Dry Biobanking as a Conservation Tool in the Anthropocene

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Abstract

Species are going extinct at an alarming rate, termed by some as the sixth mass extinction event in the history of Earth. Many are the causes for this but in the end, all converge to one entity – us, humans. Since we are the cause, we also hold the key to making the change. Any change, however, will take time, maybe too long for some species. While working on possible solutions, we also have the responsibility to buy time for those species on the verge of extinction. Genome resource banks, in the form of cryobanks, where samples are maintained under liquid nitrogen, are already in existence but they come with a host of drawbacks. Biomimicry – innovation inspired by Nature, has been a huge source for ideas. Searching Nature for ways to preserve biological systems for extended periods of time, we see that in Nature, drying rather than freezing is the method of choice. We thus argue here in favor of preserving at least part of the samples from critically endangered species in dry biobanks, a much safer, cost-effective, biobanking approach.

Keywords: Anhydrobiosis; Biobanking; Conservation; Cryopreservation; Desiccation; Freeze-drying; Lyophilization;

1. Introduction

The renowned Harvard biology professor Edward O. Wilson once said about ants, "*We need them to survive, but they don't need us at all.*" He also said, "*If all mankind were to disappear, the world would regenerate back to the rich state of equilibrium that existed ten thousand years ago. But if insects were to vanish, the environment would collapse into chaos*."

Species extinction is not new. It has been happening throughout the history of life on Earth. If one looks through the geologic record, probably over 95% of all species that have ever lived on this planet went extinct. Since speciation takes place in parallel to the process of extinction, the planet has not been depleted of its life forms (yet). Based on paleontological data, the rate of this ongoing extinction, which has been termed the 'background extinction rate' or 'pre-human extinction rate', was recently estimated 0.1 species out of every one million species per year (0.1 extinction per million species years or 0.1 E/MSY) [1]. With an estimated 15 million species on this planet, this means that, on average, one can expect to witness the extinction of about 1.5 species every year. The planet has witnessed five mass extinction events; events during which at least 75% of the estimated species present at the time went extinct over relatively short geologic time. These 'Big Five' events are the Late Ordovician event (~447-443 million years ago or MYA; estimated 86% of species lost), the Devonian event (~388-359 MYA; estimated 75% of species lost), the Late Permian event (~254-251 MYA; estimated 96% of species lost), the Late Triassic event (~208-200 MYA; estimated 80% of species lost), and the Late Cretaceous event (~68-65 MYA; estimated 76% of species lost) [2]. For some time now the planet has been dominated by human activities that have altered it in many different ways, one of which is the accelerated loss of biodiversity. It has thus been suggested naming a new, human-dominated, epoch that has

been termed the Anthropocene [3]. Differing opinions persist as to whether we, humans, are bringing about the sixth mass extinction event, and whether this event has already started [2, 4-6]. Regardless of whether the current rate of species extinction already falls within the definition of a mass extinction event or not, there seem to be unanimous agreement that the current rate of extinction is orders of magnitude higher than the background rate, with some estimates putting it at as much as 1,000 times the background rate or even more. Estimates, however, vary greatly, in part because we do not really know exactly how many species there are out there, with some estimates going as high as 50 million. We also know very, very little about the status of the vast majority of these species. Only about 1.7 million species have been described thus far, and only about 4% of these have been thoroughly assessed [7].

As it is widely agreed that the leading cause for the accelerated loss of biodiversity we witness today are us, humans [8], it is in our hands to make a change and attempt to fix things by changing our behavior before it would be too late, before we reach the tipping point beyond which everything would collapse.

2. What can we do?

In recognition of this accelerated loss of biodiversity, representatives from almost all nations of the world gathered in 1992 in Rio de Janeiro, Brazil, to reaffirm the 'Rio Declaration on Environment and Development' and to ratify the 'Convention on Biological Diversity' [9]. Since loss of biodiversity continued, the Parties to the Convention gathered again in 2002 and have "committed themselves to achieve by 2010 a significant reduction of the current rate of biodiversity loss at the global, regional and national levels as a contribution to poverty alleviation and to the benefit of all life on Earth" (https://www.cbd.int/2010-target). The year 2010 was also set by the United Nations as 'The International Year of Biodiversity'. However, with the approach of 2010, it became clear that not only the targets will not be

achieved; even some of the tools to measure progress towards these targets were still missing [10]. With this realization, the Parties to the Convention gathered again in 2010 in Nagoya, Japan, and set forth new targets to be achieved by the year 2020 – the 'Aichi Biodiversity Targets' (https://www.cbd.int/sp/targets/). The United Nations has also set the current decade (2011-2020) as the 'Decade on Biodiversity'. The recent summary of the Global Assessment Report on Biodiversity and Ecosystem Services [8] states that while progress has been made in some areas, the objectives set forth in the Aichi Biodiversity Targets, as well as the 2030 Agenda for Sustainable Development, and the 2050 Vision for Biodiversity will not be achieved based on current trajectories [e.g. 11, 12, 13]. This leaves much room for improvement.

Sitting and waiting for things to happen is not the solution. Not taking any action till proper solutions are identified, tested, and mastered is also not the right approach. Far too many species will go extinct before we find the ways to effectively protect the ecosystems in which they live. Some thousands years ago, according to the Bible, Noah built an ark and took a pair (or seven pairs of clean animals and birds) from each species so as to prevent their extinction during the Big Flood [14]. With the knowledge we have today, it is clear that with such a small founding population, most species would have gone extinct anyway or, if somehow survived, they would suffer from severe inbreeding. So, Noah's solution, if taken literally, is probably not a viable one. The alternative is to keep, under human care, large enough and self-sustaining populations from each species with sufficient genetic variability. Minimum viable population size ranges between several hundreds and several thousands, depending on the species and its distribution. With the limited resources (land, manpower, funds) and knowledge (reproductive biology, captive rearing and breeding), this option is also not a viable one for many species. Some years ago, the 'Global Seed Vault" was established on the island of Svalbard in Norway [15]. At present, seeds of about one million

varieties of food plants are stored in this underground seed bank. The seeds are maintained at -18ºC with limited access to oxygen to ensure low metabolic activity and longevity. A similar approach for wildlife conservation, termed Genetic (or Genome) Resource Banks (GRBs), has also been suggested [16-20]. Generally speaking, animal cells and tissues cannot survive and maintain their biological activity at -18ºC for extended time spans. Normally, much lower temperatures, in the range of liquid nitrogen boiling temperature (-196ºC), are required for long-term preservation. Activities in this direction took place in many places around the world. At present there is no global GRB but rather consortia of organizations, research institutions, museums, zoological gardens and such, where various samples are stored (e.g. www.frozenark.org or www.amphibianark.org). There is also limited information available as to which samples are stored at which institution so efforts to preserve sufficient representative samples from each species are practically hindered by lack of information. When species-specific cryopreservation protocols have been devised, they are often of adequate efficiency. By very rough estimate, reported attempts to cryopreserve germplasm in mammals to date cover spermatozoa from approximately 116 species (~2% of all mammals), embryos from 51 species (~0.9% of all mammals), and oocytes from just a handful of species [21-23]. Births of live offspring from cryopreserved mammalian sperm were reported in about 45 species and from cryopreserved embryos in about 25 species. However, the vast majority of the mammalian species in which live offspring from cryopreserved germplasm had been reported, are domestic species, laboratory species, and species of research and/or commercial value. Germplasm from fairly few truly wild mammalian species have been preserved. A number of causes stand behind this rather poor state of affairs. Species, by definition, are different from each other and as such, their germplasm respond differently to the cryopreservation process [24]. To overcome this, customizing the protocols for each species is necessary. As access to samples from wildlife species is limited, especially from

those that need it the most – the critically endangered species, progress has been limited and very, very slow. Knowledge about the majority of species is still rather scarce. In the absence of sufficient understanding of the anatomy, physiology, and reproductive biology of each species, progress is expected to remain very slow. To this we need to add factors such as the risk associated with the often-needed anesthesia or sedation for sample collection, the distance of the animals from a proper laboratory, and the general poor reproductive health of many individuals. So, the picture is clear – germplasm cryopreservation from wildlife (mammalian) species is destined to see very limited progress in coming years. Furthermore, cryopreservation of germplasm and tissues, and their maintenance under liquid nitrogen (LN), while effective, comes with many inherent problems. It is expensive, energy-dependent, requires dedicated facilities and trained personnel, must have continuous monitoring and uninterrupted LN and power supply, thus putting such biobanks out of reach for many countries in the developing world and small businesses. LN is also dangerous, complicates sample transport, may act as pathogen transmission medium, and its production, transport and maintenance have high carbon footprint. A cheaper, simpler, and environmentally friendlier alternative is thus highly desirable.

3. What Nature "thinks" about this?

Long term preservation of biological life forms is often required in Nature as resources are not always readily available and so, one may expect that ways to do just that have been devised and refined over evolutionary time scales. A look around indicates that while freezing as a preservation approach is possible in some regions of the world [25], the system that is by far more widespread is drying. By an almost complete removal of water from biological systems, metabolism and interactions between molecules are brought to an almost complete halt. This, however, is a reversible process so, when water becomes available again, biological activity is resumed. This process is known as anhydrobiosis, a name coined by

158 Alfred Giard in 1894 [26]. Anhydrobiosis was, however, discovered almost two centuries
159 earlier by the renown Dutch microscopist, Antony van Leewenhoek, who made the following
160 observation in 1702 [27]:

161 "…In order more fully to satisfy myself in this respect, on the third of September,
162 about seven in the morning, I took some of this dry sediment, which I had taken out of
163 the leaden gutter and had stood almost two days in my study, and put a little of it into
164 two separate glass tubes, wherein I poured some rain water which had been boiled and
165 afterwards cooled… As soon as I had poured on the water… I examined it, and
166 perceived some of the Animalcules lying closely heaped together. In a short time
167 afterwards they began to extend their bodies, and in half an hour at least an hundred
168 of them were swimming about the glass… After five or fix hours had elapsed I saw
169 two several sorts of Animalcules swimming in the water…."

170

171 It is suggested that what van Leewenhoek saw was a species of bdeloid rotifer (*Philodina*
172 *roseola*) [28]. Anhydrobiosis is not unique to rotifers. A variety of taxa, including bacteria,
173 yeast, insects, fish, and crustaceans, are known to employ this survival technique when water
174 is in short supply [29-32]. The approach is also widely used in the plant kingdom, primarily
175 in seeds and pollen [33, 34] but also in whole plants that are known as resurrection plants
176 [35]. In the absence of water, these organisms accumulate a variety of substances to protect
177 their cells, and they gradually dehydrate and often shrink in the process. Once in the dry state,
178 they can survive for long spans of time, sometimes even thousands of years, and then, when
179 water become available again, they rehydrate and return to full biological activity [36]. Study
180 of these organisms revealed some of their protective ingredients. These include disaccharides
181 (primarily trehalose and sucrose) [37], a wide array of late embryogenesis abundant (LEA)
182 proteins [38], various heat shock proteins [HSP; 39], and anhydrin [40]. As was shown in

tardigrades [41], a combination of a number of protective mechanisms and cell activity alterations is required to provide the needed protection during desiccation. The combinations seem to vary between organisms, suggesting the possibility that this unique long-term preservation technique has emerged in Nature more than once. Animals and plants studies have shown that accumulation of sugars alone is not enough to bestow desiccation tolerance. The sugars, when in high concentrations, can depress the lipid phase transition (LPT) temperature, known as Tm. Around the Tm, depending on the size and degree of unsaturation, some of the phospholipids in the membrane are in the liquid phase while other are in the gel phase. This opens the membrane to leaking and possible damage to the cells. The LPT may also lead to a process known as lipid phase separation, during which membrane components shift their position by floating through the liquid components. This, too, is a damaging process. Depression of the Tm by the sugars, thus, enhances the stability of the dried cells, and increases the viscosity and the glass transition temperature (known as Tg) in the dry state. The two disaccharides most often featured in desiccation tolerant organisms are trehalose and sucrose. These two have the highest Tg amongst all disaccharides - 73-115ºC and 52-70ºC, respectively [42, 43]. Based on the 'replacement theory' of Crowe and colleagues [29, 37], these disaccharides also replace water molecules in the lipid bilayer, thus preventing structural changes during drying. For this replacement to be stable, and so as to prevent the sugar molecules from being squeezed out during drying, some plants use flavonols [44]. Flavonols not only stabilize the placement of the disaccharides in the membranes, they also act as potent antioxidants, which further protect the membranes. In summary, for proper desiccation tolerance and membrane protection, it is imperative to increase the Tg, depress the Tm, maintain the sugar-phospholipid headgroups interaction in the dry state, protect from oxidation, in prevent individual cells from structural collapse when water is removed. When these factors achieved and water content is kept low (under 10% of dry weight), in principal,

biological samples can be maintained for extended periods of time at ambient temperature and resume biological activity upon rehydration.

4. Dry Biobanking

Dry biobanking has many advantages over cryo-biobanking. If successful, and for now there is still a big 'if' here, samples can be preserved at ambient temperatures, practically anywhere in the world, at considerably reduced cost, space, and special needs. When compared to the current cryostorage, it is also less dangerous, less prone to natural disasters, much simpler to handle and ship, and it would have a drastically lower carbon footprint. Dried cells are also simpler to handle when considering transport into space for use on space station or eventual colonization of other planets. While the general principles are, more or less, understood, their effective implementation proved to be very challenging. Attempts to dry preserve gametes (spermatozoa) date back at least 70 years to the pioneering experiments of Polge, Sherman, Saacke, Yushchenko, Nei, Bialy, Meryman and their colleagues [45-52]. Because of their small size, little water content, and highly condensed nucleus, spermatozoa were very attractive candidates. If successful, maintaining sperm samples in the dry form is a very appealing biobanking option. Drying, however, turned out to be more challenging than expected. Although early publications did report on some recovery of motility [45, 51] and even about the production of offspring following artificial insemination [52], these results could not be replicated, not even by their own authors [53, 54], or else they were the outcome of only partial and short-lived drying [45]. The attempts continued over the years but it was only after Palermo and colleagues [55] showed that viable offspring can be generated by injecting a spermatozoon into the oocyte, that Wakayama and Yanagimachi [56] took this one step further and demonstrated that lyophilized-rehydrated spermatozoa with damaged plasma membrane, so-called 'non-viable spermatozoa', can still lead to embryonic development and healthy offspring following intracytoplasmic sperm injection (ICSI). Since

then, much work has been done in the field of sperm drying, concentrating almost entirely on mice and cattle, with some studies on human and pig sperm. For all other species in which sperm drying had been attempted, less than five (in most only one or two) publications were found in the scientific literature. Several reasons can be suggested for this state of affairs. First, the field is still really in its early stages of development. The many drying techniques and approaches reported on indicate that a leading approach is still missing. Before such a protocol is found, research will probably continue in mice where it is relatively easy to generate offspring, in cattle and pigs where semen samples are routinely collected for breeding programs, and in humans where ICSI is routinely used in fertility centers so dry biobanking might find a niche there faster than in other mammals. Transfer of technology to other species would probably come once a winning protocol (e.g. motile sperm after rehydration) is developed in any of these model species. Second, in species other than domestic/lab animals, there is also the issue of sample accessibility. Those endangered species who truly need optimal biobanking coverage are also the species from which it is often most difficult to collect samples for research and for banking. After all, rare species are rare. Still a bit of activity has been done in wildlife species. In Japan, collaboration between researchers at Kyoto University and the Kyoto City Zoo has set up a wildlife sperm bank. As part of this initiative, sperm drying towards wildlife dry biobank has also been attempted [57]. Towards this goal, Kaneko and collaborators have freeze-dried sperm from chimpanzee (*Pan troglodytes*; endangered), reticulated giraffe (*Giraffa camelopardalis reticulata*; vulnerable), jaguar (*Panthera onca*; near threatened), Japanese weasel (*Mustela itatsi*; near threatened), and Ryuku long-tailed giant rat (*Diplothrix legata*; endangered). Maintenance of biological activity was demonstrated through the formation of pronuclei following injection of these freeze-dried spermatozoa into mouse oocytes and incubation *in vitro*. Attempt was also made (by other researchers) to freeze-dry the spermatozoa of fat-tailed dunnart (*Sminthopsis*

crassicaudata; least concern), member of the Dasyurids, a family of carnivorous marsupials [58]. Sperm of this species, like that of many other marsupials, can tolerate glycerol concentrations as high as 40%. However, freezing sperm form members of this family has proved challenging and post-thaw motility remain close to zero. Although the freeze-dried sperm had compromised plasma membrane, its acrosome and DNA maintained their integrity through the freeze-drying process. Two different approaches were applied to drying rhesus macaque (*Macaca mulatta*; least concern) sperm. In one approach, Meyers and colleagues tested two different vacuum drying techniques, both resulting in partial drying. They either dried the spermatozoa on filter membrane at a pressure of 711.14 mbar (atmospheric pressure is 1013.25 mbar) for five hours [59] or by drying 50 µL drops of sperm suspension at even higher pressure of 745.01 mbar for 130 min to water content of 0.3 g H_2O / 1 g dry weight [60]. For comparison, seeds, pollen, and anhydrobiotic organisms normally dry to somewhere in the range of 0.02-0.07 g H_2O / 1 g dry weight. Under these drying conditions (high pressure and short duration), residual water was rather high, making long-term preservation at ambient temperatures not feasible. Spermatozoa from the first study were used for ICSI after four hours under vacuum at room temperature and resulted in embryo development. Spermatozoa in the second study were kept under vacuum at room temperature or -80ºC for 7-10 days and then used for ICSI. Blastocyst rate was zero in the -80ºC group and 5.3% in the room temperature group compared to 40% in the control. It can be postulated that the reason for this is cryodamage caused by freezing the residual water, after the drying process. The alternative approach was to use freeze-drying [61]. Following this approach, the researchers cryopreserved the spermatozoa in pellets on dry ice, which were then transferred into liquid nitrogen. After drying for 12 h and storage in argon environment for up to two months, spermatozoa were rehydrated and used for ICSI. As in the other two studies on rhesus

macaques mentioned above, rates of activation were similar to the control but further embryonic development was compromised.

And alternative approach was taken in Israel by one of us (A. Arav) and his colleagues. Rather than drying spermatozoa, the researchers opted for freeze-drying nucleated cells that can be later used in somatic cell nuclear transfer (SCNT) to generate embryos [62]. The animals were anesthetized and blood was taken into a CPDA collection bag. Approximately 200-500 mL of blood was taken from each animal. The blood was then separated using Histopaque-1077 (Sigma, Israel) and the Mono-Nuclear Cells (MNC) were collected. Briefly, 3 mL of Histopaque-1077 were placed in a 15 mL test tube. Three mL of blood were gently placed on top to avoid mixing. The tubes were centrifuged for 30 minutes at 400 *g* without breaks. Then the MNC layer was collected into a separate 15 mL test tube and 10 mL of PBS (Ca^{2+}- and Mg^{2+}-free) was added, and the cells suspension was centrifuged for 10 minutes at 200 *g*. Then, the supernatant was discarded and the cells pellet was re-suspended with IMT-2 solution, composed of 0.945 mg/mL EGCG and 0.1 M trehalose dissolved in Ca^{2+}- and Mg^{2+}-free PBS. Cells were counted, aiming to reach a concentration of about 2×10^6 cells/mL. Two mL of cell suspension in IMT-2 solution were then put in 16 mm diameter glass tube (Manara, Israel) and frozen using the MTG-516 device (IMT Ltd., Nes Ziona, Israel). The device was set to have a temperature gradient from 5°C to -70°C, at a velocity of 0.2mm/min, resulting in a cooling rate of 5.1°C/min [63]. After freezing, the tubes were opened and inserted into a lyophilizer (Labconco, USA) having a condenser temperature of -80°C. Samples were placed near the condenser for 3.5 days. After 3.5 days the samples were heat-sealed under vacuum (Figure 1). Using this technique, samples were collected and freeze-dried from Aoudad (*Ammotragus lervia*; vulnerable), Somali wild ass (*Equus africanus somaliensis*; critically endangered), Addax (*Addax nasomaculatus*; critically endangered), Arabian leopard (*Panthera pardus nimr*; critically endangered), and mountain gazelle

(*Gazella gazella gazella*; endangered). The samples are stored at room temperature and are protected from light.

Like endangered species that are becoming extinct at stunningly accelerated rate, so do many endemic breeds of domestic animals, breeds that have developed unique adaptations and characteristics [64]. And, just like endangered species, so do endangered breeds require protection as well as banking of a variety of samples in biobanks. In our laboratory, a sperm bank for the Pagliarola, an Italian endemic and endangered sheep breed, has been created. The breed, native to the province of Teramo in central Italy, has decreased from around 350,000 animals to only 25 (21 ewes and four rams) in less than a century. The bank currently stores > 600 straws (Figure 2A) of cryopreserved sperm from two Pagliarola rams. In recent years, part of these cryopreserved samples was further processed through lyophilization and storage at room temperature (~50 vials, each with ~25 × 10^6 spermatozoa; Figure 2B). Desiccated samples, tested through ICSI, showed blastocyst rate of 10.2% compared to around 25% for ICSI with frozen-thawed sperm controls.

Sperm or MNC drying, however, is not the only option to aim for. When a species goes extinct or very near that fate, even if sperm is available, frozen or dried, oocytes would be very hard to get by. Although a handful of attempts to cryopreserve oocytes from wildlife, by slow freezing or vitrification, have been reported [21], this is not an option we can rely upon, especially since ovum pickup is invasive, often requires hormonal stimulation to increase the numbers, and normally – full anesthesia. In the absence of oocytes in sufficient numbers to generate enough embryos for transfer, and in the absence of surrogate animals to carry those embryos to term, preserved spermatozoa would be of little use. The alternative is to attempt to follow in the steps of work done by Katsuhiko Hayashi and others on *in vitro* gametogenesis. The complete process *in vitro* from embryonic stem cells or induced pluripotent stem cells (iPSCs) through primordial germ cells-like cells to oocytes that were

fertilized, transferred, and lead to pregnancy and birth of normal offspring has been reported in mice [65]. Spermatogenesis is a more complicated process, with different stages, cell types and their specific requirements, and with a need for a rather long incubation time. While various stages of the process that, if put together, cover the entire length of spermatogenesis, have been reported [see reference 66 for recent review], the complete process from somatic cell to usable spermatozoa in a dish is still to be achieved. Furthermore, the *in vitro* process is faster than the *in vivo* one, suggesting that it does not really mimic the natural gametogenesis and that culture conditions should be improved.

Some years ago, we have proposed to harness the process of *in vitro* gametogenesis to the efforts to save critically endangered species from the fate of extinction [67]. The northern white rhinoceros (*Ceratotherium simum cottoni*) had a population of only three animals at the time (now only two females left after the death of the only remaining male in March 2018). There were also cryopreserved samples (somatic cell cultures, iPSCs, tissue samples, spermatozoa) from 12 individuals. We suggested as one possible alternative to utilize this limited resource to reconstitute the population by generating iPSCs cell lines, which could then theoretically be directed to differentiate into the germ cell direction and then into male and female gametes. This work is in progress. For this subspecies, oocytes can only be collected from the last two remaining individuals – a mother and her daughter. Such collection is a complicated and risky procedure involving full anesthesia and international transport of the collected oocytes to a laboratory capable of fertilizing them by ICSI. Such a procedure was performed in August 2019 [68], during which five oocytes were harvested from each of the two females, shipped to a laboratory in Italy, and seven of those oocytes matured in vitro. Two of them became fertilized following ICSI with frozen-thawed northern white rhino sperm, developed to the blastocyst stage and were cryopreserved. For other species, with a little bit of foresight, it would be prudent to collect tissue samples from which

fibroblast cell culture can be generated and preserved. These fibroblasts can then be reprogrammed back into pluripotency to become iPSCs and from there, theoretically, into male or female gametes. And here enters dry biobanking. At least some samples from each stage – fibroblasts, iPSCs, germ cells, as well as spermatozoa collected from living or deceased males and spermatogonial stem cells extracted from testicular tissue collected from castrated to deceased males, can all be preserved in a dry form. With current technology, cellular membranes of these dried cells would be damaged, rendering these cells 'non viable'. A large number of studies, however, have demonstrated that while the cell membrane is damaged, the DNA is mostly well preserved. With relatively intact genetic load, these preserved cells can be "brought back to life" through somatic cell nuclear transfer (SCNT), a possibility demonstrated more than a decade ago [62] or, for spermatozoa, through ICSI as was first demonstrated in 1998 [56]. Besides the embryos that are generated this way and can thus be transferred into surrogate mothers, these embryos can also be a source of embryonic stem cells that can potentially be induced to commit into the germ line. SCNT has yet another advantage. The oocyte has a number of DNA repair pathways that become activated upon fertilization. These seem to be able to deal with a range of damages, sometimes even when really extensive, as we saw happening with rates of single strand DNA breaks near 100% in freeze-dried ram spermatozoa [69]. If DNA damage in lyophilized cells can be repaired, at least in part, by the oocyte, it would improve the probability of normal development further down the road. If DNA damage is extensive, one may consider even two rounds of nuclear transfers, i.e. performing a second nuclear transfer using blastomeres from the embryos produced through the first SCNT. The drawback of the SCNT approach is that at present SCNT efficiency is very low (in the range of 5%) so a huge number of trials and nuclear transfer procedures will be required to generate enough usable embryos *in vitro*. While work on *in vitro* gametogenesis is on going, we work in our lab on the process of drying somatic

cells for the purpose of eventual use in SCNT. The work progresses in several routes that would eventually converge.

During the process of cryopreservation, the cells go through several stressing stages, with possible damages inflicted at each of them – chilling injury, freezing (physical) injury, toxicity of cryoprotectants, osmotic injury, and damages during warming. So, the first question that is being asked is if freeze-drying really is our best option? Or can we follow a different drying approach and avoid freezing with its associated damages? After all, desiccation tolerant organisms in nature dry without freezing. Various research groups have attempted drying without freezing (by evaporation), including spin-drying [70, 71], microwave-drying [72, 73], air-drying [74, 75], vacuum-drying [59, 76], evaporative-drying [77, 78], convective-drying [79, 80], heat-drying [81], and vitri-drying (drying following vitrification) [82]. At our laboratories, we have also attempted drying without freezing (unpublished). Bovine and ram spermatozoa were subjected to drying in a vacuum oven with shelf temperature of 37ºC and a drying program that included two stages – four hours at 200 mbar (150 Torr) and then gradual decrease in pressure to 4 mbar (3 Torr) and maintain at this pressure overnight. For comparison, samples were freeze-dried. After rehydration, no motility was observed in either species or either drying technique but, at times, 1-2% of the freeze-dried bovine spermatozoa had intact plasma membrane when evaluated by eosin-nigrosin staining (Figure 3). Morphologically, evaluated as was previously described [83], 59.90±21.09% and 64.40±15.13% of the bovine spermatozoa and 52.9±22.1% and 75.3±16.9% of the ovine spermatozoa dried in vacuum oven and freeze-dryer, respectively were normal. Freeze drying preserved morphology significantly better than vacuum oven for ovine spermatozoa ($Z = 2.36643$, $P = 0.018$) but not for bovine spermatozoa ($Z = 0.509647$, $P = 0.610$). DNA fragmentation was evaluated by both Halomax and two-way comet assay and suggested that the DNA maintained its integrity following freeze-drying but not so after

vacuum oven drying. In the vacuum oven group there were over 30% double strand breaks and up to 100% single strand breaks, whereas level of double strand breaks following freeze-drying (~3%) was similar to that after freezing alone. Single-strand breaks, however, were a bit elevated in bull sperm (18%) but almost 100% in ram sperm [69] (Figure 4). This suggests difference in species sensitivity to assume iatrogenic damage, a fact that is probably related to the level of protection linked to the protamines' structure [84]. Still, as we have reported for freeze-dried ram spermatozoa [69], even with the very high single strand DNA breaks, fertilization still occurred following ICSI with rehydrated spermatozoa and around 6% blastocysts have developed (Figure 2C,D). While thoughts of liquid drying were not (yet) abandoned, at least for now freeze-drying seems to perform better.

One of the characteristics of anhydrobiotic organisms is that during the process of desiccation they accumulate large quantities of disaccharides, LEA and other proteins, and other protective components inside their cells. These large molecules do not readily permeate through the plasma membrane so solely placing the cells in a media enriched with such xeroprotectants is probably not enough, even if something does diffuse inside. For desiccation-sensitive cells, these should probably be actively loaded into the cells to ensure their protection also from within. To do that, a number of approaches can be found in the scientific literature. One way is to genetically modify the cells [79] or to transfect them with the required gene(s) [71]. While these may be viable options for proof-of-concept attempts, such techniques would not be suitable for routine clinical use. Others suggested loading the cells with these xeroprotectants by utilizing the transient permeability of the membrane during its lipid phase transition [85]. Alternatively, the membrane can be made temporarily permeable by using haemolysin [86] or through electroporation [87]. Yet another approach that researchers are taking is to turn these protective agents into lipid-soluble molecules so they can freely diffuse in and out of the cells [88]. Most studies, however, seem to rely on

simple diffusion of the protective agents through the membrane or hope that external protection would be sufficient.

Another aspect of drying that is being studied is the imbibition stage. In Nature, drying is normally very slow, allowing the organism time to prepare the cells for the anhydrous state. Rehydration, on the other hand, can be assumed to be rather fast. For example, when rain comes, the organism is exposed to water and rehydrates. In the lab the process works differently. Drying is relatively fast, done under stressful conditions, and the dried cells do not have the ability to generate the needed protective molecules. If drying is so different from the way it is done in Nature, maybe rehydration should be different too? Maybe the notion of "just add water" is wrong? Can one tell if the damages inflicted on the cells happened during drying or maybe they are the result of the fast rehydration? We know that one of the damaging mechanisms in cryopreserved cells is the abrupt and faster-than-desired movement of water down the osmotic gradient into the cells upon thawing. So, maybe a similar process happens during rehydration? Once dried, the cells' membrane is crumpled as the cells have shrunk in volume. If re-expansion is too fast, such fragile membranes can easily break, resulting in the compromised membranes we view in rehydrated cells. One way to slow down the rate of water movement into the cells is to expose them gradually to decreasing osmotic pressure, just like we do after warming vitrified oocytes and embryos. Oocytes and embryos, however, are large and can be easily picked and transferred from one solution to the next, an impossible task when dealing with many thousands of cells. An alternative approach we have been exploring is gradual rehydration by placing the dried samples in a humidity chamber. Preliminary results from work on sheep cumulus cells suggest that this slow and gradual rehydration procedure result in better morphology of the rehydrated cells.

5. So, what now?

If one looks at the history of wildlife conservation, the use of assisted reproductive technologies (ART) is extremely limited, and has not become a routine in management of wildlife species with the exception of the black-footed ferret (*Mustela nigripes*), the giant panda (*Ailuropoda melanoleuca*), and, to a lesser extent, in the Asian elephant (*Elephas maximus*). Conservation, however, should be far-sighted, and samples collected today should be preserved not only with current technologies and capabilities in mind, but also with those techniques currently under development (e.g. synthetic biology) and the yet unknown future ones. Cryopreserving samples from a large number of individuals from each endangered species to ensure coverage of the population genetic diversity and maintaining them under liquid nitrogen for yet unknown duration of time would probably be beyond the currently available resources and in some countries practically impossible for lack of stable liquid nitrogen and power supply. The alternative of preserving dried samples at ambient temperature is attractive. As the drying technology is still under development and as the protocol that will ensure return to biological activity after rehydration is still to be discovered, it might be prudent for now to preserve samples both by cryopreservation and drying. Once the drying technology has matured, this approach can be changed in favor of routine dry biobanking.

6. Acknowledgements

The research presented received funding from MIUR/CNR and Program FIRB Grant agreement No. B81J12002520001 “GenHome”, as well as from the European Union’s Horizon 2020 research and innovation program under the Marie Skłodowska-Curie Actions “Individual Fellowships (IF)” – DRYSTORE, Grant agreement No. 749537 and "Research and Innovation Staff Exchange (RISE)" – DRYNET, Grant agreement No. 734434, and

479 under the Twinning Action “EraofArt”, Grant agreement No. 698165. The somatic cells
480 freeze-drying for somatic cell nuclear transfer (SCNT) by AA was done under FertileSafe
481 funding.
482 Part of the unpublished work described in this paper was performed by JS while still working
483 at the Leibniz Institute for Zoo and Wildlife Research in Berlin, Germany.

484 7. Conflict of Interest

485 AA is consulting to Lyolife and is the founder of FertileSafe. All other authors declare no
486 actual or potential conflict of interest to disclose.
487
488

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9. Figures Legends

Figure 1. Freeze-dried nucleated blood cells. Blood samples collected from various endangered species in Israel were processed, the nucleated cells were separated, frozen by the directional freezing technology, lyophilized and stored under vacuum are room temperature in the dark. Shown here desiccated samples of cells from (left to right) Somali wild ass, Aoudad, and Addax.

Figure 2. Pagliarola ram freeze dried spermatozoa. (A) Straws of cryopreserved Pagliarola semen in the cryobank; (B) Dry biobank of lyophilized Pagliarola spermatozoa. (C) 2-Cell stage embryos at 24h after intracytoplasmic sperm injection (ICSI) with freeze-dried spermatozoa. (D) A blastocyst derived from ovine oocyte injected with freeze-dried spermatozoa at day 7 of *in vitro* embryo culture. Scale bar in C and D represents 100 μm.

Figure 3. Membrane integrity in freeze-dried spermatozoa: Membrane integrity in Freeze-dried bull spermatozoa. Shown in this figure a bovine spermatozoon with intact plasma membrane (left, termed “Live”) and a spermatozoon with damaged membrane (right, termed “Dead”) following freeze-drying and rehydration. Samples were stained with eosin-nigrosin and evaluated on a Keyence BZ-8000 microscope equipped with an x60 oil immersion lens.

Figure 4. Sperm DNA fragmentation after Halomax-test. a, b) Showing fragmented and unfragmented bull spermatozoa (a: cryopreserved; b: lyophilized). c, d) Showing fragmented and unfragmented ram spermatozoa (c: cryopreserved; d: lyophilized). Panels e, f, g, and h are magnified details from a, b, c, and d, respectively. Note that normal bull spermatozoa display morphologically equivalent baseline haloes in both treatments (compare e with f).

718 Large haloes of dispersed chromatin represent spermatozoa containing fragmented DNA.
719 Note that in ram, the halo morphology observed in putative normal spermatozoa differs
720 between the cryopreserved (g and sperm 1 in panel c) and lyophilized (h and sperm 1′ in
721 panel d) treatments. The baseline halo is no longer visible. Additionally, spermatozoa
722 containing fragmented DNA (large halo in panel c) tend to diminish after lyophilization
723 (arrow in panel d).
724 All figures were captured under fluorescence microscopy and were electronically filtered to
725 enhance differences.
726