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# Genome Banking for Vertebrates Wildlife Conservation

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## 1. Introduction

About 140 years ago, Charles Darwin wrote in his book *The Descent of Man, and Selection in Relation to Sex* the following prediction: “The slowest breeder of all known animals, namely the elephant, would in a few thousand years stock the whole world.” (Darwin, 1871). Unfortunately, primarily due to human activities, this prediction will probably not come true. Sadly, not only elephants face the risk of extinction. The number of species listed as endangered is on the rise. The Species Survival Commission (SSC) of the International Union for Conservation of Nature and Natural Resources (IUCN) continuously monitors the planet’s fauna and flora and launches the IUCN Red List of Threatened Species (<http://www.iucnredlist.org>). As of the end of 2010, there were 5491 species of mammals described (IUCN, 2010). Of these, 1,131 species (21%) are now listed as endangered to some degree. In addition, there are 324 species listed as near threatened and another 836 species for which data is deficient and thus could be at risk. Adding all these numbers together, about 42% of the planet’s mammalian species are at some level of threat for extinction. The list also reports on 76 species (1.4%) of mammals that became extinct in recent years and two more species that are extinct in the wild and whose survival completely depend on *ex situ* breeding programs. The situation is not distinctively different in other classes of the vertebrata subphylum or in the other subphylums of the animal kingdom. If anything, it is even worse for some such as the reptiles (21% endangered), amphibians (30%), fish (21%) or among the invertebrates: insects (22%), mollusks (41%), crustaceans (28%), anthozoa (corals and sea anemones; 27%) or arachnids (58%). With each extinct species, the stability of the entire ecological system surrounding it and the food chain of which it is an integral part is shaken. Such shaking may lead to the co-extinction of dependent species (Koh et al., 2004).

In 1992 the Convention on Biological Diversity (CBD) was ratified at the United Nations Conference on Environment and Development in Rio de Janeiro. Ten years later, during the 6<sup>th</sup> meeting of the Conference of the Parties to the CBD in 2002, it was agreed “to achieve by 2010 a significant reduction of the current rate of biodiversity loss at global, regional and national level as a contribution to poverty alleviation and to the benefit of all life on Earth” (Convention on Biological Diversity, 2002). However, 2010, which was named by the United Nations as the “Year of Biodiversity”, has arrived and gone and this target not only has not

been met, even some of the indicators needed to measure progress (or regress) have not yet been developed or fully implemented (Walpole et al., 2009).

Based on paleontological data, of the total biota of about 10 million species, the natural or background extinction rate is approximately 1 to 10 species per year (Reid & Miller, 1989). This may be divided into species with restricted ranges for which extinction rate might be higher and those with widespread ranges for which it is considerably lower (Pimm et al., 1995). The expected extinction rate amongst all bird and mammal species is about one species every 100 to 1,000 years, yet the current extinction rate for these and other groups is about one species per year, which is 100 to 1,000 times the natural rate by some estimates (Reid & Miller, 1989; Pimm et al., 1995; Ceballos & Ehrlich, 2002; IUCN, 2004; Living Planet Report, 2008) and even as high as 10,000 times by others (Mace et al., 2005). One of the major problems behind these predictions is that we do not really know how many species are there and this is primarily true for many understudied taxonomic groups (e.g. bacteria, marine invertebrates, insects) and endemic species in many parts of the world, which by them being endemic to limited habitats face much higher risk of extinction (Pimm et al., 1995; Hunter, 2011). Earth history has witnessed 5 major events of mass extinctions in which a significant fraction of the diversity in a wide range of taxa went extinct within relatively short period (Erwin, 2001). The last, and probably the most well known episode, took place during the late Cretaceous era, approximately 65 million years ago, when the dinosaurs became extinct. The current dramatically accelerated rate of species extinction has been likened to these events and was termed 'the sixth mass extinction event in the history of life on Earth' (Chapin et al., 2000; Wake & Vredenburg, 2008). Various studies have demonstrated the severity of this accelerated extinction process on both the population level (Ceballos & Ehrlich, 2002) and the global biodiversity level (Living Planet Report, 2008; Rockstrom et al., 2009). Not all researchers agree with the definition of mass extinction (Barnosky et al., 2011) but all agree that the current extinction rate is far too fast. Whether we call it mass extinction or not, the cause for the current accelerated extinction rate is anthropogenic in essence, resulting from six major human interference categories: (i) habitat loss or fragmentation, (ii) over exploitation, (iii) species introduction (exotic species and diseases), (iv) pollution of water, soil and air, (v) global warming, and (vi) increasing atmospheric carbon dioxide level and the consequential acidification of the oceans. Based on different projections such as climate change, human population growth or deforestation rate, predictions suggest that large chunks of the world's biodiversity is destined to disappear (Reid & Miller, 1989; Ehrlich & Wilson, 1991; Thomas et al., 2004). For the sake of the entire ecosystem stability and for our and future generations' well being, and because we are the leading driver behind this accelerated decline in biodiversity, it is our obligation to try and slow down the current extinction rate. This, however, is not going to happen overnight, probably not even over a single generation time. So as to "buy time", the establishment of genome resource banks (GRB), which will store and manage collections of gametes (sperm and oocytes), embryos, tissues and organs of endangered species, has been proposed (Veprintsev & Rott, 1979; Benirschke, 1984; Wildt, 1992). By gathering such collections, at the moment primarily through cryopreservation, these institutions, among other services, fulfill their function as a mean to extend the reproductive lifespan of individuals beyond their biological life and prevent the loss of valuable individuals to the gene pool. Several such GRBs are already in existence. These include for example the

Frozen Ark Consortium (<http://www.frozenark.org/>; Clarke, 2009), the Amphibian Ark (<http://www.amphibianark.org/>) and the Biological Resource Bank of Southern Africa's Wildlife (Bartels & Kotze, 2006). Long-term preservation of such biological material is almost entirely a matter of how water therein is dealt with. Plant seeds, whose water content is very low, can easily be preserved at relatively high subzero temperatures of  $-20^{\circ}\text{C}$  to  $-30^{\circ}\text{C}$  (Ruttimann, 2006) whereas water content in animal tissues and cells is generally very high, in the range of 80%, thus requiring special handling.

About 70 years ago, the late Ernst W. Mayr coined one of the currently leading definitions for a species: "Species are groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups" (de Queiroz, 2005). The uniqueness of reproduction is thus central to the definition of species. It is therefore only natural that we find a wide variety of unique reproductive traits across species. These variations can come in a range of different forms, be it the anatomy of the genital system, morphology of gametes, presence or absence of accessory glands, mechanisms of ovulation, variations in the active hormones, duration of reproductive cycle and gestation and many other aspects of reproductive biology. It is also not surprising that great differences between species are found when it comes to the reaction of their gametes, embryos and tissues to the process of cryopreservation. Thus, when a successful cryopreservation protocol has been devised for a certain species, it will not necessarily be successful in other members of the same family, not to mention species that are phylogenetically further apart. In addition, to date the process of cryopreservation and the mechanisms that cause chilling- and cryodamages are not fully understood. Each new species we approach is thus a *terra incognita* and should be thoroughly studied before a successful protocol can be developed, if at all. Model species are often used for the development of basic techniques but in the end vast experimentation should be conducted in the target species. While this is relatively simple in domestic and laboratory animals, when a rare and endangered species is the target, opportunities to obtain gametes and other relevant cells and tissues are rare and far apart in terms of time and space, making progress extremely slow or practically impossible.

## 2. The male

The male's gametes are produced in very large numbers and are relatively easy to obtain. A wide variety of collection methods have been devised, including: 1) post-coital vaginal collection, either directly (e.g. O'Brien & Roth, 2000) or with the aid of intra-vaginal condom or vaginal sponge (e.g. Bravo et al., 2000), 2) artificial vagina (e.g. Gastal et al., 1996; Asher et al., 2000), 3) manual stimulation of either the rectum (e.g. Schmitt & Hildebrandt, 1998; Schmitt & Hildebrandt, 2000), the abdomen (Burrows & Quinn, 1937), or through stimulation of the penis (e.g. Schneiders et al., 2004; Melville et al., 2008), 4) electroejaculation (e.g. Hermes et al., 2009b), 5) pharmacologically-induced ejaculation by oral imipramine and intravenous xylazine (McDonnell, 2001) or through urethral catheterization after medetomidine administration (Zambelli et al., 2008), 6) aspiration from the cauda epididymis (e.g. Moghadam et al., 2005), and 7) semen retrieval from the cauda epididymis and proximal portion of the vas deference following castration or post mortem (e.g. Jewgenow et al., 1997; Saragusty et al., 2006; Keeley et al., 2011). Whereas techniques one to three above are relatively close to natural ejaculation, they require easy access to the

animal and excessive training (e.g. Robeck & O'Brien, 2004) and are thus limited to only a handful of species and individuals. The pharmacological techniques (5) and aspiration from the epididymis (6) are too invasive to be frequently used, and extraction from the epididymis (7) is a one-time technique, which is often used as a gamete rescue procedure. Epididymal sperm extraction and preservation is a well-documented collection technique. Probably the main advantage of this method is that it enables us to collect sperm post mortem and, if stored, it can be used to extend the reproductive "life span" of that individual. When dealing with endangered species, this may enable us to preserve the spermatozoa of wild and genetically valuable captive males who die in an accident or otherwise. The spermatozoa accumulated in the cauda epididymis is already mature and fertile (Foote, 2000) making it a useful source. Several methods were described as to how to extract the sperm out of the cauda epididymis. These include squeezing the cauda epididymis (Krzywinski, 1981), making cuts in the cauda epididymis (Krzywinski, 1981; Hishinuma et al., 2003; Martinez-Pastor et al., 2006; Saragusty et al., 2006), cutting and squeezing (Quinn & White, 1967), extrusion by air pressure (Kikuchi et al., 1998; Ikeda et al., 2002) and flushing the vas deferens (Martinez-Pastor et al., 2006). Flushing the vas deferens, when compared with the cutting method (Martinez-Pastor et al., 2006), was showed to be superior, yet it seems to be less suitable for field work. For epididymal sperm extraction, spermatozoa stored chilled within the epididymis seem to survive better and for longer periods than those stored in an extender (Ringleb et al., 2011). Still, for *in vivo* sperm collection, electroejaculation became by far the most frequently used method in wildlife species. To be successful, one would need a suitable probe, which often needs to be specifically designed for the animal to be collected based on preliminary knowledge of its anatomy (Hildebrandt et al., 2000; Roth et al., 2005). Even so, ejaculates often come with urine contamination (e.g. Anel et al., 2008) or they may come with or without the relevant secretions from all accessory glands. In elephants this is manifested by occasional ejaculates with very sticky consistency indicating that high level of secretions from the bulbourethral gland are present (personal observation) whereas in rhinoceros it is manifested by high viscosity of the ejaculate (Behr et al., 2009b). In rhinoceros, measuring alkaline phosphatase in the ejaculate was suggested as a mean to identify true ejaculates (Roth et al., 2010). Despite its wide use and success in many species, there is one major drawback to electroejaculation that limits its use on a frequent basis. To conduct electroejaculation, the animal needs to be anesthetized, something many zoos would rather avoid when possible. The need to anesthetize the animal makes it impossible to collect from the same individual on a regular, frequent basis. Anesthesia may also affect the collection procedure (Santiago-Moreno et al., 2010) and the quality of the collected sample (Campion et al., 2011). One should also keep in mind that the collection technique itself may effect the composition of the ejaculate and therefore its quality (Christensen et al., 2011). Thus, the development of preservation protocols for wildlife species progress slowly and often rely on relatively small number of individuals, repeats, and/or ejaculates.

Sperm evaluation also requires understanding of the species under study as sperm competition, for instance, is a major driver behind the wide variety of sperm traits, morphologies and behaviors found in nature (Tourmente et al., 2011). In primates, semen can be as thick as paste, which requires liquefaction and extraction of the cells into a diluent (e.g. Oliveira et al., 2010). In camelids, possibly due to the absence of vesicular glands,



sperm is also fairly viscous but it can be enzymatically liquefied (Bravo et al., 2000). Similar enzymatic liquefaction was also helpful when attempting to separate rhinoceros sperm from the seminal plasma, something that cannot be done efficiently with centrifugation alone in some of the ejaculates (Behr et al., 2009b). The volume and concentration also vary by several orders of magnitude among species. In the naked mole rat (*Heterocephalus glaber*) only 5 to 10  $\mu\text{L}$  of sperm can be collected with cells in the hundreds to thousands at the most, many of which are morphologically abnormal (unpublished data). In the European brown hare (*Lepus europaeus*) or the Asiatic black bear (*Ursus thibetanus*), volume of semen collected by electroejaculation is often in the range of 1 mL or less with concentrations that at times can exceed  $10^9$  cells/mL (personal observations; Chen et al., 2007). Low volume of up to a few mL and low concentration of few millions per mL is often the case in felids both in captivity and in the wild (Barone et al., 1994; Morato et al., 2001). In the pygmy hippopotamus (*Choeropsis liberiensis*) the sperm-rich fraction can be extremely concentrated. In one case we found as much as  $9.85 \times 10^9$  spermatozoa per mL (Saragusty et al., 2010a). In some other animals volumes can be very large. In boar, donkey or elephant semen can exceed 100 mL with concentrations of several hundred million cells per mL (unpublished data and e.g. Saragusty et al., 2009e; Contri et al., 2010). Initial motility is expected to be low in sperm collected from the epididymis, as epididymal sperm is immotile in most mammals. This is likely to change after a short incubation time in a suitable media. As many of the cells in the epididymis did not complete their maturation process at the time of extraction, cytoplasmic droplets can be highly prevalent (Saragusty et al., 2010b). Some specific characteristics were also noted in certain species. For example the seminal plasma pH of the black flying fox (*Pteropus alecto*) or the snow leopard (*Panthera uncial*) is high (8.2 and 8.4, respectively) (Roth et al., 1996; Melville et al., 2008) or in the Asian elephant (*Elephas maximus*) osmolarity of the seminal plasma is low, at around 270 mOsm/kg (Saragusty et al., 2009e). Such characteristics demonstrate the need to verify multiple aspects of the semen so that suitable diluents can be made. One should always keep in mind that when dealing with endangered species, many were pushed into a bottleneck situation, resulting in highly inbred populations. Inbreeding comes with a very high price with respect to the soundness of the reproductive system. This can be manifested in sperm quality (Roldan et al., 1998; Gomendio et al., 2000; Ruiz-Lopez et al., 2010) and in the outcome in term of litter size and survival (Rabon & Waddell, 2010). Once proper sample of sufficiently good quality is in hand, there are several options for its preservation.

## 2.1 Semen freezing

Probably the most popular preservation technique is slow freezing of semen or the cells therein. Spermatozoa are generally small in size and thus have low surface to volume ratio, an important factor in cryopreservation, which influences the movement of cryoprotectants and water in and out of the cells. They also have highly condensed and thus stable nucleus and little cytoplasm, making them relatively easy to freeze. Although problems are still numerous and even after more than 60 years of extensive research, propelled primarily by that related to human infertility and livestock and laboratory animal production, our knowledge about the exact mechanisms that eventually lead to success, failure or anywhere in-between is still very limited (Saragusty et al., 2009a). Thus, much of the progress in this field has been primarily empirical in nature (e.g. Saragusty et al., 2009e). Evolution made

each species unique in many respects, one of which is the sperm that comes in different shapes, sizes, membrane composition, and sensitivity to chilling, osmotic pressure, pH and more. This means that any new species is an enigma and the specific characteristics of its spermatozoa and seminal plasma and their interaction with various components of freezing extenders and stages of the freezing and thawing process should all be verified. While this can be done relatively easy in domestic and laboratory animals where samples are ample and easy to get, conducting such studies in endangered species is very difficult. Opportunities to obtain samples are rare and often far apart in terms of time and space. Such samples are thus very valuable and using them for experiments rather than for banking would be a waste of important genetic material. Still, to date, semen from probably upward of 200 species from all five major classes of the Vertebrata subphylum (mammals, birds, fish, reptiles and amphibians) have been cryopreserved. When approaching a new species, several hurdles must be overcome before a successful cryopreservation protocol can be developed. The first step is to determine the specific characteristics of its spermatozoa and seminal plasma mentioned above. The next step would be to determine the composition of the freezing extender. Sensitivity to chilling-, freezing- and thawing-associated damages and cryoprotectant-associated toxic or osmotic damages is species-specific and often even individual-specific (Thurston et al., 2002). Similarly, sensitivity to various aspects of sperm handling in preparation for cryopreservation should also be taken into account. In some species it is better to remove the seminal plasma by centrifugation before freezing [e.g. goat, boar, elephant (Saragusty et al., 2009e; unpublished data)] while in others this is not required [e.g. hare or cattle (Hildebrandt et al., 2009; Saragusty et al., 2009c)]. When the seminal plasma is removed, at times adding at least some back after thawing is needed to facilitate fertilization [e.g. camels (Pan et al., 2001)]. Centrifugation is also used for selection of live, morphologically normal cells. When doing so, one needs to understand the basic species-specific sperm characteristics. For example, in opossum (*Monodelphis domestica*) sperm tend to team into pairs to enhance swimming speed (Moore & Taggart, 1995) or in deer mice (genus *Peromyscus*) sperm form large aggregates (Fisher & Hoekstra, 2010). One should also keep in mind that the fast forward moving population is not necessarily the right one to choose because in some species the slow and steady ones are the cells to eventually win the race (Dziminski et al., 2009). Some species are highly sensitive to glycerol (e.g. mice, boar) while others require concentrations as high as 28% for freezing to be successful, with even higher concentrations to maintain high DNA integrity (e.g. in marsupials: Johnston et al., 1993; Czarny et al., 2009a). In some cases insemination can be done with the thawed sample [e.g. cattle, rhinoceros (Hermes et al., 2009b)] while in others the glycerol should be removed or else fertilization does not occur (e.g. Poitou donkey: Trimeche et al., 1998). So, on the way to developing a successful cryopreservation protocol, species-specific characteristics should be identified and techniques to protect the cells from all these damaging mechanisms should be devised (Zeron et al., 2002; Saragusty et al., 2005; Pribenszky et al., 2006; Saragusty et al., 2009b; Pribenszky & Vajta, 2010). Several cryopreservation techniques were described. These can be divided into field-friendly and -unfriendly ones. The field-friendly techniques include the pellet method [placing a sample drop of ~200  $\mu$ L directly on carbon dioxide ice ("dry ice")] (Gibson & Graham, 1969), the dry-shipping container technique (Roth et al., 1999), freezing in cold ethanol (Saroff & Mixner, 1955) or in liquid nitrogen vapor (Sherman, 1963; Roussel et al., 1964). The last two

being a bit less field-friendly as they require bringing the cold ethanol or liquid nitrogen to the site of work. Still, freezing in liquid nitrogen vapor is currently the most popular one amongst the low-tech, equipment-free freezing techniques. The more sophisticated and more laboratory-bound techniques include the controlled-rate freezing machines (Landa & Almquist, 1979) and the directional freezing machine (Arav, 1999; O'Brien & Robeck, 2006; Si et al., 2006; Saragusty et al., 2007; Reid et al., 2009). When the initial sample is of very poor quality or with very small number of cells, small cell-number or single cell cryopreservation techniques may become useful. Starting in the late 1990's (Cohen & Garrisi, 1997; Cohen et al., 1997), reports on several single sperm cryopreservation techniques showed up in the scientific literature (Walmsley et al., 1998; Gil-Salom et al., 2000; Gvakharia & Adamson, 2001; Just et al., 2004; Herrler et al., 2006; Isaev et al., 2007; Koscinski et al., 2007; Woods et al., 2010). Using these various techniques, researchers reported a wide range of outcomes and recovery efficiency. Time will tell which of these technologies, or others that are currently under development, will emerge as the leading technique that will gain a foothold in sperm banks. Naturally, when sperm banking is considered, single sperm cryopreservation is an option to be considered only if all other possibilities were exhausted. When banking sperm from wildlife, the aim is to bank large number of cells from large number of individuals to ensure availability and variability.

About three decades ago, the thus far only technique that has reached commercial level made it possible to sort sperm according to the sex chromosome they carry (Johnson et al., 1987a; Johnson et al., 1987b). This technique has been tested in various wildlife species such as elephants, rhinoceros, dolphins and non-human primates (O'Brien et al., 2004; O'Brien & Robeck, 2006; Behr et al., 2009a; Behr et al., 2009b; Hermes et al., 2009a). Sperm sorting machines, however, are very expensive, scarce and usually situated far away from where the sperm donor and recipient are located. For this, the double freezing technique has been developed (Arav et al., 2002; Hollinshead et al., 2004; Maxwell et al., 2007; Saragusty et al., 2009c; Montano et al., 2010). This technique allows collection and cryopreservation of sperm sample near the donor, transportation of the frozen sample to the sorting center, thawing it for sorting and then freezing the sorted sample for transportation to the recipient. In this respect the advantage of large volume freezing at the sperm donor site is clear.

Although advances were made over the six decades of sperm cryopreservation history, the basic model that will predict behavior of spermatozoa during cryopreservation is still to be devised. Current knowledge is lacking in many respects and thus, when approaching a new species, much empirical work, often based on trial and error, should be conducted. Thanks to the large number of cells in each ejaculate, these can be split into several treatment modalities, thus speeding up the freezing protocol development process. Once better understanding is attained, and predictions can be made for sperm behavior under various freezing-associated conditions, probably the right course to be taken will be tailor-made, individual-based cryopreservation. This will help overcoming considerable differences between males in response to cryopreservation (Thurston et al., 2002; Saragusty et al., 2007; Loomis & Graham, 2008). However, despite all hurdles, and certainly since ICSI made sperm motility and membrane integrity obsolete, sperm banking under liquid nitrogen is probably the most widely used technique in gametes and tissue banking for reproduction preservation. Success in post-thaw survival, and often also in offspring production, has been



demonstrated in many vertebrate species. And yet, there are other options to preserve male fertility.

## 2.2 Semen vitrification

Ice crystals, both outside and even more so – inside, can be very damaging to any frozen cell or tissue. To avoid ice formation and to minimize the pre-freezing chilling damages, vitrification can be used. Vitrification, also known as ice-free cryopreservation, is a process in which liquid is transformed into an amorphous, glass-like solid, free of any crystalline structures (Luyet, 1937). A major advantage of vitrification over slow freezing is its low-tech, low cost, simple to use, suitable for the field character. For vitrification to be successful, however, much experience in sample handling before cooling and after warming and in loading the sample into or onto the carrier system, are needed. Probability of vitrification depends on the interaction between three factors – cooling rate, sample volume and its viscosity, according to the following general relationships (Saragusty & Arav, 2011):

$$\text{Probability of Vitrification} = \frac{\text{Cooling rate} \times \text{Viscosity}}{\text{Volume}} \quad (1)$$

Thus, to achieve the state of vitrification, very high viscosity (usually attained through high concentrations of cryoprotectants or low water content), and/or very high cooling rates and/or very small volumes are needed. Since the high cryoprotectant concentrations needed are beyond what spermatozoa from most species can tolerate, the vitrified volume is usually being considerably reduced and techniques to achieve high cooling rate with adequate heat transfer throughout the sample are devised. For example, using the cryo-loop vitrification technique, it was calculated that cooling rate as high as 720,000°C/min has been achieved (Isachenko et al., 2004) or with quartz capillaries, cooling rates of around 250,000°C/min were reported (Risco et al., 2007; Lee et al., 2010). The technique, though, have several major drawbacks when sperm banking is considered: 1) The small volume that can be vitrified (at best, presently only a few microliters of semen suspended in vitrification solution) is way too small for banking for species conservation purposes and vitrifying large number of samples from each individual is not practical. 2) The small volume, and thus the small sperm number, make vitrified samples impractical for use in artificial insemination or even in standard IVF. Its optimal utilization is through ICSI, a technique that requires specialized equipment and expertise not available in most laboratories dealing with wildlife, and ICSI has not yet been developed for most species. 3) The risk of contamination through the liquid nitrogen prevails in many of the currently available vitrification carrier devices, which are open systems. 4) High permeable cryoprotectant concentrations (up to 50% compared to 3-7% in slow freezing in most species) are still needed in many of the vitrification protocols despite the reduction in volume. Such concentrations are both toxic and cause osmotic damages to the cells. To overcome this, permeating cryoprotectant-free vitrification techniques were developed through a sizable increase in cooling rate (Nawroth et al., 2002; Isachenko et al., 2003; Merino et al., 2011). Sperm vitrified this way can maintain motility (Isachenko et al., 2004) and resulted recently in human live birth (Sanchez et al., 2011).

### 2.3 Sperm drying

Storage of cryopreserved samples under liquid nitrogen is very demanding in terms of maintenance, storage space, storage equipment, specially trained personnel and associated costs. Resulting from the need for constant liquid nitrogen supply in large quantities, such storage facilities have very high carbon footprint. The possibility of discontinuation of the liquid nitrogen supply due to human (e.g. conflict, strike) or natural (e.g. earthquake, hurricane) put these facilities at a constant risk. An alternative that would minimize all these is the dry storage. Drying of cells can be done by either freeze-drying or convective-drying. Freeze-drying is achieved by sublimation of the ice after freezing the sample to subzero temperatures. Convective drying, on the other hand, is achieved by placing the sample in a vacuum oven at ambient temperatures. Sperm drying is, however, damaging to cellular membrane and rehydrated cells are often devoid of biological activity, motility and viability. Some degree of chromosomal damage may also take place due to endogenous nucleases. Attempts to freeze-dry spermatozoa were first reported about six decades ago on animal (Polge et al., 1949; Sherman, 1957; Yushchenko, 1957; Meryman & Kafig, 1959) and human (Sherman, 1954) sperm. Most researchers, however, consider all these early reports, dubious. The definitive proof that freeze-dried spermatozoa retain genetic integrity was established only when microsurgical procedures for bypassing the lack of motility of freeze-dried spermatozoa were developed, and normal mice were produced by intracytoplasmic sperm injection (ICSI) of freeze-dried sperm (Wakayama & Yanagimachi, 1998). To date, embryonic development after ICSI with freeze-dried sperm heads has been reported in humans (Katayose et al., 1992; Kusakabe et al., 2008), hamster (Katayose et al., 1992), cattle (Keskinetepe et al., 2002; Martins et al., 2007), pigs (Kwon et al., 2004), rhesus macaque (Sanchez-Partida et al., 2008), cats (Moisan et al., 2005; Ringleb et al., 2011) and fish (Poleo et al., 2005), and live offspring were reported in mice (Wakayama & Yanagimachi, 1998; Kaneko et al., 2003; Ward et al., 2003), rabbits (Yushchenko, 1957; Liu et al., 2004), rat (Hirabayashi et al., 2005; Hochi et al., 2008), fish (Poleo et al., 2005) and horses (Choi et al., 2011). Storage at room temperature would be ideal, and at least for mid-range duration it appear to be fine (3 years storage of somatic cells; Loi et al., 2008a). High-temperature storage, however, might be damaging to DNA integrity according to some (Kaneko & Nakagata, 2005; Hochi et al., 2008) but not all (Li et al., 2007; Klooster et al., 2011) researchers. These differences may be due to differences in the drying technique or related to differences between species (Li et al., 2007; Klooster et al., 2011; Kusakabe & Tateno, 2011).

While there are many reports on freeze-drying of sperm and other relevant cells, those on convective drying are scarce. Some researchers, however, consider convective drying to be the better option for the fact that it does not involve the freezing step, thus avoiding freezing-associated damages. This technique has been used to dry fibroblasts, and spermatogonial and hematopoietic stem cells (Katkov et al., 2006; Meyers, 2006).

Regardless of the drying process used, for now sperm drying will usually be placed way behind sperm freezing or vitrification because of the loss of motility and viability, and the need for ICSI. Thus, sperm drying is still to be demonstrated in true wildlife species.

### 2.4 Liquid phase semen short- to mid-term storage

In many cases, sperm can be collected in the field, away from any fully equipped andrology and cryobiology laboratory or a source for liquid nitrogen, and transferring the samples to a

facility for processing may take time. For such cases, or when the sample is destined to be used but not immediately, short- and mid-term supra-zero preservation techniques may help. Nature regularly preserves sperm for months to years in a wide variety of species including members of all vertebrate classes (Holt & Lloyd, 2010; Holt, 2011). The location nature has elected is within the female's reproductive tract. This ability has been described in many species and has been investigated in a few. To date the mechanism has not been discovered although a possible direction has recently emerged. In the greater Asiatic yellow bat (*Scotophilus heathii*), with a regular gap of several months between mating and fertilization, it was shown recently that sperm storage is regulated by androgens (Roy & Krishna, 2011). Administration of flutamine, an androgen antagonist, resulted in loss of sperm storage ability in treated females. It was also suggested that sperm storage duration and survival is the outcome of interplay between expression of B-cell lymphoma 2 (Bcl-2) – an anti-apoptotic factor, and caspase-3 – a promoter of apoptosis. In the absence, as yet, of clear knowledge on how nature does it, *in vitro* techniques were devised in an attempt to achieve this long-term fresh storage goal. In some species, such as the pig, chilled storage is the most widespread method of preservation as thus far sperm cryopreservation has provided only mediocre post-thaw results. When planning on extended chilled storage, several sperm energy-metabolism aspects should be taken into consideration. Both glycolysis and the Krebs cycle play an important role in sperm energy metabolism. Sperm from various species stored in a range of solutions, osmolarities and storage temperatures, were shown to be functional when inject into oocytes after storage of weeks to several months (Kanno et al., 1998; Van Thuan et al., 2005; Riel et al., 2007; Riel et al., 2011). An alternative is to simply leave the spermatozoa inside the epididymides and keep these at 4°C. This epididymal preservation option was demonstrated to produce good results in dogs (Yu & Leibo, 2002), bovine (Martins et al., 2009), gazelles (Saragusty et al., 2006), ram (Tamayo-Canul et al., 2011) and many other species. Short-term epididymal preservation has many advantages when dealing with wildlife. Animals usually have the “tendency” to die at inconvenient time or location. The ability to preserve spermatozoa within the epididymis, till it is transported to a laboratory for processing, helps us buy time for rescue procedures. This can easily be done by non-experts (zoo or park employees for example) by simply cutting off the testicles, putting them in 0.9% saline and keeping them in the refrigerator. Motility preservation for several days can also be done with ejaculated sperm in egg yolk based extenders. For instance, we have recently showed that pygmy hippopotamus (*Choeropsis liberiensis*) spermatozoa preserved some motility for 3 weeks when suspended in the Berliner Cryomedium basic solution (a TEST-egg yolk based extender) (Saragusty et al., 2010a) or, in humans, sperm suspended in PBS supplemented with salts, BSA, antibiotics and glucose had about 15% motility and over 40% viability after 10 days at room temperature (Amaral et al., 2011). During such storage, the reduced metabolism and biological activity, the disintegration of dead cells or the presence of leukocytes in the suspension, all result in the release of reactive oxygen species (ROS) and other damaging components into the solution (Whittington & Ford, 1999). Removal of the leukocytes and periodic exchange of solution should thus be beneficial to the stored cells and extend their life.

## 2.5 Preservation of other male reproductive-related cells

Spermatozoa, however, can only be potentially retrieved from adult, relatively healthy, individuals but not from sick, azoospermic, or prepubertal ones, and often these carry valuable genetic material that, if not preserved, will be lost for the population. Thanks to

ICSI, even early developmental stages such as elongating or elongated spermatids can be utilized for fertilization. Such cells as testicular spermatozoa and earlier developmental stages can be extracted using testicular sperm extraction (TESE) techniques, and then used through ICSI to fertilize oocytes (Schoysman et al., 1993; Devroey et al., 1995; Kimura & Yanagimachi, 1995; Hewitson et al., 2002). These early-stage cells can be used fresh but they can also be cryopreserved and used at a later stage when needed (Hirabayashi et al., 2008).

Cells of even an earlier developmental stage than the spermatocytes and spermatids are the spermatogonium or spermatogonial stem cells, which can be collected from any male, including infants and juveniles. Infant mortality rate is known to be relatively high in many populations (e.g. Howell-Stephens et al., 2009; Saragusty et al., 2009d) so methods to preserve germ cells from valuable individuals is certainly called for. Spermatogonial stem cells transplantation was first reported in mice (Brinster & Zimmermann, 1994) when it was demonstrated that such transplantation can lead to spermatogenesis. The transplantation technique was later extended to other species such as pigs (Honaramooz et al., 2002), bovine (Izadyar et al., 2003), goats (Honaramooz et al., 2003a; Honaramooz et al., 2003b), cynomolgus monkeys (Schlatt et al., 2002a), and recently to felids as well (Silva et al., 2011). Xenogeneic transplantation, usually from other mammals to nude, immune-deficient mice, has also been reported. However, the further apart (phylogenetically) the donor and recipient species are, the more difficult it becomes. Using this technique, isolated donor testis cells are infused into the seminiferous tubules of the recipient whose testes have been depleted of all germ cells (by irradiation or chemotherapy). The spermatogonial stem cells establish themselves in the testis and through spermatogenesis, produce spermatozoa carrying the donor genetic material. In 2006 the proof that such xenotransplanted cells can actually produce normal, functioning spermatozoa was reported (Shinohara et al., 2006). In their study, spermatogonial stem cells collected from immature rats were transplanted into chemically sterilized mice and the spermatozoa or spermatids collected from the recipient mice produced normal, fertile rat offspring, both when freshly used and following cryopreservation. The donor stem cells can also be grown in culture to generate more cells for transplantation (Nagano et al., 1998) and they can be cryopreserved for future use (Avarbock et al., 1996). Under very complex *in vitro* culture conditions, and with very low efficiency, morphologically normal and even motile spermatozoa were generated from spermatogonial stem cells (Feng et al., 2002; Hong et al., 2004; Stukenborg et al., 2009).

## 2.6 Testicular tissue cryopreservation

Tissue cryopreservation is more complex than cellular preservation because tissue is composed of more than one cell type and thus of different water and cryoprotectant permeability coefficient values and different sensitivities to chilling and osmotic challenges. Tissue is also larger in volume and thus cryoprotectant penetration is difficult and heat transfer is not uniform, putting the center of the sample at greater risk of intracellular ice formation and death. This is true for testicular tissue, ovarian tissues and many other types of tissues and whole organs. Testicular tissue preservation can be done in one of three basic forms. The tissue can be cryopreserved for future use, it can be cultured *in vitro* for short to mid-term preservation or it can be transplanted. When preserved in the cryopreserved form, one can freeze the whole organ or even the entire animal. Recently it was demonstrated that



spermatozoa or spermatids retrieved from reproductive tissues (whole testes or epididymides) frozen for up to one year at  $-80^{\circ}\text{C}$  or from whole mice frozen at  $-20^{\circ}\text{C}$  for up to 15 years, can produce normal offspring when used, through ICSI, to fertilize mature oocytes (Ogonuki et al., 2006). This success followed a previous, failed, attempt to cryopreserve the entire testis (Yin et al., 2003). The other option is to cryopreserve testicular tissue slices. This technique is widely used today in both adult and pediatric human medicine as a mean to preserve fertility of patients undergoing cancer treatments. To cryopreserve the tissue, it is cut into tiny pieces, usually in the range of  $1\text{--}2\text{ mm}^3$  to ensure cryoprotectant penetration, efficient heat transfer and eventual successful grafting. Other alternatives that have been proposed are to mince the tissue and then suspend it in freezing extender to achieve better cryoprotection (Crabbe et al., 1999) or to cut the testicular tissue into thin stripes (e.g.  $\sim 9 \times 5 \times 1\text{ mm}$  in sheep) to increase the total number of seminiferous tubules in each graft (Rodriguez-Sosa et al., 2010). Although such tissue samples can be obtained from every individual, infant, juvenile or adult, almost all successful studies to date used immature tissue (Ehmcke & Schlatt, 2008). Like in semen cryopreservation, there are differences between species in the reaction of their testicular tissue to cryoprotectants, chilling and cryopreservation (Schlatt et al., 2002b). The preserved testicular tissue can be handled in several ways. From these tissues, spermatozoa, spermatocytes and round and elongated spermatids can all be retrieved and used to fertilize oocytes through ICSI (Hovatta et al., 1996; Gianaroli et al., 1999). Testicular tissue can also be transplanted back to the donating individual (autografting), to another individual of the same species (allografting) or to individual of a different species, usually to nude or immunodeficient mice (xenografting). After transplantation, the graft may be lost due to tissue rejection or ischemia. If it manages to survive the critical first few days, blood supply will reach the graft, it will be supported by the recipient system and, after some time, will start producing spermatozoa, which can be harvested by surgical excision of all or part of the graft (Schlatt et al., 2002b). Although dependent on the recipient system for support, the spermatogenesis cycle length is assumed to be inherent to the spermatogonial stem cells, which are expected to preserve the donating species spermatogenesis length (Zeng et al., 2006). However other studies showed that in some species, the process is accelerated when their testicular tissue was xenografted into mice (rhesus monkeys; Honaramooz et al., 2004) while in others it is not (domestic cat; Snedaker et al., 2004). Acceleration, when identified, bears special interest for species preservation as it can shorten generation time and thus speed up population growth. This acceleration, however, may also mean abnormal spermatogenesis process that produces abnormal gametes. The sperm produced this way does not go through epididymal maturation process so the only way it can be utilized is by ICSI (Shinohara et al., 2002). One should also keep in mind that it is very costly to keep immunodeficient mice and handle them under germ-free conditions and, of course, repeated transplantations from one mouse to another are required to maintain viable tissue for many years. Still, testicular tissue cryopreservation was done in several species and pregnancies were achieved in mice (Schlatt et al., 2002b; Shinohara et al., 2002), rabbit (Shinohara et al., 2002), human (Hovatta et al., 1996), Djungarian hamsters (Schlatt et al., 2002b) and marmoset monkeys (Schlatt et al., 2002b), to name a few. Testicular tissue can also be cultured *in vitro* to give rise to mature and competent cells. Culture conditions, however, are very complex and, until recently, attempts were encouraging but still unsuccessful (Gohbara et al., 2010). Earlier this year,

generation of offspring from such tissues was demonstrated (Sato et al., 2011). In that study, neonatal mouse testicular tissue cultured *in vitro* for over two months (with or without being previously cryopreserved), generated fully competent spermatids and spermatozoa, which led to embryonic development and healthy and reproductive-active offspring production. This exciting development still needs to be evaluated in terms of accuracy of the genetic profile and absence of aneuploidy in haploid cells (Cheung & Rennert, 2011) as well as its applicability to other species. However, the fact that healthy and fertile offspring were produced is very encouraging.

3. The female

In comparison to the male, females’ gametes pose several difficulties when it comes to preservation (Table 1). Very small number of gametes is progressing to the more advanced developmental stages during each cycle, and at best only a handful mature and ovulate. When dealing with rare and endangered species in which the number of available individuals for research is extremely limited and often spatially and temporally far apart, progress is very slow and limited by the small numbers. Oocytes and embryos are orders of magnitude larger than spermatozoa, thus bringing down the ratio between surface area and volume. The outcome is slower movement of water and cryoprotectants across the cellular membrane and elevated risk for intracellular ice formation. Unlike in males, *in vivo* collection of oocytes, and to a lesser extent - embryos, is an invasive procedure requiring anesthesia or sedation. Although production of new oocytes exists even in adulthood (Niikura et al., 2009; Tilly et al., 2009), it is very minimal. So, in general terms, the female can be considered as if it is born with a limited life-long supply. Males on the other hand produce sperm continuously, throughout their adult life, sperm that can be collected relatively easy almost any time. All these differences contribute to the fact that while the number of species in which sperm was cryopreserved is in the hundreds, the number of species in which embryo cryopreservation was reported (not all successful) is currently less than 50 and the number of species in which oocyte cryopreservation was attempted is far less than that.

	Male	Female
Gamete size	<10µm (head)	10s-100s µm (species specific)
Numbers	Millions to billions	Few at a time
Production	Continuous	Very limited new production
Accessibility	Easy to collect	Invasive procedure
Collection	Almost any time	In estrous (need monitoring)

Table 1. comparison between male and female gametes in relation to gamete cryopreservation.

Female gametes can be collected at different time points in their maturation process: 1) as mature oocytes, following ovulation (natural or chemically induced), 2) as mature and immature oocytes, by ovum pick up, either transabdominally, transvaginally or

transrectally. This can be done during natural estrus cycle or following chemical stimulation, 3) at all developmental stages, mostly immature, following ovariectomy, either when neutering the animal or post mortem, a possibility with time constraint because deterioration is fast *in vitro* and even faster *in vivo* – reasonable quality oocytes can be harvested only up to ~24h after the removal of the ovaries if they were held at 4°C (Wood et al., 1997; Cleary et al., 2001; Personal experience), 4) after fertilization (natural or by artificial insemination), as embryos. This can be done at any stage prior to implantation. The collected oocytes can be at any level of maturation including oocytes found in primordial, preantral or antral follicles, each presenting its own special requirements and sensitivities. Harvesting and preserving oocytes is almost pointless if all other associated assisted reproductive technologies – *in vitro* maturation, *in vitro* fertilization, *in vitro* culture and embryo transfer, are not mastered (at present or in the future) to support it. Female fertility preservation can be done through preservation of oocytes and/or embryos at various developmental stages, as well as by preservation of ovarian tissue or entire ovaries, all of which will be discussed in details in the following sections.

### 3.1 Oocyte cryopreservation

For decades it was believed that females are born with their life supply of oocytes in their ovaries, all dormant at a very early maturation stage (Zuckerman, 1951). This dogma, however, was recently challenged by a number of studies suggesting that the female gonads retain the ability to regenerate oocytes throughout adulthood, albeit at a very limited number (e.g. Niikura et al., 2009; and reviewed in Tilly et al., 2009). The vast majority of oocytes, however, is already in the ovaries at birth and remains dormant at a very early stage of maturation to adulthood and beyond. Once the female reaches puberty, one or more cohorts of oocytes are selected at each estrus cycle to progress in the maturation process and, depending on the species, one or several oocytes are ovulated. The remaining oocytes in these selected cohorts degenerate or luteinize to form accessory corpora lutea. To be fertilized, an oocyte needs to overcome the meiotic block and progress to the metaphase II (MII) stage of maturation or else only very few oocytes will fertilize (Luvoni & Pellizzari, 2000). Thus, an *in vitro* maturation procedure should be in hand to handle immature oocytes. This process is currently developed for only a handful of species and even for these success is often fairly limited (Krisher, 2004). Furthermore, collection of immature oocytes disrupts the natural maturation process and thus compromises the quality of the oocytes even if they are later matured *in vitro*. During oocyte maturation and follicular growth, the oocyte accumulates large quantities of mRNA and proteins needed for the continuation of meiosis, fertilization and embryonic development. In the absence of the entire supporting system in the *in vitro* culture, production of some of these needed components is hampered. The resulting mature oocytes are therefore of inferior quality when compared to *in vivo* matured oocytes. In seasonal animals, oocytes collected out of the season may show resistance to IVF (Spindler et al., 2000; Berg & Asher, 2003; Comizzoli et al., 2003). In red deer for example, while about 15% of cleaved oocytes collected during the season (April-July) developed *in vitro* to blastocysts, none have developed if collected after July (Berg & Asher, 2003). Comizzoli et al. (2003) showed that anti-oxidants and FSH in the culture media can overcome this problem in the domestic cat model they have studied. Naturally, *in vitro* fertilization and culture should also be developed so that embryos can be generated for transfer. During the development of such techniques, as well as in those cases when conspecific oocytes are not available, interspecific IVF can be considered. This was done, for example between the mouflon (*Ovis orientalis musimon*) and the domestic sheep

(Ptak et al., 2002) or between some small cat species and the domestic cat (Herrick et al., 2010) or even between a cat and a mouse (Xu et al., 2011). To enhance the number of oocytes collected at any ovum pick-up procedure, hormonal stimulation can be used. This, however, will result in both mature and immature oocytes and the quality of both may be compromised (Blondin et al., 1996; Moor et al., 1998; Takagi et al., 2001). Although to date no morphological or other method is able to accurately predict which oocytes have optimal developmental potential (Coticchio et al., 2004), it is clear that oocyte quality is a major determining factor in the success of IVF (Coticchio et al., 2004; Krisher, 2004; Combelles & Racowsky, 2005), early embryonic survival, the establishment and maintenance of pregnancy, fetal development, and even adult disease (reviewed in Krisher, 2004). Once all these hurdles have been overcome and while keeping in mind the importance of oocyte quality, the next major hurdle to overcome is oocyte cryopreservation.

Oocytes are very different from sperm or embryos with respect to cryopreservation. Oocytes (and embryos) are in the range of three to four orders of magnitude larger than spermatozoa, thus substantially increasing their surface-to-volume ratio and making them sensitive to chilling and susceptible to intracellular ice formation (Arav et al., 1996; Zeron et al., 1999; Chen & Yang, 2009). Oocytes at the MII stage also have a formed fuse that is chilling-sensitive (Chen & Yang, 2009) and their plasma membrane has low (temperature dependent) permeability coefficient, thus making the movement of cryoprotectants and water slower (Jackowski et al., 1980; Ruffing et al., 1993). This, however, may vary between species. Membrane permeability increases after fertilization (Jackowski et al., 1980) and seem to be higher in morula/blastocyst stages as compared to earlier embryonic stages (Jin et al., 2011), thus contributing to the fact that embryos are easier to cryopreserve. The oocyte cytoskeleton is highly sensitive to chilling and gets disorganized at suboptimal temperatures (Trounson & Kirby, 1989). Oocytes also have high cytoplasmic lipid content which increases chilling sensitivity (Ruffing et al., 1993). They have less submembranous actin microtubules (Gook et al., 1993) making their membrane less robust. The meiotic spindle, which has formed by the MII stage, is very sensitive to chilling and may be compromised as well (Ciotti et al., 2009) resulting in uneuploidy (Sathananthan et al., 1988) and oocytes are more susceptible to the damaging effects of reactive oxygen species (Gupta et al., 2010). Many of these parameters change after fertilization, making embryos less chilling sensitive and easier to cryopreserve (Jackowski et al., 1980; Gook et al., 1993; Fabbri et al., 2000). Despite many advances in the field of cryopreservation, oocyte (ovulated, mature or immature) cryopreservation still has a long way to go before it can be routinely utilized in many species. Even in human medicine, fewer than 200 births resulting from cryopreserved oocytes were reported as of 2007 (Edgar & Gook), a number that went up to around 500 by 2009 (Nagy et al.). Yet, despite all these difficulties, some success in oocyte cryopreservation has been reported.

Two main cryopreservation techniques are used for oocyte cryopreservation – slow (equilibrium) freezing and vitrification. In slow freezing, oocytes are exposed to permeating cryoprotectants in the range of 1.0-1.5 M and are frozen, following equilibration and seeding, at a rate of 0.3°C to 0.5°C per minute down to -30°C or lower. Once at the desired temperature they are plunged into liquid nitrogen to vitrify the intra- and extracellular still unfrozen compartments and for storage. Attempts to improve outcome by altering the components of the freezing extender (e.g. replacing sodium chloride with choline chloride; Stachecki et al., 1998a; Stachecki et al., 1998b; Quintans et al., 2002) suggest that there is still some room for improvements in the standard techniques widely in use. Vitrification usually exposes the oocytes to substantially higher concentration of cryoprotectants, in the range of



5.0 to 7.0 M, and cryopreservation is done at cooling rates of 2,500°C per minute or more, depending on the technique used. Vitrification can, however, be achieved even at cryoprotectant concentrations similar to those used for slow freezing if sample volume is small enough and/or cooling rate is high enough to achieve vitrification. One advantage of vitrification over slow freezing, when oocytes are concerned, is the higher survival rate that the fast cooling facilitates. To achieve very high cooling rates, a wide variety of carrier systems were developed (reviewed by Saragusty & Arav, 2011). The small-volume sample, with the carrier, is plunged directly into liquid nitrogen or nitrogen slush. For vitrification to be successful, one should be highly experienced in handling the oocytes throughout the dilution process and in loading them onto or into the carrier system. By cooling liquid nitrogen from its boiling temperature (-196°C) to close to its freezing temperature (-210°C), nitrogen slush is formed. Vitrification in slush gives at least two major advantages. When a sample is inserted into liquid nitrogen, the nitrogen boils and forms an insulation vapor layer around the sample (the Leidenfrost effect). Boiling is considerably reduced when slush is used. Slush also significantly increases the cooling rate. Several studies have demonstrated the superiority of slush over liquid nitrogen (Arav & Zeron, 1997; Isachenko et al., 2001; Beebe et al., 2005; Santos et al., 2006; Lee et al., 2007; Criado et al., 2010) but some found little or no difference (Martino et al., 1996; Cuello et al., 2004; Cai et al., 2005) (Table 2). When cooled at such high cooling rates, oocytes spend very short interval at their lipid phase transition temperature, thus avoiding, or at least minimizing, chilling injury (Arav et al., 1996). Vitrification also reduces the loss of mRNA from the cryopreserved oocytes (Chamayou et al., 2011), mRNA that is crucial for embryonic development and beyond.

The first human pregnancy from cryopreserved (by slow freezing), *in vitro* fertilized oocyte was reported in 1986 (Chen, 1986) following success in other (laboratory) species that came a few years earlier, such as mice (Whittingham, 1977) and rat (Kasai et al., 1979) oocytes cryopreserved to -196°C or mice oocytes frozen to -75°C (Parkening et al., 1976). Still, despite several decades of research and many advances in the field, success is very limited and oocyte cryopreservation is still labeled as experimental even in human medicine (Noyes et al., 2010). Cryopreservation can cause cytoskeleton disorganization (Trounson & Kirby, 1989), chromosome and DNA abnormalities (Van Blerkom, 1989), spindle disintegration (Pickering & Johnson, 1987), plasma membrane disruption (Van Blerkom, 1989) and premature cortical granule exocytosis with its related zona pellucida hardening, making it impermeable to spermatozoa (Johnson et al., 1988). It also hamper, at least to some extent, the ability of oocytes to mature *in vitro* after thawing/warming (Rao et al., 2011). When comparing these parameters, in addition to survival rate, oocyte cryopreservation by vitrification seem to be superior to slow freezing, which explains why oocyte vitrification is gradually replacing slow freezing as the leading technique of preservation. Either open or closed carrier systems are used for vitrification. The closed systems are more secure while the open systems can provide higher cooling rates by direct exposure of the sample to liquid nitrogen. The large number of carrier systems (see Saragusty & Arav, 2011 for a most current list) suggests that the field is still developing and even decision if the open or the closed system is better is still under debate. While in most carrier systems, the volume that enables vitrification limits the number of oocytes that can be contained in it to just a few, some carrier systems such as the electron microscope grid (Steponkus et al., 1990) or nylon mesh (Matsumoto et al., 2001) allow simultaneous vitrification of a large number (as many as 65 in one study) of oocytes. Most reports on oocyte vitrification are, however, sporadic in nature and usually on small number of oocytes. The open system [Cryotop and Open Pulled Straw (OPS)] was used to vitrify germinal vesicle-stage oocytes of the minke whale

(*Balaenoptera bonaerensis*) with the Cryotop producing better results in post-warming morphology and rate of maturation (Iwayama et al., 2005) and both carrier systems produced better results compared to an earlier attempt to cryopreserve minke whale oocytes by slow freezing (Asada et al., 2000). Oocytes of the Mexican gray wolf (*Canis lupus baileyi*) and the domestic dog were also vitrified recently using the Cryotop carrier system (Boutelle et al., 2011). Post warming viability was 61% of intact dog oocytes and 57% of intact wolf cells. Open systems were also used to vitrify granulosa-oocyte complexes (GOC) from primary follicles of marsupials. In two different studies the fat-tailed dunnart (*Sminthopsis crassicaudata*) (Czarny et al., 2009b) and the Tasmanian devil (*Sarcophilus harrisii*) (Czarny & Rodger, 2010) GOC were vitrified in self-made OPS. Post-warming viability was about 70% in both studies. Immature oocytes of the lowland gorilla (*Gorilla gorilla gorilla*) were also cryopreserved, using slow freezing. Of the thawed oocytes, 4/6 were morphologically degenerated, one arrested at the GV stage and the other progressed to the MI stage and then arrested (Lanzendorf, 1992). Immature oocytes of chousingha (*Tetracerus quadricornis*) were also vitrified using the OPS as a carrier system but post warming maturation rate (29.4%) was considerably lower than that of fresh oocytes (69.3%) (Rao et al., 2011). What unifies all these studies is the fact that only small number of oocytes were cryopreserved and only *in vitro* post thaw / warming evaluations were conducted.

Species	Model	Liquid nitrogen	Nitrogen slush	Sig.	Refernce
Bovine	MII oocytes	40% cleavage	25% cleavage	NS	(Martino et al., 1996)
Bovine	MII oocytes	28% cleavage	48% cleavage	P<0.05	(Arav & Zeron, 1997)
Ovine	GV-oocytes	25% survival	5% survival	P<0.05	(Isachenko et al., 2001)
Porcine	Early blastocysts	77% survive	95% survive	NS	(Cuello et al., 2004)
Rabbit	MII oocytes	83% survive	82% survive	NS	(Cai et al., 2005)
Porcine	Blastocysts	62% survive	83% survive	P<0.05	
Bovine	MII oocytes	39% survive	48% survive	P<0.05	(Santos et al., 2006)
Mouse	4-cell embryo with biopsy	50% survive	87% survive	P<0.05	(Lee et al., 2007)
Mouse	Blastocysts	10% survive	54% survive	P<0.05	(Yavin et al., 2009)
Rabbit	Morulae	83% develop	92% develop	P<0.05	(Papis et al., 2009)
Mouse	MII oocytes	45% survive	90% survive	P<0.001	(Lee et al., 2010)
Human	MII oocyte	56% survive	92% survive	P<0.05	(Criado et al., 2010)

NS = not significant.

Table 2. When oocytes or embryos from various species were vitrified in liquid nitrogen slush in comparison to regular vitrification, results either showed no difference or, more frequently, that slush was superior.

Immature oocytes seem to be less prone to damages caused by the chilling, freezing and thawing or warming procedures (Arav et al., 1996) and they, too, can be cryopreserved by slow freezing (Luvoni et al., 1997) or vitrification (Arav et al., 1993; Czarny et al., 2009b). Preantral oocytes can be preserved inside the follicle and about 10% seem to be physiologically active after thawing and one week of culture. Of over 16,000 small preantral oocytes recovered from the ovaries of 25 cats, 66.3% were intact after thawing (Jewgenow et al., 1998). Before freezing 33.9% of the follicles contained viable oocytes while after thawing there were 19.3% if frozen in Me<sub>2</sub>SO and 18.5% if frozen in 1,2-propanediol. However, culture conditions that will allow these oocytes to grow and reach full maturation are still largely unknown despite attempts in several species (Jewgenow et al., 1998; Nayudu et al., 2003). For example, in the marmoset monkey, oocytes collected from secondary pre-antral follicles of either mature or pre-pubertal females were able to develop *in vitro* to the polar body stage but could not complete the maturation process (Nayudu et al., 2003). The exception is the mouse, in which this was done and embryos were produced following IVF of frozen-thawed primary follicles matured *in vitro* and live young were born after embryo transfer (Carroll et al., 1990). Some, very limited, success was also reported in cats, where following vitrification in 40% ethylene glycol, 3.7% of the *in vitro* matured oocytes were able to develop to the blastocyst stage following IVF (Murakami et al., 2004). The problems associated with maturation of early-stage oocytes *in vitro* are the need to develop the complex endocrine system that support the development at different stages, other culture conditions that will ensure survival (oxygen pressure for example) and, in many species, the duration of time required to keep the follicles in culture – 6 months or more. An alternative to isolated oocyte cryopreservation is cryopreservation of individual primordial follicles and later transplanting them to the ovarian bursa, where they can mature and eventually produce young offspring following natural mating as was shown in mice (Carroll & Gosden, 1993).

Liquid-phase sperm preservation is relatively simple. Doing the same with oocytes was, until recently, much more challenging. A recent report on pig oocytes, however, has demonstrated ambient-temperature (27.5°C) preservation for 3 days with as many as 65% of the GV oocytes maintaining viability and developmental competence (Yang et al., 2010). This study demonstrated that oocyte preservation without freezing for several days is possible and relatively simple. This is of great importance for wildlife as cryopreservation or IVF of oocytes collected from dead animals in the field often cannot be done on the spot. The ability to keep oocytes alive while transporting them to the laboratory will considerably increase the number of possibilities.

### 3.2 Embryo cryopreservation

As discussed earlier with regards to oocytes, the vast difference in size, components and associated structures between spermatozoa on the one hand and oocytes and embryos on the other make cryopreservation of the latter much more complex. The issue of intracellular ice formation becomes a major concern, even at relatively slow cooling rates. To avoid this from happening, small volume cryopreservation and either high cryoprotectant concentration coupled with very fast cooling rate to achieve a state of vitrification or lower cryoprotectant concentration and slow cooling rate (slow freezing) are utilized. The first report on fertilized eggs cryopreservation was on rabbit fertilized ova frozen to -79°C (Ferdows et al., 1958). Some of these cryopreserved ova resulted in pregnancies after

thawing and transfer. This abstract, however, seem not to have been followed by a full peer-reviewed manuscript so it is not clear if those zygotes really froze and resulted in pregnancies. In 1971 another report on successful mouse embryo cryopreservation to  $-79^{\circ}\text{C}$ , using 7.5% polyvinylpyrrolidone (PVP) as cryoprotectant, was published, reporting post-thaw *in vitro* development to blastocysts and *in vivo* development to day-18 fetuses (Whittingham, 1971). Several researchers tried to repeat these results but none was successful (Whittingham et al., 1972; Wilmut, 1972; Ashwood-Smith, 1986; Leibo & Oda, 1993). The real start of the embryo cryopreservation era can therefore be considered as the year 1972. During that year two groups reported successful cryopreservation of mouse embryos to  $-196^{\circ}\text{C}$  (Whittingham et al., 1972; Wilmut, 1972). These reports came more than two decades after Polge et al. (1949) reported their chance observation that led to successful freezing of spermatozoa and opened a new era in cryobiology and assisted reproduction. Despite the decades that went by and numerous studies attempting a plethora of protocols and combinations of cryoprotectants, it is amazing to note that besides modification to cooling rate that came a few years later (Willadsen et al., 1976; Willadsen et al., 1978), the same basic protocol is still in vast use today. From conservation standpoint, embryo cryopreservation has the advantage of preserving the entire genetic complement of both parents. Naturally, a number of both male and female embryos should be stored to ensure representation of both sexes and a wide genetic diversity. Since sexing each embryo before cryopreservation is not practical, a large number of embryos should be preserved to increase the probability for sufficient representation of embryos from both sexes. Cryobanking of embryos can thus help establishing founder population with the aim of eventual reintroduction into the wild (Ptak et al., 2002) or revive isolated small population. However, while millions of offspring were born following the transfer of cryopreserved embryos in humans, cattle, sheep and mice, success is very limited in many other, even closely related species. To date the number of species in which embryo cryopreservation has been reported stands at less than 50 mammals (human, domestic and laboratory animals included), with live birth achieved in only about half of them (Table 3). There are also a few reports on non-mammalian embryo cryopreservation, all of them in fish (Table 3). Looking through the table, one can see that the majority of species in which embryo cryopreservation led eventually to pregnancy and live birth are domestic, companion, and laboratory species and species of commercial value. Only very few are truly wildlife species. Much of the knowledge gained came from studies on model animals since endangered species are too rare and studying them directly is often too difficult or practically impossible. By definition, however, each species has a unique reproductive specialization so, no matter how close we get with the aid of model animals, we must in the end gain access to the target species and verify that what worked in the model also works in the target. For example, studies on the domestic cat helped develop various technologies, which were later used in non-domestic cats (Dresser et al., 1988; Pope et al., 1994; Pope, 2000), or cattle served as a model for other ungulates (Dixon et al., 1991; Loskutoff et al., 1995). Too often direct adaptation is not possible and either adjustments to protocols or complete revision are required, forcing researchers to settle for small animal study population, at times comprised of a single animal (e.g. Robeck et al., 2011), and samples that are hard to come by. As in the case of oocytes, slow freezing and vitrification are currently used for embryo cryopreservation. Unlike oocytes, however, slow freezing has been producing good results so vitrification does not occupy as important a role in embryo cryopreservation as it does with oocytes. Two main sources of embryos can be considered – *in vivo* produced embryos and those



produced *in vitro*. These two embryo groups can develop *in vivo* to produce live offspring but the *in vivo* produced embryos seem to be superior to the *in vitro* ones in many respects, including their sturdiness and ability to survive cryopreservation (Rizos et al., 2002). Obtaining *in vivo*-produced embryos from an endangered species for cryopreservation is a difficult ethical question. If pregnancy has already occurred, shouldn't we let it proceed? Still, because of their superiority, *in vivo*-produced embryos were used in many of the studies on embryo cryopreservation in wildlife.

Species	Procedure	Outcome	References
<b>Primates</b>			
Human ( <i>Homo sapiens</i> )	4 to 8-cell, freezing, 4 to 16-cell freezing	Pregnancy, Pregnancy to term	(Trounson & Mohr, 1983; Zeilmaker et al., 1984)
Baboon ( <i>Papio</i> sp.)	<i>In vivo</i> -produced 6-cell to blastocyst, freezing	Pregnancy to term	(Pope et al., 1984)
Marmoset monkey ( <i>Callithrix jacchus</i> )	<i>In vivo</i> -produced 4 to 10-cells and morulae freezing	Pregnancy to term	(Hearn & Summers, 1986; Summers et al., 1987)
Cynomolgus monkey ( <i>Macaca fascicularis</i> )	IVF, 4 to 8-cell freezing 2 to 8-cell vitrification	Pregnancy <i>In vitro</i> survival	(Balmaceda et al., 1986; Curnow et al., 2002)
Rhesus macaque ( <i>Macaca mulatta</i> )	IVF, early-stage freezing ICSI blastocysts vitrification	Pregnancy to term  Pregnancy to term	(Wolf et al., 1989; Yeoman et al., 2001)
Hybrid macaque [pig-tailed ( <i>Macaca nemestrina</i> ) & lion-tailed ( <i>M. silenus</i> )]	IVF, 2-cell freezing	Pregnancy to term	(Cranfield et al., 1992)
Western lowland gorilla ( <i>Gorilla gorilla gorilla</i> )	IVF, 2-cell freezing	Not reported	(Pope et al., 1997a)
<b>Ungulates</b>			
Bovine ( <i>Bos taurus</i> )	<i>In vivo</i> -produced blastocysts freezing	Pregnancy to term	(Wilmut & Rowson, 1973; Willadsen et al., 1978)
Sheep ( <i>Ovis aries</i> )	<i>In vivo</i> -produced morula and blastocyst freezing	Pregnancy to term	(Willadsen et al., 1974, 1976)
Goat ( <i>Capra aegagrus</i> )	<i>In vivo</i> -produced morula and blastocyst freezing	Pregnancy to term	(Bilton & Moore, 1976)

Species	Procedure	Outcome	References
Horse ( <i>Equus caballus</i> )	<i>In vivo</i> -produced blastocysts freezing	Pregnancy to term	(Yamamoto et al., 1982; Slade et al., 1985)
African eland antelope ( <i>Taurotragus oryx</i> )	Details not provided	Stillbirth	(Kramer et al., 1983; Dresser et al., 1984; both cited in Schiewe, 1991)
Arabian Oryx ( <i>Oryx leucoryx</i> )	<i>In vivo</i> -produced morula freezing	Transferred but no pregnancy	(Durrant, 1983)
Gaur ( <i>Bos gaurus</i> )	<i>In vivo</i> -produced blastocysts freezing	Transferred to both cow and gaur. Pregnancy at day 135 in cow	(Stover & Evans, 1984; Armstrong et al., 1995)
Bongo ( <i>Tragelphus euryceros</i> )	<i>In vivo</i> -produced blastocysts freezing	Transferred but outcome not reported	(Dresser et al., 1985)
Swine ( <i>Sus domestica</i> )	<i>In vivo</i> -produced blastocysts freezing to -35°C and -196°C	Pregnancy to term from -35°C, no pregnancy from -196°C	(Hayashi et al., 1989)
Scimitar-horned Oryx ( <i>Oryx dammah</i> )	<i>In vivo</i> -produced morula and blastocysts freezing	Transferred but no pregnancy	(Schiewe et al., 1991a)
Red deer ( <i>Cervus elaphus</i> )	<i>In vivo</i> -produced blastocysts freezing	Pregnancy by ultrasound	(Dixon et al., 1991)
Suni Antelope ( <i>Neotragus moschatus zuluensis</i> )	8-cell freezing	Transferred but no pregnancy	(Schiewe, 1991)
Water buffalo ( <i>Bubalis bubalis</i> )	<i>In vivo</i> -produced morula and blastocysts freezing	Pregnancy to term	(Kasiraj et al., 1993)
Fallow deer ( <i>Dama dama</i> )	<i>In vivo</i> -produced morula and blastocysts freezing	Pregnancy by ultrasound at day 45.	(Morrow et al., 1994)
Domestic donkey ( <i>Equus acinus</i> )	<i>In vivo</i> -produced blastocysts freezing	Outcome not reported	(Vendramini et al., 1997)
Dromedary camel ( <i>Camelus dromedarius</i> )	<i>In vivo</i> -produced blastocysts freezing and vitrification	Freezing – pregnancy by ultrasound, vitrification – pregnancy to term	(Skidmore & Loskutoff, 1999; Nowshari et al., 2005)

Species	Procedure	Outcome	References
Wapiti ( <i>Cervus canadensis</i> )	Details not mentioned	Pregnancy to term	(cited in Rall, 2001)
European mouflon ( <i>Ovis orientalis musimon</i> )	IVF blastocysts vitrification	Outcome not reported	(Ptak et al., 2002)
Llama ( <i>Lama glama</i> )	<i>In vivo</i> -produced blastocysts freezing and vitrification	Pregnancy by ultrasound after vitrification	(Aller et al., 2002; Lattanzi et al., 2002)
Wood bison ( <i>Bison bison athabasca</i> )	IVF morula and blastocysts vitrification	Not evaluated	(Thundathil et al., 2007)
Sika deer ( <i>Cervus nippon nippon</i> )	IVF blastocysts freezing	Pregnancy to term in red deer surrogate hind	(Locatelli et al., 2008)
Carnivores			
Domestic cat ( <i>Felis catus</i> )	<i>In vivo</i> -produced blastocysts freezing	Pregnancy to term	(Dresser et al., 1988)
African wildcat ( <i>Felis silvestris</i> )	IVF morula and blastocysts freezing	Pregnancy to term in domestic cat	(Pope et al., 2000)
Siberian Tiger ( <i>Panthera tigris altaica</i> )	IVF 2 to 4-cell freezing and vitrification	<i>In vitro</i> development of vitrified only	(Crichton et al., 2000; Crichton et al., 2003)
Blue fox ( <i>Alopex lagopus</i> )	Frozen and vitrified embryos, stage and source not mentioned	Both transferred and implanted but not carried to term	(cited in Farstad, 2000a)
Ocelot ( <i>Leopardus pardalis</i> )	IVF (stage not reported) freezing	Pregnancy to term	(Swanson, 2001, 2003)
Tigrina ( <i>Leopardus tigrinus</i> )	IVF 2 to 8-cell freezing	Not evaluated	(Swanson et al., 2002)
Bobcat ( <i>Lynx rufus</i> )	<i>In vivo</i> -produced blastocyst freezing	Transferred but no pregnancy	(Miller et al., 2002)
European Polecat ( <i>Mustela putorius</i> )	<i>In vivo</i> -produced morula and blastocysts freezing and vitrification.	Pregnancy to term in both cryopreservation techniques	(Lindeberg et al., 2003; Piltti et al., 2004)
Caracal ( <i>Felis caracal</i> or <i>Caracal caracal</i> )	IVF day 5 to 6 freezing	Pregnancy to term	(cited in Swanson, 2003; Pope et al., 2006)
Geoffroy's cat ( <i>Felis geoffroyi</i> )	Source and technique not mentioned	Outcome not mentioned	(Swanson & Brown, 2004)

Species	Procedure	Outcome	References
Serval ( <i>Leptailurus serval</i> )	IVF morula and blastocysts freezing	Transferred but no pregnancy	(Pope et al., 2005)
Dog ( <i>Canis lupus familiaris</i> )	<i>In vivo</i> -produced 1-cell to blastocyst vitrification	Pregnancy to term from 8 to 16-cell embryos	(Suzuki et al., 2009)
Clouded leopard ( <i>Neofelis nebulosa</i> )	IVF and ICSI day-five freezing	Transferred but no pregnancy	(Pope et al., 2009)
Glires			
European rabbit ( <i>Oryctolagus cuniculus</i> )	Fertilized ova frozen to -79°C, later 4 to 16-cell and morula freezing to -196°C and vitrification.	Confirmed pregnancy and later pregnancy to term by freezing and <i>in vitro</i> survival for vitrification	(Ferdows et al., 1958; Bank & Maurer, 1974; Whittingham & Adams, 1974, 1976; Popelkova et al., 2009)
Mouse ( <i>Mus musculus</i> )	<i>In vivo</i> -produced 8-cell freezing	Live fetuses to term	(Whittingham et al., 1972; Wilmut, 1972)
Rat ( <i>Rattus norvegicus</i> )	<i>In vivo</i> -produced 2 to 8-cell freezing and blastocysts vitrification	Confirmed pregnancy on day 18 for freezing, pregnancy to term for vitrification	(Whittingham, 1975; Kono et al., 1988)
Syrian hamster ( <i>Mesocricetus auratus</i> )	<i>In vivo</i> -produced 1-cell to morula freezing and 1 to 2-cell vitrification	Confirmed pregnancy on day 14 for freezing and pregnancy to term for vitrification	(Ridha & Dukelow, 1985; Lane et al., 1999)
Mongolian gerbil ( <i>Moriones unguiculatus</i> )	<i>In vivo</i> -produced 2-cell, morula and blastocyst vitrification	Pregnancy to term	(Mochida et al., 2005)
Marsupials			
Fat-tailed dunnart ( <i>Sminthopsis crassicaudata</i> )	<i>In vivo</i> -produced day 2 to 4 freezing and vitrification	<i>In vitro</i> survival in both systems	(Breed et al., 1994)



Species	Procedure	Outcome	References
Others (fish)			
Zebrafish ( <i>Danio rerio</i> )	6-somite and heartbeat stage vitrification	No viability	(Zhang & Rawson, 1996)
Turbot ( <i>Psetta maxima</i> )	Tail bud and tail bud free stages vitrification	No viability	(Robles et al., 2003)
Flounder ( <i>Paralichthys olivaceus</i> )	14-somite to pre-hatching stage vitrification	Post-warming hatching	(Chen & Tian, 2005)
Gilthead seabream ( <i>Sparus aurata</i> )	Tail bud and tail bud free stages vitrification	No viability	(Cabrita et al., 2006)
Red seabream ( <i>Pagrus major</i> )	Heart beat stage vitrification	No viability	(Ding et al., 2007)
Cascudo preto ( <i>Rhinelepis aspera</i> )	Blastoporous closing stage freezing to -8°C for 6h	Post-thaw hatching	(Fornari et al., 2011)

Table 3. Embryo cryopreservation in vertebrates. The table make it clear that attempts were made almost only in mammals and success in terms of pregnancy carried to term was achieved almost only in domestic, laboratory or companion species and species of commercial value.

3.2.1 Mammals

3.2.1.1 Non-human primates

The number of cryopreserved human embryos successfully transferred since the first report on birth resulting from a transfer of a frozen-thawed embryo (Trounson & Mohr, 1983) is probably over half a million. Yet, despite the fact that non-human primates are used as laboratory models for humans in many studies, progress in primate embryo cryopreservation has been very limited (Mazur et al., 2008) and reports are scarce but with promising results. Observing the progress in non-human primate embryo cryopreservation, it seems that in this field humans act as models for other primates rather than the other way around. The first report on the birth of a non-human primate (baboon; *Papio* sp.) following transfer of frozen-thawed embryo came in 1984, about a year after similar report in humans (Pope et al., 1984). Six *in vivo* produced embryos were retrieved and frozen using glycerol as cryoprotectant. All six embryos survived the freeze-thaw procedure and resulted in two pregnancies (33.3%) after being transferred to six recipients. A similar report on cryopreservation of *in vivo* produced embryos in marmoset monkey (*Callithrix jacchus*) showed higher pregnancy rates (Hearn & Summers, 1986; Summers et al., 1987). In one of these studies, for example, 70% (7/10) of cryopreserved four- to 10-cell embryos and 56% (5/9) of cryopreserved morulae resulted in pregnancies (Summers et al., 1987). Five pregnancies of the first and four of the latter were carried to term resulting in six babies in each group. These authors noted that 1.5M Me<sub>2</sub>SO was superior to 1.0M glycerol; the latter

causing severe osmotic damage. Relying on success in IVF followed by embryo transfer (Balmaceda et al., 1984), pregnancies resulting from frozen-thawed IVF-produced embryos in cynomolgus monkeys (*Macaca fascicularis*) were reported (Balmaceda et al., 1986). Fifty-six cynomolgus macaque embryos were cryopreserved at the four- to eight-cell stage using 1.5 M Me<sub>2</sub>SO as cryoprotectant and the slow-freezing technique. After thawing, 39 embryos (70%) were still viable. Of these, 25 were transferred to nine synchronized recipients 24 to 48 h after ovulation, resulting in three pregnancies. Report on pregnancy carried to term from frozen-thawed transferred embryo in the rhesus macaque (*Macaca mulatta*) came not too long after that (Wolf et al., 1989). Using hormonal stimulation to achieve superovulation, oocytes (68% mature) were retrieved and inseminated *in vitro*. Embryos were then cryopreserved at the three- to six-cell stage following a propanediol-based freezing protocol, originally developed for humans. Embryo post-thaw survival was high (100%; 11/11). After transferring two embryos to each of three recipients during the early luteal phase of spontaneous menstrual cycles, one pregnancy was achieved and was carried to term. The same group also attempted *in vitro* maturation (IVM) of oocytes prior to IVF, freezing and transfer (Lanzendorf et al., 1990). Oocytes collected at the germinal vesicle (GV) stage did not fertilize *in vitro* and fertilization rate of those collected at the metaphase I (MI) stage was low (32%), even if these were matured *in vitro* to the metaphase II (MII) stage. Fertilization rate of oocytes collected at the MII stage was high (93%) and eight embryos frozen and transferred at the two- to six-cell stage to four recipients (two embryos to each) resulted in three pregnancies culminating in the delivery of three twins. Cross-species IVF was also attempted using *in vitro*-matured oocytes from the non-endangered pig-tailed macaque (*Macaca nemestrina*) and sperm from the endangered lion-tailed macaque (*M. silenus*) (Cranfield et al., 1992). Of the 65 oocytes collected, 25 (38%) were fertilized and 15 (24%) have developed to good quality embryos. These embryos were cryopreserved in propanediol-based extender and the slow freezing technique. Nine embryos were transferred to naturally cycling *M. nemestrina* foster mothers, one of which delivered a healthy hybrid male infant. In Western lowland gorilla (*Gorilla gorilla gorilla*), associated *in vitro* techniques (IVM, IVF, IVC) were adopted successfully from humans (Pope et al., 1997a). Of eight embryos at the two-cell stage produced *in vitro*, three were transferred to a single female, leading to a pregnancy and birth of a female infant. The other five embryos were cryopreserved in 1.5 M 1,2-propanediol containing cryoprotectant. Regrettably, cryopreservation outcome was not reported.

Vitrification is a good alternative to the slow freezing. Following the lead of human and laboratory and farm animals' embryo cryopreservation, the use of vitrification was attempted and compared to slow freezing in non-human primates as well (Yeoman et al., 2001; Curnow et al., 2002). Early-stage (two- to eight-cells) cynomolgus macaque embryos were used to compare vitrification using open pulled straw (OPS) as a carrier system to slow freezing (Curnow et al., 2002). Vitrification proved to be inferior to slow freezing in cell survival rate (18 to 29% vs. 82%), embryo survival (26 to 32% vs. 90%) and cleavage rate (29 to 38% vs. 83%). In another study, on rhesus monkey blastocysts cryopreservation, vitrification using the cryoloop as a carrier system was compared to slow freezing (Yeoman et al., 2001). Embryos were produced *in vitro* by ICSI into mature oocytes and then *in vitro* cultured to the blastocyst stage. Cryopreservation was carried out by either the slow freezing technique or vitrification using two different cryoprotectant combinations – 2.8M

Me<sub>2</sub>SO with 3.6M EG (combination A) or 3.4M glycerol with 4.5M EG (combination B). Similar results were achieved when blastocysts were cryopreserved by slow freezing [8/22 (36.4%) embryos survived and 1/22 (4.5%) hatched following co-culture] or combination A of cryoprotectants [6/16 (37.5%) embryos survived and 1/16 (6.3%) hatched]. In comparison, using vitrification with cryoprotectant combination B 28/33 (84.8%) of the blastocysts survived and 23/33 (69.7%) hatched. This last study not only achieved high embryonic survival using vitrification, it has also demonstrated the suitability of this technique to overcome the problem of advanced-stage embryo preservation. Six embryos vitrified with cryoprotectant combination B and transferred to three recipients (two to each) resulted in a twin pregnancy carried to term.

### 3.2.1.2 Ungulates

Embryo cryopreservation has reached a commercial level in the cattle industry and to a lesser extent in sheep and goats. According to a report of the International Embryo Transfer Society (IETS), 297,677 *in vivo*-derived frozen-thawed bovine embryos were transferred in 2008 worldwide, representing 55.2% of all transferred *in vivo*-derived bovine embryos in that year (Thibier, 2009). There were also 26,914 frozen-thawed IVF embryos, comprising 10.6% of all transferred bovine IVF embryos in 2008. The actual numbers are most probably much larger since not all transfers are reported to IETS. Major Asian countries such as China, India, Korea and Thailand as well as some of the South American countries did not report their activities to IETS and the reports from Oceania are only partial. At least four important factors are responsible for this success: availability of almost unlimited number of oocytes for research, the possibility to collect *in vivo* produced embryos non-surgically and without the need for anesthesia or sedation, the availability of financial resources to finance overwhelming body of studies and the needs of the cattle industry. Because none of these factors is helping to push studies on endangered ungulates, situation is dramatically less developed in other species of this group. Statements in reviews on assisted reproductive technologies in non-domestic ungulates from only a decade ago were to the effect that by that time only one successful embryo cryopreservation has been achieved (Holt, 2001). Non-domestic ungulates usually do not show discernable signs of estrous and their receptive period is fairly short. This requires a thorough understanding of the estrus cycle endocrine activity, methods for its monitoring in each species under study and the development of species-specific hormonal administration for ovarian stimulation. As in all other wildlife species, one should always keep in mind that what works for one species not necessarily will also work for another, even closely related species. For example, the bovine IVC protocol works well for the water buffalo (*Bubalis bubalis*) but when this protocol was used for the African buffalo (*Syncerus caffer*), embryos did not develop beyond the morula stage (Loskutoff et al., 1995). Hormonal monitoring can be achieved non-invasively through fecal or urine analysis but even developing such techniques is not always eventless and not always successful (Paris et al., 2008). Hormonal administration requires stress-afflicting activities such as repeated darting, general anesthesia or movement restriction by chute. Thus, progress in this field has been slow and efficiency in *in vitro* technologies (IVM, IVF, IVC) has been low. For example, in the Kudu (*Tragelaphus* sp.), of 397 oocytes collected, 79 zygotes cleaved yet only two blastocysts were achieved (0.5%) (Loskutoff et al., 1995). Another example is the Mohor gazelle (*Gazella dama mhorr*) in which embryos produced by IVF with frozen-thawed semen did not develop beyond the six- to eight-cell stage (Berlinguer et al., 2008). These studies suggest that while embryo cryopreservation is a

technology worthwhile pursuing, other associated technologies should also reach a level of maturation to support it.

The European mouflon (*Ovis orientalis musimon*) is a wild sheep threatened by extinction. During the efforts to develop the necessary assisted reproductive technologies, the domestic sheep was used as a model. Using 25% glycerol and 25% ethylene glycol as cryoprotectants, *in vitro* produced embryos at the expanded blastocyst stage were vitrified (Ptak et al., 2002). Twenty blastocysts were transferred to domestic sheep foster mothers (two embryos each). At 40 days, seven of the sheep were pregnant and three carried the pregnancy to term, delivering four normal mouflon offspring. In another study, *in vivo* produced embryos were vitrified following embryo vitrification protocol developed for sheep (Naitana et al., 1997; Naitana et al., 2000). Of the five vitrified blastocysts, four survived and were transferred to four synchronized domestic sheep ewes, two of which became pregnant and one pregnancy was carried to term. The domestic sheep, and in part the cow as well, acted as a model for the scimitar-horned Oryx (*Oryx dammah*) as well. After developing the needed methods, including embryos collection, cryopreservation and transfer, in the sheep, the gained knowledge was used in the scimitar-horned Oryx. *In vivo* produced embryos were frozen in propylene glycol or glycerol but no specific results were reported (Wildt et al., 1986). In another, later study performed on scimitar horned Oryx embryos, thirty late morula- to blastocyst-stage embryos were frozen in cryoprotectant containing Me<sub>2</sub>SO, glycerol, or propylene glycol, 10 embryos in each (Schiewe et al., 1991a). Survival was higher in the Me<sub>2</sub>SO and glycerol groups. Although the majority (67%) of *in vitro*-cultured embryos developed into hatched blastocysts after 48 h, no pregnancies were established following nonsurgical (n = 8) or laparoscopic (n = 1) transfer of the remaining transferable embryos. Another Oryx species in which an attempt to cryopreserve embryos was made is the Arabian Oryx (*Oryx leucoryx*). Morula-stage *in vivo*-produced embryos were collected and one was frozen in 1.5M Me<sub>2</sub>SO. After thawing, the embryo was rated as having a good quality grade. It was transferred to a scimitar-horned Oryx foster female but failed to produce a pregnancy following surgical transfer (Durrant, 1983). Another failed attempt concerns cryopreservation of suni antelope (*Neotragus moschatus zuluensis*) eight-cell stage embryos (N. Loskutoff, personal communication cited in Schiewe, 1991). Of the 18 embryos frozen, nine completely degenerated after thawing. The other nine embryos were transferred by laparoscopy despite the fact that all of them exhibited partial blastomere degradation. No pregnancies were achieved. Attempts were also carried out to freeze *in vivo* produced embryos of African eland antelope (*Taurotragus oryx*) and bongo (*Tragelaphus euryceros*) using glycerol as cryoprotectant. Post-thaw evaluations indicated that six of seven eland (Dresser et al., 1984) and bongo (Dresser et al., 1985) embryos were considered viable and of good enough quality for transfer. Damage to the zona pellucida was noted in one of the eland embryos. Only one pregnancy was carried to term but resulted in a stillborn eland offspring due to dystocia. This attempt was followed by subsequent transfer attempts that resulted in a live eland offspring (B.L. Dressen, personal communication cited in Schiewe, 1991).

The red deer (*Cervus elaphus*), an animal of commercial value in various parts of the world, can also act as a model animal for other closely related species. Slow freezing of red deer *in vivo*-produced embryos in 1.4M glycerol followed by embryo transfer in another country resulted in pregnancy rate ranging between 50 and 72% in different farms, with an average pregnancy rate of 61.2% (153/247) (Dixon et al., 1991). In another study, slow freezing was



compared to vitrification by the OPS technique and fresh embryos as control (Soler et al., 2007). Pregnancy rates were 64.3% (18/28), 53.3% (8/15) and 70.0% (7/10) for fresh, vitrified and frozen embryos, respectively. The knowledge accumulated through experiments on red deer was used to freeze embryos from fallow deer (*Dama dama*) (Morrow et al., 1994). *In vivo*-produced embryos resulting from AI were collected surgically from fallow deer and transferred either fresh or following cryopreservation to recipients. Pregnancy rate of frozen-thawed embryos was half that of fresh (26% vs. 53%) and the overall efficiency of the program was low (0.9 to 1.0 surrogate pregnancy per donor). Another deer species in which embryos cryopreservation was attempted is sika deer (*Cervus nippon nippon*). Here, too, the protocol developed for the red deer (Dixon et al., 1991) was used. Of 142 oocytes collected following chemical synchronization, 57 (40.1%) cleaved after IVF and 14 of them reached the blastocyst stage. These embryos were cryopreserved by slow freezing and were later transferred (two per recipient) to synchronized red deer hinds. One of the seven recipients delivered a healthy young sika deer fawn after 224 days of pregnancy (Locatelli et al., 2008).

The domestic cow has acted as a model for other members of the Bovinae subfamily. The gaur (*Bos gaurus*), a member of this subfamily living in the forested areas of South and South East Asia is classified in the IUCN red list as vulnerable. Following protocols developed for the cow, nine *in vitro* produced blastocysts were cryopreserved. One embryo was transferred to a domestic cow which was confirmed pregnant on day 135 (Armstrong et al., 1995). Cryopreservation of gaur embryos was reported more than a decade earlier (Stover & Evans, 1984) however that report did not elaborate on the freezing protocol nor was any information provided as to the outcome of the procedure. Another member of this subfamily is the wood bison (*Bison bison athabasca*), a sub species of the North American bison. Using IVM, IVF, IVC and vitrification protocols developed for bovine, *in vitro*-produced embryos were vitrified (Thundathil et al., 2007). Regrettably, protocols that works very well for cattle, gave fairly poor results in wood bison. Only 6.9% (11/160) of the embryos reached the blastocyst stage. Morula-stage (n=27) and blastocyst-stage (n=6) embryos were vitrified. Disappointingly, the researchers failed to report on the evaluation of the embryos after warming.

Camelids are seasonal breeders and induced ovulators. *In vivo*-produced embryos of dromedary camel (*Camelus dromedarius*), collected at the blastocyst stage, were vitrified. Post-warming survival and intact morphology were high (92%) and following transfer of 45 embryos (20 during the breeding season and 25 off-season), three pregnancy were obtained, one of which was carried to term (Nowshari et al., 2005). This report follows a previous one in which cryopreserved embryos did not lead to a pregnancy after transfer (Skidmore & Loskutoff, 1999). Among the South American camelids, attempts have reached some level of success in the Llama (*Lama glama*) whose embryos were found to be three- to five-fold larger than bovine embryos of the same stage (Lattanzi et al., 2002). In one attempt, *in vivo* produced hatched blastocysts were either vitrified or frozen slowly (Lattanzi et al., 2002). After 24 h of *in vitro* culture, 64% (21/33) of the vitrified embryos and in 63% (12/19) of the slow freezing embryos re-expanded. In another attempt to vitrify llama embryos, 10/40 embryos re-expanded after warming (von Baer et al., 2002). Three fresh-chilled and two vitrified-warmed embryos were transferred to synchronized recipients but only one of the fresh embryos resulted in a pregnancy. In yet another report from about the same time, by a different group, success was achieved. *In vivo* produced embryos were collected non-surgically and vitrified at the expanded blastocyst stage. Eight embryos were transferred after warming to four recipients (two embryos, each) and two of them became pregnant, delivering two offspring (Aller et al., 2002).

As for other members of this group, some but very modest success have been reported on cryopreservation of domestic species like the horse (Yamamoto et al., 1982; Slade et al., 1985; Barfield et al., 2009; Choi et al., 2009) and swine (Nagashima et al., 1995; Dobrinsky et al., 2000) but very little success have been reported in other species.

### 3.2.1.3 Carnivores

The order Carnivora includes two suborders – Caniformia (dog-like species) and Feliformia (cat-like species). Similar to cows among the ungulates, the domestic dog (*Canis lupus familiaris*) and cat (*Felis catus*) are representatives of these two suborders and are highly accessible in terms of their frequent use as laboratory animals and the availability of large number of ovaries from neutered or euthanized animals. Still, despite these similarities and their being members of the same order, embryo cryopreservation and all associated technologies are highly developed for cats but lagging far behind in dogs. The domestic cat was found to be a very suitable model for other felid species, which may partially explain why things are more advanced among felids. Felines are induced ovulators (the release of LH that leads to ovulation is induced by mating) and mostly seasonal breeders. The first report on successful IVF and IVC to the blastocyst stage in a cat came in 1977 (Bowen, 1977). Eleven years later the first in-depth study on cat IVF and the first report on birth of live kittens after embryo transfer of cryopreserved, *in vivo*-derived embryos at the morula stage were published (Dresser et al., 1988; Goodrowe et al., 1988). Cryopreservation was carried out using the slow freezing technique and glycerol as cryoprotectant. However, success rate of embryo transfer was relatively low (14.4%, 17/118), most probably because all thawed embryos were transferred, regardless of their grade. Subsequently, production of offspring after transfer of *in vitro*-derived embryos from *in vivo* and *in vitro* matured oocytes and with or without post thaw culture were described (Pope et al., 1994; Wolfe & Wildt, 1996; Pope et al., 1997b; Wood & Wildt, 1997; Pope et al., 2002). Recently it was suggested that removing some of the lipids from the embryo before cryopreservation, a process known as delipidation, result in higher survival rate and higher rates of post-thaw development to morula and blastocyst stages (Tharasanit & Techakumphu, 2010).

Differences between the domestic cat and other feline species still exist and transfer of knowledge is not entirely straightforward. Still, following the success in the domestic cat, maturation and *in vitro* fertilization of oocytes from a large number of feline species was demonstrated (Johnston et al., 1991). This included tiger (*Panthera tigris*), lion (*Panthera leo*), leopard (*Panthera pardus*), jaguar (*Panthera onca*), snow leopard (*Panthera uncia*), puma (*Felis concolor*), cheetah (*Acinonyx jubatus*), clouded leopard (*Neofelis nebulosa*), bobcat (*Lynx rufus*), serval (*Felis serval*), Geoffroy's cat (*Felis geoffroyi*), Temminck's golden cat (*Felis temmincki*), and leopard cat (*Felis bengalensis*). A total of 846 oocytes were recovered from ovaries of 35 individuals from these 13 species, 508 of them were of fair to excellent quality, yet only 4 (0.8%) cleaved – one of leopard using homologous sperm and three of puma using domestic cat sperm. Matured oocytes were achieved in all but fertilization was not achieved in jaguar, cheetah, clouded leopard, bobcat and Temminck's golden cat. In another study, on puma, 6/25 recovered oocytes fertilized and five of them cleaved (Jewgenow et al., 1994). Success in IVF came at about the same time in other species, e.g. in the tiger (Donoghue et al., 1990), the Indian desert cat (*Felis silvestris ornata*) (cited in Pope, 2000) or leopard cat (*Felis bengalensis*) (Goodrowe et al., 1989). Pope (2000) also mentions IVF/ET in African wild cat (*Felis silvestris lybica*) but pregnancy here ended with stillbirths.

Over the past decade or so, several reports on embryo cryopreservation in felids appeared in the scientific literature. Some investigators, using the domestic cat as a surrogate mother for frozen-thawed embryos of similar-sized wild feline species, produced offspring of ocelot (*Felis pardalis*) (Swanson, 2001) and the African wild cat (Pope et al., 2000). Transfers of frozen-thawed embryos to conspecific recipients have often failed to produce live offspring. In clouded leopard, no pregnancies were achieved with either frozen-thawed or control embryos (Pope et al., 2009). Similarly, frozen-thawed morula-stage cervel embryos failed to result in pregnancies after transfer (Pope et al., 2005). In the bobcat (*Lynx rufus*), out of three transferred embryos – two fresh and one frozen-thawed, one pregnancy (from a fresh embryo) was achieved (Miller et al., 2002). Failure, however, was not a universal phenomenon. In the ocelot (*Felis pardalis*) over 80 IVF embryos, representing 15 founders of the North American population of this species were cryopreserved for safekeeping (Swanson, 2003) and two pregnancies were established following laparoscopic transfer of frozen-thawed embryos (Swanson, 2006). IVF was also carried out in tigrina (*Leopardus tigrinus*), another South American wild felid, and the resulting embryos (n=52) were cryopreserved (Swanson et al., 2002). Regrettably, the researches failed to report on post-thaw evaluation. In caracal (*Felis caracal*) from 452 recovered matured oocytes, 297 embryos were produced. Additional 16 embryos were produced following IVM of 83 oocytes. A total of 109 embryos were cryopreserved using slow freezing. Of nine recipients, three became pregnant and three kittens were delivered (Pope et al., 2006). Vitriification was also attempted in wild felids and was shown to produce superior results as compared to slow freezing. Siberian tiger (*Panthera tigris altaica*) oocytes were collected by laparoscopy from chemically stimulated ovaries. Following IVF with frozen-thawed sperm and IVC to the 2- to 4-cell stage, embryos were cryopreserved by either slow freezing or vitrified. None of the slow freezing embryos survived (0/89). From those vitrified, 46% (32/70) survived (Crichton et al., 2000; Crichton et al., 2003).

Whereas some success has been achieved in felids, situation is lagging far behind in canids and progress has been slow (Farstad, 2000a, b). Associated ART techniques such as IVM, IVF and IVC still face many difficulties and outcome is often unpredictable, most probably because *in vitro* culture media and conditions are not optimized for this group (Rodrigues & Rodrigues, 2006; Mastromonaco & King, 2007). In the vast majority of the studies, dog zygotes did not progress to the advanced embryonic developmental stages – morula and blastocyst (Rodrigues & Rodrigues, 2006). The first successful embryo cryopreservation in dogs, leading to pregnancy after ET, was reported only in 2007 (Abe et al., 2007) and pup delivery following embryo cryopreservation came two years later (Suzuki et al., 2009). This success was later repeated with *in vivo*-produced embryos using vitrification as the cryopreservation method (Abe et al., 2011). Canine females are unique in their reproductive cycle in the fact that the ovulated oocytes are still immature and their maturation may take two or more days (estimated at 48 to 60 h) while in the distal uterine horn. Also unique is the fact that luteinization and the increase in progesterone actually occur before ovulation (Reynaud et al., 2005; Chastant-Maillard et al., 2011; Concannon, 2011). The extra-follicular maturation process has proved hard to mimic and to date *in vitro* maturation and fertilization are not yet developed in dogs. The bitch anatomy makes retrieval of *in vivo*-produced embryos very difficult, leading researchers to resort to a complete surgical removal of the uterus and associated structures, a procedure that limits its application. From the same reason, embryo transfer was also done surgically until the recent development of a non-surgical technique (Abe et al., 2011). Canine oocytes and early-stage embryos also have

high lipid content (Reynaud et al., 2005) making their cryopreservation challenging. These multiple factors are responsible for the slow progress in ART developments in canids. Despite extensive search, the only report on embryo cryopreservation in a non-domestic canid found in the scientific literature is a few words on a trial with blue fox (*Alopex lagopus*) embryos. These were cryopreserved by slow freezing and vitrification and were later transferred to recipients. Although no live pups were achieved, two implantation sites from each of the two cryopreservation techniques were found. (Personal communication with H. Lindeberg, cited in Farstad, 2000a).

Some progress has also been reported in other carnivore families. In the Mustelidae family, a member of the caniformia suborder, some species are of commercial value, primarily in the fur industry. These include, for example, the European polecat (*Mustela putorius*) and the American mink (*Mustela vison* or *Neovision vison*). Other members in this family are listed as endangered or critically endangered species, including the black-footed ferret (*Mustela nigripes*) and the European mink (*Mustela lutreola*). The species of commercial value can thus act as models for developing reproduction technologies and for gaining needed knowledge on specific attributes of the Mustelidae family. European polecat, for example, acted as a model for the European mink and the first successful embryo cryopreservation in this family was reported in this species (Lindeberg et al., 2003). Surgically recovered *in vivo*-produced European polecat embryos were cryopreserved by slow freezing and resulted, following surgical transfer, in 3/8 pregnancies and nine pups were delivered out of a total of 93 embryos transferred (9.7%). A second paper by the same group (Piltti et al., 2004) reported on the first successful embryo vitrification in carnivores. Out of 98 European polecat *in vivo*-produced embryos at the morula and blastocyst stages, 50 survived and were transferred to four recipients. Two of the recipients delivered a total of eight pups, a success rate similar to that of slow freezing (8/98; 8.2%). Further improvements came when a different vitrification technique, pipette tip, was used. Using this technique, 43.6% (44/101) of the embryos survived vitrification and resulted in live births (Sun et al., 2008). Vitrified embryos that were cultured for two or 16 h before transfer resulted in success rate (71.3% and 77.4% live births, respectively) similar to that of the control (79.3%) and significantly higher than in embryos cultured for 32 h (25%) and 48 h (7.8%).

#### 3.2.1.4 Glires - rodents and lagomorphs

Mouse was the first animal in which embryo cryopreservation was reported (Whittingham et al., 1972; Wilmut, 1972). Since then work on glires has largely concentrated on mice, rats, gerbils, hamsters and rabbits - all species in extensive laboratory use. The major cryoprotectant used for freezing embryos in this group is Me<sub>2</sub>SO. Although vitrification seems to be gradually taking the lead and many studies claim similar results to fresh controls, a recent meta-analysis found that vitrification is still inferior to fresh embryos (Manno III, 2010). It also found that a variety of covariates are associated with vitrified but not fresh embryos. These include issues such as the time lapse between hCG treatment and embryo cryopreservation, maternal age, and the time from hCG treatment to post-warming assessment. These and possibly other factors might be the result of heterogeneity of conditions of the studies included in such analysis but they can also be real factors arising from the process of cryopreservation. In rabbits, using *in vivo* produced embryos and either slow freezing (Bank & Maurer, 1974; Whittingham & Adams, 1974, 1976) or vitrification (Popelkova et al., 2009; Mocè et al., 2010), resulted in fairly high survival (up to 83%) and pregnancy (up to 92%) rates. However, rate of young born was still relatively low, in the range of 7 to 17% (Bank



& Maurer, 1974; Whittingham & Adams, 1974, 1976). In rats both slow freezing and vitrification were attempted, with considerably better results in the latter. *In vivo* produced embryos at the two-, four- and eight-cell stages were recovered and frozen with 3.0M Me<sub>2</sub>SO. Post-thaw normal morphology recovery rate ranged between 65% and 68%. Rate of embryos carried to term, however, was low - 11% for two-cell embryos, zero for four-cell embryos and 9% for eight-cell embryos (Whittingham, 1975). In contrast, in the vitrification study, 79% (117/149) of the vitrified *in vivo* produced blastocysts were morphologically normal after warming. These were split between *in vitro* culture (n=48) and transfer to recipient rats (n=69). All cultured embryos progressed to expanded and hatched blastocysts and of the 69 embryos transferred, 41% (n=28) resulted in live pups (Kono et al., 1988). The golden hamster, also known as the Syrian hamster (*Mesocricetus auratus*), is another member of this group in frequent use as a laboratory research subject. *In vivo* produced embryos at the one- and two-cell stages were flushed and vitrified by the cryoloop technique (Lane et al., 1999). Of 216 vitrified two-cell embryos, 54.2% continued development to the morula/blastocyst stage after warming. Such embryos were transferred to two recipients who delivered 6 pups. In another study, *in vivo* produced embryos at the eight-cell stage were vitrified in 250µL straws, following the technique developed for mouse embryos (Mochida et al., 2000). This study evaluated only *in vitro* development and this was fairly poor, as only two out of 37 embryos developed to the blastocyst stage. Similar to the hamster, *in vivo*-produced Mongolian gerbil (*Mesocricetus auratus*) embryos were vitrified in 250µL straws (Mochida et al., 1999). Following vitrification, 155 embryos developed to the blastocyst stage were transferred to 10 synchronized females, 3 of which became pregnant and delivered 15 pups (9.7%). In a follow-up study by the same group it was shown that embryos at later developmental stages (four-cell, morula and blastocyst) can also be vitrified and result in very high post-warming normal morphology (ranging between 87% and 100%) (Mochida et al., 2005). In this last study, after transfer into recipient females, 3% (4/123), 1% (1/102), 5% (4/73), and 10% (15/155) of embryos developed to full-term offspring from vitrified-warmed early two-cell embryos, late two-cell embryos, morulae, and blastocysts, respectively. The general tendency in all glires seem to be the same - post-thaw/warming *in vitro* quality of the embryos is good but when transferred to recipient females, only around 10% of transferred embryos develop to term. The study by Kono and colleagues (1988) with the reported 41% pups delivered is the exception to this rule. When vitrification was attempted, it seems to result in better outcome.

### 3.2.1.5 Marsupials

Marsupials are very different from eutherian mammals in many respects, attributes related to their oocytes is one of them. Their oocytes are about twice as large as those of humans (about 200 to 250 µm vs. 100 to 120 µm in humans) (Rodger et al., 1992; Breed et al., 1994). The size is probably that large because of the very large yolk sac that occupies much of the cell volume. The much larger volume and the large yolk compartment make their cryopreservation even more difficult than that of the already hard-to-cryopreserve eutherian oocytes. The alternative is to cryopreserve embryos and in that direction only a single report was found (Breed et al., 1994). In that study, *in vivo*-produced embryos of the carnivorous fat-tailed dunnart (*Sminthopsis crassicaudata*) were cryopreserved by slow freezing or vitrification. Me<sub>2</sub>SO proved to be not suitable for vitrification of embryos in this species as none of the embryos vitrified with this cryoprotectant cleaved after warming. Embryos cryopreserved by slow freezing or vitrification (with ethylene glycol as cryoprotectant) had similar cleavage rates or 17% and 18%, respectively. Even when morphological examination

found embryos to be normal, examination by electron microscopy revealed multiple damages to intracellular components.

### 3.2.2 Cetaceans

Only very few studies have reported attempts at cryopreservation of marine mammals oocytes and the only ones I was able to locate were on the common minke whale (*Balaenoptera acutorostrata*). These include studies on both slow freezing (Asada et al., 2000; Asada et al., 2001) and vitrification (Iwayama et al., 2005; Fujihira et al., 2006). To date, no study reporting embryo cryopreservation in cetaceans has been published (O'Brien & Robeck, 2010).

### 3.2.3 Non-mammal vertebrates

Whereas embryo cryopreservation in mammals shows some success, at least in those extensively studied species, situation lagging far behind in all other vertebrates (fishes, birds, reptiles and amphibians). It is true that considerably less efforts have been invested in embryo cryopreservation in most members of these groups, but the more important cause is the different structure embryos in these vertebrates have, difference that complicates their cryopreservation. From the little that has been done in these vertebrates, the vast majority of studies were done on fish (primarily the zebrafish; *Danio rerio*) and to a lesser extent also in amphibians – the two classes with the smaller oocytes among the non-mammalian vertebrates. The ensuing discussion will therefore be primarily on fishes as representatives for these classes. When sex chromosomes are the determination method, as is the case in most vertebrates, either the male or the female can be the heterogametic sex. In mammals the male carry both X- and Y-chromosomes while the female carries two copies of X-chromosome. In birds, on the other hand, it is the female that carry the Z- and W-chromosomes while the male carries two copies of the Z-chromosome. In fishes and amphibians both systems can be found. To have both chromosomes represented, one should aim to at least preserve enough gametes of the heterogametic sex. In many of the non-mammal species this means preserving the female's gametes, which, as will be discussed here, is problematic. Several attributes differentiate oocytes in these classes from those of mammals. To start with, they are considerably larger, resulting in lower surface area to volume ratio. For example, while the diameter of human oocyte is ~120  $\mu\text{m}$  or that of the mouse is ~80  $\mu\text{m}$ , oocyte of the zebrafish is ~750  $\mu\text{m}$  (Selman et al., 1993) or that of the marsh frog (*Rana ridibunda*) is ~1,400  $\mu\text{m}$  (Kyriakopoulou-Sklavounou & Loumbourdis, 1990), oocytes of the American alligator (*Alligator mississippiensis*) are ~4,000  $\mu\text{m}$  (Uribe & Guillette, 2000), those of the pink salmon (*Oncorhynchus gorbuscha*) in the range of 5,150 to 6,340  $\mu\text{m}$ , and the sizes go even higher in snakes such as kingsnakes (genus: *Lampropeltis*) with diameter of about 22,000  $\mu\text{m}$  (Tryon & Murphy, 1982), and birds like the Japanese quail (*Coturnix coturnix japonica*) – 17,000 to 19,000  $\mu\text{m}$  (Callebaut, 1973) or the domestic chicken (*Gallus gallus domesticus*) with a diameter of about 35,000 to 40,000  $\mu\text{m}$  (Schneider, 1992). The consequence of this is relatively poor water and cryoprotectant movement across the cellular membrane during chilling, freezing and thawing. The difference in size also means considerably larger volume of water to vitrify, thus greatly increasing the risk for intracellular ice formation and cell death. Fish embryos contain a large yolk compartment, enclosed in the yolk syncytial layer (YSL). The behavior of the yolk during freezing defer

from the behavior of other embryonic compartments, making freezing very complex. These embryos have at least three membrane structures (YSL, plasma membrane of the developing embryo and the chorionic membrane which surrounds the perivitelline space) (Kalicharan et al., 1998; Rawson et al., 2000). Each of these membranes has a different permeability coefficient for water and cryoprotectants, resulting, for example, in water permeability in the range of one order of magnitude lower in fish embryos compared to other animals - 0.022 to 0.1  $\mu\text{m} \times \text{min}^{-1} \times \text{atm}^{-1}$  in zebrafish (Hagedorn et al., 1997a) compared to 0.722 in *Drosophila* (Lin et al., 1989) or 0.43 in mice (Leibo, 1980). To complicate things even further, the different embryonic compartments have different water content and different osmotically inactive water content (Hagedorn et al., 1997b). Since the chorionic membrane can be removed enzymatically (by pronase) and its removal does not hinder embryonic development (Hagedorn et al., 1997c), Hagedorn et al. (1997a) suggested that the YSL was the primary barrier to cryoprotectants resulting in the yolk sac reaching lower levels of cryoprotection compared to other embryonic compartments. Using magnetic resonance microscopy, they have shown that while no cryoprotectant injected into the yolk was able to leave, some cryoprotectant was able to enter the blastoderm (Hagedorn et al., 1996). Attempts to solve this permeability issue by adding aquaporin 3 water channels to the zebrafish embryonic membranes (Hagedorn et al., 2002) or inserting cryoprotectants into the yolk by microinjection (Janik et al., 2000) were unsuccessful. Efforts to test various permeating and non-permeating cryoprotectants including methanol,  $\text{Me}_2\text{SO}$ , glycerol, 1,2-propanediol, PG, EG, trehalose, and sucrose also took place. Embryos were shown to be very sensitive to glycerol and EG at a concentration of 1.5M, but less so to methanol,  $\text{Me}_2\text{SO}$  or PG (Hagedorn et al., 1997c). Studies also showed that later-stage embryos were less chilling sensitive than early-stage ones and thus probably more suitable for cryopreservation (Zhang & Rawson, 1995). However, attempts to cryopreserve fish embryos by slow freezing or vitrification generally met with lack of success. (reviewed in Robles et al., 2009). For instance, when intact embryos were cryopreserved by slow freezing, only about 2% of the cells in them survived the process (Harvey, 1983). Attempts were also carried out to cryopreserve amphibian (the frog *Xenopus*) oocytes with similar lack of success (Guenther et al., 2006; Kleinhans et al., 2006).

So, if oocytes and embryos are not an option at the moment, the alternatives are blastodermal cells and primordial germ cells. These cells can be cryopreserved by slow freezing (Naito et al., 1992; Naito et al., 1994) or vitrification (Kohara et al., 2008; Higaki et al., 2010) with good overall post-thaw/warming viability. Goose blastodermal cells, cryopreserved by slow freezing resulted in relatively low survival rate of 25% or less, depending on the cryovial used (Pataki Varkonyi et al., 2007). In another study, quail blastodermal cells were isolated, cryopreserved and the thawed viable cells were used to create quail-chicken chimeras (Naito et al., 1992). Chicken primordial germ cells had survival rate of  $85.8 \pm 1.2\%$  and  $91.2 \pm 2.8\%$  for vitrified-warmed and frozen-thawed cells, respectively with no significant difference between treatments and the control (Kohara et al., 2008). Blastodermal cells can be used to create chimeras, which are organisms made out of cells from two or more donors with different genetic background. Using this system, duck blastodermal cells were injected into the subgerminal cavity of same stage gamma-irradiated chicken embryo to produce duck-chicken chimeras (Li et al., 2002). These chimeras were mated with ducks to produce six duck hatchlings (out of 622 eggs collected) indicating that, albeit at low efficiency, this system can produce offspring of the

blastodermal cells donor. The alternative, which seems to have higher potential from conservation point of view, is the preservation of primordial germ cells. These can later be allo- or xenotransplanted to produce viable offspring of the donor. As a demonstration of concept, primordial germ cells from pheasant (*phasianus colchicus*) were injected into the bloodstream of domestic chicken (*Gallus gallus domesticus*) embryos to produce pheasant-chicken chimeras (Kang et al., 2008). Back-crossing chimera males with pheasant females produced 10 pheasant chicks with an efficiency of 17.5%. Chimera offspring were also generated in zebrafish by transplanting GPC from various sources including vitrified embryoid, an aggregate of cells derived from embryonic stem cells (Kawakami et al., 2010). The male chimeras were then mated with normal females through natural spawning to produce offspring.

In conclusion, cryopreservation of embryos in the few mammalian species in which it was attempted shows some, though very limited, success. The situation is much less advanced in all other vertebrates (fish, birds, reptiles and amphibians) where noticeably less efforts have been invested and the challenges are often considerably more complex. In comparison to mammals, embryos in all these classes are usually larger in volume, with large amount of yolk and multiple membranes showing varying permeability to water and cryoprotectants. All these make embryos in these classes highly susceptible to chilling injury and, with the currently available knowledge and techniques, make their cryopreservation extremely complicated and often practically impossible. The alternative approach, at least for now, would therefore be to preserve blastodermal cells and primordial germ cells, which can be transplanted into host embryos to produce offspring.

### 3.3 Ovarian tissue cryopreservation

Cryopreservation of ovarian tissue has several advantages over oocyte or embryo cryopreservation, but it also comes with its unique complications. As was discussed earlier, in the section on testicular tissue cryopreservation, tissue is a complex structure and thus presenting many difficulties with respect to cryopreservation. Ovarian tissue is available at any time, season, stage in cycle, and age – from fetus to old to deceased. It contains large number of oocytes and, to overcome the problems associated with *in vitro* development and maturation, it can be implanted so that this can take place *in vivo* (Candy et al., 1995) or after partial development *in vivo*, oocytes can be retrieved and matured *in vitro* (Liu et al., 2001). Ovarian tissue also contains premeiotic germ cells, even in aged animals whose ovaries are otherwise devoid of follicles (Niikura et al., 2009). By transplanting such ovaries into recipient young adult animals can help generate new follicles. Attempts to cryopreserve ovarian tissue were reported already in 1951 (Smith & Parkes), only two years after the same group discovered the protective effect of glycerol during freezing (Polge et al., 1949). The first live birth following ovarian tissue freezing and transplantation was reported in mice, in which the tissue was frozen to -79°C (Parrott, 1960). Grafts can be transplanted to the owner of the tissue (autotransplantation), to another member of the species (allotransplantation) or to a member of a different species (xenotransplantation). All three possibilities were successfully used to support follicular development in grafted tissue. When it comes to wildlife conservation, ovarian tissue will not be used in a similar manner to the way it is used in human medicine, namely retransplanted into its donor. Rather, these cryopreserved tissues will be used to collect oocytes by isolation and maturation *in vitro* or by transplanting them to immune deficient host animals (usually mice or rats) that will support oocyte



development *in vivo*. Although ovarian tissue grafting is usually done under the kidney's capsule where ample of blood vessels are found, other locations like subcutaneous grafting for easy access have also been reported (Cleary et al., 2003). Transplantation can be to either female or male recipient (Weissman et al., 1999; Snow et al., 2002) and, interestingly, in a study on human ovarian cortex transplantation to non-obese diabetic-severe combined immune deficiency (NOD-SCID) mice, more males (76.5%, 13/17) supported follicular development than females (30%, 6/20) (Weissman et al., 1999). In another study, while more xenografts were retrieved from females, the number of oocytes recovered from each xenograft was higher in those transplanted to males (Snow et al., 2002). Oocytes developed in males, however, showed reduced fertilizing ability and none of the transferred embryos resulted in implantation. The tissue, cut of its blood supply from harvesting till about 48h after transplantation, needs to rely on its surrounding for supply of oxygen and nutrients and removal of CO<sub>2</sub> and other wastes. If not completely lost or rejected, ischemia can thus lead to the death of more than half of the follicles in the graft (Candy et al., 1997). The surviving follicles, though may grow and develop after transplantation, often contain oocytes of suboptimal quality (Kim et al., 2005). Transplanted ovarian tissue, like any transplanted tissue, carries the risk of transmitting diseases from donor to recipients, a risk that is greatly elevated by the need to use immune-deficient recipients to reduce the risk of graft rejection. The alternative to grafting is growing the follicles to maturation *in vitro*. This, however, has been demonstrated thus far only in mice where primordial follicles (Eppig & O'Brien, 1996) or primary follicles (Lenie et al., 2004) were cultured successfully *in vitro*.

The standard cryopreservation protocol, which seems to work for many different species, is cryopreservation of ovarian cortical tissue slices with a size of 1 to 2 mm<sup>3</sup> in cryoprotective solution containing Me<sub>2</sub>SO, ethylene glycol or 1,2-propanediol. The tissue and the cryoprotective solution are equilibrated at 0°C and then again at -5 to -7°C. Seeding to initiate extracellular freezing is performed and the sample is then cooled at a slow and constant rate of 0.3°C to 0.5°C/min till somewhere between -30°C and -80°C, before being plunged into liquid nitrogen for storage (for review see Paris et al., 2004). An alternative technique proposed a few years ago does not require expensive equipment and is suitable for work under field conditions (Cleary et al., 2003). Following this technique, equilibration is performed on ice, and the sample is then placed in a passive freezing device that is placed on dry ice. Using this device, a cooling rate of about 1°C/min can be achieved. This is faster than optimal cooling rate but still tolerable. When freezing wombat (*Vombatus ursinus*) ovarian cortical tissue slices this way, 134 ± 32 intact follicles per graft were found compared to 214 ± 55 for the controlled-rate freezing machine.

Cryopreserved ovarian tissue, which was later auto-, allo- or xenografted, has been done in a variety of species including humans (Weissman et al., 1999; Gook et al., 2001; Gook et al., 2003; Donnez et al., 2004), non-human primates - rhesus macaque (*Macaca mulatta*) (Lee et al., 2004), cynomolgus macaque (*Macaca fascicularis*) (Schnorr et al., 2002) and common marmoset (*Callytrix jacchus jacchus*) (von Schönfeldt et al., 2011), bovine (Herrera et al., 2002), sheep (Gosden et al., 1994), cats (Gosden et al., 1994; Jewgenow et al., 1997; Bosch et al., 2004; Jewgenow & Paris, 2006; Luvoni, 2006), mice (Parrott, 1960; Liu et al., 2000; Liu et al., 2001), rabbits (Almodin et al., 2004), common wombat (*Vombatus ursinus*) (Wolvekamp et al., 2001; Cleary et al., 2003), African elephant (*Loxodonta Africana*) (Gunasena et al., 1998), Amur leopard (*Panthera pardus orientalis*) and African lion (*Panthera leo*) (Jewgenow et al., 2011), tammar wallaby (*Macropus eugenii*) (Mattiske et al., 2002), and Fat-tailed dunnart

(*Sminthopsis crassicaudata*) (Shaw et al., 1996). The last two are of special interest as they demonstrate that even when xenografting between species so phylogenetically distant as marsupials and mice, the graft is still supported and oocytes can develop. Primordial oocytes in ovarian tissue are probably less prone to cooling and cryopreservation damages when compared to mature ones because they are smaller in size and they lack zona pellucida. Still, recovery rate is low. In cats, for example, only 10% of the follicles survived freezing, thawing and transplantation-associated ischemia (Bosch et al., 2004). To overcome this low harvesting rate, multiple grafts are required.

The alternative cryopreservation approach that has been applied to gametes and embryos, namely vitrification, has been applied to ovarian tissue as well. Naturally, to achieve good cryoprotectant penetration and proper heat transfer the sample should be thin enough, normally in the range of 1 mm or less. Several groups have experimented with this approach, cryopreserving tissue samples from humans (Isachenko et al., 2009), mice (Salehnia et al., 2002), sheep (Baudot et al., 2007), pig (Gandolfi et al., 2006), cow (Kagawa et al., 2009), goat (Santos et al., 2007), dog (Ishijima et al., 2006) and cynomolgus and rhesus macaques (Yeoman et al., 2005). The general trend in recent years is for similar outcome from slow freezing and vitrification (see recent review by Amorim et al., 2011)

### 3.4 Whole ovary cryopreservation

Cryopreservation of large volumes, including whole organs, involves several aspects, which make any attempt at cryopreservation a challenge (Arav & Natan, 2009). These difficulties include: 1) the need for efficient heat transfer throughout the tissue. When a thick tissue or whole organs are involved, this is very difficult to accomplish, 2) the need for efficient cryoprotectant penetration to all cells in the tissue. This is challenging because of the tissue thickness and because different cell types in it have different permeability coefficients and different sensitivities. Excessive exposure time may be damaging to some cells in the tissue due to cryoprotectant toxicity while shorter time might not provide sufficient protection to others. Thus, the optimal time slot is to be identified, 3) supercooling (cooling below the solution's freezing point without crystallization) may take place in some parts of the tissue. This may lead to damages from uncontrolled intra- and extracellular ice formation once crystallization occurs, 4) attaining homogenous cooling rate while avoiding the excessive build-up of toxic concentrations of cryoprotectants, 5) during cryopreservation, latent heat is released from the solution. This released heat can induce recrystallization and extend the isothermal stage, resulting in the development of a large temperature difference between the tissue/organ and the surrounding. This may lead to faster-than-optimal cooling once all latent heat has been released, 6) recrystallization may also occur during thawing because of inhomogeneous warming of the sample. Still, if these issues can be overcome, whole ovary presents one very important advantage over ovarian tissue when it comes to cryopreservation. One of the major problems with cryopreserving ovarian cortical tissue is the ischemia the graft goes through when transplanted. This ischemia cause both graft loss and death of large portion of the follicles within surviving grafts. Cryopreserving whole ovary, including its vascular pedicle, can ensure blood supply as soon as the organ has been transplanted (Bromer & Patrizio, 2009). For the grafted ovary to become fully functional, both ovaries of the recipient should be removed (Liu et al., 2008). Grafting the ovary can be done to its natural position or to any other location in the body that may provide easy

access. Of course ovary transplanted to another location can produce oocytes that should be harvested for use *in vitro*. First whole ovary cryopreservation reported was in sheep (Revel et al., 2001; Revel et al., 2004). This report used directional freezing technique, which is claimed to provide a solution to many of the issues involved in large volume cryopreservation mentioned above (Arav & Natan, 2009). Most other cryopreservation experiments used controlled-rate freezing equipment to achieve the desired very slow ( $\sim 0.1^{\circ}\text{C}/\text{min}$ ) cooling rate needed. This first report was followed by reports on cryopreserving ovaries of various other species such as rats (Wang et al., 2002; Qi et al., 2008), mice (Liu et al., 2008), bovine (Arav, 2003), pigs (Imhof et al., 2004), human (Bedaiwy et al., 2006) and another study on sheep (Onions et al., 2009). In some of these studies, pregnancies were achieved and live young were produced. Interestingly, to date transplantation of cryopreserved whole human ovary has not been reported (Bromer & Patrizio, 2009) despite the fact that ovarian transplantation has been in practice for several years now and whole human ovary cryopreservation was attempted by several researchers.

Although vitrification is an attractive procedure for cryopreservation of whole ovaries, the current knowledge in cryobiology is insufficient to overcome the multiple problems involved in large volume vitrification (Fahy et al., 1990), primarily when tissue, rather than suspension, is involved. Keeping in mind the relationship between the three factors determining the probability of vitrification mentioned earlier (see section on semen vitrification and also Saragusty & Arav, 2011), to avoid cryoprotectant toxicity, very high cooling rates and very small sample volume are needed. Attempts at whole ovary vitrification did take place and in some cases, when the ovaries were sufficiently small, were even successful. An attempt to vitrify whole sheep ovary resulted in complete loss of all follicles (Courbiere et al., 2009). On the other hand, in studies on mice and rats, vitrification of whole ovary was successful (Migishima et al., 2003; Hoshina et al., 2009). One study showed acceptable post warming viability by *in vitro* evaluations of mice ovaries (Migishima et al., 2003). In another study follicular growth was demonstrated after autotransplantation under the kidney capsule of vitrified warmed rat ovaries (Sugimoto et al., 2000). In yet another study, live offspring were produced when the donor mice were transgenic so that their ovaries expressed anti-freeze protein type III as an additional mean of cryoprotection (Bagis et al., 2008).

With the big potential whole ovary cryopreservation holds for wildlife conservation, this procedure is yet to be reported in any animal other than laboratory or domestic species.

#### **4. Options equally good for both males and females**

Some options, as will be discussed in the following sections, are available for both sexes. These options are still largely experimental in nature, their efficiency is often low and they require well equipped laboratories with highly experienced staff so their widespread implementation in wildlife conservation is probably still years down the road. They are, however, worthy of mentioning because of the great potential they hold. These, and many of the options described in the previous sections, are not and may never become widely used techniques. They are also nowhere near the decades old slow freezing and vitrification and so, to be on the safe side one should probably opt for cryopreservation of gametes and embryos using one of the available techniques. However, by definition endangered species are species whose global population is small and declining. This means that with time the genetic diversity of such populations is dwindling. If we do not set up collections of samples

(gametes, embryos, somatic cells, or anything else we can put your hands on) of the genetic diversity, and just sit and wait for some new technology to come by or for breakthrough in one of the still experimental technologies at hand, genetic diversity within species and possibly entire species will be lost for ever. We should therefore aim to create banks that will hold samples from each endangered species on earth and of as wide a diversity of genetic make up as possible in each. Cryopreservation is a more mature technology for this purpose but many other options are advancing and may one day play an important role in long-term banking for wildlife conservation. New and much better technologies may emerge with time but we cannot sit and watch species going extinct and take no action. Collections should be created with any and all possible technologies in mind.

#### 4.1 Somatic cells cryopreservation for SCNT

To produce embryos *in vivo* or *in vitro*, conspecific spermatozoa and good quality oocytes are required, both or either of which often prove very difficult to obtain. An alternative that can circumvent this, at least in part, is preservation of somatic cells, to be later used for somatic cell nuclear transfer (SCNT, Wilmut et al., 1997). In SCNT, also known as cloning, nucleus of a somatic cell is microinjected into enucleated oocyte, which is then grown *in vitro* and can be later transferred to recipient females for development to term, with or without a cryopreservation step in between. Somatic cells from a wide variety of sources can be used for this purpose. Such diverse sources include cells from tissues preserved without cryoprotectant at -80°C for more than a decade, or cells from tissues kept at -20°C for as long as 16 years (Hoshino et al., 2009), cells isolated from mummified animals (Kato et al., 2009), freeze-dried somatic cells (Loi et al., 2008a; Ono et al., 2008; see next section), semen-derived somatic cells (Nel-Themaat et al., 2008a; Nel-Themaat et al., 2008b; Liu et al., 2010), cells collected postmortem (Oh et al., 2008), cell line (Campbell et al., 1996), and of course both fetal and adult cells are suitable for this purpose (Wilmut et al., 1997). SCNT has indeed an obvious potential for the multiplication of rare genotypes (Corley-Smith & Brandhorst, 1999; Loi et al., 2008a; Loi et al., 2008b), but its wide application is prevented by the currently low efficiency in terms of offspring outcome. To date, successful cloning was reported in sheep (Campbell et al., 1996; Wilmut et al., 1997; Loi et al., 2008a; Loi et al., 2008b), cow (Cibelli et al., 1998), mice (Wakayama & Yanagimachi, 1998), goat (Baguisi et al., 1999), pigs (Polejaeva et al., 2000), cats (Shin et al., 2002), dogs (Jang et al., 2007), rabbits (Chesne et al., 2002), ferrets (Li et al., 2006), mule (Woods et al., 2003), horse (Galli et al., 2003), gaur (*Bos gaurus*) (Lanza et al., 2000), buffalo (*Bubalus bubalis*) (Lu et al., 2005; Shi et al., 2007), mouflon (*Ovis orientalis musimon*) (Loi et al., 2001), African wild cat (*Felis silvestris libica*) (Gómez et al., 2003), wolves (*Canis lupus*) (Kim et al., 2007), mountain bongo antelope (*Tragelaphus euryceros isaaci*) (Lee et al., 2003) and eland (*Taurotragus oryx*) (Nel-Themaat et al., 2008b). When dealing with already extinct species, we can anticipate survival of nucleus DNA but not for viable oocytes. The only hope is then to use oocytes from closely related species. Interspecies SCNT (ISCNT), performed by injecting the nucleus from one species into the oocyte of another has also been carried out in a variety of species (for a recent review see Loi et al., 2011a). These include ISCNT from the endangered mouflon to a domestic sheep (*Ovis aries*) (Loi et al., 2001), from red panda (*Ailurus fulgens*) to rabbit (Tao et al., 2009), from sand cat (*Felis margarita*) to domestic cat (Gómez et al., 2008), from Canada lynx (*Lynx canadensis*) to both domestic cat and caracal (*Caracal caracal*) (Gómez et al., 2009), from water buffalo (*Bubalus bubalis*) to cow (*Bos taurus*) (Srirattana et al., 2011), and most strikingly – from a



15,000 year-old woolly mammoth (*Mammuthus primigenius*) to a mouse (Kato et al., 2009). While this technique holds much promise for the resurrection of extinct species and saving those on their way there (Loi et al., 2011b), with the exception of a few sporadic instances, all these attempts at ISCNT did not result in live offspring. Cryopreservation of reproductive tissue or any other viable body tissue or, alternatively, of *in vitro* grown cell cultures is routinely done in many places around the world and enough cells survive the process to be used in SCNT. Furthermore, obtaining tissue samples is usually much simpler than collecting gametes or embryos, so a larger and more diverse collection can be accumulated.

While SCNT has the advantage that no genetic drift takes place because recombination does not occur, when considering SCNT for wildlife species preservation, several important issues should be taken into consideration. First, as mentioned above, suitable enucleated oocytes are required. The availability of such oocytes and the ability to access them should thus be part of the program (Loi et al., 2011b). If conspecific oocytes are not available, the issues of mitochondrial inheritance and nucleus-cytoplasmic incompatibility become a problem and ways to overcome these should be sought for. When the donor and recipient are close enough, some of the donor mitochondria get transferred as well (Gómez et al., 2009; Srirattana et al., 2011). As was demonstrated for the famous sheep, Dolly, the telomere is shorter following SCNT (Shiels et al., 1999). Interestingly, it was recently shown that cloned cows with short telomeres produce normal and healthy offspring with normal telomere length following artificial insemination with sperm from normal bulls (Miyashita et al., 2011). This study suggests that cloning does not interfere with the eventual function of the germ line. Cloned offspring, however, are known to show elevated prevalence of developmental abnormalities and high mortality rate, issues that should be kept in mind when initiating a cloning program (e.g. Lanza et al., 2000). One should also keep in mind that the spermatozoa carry more than just genetic material. They come with a whole load of epigenetic factors important for proper embryonic development (Yamauchi et al., 2011). These are missing when SCNT is performed and might be one of the causes behind the relatively low efficiency of the process. As with cryopreservation of other cells and tissues, storage space and costs and environmental impact are major issue pertaining to liquid nitrogen storage so a cheaper alternative would be very attractive for long-term conservation purposes.

#### 4.2 Somatic cell drying for SCNT

In tissue banking, as in the banking of germ cells and embryos, storage and maintenance costs are always an issue because of the properties of liquid nitrogen. Seeds of plants, having low water content are relatively easy to preserve at high subzero temperatures (-20 to -30°C). With water content of about 80%, preservation of gametes and embryos in the animal kingdom is complicated and species-specific. The use of large quantities of liquid nitrogen for cryopreservation and storage also has its toll on the environment, as the production of liquid nitrogen is energy-intensive, resulting in the release of large quantities of carbon dioxide. An alternative to cryopreservation of somatic cells, then, can be to dry them and store the dry cells at room temperature. While, as was discussed earlier, sperm drying has been achieved in a number of species, the parallel in females, namely oocyte drying, is yet to be demonstrated. Somatic cell drying is thus the way to go when long-term storage for females or of the entire genetic complement is desired. In this respect, the use of sheep freeze-dried somatic cells for SCNT was recently demonstrated (Loi et al., 2008a; Loi et al., 2008b). In their report, utilizing the directional freezing technology, freeze-dried

granulosa cells, kept at room temperature for 3 years, were used to direct embryonic development following nuclear transfer into *in vitro* matured enucleated oocytes. The reconstructed oocytes initiated cleavage at similar rates to control embryos generated using fresh granulosa cells. Microsatellite DNA analysis of the cloned blastocysts matched perfectly with the lyophilized donor cells. Later, these results were confirmed by other researchers studying mouse granulosa cells (Ono et al., 2008), human hematopoietic stem and progenitor cells (Buchanan et al., 2010) or porcine fetal fibroblasts (Das et al., 2010). These studies demonstrate for the first time that dry cells maintain the development potential when injected into enucleated oocytes. Naturally, we still have a long way to go before live offspring will be generated using this technology but the potential is there.

### 4.3 Stem cell preservation

Embryos can be a source for primordial germ cells (PGC) which, as was shown in the zebrafish, can be vitrified, warmed and then transplanted into sterilized recipient blastulae to differentiate into males and females that produced gametes carrying the genetic material of the transplanted PGC donor (Higaki et al., 2010). Such PGC can be transplanted, along with gonadal somatic cells, and develop into normal male or female gonadal tissue with normal spermatogenesis or oogenesis. Both mouse round spermatids and GV oocytes derived from such tissues were able to direct embryonic development to term following ICSI (Matoba & Ogura, 2010). In a recent study on felids (Silva et al., 2011) it was shown that such germ line stem cells can be transplanted to the gonads of a different species and still develop normal early stage gametes. In that study, ocelot (*Leopardus pardalis*) spermatogonial stem cells were transplanted into domestic cat testis and thirteen weeks later ocelot spermatozoa were retrieved from the cat's epididymis.

Going even earlier in the development timeline, embryos can be a source for stem cells. Embryonic stem cells, being pluripotent, can differentiate *in vivo* or *in vitro* into germ cells. They can also be used for nuclear transfer. So, they, too, can be considered an optional venue. In a study on mice, transplanted embryonic stem cells were able to form testicular tissue structures and direct spermatogenesis (Toyooka et al., 2003). These cells, which can be isolated from embryos, can also be cryopreserved (Thomson et al., 1998; Toyooka et al., 2003) or vitrified (Reubinoff et al., 2001; He et al., 2008). Such stem cells can also be derived from embryos generated by nuclear transfer of freeze-dried cells (Ono et al., 2008). Embryonic stem cells can also be derived from isolated blastomeres, and blastomeres can also be cryopreserved individually by inserting them into emptied zona pellucida and then vitrifying them (Escriba et al., 2010). If embryonic stem cells are not available, somatic cells can be induced to become embryonic stem cells-like (Takahashi & Yamanaka, 2006), also known as induced pluripotent stem cells or iPS cells (for recent review see: Cox & Rizzino, 2010). Being pluripotent in nature, they are also germ line competent (Okita et al., 2007) and as such can give rise to germ cells of both male and female.

The fantastic options mentioned above are theoretical and speculative in nature when it comes to wildlife preservation as currently these techniques are in their infancy and were adapted thus far only to laboratory animals, and even in these the unknown is still vast.

## 5. Conclusion

With the dramatically accelerated species extinction rate we see in recent decades, it is our obligation to seek any possible venue to bring this biodiversity loss to a halt and, while

attempting to do so, to seek ways to safe-keep gametes, embryos and somatic cells from (ideally) all species on Earth. To our great disadvantage from a cryobiologist standpoint, species are different from each other and preservation techniques almost invariably require species-specific customization. As was discussed here, there are many options for 'putting life on hold'. Cryopreservation is by far the most advanced and widely used technique that has lead to the establishment of several genome resource banks. Within cryopreservation, slow freezing currently holds the leading role but at least for oocytes, and slowly for embryos too, vitrification is gradually replacing it to become the cryopreservation technique of choice. Due to its small size, condensed DNA and little cytoplasm, spermatozoa are relatively easy to cryopreserve and this was already done in hundreds of species. Oocytes and embryos are much more difficult to obtain in large enough numbers to develop the needed protocols and, because of their large size, more difficult to cryopreserve. It is thus not surprising that oocytes or embryos of only a handful of wildlife species have been cryopreserved. An array of other options, including gonadal tissue and whole gonads cryopreservation, freeze-drying of spermatozoa and somatic cells, SCNT and ISCNT, to name just a few, are largely in the developmental or experimental stage and, if matured and improve in efficiency, they hold great promise and will become highly attractive to wildlife conservation and other fields concerned with 'putting life on hold'. These techniques will not replace the basic and well studied equilibrium freezing and vitrification but will help in supporting them as well as in handling cases in which routine cryopreservation cannot be done. While waiting for these and future technologies to mature and improve in efficiency we should strive to preserve whatever we can – gametes, embryos, gonadal tissue, whole gonads, somatic cells, and stem cells – anything we can. Such collections should be from sufficient number of representatives of each species so that we will be well prepared in the unfortunate event that a need will arise. As Benirschke (1984) put it: 'You must collect things for reasons we don't yet understand.'

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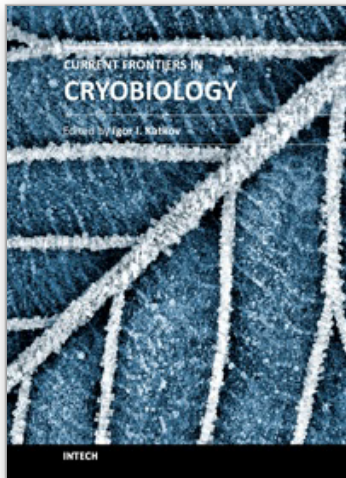
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## **Current Frontiers in Cryobiology**

Edited by Prof. Igor Katkov

ISBN 978-953-51-0191-8

Hard cover, 574 pages

**Publisher** InTech

**Published online** 09, March, 2012

**Published in print edition** March, 2012

Almost a decade has passed since the last textbook on the science of cryobiology, *Life in the Frozen State*, was published. Recently, there have been some serious tectonic shifts in cryobiology which were perhaps not seen on the surface but will have a profound effect on both the future of cryobiology and the development of new cryopreservation methods. We feel that it is time to revise the previous paradigms and dogmas, discuss the conceptually new cryobiological ideas, and introduce the recently emerged practical protocols for cryopreservation. The present books, "Current Frontiers in Cryobiology" and "Current Frontiers in Cryopreservation" will serve the purpose. This is a global effort by scientists from 27 countries from all continents and we hope it will be interesting to a wide audience.

### **How to reference**

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Joseph Saragusty (2012). Genome Banking for Vertebrates Wildlife Conservation, Current Frontiers in Cryobiology, Prof. Igor Katkov (Ed.), ISBN: 978-953-51-0191-8, InTech, Available from:  
<http://www.intechopen.com/books/current-frontiers-in-cryobiology/genome-banking-for-vertebrates-wildlife-conservation>

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