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Biobanking and fertility preservation for rare and endangered species

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Abstract

For more than 25 years, systematic gathering and cryo-storage of biomaterials from diverse wild species have been ongoing to save gene diversity and improve captive (*ex situ*) and wild (*in situ*) animal management. Cryo-storage of biomaterials offers broad opportunities - from helping understand the fundamental biology of unstudied species to enhanced conservation breeding, genomics and veterinary medicine. While promoted for decades, the banking of germplasm, tissue, blood and DNA from wildlife species only recently has been considered by some to be a core function of animal conservation programs. Importantly, reproductive biotechnologies and fertility preservation are critical tools for saving and maintaining endangered species and are tightly related to biobanking. Some successes have been reported with the use and integration of artificial insemination (with fresh or frozen-thawed semen) in conservation programs. However, not a single wild species is currently managed through oocyte freezing or embryo-based technologies. This is primarily due to the lack of knowledge of species biology, as well as inadequate facilities, space, expertise, and funding needed for their successful application. More fundamental studies on animal reproductive biology as well as more fertility preservation options are needed with all parties involved (reproductive technologists, zoo biologists and conservationists) adopting parallel efforts to sustain wild populations and habitats

Keywords: biobanking, conservation breeding, endangered species, fertility preservation.

Introduction

Understanding and sustaining biological and genetic diversity is a social, cultural, scientific and economic imperative that is key to adaptation and survival in a human-dominated environment (Hooper *et al.*, 2012). It is apparent that the earth's biodiversity (its wealth of diverse species) is under assault by habitat degradation and loss, overexploitation, pollution, emerging diseases, invasive alien animals and plants and climate change (Wildt *et al.*, 2010). Besides putting the existence of species at risk, these hazards lead to small, fragmented animal populations that reduce resiliency and adaptability to change, often through the loss of genes that control integrity and fitness. Once a genetic resource disappears, it cannot be recovered. Raven and Wilson, 1992 stated that 'biological diversity is the key to the maintenance of life as we know it'. It is the planet's life support system, regulating local climate and atmospheric quality while absorbing pollutants, protecting watersheds and generating and maintain soils (Monfort, 2014). Most of all, ensuring the long-term protection of all species and their genotypes helps maintaining an environmentally functional, healthy planet (Wildt *et al.*, 2010).

The value of wild animal biobanks and reproductive biotechnologies

Understanding and sustaining a biodiverse planet is a critical task. Historically, genetically diverse species have been preserved by protecting large-size natural habitats, a strategy that, while ideal, is insufficient given our growing global human population that now exceeds 7 billion people. Resource demands by humans doom the idea that all species can survive sustainably and undisturbed in nature. Zoos and aquaria are not the answer due to severely limited spaces in their restricted urban environments - too few acres to manage enough animals. This is now fact as most structured breeding programs in zoos are failing to meet demographic and genetic goals, including retaining at least 90% of existing gene diversity (Monfort, 2014). Thus, while governments determine how to protect and restore habitat, and zoos explore alternative conservation approaches (for example, the advantages of large breeding centers; Wildt *et al.*, 2012), there is a crucial unfilled gap - protecting the extant genomes of living species that already are under threat, or are likely to be so soon.

This need actually may be envisioned as an enormous opportunity that can be addressed by establishing and using wildlife biobanks - organized collections of living biomaterials. The value of maintaining data-rich biological samples, including microorganisms, DNA, somatic cells, tissues, blood products, germplasm and embryos, has long been recognized for human health care and agro-industries and is a fundamental component of most basic scientific research (Baker, 2012).

The idea that these genome banks should exist for more than humans, livestock and crops is not new. The U.S. National Academy of Science declared in 1978 that 'what is done for domestic species should be done for all species'. Similar proclamations also were made decades ago by the U.S. Agency for International Development, the U.S. Congress' Office of Technology Assessment and the National Science Foundation. Subsequent advocacy and sound justifications have been provided by various laboratories (Comizzoli *et al.*, 2012; Comizzoli and Holt, 2014). First, there is the 'insurance factor', that is, protecting what we have now - all species and all existing gene diversity. Small populations of endangered species are especially vulnerable to events beyond inbreeding depression,

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including environmental catastrophes and epidemics. This is especially relevant as most of the earth's biodiversity exists in underdeveloped regions that are particularly sensitive to epizootics and drastic shifts in social and political structure. These resources deserve immediate and thorough protection. Second, having repositories of biomaterials, especially germplasm, can support conservation breeding programs where the goal of producing healthy and sustainable insurance populations is only possible in the face of adequate gene diversity. Currently, such programs exclusively rely on the expensive and unsafe movement of wild animals from one zoo to another for breeding. With biobanks and assisted reproductive technologies (i.e., artificial insemination [AI] and embryo transfer), only germplasm and embryos are moved to maintain the same levels of heterozygosity. Availability of germplasm in the repository also extends the generation interval of individual animals indefinitely, to be re-derived and infused into the living population at any time, 5, 20 or more than 100 years from now. The result is decelerating natural losses in diversity as a result of genetic drift. At the same time, managing a portion of the species as frozen germplasm reduces space needs. For example, even partial reliance on AI with frozen semen could reduce the number of living animals required in zoos and breeding centers by as much as 50% (Wildt *et al.*, 2010).

There now are real-life illustrations of using biobanks for conservation breeding. The iconic giant panda is routinely managed in *ex situ* collections and on a large-scale in China using AI with fresh and frozen-thawed spermatozoa (Comizzoli *et al.*, 2009). The black-footed ferret, once the most endangered species in North American, has been recovered by a combination of natural mating and AI (Howard and Wildt, 2009), including with sperm that has been frozen and stored for up to 2 decades. A litter of cheetah cubs was produced in a North American zoo by importing frozen sperm from a wild captured male in Africa (Comizzoli *et al.*, 2009). There also are many examples of 'milestone' births using frozen-thawed spermatozoa or even embryos (Saragusty and Arav, 2011; Comizzoli *et al.*, 2012) with the incidence of success completely dependent on having an excellent understanding of the details of the target species' reproductive physiology (Wildt *et al.*, 2010; Comizzoli and Wildt, 2013).

The components of the most valuable wildlife biobanks, of course, extends beyond reproductive cells to include tissues, cell lines, blood products and DNA, all highly relevant to the study and maintenance of biodiversity. Quantifiable amounts of genetic diversity can be determined for every sampled individual to help make informed conservation management decisions as well as improve our understanding of the processes underlying patterns of gene flow, selection and mating (Comizzoli *et al.*, 2009). Blood samples can be screened for clinical chemistries to provide new data on species norms or as sentinel information to identify onset and, eventually, cause of disease outbreaks to speed remedial actions. Most importantly, properly organized biobanks can provide open access to qualified researchers who normally work outside the conventional mainstream of wildlife conservation biology. This has the potential for generating vast amounts of additional basic and applied information, especially as advantages of the new 'omics' technologies are realized and directed to stored samples. Genomes of thousands of organisms, including bacteria, archaea and many fungi, animals and plants have been sequenced to begin more thoroughly documenting the earth's abundant bio- and genetic diversity (Tanabe and Toju, 2013). Genomic data are being annotated, augmented and refined through transcriptomics, proteomics and metabolomics to give us detailed pictures of messenger RNA, protein and metabolite systems and the mechanisms that are controlling life (Baker, 2013).

Examples of wildlife species biobanking associated with reproductive biotechnologies

While there are large facilities in the USA, other countries also manage biobanks with significant collections representing diverse species and specimens. Various biobanking activities at the Smithsonian were conducted originally by investigators within individual units, but the importance of managing an institution-wide repository now is fully recognized and appreciated, including by administrative leadership. These collective activities currently are conducted under the Pan-Smithsonian Cryo-Initiative that has a mission to promote collaborative stewardship of, and access to, Smithsonian's frozen collections. The advantages of such a coordinated approach have been significant, including creating stronger justification for more core budget resources for collection and storage as well as for enhanced equipment, staffing, barcoding of individual samples and database development. The latter involves metadata related to sample type, date and locality of collection, Geographical Information System references, collector, voucher information, DNA sequencing, and freezer location (as well as sample location in freezer), among other information items. The Smithsonian is rapidly moving to ensuring that all involved units meet similar best practices in cryo-collection management.

Certain wildlife biobanks have unique characteristics and/or constituencies. For example, the Frozen Ark, launched in 1996 (www.frozenark.org), has a mission to inventory and preserve the genetic material of threatened animal species, preferably in the form of living (including somatic) cells. This consortium has a membership platform comprised of zoos, aquaria, museums and universities from the United Kingdom, USA, Australia, India and other countries and has implemented an organized, internationally-linked and properly catalogued repository of genetic material (Clarke, 2009). Currently, member institutions manage more than 48,000 samples representing frozen tissues, somatic cell cultures and DNA from at least 5,500 animal species (www.frozenark.org). Some of the newer contributors, such as the German Cell Bank for Wildlife 'Alfred Brehm' (Cryo-Brehm), are contributing stem cells to the collection repertoire

(www.emb.fraunhofer.de/en/Uebersichtsindex/cellbank_cryo-brehm.html). Therefore, while early efforts in wildlife biobanking were largely focused on spermatozoa and embryos (Comizzoli and Holt, 2014), more recent activities envision significant, near-term opportunities with non-germinal cells. This is logical given significant advancements made in nuclear transfer and stem cell technologies, with somatic cells having potential to be used directly or indirectly for offspring production. The ability to reprogram differentiated somatic cell nuclei into embryonic or germinal cell lineages triggered the original interest in storing somatic genomes about a decade ago (Mastromonaco *et al.*, 2014). While the technology to convert these cells and DNA into living young has not advanced sufficiently to contribute to 'real-life conservation', there are some enticements to justify continuing such a collection/storage strategy. For example, using new somatic cell manipulations, Ben-Nun *et al.*(2011) have produced embryoid bodies derived from induced pluripotent stem cells (iPSCs) in the silver-maned drill (a non-human primate) and the nearly extinct northern white rhinoceros, the first such cases of induced pluripotency in adult fibroblasts. While many steps away from producing a living youngster, the technique advancements and the new knowledge only have been possible because the raw biomaterials (i.e., germ cells, somatic cells) were available in a biobank. Without this preemptive effort, these genomes would be lost forever, including to whatever new technical approaches may be on the horizon to retain species and genetic diversity and integrity. The same philosophy holds for storing cell lines, the 'gold standard' resource, for genomic studies and the eventual understanding of proteins and epigenetic factors that are regulating unique gene expression (Baker, 2013). These same thought processes are finding their way into the plant community.

Current challenges and prospects

These eclectic biorepositories also are essential for continuing to sort the many challenges remaining in effectively using germplasm and embryos to actually propagate and conserve thousands of species. To-date, most such research has been sperm- and embryo-centric, so there is additional need to focus on female genetic material, especially ova and oocytes (Comizzoli *et al.*, 2012) as well as their components (i.e., the germinal vesicle; Graves-Herring *et al.*, 2013). Due to their size and complexity, ova/oocytes present special cryopreservation challenges. Furthermore, while most research emphasis has been placed on mammals, some of the most interesting issues surround the question of how to store the essential reproductive elements of other taxa, for example, viable fish and amphibian germplasm that are fundamentally complex and cryo-sensitive. In such cases, these problems are being tackled by a host of novel approaches, including freezing gonadal germ cells that later are revived in other individuals of the same or even a closely-related species (Comizzoli *et al.*, 2012). However, it has been possible to recover and successfully freeze-thaw mature spermatozoa in at least one toad species (Kouba *et al.*, 2013). The priority now is developing fertilization methods *in vitro*, which would allow mass tadpole production for reintroductions into nature, thereby allaying some of the conservation challenges related to the deadly chytrid epidemic that has been responsible for multiple frog species extinctions in the past decade (Clulow *et al.*, 2014). As another example, investigators from our laboratory have successfully processed and then cryopreserved stem cells from coral species, taxa that are experiencing mass die-offs in the world's oceans. After storage in liquid nitrogen, these cells have been thawed and used to create living offspring that would be suitable for repopulating restored marine habitat (Hagedorn and Spindler, 2014).

In sum, there are substantial ongoing activities throughout certain parts of the world in the collection and storage of many biomaterials from non-human, non-livestock and non-laboratory animals. While most of the emphasis has been on collecting, there is evidence that these specimens are biologically viable. However, clearly there is the need for more research to ensure (1) that samples are being processed appropriately (i.e., after documenting basic cryobiological properties) and (2) that we understand the detailed physiology of every species to ensure that the specimen can be used to give information or produce a healthy offspring (Comizzoli and Wildt, 2013).

Finally, of course, a commonality of all banking initiatives is the financial capacity to sustain the repository forever. Good stewards of every such effort are preoccupied with apprehension of inadequate support and the collection being 'orphaned' or lost at some point. The advantage of human biorepositories is the commercial incentive associated with an enhanced or rescued human condition. However, profit-related enticements are illegal for wildlife (including their biomaterials) to prevent trafficking of rare species. Therefore, the support for wildlife biobanks must be based on justifications (as articulated above) for preserving bio- and genetic diversity, analogous to why we would support wild animals and plants in parks, reserves, zoos, aquaria and botanical gardens. Growing international awareness that these samples are a form of national asset or wealth (especially if they or the genes within can be exploited commercially) has produced a culture where national governments are keen to prevent biomaterials exports, even for research. This has created some difficulties for biobanks and museums that either cannot accept samples that do not have appropriate legal provenance or, worse, must discard historical, already in-house samples that lack requisite paperwork. The upside is that there is a growing realization about the importance of sovereign, self-interests that hopefully will ensure new means of equitable and fair resource protection and use, including building more biobanking capacity in underdeveloped countries.

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