonic NaCl solutions were used to remove surface associated proteins and glycoproteins (Wishart and Steele, 1992). Reduced fertilization was attributed to impaired ability of spermatozoa to enter or remain within the SST.

Because of the metabolic requirements and physical composition of spermatozoa and seminal plasma, in vitro storage of turkey spermatozoa is highly susceptible to lipid peroxidation. Chicken and turkey spermatozoa contain high amounts of polyunsaturated fatty acyl groups (Ravie and Lake, 1985) and spontaneous peroxidation occurs during in vitro semen storage in both species (Fujihara and Koga, 1984; Cecil and Bakst, 1993). In turkey semen, malonaldehyde (MAL) which is a by-product formed during peroxidation)

increased with length of in vitro storage (Cecil and Bakst, 1993). Froman and Thurston (1981) observed lower superoxide dismutase (SOD) activity in turkey semen compared to chicken semen and speculated that turkey spermatozoa are more susceptible to oxygen toxicity during in vitro storage. Wishart (1984) demonstrated that lipid peroxidation differs in individual roosters. Males ranked high in rates of lipid peroxidation produced no fertile eggs after 5 h in vitro storage. Yet, these same males had similar fertility rates to low lipid peroxide males with fresh inseminations (Wishart, 1984). MAL production was found to be 10-fold higher in toms after 26 weeks of semen production compared to males after 2 weeks of semen production (Donoghue and Donoghue, 1997). As alluded to in the discussion of spermiophages, semen from individual toms is generally pooled for long-term storage. Free radical generation is autocatalytic (Burton and Ingold, 1988), so mixing semen from a high lipid peroxidation-producing tom could result in damage to the entire pooled sample. Addition of antioxidants, such as vitamin E, BHT or Tempo to in vitro stored turkey semen resulted in maintenance of sperm membrane integrity and motility five-fold higher compared with controls after 48 h storage (Donoghue and Donoghue, 1997). Vitamin E added to the chicken semen has been shown to improve the fertilizing ability of 24-h stored semen over controls (Blesbois et al., 1993). Evaluation and exclusion of semen from high lipid peroxide-producing toms before pooling semen or development of a defense system against lipid peroxide damage may be of practical importance to improve the extended liquid storage of turkey semen.

Improvement of long-term liquid storage procedures for poultry could have significant impact on the industry. The development of new assays to evaluate sperm function in vivo gives us the opportunity to understand how spermatozoa are compromised after in vitro storage. These assays also allow for the development and evaluation of new diluents for long-term storage that will lead to improved efficiency of storage systems for poultry.

5. Frozen storage of semen

Frozen storage of avian semen has a long-established pedigree; chickens were the first animals to be produced from spermatozoa frozen in the presence of glycerol in 1951 (Polge, 1951). In the ensuing decades, we might then have expected to see considerable progress in the development of this technology and its application to the highly successful poultry industry. The latter is certainly not true — despite recent efforts to demonstrate how such systems could be economically viable in the broiler breeder industry (see Hammerstedt, 1995), there is little or no commercial use of frozen-stored poultry spermatozoa. The reason for this is the limited, and variable, fertilizing ability obtained from frozen-thawed poultry spermatozoa — even for chickens, in which species the technology is most advanced.

The comparatively poor rate of fertilizing ability obtained from avian, compared to mammalian, spermatozoa may, in part, result from a greater sensitivity of avian spermatozoa to the freezing/thawing process (see Hammerstedt and Graham, 1992), but is more likely to be a function of the system of sperm transport and storage in the hen

reproductive tract. Avian spermatozoa are normally inseminated into the lower vagina from where, even with untreated spermatozoa, only 1–2% are able to reach and enter the SST at the uterovaginal junction, where they are subsequently stored for days or weeks before fertilization (see Bakst et al., 1994). Thus, in comparison to mammalian spermatozoa, the post-thaw survival of avian spermatozoa is obliged to be particularly efficient.

Variations in levels of fertility from different studies has been a constant feature of poultry semen cryopreservation work. Whilst it is true that some biological variation in sperm or hen function has been shown between strains of chickens (Ansah and Buckland, 1983; Buss, 1993; Alexander et al., 1993), other factors such as the dose of inseminated spermatozoa, the frequency of insemination and, less obviously, the depth of intravaginal insemination have considerable effects on resultant fertility and are not always clearly reported — or repeated. All of these problems are compounded by, in all but a few reports, an inappropriate quantitative comparison of the fertilizing ability of control and frozen-thawed spermatozoa (see below). Thus, in assessing the efficacy of different freezing systems, this review will concentrate on those experiments in which comparisons between protocols have been done concurrently, rather than list levels of fertility achieved by isolated studies.

Despite the rather pessimistic introduction to avian semen freezing technology, a range of protocols have been developed and have been applied successfully to breed several avian species. These are described in several reviews: Lake (1986), Bakst (1990), Busch et al. (1991), Hammerstedt and Graham (1992), Bellagamba et al. (1993), Hammerstedt (1995), and Surai and Wishart (1996). Since by far the most work has been performed on chicken spermatozoa, the following sections will review freezing technology as applied to that species, before considering its application to other domesticated and non-domesticated avian species.

5.1. Fertilizing ability

Systems for demonstration of the fertilizing ability of frozen-thawed spermatozoa are invariably arranged — in terms of insemination dose, frequency and site of insemination — so that the proportion of fertile eggs laid by inseminated hens ('% fertility') over a given period is around, or greater than, 50%. For example, 53% fertility during 1 week after a single 'deep' intravaginal insemination of around 600 million spermatozoa (Lake and Stewart, 1978a,b) or 80–94% fertility by insemination 3–4 cm into the vagina of around 400 million spermatozoa every 3 days for several weeks (Kurbatov et al., 1984). Under the same conditions, hens inseminated with unfrozen spermatozoa would lay approaching 100% fertile eggs. However, this gives us little idea of the innate loss of fertilizing ability resulting from freezing, since unfrozen spermatozoa would also produce around 100% fertility when inseminated at smaller doses or less frequently. On the other hand, by comparing the insemination dose of fresh and frozen-thawed spermatozoa which invoked 50% fertility in inseminated hens, Wishart (1985) was able to show that cryopreservation using glycerol reduced the fertilizing ability of chicken spermatozoa to 1.6% of fresh, untreated spermatozoa. The severe loss in fertilizing ability of frozen-thawed chicken spermatozoa was also demonstrated by Tajima et al.

(1989) using competitive insemination of spermatozoa from two different strains of chickens. In experiments involving concurrent insemination of equal quantities of spermatozoa from both strains into the same hens, these authors reported that the progeny derived from frozen spermatozoa were only 20% as frequent as those from unfrozen but glycerolated and centrifuged spermatozoa. The conclusion from these studies is that the freeze-thawing process itself results in 80% loss of fertilizing ability, whilst the process of glycerolization, dilution, centrifugation and resuspension causes a further loss of approximately 18%. These estimations were also given credence by the finding that the numbers of frozen-thawed spermatozoa found in the OPL of laid eggs from inseminated hens was 2.2% of that of untreated spermatozoa (Alexander et al., 1993).

In addition to the above problems with the 'scale' of fertility, we have the problem of the inherent variation in the hen's response to insemination, in terms of the proportion of fertile eggs that she subsequently lays. This is, in turn, a function of the number of spermatozoa which she retains in her oviduct and transfers to the egg at fertilization and is true not only with respect to individual hens, but with respect to different inseminations made into the same hen within a period of a few days (Wishart et al., 1992). This means that large numbers of hens must be inseminated to demonstrate significant differences in the fertilizing ability of two samples of frozen-thawed semen.

Thus, in the interests of both economy and avoidance of the inherent problems of measuring fertility, in vitro assays of sperm function after freeze-thawing have been sought.

5.2. Quality assays of spermatozoa

Some parameters which have been used to assess the function or status of frozen-thawed chicken spermatozoa in vitro are shown in Table 1. For each parameter, the examples have been selected only on the basis that they cover three decades, different laboratories and different systems for both measurement of the individual parameters

Table 1
Maintenance of sperm function after frozen storage

Parameter	% untreated levels	Reference
Morphology (light microscopy)	68; 55; 41; 44	Lake, 1968; Westfall and Harris, 1975; Haije 1990; Seigneurin and Blesbois, 1995
Morphology (electron microscopy, EM)	23	Bakst and Sexton, 1979
Motility	58; 56; 24; 53; 34.	Bakst and Sexton, 1979; Westfall and Harris, 1975; Wishart and Palmer, 1986; Ochkur, 1990; Seigneurin and Blesbois, 1995
Hypo-osmotic swelling test	62	Gill et al., 1996
Oxygen utilization rate	48	Scott et al., 1980
ATP levels	26	Wishart and Palmer, 1986
Dye reduction potential	31	Robertson et al., 1997
Binding to IPVL proteins in vitro	18	Cramer et al., 1994
Penetration of IPVL in vitro	8.5	Robertson et al., 1997

and for storage of spermatozoa. Despite this, the percentage of spermatozoa shown to survive freeze-thawing — or the percentage of the parameter which survives within a group of spermatozoa — is remarkably similar in most cases, at around 40–50%.

Of course, these parameters grossly overestimate the fertilizing ability of frozen-stored spermatozoa, which is reduced to 1–2% under even the most favorable conditions (see above). This may reflect the fact that the in vitro methods generally only measure one parameter, whilst fertility is multifactorial; or that the parameters are measured soon after thawing, and the spermatozoa are required to survive for prolonged periods before fertilization or that the techniques are too insensitive to measure more subtle damage. All of these may be true: the more detailed EM studies show more morphological damage than light microscopic morphological studies; prolonged incubation of frozen-thawed spermatozoa in vitro results in considerable loss of fertilizing ability (Wishart and Palmer, 1986); and more complex functions such as the ability to hydrolyze holes in the IPL in vitro are more compromised than simpler parameters such as dye reduction, studied in the same samples (Robertson et al., 1997).

Because of their poor correlation with fertilizing ability, the use of in vitro techniques for monitoring cryodamage in chicken spermatozoa has often been avoided, or at best given little credence. The problems have been two-fold: the limitations of the techniques themselves, as discussed above, and a lack of understanding of the scale of fertility. Perhaps with the recent elucidation of the quantitative relationship between transport and storage of spermatozoa in the oviduct, sperm–egg interaction and fertility, as well as the increasing range of sperm-assay techniques becoming available (see Wishart, 1995a), some confidence will be restored in the use of in vitro screening systems for frozen-thawed spermatozoa. They may underestimate true fertilizing ability, but they are more reliable than in vivo tests of sperm function and, especially in these days of financial stringency in research, they are the only practical way of dealing with the large numbers of variables which damage spermatozoa during frozen storage.

5.3. Cryoprotectants

The range of cryoprotectants used for chicken spermatozoa includes glycerol, dimethylsulphoxide, dimethylacetamide, ethyleneglycol, dimethylformamide, and propyleneglycol (see Hammerstedt and Graham, 1992; Surai and Wishart, 1996). Of these, only the first four, which have been used most extensively, will be considered further here and consideration of the efficacy of these cryoprotectants will be restricted to comparative experiments only. Because of these omissions, some proponents of the use of these different cryoprotectants are given in Table 2.

In comparing the efficacy of different compounds for their cryoprotective activity, or for their toxicity towards chicken spermatozoa, several factors should be taken into consideration: cryoprotectant concentration, equilibration temperature, equilibration time, freezing rate, freezing method and post-thaw treatment. Rarely are these parameters repeated between two studies, so that in the interest of clarity, the results given in Table 3 ignore such experimental differences. Additionally, the ranking of the results of these different studies does not assume statistical significance. However, despite these inconsistencies, a pattern clearly emerges, showing that the most toxic and least effective

Table 2
Examples describing the use of four cryoprotectants

Cryoprotectant	Researchers (country)	Reference
Glycerol (Gly)	Lake/Wishart (UK)	Lake et al., 1981; Wishart, 1995b
	Watanabe/Terada/Maeda (Japan)	Watanabe and Terada, 1980; Terada et al., 1989
	Ogasawara/Buckland/Harris; Hammerstedt/Buss/Graham (USA)	Ansah and Buckland, 1983; Bacon et al., 1986; Buss, 1993
Ethyleneglycol (EG)	Kurbatov/Tselutin (USSR)	Kurbatov et al., 1980
Dimethylacetamide (DMA)	Kurbatov/Tselutin (USSR)	Kurbatov et al., 1984; Tselutin et al., 1995
	Schramm (Germany)	Hubner and Schramm, 1988
Dimethylsulphoxide (DMSO)	Sexton/Bakst (USA)	Bakst and Sexton, 1979

cryoprotectant is generally found to be DMSO, with EG probably less effective than DMA. The least toxic and most effective cryoprotectant is glycerol, ironically, because this cryoprotectant is, uniquely, contraceptive for intravaginally inseminated chicken (and turkey) spermatozoa (see Hammerstedt and Graham, 1992).

The nature of the contraceptive mechanism by which glycerol acts has been discussed at length in earlier reviews (e.g. Hammerstedt and Graham, 1992; Bellagamba et al., 1993) and remains obscure. Evidence that spermatozoa retain motility in vitro at room temperature in the presence of glycerol and that the fertilizing ability of glycerolated spermatozoa is negligible when inseminated intravaginally, but not by intrauterine or intramagnal routes, has been taken to signify a glycerol–sperm–vaginal mucosa interaction (see Bellagamba et al., 1993). However, since transport and storage of spermatozoa is much more efficient from sites beyond the vagina (see Bakst et al., 1994), and since spermatozoa are damaged by the presence of glycerol at physiological temperatures (Lake, 1968), then the 'contraceptive' effect may yet prove to be simply an interaction

Table 3
Efficacy and toxicity of different cryoprotectants towards chicken spermatozoa

Reference	Parameter	Efficacy ranking
Sexton, 1973	motility before freezing	Gly > DMSO > EG
	fertility before freezing	DMSO > EG > Gly
Tereshchenko, 1985	integrity before freezing	Gly = DMA > DMSO
Tereshchenko, 1988	integrity before freezing	DMA = Gly > EG > DMSO
Ochkur, 1990	motility before freezing	Gly > EG > DMA > DMSO
Lake and Ravie, 1984	fertility before freezing	DMA > DMSO > EG
Maeda et al. (1984)	morphology after freezing	Gly > EG > DMSO
Sakhatsky et al., 1988	integrity after freezing	Gly > EG = DMSO
Lake and Ravie, 1984	fertility after freezing	DMA > EG or DMSO
Kurbatov et al., 1986	fertility after freezing	EG > DMSO
Hubner and Schramm, 1988	fertility after freezing	DMA > EG
Tajima et al., 1990	fertility after freezing	Gly > DMSO > DMA
Haije, 1990	fertility after freezing	Gly > DMSO

of glycerol and spermatozoa at temperatures of around 40°C. This interaction may be classic osmotic damage or may involve alteration to surface-associated proteins of spermatozoa (for discussion, see Hammerstedt and Graham, 1992; Steele, 1992).

In practical terms, removal of glycerol from frozen-thawed spermatozoa at 5°C is achieved by dialysis or by centrifugation and resuspension. Both methods have disadvantages; dialysis tends to be time-consuming, whilst centrifugation itself causes damage (Steele, 1992). Recently, processing, storage, and removal of glycerol by dialysis has been simplified by the introduction of a semi-permeable container, the Cryocell (Buss, 1993).

The non-penetrating cryoprotectants trehalose (Terada et al., 1989) and methyl cellulose (Philips et al., 1996) have been used in combination with glycerol to ameliorate osmotic damage to chicken spermatozoa during freezing. The former seemed to improve post-thaw fertility and the latter, whilst having no discernable effect on post-thaw fertility, did reduce the contraceptive action of glycerol on unfrozen spermatozoa.

5.4. Freezing systems

In general, semen is cooled to around 5°C before dilution or equilibration with cryoprotectant solution. Samples are then held at this temperature for around 10 min before cooling further. Examples of the various methods are given in Table 4.

It is unfortunate that there are no published examples where any one of the above methods has been compared concurrently with another, except that no difference was

Table 4
Freezing systems

System	Principle	Example reference
Pellet formation 1	0.2 ml volumes of semen dropped onto a depression in solid CO_2, then transferred into liquid nitrogen	Watanabe and Terada, 1980; Terada et al., 1989
Pellet formation 2	0.2 ml semen dropped onto a fluoroplastic plate held in liquid nitrogen vapor at −70°C then transferred into liquid nitrogen	Kurbatov et al., 1984; Tselutin et al., 1995
Pellet formation	0.2 ml volumes of equilibrated semen are dropped directly into liquid nitrogen	Yerashevich, 1990; Tselutin et al., 1995
Rotation method	1 ml volumes within a 10 ml capacity glass vial are rotated in liquid nitrogen vapor at −70°C before plunging into liquid nitrogen	Kurbatov et al., 1984
Programmable freezer	Samples within glass ampoules[a] plastic cryovials[b] or straws[c] are cooled from 5 to −35°C at between 1 and 10°C/min, before plunging into liquid nitrogen	Lake and Ravie, 1984[a]; Wishart, 1995b[b]; Seigneurin and Blesbois, 1995[c]

found in the resultant fertility between semen frozen-thawed in glass cryovials and in plastic straws (Ravie and Lake, 1984).

Theoretical studies have predicted that the probability of ice crystal formation in chicken spermatozoa is unlikely to be affected by rates of cooling up to several thousand degrees centigrade (Ravie and Lake, 1982); however rates of −5°C, −7°C and −10°C per min appear to maintain fertility better than −1°C per min. Furthermore, we find (GJW, unpublished work) that freezing of spermatozoa from 5°C to −35°C in the presence of glycerol or DMA maintains the ATP levels of spermatozoa considerably better than dropping samples directly into liquid nitrogen.

Thawing systems involve plunging samples from −196°C into an alcohol bath at 5°C, this temperature being appropriate for dilution or dialysis to remove glycerol (Wishart, 1995b), and placement, briefly, into a 75°C bath (Kurbatov et al., 1988). Customized systems for thawing frozen chicken semen have been described (Buss, 1993; Tselutin et al., 1995).

5.5. Frozen storage of semen from other species of poultry

Chicken, turkey, drake and gander spermatozoa are morphologically similar, so that from a biophysical perspective the stresses on these spermatozoa during freezing should be similar, although chicken and turkey spermatozoa are metabolically different (see Lake and Wishart, 1984). However, Bakst and Sexton (1979), using DMSO, and Lake (Lake, personal communication), using glycerol, were able to produce acceptable levels of fertility in chickens, but no fertile eggs at all in turkeys using the same freezing technology. This may reflect a greater susceptibility of turkey spermatozoa to the freeze-thawing process itself, or it may be because turkey spermatozoa are subjected to more stringent conditions in the female reproductive tract: the rate of release of turkey spermatozoa from the SST is around 30% of that of chicken spermatozoa (Wishart, 1995a). Thus, theoretically, three times more turkey spermatozoa need to be inseminated so that equivalent numbers of spermatozoa from the two species reach the egg. However, fertility has been obtained with frozen-stored turkey spermatozoa using all four cryoprotectants listed in Table 2 and all methods used in Table 3 (e.g. Nelson et al., 1980; Sexton, 1981; Schramm and Hubner, 1988; Kurbatov et al., 1988; see Surai and Wishart, 1996).

Goose spermatozoa have been successfully frozen by the pellet and rotation methods with EG, DMA and DMSO (Kurbatov et al., 1988) and drake spermatozoa by the pellet and rotation methods with DMA (Kasyanenko and Kurbatov, 1986; see also Kurbatov et al., 1986; Surai and Wishart, 1996).

6. Frozen storage of semen from non-domesticated species

Freezing of semen from non-domesticated species has recently been reviewed by Gee (1995). The few species in which offspring have been produced are listed in Table 5. These, inevitably, include species with some value as pets, game or other recreational

Table 5
Non-domesticated species bred from frozen-thawed spermatozoa

Species	Cryoprotectant	Reference
American kestrel	glycerol	Brock et al., 1983
Sandhill crane	DMSO	Gee et al., 1985
Peregrine falcon	glycerol	Parkes et al., 1986
Budgerigar	glycerol	Samour et al., 1988
Aleutian Canada goose	DMSO	Gee and Sexton, 1990
Pheasant	DMSO	Durrant and Burch, 1991

use, such as falconry as well as endangered species in which there is local or national interest and funding. The morphology of spermatozoa from the species listed in Table 5 varies from the sauropsid spermatozoa of the pheasant, goose and sandhill crane, to the passerine type, with its helical nucleus (sparrow and budgerigar) and the raptor type, with its more rounded nucleus, so that simple morphology does not seem to be a barrier to successful frozen storage of avian spermatozoa. The major effort for extending this technology to other non-domesticated avian species (apart from funding!) is more likely to be the development of husbandry systems suitable for captive breeding of given species by AI, rather than the development of a protocol for freezing of semen.

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