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PERSPECTIVE

GENOME RESOURCE BANKING FOR WILDLIFE CONSERVATION: PROMISES AND CAVEATS

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Abstract

The value of cryopreserved germplasm in agriculture, aquaculture and medicine was recognized in the mid-twentieth century following the discovery in the late 1940s of a method for recovering viable spermatozoa after freeze-thawing. Sir Alan Parkes (a founder of cryobiology as a discipline) remarked that "time and space has been abolished for cattle breeding", a phrase that continues to summarise the potential value of the Genetic Resource Bank (GRB) concept for all species. The underlying principle behind these remarks was based on the recognition that spermatozoa could remain viable for many years, and still achieve pregnancies even long after the semen donor had died. Nowadays, live mammalian embryos, amphibian spermatozoa and cultured somatic cells can also be stored for future use in conservation breeding programmes, where the overarching aim is to mitigate the deleterious impacts of inbreeding on the fitness and survival of populations. Revolutionary advances in the cryobiology of coral spermatozoa, embryos and larvae are also helping to counter the damaging effects of climate change and toxic chemicals on coral reefs. In this article we review the ways in which GRBs can contribute to global conservation activities, noting that species-specific biological differences can limit the success of standard animal breeding technologies such as artificial insemination and embryo transfer. These limitations mean that there is still a need for the development of novel, and possibly species-specific, GRB technologies.

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Keywords: amphibians; cloning; corals; embryos; oocytes; spermatozoa; stem cells.

INTRODUCTION

For many years, cryobiologists with an interest in animal conservation have proposed that by conserving genetic materials such as gametes and embryos in the frozen (or desiccated) state, it should eventually be possible to use them in support of wildlife conservation in general. In principle, the main value of using such preserved materials lies in capturing and preserving a "genetic snapshot" of an animal population before it becomes rare and genetically depauperate, then being able to restore genetic diversity to the population when

and if required. This model, widely known as genetic resource or germplasm banking (GRB), or Biobanking, mimics the widespread practices used in other scenarios such as commercial cattle breeding, aquaculture and biomedical research, where stocks of frozen sperm and embryos from genetically valuable breeds, strains or individuals can be provided to breeders or researchers on request.

The distinctions between in situ and ex-situ conservation have undoubtedly become more blurred since the early 1990s, and some authors have even argued that, with some exceptions, animal populations can no longer be regarded as existing under truly wild conditions (1). The relentless growth of human populations has meant that former natural environments have frequently been converted into managed and possibly fragmented reserves, with the result that many free roaming animals, especially mammals, are unable to migrate and find unrelated mates. As a discipline, wildlife conservation should therefore be seen as an integrative set of activities, not restricted by false boundaries. In keeping with this idea, two articles published in 2013 and 2021 respectively (2) and (3) advocated using the phrases "One Plan" or "One conservation" to emphasise the need for the holistic integration of sustainability, in situ and ex situ conservation for the restoration of ecosystems.

Back in 2001, Peter Bennett (4) and the late David Wildt (5), among others, anticipated that habitat fragmentation would result in the genetic isolation of small populations, and would therefore represent a problem for the maintenance of genetic diversity. They suggested that genome resource banks (GRBs; i.e., collections of frozen gametes, embryos, fibroblasts, blood serum and other genetic materials), now widely known as "biobanks" could help. They envisaged that GRBs could function in the same way as "wildlife corridors" (6), which naturally permit animals to move between areas that are otherwise isolated, and thus allow natural gene flow to occur (Fig. 1). An essential requirement for this plan to work involves not only the development of effective and efficient reproductive technologies for sperm and embryo cryopreservation, but reliable techniques for using the frozen materials for animal breeding. Unfortunately, although the cryopreservation techniques have been optimized for use with a few domestic animals,

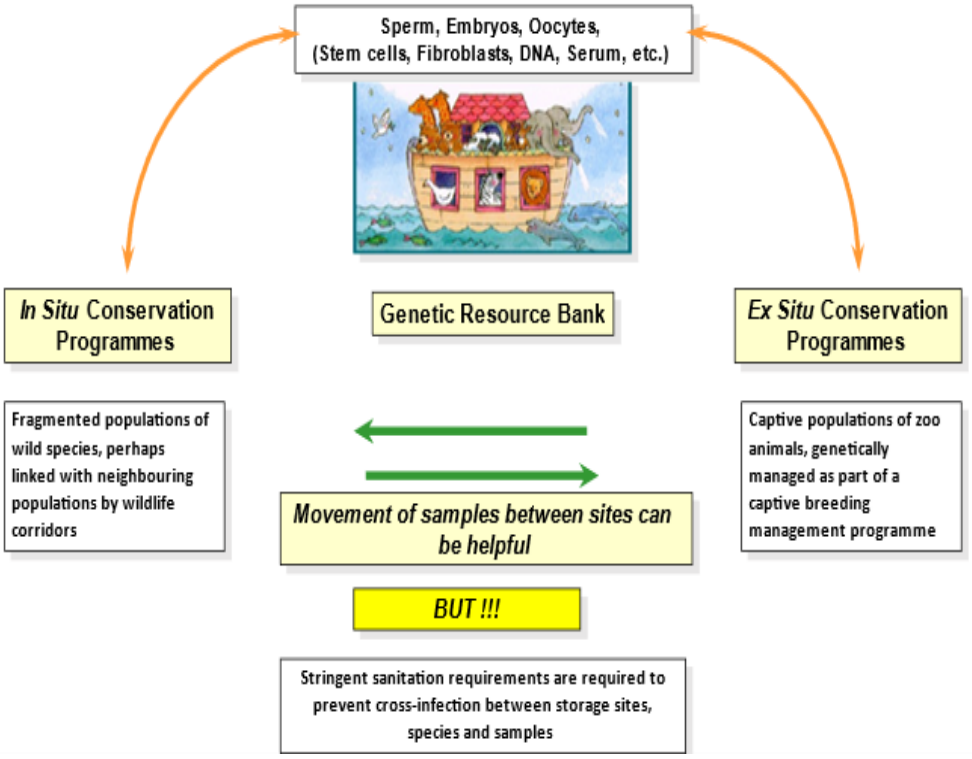

Figure 1. Schematic diagram illustrating the potential interactions between stored genetic material in a GRB and both in situ and ex situ conservation programmes. Note the need for stringent sanitary storage conditions.

notably cattle, sheep, goats and chickens, it usually requires a major research undertaking to translate the techniques to a previously unstudied wild species. This is because, despite advances in our understanding of cryobiological theory and how to mitigate cellular damage, the biological differences between species are a major impediment to the development of preservation methods that apply across different species (7). This problem was recognized many years ago by Wildt (5), a long-standing enthusiast and ambassador for the establishment and use of GRBs, who delighted in pointing out that "a cheetah is not the same as a cow"! Nevertheless, the principle of valuing GRBs for providing links genetic linkages between in situ and ex-situ conservation actions remains valid.

IMPLEMENTATION AND GENETIC VALUE OF GRBs

It is no easy matter to implement a practical programme that supports wild species in situ through the use of frozen semen, artificial insemination (AI), and the establishment of a GRB, but one project stands out as a success. In 1981 conservation biologists in the USA realized that the only ferret species endemic to north America, the black-footed ferret (*Mustela nigripes*) was nearly extinct. Its population had been almost completely destroyed by the extensive poisoning and hunting of their prey species, the prairie dog. A small population of 18 animals was discovered in Wyoming and eventually moved into a captive breeding facility to protect them from ultimate devastation by canine distemper and sylvatic plague. With advice from various conservation bodies, a plan to propagate the species by natural breeding and the use of reproductive technology was devised and established. Assisted reproductive technologies for the black-footed ferret had never previously been developed, initial studies to establish methods for semen collection and cryopreservation. An extensive study of semen cryopreservation techniques showed that freezing sperm samples as pellets on dry ice, using an egg yolk/lactose cryodiluent worked well. These techniques, including the technically demanding laparoscopic AI method, were devised using black-footed ferrets as well as domestic ferrets as a model species (8). Like many other wild species, the black-footed ferret is a seasonal breeder, with the mating season restricted to the months of March to June. As they are also "induced ovulators", which means that ovulation only occurs after a natural mating, the researchers had to find a way to induce ovulation artificially by the use of an injection of the hormone, human chorionic gonadotrophin (hCG). This project was very successful, and sufficient black-footed ferrets were bred that they could be reintroduced to their natural habitats. It is estimated that more than 8000 ferrets have been bred in captivity and more than 4000 reintroduced across eight US states (8, 9). The early development of a GRB to accompany the black-footed ferret project was aimed at capturing as much genetic diversity as possible within the remnant population, with the aim of restoring it in the future. This strategy has also paid off in terms of genetic diversity: recent genetic analyses have shown that those ferrets born after AI with frozen-stored semen show lower inbreeding coefficients than those bred without that input (9, 10).

The eventual development of reliable, even if complex, methodologies was crucial to the success of the black-footed ferret programme. That degree of reliability is extremely important, considering that frozen semen samples and embryos in a GRB are expected to remain completely viable, even if stored for several decades or more. With a small number of notable exceptions, including the black-footed ferret, there have been insufficient births arising from the assisted reproduction of a wild species to permit the assessment of reliability. The Giant panda breeding programme in China has, however, amassed sufficient data to examine success rates of AI with frozen semen and to compare them with natural breeding outcomes (11). An analysis published in 2012 found that the success rate of AI with frozen semen up to the year 2011 was about 25%; (5/20 AI events with cryopreserved semen resulted in live offspring). This is slightly higher than a later estimate (18.5%) published in 2017 (11), which was based on 65 AI events, carried out using both fresh and cryopreserved semen, but much lower than the birth rate of 60.7 % obtained after natural mating. Critical examination of the factors determining AI success in the giant panda (11-14) has revealed that the quality of frozen semen is less important that the timing of the insemination itself. As with many other species, matching the insemination procedure and the occurrence of ovulation requires precise information about ovarian status. The lifespan of

frozen-thawed semen within the female reproductive tract may be very short (possibly less than 4 hours in some species, and therefore if ovulation occurs 5 hours or more after insemination, fertilization would not take place. While information about ovulation status can be obtained using ultrasound scanning in readily handled domestic species, this is not practical in most wild species that, of necessity, require regular sedation or anaesthesia for such examinations [See, for example, a recent study of brown bear ovarian dynamics (15)]. Successful AI in the giant panda is known to depend on very precise estimations of ovarian status, either by the regular examination of cellular morphology in vaginal smears (16) or the regular (8-hour intervals) measurement of urinary oestrogen, progesterone and luteinizing hormones. Greatest success using the hormonal measurement method was obtained when AI was performed within 40 hours of the decline in urinary oestrogen concentration.

Although the successful use of frozen semen and AI in the Giant panda did not immediately contribute to in situ conservation, it will have considerable value for the genetic management of panda populations. At present there are seven ex-situ centres for giant panda breeding in China (17), a captive population of about 350 animals and a wild population that is split across 6 isolated sites. If left alone this fragmented situation is predicted to lead towards eventual genetic drift, hybridization and fitness differences between animals from the isolated areas (17). The judicious use of AI with the transportation of frozen semen between isolated sites could clearly help to correct some of these problems. Admittedly, it would be difficult in practice to capture and inseminate wild females, especially given the need for precise AI timing, but the collection and cryopreservation of semen from wild pandas, thus extending the genetic value of a GRB, may be a possibility. Apart from the Giant panda population within China, there are a number of captive giant pandas in zoos around the world, some of which have been bred using AI (13, 18-20). These isolated animals form a cohort that is potentially vulnerable to inbreeding depression, unless they are physically moved around the world as participants in a captive breeding programme. The physical stress imposed by such typically long-distance transportation can, and to some extent is, avoided by the exchange of frozen semen samples instead of whole animals. These observations emphasize the valuable supportive role that well-resourced and planned GRBs are, and will be, able to play in animal conservation and breeding if given the chance.

GRBs IN PROGRESS

Over the past few decades, a number of other GRB projects that aim to facilitate gene flow between ex-situ and in-situ situations have been established with varying degrees of success. One example concerns the ocelot, *Leopardus pardalis,* a wild feline species endemic to the southern USA, regions of Mexico and Brazil (21). Habitat fragmentation has progressively resulted in the genetic isolation of these animals (22), while at the same time there are a number of captive animals in zoos. Background studies have established electroejaculation as a routinely successful method of obtaining viable spermatozoa from ocelots (23) and the feasibility of using freshly collected and frozen–thawed spermatozoa for laparoscopic AI has been demonstrated (24). In addition, ocelot embryos have been produced following IVF and kittens born following the transfer of frozen–thawed embryos (25). The techniques could therefore be exploited within a systematic ocelot GRB, but this has yet to happen.

So far, we have focused on the use of cryopreserved semen samples, and to some extent it has been a fortunate coincidence that sperm cryopreservation methods were successful enough for use with the black-footed ferret and Giant panda projects. This is not always the case, and gamete biologists are currently attempting to make sense of the sometimes profoundly different cryobiological differences that can interfere with their plans. One outstanding and unsolved problem concerns the establishment of a cryopreservation method for marsupial spermatozoa. There are about 300 living marsupial species (26), many of which are of special interest in terms of conservation and the establishment of reliable methods for assisted reproduction. Unfortunately, despite a significant number of research projects targeted on the development of assisted reproduction and semen cryopreservation in marsupials (reviewed by Rodger et al (27, 28) only one, focused on the koala, has succeeded in developing a reliable method for AI (29, 30). To date this method, which required considerable background

research into female anatomy and endocrinology, has resulted in the birth of 34 koala babies following the insemination of fresh or chilled semen. However, as with other marsupials, the cryopreservation of koala spermatozoa has remained an intractable problem and it is questionable whether traditional AI with cryopreserved semen in these species will ever be a practical possibility. There are strong arguments in favour of developing successful semen cryopreservation and banking methods for many of the threatened marsupial species, but to date this has not proved possible. The problems encompass not only the sperm cryopreservation methods themselves, but also the extraordinary range of adaptations in reproductive biology that have evolved among marsupials.

Understanding reproductive anatomy in female marsupials is a challenge in itself, being different from that of the eutherian mammals. The caudal region of the female reproductive tract possesses two lateral vaginae, each of which opens into a urogenital sinus that also receives the urethra (31). Artificial insemination therefore requires a detailed understanding of sperm transport. Moreover, fertilization and embryonic development in marsupials show many differences from the same processes in the eutherian mammals; the main points of these differences are ably summarized in two reviews (32, 33). Marsupial oocytes are much larger (150-200 µm diameter) than those of the eutherians (typically about 100 µm diameter), and after fertilization (34) the zygotes exhibit visible polarity as an early indication of embryonic cell commitment. The polarity predicts the eventual separation into two cell types, pluriblast (equivalent to the inner cell mass of the mouse), the trophoblast, and ultimately the alignment of the body axis. The morphology and physiology of marsupial spermatozoa are also highly variable between species (35), but it is notable that sperm morphology within a single species can either be highly pleiomorphic, as in the koala (*Phascolarctos cinereus*) or show very little variability (e.g., macropodidae; i.e., kangaroos and wallabies). In addition, the new world marsupials endemic to south America and the southern USA, have evolved an unusual reproductive specialization whereby their spermatozoa become “paired” during maturation in the epididymis (36); the pairing involves very specific and stable interactions between the flattened surfaces of the two sperm heads, a situation in which the two sperm flagellae beat in synchrony, and progressive motility is linear and very rapid (37, 38).Given the seemingly insoluble problems of sperm cryopreservation in marsupials, and the urgent demand for a practical method of providing genetic support for (at least) some the threatened species, John Rodger has argued that the spermatozoa should at least be frozen and stored (27) in case they become usable in the future. Oocyte vitrification has been proposed as a feasible strategy for the establishment of a GRB (39). Czarny and Rodger (39) have shown that 70% of Tasmanian devil (*Sarcophilus harrisii*) oocytes remained viable after vitrification. This is a significant result as this species is currently the only mammal being decimated by a transmissible form of facial tumour that is spread when these aggressive animals bite one another (40, 41). Considerable effort is currently being invested in Tasmanian devil conservation (42, 43), with the establishment of a disease-free insurance population, split across a number of isolated sites (44).

GRBs AND THE GLOBAL AMPHIBIAN EXTINCTION CRISIS

The joint impact of ranavirus and fungal diseases, especially the chytrid fungus *Batrachochytrium dendrobatidis*, is currently bringing about the extinction of many, already threatened, frog, toad, newt and salamander species in Australia, Europe, North and South America and elsewhere (45-47), and about 200 species have already disappeared completely. As it is not economically viable to hold disease-free rescue populations indefinitely in biosecure facilities, there has been an upsurge of interest in assisted breeding techniques for amphibians, including the collection and cryopreservation of spermatozoa, as well as the collection of eggs and the production of tadpoles. It is feasible to stimulate amphibian sperm and egg production by hormonal induction in males and females, and as fertilisation and embryonic development take place in water, there is no need for complex IVF or embryo transfer methods. The establishment of species-specific hormonal protocols, mostly involving gonadotropin treatments that stimulate sperm and egg release, have now been published extensively (48-50). The technologies have clearly developed rapidly

over the past two decades and are ready for use if required by managers. In fact, a recent review (51) explains that hundreds of thousands of tadpoles have already been produced and released into nature.

In principle, these advances, which have been evaluated in a number of different amphibians, offer potential and impressive conservation benefits at the population level. One particular study (50), focused on the endangered Oregon spotted frog (*Rana pretiosa*), showed that supplementing a captive breeding programme by the regular use of cryopreserved spermatozoa every generation, would significantly reduce inbreeding in the colony, thereby improving the health of the whole population. Although amphibian propagation methods have been well established and integrated into biomedical research programmes, and centralised facilities exist for the distribution of eggs, egg extracts, embryos and frozen *Xenopus* spermatozoa (See https://xenopusresource.org/), the survival rate of tadpoles released into the wild is probably species-dependent and largely unknown. There is some evidence from a study of Fowler toads (*Anaxyrus fowleri*) that tadpoles and metamorphs derived from cryopreserved spermatozoa are morphologically smaller than normal (52; the significance of this effect has yet to be determined.

ALTERNATIVES TO SPERM-BASED GRBs

Collecting and holding frozen or vitrified oocytes, embryos or even fragments of ovarian cortex that contain primordial oocytes from threatened wild species is a valuable, but technically demanding approach to the establishment of a GRB (53-55). Nevertheless, a European project (Felid-Gamete-Rescue-Project), organised and run by the Leibniz-Institute for Zoo and Wildlife Research in Berlin has recently reported that during the period 2007-2017 they retrieved 1110 oocytes from 62 out of 74 female felids (84%), of which 277 (25%) oocytes were matured in vitro. Forty-seven embryos were generated by in vitro maturation and fertilisation, among them nine very valuable embryos from Asiatic golden cats (*Catopuma temmincki*) and Northern Chinese leopards (*Panthera pardus japonensis*). This project relies on the cooperation of European zoos and wildlife parks, which donate ovaries from animals that either died or underwent castration or euthanasia because of a medical condition.

The rising incidence of testicular cancer in humans has prompted an upsurge of interest in the feasibility cryopreserving and storing small samples of testicular tissue from patients undergoing invasive therapies in the expectation that they can eventually be used for the restoration of fertility (56). The potential value of these techniques for animal conservation has also shown promise when tested for use with various animal species [for review, see (57)]. Recent publications have reported the isolation and survival of mammalian testicular stem cells (spermatogonia) from alpaca, *Vicugna pacos*, (58), grey wolf, *Canis lupus* (59) and collared peccaries, *Pecari tajacu* (60). There has been similar interest and success in stem cell transfer between fish species, both for use in aquaculture and for the propagation of threatened fish species (61-66). This is particularly significant for the preservation of fishes, given that fish oocytes are still impossible to preserve by freeze-thawing.

ALTERNATIVES TO CRYO-GRB

Our scientific community still relies on expensive, low temperature methods for preserving biomaterials – from DNA samples, to blood products, to germ cells and reproductive tissues. However, electricity and liquid nitrogen are costly and not always readily available in many regions of the world designated as hotspot of biodiversity. Rather than relying on subzero-temperatures to suspend cellular and molecular activities, mimicking a natural phenomenon called ‘anhydrobiosis’ is a promising alternative. This mechanism is exploited by certain frogs, nematodes, tardigrades, insects and brine shrimp to survive extreme cellular water loss (67). During desiccation, these organisms synthesize and accumulate disaccharides (mainly trehalose) intracellularly. These sugars replace water within the cells and then convert to a glass state – a high viscosity liquid that immobilizes enzymes and prevents biochemical activities – at ambient temperatures. Dehydration of somatic cells by lyophilization (freeze-drying) has provided encouraging results (68). Similar approaches have been successfully applied in the domestic cat model to egg nuclei, sperm cells, ovarian biopsies, and testicular tissues (69-72).

These encouraging results clearly show that structures and, more importantly, functions of gametes and gonadal tissues can be suspended in trehalose glass and potentially be preserved for the long-term at room temperatures.

CRYOPRESERVATION AND CORAL REEF RESTORATION

The degradation of coral reefs is a serious and ongoing consequence of several processes including global warming by greenhouse gases, pollution, ocean acidification and inappropriate land use, fishing and mining practices. As a result, the corals themselves undergo bleaching, stress, increased susceptibility to diseases and ultimately a significant risk of extinction (73, 74). Cryopreservation has been seen as a tool for preserving frozen coral spermatozoa, embryos and tissue fragments (75), and a number of focused gene banks have been established around the world (Caribbean, Hawaii, French Polynesia and the Great Barrier Reef) (75). Hagedorn's review (75), published in 2019, stated that spermatozoa from 31 different coral species had been cryopreserved successfully using a standard technique involving dimethylsulphoxide as the cryoprotectant, and that a novel technique for the successful cryopreservation of coral larvae had been developed (76). A recent study carried out with a Caribbean coral species, *Diploria labyrinthiformis* (77), showed that cryopreserved spermatozoa with relatively poor motility were able to fertilise fresh oocytes, produce viable larvae and contribute to a genetic rescue project. Compared to other organisms there is an added complication to the cryopreservation of coral cells in that many co-exist with dinoflagellate symbionts [see (78) for review], whose biochemical functions are also being compromised by climate change, toxic chemicals and other environmental problems. This multidimensional problem, which includes the development of suitable methods for culturing corals in vitro, is nevertheless, being addressed successfully (75, 79, 80).

THE ETHICS AND REALITY OF SPECIES CONSERVATION THROUGH CRYOBIOLOGY

Advances in both cryobiology and animal biosciences have led to the development of an array of unorthodox possibilities for species propagation. Foremost among these is the ability to replicate genetic traits via cloning, where cell nuclei from an animal with desired genetic traits can be inserted into recipient, but enucleated, oocytes and the develop into fully grown individuals. This technology is well known, and was announced to the world by the birth of several sheep, including Dolly, cloned using mammary cell nuclei (81). Since then, many scientists, not to mention journalists, have argued that the somatic cells such as fibroblasts and induced pluripotent stem cells (iPSCs) (82), that are already cryopreserved and stored in GRBs, could be used for breeding threatened species via various cloning technologies (83-86). This view is supported by the successful application of cloning for several domestic mammals, including horses, rabbits, mice and dogs, but it is also widely recognised (87) that the cloning procedure itself has a low success rate and that cloned mammals frequently suffer from developmental abnormalities (88-90).

Proponents of cloning and iPSC technologies have gone further and proposed that archived tissues, iPSCs and other genetic materials, could theoretically be used to resurrect (or de-extinct) species that have gone extinct in the recent or distant past (85, 91, 92). This suggestion has sparked considerable debate among conservation biologists, ethicists and philosophers about the merits, or otherwise, of following this path. Apart from pointing out that current de-extinction approaches typically involve the production of inter-species hybrids containing mitochondrial DNA from one species alongside nuclear DNA from another, thus causing potential incompatibilities and mitochondrial disease (93), space prevents us from analysing the harms and benefits of this and other approaches. Several detailed, and often conflicting, harm-benefit analyses have, however, been published recently (94-96).

One extremely important aspect of using cells and tissues for breeding threatened species, whether or not they have been cryopreserved, is the avoidance of disease transmission and cross-infection. The agricultural industry observes strict hygiene protocols, especially when

processing, importing or exporting germplasm such as embryos and spermatozoa. This includes the recognition that liquid nitrogen storage tanks may contain unwanted bacteria and viruses (97), and unless sterilized using tested and approved methods, could devastate a country's dairy or pig industry if left uncontrolled. In the case of GRBs for wild species, the same principles apply and, as pointed out in a recent review (98), resurrected species may either be incompatible with modern parasites of all kinds (e.g., arthropods, bacteria, viruses, etc.), or alternatively may lack the beneficial interactions they previously obtained from their microbiomes. Evidence from the current global devastation of amphibian populations by pathogenic fungal and viral diseases (45-47), showed that disease transmission can occur between sites, within countries, if insufficient attention was paid to the disinfection of vehicles, tyres and clothing. This underlines the need for care when designing and implementing conservation programmes and, in principle, highlights the similarities in approach between the use of GRBs and species reintroduction/translocation programmes (99).

CONCLUSIONS

Conservation biology is, by definition, an international activity that involves practitioners in many countries. This means that for conservation breeding purposes, the genetic materials stored in GRBs are often seen as resources to be shared between organisations across the world. In practice this idealistic approach is, however, unrealistic for several reasons. The avoidance of disease transmission is an obvious necessity, but another is the protection of those genetic resources against the unfair exploitation of intellectual property rights. Nations regard the biodiversity within their own territories as "their own to exploit", a principle which is now enshrined within an international treaty known as the "Nagoya protocol" (100). This treaty obliges researchers, managers and industrial companies to respect this principle, and means that the international transport of frozen gametes, embryos and stem cells and other animal components requires the negotiation of binding agreements between the sending and receiving countries. The use of GRBs for "within-country" animal breeding programmes is therefore more practical and easier than programmes involving import and export.

With these considerations in mind, it is worth emphasizing that the current enthusiasm for exploiting existing germplasm collections as source material for de-extinction projects involving the use of cloning with somatic cells, and even resynthesized DNA (e.g., from ancient Woolley mammoth DNA), is an undeniably complex practical, as well as scientific, prospect.

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