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# Reproduction biotechnologies in germplasm banking of livestock species: a review

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## Summary

Many biotechnologies are currently used in livestock breeding with the aim of improving reproductive efficiency and increasing the rate of genetic progress in production animals. Semen cryopreservation is the most widely used cryobiotechnology, although vitrification techniques now allow embryos and oocytes to be banked in ever-increasing numbers. Cryopreservation of other types of germplasm (reproductive tissue in general) is also possible, although the techniques are still in the early stages of development for use in livestock species. Although still in their infancy, these techniques are increasingly being used in aquaculture. Germplasm conservation enables reproductive tissues from both animals and fish to be preserved to generate offspring in the future without having to maintain large numbers of living populations of these species. However, such measures need careful planning and coordination. This review explains why the preservation of genetic diversity is needed for livestock and fish, and describes some of the issues involved in germplasm banking. Furthermore, some recent developments in semen handling leading to improved semen cryopreservation and biosecurity measures are also discussed.

Keywords: Biodiversity, Endangered breeds, Epigenetics, Fish, Livestock

## Introduction

Animal reproduction has a central role in the production efficiency and genetic improvement of domestic animals. Therefore, advances in reproductive technologies to maximize reproductive efficiency will be crucial to meeting future demands of an increasing global population (food security), as well as helping to overcome future difficulties in animal production wrought by climate change. Modern techniques of reproduction encompass various assisted reproduction technologies (ART), as well as technologies such as genomic selection that are applied in animal breeding to identify animals with superior genotypes (Veerkamp & Beerda, 2007). While ART enables us to bring gametes together to establish a pregnancy, genomic selection is used to identify which individuals should be selected as a source of those gametes (Humblot *et al*., 2010). Epigenetics, a related area of study, endeavours to explain environmental effects on DNA that affect expression of genes and therefore the phenotype of the resulting offspring (Urrego *et al*., 2014). The key to these technologies is that a variety of methods can be combined to improve reproductive efficiency in breeding animals, in an effort to obtain optimal reproductive performance while maximizing usage of natural resources. Cryopreserving semen and embryos is one way of maximizing the availability of reproductive material, facilitating reproductive procedures independently of time and geographic location. However, germplasm banking is not restricted solely to gametes or embryos but refers to the cryopreservation of reproductive cells in general

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(Woelders *et al*., 2012). The application of these techniques in livestock husbandry is the subject of this review.

## Materials and Methods

The literature used in this review has been sourced via PUBMED and Web of Science. Search terms included germplasm, cryopreservation, vitrification, aquaculture, rare breed conservation, and ART.

## Why preserve genetic biodiversity?

Genetic biodiversity allows animals to adapt to different environments, both to changes imposed on animals by mankind in the guise of management or husbandry practices and, in the wider sense, to diseases and climate change (Woelders *et al*., 2012). Selecting only high-producing genotypes may be counter-productive in the long-term if fertility is thereby impaired, as seems to have been the case for dairy cattle (Rodriguez-Martínez *et al*., 2008). Conservation of animal genetic resources is essential to provide the genetic biodiversity required to allow animals to adapt to future changes in the environment. As far as farm animals are concerned, this means conserving our indigenous breeds; currently some 20% of livestock breeds are at risk of extinction and data are lacking for a further 36% of breeds. Rather than trying to maintain viable populations of each breed of livestock species (*in situ* conservation), a more practical method of maintaining genetic diversity is to cryopreserve germplasm (*ex situ* conservation). Thus by conserving existing genetic diversity, the basis for selecting 'desirable' genotypes in the future can be maintained.

With the rapid expansion in global aquaculture, there has been an increased emphasis on the development of reproductive technologies directed towards increased production efficiency in fish farming (Weber & Lee, 2014). While procedures for sperm cryopreservation have been developed for most major aquaculture species, successful sperm management for many species is currently constrained by the high variability of sperm cryo-resistance, a lack of appropriate biosecurity safeguards, and the absence of cryopreservation technologies sufficiently validated for large-scale aquaculture (Martínez-Páramo *et al*., 2017). In contrast to mammalian vertebrates, fish spermatozoa remain immotile in seminal plasma (Dadras *et al*., 2017), with motility being activated following release into the external environment (sea or freshwater). Following activation, spermatozoa from most fish species remain motile for a short time period, often 1 min or less. To address these challenges, protocols for fish semen processing have focused on developing species-specific extenders to facilitate the collection and storage of inactivated spermatozoa (Tiersch *et al*., 2007). Recent studies have indicated that temperature affects the lipid composition of motile carp sperm, with greater temperatures being associated with reduced lipid content, increased sperm curvilinear velocity and a decreased duration of the period motility (Dadras *et al*., 2017).

## Assisted reproduction technologies

The technologies involved in mammalian ART include a number of methods of bringing together spermatozoa and oocytes, such as artificial insemination (AI), gamete intra-fallopian transfer (GIFT), *in vitro* fertilization (IVF) and embryo transfer (ET), intra-cytoplasmic sperm injection (ICSI). To facilitate carrying out these techniques, other technologies have been developed such as sperm preservation and cryopreservation, and *in vitro* maturation (IVM) of oocytes (usually from slaughterhouse material), for subsequent IVF and ET.

By far the most widely used ART for breeding food production animals is AI using fresh or frozen semen. The availability of preserved semen (either cooled or cryopreserved) enables semen to be inseminated into females that are isolated from the male, thus facilitating both genetic improvement and disease control. If frozen, the semen can be used at any time, including after the death of the donor. This feature is particularly important when rare breeds are concerned as the chances of having access to fresh semen exactly when it is needed for insemination are low. However, it is not always possible to collect semen from all individuals, particularly those that have not been trained to ejaculate into an artificial vagina. In some cases, the collection of epididymal spermatozoa after castration or death is feasible (Woelders *et al*., 2012) and can yield high numbers of useable spermatozoa e.g. from rams.

Use of AI has the disadvantage that it focuses mainly on the genetic component of the male; ET should enable genetic progress to be made more rapidly than AI as it enables both the male and female genetic components to be transferred. Worldwide, ET is increasing considerably within the bovine livestock sector, mainly due to an increase in the transfer of *in vitro* produced (IVP) embryos. Figures from 2012 indicate a current annual transfer of around 500,000 embryos produced by superovulation and 400,000 by *in vitro* maturation (Galli *et al*., 2014). The vast majority

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of these transfers is occurring in South America, especially Brazil.

Some *in vitro* technologies are better suited to particular species than others. The increasing use of bovine IVP embryos is indicative of the success of the technique in this species. However, in horses it has not been possible to develop a reliable method for equine IVF. Thus ICSI is more commonly used than IVF for equids, although the need for specialized equipment and highly trained personnel limit the use of this ART. Conversely, ICSI is not commonly used for bovine oocytes because of technical difficulties, such as inadequate oocyte activation (Malcuit *et al*., 2006) or cytoskeletal damage, e.g. from the large-diameter pipette needed to accommodate the bull spermatozoon (Galli *et al*., 2003). In other species, the lack of access to oocytes is a limiting factor in the potential development of an ET program. Protocols for cryopreserving embryos may not be suitable for oocytes, even in the same species; further research is needed to develop better methods of preserving oocytes. Thus it is important not only to choose the technique to fit the species but to develop as many forms of ART as possible, to increase the chances of success in any given species.

## Germplasm banking

The term 'germplasm' encompasses all reproductive cells that, singly or in combination, lead to the production of offspring. The most commonly encountered form of germplasm banking is sperm cryopreservation, which has been used in livestock breeding for more than 50 years and is considered to be the backbone of modern dairy cattle production. It is also the most readily applicable biotechnology as spermatozoa are the reproductive cells that are most easily obtained and numerous. In contrast, obtaining oocytes is invasive, their numbers are limited and maturation is cycle-dependent in many livestock species. Whereas sperm cryopreservation protocols exist for approximately 300 species, protocols for oocytes and embryos have been developed for fewer than 50 species. Other sources of germplasm include spermatids, spermatogonial stem cells, testicular and ovarian tissue, as well as somatic cells for nuclear transfer.

### Sperm cryopreservation

The technique for sperm cryopreservation in the presence of a permeating cryoprotectant, glycerol, was developed by Polge *et al*. (1949) in the middle of the 20th century. Despite many decades of research the basic principles of this technique remain unchanged. In brief, the method for cryopreservation of semen is as follows: in some species, e.g. boar and stallion, the semen is centrifuged in extender first to remove most of the seminal plasma. The spermatozoa are cooled slowly in a hyperosmotic solution containing cryoprotectants to minimize ice-crystal formation. Water is drawn out of the sperm cells while permeating cryoprotectants enter (Watson, 1995). Ice-crystal formation cannot be prevented completely, nor the increasing solute concentration caused by loss of water, leading to a certain amount of structural damage. Thus, only a proportion of the spermatozoa survive cryopreservation, depending on the individual and species.

Slight variations to this protocol have been made over the intervening decades, such as substituting plant-derived lecithins (Leite *et al*., 2010) or liposomes (Röpke *et al*., 2011), for egg yolk or milk in the cryopreservation extender, to avoid including material of animal origin. Glycerol is still the main cryoprotectant used for freezing spermatozoa for most mammalian species although attempts have been made to find substances that are less toxic, with various amides attracting attention (Alvarenga *et al*., 2005). However, there seems to be considerable variation within species regarding the suitability of different agents: a combination of dimethylformamide and glycerol was reported to be useful for stallion semen (Álvarez *et al*., 2014), although dimethylformamide was not superior to glycerol for freezing bull semen (Forero-González *et al*., 2012). Moreover, including dimethylacetamide and glycerol as the cryoprotectant for boar spermatozoa did not offer any advantages over glycerol alone (Yang *et al*., 2016).

Recent advances in sperm cryopreservation have examined the effects of seminal plasma on sperm membranes, particularly in species in which there is considerable individual variation in sperm freezability. For instance, it was observed that adding seminal plasma from boars that were known to be good freezers improved motility and membrane integrity of other boars′ spermatozoa (Hernández *et al*., 2007). Similar experiments with stallion spermatozoa, however, have achieved contradictory results; in some cases sperm quality is improved (e.g. Moore *et al*., 2005) and in other cases there was no effect (e.g. Mari *et al*., 2011).

The effects of other additives have also been studied. The survival of both stallion (Moore *et al*., 2005) and bull spermatozoa (Amorim *et al*., 2009) has been shown to be improved by including cholesterol in the cryopreservation medium. Various antioxidants have been shown to improve post-thaw sperm quality (Lindemann *et al*., 1988) e.g. glutathione peroxidase (Sławeta *et al*. 1988), and interest in other antioxidants has blossomed recently. Plant-derived substances with antioxidative activity such as rosmarinic acid have

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**Table 1** Comparison of slow freezing and vitrification for embryos and oocytes

| Slow freezing | Vitrification |
|---|---|
| Equilibrate embryos in hyperosmotic solution with cryoprotectants | Solidification without ice-crystal formation (amorphous vitreous state) |
| Slow rate of cooling (0.2–2.0°C/min) to approximately −70°C | High concentrations of osmotic substances e.g. sucrose, fast cooling rates |
| Stored in liquid nitrogen | Stored in liquid nitrogen or at −80°C; variety of storage devices |

been used for boar spermatozoa (Luño *et al.* 2014). Virgin coconut oil was found to enhance post-thaw motility, morphology, membrane integrity and acrosome status of bull spermatozoa (Tarig *et al.*, 2017). The flavonol quercetin, which is thought to suppress formation of superoxide and peroxy radical formation, as well as chelating iron, was reported to improve post-thaw motility and DNA integrity of stallion spermatozoa (Gibb *et al.*, 2013). These are only some examples of recent attempts to improve post-thaw sperm quality with various additives.

## Embryos and oocytes

Embryos (and, to some extent, oocytes) can be frozen using permeating cryoprotectants and slow freezing protocols in programmable cell freezers, in a similar manner to spermatozoa. Vitrification, particularly of oocytes, is in many cases more successful than conventional slow freezing. Oocytes are particularly sensitive to slow freezing because of the low permeability of the plasma membrane to cryoprotectants but also because of premature cortical granule exocytosis leading to zona hardening, which renders them impenetrable by a spermatozoon for subsequent fertilization (Arav, 2014). With vitrification, however, the cells are placed in a solution of high osmolarity but without permeating cryoprotectants, and are then plunged directly into liquid nitrogen. As no ice-formation takes place and the transition to a glass-like state occurs very rapidly, structural damage is minimized (Arav, 2014). Table 1 provides a comparison of the two techniques. Despite the success with vitrification of human oocytes, those of cattle are proving more problematic, which may be due to the amount of cytoplasmic lipids and/or the composition of the plasma membranes (Sprícigo *et al.*, 2015).

Vitrification has also been attempted with spermatozoa. The problems encountered with cryopreservation-induced sperm damage are well known and include physical damage from ice-crystal formation and dehydration, damage to the sperm mitochondria arising from cold-shock, damage to the plasma and acrosomal membranes (capacitation-like damage) and DNA, as well as toxicity of cryoprotectants (Agha-Rahimi *et al.*, 2014). Theoretically, these problems could be circumvented by vitrification, in which there is no ice-crystal formation and very high rates of cooling are employed that are considered to be too rapid for cold-shock to occur (Isachenko *et al.*, 2012). However, to date, the only species in which sperm vitrification is reported to be successful is the human (Isachenko *et al.*, 2012). Considerable chromatin damage was induced in dog spermatozoa by vitrification, much more so than by conventional freezing (Sánchez *et al.*, 2011). Even with human spermatozoa there are conflicting reports about whether vitrification offers any advantages over conventional slow freezing (Agha-Rahimi *et al.*, 2014). The size of the sperm head appears to be a crucial factor in determining cryosensitivity during vitrification, with smaller sperm heads being able to withstand the severe osmotic effects better than the larger, paddle-shaped sperm heads of most livestock species. Therefore, further developments are needed before this technique will be able to provide a real alternative to slow freezing of spermatozoa.

## Other germplasm

Recent research has examined the possibilities of transplanting spermatogonial stem cells, or testicular or ovarian tissue following cryopreservation. There are isolated reports of successful transplantation of spermatogonial stem cells in mice (Gouk *et al.*, 2011), and of bovine testicular tissue into recipient bulls (Herrid *et al.*, 2006); both techniques are reported to produce viable spermatozoa. The pattern of donor sperm production in the recipient bulls showed a decline in the proportion of spermatozoa from the donor up to 6 months following transplantation (Herrid *et al.*, 2006). Donor-derived spermatozoa were detected in semen from 2% recipient goats for 5 to 8.5 months; in rams 1–30% spermatozoa were donor-derived for up to 30 months and 15% of the progeny from one of these recipient rams were donor-derived (Herrid & McFarlane, 2013). Post-transplantation production of ram spermatozoa of donor origin for at least 5 years was reported in other studies (Stockwell *et al.*, 2013). These studies show that such transplantation is possible, even if further research is needed to refine the technique.

Interestingly, cryopreservation of testicular tissue requires a different cryoprotectant from those commonly used for spermatozoa or for embryos, dimethyl sulphoxide (DMSO) for tissue (Wu *et al.*, 2011), as opposed to glycerol for spermatozoa and propanediol or ethylene glycol for embryos. Although convenience dictates that one protocol is optimal for any given

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species, it appears that the best results are obtained if the protocol is tailored to fit the gametes of each individual.

Cryopreserved ovarian tissue has apparently been transplanted successfully in mice and human patients as a means of retaining fertility despite chemotherapy. Similar studies have been attempted with bovine, ovine and caprine tissue. The use of *in vitro* follicular culture with cryopreserved tissue is preferred to transplantation in post-pubertal human patients because it helps to reduce the risk of reintroducing cancer cells into the patient. Again, the use of DMSO as the cryoprotectant for ovarian tissue gives much higher success rate than the use of glycerol (Lunardi *et al*., 2012); it is hypothesized that the smaller molecular size of the cryoprotectant enables it to permeate the tissue more readily than larger molecules. An alternative method, vitrification of the tissue, also gives better results than slow freezing.

### Fish germplasm

While significant advances have been made in the cryopreservation of mammalian oocytes and embryos the situation is far less advanced in all other vertebrates, including teleost fish. In comparison with mammals, oocytes and embryos of fish are substantially larger in volume (Seki *et al*., 2011) and are characterized by low membrane permeability, resulting in restricted movement of both water and cryoprotectants across cellular membranes during chilling, freezing and thawing (Saragusty & Arav, 2011). Further, the oocytes and embryos of fish typically contain large yolk stores. The behaviour of yolk during cryopreservation differs markedly from that of other embryonic compartments, making cryopreservation very complex (Seki *et al*., 2011). As a consequence of these characteristics, the development of successful and reproducible procedures for the cryopreservation of oocytes and embryo from non-mammalian vertebrates, including fish, is extremely complex and remains one of the main challenges in the field of cryobiology.

## Primordial germ cells (PGCs)

The conservation of both maternal and paternal genetic information is essential for conservation initiatives (germplasm banking). As successful cryopreservation of both oocytes and embryos in teleost fish remains prohibitively difficult, attention has switched to preserving alternative forms of germplasm, notably PGCs, which are the precursors of both oogonia and spermatogonia. PGCs are formed during the early stages of embryonic development and, in fish, these cells undergo active proliferation following migration to the genital ridges. Recent studies have now demonstrated that functional spermatozoa and eggs can be obtained following the transplantation of either PGCs or functionally similar spermatogonial stem cells in a number of fish species (Okutsu *et al*., 2006; Kobayashi *et al*., 2007; Morita *et al*., 2012). For example, thawed PGCs, isolated from adult rainbow trout (*Oncorhynchus mykiss*), when transplanted into the peritoneal cavity of newly hatched embryos differentiated into mature spermatozoa and eggs in the recipient gonads. Furthermore, the donor-derived spermatozoa and eggs obtained from the recipient fish were able to produce normal offspring.

Interest in PGC transplantation techniques increased further after it was demonstrated that PGCs from one species could be successfully transplanted into the embryo of another, in which they proliferate, yielding viable gametes of the donor species. This procedure, termed surrogate reproduction, was first demonstrated with trout PGCs successfully transplanted into triploid masu salmon (*Oncorhynchus masou*) embryos (Okutsu *et al*., 2007). The principle of this reproductive technology is relatively simple. Progenitor cells of eggs and sperm, either PGCs or spermatogonia, are isolated from the target species and transplanted into a closely related recipient species. Upon maturation, donor-derived eggs and sperm are then produced in the recipient (Fig. 1). Surrogate broodstocking has major implications to both aquaculture production and conservation, especially as studies now show that PGCs can be transplanted across species, genus, and even family barriers (Saito *et al*., 2010, Yoshizaki *et al*., 2012). There is now considerable interest in applying surrogate broodstock technologies in the aquaculture of species that are difficult or costly to breed in captivity, such as the sturgeons (family Acipenseridae) and the bluefin tuna (*Thunnus thynnus*). For example, transplantation of germ cells from the valuable bluefin tuna into the earlier maturing and easier to maintain chub mackerel (*Scomber japonicus*) is being viewed as a possible answer to the aquaculture production of this difficult species (Yoshizaki *et al*., 2012). It is envisaged that both conservation and efficient utilization of genetic resources (aquaculture) can now be more efficiently achieved in fish through surrogate production combined with the cryopreservation of PGCs.

Given the current limitations in the ability to freeze fish oocytes and embryos there is increasing interest in cryobanking PGCs and spermatogonia of threatened fish species. Thus PGCs have been successfully transplanted from the critically endangered Chinese sturgeon to a closely related but not endangered species, the Dabrey's sturgeon (Ye *et al*., 2017). While there is still much work to do to develop this

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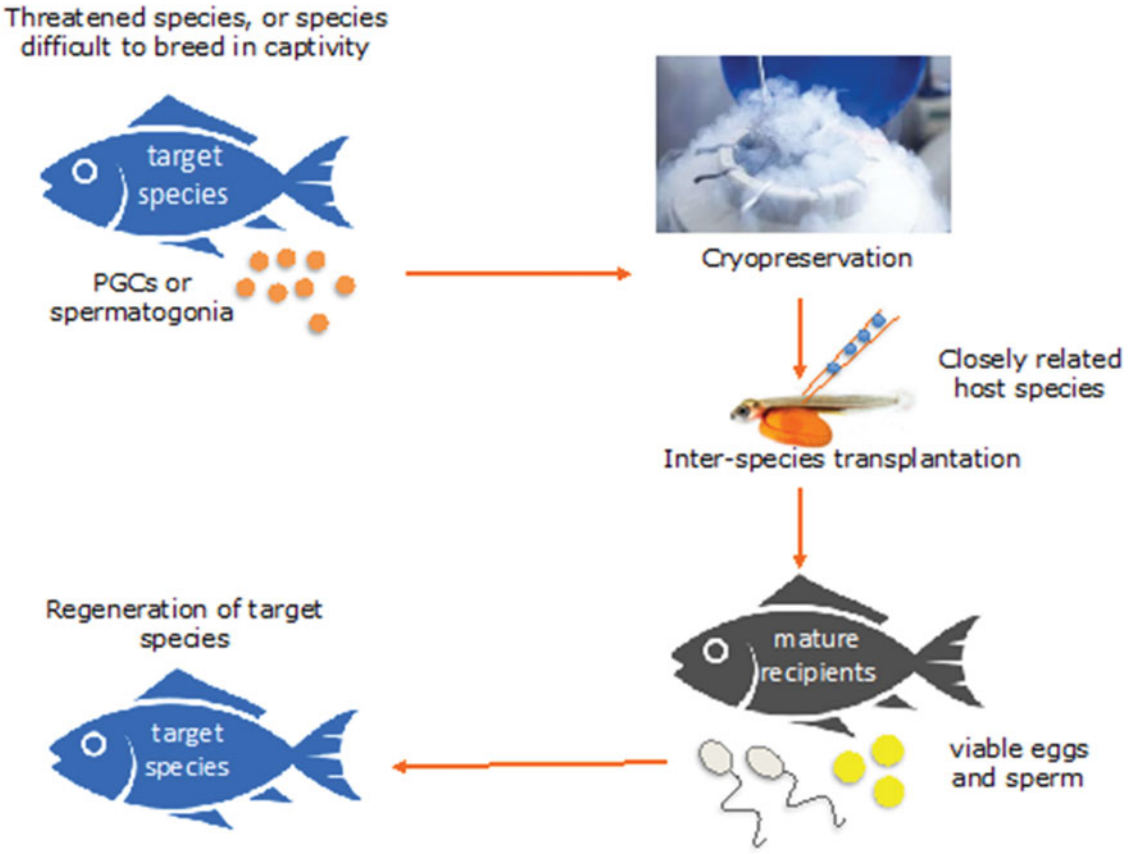


**Figure 1** Principle of surrogate reproduction in fish. Conservation of a threatened species can be achieved by transplanting frozen PGCs or spermatogonia into recipient embryos of a closely related species (adapted from Yoshizaki *et al.*, 2012).

technique, these results show that transplantation of PGCs, and their survival in the recipient, is possible.

While public concern has focused primarily on the conservation of terrestrial species, especially iconic mammalian species, aquatic species including teleost fishes are considered more vulnerable to anthropogenic impacts. Indeed, according to the IUCN Red List of Threatened Species (International Union for Conservation of Nature, 2016), 58 ray-finned fish species (Actinopterygii), as well as one lamprey species, have become extinct in recent years. As such, further advances in the cryopreservation of fish germ cells and reproductive technologies such as surrogate broodstocking will be a major benefit not only to aquaculture, but also for the conservation of threatened fish species (Comizzoli & Holt, 2014). The principle of surrogate reproduction in fish is depicted in Fig. 1.

## Pitfalls with germplasm cryopreservation

Although there are many indications for the use of germplasm banking in livestock production, major technological limitations still exist in its application to all types of germplasm and to all species. Cryopreservation tends to decrease the viability and fertilizing capability of germplasm, and this is more so where pieces of tissue are frozen rather than gametes. In addition, there may be epigenetic changes brought about by the cryoprotective agents and techniques used (Urrego *et al.*, 2014) which cannot be studied effectively until enough offspring are produced from these procedures to provide a representative population. A complicating factor in such studies with rare breeds or endangered species is the lack of suitable breeding animals to provide gametes, or to test the effect of proposed changes in handling procedures on fertility (Woelders *et al.*, 2012).

Species-specific protocols may be needed as existing ones cannot be extrapolated to all other species. Tissue-specific protocols may be needed: for example, cryopreserving epididymal spermatozoa may require a different protocol to ejaculated samples. Cryopreserving embryos allows the full genetic complement of both sire and dam to be preserved whereas sperm freezing preserves only the male

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genetic component, but the transfer of the material to recipient animals may be technically more demanding for embryos than for spermatozoa, depending on the species. In many sheep, for example, it is not possible to pass a catheter through the cervix; while semen can be deposited vaginally, deposition of embryos requires a surgical or laparoscopic approach, which is more technically demanding than vaginal deposition of semen. In addition, awareness of biosecurity has increased considerably over the last few decades; it is now known that not only must the donor animals be free of disease but also that material of animal origin in the cryopreservation medium should be avoided or, when this is impossible, the material should be from specific pathogen-free sources. Furthermore, liquid nitrogen itself may act as a vector for transmitting pathogens (Bielanski & Vajta, 2009). Relying on antibiotics to control bacterial contamination is not a sustainable course of action and may result in many of the stocks of frozen germplasm being unusable in the future if legislation on antimicrobials changes. Use of cryopreserved tissues (as opposed to gametes or embryos) necessitates surgical transplantation of the tissue to the recipient, which is not a trivial procedure.

## Cloning

The cloning of domestic livestock by somatic cell nuclear transfer (SCNT) has been possible for several years but the procedure remains technically demanding and expensive. In this procedure, a donor cell is injected into, and fused with, a mature, good quality, enucleated oocyte, followed by activation and culture of the product. However, there are high rates of pregnancy loss and also abnormal placental development and pathologies during the neonatal period, which is a major limiting factor in the adoption of this technique. As biobanking of somatic cells is increasing around the world, material from rare livestock breeds could be available for SCNT. While the technology is still in its infancy for conservation purposes with low success rates in wild species (Holt, 2008), it is possible that it could provide a means of rescuing rare livestock breeds because of the availability of oocytes and recipient females for the cloned embryos among production animals. Several wild species of cattle and sheep have already been cloned (Ryder & Benirschke, 1997; Critser *et al.*, 2003; Holt *et al.*, 2004). However, it should be noted that many attempts are needed in order to obtain some success; only 1–5% of porcine SCNT embryos develop into piglets (Grupen, 2014). Production of offspring unfortunately does not mean that a healthy adult animal, capable of reproducing, will result (Holt, 2008). Furthermore, the production of a few cloned individuals is unlikely to conserve much genetic diversity; an extensive biobank of material from existing individuals would be needed to ensure that as much as possible of the current gene pool is retained. Such biobanking for all livestock species is likely to require considerable commitment in time and resources.

## Epigenetics

Epigenetic changes can be defined as stable alterations of DNA-associated molecules that may cause gene expression to be altered. This alteration is brought about by changes in methylation that affect gene expression (Jovanovic *et al.*, 2010; Munro *et al.*, 2010). It is now known that factors such as nutrition (under- or over-nutrition) can affect methylation, as can various environmental toxins (Schagdarsurengin & Steger, 2016). It is not known, however, if sperm handling procedures such as preservation or cryopreservation, may have an epigenetic effect. Certainly the more handling procedures to which germplasm is subjected, the more chances there are that an epigenetic effect will occur. Speculation exists of a link between various ART and imprinting disorders, occurring via altered DNA-methylation patterns and histone coding (Urrego *et al.*, 2014).

Epigenetic effects can influence reproductive efficiency through alterations in the viability of embryos, foetuses and the neonate, and also through control of endometrial gene expression which may alter implantation. They may also influence health, especially the occurrence of cancer through regulation of proto oncogenes and suppression of tumour suppressor genes, and phenotypic performance for a variety of functions or traits. Some epigenetic modifications can even be transmitted to subsequent generations (Schagdarsurengin & Steger, 2016).

## Recent advances in sperm handling for livestock species

The spermatozoa of some species, e.g. stallions, survive cryopreservation better if most of the seminal plasma is removed before the cryoprotectant is added (Loomis & Graham, 2008). Further improvements can be seen if living spermatozoa are separated from dead and dying cells prior to freezing (Hoogewijs *et al.*, 2011). Various methods have been advocated for selecting viable spermatozoa and removing them from seminal plasma (Morrell & Rodriguez-Martínez,

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2016) but, to date, only colloid centrifugation of stallion spermatozoa seems to be feasible for field use. These methods select spermatozoa based on certain functional attributes, such as motility or intact plasma or acrosomal membranes and are, therefore, believed to mimic the selection occurring in the female reproductive tract (Suarez, 2007). Some of these techniques will now be described briefly.

In migration techniques, e.g. 'swim-up', motile spermatozoa swim away from the bulk of the sample to an area containing few spermatozoa. Passage through glass wool or Sephadex gel physically impedes spermatozoa with reacted acrosomes, damaged membranes or abnormal morphology. Colloid centrifugation selects motile spermatozoa with intact membranes and also those with mature chromatin from those with immature or damaged chromatin. Magnetic activated cell sorting (MACS) has been used in combination with a marker, such as annexin V, to remove apoptotic spermatozoa from the sample. Annexin V has a high affinity for phosphatidylserine, which is thought to be externalized on the sperm plasma membrane in the early stages of apoptosis. When conjugated to magnetic microspheres, annexin V can bind to, and retain, apoptotic spermatozoa within an affinity column. This selection technique has been reported for processing of human spermatozoa for fertility treatments (reviewed by Gil *et al.*, 2013) but has not been widely adopted for processing of animal spermatozoa, apart from occasional research reports, e.g. for rabbit (Vasicek *et al.*, 2013), bull (Faezah *et al.*, 2014) and Senegalese sole spermatozoa (Valcarce *et al.*, 2016). The method may not be amenable for processing large volumes of semen.

One selection technique that is showing promise in the preparation of spermatozoa for ART is colloid centrifugation. This technique has been available for preparing spermatozoa, mainly human spermatozoa for fertility treatments or bovine spermatozoa for IVF, for at least 20 years but the technique has only been adopted more generally for sperm preparation with the development of single layer centrifugation (SLC) in the last few years (Morrell & Wallgren 2011). The chronology of these techniques is shown in Table 2. The reasons for this late adoption were mainly that the method first described – density gradient centrifugation – was considered to be too impractical for use when preparing large volumes of semen or for ejaculates with a high sperm concentration. The development of a technique using only a single layer of colloid has revolutionised sperm preparation as it is much easier to use and allows the technique to be scaled up to process large volumes of semen (Morrell *et al.*, 2009). This technique was named single layer centrifugation by its developer, one of the authors of the present paper (Morrell & Rodriguez-Martínez, 2011).

Centrifugation of sperm samples through a colloid allows robust spermatozoa i.e. motile spermatozoa with normal morphology, intact plasma membranes and intact chromatin, to be selected from the rest of the ejaculate. The colloid works by presenting a physical barrier to the passage of immotile spermatozoa or those with abnormal morphology and damaged membranes; thus such spermatozoa are prevented from pelleting during centrifugation. Spermatozoa are also separated from seminal plasma and any non-sperm cells in the semen, such as epithelial cells from the lining of the reproductive tract or bacteria contaminating the ejaculate during semen collection. Removal of dead or dying spermatozoa and cellular debris aids survival of living spermatozoa and may also improve sperm cryosurvival in some species (Hoogewijs *et al.*, 2011; Martínez-Alborcia *et al.*, 2013). Various ready-to-use species-specific colloid formulations have been produced; these formulations are adjusted according to the semen characteristics of different species and therefore it is important to use the correct formulation for the species in question.

Apart from improving sperm survival there are other reasons for using colloid centrifugation to prepare sperm samples: if the spermatozoa are to be used for IVF, they should first be separated from seminal plasma which contains decapacitating factors, and from cryoprotectants (Morrell & Rodriguez-Martínez, 2009). Traditionally this separation has been achieved with a technique known as 'swim-up', wherein motile spermatozoa swim away from the mixture of seminal plasma and cryoprotectant into fresh medium. However, this method does not select spermatozoa with normal morphology or with intact chromatin, and usually only 10–20% of the spermatozoa are recovered (Hallap *et al.*, 2004). Colloid centrifugation with Percoll was suggested as a means of selecting the best spermatozoa for IVF as well as separating them from the seminal plasma but lost favour when it was reported that some batches of Percoll were toxic to spermatozoa (Avery & Greve, 1995). A further disadvantage of the original Percoll protocol was that it did not take into account species differences in semen characteristics (physical properties such as osmolarity and pH, as well as chemical composition) that affect the yield of spermatozoa recovered after the centrifugation (Morrell *et al.*, 2011). The SLC technique with our colloid has also been used to separate sturgeon spermatozoa from cryomedium (Y. Horokhovatskyi & B. Dzyuba, unpublished data).

Colloid centrifugation selects sperm with intact chromatin, which can be important in species such as horses and some exotic animals. Spermatozoa with

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**Table 2** Chronology of colloid centrifugation as a sperm preparation technique

| Time | Density gradient centrifugation | Single layer centrifugation |
|---|---|---|
| 1980s | Percoll gradients used to prepare human sperm for IVF (e.g. Bolton & Braude, 1984; Marrs *et al.*, 1988) | |
| Early 1990s | Percoll gradients made from polyvinyl propylene (PVP)-coated silica and various buffers used for spermatozoa of various species e.g. human (Serafini *et al.*, 1990); boar (de Vries & Colenbrander, 1990) | |
| Late 1990s | Used for bull spermatozoa; problem ejaculates improved, normal ejaculates not improved. Not considered to be useful for preparing animal semen for AI but used to prepare sperm samples for IVF | Sharma *et al.* (1997) used only one layer of Percoll for human spermatozoa; problem ejaculates improved, normal ejaculates not improved |
| Late 1990s | Percoll falls into disfavour because of alleged toxicity of PVP to spermatozoa (Avery & Greve, 1995); high endotoxin levels reported occasionally (Mortimer, 2000). Silane-coated silica colloids replace PVP-coated silica for human sperm preparations | |
| Early 2000 | Can reduce bacterial load in human sperm samples (Nicholson *et al.*, 2000) | Percoll used as a single layer for stallion semen; problem ejaculates improved, normal ejaculates not improved |
| 2008 | Attempts to scale-up DGC reportedly not successful (Edmond *et al.*, 2008) | Silane-coated silica colloids used for stallion semen; improvements in sperm quality observed (Morrell *et al.*, 2008a,b); |
| 2009 | Mini-Percoll gradient used for small volumes of semen for IVF (Machado *et al.*, 2009) | Scaled up SLC with silane-coated silica colloids developed for normal bull and boar semen (Morrell *et al.*, 2009) |
| 2011 | | Method scaled up into 500 ml tubes for normal boar semen (Morrell *et al.*, 2011). Bacteria removed from some sperm samples (Morrell & Wallgren, 2011). Virus titre can be reduced considerably (Blomqvist *et al.*, 2011). Improves cryosurvival of stallion spermatozoa (Hoogewijs *et al.*, 2011) |
| 2012, 2013 | | Post-thaw boar sperm quality improved if SLC done prior to freezing |
| 2016 | | Mini-SLC for small volumes of semen gives better results if done in 15 ml tubes compared with 1.5 ml tubes (Abraham *et al.*, 2016) |
| 2017 | | Post-thaw bull sperm quality improved if SLC done prior to freezing |

damaged chromatin are still capable of moving and of fertilizing oocytes, which means that they effectively compete with normal spermatozoa. At some stage after fertilization, however, development is halted and the pregnancy fails. In AI trials, SLC-selected stallion sperm samples produced more pregnancies than their corresponding control samples (Morrell *et al.*, 2014), which may be due, at least in part, to the selection of spermatozoa with intact chromatin. In addition, colloid centrifugation separates spermatozoa from pathogens in the ejaculate, which may enable a reduction in the use of antibiotics (Morrell & Wallgren, 2014). The inclusion of antibiotics in semen extenders represents a non-therapeutic use of antimicrobials, which is not consistent with current policies to reduce antibiotic usage. It has become apparent that using any antibiotics can lead to the development of resistance in bacterial species other than the target species, which is readily transferred to bacterial populations in other animal species, including potentially humans. As such, the application of procedures such as SLC will result in increased biosecurity, and importantly will facilitate the reduction in antibiotic use in trading and use of animal semen.

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## Conclusions and future perspectives

Conservation of genetic diversity in livestock breeds is needed to ensure that there is sufficient genetic flexibility to meet future environmental or husbandry challenges. The most practical way to implement this conservation is by cryopreserving semen and embryos, because functional techniques already exist for these procedures in most livestock species and the thawed material can be transferred to recipient animals by non-invasive means, at least in some species. Further research is needed to develop non-invasive transfer methods for the remaining livestock species. Cryopreservation of oocytes is less common because of the difficulty in obtaining material and in overcoming structural differences between oocytes and embryos. In addition, the development of methods for preserving testicular and ovarian tissue should be continued, especially if the tissue fragments can be used for the *in vitro* generation of gametes in the future rather than being transferred to recipient animals. Ovarian and testicular tissues constitute a wealth of untapped, arrested or developing germ cells, most of which never participate in fertilization. Advances in our ability to preserve gonadal tissues, and to successfully mature early stage oocytes and spermatogonia in culture or by xenografting could provide an unlimited source of germplasm to generate embryos, including from animals that are prepubertal, outside their breeding season, nearing the end of their reproductive lifespan or that die unexpectedly. The use of gonadal tissue as a source of germplasm, for use in both ARTs and genetic resource banking, would be particularly beneficial to non-mammalian vertebrates (fishes, birds, reptiles and amphibians), due to the current lack of success in cryopreserving both oocytes and embryos in these animals groups. Finally, sperm cryosurvival can be enhanced by simple handling procedures such as SLC that not only select the most robust spermatozoa but also may improve the chances of fertilization and pregnancy.

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## Conflict of interest

JMM is the inventor and one of the patent holders of a colloid formulation and the technique of SLC mentioned here.

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