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Specific features of *in vivo* and *in vitro* sperm storage in birds

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This review focuses on some of the main features of sperm selection and storage in birds mainly on the basis of studies performed in poultry species, with emphasis on the initial selection of sperm at the female vagina level prior to migration towards the sperm storage tubules. Sperm originating from low-quality males or subjected to inappropriate in vitro *storage conditions are rapidly discarded, resulting in impaired fertility in corresponding flocks. In the absence of accessible and appropriate technology for matching the 'storing' potential of sperm in the oviduct, conditions for prolonged sperm storage under a liquid (through the use of semen extenders) or a solid state (cryopreservation) have received only limited attention, despite their potential interest to facilitate male and female management in poultry flocks. Despite this, technology for short-term liquid storage is currently used in turkeys, guinea fowl and muscovy ducks and also in progress in chickens. In addition, technology for cryopreservation of avian semen has become available for some species (chicken, goose) to facilitate the management of genetic resources, including the preservation of rare and economically important breeds.*

Keywords: cryopreservation, *in vitro* storage, sperm, sperm selection, sperm storage tubules

Introduction

Birds have developed original reproductive processes, many derived from specific physiological features which form part of the adaptative strategies of flying animals to highly variable climatic conditions and environments. One of these features is oviparity, which has evolved for the production of telolecitic, hard-shelled eggs, enabling the autonomous development of the embryo in a highly protected micro-environment. Among other features, the presence of sperm storage tubules (SSTs; see review in Bakst *et al.*, 1994) in the single avian oviduct dedicated to the prolonged storage of sperm provides 'adaptative freedom' to the female to sustain fertilising potential even in the absence of males. Another specific feature of reproduction in males of avian species is the relatively short interval of time required for B-type spermatogonia to evolve into spermatozoa compared with mammals. For example, in the chicken, guinea-fowl, duck and turkey, the duration of spermatogenesis is approximately 14 days (De Reviers, 1968; Marchand *et al.*, 1977; Brillard, 1981; Noirault *et al.*, 2006), which provides males with extended potential to produce large quantities of gametes within a given period of time. Following their evacuation from the rete testis, avian sperm are rapidly transported through the epididymis to the vas deferens, where they are stored for a limited period of time (e.g. 2 to 3 days in the chicken, De Reviers, 1981) prior to being exported at ejaculation. Contrary to mammalian species, there is no sexual accessory gland in the vas deferens of birds.

At mating, sperm are either deposited into the cloaca, 'cloacal kiss', in species equipped with a vestigial penis (chicken, turkey) or introduced into the mid-vagina in species with a retractable penis (ostrich, duck, goose). Sperm are then subjected to a drastic process of vaginal selection prior to being stored in SSTs. The *in vivo* duration of storage in the SSTs and therefore the duration of the fertile period after a single mating or artificial insemination (AI) vary between species, breeds and individuals. The maximum duration represents the number of days during which a female may lay fertile eggs following a single insemination (Brillard, 1993). The duration of the fertile period is up to 12 days in the Japanese quail (Sittmann and Abplanalp, 1965; Reddish *et al.*, 1996), 21 days in the chicken and guinea-fowl (Brillard, 1993) and over 70 days in the turkey (Lorenz, 1950). Storage of spermatozoa in the female tract has been used to develop original strategies of reproduction. Thus, the choice of sires with high reproductive potential based on the preferential selection and storage of their sperm has probably developed in wild species (Birkhead and Brillard, 2007). Duration of the fertile period is of practical interest in domesticated birds to select females on the basis of their

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reproductive potential (Brillard *et al.*, 1998) and also to establish AI intervals (Brillard *et al.*, 1989). The processes of *in vivo* sperm storage in the oviduct are also a challenge to the development of appropriate methodologies to preserve sperm *in vitro*, as avian sperm must still undergo the normal processes of selection and prolonged storage in the SSTs following *in vitro* storage. Procedures to store poultry semen *in vitro* were first developed to facilitate the management of breeder flocks subjected to AI and also to transport genetic material to distant locations without the need for setting up of local breeder male facilities. However, despite the extensive efforts of poultry physiologists and biotechnology companies over recent years, poultry semen can currently be kept in a liquid state ($>0°C$) for only a few hours without significant loss of fertilising potential. In addition to the preservation of semen in a liquid state, cryopreservation has also been successful in a limited number of avian species. Cryopreservation is considered to be an alternative strategy to limit the erosion of genetic diversity (Dohner, 2001). It is also of major interest for the preservation of endangered breeds as it greatly facilitates the development of international programmes to assess genetic biodiversity. Recent advances in cryopreservation technology for poultry semen have resulted in the emergence of cryobanking, which is now being developed in an increasing number of countries (see Blackburn, 2006; Woelders *et al.*, 2006; Blesbois, 2007; Blesbois *et al.*, 2007).

In the present review, we focus on certain major aspects of sperm storage in birds, including morphological and functional features of the oviduct and the consequences on reproduction strategies. The 'state of the art' of *in vitro* sperm storage is also presented, with special emphasis on current and future applications in poultry.

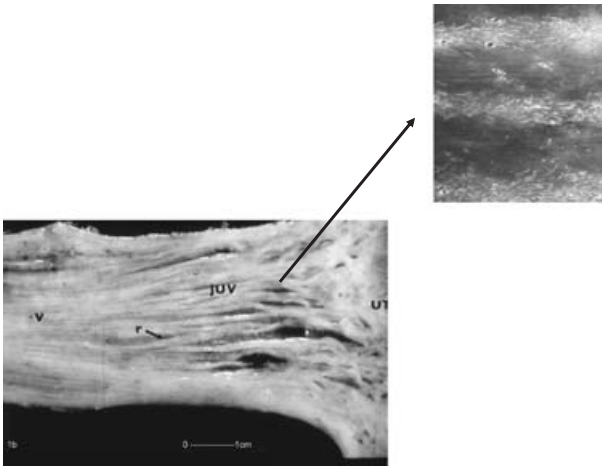

Figure 1 Location and orientation of the utero-vaginal sperm storage tubules of the hen ($\times 10$). Sperm storage tubules are cylindrical, usually non-branched structures. They are distributed along the folds of the internal mucosa of the utero-vaginal junction and of the lower infundibulum. The photograph on the left was kindly provided by Dr M. R. Bakst.

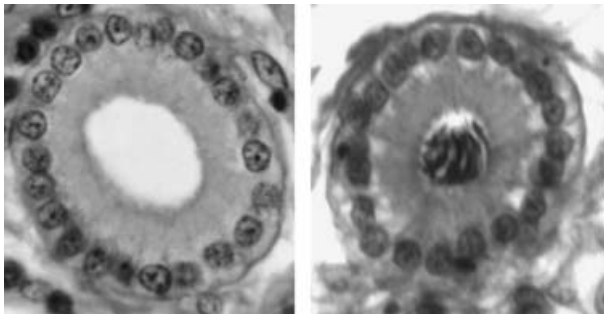

Figure 2 Transverse sections of hen sperm storage tubules (SSTs): SSTs without sperm on the left and SSTs with many sperm in the lumen on the right (mean external diameter 30 to 50 μm).

In vivo sperm storage in the avian oviduct

Females of avian species share with reptiles and hymenoptera the presence of structures located in the oviduct in which sperm can be stored for prolonged periods of time. These SSTs provide a specific micro-environment to sustain sperm survival and fertilising potential. Their presence has now been identified in all avian species studied to date, including domesticated birds (see review by Briskie and Montgomerie, 1993). Two sites devoted to sperm storage have been recognised in the avian oviduct, one located in the upper portion of the vagina at the utero-vaginal junction (UVJ) and the other in the lower portion of the infundibulum (infundibular SSTs). While both storage sites have proved functional, it is generally recognised that UVJ-SSTs are the main sperm storage site in poultry as most sperm stored for prolonged periods of time are located at this site. SSTs are cylindrical, generally non-branched structures found in the folds of the upper vaginal mucosa (Figures 1 and 2). Their numbers are species-dependent, ranging from less than 200 to 500 in passerines (Briskie, 1996) to 2000 to 3000 in the chicken (Brillard *et al.*, 1998) and up to 20 000 in the turkey (Goodrich-Smith, 1977). Studies in the chicken have demonstrated that the number of SSTs located at the UVJ is genetically controlled. Thus, hens selected for their capacity to produce high or low numbers of hatched chicks have high or low numbers of SSTs (Brillard *et al.*, 1998). Selection for this trait may therefore be of interest to sustain fertility in poultry breeder flocks.

Mechanisms of sperm selection

Upon deposition in the cloaca or vagina, avian sperm are subjected to a complex series of selection mechanisms acting both as potent postcopulatory prezygotic barriers to heterospecific fertilisation (Birkhead and Brillard, 2007) and, more generally, as biological filters impairing the migration and storage of low-quality or damaged spermatozoa (Bakst *et al.*, 1994). Sperm motility is thus required

for sperm storage in the SST. It has long been accepted that sperm that reach the SSTs must be motile (reviewed by Bakst *et al*., 1994). A more recent study by Froman (2003) also suggested that sperm stay motile during their stay in the SSTs where they have to maintain position against a fluid current generated by SST epithelial cells. To stay active at this stage, they are believed to metabolise exogenous fatty acids released from lipid-laden epithelial cells. Moreover, this author suggested that motile sperm emerge from the SST when their velocity declines to a threshold at which retrograde movement begins.

The initial sperm selection of avian sperm in the vagina is a pre-requisite to their penetration into the SSTs. Thus studies performed in chickens and turkeys have revealed that only about 1% of the initial sperm population deposited intravaginally reaches the SSTs (Brillard and Bakst, 1990; Brillard, 1993). As only 1% of the sub-population of pre-selected sperm can be recovered in the perivitelline membrane of eggs, it can be postulated that the overall population of pre-selected sperm transported to the sites of fertilisation does not exceed 1/10 000th of the initial population deposited intravaginally. The intensive selection of sperm occurring in the avian oviduct appears to be at least partly responsible for the relatively low percentages of early embryo mortality observed in birds compared with mammals. However, despite repeated efforts to locate and then assess the overall efficacy of prezygotic sperm selection in the avian female genital tract (see Birkhead and Brillard (2007) for review), a comprehensive description of the mechanisms involved in each region is yet to be attempted. Based on the current body of knowledge available, the first vaginal barrier is of a mechanical nature: it limits sperm migration to those capable of progressive motility, therefore rejecting dead and immobile sperm and sperm with impaired motility (Allen and Grigg, 1957). A second barrier, also located in the vagina, is a mechanism of sperm recognition and selection based on membrane surface characteristics. A study in the chicken showed the presence of an immunological identification of sperm impeding their migration towards the SSTs (Steele and Wishart, 1992a). More than 80% of the sperm population recovered from washings performed in the vaginal portion of the oviduct in this study had bound immunoglobulin (A or G) on the flagellar portion of their plasma membrane, compared with only 7% of the sperm present in the SSTs. The above report was the first direct demonstration of an immunological process in sperm selection occurring in the vaginal portion of the oviduct in the chicken and likely to occur in other avian species. Additional experiments in the chicken revealed the existence of an immunological, species-specific barrier located in the vagina, acting as a potent biological barrier to heterospecific fertilisation (Steele and Wishart, 1992b). Once recognised and trapped as described above, unselected sperm populations are rapidly imbedded in mucosal secretions on the surface of the vaginal epithelium (Figure 1) and then evacuated towards the cloaca.

Physiological bases to maintaining sperm viability and fertilising potential in vivo

Sperm that penetrate the SSTs reside in a lumen surrounded by a single layer of polyhedric cells with a large, basal and spherical nucleus distributed in a columnar manner (Bakst, 1981). Studies by Gilbert *et al*. (1968) showed that cells located in the body and blind extremity of the tubule are of a non-ciliated type. By contrast, those distributed at the opening (near the vaginal mucosa) are equipped with cilia beating counterclockwise (M. R. Bakst and J. P. Brillard, unpublished observations). In the presence of sperm entering the SSTs, the coordinated motion of cilia appears to facilitate sperm penetration into the SST lumen. One important aspect of prolonged sperm storage in the SSTs is the local metabolic regulation involved in the processes of maintaining selected sperm populations in a functional state. While additional information is still needed to propose a fully comprehensive description of such regulation, it can be postulated that the following requirements must be met:

(a) provision of sufficient molecules to sustain sperm metabolism over prolonged periods;
(b) prevention of damage caused by peroxidation to cellular components, with special emphasis on plasma membranes;
(c) permanent renewal or recycling of the micro-milieu surrounding sperm in the SST lumen to evacuate products of catabolism.

Regarding the first requirement, early studies performed mainly in the chicken and turkey have shown that the cytoplasm of SST cells contains lipidic components (cholesterol esters, complex lipids) varying in quantity according to the functional status of the oviduct (Fujii, 1963; Gilbert *et al*., 1968). It is likely, even though not directly demonstrated, that these lipids act as metabolic substrates to maintain the functional integrity of spermatozoa during their *in vivo* storage (Bakst, 1981; Renden *et al*., 1981). Additional studies have also established that glycogen is present in abundance in the cell cytoplasm of chicken SSTs but absent in the turkey, quail and duck (Bakst, 1981; Bradley, 1982). Its exact role remains to be established, but it can be considered as a source of energy accessible to sperm during *in vivo* storage.

Meeting the second requirement relies mainly on the presence of large quantities of polyunsaturated fatty acids (PUFAs), which are part of the components of the sperm plasma membrane (reviewed by Blesbois and Hermier, 2003). The high frequency of double bonds in PUFAs makes them highly susceptible to damage caused by peroxidation (Surai, 2002), a major cause of increased membrane permeability and of damage to the plasma membrane (Williams *et al*., 1998; Halliwell and Gutteridge, 1999). Recent studies in the chicken have shown that a complex antioxidant system including Se-dependent enzymes (e.g. GSH-Px) is present in the SSTs to prevent or repair membrane damage caused by peroxidation during storage inside the SSTs (Bréque *et al*., 2003). The efficacy of the system is

maximal during the first part of the reproductive season but declines thereafter. It can be partly compensated by Se and/or vitamin E dietary supplementation (Bréque *et al.*, 2006).

The third requirement is the evacuation of degradation products by renewal/recycling of the milieu present in the SSTs lumen. Studies performed in the turkey (Zaniboni and Bakst, 2004) have shown the presence of aquaporins (AQP), a group of small membrane proteins functioning as water channels facilitating the movement of water in cellular tissues (Agre *et al.*, 1993; Nishimura and Fan, 2002). According to Zaniboni and Bakst's observations, AQP are mainly located in the apical portion of SSTs. Although not directly demonstrated, it is likely that AQP participate in the processes of elimination of sperm catabolites accumulated in the lumen during their prolonged storage in the oviduct.

In vitro storage of semen

In contrast to *in vivo* storage in the SSTs, *in vitro* storage of semen is a non-physiological process, practiced in many animal species to maintain male gametes in a functional stage. In species with internal fertilisation, male gametes are usually rapidly transported into the female tract (generally the vagina) at copulation. In contrast, AI technology needs preservation of sperm viability and fertilising potential under *in vitro* conditions over short or extended periods of time. AI practice in the poultry industry expanded for selection and multiplication purposes over the second half of the 20th century but its justification for the production of broiler-type chicks remains species specific. For example, the turkey industry switched from natural mating to AI in the 1960s because of the strong negative phenotypic correlation between breast widths and mating potential (reviewed by Krueger, 2003). AI has also become increasingly popular to produce guinea fowl chicks. In this species, the technique was first developed because the guinea fowl hens used in selection were not adapted to trap nesting conditions (hens enter their nest but cannot get out). AI has recently become extensively used to produce mule ducklings (muscovy ♂ × pekin ♀), a consequence of the repeated problems of mating behaviour encountered in heterospecific breeder flocks. AI is now widespread in the chicken industry for selection purposes, but it has also become increasingly popular to produce broiler chicks in regions with low labour costs (Blesbois *et al.*, 2006a and b).

In addition to its extended use in the poultry industry, *in vitro* semen storage has also emerged as a route for the management of biodiversity that is complementary to *in situ* management. It has been developed in some western countries with the support of national public organisations involved in the protection of rare breeds and endangered species (e.g. in France: Genetic Resources Committee).

In order to satisfy the various fields of application of AI in poultry, handling procedures have been progressively developed aiming at the short- and long-term preservation of sperm viability and functionality. *In vitro* storage of semen in a liquid state is mainly used for poultry industry purposes, while semen cryopreservation has become the main form of reproductive biotechnology now available for the preservation of biodiversity. For this last purpose, complementary studies involving the cryopreservation of embryonic cells are also in progress (Tajima, 2002; Van de Lavoir *et al.*, 2006). However, these methods, mainly focusing on the production of transgenic birds, are not sufficiently effective and are too costly for large genetic conservation programmes (Petite, 2006).

Sperm collection

Careful use of semen collection procedures is essential to achieve good results from AI operations in poultry. Only two of the various methods described in the literature have proved to be valuable for practical use in the industry. The first, commonly known as the 'massage' technique, is derived from the original dorso-abdominal massage described by Burrows and Quinn (1937) for application in chicken and turkey. It is currently used in galliforms, goose and pekin ducks while the other, which requires a teaser, is extensively used in muscovy ducks for mule duck production. Whatever the technique, the initial 'quality' of ejaculates is primordial to achieve high fertility, which itself also depends on effective sperm storage and therefore on sperm acceptance by the female tract. Besides the high inter-individual variability of semen quality encountered in poultry species, the degree of semen contamination by faeces and urates also has an important role in subsequent fertility performance. In addition, the male cloaca secretes a glucose-rich liquid called 'transparent fluid'. This fluid has been described as stimulating sperm metabolism but also impairs *in vitro* storage (reviewed by Lake, 1986; Etches, 1996). This deleterious effect requires that ejaculates contaminated with transparent fluid should be discarded at the time of collection. It should be remembered at this point that none of the various techniques available for handling poultry semen in a liquid or solid state can rehabilitate ejaculates initially classified as being of 'poor quality'.

Storage of avian sperm in a liquid state

Depending on species, neat semen may be stored *in vitro* at an ambient temperature for 5 to 15 min without significant loss of fertilising potential. However, sperm susceptibility under undiluted conditions is extremely high in species such as the pheasant and guinea-fowl, reducing the value of this fairly 'straightforward' practice in these species. Moreover, the generally high numbers of females routinely inseminated in breeder flock operations require repeatable and reliable practices to facilitate proper coordination between semen collection and AI. In this case, semen diluents are fully justified to sustain sperm viability and fertilising potential for limited periods of time. It should be pointed out that most sperm diluents currently in use in poultry contain components naturally found in seminal plasma. As such, their composition is closer to the fluids found in the

vas deferens than in the female genital tract. Indeed, the process of *in vivo* storage of sperm in the female reproductive tract is not sufficiently documented to support the fabrication of diluents.

Among other characteristics, semen diluents must provide relatively constant physico-chemical stability to sperm during *in vitro* storage, including iso-osmotic and nearly neutral pH conditions. This is generally achieved by mixing balanced salt solutions in the pH range 7.0 to 7.4 depending on species. Simple energy substrates as well as antioxidants and antibiotics may also be added (Christensen, 1995; Thurston, 1995).

At the time of ejaculation, avian sperm contain very few intra-cellular energy reserves but repeated studies have demonstrated that they can access various types of energy metabolites present in the extra-cellular medium to ensure their basal metabolism and motility. The energy metabolites available to sperm vary between species, but they generally consist of sugars (mainly fructose; McIndoe and Lake, 1973; Sexton, 1974) and fatty acids (Scott *et al.*, 1962). Early studies (El Zayat and Van Thienhoven, 1960) also demonstrated that chicken spermatozoa may metabolise limited quantities of glutamic acid present in high amounts in seminal plasma. More generally, it has been reported that aerobic metabolism occurs in all avian species studied to date, but sperm from some species (e.g. the chicken but not the turkey) may also metabolise glucose under anaerobic conditions, at least *in vitro* (Wishart and Carver, 1984). As a consequence, energy substrates may be added to diluents in order to prolong sperm viability and activity. However, even in these conditions the fertilising potential of sperm kept *in vitro* at body-like temperatures (41°C for chicken) cannot be maintained over many minutes. In order to improve *in vitro* storage conditions, sperm metabolism must be decreased. This is generally achieved by decreasing temperature (Lake and Ravie, 1982; Giesen and Sexton, 1983). However, the decrease must be progressive (e.g. 0.5°C/min) in order to prevent adverse cold shock effects (Clarke *et al.*, 1982; Wishart, 1984). The general principle guiding the choice of storage temperature conditions for avian semen is 'the longer the duration of storage, the lower the temperature', on the understanding that the lowest temperature should never go below 2°C to 5°C (storage scheduled for 24 h) to prevent local freezing points in the vials (Blesbois, 2003). Another important aspect in preserving avian semen in a liquid state is its dilution rate. It is generally considered that optimal dilution rates should be increased when the duration of *in vitro* storage increases (from a minimum of two volumes of semen for one volume of diluent (2 : 1) to a maximum of one volume of semen for three volumes of diluent; Sexton, 1977, 1978 and 1982). This practice not only reduces the deleterious factors present in seminal plasma (e.g. faeces, urates, sperm catabolites), but also ensures the presence of protective elements naturally present in ejaculates (Blesbois, 1990). In the absence of accessory glands in the deferent ducts of birds, seminal plasma contains molecules secreted (or excreted) at various locations of the vas deferens. They include lipoproteins (Blesbois and Hermier, 1990), a variety of proteins (the main protein being a serum-like albumin: Blesbois and Caffin, 1992), several sugars (including fructose but not glucose) and many other biological co-factors (Etches, 1996). Overall, the action of seminal plasma is complex during *in vitro* storage. It contains oligo-elements, lipid peroxides and phospholipases that are deleterious to *in vitro* storage (Blesbois and Mauger, 1989; Blesbois *et al.*, 1993; Douard *et al.*, 2004a). In contrast, the anti-oxidant enzymes and a fraction of high molecular weight also present in the seminal plasma are beneficial to the *in vitro* storage of avian sperm (Blesbois and de Reviers, 1992; Surai *et al.*, 1998). However, the presence of various toxic components, either naturally present in seminal plasma or added to it at the time of ejaculation or as the result of sperm catabolism, results overall in improved conditions of *in vitro* storage if the initial seminal plasma is discarded (Blesbois, 1990; Blesbois and Hermier, 1990; Douard *et al.*, 2005) or replaced by dialysed seminal plasma (Blesbois and De Reviers, 1992; Iaffaldano and Meluzzi, 2003).

Another aspect of sperm storage conditions over limited periods of time (temperature >0°C) is the need to protect sperm membranes against free radicals. The addition of antioxidants such as vitamin E and selenium to semen diluents has been found effective to counteract the action of free radicals (Blesbois *et al.*, 1993; Dimitrov *et al.*, 2007). Peroxidation occurs more rapidly under quite high storage temperatures (15°C to 41°C, Donoghue and Donoghue, 1997; Douard *et al.*, 2004b), and when males are ageing (Douard *et al.*, 2003).

However, even under optimal storage conditions, sperm functionality and viability decline slowly over the first 48 h of *in vitro* storage (Donoghue and Walker-Simmons, 1999). The degradation is accompanied by significant losses of the main phospholipids of the plasma membrane (especially those constituting its outside leaflet: Blesbois *et al.*, 1999; Douard *et al.*, 2000), disruption of the acrosome and finally DNA fragmentation (Kotlowska *et al.*, 2007). On the basis of the kinetics of these events, some hypotheses can be proposed to improve the composition of semen diluents in poultry. For example, the action of phospholipases A2 and B of sperm may induce *in vitro* plasma membrane degradation, which then produces intra-cellular dysfunction (Douard *et al.*, 2004a). This may result in the rejection of damaged sperm during the processes of selection exerted by the female tract prior to their acceptance into the SSTs. We therefore suggest that the addition of phospholipase inhibitors could counteract plasma membrane degradation during the *in vitro* storage of poultry semen.

To conclude, improvements in methods and technology dedicated to short-term storage of avian spermatozoa will be needed to accompany the development of AI practice in birds. In poultry species such as the chicken, AI is developing rapidly in several geographic regions (Asia, India and Eastern Europe). This is accompanied by an increased demand for working procedures to maintain the fertilising

potential of ejaculates for 6 to 24 h under field conditions. Although this can be reasonably expected in highly fertile breeds, it will remain a challenge for others due to the genetic dependence of most fertility traits, including semen quality (Reddy and Sadjadi, 1990) and sperm storage potential (Brillard *et al*., 1998). In such breeds, the initial quality of semen and *in vitro* sperm storage technology will remain critical.

Semen cryopreservation

Semen cryopreservation in birds has been a subject of intense scientific interest for about 100 years (reviewed by Luyet and Gehenio, 1940) but its extensive use for large field operations remains unrealistic, partly due to the high cost of using frozen compared with fresh semen and partly due to the difficulty of obtaining freeze/thaw avian semen without significant loss of fertilising potential. As previously described, and in contrast to mammals, avian sperm can normally remain in the oviduct for prolonged periods of time prior to fertilising an oocyte. In the case of sperm treated for cryopreservation, damage caused to plasma membranes results in a dramatic increase in sperm rejection prior to storage in the SSTs, thus preventing subsequent fertilisation (Wishart, 1985; Seigneurin and Blesbois, 1995; Chalah *et al*., 1999). Such impairment of fertility parameters may be partly compensated by increasing the number of sperm deposited at each insemination and also by increasing the frequency of insemination (e.g. two times per week *v.* one time per week in chickens). Surprisingly, embryo mortality following insemination with frozenthawed spermatozoa is generally well below that observed with fresh semen, at least in the chicken (Seigneurin and Blesbois, 1995). This indicates that freezing/thawing procedures may 'emphasise' the natural processes of sperm selection exerted by the lower oviduct prior to their acceptance in the SSTs.

As for liquid storage, high initial quality of semen is required to cryopreserve avian sperm successfully as they must face a succession of thermal, osmotic and mechanical stresses prior to accessing the storage sites. Indeed, avian sperm share properties that are common to sperm of most animal species with internal fertilisation (Massip *et al*., 2004). Their low cytoplasmic content and relatively large surface of membranes exposed to adverse micro-environmental conditions (Lake, 1984; Etches, 1996) explain why the overall biophysical characteristics of membranes, including resistance to osmotic shock and membrane fluidity, appear critical for the success of cryopreservation (Blanco *et al*., 2000; Blesbois *et al*., 2005, 2006a and b). As a consequence, membrane damage induced by cryopreservation also results in impaired motility and decreased concentrations of various metabolic factors, including ATP concentration (Long, 2006). Limitation of membrane damage induced by cryopreservation has been attempted using specific diets to modify the lipid composition of the sperm membrane, including its cholesterol/phospholipid ratio and fatty acid composition (Ansah and Buckland, 1982; Blesbois *et al*., 1997). Membrane fluidity measured in freshly collected sperm has recently been shown to be a reliable predictor of sperm freezing potential in the chicken (Blesbois *et al*., 2006a).

Freezing procedures developed to cryopreserve avian semen are close to those currently in use for mammals. Interestingly, studies performed to find the best cryoprotectant agent, the best combination of freezing and thawing temperature curves, and the least deleterious and most traceable system of semen packaging have been legion, probably because of the disappointing results reported in these species. They include comparisons of internal (glycerol, dimethyl-sulphoxide, dimethyl-formamide, dimethyl-acetamide, ethylene-glycol) and external cryoprotectants (e.g. polyvinyl-pyrolidone), rapid or slow freezing/thawing rates and pellet or straw packaging. A 'historical' picture of the progress and still unanswered questions concerning avian sperm cryopreservation can be obtained from several reviews published in the last few years by Hammerstedt (1995), Surai and Wishart (1996), Donoghue and Wishart (2000), Massip *et al*. (2004) and Blesbois (2007). Briefly, several workable methods are currently available for use in the chicken with different internal cryoprotectants (Figure 3), while results in other species remain inconsistent or insufficient. We will not extensively review the freezing-thawing procedures here because they can be found in the above studies. We focus instead on two points that differ from practices currently used for the cryopreservation of mammalian sperm, i.e. use of the egg yolk or of one of its synthetic substitutes and the glycerol effect.

Egg yolk alone or in combination with milk has long been a major component of freezing diluents used for mammalian sperm because of its protective effect on plasma membranes repeatedly subjected to physical–chemical stress during freezing/thawing procedures. In contrast, early reports by Jasper (1950) and Fewlass *et al*. (1975) indicated that the addition of chicken egg yolk to semen diluents decreased sperm respiration rates and fertilising potential in the chicken. The reasons for these decreases remain unclarified but the replacement of chicken egg yolk by turkey egg yolk resulted in the disappearance of the deleterious effect on fertility caused by chicken egg yolk. Furthermore, a diluent containing glucose and chicken egg yolk was successfully developed for gander sperm (Tai *et al*., 2001). It can be hypothesised that intra-species interactions of an unknown nature between the egg yolk and sperm occur in a given species. However, even in the absence of toxicity, a beneficial effect of egg yolk on avian sperm remains to be established. The preparation of freezing extenders for avian semen has therefore long been based on precisely defined synthetic compounds.

The case of glycerol is also complex. Glycerol is probably the most efficient and least toxic cryoprotectant for chicken sperm (Chalah *et al*., 1999; Tselutin *et al*., 1999) but its introduction into the vagina with sperm results in the total absence of fertilisation if present at final concentrations $\geqslant$2% in the semen dose (Lake, 1986). In order to

(a)	Cryoprotectant	Authors
Chicken semen	DMA Glycerol DMF DMSO	Tselutin *et al.*, 1999 Seigneurin and Blesbois, 1995 Schramm, 1991 Sexton, 1980
Turkey	DMA Ethylene-glycol	Blesbois and Grasseau, 2001 Graham *et al*., 1984
Gander semen	DMF DMA	Lukaszwicz *et al.*, 2001 Taï *et al.*, 2001
Duck semen	DMA	Tselutin *et al.*, 1995

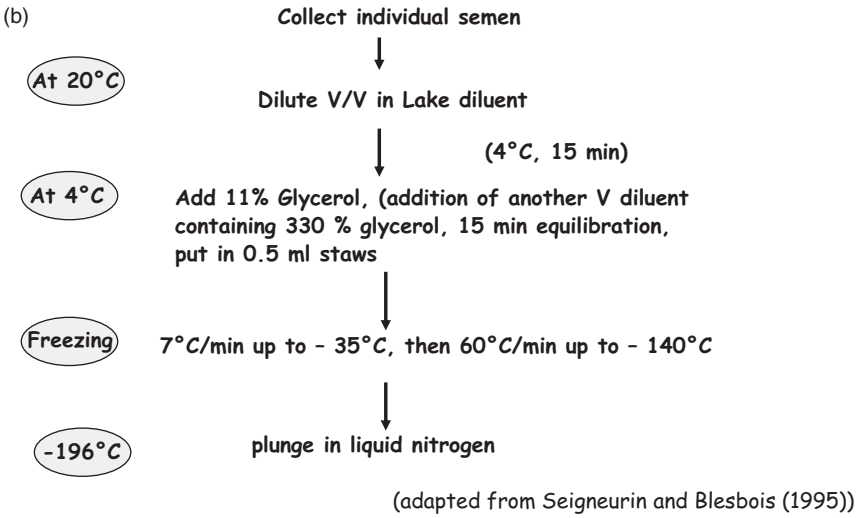

Figure 3 Semen freezing procedure: (a) examples of cryoprotectants used for different species; (b) a procedure of semen freezing currently in use for sperm cryobanking in chicken.

counteract this 'contraceptive' effect, it is therefore necessary to undertake its slow removal prior to insemination. Among the hypotheses to explain the contraceptive effect of glycerol (reviewed by Hammerstedt and Graham, 1992), it has been suggested that damage could be created by a sudden osmotic imbalance across plasma membranes initiated by the addition of a glycerol-free medium to glycerol-permeated sperm within seconds before insemination. Other hypotheses include the rapid degradation of the cytoskeleton of sperm following the thawing procedure. Direct effects of glycerol on bio-energy balance, increased membrane permeability to toxic components and direct changes in the membrane lipid bi-layer have also been suggested.

Interestingly, AI in birds is usually intravaginal to prevent dysfunction of the laying cycle. This practice is different from that found in most mammalian species in which intrauterine insemination is the general rule. In avian species, the contraceptive effect of glycerol is maximal when semen is deposited in the vagina or at the utero-vaginal junction but it disappears in the case of insemination performed in the upper oviduct. This strongly suggests that, depending on the site of its deposition, glycerol may act as a stimulating factor for a barrier of unknown nature, impairing sperm migration to the storage sites. Despite this, the use of glycerol as cryoprotectant, combined with its slow, controlled removal within minutes prior to insemination has been shown to be effective even for the cryopreservation of chicken sperm originating from subfertile lines (Blesbois *et al*., 2007). These results demonstrate the potential of sperm cryopreservation for *ex situ* management of genetic resources in chickens. At least three national programmes are currently being developed to cryopreserve chicken sperm from rare and endangered breeds, including the French National Cryobank of Domestic Animals www.cryobanque.org), the North America National Animal Germplasm Programme (www.ars-grin.gov/animal) and the

Netherlands programme managed by the Netherlands Centre of Genetic Resources (www.cgn.wur.nl/uk). In France, four lines of geese, muscovy and pekin ducks have also been added to the French Cryobank so far this year. Despite these efforts, the development of cryobanking for species other than the chicken remains extremely limited. This is a consequence of the lack of knowledge required to develop the appropriate technology for the cryopreservation of sperm in these species, another challenge for the years to come.

Conclusion

Among the physiological adaptations developed in avian species to maintain full reproductive potential over the entire reproductive season, the existence of highly challenging conditions of sperm selection combined with the presence of specialised sites for the prolonged storage of sperm in the oviduct is quite unique among homoeotherms. Although far from exhaustive, a significant body of information has now emerged from comprehensive studies performed in this field. From a practical standpoint, such information should be considered as a reliable scientific resource for the development of alternative strategies dedicated to the short-term preservation of sperm in domesticated avian species. For example, the processes of high initial selection of sperm prior to their prolonged *in vivo* storage appear to play a crucial role in the maintenance of high fertility and low embryo mortality. Such processes have, with a few exceptions, received only limited attention to date to improve the efficacy of handling procedures in poultry (Hulet *et al.*, 1995). Initial sperm selection may also be essential to improve the overall efficacy of semen cryopreservation in avian species, a tool currently available for only a limited number of species despite its obvious value for the management of avian genetic resources and, more generally, for the preservation of biodiversity.

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