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REVIEW

Sperm cryopreservation in wild animals

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Abstract Extinction of a species represents the loss of a resource evolved through eons of mutations and natural selection. Reproductive technologies, including artificial insemination, embryo transfer, in vitro fertilization, gamete/embryo micromanipulation, semen sexing, and genome resource banking (GRB) have all been developed with the aim of solving existing problems and preserving genetic material for conservation purposes. Although protocols from domestic or non-threatened related species have been extrapolated to nondomestic and endangered species, usually these reproductive technologies are species-specific and inefficient in many nondomestic species because of insufficient knowledge on their basic reproduction biology and the need for species-specific customization. Since spermatozoa are usually more accessible and come in large numbers compared to oocytes and embryos, they are considered the primary cell type preserved in most emerging GRBs. For this purpose, semen from endangered species is currently cryopreserved to avail long-term storage. Due to the intractability of most exotic species, semen collection without chemical restraint is limited to only a handful of species and individuals. Viable epididymal spermatozoa can be obtained from dead or castrated animals, but this resource is limited. Electroejaculation, artificial vagina, abdominal massage, and/or transrectal, ultrasound-guided, massage of the accessory sex glands of living animals are viable alternative methods of semen collection. The ultimate goal is to adapt and optimize collection and cryopreservation protocols for each species, making it feasible, among other things, to collect gametes in the wild and introduce them into captive or isolated populations to increase genetic diversity. Recent advances in these fields have allowed the establishment of GRBs for many threatened species.

Keywords Biodiversity · Conservation · Cryopreservation · Endangered species · Freezing · Vitrification

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Introduction

Conservation status

Current extinction rate is estimated to be up to 1,000 times higher than the natural, or background, extinction rate, which means that, in the next few decades, up to 30 % of species, including many mammals, birds, reptiles, fish, and amphibians, may go extinct (Rockström et al. 2009; Pimm et al. 1995). Extensive habitat fragmentation or destruction, hunting and poaching, over exploitation, deforestation, introduction of invasive species, pollution of air, water and soil, climate change—including global warming, ocean acidification, and interference with the nitrogen cycle—have all contributed to diminished wild populations in a huge number of species, turning them into small and highly fragmented populations, resulting in a growing number of species being classified as "Endangered" (Reid and Miller 1989; Saragusty 2006). In addition to in situ conservation efforts, captive breeding and assisted reproductive technologies represent good tools in support of the struggle to protect species from going extinct.

Captive breeding plays an important role in conservation of species that may not survive in the wild, and as a breeding stock for reintroduction of individuals to repopulate dwindling or extinct populations. Captive populations are established for many reasons, including conservation, exhibition of interesting species, and research. Establishing self-sustaining captive populations as a means to protect species from the risk of extinction is an important contribution of zoos to conservation. A major goal of captive breeding programs is to preserve as much genetic diversity as possible in order to guarantee healthy survival of each species. However, to maintain sufficient numbers of animals to preserve satisfactory genetic variability of a species to ensure long-term genetic health is beyond the ability of zoos for most species. Notter and Foose (1985) calculated that nearly 50,000 individuals would be required to maintain 99 % of a species' genetic diversity for 1,000 generations. Even if one goes by much more loose estimates aimed at maintaining only about 90 % genetic diversity for a couple of 100 years, still several hundred animals are required in many vertebrate species (Traill et al. 2007). Due to space limitations in zoos and animal parks, it is impossible to maintain such large living populations. Long-term germplasm (gametes and embryos) dynamic storage can be considered as a possible way to preserve this ideal genetic diversity while minimizing holding space requirements and increasing the number of species that can be maintained through captive breeding (Wildt 1992; Wildt et al. 1997; Holt et al. 2003; Holt and Lloyd 2009).

Another advantage of captive breeding programs and germplasm storage by cryopreservation is the possibility to use genetically important individuals, via their spermatozoa, oocytes, or embryos, even when separated by long distances (Holt and Lloyd 2009; Hildebrandt et al. 2012) or after their death, thus reducing the risks associated with inbreeding (Lacy 1987; Lacy 1997; Roldan et al. 2006; Holt and Lloyd 2009). However, the success or failure of captive breeding programs and germplasm conservation depends on the further use of cryopreserved gametes and embryos for artificial insemination (AI), in vitro fertilization (IVF), and embryo transfer (ET).

Methods of assisted reproduction widely used in humans and domestic and laboratory animals are being applied to nondomestic species (Silber et al. 2013). Techniques can be transferred from well-known species to related but less studied species and may lead to improvements in their conservation. However, even if it is possible to develop protocols based on such model species, due to the uniqueness of each species, reproductive protocols must be adapted to the target species. Species vary in the anatomy of their genital system, morphology of their gametes, presence or absence of various male accessory glands, ovulation mechanisms, active hormones, duration of the reproductive cycle and gestation, and many other aspects of reproductive biology. We therefore should not be surprised to find differences between species in the reaction of their gametes, embryos and tissues to the cryopreservation process. The use of model species (usually more accessible) is common and useful, but, in the end, experimentation should be conducted in the target species. While this is relatively simple in domestic and laboratory animals, opportunities to obtain gametes and other relevant cells and tissues from endangered species are rare and far apart in terms of time and space, making progress extremely slow or, at times, practically impossible (Saragusty 2006).

There are some issues or peculiarities associated with sampling in wildlife. To start with, as described above, every new species is an enigma as far as key details of its anatomy or physiology are concerned. Due to the fact that the number of available individuals for sampling is usually very small, sampling opportunities are very limited and normally far away from the laboratory. It is also important to note that many wild species are seasonal, making sampling unpredictable in terms of quality and quantity. An additional concern when working with wild species, and due to the intractability of many of them, is that sampling often requires the use of general anesthesia, a risky procedure when it comes to wild animals that are very good at concealing health issues. Anesthesia protocols require species-specific customization, and the use of anesthesia makes repeated and frequent sampling impossible.

There is no doubt that semen cryopreservation, coupled with artificial insemination, has become the main tool for threatened species in ex situ assisted reproduction and research programs due to its advantages as long-term conservation tool and for facilitating transport of frozen semen samples rather than the stress-susceptible animal. Hundreds of studies describing sperm cryopreservation in threatened species have been published to date (e.g., Fickel et al. 2007; Saragusty and Arav 2012). Spermatozoa represent the primary cell types among germplasm preserved in genome resource banks (GRBs) due to its accessibility compared to oocytes or embryos, and, in all vertebrate species other than mammals, it being the only germplasm that can presently be cryopreserved (Saragusty 2006). Sperm cryopreservation has been widely developed in mammalian, fish, and avian species. In other vertebrate species (amphibians and reptiles), there is a growing interest; and development of these techniques for conservation management is in progress (Kouba and Vance 2009; Robles et al. 2009).

Interest in zoological gardens

Zoological gardens play an important role in species conservation by educating the general public, breeding endangered animals, and maintaining frozen repositories of cells and tissues from a wide variety of species. The goal of most endangered species captive breeding programs is to establish captive populations that are large enough to be demographically stable and genetically healthy. In order to accomplish

this goal, the most important objectives to have in mind are: (a) ensure successful reproduction, (b) protect the population against diseases, and (c) preserve the gene pool to help avoiding problems associated with inbreeding. Some captive breeding programs may also be interested in eventual reintroduction of animals back to the wild, although not all agree that captive breeding and reintroduction are the right ways to tackle populations decline and local extinction (Hunter et al. 2013). One of the main problems associated with captive breeding is diminishing genetic diversity in the remaining mating stocks. Germplasm cryopreservation means that, at least to some degree, diversity is stocked away and can be reintroduced into the population at a later date by thawing gametes from animals that are not sufficiently represented in the population and are not directly related to their living mates. Since zoos normally have space limitations and cannot maintain large-enough populations needed to keep them genetically healthy and sufficiently diverse, the use of GRBs and reproductive technologies are important supportive tools.

Another important issue relevant to captive breeding of wildlife is the fact that reproductive biology is well characterized for only a very small number of species, almost all of them are domestic or laboratory species and species of economic or scientific importance. This is considered a big issue in the application of reproductive techniques such as sperm cryopreservation, synchronization of ovulation, or artificial insemination (Loskutoff 1998; Wildt 1990; Wildt et al. 2001). The use of model species, such as domestic animal or more accessible wild species, coupled with increased understanding of sperm cryobiology, can help in the process of developing protocols to assist in the preservation of semen from endangered wild species (Wildt et al. 1995, 2001). Work with samples collected from the target species, however, will eventually have to be done to ensure suitability of the developed protocol.

The creation of GRB programs, such as the Frozen Ark Consortium (www.frozenark.org) (Clarke 2009) and the Amphibian Ark (www.amphibianark.org), or species-specific repositories of frozen semen such as those for the European bison (*Bison bonasus*) (Sipko et al. 1997), tiger (*Panthera tigris*) (Wildt et al. 1993, 1995), or the mhorr gazelle (*Gazella dama mhorr*) (Holt et al. 1996), all strive to keep genetic material and viable gametes from threatened species and, while doing so, demonstrate the importance of GRBs.

Due to the huge amount of work and money needed to support the creation of GRBs for all species, together with the practical benefit of using the preserved material in its natural environment, it is clearly more appropriate that each country will focus on species and breeds of local interest (Holt and Pickard 1999). To that extent, article 6 of the Convention on Biological Diversity (CBD) calls on all signatory nations to prepare National Biodiversity Strategy and Action Plan (NBSAP) and to establish, regulate, and manage collection of biological resources from natural habitats for ex situ conservation purposes (Convention on Biological Diversity 1992). A clear example of such efforts of research groups on local species is the activity concerned with marsupial conservation in Australia under the organization of the Animal Gene Storage and Resource Centre of Australia (AGSRCA), one of the members of the Frozen Ark Consortium (Holt and Pickard 1999; Clarke 2009).

The Leibniz Institute for Zoo and Wildlife Research (IZW), located in Berlin, Germany, another member of the Frozen Ark Consortium, has a cryobank that stores oocytes, embryos, and epididymal and ejaculated spermatozoa, as well as somatic cells of large variety of mammalian species. For example, the bank stores semen samples from Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephants, Indian (*Rhinoceros unicornis*), white (southern: *Ceratotherium simum simum*; northern: *C. simum cottoni*) and black (*Diceros bicornis*) rhinoceroses, various deer species, giant panda (*Ailuropoda melanoleuca*), brown bear (*Ursus arctos*), a large collection of wild felids, lagomorphs, primates, and many others (Fickel et al. 2007). The cryobank of the Spanish National Institute for Agricultural Research (INIA) maintains samples of sperm from several threatened avian species and somatic cells from bucardo (*Capra pyrenaica pyrenaica*), the now extinct Pyrenean ibex species. These samples may be used in the future when trying to resurrect this species through interspecies somatic cell nuclear transfer (Folch et al. 2009). High survival rates of germplasm are obtained in some but not all species (Blottner 1998; Asher et al. 2000; Leibo and Songsasen 2002; Swanson and Brown 2004). Further research towards improving current protocols as well as research aimed at establishing AI, IVF, or intracytoplasmic sperm injection (ICSI) using cryopreserved gametes is needed. In non-mammalian vertebrate species, conservation of primordial germ cells (PGCs) opens new possibilities, especially since thus far only male gametes can be cryopreserved, and since in birds and many reptilian, amphibian, and fish species the female is the heterogametic sex.

Interest in wildlife farming

There are obvious economical and conservational reasons for developing germplasm banks (Holt and Pickard 1999). One of which is the fact that many wild species have expanding utility prospects (Fletcher 2001). Their use as farmed species has increased notably in recent years for production of game meat, fur, and leather, as well as for hunting purposes. In the context of domestication of some of these species, semen cryopreservation, AI, and other assisted reproductive techniques have been successfully applied (Fennessy et al. 1990; Fukui et al. 1991; Morrow et al. 1994; Ponce et al. 1998; Asher et al. 1999, 2000; Malo et al. 2005; Zomborszky et al. 1999; von Baer et al. 2002; Lattanzi et al. 2002; Berg and Asher 2003;

Williams et al. 2004; Tibary et al. 2007; Amstislavsky et al. 2012). Development of assisted reproductive techniques adapted to these species is needed in order to increase productivity, facilitating selection of desirable traits such as meat quality and quantity, and for antler/horn size and beauty (for hunting). It is also useful as a mode to preserve, through germplasm banking, interesting subspecies and breeds, or selected varieties. The task of creating specific protocols for such wild ruminants, camelids, or carnivores still require further efforts, as although many techniques used in domestic livestock can be applied to some of these species (Asher et al. 2000; Garde et al. 2003; Martinez-Pastor et al. 2005a; Santiago-Moreno et al. 2009a, 2010a, 2013), species-specific adjustments must still be carried out to optimize cryosurvival. Indeed, even within the same genus, for example among cervid species, different species were shown to require different protocols for sperm cryopreservation (Cheng et al. 2004).

Genetic material recovery and processing methods

Species-specific sperm characteristics

There are many species-specific sperm characteristics in wildlife. For example, ejaculate volume and its concentration vary greatly between species. Large-volume ejaculates exceeding 100 mL of concentrated sperm can be collected from elephants, boar, or donkey (Saragusty et al. 2006, 2009) compared to small volume of up to just a few microliters in the naked mole rat (*Heterocephalus glaber*) or in species of the *Fukomys* genus (Bathyergidae, Rodentia) (Saragusty et al., unpublished data). Concentration can also vary greatly between several billions of spermatozoa per milliliter collected, for example, from the pygmy hippopotamus (*Choeropsis liberiensis*) (Saragusty et al. 2010a) or occasionally in the Asian black bear (*Ursus thibetanus*) (Chen et al. 2007) to the normally low concentration found in samples collected, for example, from some felids (Morato et al. 2001; Chen et al. 2007; Gañán et al. 2009).

Depending on the species, spermatozoa differ in size and shape, possibly as an adaptation to sperm competition or environmental factors. Marsupials may be a good example for species-specific sperm characteristics. As mentioned by Moore and Taggart (1995), spermatozoa in all American marsupials, with the exception of one species (*Dromiciops australis*) (Temple-Smith 1987), form pairs during epididymal maturation (Biggers and Creed 1962; Temple-Smith and Bedford 1980). Once the spermatozoa reach the distal epididymis, they rotate, aligning their heads to form pairs (Moore and Taggart 1995). This pairing turns the two spermatozoa into biflagellate unit, probably with enhanced swimming abilities (Phillips 1972). It was thus suggested that the mechanism has evolved to increase the number of successful spermatozoa reaching the fertilization site along the marsupial female reproductive tract (Moore and Taggart 1995).

Sexual strategies in animals (polygyny, monogamy) also influence sperm characteristics. In mammalian and avian polygynous species, sperm competition dictates high-quality sperm ejaculates, with high motility and low rate of sperm abnormalities. In contrast, sperm from monogamous species usually show high rate of sperm abnormalities (Birkhead 2000).

Collection techniques

There are many semen collection techniques. The most appropriate one should be chosen depending on the physiology and anatomy of the target species as well as on the circumstances and the specific individual involved (Santiago-Moreno et al. 2010b). In the subsections below, we describe the more common techniques used in mammals, with the last subsection dedicated to the other vertebrate taxa.

Artificial vagina

Semen collection with an artificial vagina is the most popular technique used in domestic animals, mainly in cattle (Bhattacharyya et al. 2009) and horses (Love 1992), but also in rams (Marco-Jiménez et al. 2008), goats (Roca et al. 1992), and rabbits (Amann and Foote 2004). Artificial vagina offers the advantage of frequent sampling without the stress of chemical or physical restraint. However, the conditioning required to train a male for service excludes its utility in most wild animals. Still, semen collection using artificial vagina has been performed in some wild species such as wild ruminants maintained in captivity like red deer (*Cervus elaphus*) (Deen et al. 2003; Gizejewski 2004; Giuliano et al. 2008), european moose (*Alces alces*), fallow deer (*Dama dama*) or reindeer (*Rangifer tarandus tarandus*) (Asher et al. 2000), Iberian ibex (*Capra pyrenaica*) and European mouflon (*Ovis orientalis musimon*) (Berlinguer et al. 2005; Santiago-Moreno et al. 2010a), and other species like Asian elephant (Kitiyanant et al. 2000), nondomestic cats [Indian desert cat (*Felis silvestris ornata*), jungle cat (*Felis chaus*), fishing cat (*Felis viverrinus*), and black-footed cat (*Felis nigripes*)] (Pope et al. 1993), Grevy's zebra (*Equus grevyi*) (Crump and Crump 1994), and non-human primates [chimpanzee (*Pan troglodytes*), orangutan (*Pongo pygmaeus*), and marmoset monkey (*Callithrix jacchus*)] (Morrell and Hodges 1998).

Postcoital semen collection

Another option that does not require restraint, at least not of the male, is collection of postcoital spermatozoa. Although

only part of the ejaculate can be collected this way, it has the advantage of being a natural ejaculate. The semen can be collected directly from the female reproductive tract, as was performed in the Sumatran rhinoceros (*Dicerorhinus sumantrensis*) (O'Brien and Roth 2000), South American camelids (*Lama glama*, *Vicugna pacos*) (Adams et al. 2009), non-human primates (marmoset, chimpanzee) (Morrell and Hodges 1998), and Asian elephant (Landowski and Gill 1964). It can also be collected with the aid of vaginal condom, as was performed previously in llama (Bainbridge and Jabbour 1998; Bravo et al. 2000).

Electroejaculation

Electroejaculation under surgical anesthesia has become the standard collection technique in the vast majority of wild mammalian species because many of them are intractable. The technique was used successfully in a very wide range of species. Still, the electroejaculation technique presents some issues that should be kept in mind. By their nature, wild animals are very good at concealing any internal health issue they may have till they cannot hide it any longer. Anesthesia is therefore a big gamble when it comes to wild animals and as such a risky procedure zoo veterinarians strive to avoid whenever possible. Furthermore, anesthesia may have lingering effects on wild animals just as in any other animal, so frequent collections, even in healthy animals, are not recommended. One should also remember that anesthesia can affect the quality of the ejaculate (Giulini et al. 2004; Durrant 2009; Santiago-Moreno et al. 2010b) and the collection procedure (Campion et al. 2012).

Several factors are very important to have in mind when designing an anesthesia protocol for electroejaculation. All aspects of the electroejaculation process (e.g., voltage or number of stimulations required for erection and ejaculation) may be affected by the species involved and the specific animal's plane of anesthesia (Gould et al. 1978; Durrant 1990). Some tranquilizers and anesthetics are contraindicated for electroejaculation (Meltzer et al. 1988; Tecirlioglu et al. 2002; Santiago-Moreno et al. 2010b), and even the doses of anesthetics may be an issue, as similar doses do not always have the same effect because of the physiology of the species or the stress level of the specific animal. The influence of anesthesia on electroejaculation must be investigated so as to be adapted for the species involved and adjusted as needed during the procedure in order to facilitate good immobilization, and provide optimal outcome for the animal and the procedure (Santiago-Moreno et al. 2010b, 2011).

The response to the electroejaculation stimulus can vary not only between species or between males from the same species, but even between collections from the same male. Therefore, modifications to the protocol during the process, based on the male's responses, may be needed, even if one strives to follow the same stimulation protocol in all electroejaculation procedures (Wildt et al. 1983). It is important to develop a specific collection protocol for the target species, but it is no less important to have an adequate probe, sometimes specially designed, for each species collected (Schmitt and Hildebrandt 2000; Roth et al. 2005).

Electroejaculation has been performed in a large number of wild species including: rhinoceroses (Hermes et al. 2005), nondomestic ungulates (Watson 1976; Asher et al. 1990, 2000; Cassinello et al. 1998; Holt 2001; Santiago-Moreno et al. 2009a), Asian black bears (Okano et al. 2006), various mole rat species (Hildebrandt et al., unpublished data), elephants (Howard et al. 1984, 1986), wild feline species [South African cheetah (*Acinonyx jubatus jubatus*), clouded leopard (*Neofelis nebulosa*), snow leopard (*Panthera uncia*), leopard (*Panthera pardus*), and puma (*Puma concolor*)] (Wildt et al. 1983, 1986, 1988; Roth et al. 1994; Howard et al. 1997), and non-human primates [chimpanzee, marmoset monkey, and rhesus monkeys (*Macaca mulatta*)] (Morrell and Hodges 1998).

Transrectal massage

An interesting alternative for noninvasive semen collection is the use of rectal manual stimulation of the accessory sex glands. It has been previously performed in humans (Fahmy et al. 1999), elephants (Schmitt and Hildebrandt 1998, 2000), and rhinoceros (Schaffer et al. 1998). This technique can be performed more often as, unlike electroejaculation, it does not require anesthesia. It can also be used to reduce the number and intensity of stimulations required during electroejaculation. In nondomestic ungulates, transrectal ultrasound-guided massage of the accessory sex glands (TUMASG), with or without a small number of electrical stimulations was reported (Santiago-Moreno et al. 2013). Ejaculates can also be obtained by voluntary semen collection through stimulation to the perineal area, as was done for example in the bottlenose dolphin (*Tursiops truncatus*) (Robeck and O'Brien 2004).

Miscellaneous alternative techniques

Some additional alternative techniques may include sperm collection through urethral catheterization after anesthetic level medetomidine administration as previously described in the domestic cat (Zambelli et al. 2008), stimulation of the penis done in some primates (McDonnell 2001; Schneiders et al. 2004; Melville et al. 2008), or pharmacologically induced ejaculation by oral imipramine and intravenous xylazine reported to have moderate success in stallions (McDonnell 2001).

Semen retrieval postmortem or following castration

Epididymal sperm collection is an important source of spermatozoa. Its main advantage in endangered species is that it allows maintenance of genetic variability that otherwise would be lost (Garde et al. 2006; Saragusty 2006). The cauda epididymis stores spermatozoa that are already mature and capable of fertilizing oocytes (Foote 2000). Epididymal sperm is normally collected from excised testicles either following the death of the animal or after castration. The time between the death of the animal or the castration procedure and sperm recovery is a very important factor with respect to the quality of the sperm recovered (Martinez-Pastor et al. 2005b, c; Santiago-Moreno et al. 2006). Maintaining the excised cauda epididymides, the whole testicles, or even the entire animal chilled can prolong survival of the spermatozoa. When testicles are removed from the body or when cauda epididymides are removed from the testicles, it is best to maintain them in saline or other physiologic solution to prevent drying and excessive damage to the tissue and the spermatozoa within it. Depending on the size and shape of the testis, the working conditions, and the experience of the operator, one may choose between several sperm extraction techniques. These include squeezing the cauda epididymis (Krzywinski 1981), making cuts (Krzywinski 1981; Saragusty et al. 2006; Santiago-Moreno et al. 2007), cutting and squeezing (Quinn and White 1967), extrusion by air pressure (Kikuchi et al. 1998), and retrograde flushing from the vas deferens (Santiago-Moreno et al. 2009b), which can be performed using a specific media (Santiago-Moreno et al. 2010b). Postmortem and postcastration semen collection has been performed in a large number of species including rhinos (Saragusty et al. unpublished data), common hippopotamus (*Hippopotamus amphibius*) (Saragusty et al. 2010b), Tasmanian devil (*Sarcophilus harrisii*) (Keeley et al. 2011), pygmy hippopotamus (*Choeropsis liberiensis*) (Saragusty et al. 2010a), nondomestic felids [tiger, lion (*Panthera leo*), puma, cheetah, leopard, jaguar (*Panthera onca*)] (Jewgenow et al. 1997), non-human primates [marmoset monkey, lowland gorilla (*Gorilla gorilla gorilla*)] (Morrell and Hodges 1998), and nondomestic ungulates [Burchell's zebra (*Equus burchelli*), bontebok (*Damaliscus pygargus pygargus*), blesbok (*Damaliscus pygargus phillipsi*), tsessebe (*Damaliscus lunatus*), impala (*Aepyceros melampus*), sable antelope (*Hyppotragus niger*), nyala (*Tragelaphus angasii*), and mountain reedbuck (*Redunca fulvorufula*)] (Holt 2001).

Epididymal spermatozoa, however, are not exposed to the seminal fluids and the many important components therein. This may hamper or partially challenge their fertilizing ability. Thus, in some species, it was found that adding seminal fluids before insemination increases fertilization success rate (Pan et al. 2001; Adams et al. 2005; Okazaki et al. 2012). One should also keep in mind that in most mammals, epididymal sperm are immotile and may require time and proper media to induce motility.

Collection methods in other vertebrate taxa

The first method applied in avian semen collection was the massage technique in roosters (Burrows and Quinn 1937). This technique requires restraining the bird by holding its legs. Repeated massage with rapid movements along the backbone towards the tail, abdomen, and behind the wings is required. The male responds with an erection of its copulatory appendage. The handler gently squeezes along the sides of the cloacae, collecting the semen from the *ducti deferentis* into a container. This technique is commonly used in wild avian species, but should be performed with care due to the stress that the capturing, handling, restraining, and massaging procedures cause (Gee 1995). Manual abdominal massage collection has been described in several species, and collecting semen this way from small passerines is relatively easy (Birkhead et al. 2005). The abdominal massage technique may need to be adapted depending on the reproductive anatomy of the species collected. Thus, adaptations in massage collection have been made for waterfowl, ratites, guans, and tinamous. All these species have a penis-like copulatory appendage (Cooper 1977). For birds producing limited ejaculate volume, the operator must evert the phallus early in the collection process, and a suction device is often used to avoid losing semen on the phallic surface (Gee and Sexton 1990). Semen from larger-sized individuals (i.e. cranes, storks, eagles) is recovered with the bird in a standing position (Gee and Temple 1978).

In wild avian species, the use of phantom-imprinted birds is also a popular semen collection technique. This method was first employed by falconers using sexually imprinted birds (Boyd and Schwartz 1983). Birds are trained to copulate on special devices so that no animal handling is needed throughout the process. The advantages of this technique are reduction of stress and risk of trauma, and reduction of ejaculate contamination with feces or urine. However, seminal volume varies significantly among individuals and collections, and some birds perform copulatory behavior but fail to ejaculate or produce only few or no spermatozoa. The use of an artificial vagina in the Muscovy duck (*Cairina moschata*) (Gvaryahu et al. 1984), or a "dummy" female in the Houbara bustard (*Chlamydotis undulata*) (Saint Jalme et al. 1994) improved the collection results in these species. Another possible method of semen collection in birds is electroejaculation. This method, though relatively uncommon in birds, has been reported in ducks and geese (Samour et al. 1985), in pigeons (Betzen 1985), and in a variety of psittacines (Harrison and Wasmund 1983).

Collection of fish semen, also known as milt, has been performed for many decades using the same basic technique

known as stripping. To perform this technique, the fish is either anesthetized or killed. It is then held so that its head is positioned higher than its tail with its abdomen facing ventrally. Application of gentle pressure and massage along the abdomen from the ventral fin caudally will then result in release of the semen. Females are spawned by the same method. Caution however should be taken to avoid contamination of the sample by water, blood, urine or feces. Because of the risk of contamination, alternative techniques were also developed. Using suction devices, it was reported that larger sample volumes with no contamination could be obtained (Graybill 1968). Such suction techniques, however, did not become popular and are in only limited use. If the animal is killed, its testicles can also be removed by carefully opening the abdomen. The testes may then be cleaned from blood vessels, rinsed, and then sliced open so that the milt can be squeezed out.

For many years amphibian semen collection required killing the animal and removing its testicles for further processing. The excised testes are macerated in physiological buffer to release the sperm. The tissue debris is then removed to obtain the sperm. This method, however, is not ideal when it comes to endangered species. Alternative techniques that can spare the animal's life were therefore called for. In one such technique, used in salamanders for example, joining a male and a female during the courtship season will often result in the male releasing a number of spermatophores that can be collected from the water (Figiel 2013). Each spermatophore contains a large number of spermatozoa. Alternatively, the animal can be given hCG to induce spermiation and then the sperm can be collected using abdominal massage (Mansour et al. 2011). Another hormonally induced spermiation method used in amphibians is the administration of Luteinizing Hormone-Releasing Hormone analog (LHRHa) intraperitoneally. Sperm can then be collected from the urine in relatively large quantities (Shishova et al. 2011).

In reptiles, like in fish, semen is most often collected by abdominal massage. The region of the cloacae is cleaned and rinsed to avoid contamination. Repeated gentle massage of the caudal third of the abdomen (cranial to the cloacae) in a caudal direction can lead to the release of semen that can then be collected with a syringe (without needle, of course) from the papilla inside the cloacae. To facilitate easier collection, subcutaneous injection of local anesthetics (e.g., 1 % lidocaine) can help in relaxing the cloacal region (Zacariotti et al. 2007). The abdominal massage technique has been applied to snakes, lizards, and other small reptiles. When reptiles are small, instead of a syringe to collect the minute quantity of sperm, a capillary can be used (Molinia et al. 2010). In larger reptiles such as alligators, crocodiles, and turtles, electroejaculation can also be used.

Sperm cryopreservation methods

Sperm cryopreservation is the process of cooling spermatozoa to very low sub-zero temperatures (normally to liquid nitrogen boiling temperature of −196 °C) for prolonged storage. To achieve that while maintaining acceptable viability of the preserved spermatozoa, dilution in cryoprotective agents is normally required. Species-specific customization of freezing extenders is aimed at providing protection for spermatozoa against damages caused by the chilling, freezing, and thawing processes. The most adequate extender must therefore be used for each species (Curry 2000; Watson 2000; Yoshida 2000; Leibo and Songsasen 2002; Thurston et al. 2002a). Mode and degree of cryoinjuries strongly depend on sperm physiology and the species involved, as differences are noted even between closely related species or individuals of the same species (Thurston et al. 2002a, b). For example, Asian elephant spermatozoa are more chilling sensitive than spermatozoa of the African elephant (Swain and Miller 2000). Egg yolk (or its substitutes) concentration, the amount of glycerol or other cryoprotective agents (CPAs), and the presence of sugars, buffers, antibiotics, and, at times, some other additives, in the cryodiluent, are all very important factors to keep in mind in the process of devising freezing extender, remembering the variability between species (Loskutoff et al. 1996; Leibo and Bradley 1999; Holt 2000; Salamon and Maxwell 2000; Watson and Holt 2001; Cheng et al. 2004; Fernández-Santos et al. 2006, 2007). In addition, the types of buffering additives used and the interactions of these with other ingredients in the extender may affect the rate of sperm viability after thawing. These interactions, too, can be species-dependent. Cryoprotectants may be membrane permeable (e.g., glycerol, ethylene glycol, or DMSO) or impermeable (e.g. sucrose, trehalose, raffinose, PVP). These CPAs change the properties of the cellular membrane and intracellular aqueous phase, decrease osmotic stress, cause moderate dehydration while protecting from excessive dehydration and cellular membrane collapse, and prevent intracellular ice formation (Gao et al. 1993, 1995; Saragusty et al. 2005; Johnston et al. 2006). These cryoprotectants also increase the viscosity of the solution and thus the glass transition temperature. Macromolecules such as lipoproteins, proteins, and phospholipids present in the extender can stabilize the plasma membrane and often increase its fluidity while decreasing its thermotropic phase transition temperature, thus protecting the cells during chilling, freezing, and to the associated osmotic changes (Zeron et al. 2002; Saragusty et al. 2005). Glycerol is considered the most effective cryoprotectant for spermatozoa of the vast majority of mammalian species. Sensitivity to this cryoprotectant, however, varies greatly between species. For example, marsupial species require very high concentration of glycerol (>14 %) (Johnston et al. 1993, 2006; Czarny et al. 2009; Keeley et al. 2012), whereas other species, such as boar or mice are highly

sensitive to glycerol (Koshimoto et al. 2000; Sztein et al. 2001; Gutiérrez-Pérez et al. 2009). In avian species, although glycerol is usually regarded as a suitable cryoprotectant for spermatozoa, it has its biological limitations, e.g., it has a contraceptive effect in certain species (Hammerstedt and Graham 1992). Dimethylacetamide (DMA) seem not to have such contraceptive effects, and good quality and fertility rates were achieved with turkey and sandhill crane (*Grus canadensis*) frozen-thawed sperm using this cryoprotectant (Blanco et al. 2012). In other taxa (fish, amphibians, reptiles) methanol, DMSO, ethylene glycol, or dimethylformamide in concentrations ranging between −2 and 25 % were all reported, in addition to glycerol (Millar and Watson 2001; Tiersch 2006; Shishova et al. 2011; Zacariotti et al. 2011; Johnston et al. 2014).

Three main processes are used for sperm cryopreservation: very slow freezing, slow freezing, and vitrification. The very slow freezing technique consists of progressive sperm cooling over a period of >4 h. Freezing by this technique is performed by slowly decreasing the temperature of the sperm sample first in the refrigerator to 5 °C, and then by placing the sample in a cryobox and chilling it down from 5 to −80 °C over 2 to 4 h. After that, the sample is plunged into liquid nitrogen for storage (Thachil and Jewett 1981). The optimal cooling rate must be adapted from species to species.

The slow freezing technique is arguably the most widely used one. This technique is based on the notion that spermatozoa, like any other cell type, react in an inverted U-shaped mode to cooling rate (Mazur 1977; Arav and Saragusty 2013). Outside the optimal range, at which high survival is achieved, a progressive decline in survival rate is noted with progressive increase or decrease in cooling rate. As a result of the slow cooling rates used, intracellular water has sufficient time to exit the cells, thus allowing osmotic equilibration and prevent the risk of intracellular ice formation. In the slow freezing technique, as in the very slow freezing technique, the sample, suspended in freezing extender, is first cooled slowly to about 5 °C and then frozen over a duration of up to several minutes to the desired temperature (ranging between about −30 and under −100 °C, depending on the protocol) before it is plunged into liquid nitrogen for storage. Two main slow freezing methods currently lead the field. One is the liquid nitrogen vapor phase equiaxed freezing and the other is the directional freezing. Inside nitrogen vapors there is a thermal gradient as a function of the distance from the liquid nitrogen surface and the volume of the liquid below. For liquid nitrogen vapor freezing, samples are either placed horizontally at a predefined distance above the liquid nitrogen surface to achieve the desired cooling rate or they are placed inside programmable controlled-rate freezing device. The distance to the surface of the liquid nitrogen and the duration of exposure depend on the species. Controlled-rate freezers use a plate to hold the samples, a plate that is cooled by liquid nitrogen. Once programmed, the machine follows the program to obtain the required cooling ramp, using liquid nitrogen as the cooling agent. When the freezing process is completed, the samples are removed and stored in liquid nitrogen. The directional freezing technique (Arav et al. 2000) relies on controlled progression of the sample down a predefined temperature gradient. This permits accurate control over heat dissipation and ice crystal propagation, morphology, and velocity throughout the freezing process. Directional freezing has been used for cryopreservation of spermatozoa from a very wide range of species (see Arav and Saragusty 2013 for a recent review). This technique is useful for freezing samples in volumes ranging from the standard 0.25 mL plastic straws to glass tubes of 12 mL. Directional freezing has also been used to vitrify oocytes and embryos (Arav 1989; Rubinsky et al. 1991, 1992), and to freeze tissue slices and even whole organs (Arav et al. 2007; Gavish et al. 2008; Arav and Natan 2012; Maffei et al. 2014).

Alternative methods were also reported. These include, for example, freezing inside a dry shipper, in methanol that was chilled with dry ice, or freezing by placing the sample in a −80 °C freezer (Beesley et al. 1998; Sargent and Mohun 2005; Viveiros et al. 2012; Figiel 2013).

Vitrification is solidification of the sample without formation of crystals. At the end of the very slow or slow freezing techniques mentioned above, when the viscosity of the intracellular milieu and the extracellular unfrozen fraction is sufficiently high, vitrification will occur. In the absence of vitrification, the frozen cells will not survive, as ice crystals will disrupt the cellular membranes resulting in death of the cells. To achieve vitrification, a balance between sample viscosity, volume, and the cooling rate should be achieved. To achieve fast vitrification, one should increase the cooling rate and/or the viscosity of the sample and/or decrease the vitrified volume. As achievable high cooling rates are limited, one has to decrease the vitrified volume and increase the solution's viscosity. Both of these are problematic when it comes to vitrifying spermatozoa. When vitrifying oocytes or embryos, very high concentrations (normally in the range of 30 to 40 %) of vitrificants (e.g., sucrose, trehalose, DMSO, ethylene glycol) are used. This is possible without killing the cells because oocytes and embryos are large so they can be picked out of the solution in preparation for vitrification and upon warming and transferred through a series of solutions for gradual dehydration or back to physiological osmolarities, respectively. Because of the size and number of spermatozoa, it is impossible to do the same fast enough with these cells. Volume-wise, oocytes and embryos are vitrified in very small volumes in the range of 1 μL or less. The number of spermatozoa that can be vitrified in such a volume is very small and so, to compile an insemination dose, one will have to vitrify a huge number of samples. Still, in some cases, vitrification of small number of spermatozoa in very small volume can be beneficial and

experiments in this direction were conducted (Isachenko et al. 2003; Chang et al. 2008; Merino et al. 2011; Endo et al. 2012).

Conclusions

Development of assisted reproductive technologies in domestic animals is difficult enough to carry out. Performing this in wildlife, especially in endangered species, has the additional limitation of accessibility to the animals, availability of animals for collection, their distance from the laboratory, and sampling frequency. These factors increase the difficulty of developing new technologies in wildlife. These difficulties thus explain why procedures such as cryopreservation of gametes and embryos, embryo production, and embryo transfer are far from optimum in many nondomestic species. The use of model species to learn basic processes and develop protocols for gamete cryopreservation or embryo transfer is a valuable tool when it comes to developing and improving these techniques for use in endangered species. However, it is necessary to eventually customize these in vitro and in vivo protocols to each target species (Leibo and Songsasen 2002). There is no doubt that "buying time" by storing germplasm, and thus genetic diversity, under liquid nitrogen is beneficial to the process of gaining and refining the knowledge with respect to embryology and reproductive biology of any nondomestic species studied. It is also very important to step-up efforts concerning animal welfare in association with anesthesia, sedation, animal management techniques, and sample collection and transfer protocols. In conclusion, we have within our reach useful tools to help preserve genetic diversity while working on ways to stop or, at least, slow down the alarming decline in global biodiversity, for which we have an ethical responsibility. Further teamwork research, involving scientists and zoological institutions, may help focus on advancing the knowledge on endangered wildlife species. There is no doubt that more efforts are needed, but optimistically, recent years have seen an increase in research programs on threatened and endangered species conservation, a trend we hope will continue and expand.

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