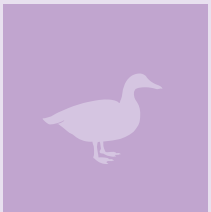
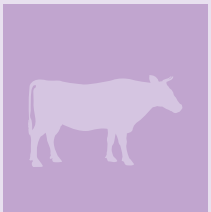
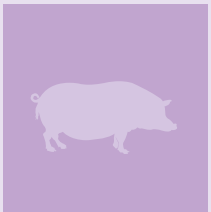
SECTION 1

Confirming the decision to cryoconserve

Confirming the decision to cryoconserve

Conservation of animal genetic resources for food and agriculture (AnGR) may be undertaken for a number of reasons. In developed countries, traditions and cultural values are important driving forces in the conservation of breeds at risk and the emergence of niche markets for livestock products. However, in developing countries the immediate concerns are food security and economic development.

In general terms, objectives for AnGR conservation fall into the following categories:

- Domestic animal diversity should be maintained for its economic potential in allowing the livestock sector to respond to changes in agro-ecosystems, market demands and associated regulations, availability of external inputs, disease challenges or a combination of these factors.
- Domestic animal diversity plays an important social and cultural role. Loss of typical breeds, therefore, means a loss of cultural identity for the communities concerned and the loss of part of the heritage of humanity.
- Domestic animal diversity is an integral part of the environment in a range of production systems. The loss of this diversity would increase instability and risk in these production systems and reduce their ability to respond to changes. Maintenance and development of adapted breeds are critically important in ensuring that food security can be achieved sustainably without adverse environmental impact.
- Domestic animal diversity should be conserved for research and training. This may include basic biological research in genetics, nutrition, reproduction, immunology and adaptation to climatic and other environmental changes.

The specific objective or objectives for conserving a given AnGR will influence the strategy employed to conserve it. Conservation strategies can be categorized either as *in situ* conservation (in which animals are maintained within the environments or production systems in which they were developed) or as *ex situ* conservation (all other cases). The latter can be further divided into *ex situ – in vivo* conservation and cryoconservation.

IN SITU CONSERVATION

In the context of domestic animal diversity, *in situ* conservation primarily involves the active breeding of animal populations for food and agricultural production in such a way that diversity is optimally utilized in the short term and maintained for the longer term. Activities pertaining to *in situ* conservation include performance recording schemes, development of breeding programmes and management of genetic diversity within populations. *In situ* conservation also includes steps taken to ensure the sustainable management of ecosystems used for agriculture and food production. These various aspects of *in situ* conservation are

discussed in detail in the guidelines on In vivo *conservation of animal genetic resources* (forthcoming) and on *Breeding strategies for sustainable management of animal genetic resources* FAO (2010*)* in this series.

EX SITU CONSERVATION

In the context of domestic animal diversity, *ex situ* conservation means conservation away from the habitat and production systems where the resource developed. This category includes both the maintenance of live animals and cryoconservation.

Ex situ – in vivo conservation

Ex situ – in vivo conservation is *ex situ* conservation in which germplasm is maintained in the form of live animals. As in the case of *in situ* conservation, it is accepted that improvement and natural selection may alter gene frequencies in the conserved population. A key question with respect to this strategy is whether or not long-term finances and commitment are available to maintain generations of animals to the standards required for successful conservation (i.e. with a sufficiently large population size). More details on *ex situ – in vivo* conservation can be found in the forthcoming FAO guidelines In vivo *conservation of animal genetic resources*.

Cryoconservation

Cryoconservation is the collection and deep-freezing of semen, ova, embryos or tissues for potential future use in breeding or regenerating animals. A key question with respect to cryoconservation is whether, in the short term, the facilities and expertise required for collecting the samples can be financed and put in place. The logistics and costs of establishing and maintaining storage facilities will need to be addressed before the cryoconservation scheme is set up.

COMPLEMENTARY ROLES OF *IN SITU* AND *EX SITU* CONSERVATION

The Convention on Biological Diversity[1] emphasizes the importance of *in situ* conservation and considers *ex situ* conservation to be an essential complementary activity.[2] *In situ* and *ex situ* conservation are complementary rather than mutually exclusive. The exact conservation strategy adopted will depend on the specific objectives. *In situ* and *ex situ* strategies differ in their capacity to achieve different objectives.

From a general point of view, *in situ* conservation is often regarded as the preferable method because it ensures that breeds are maintained in a dynamic state. This may be true when the dynamics of a breed are characterized by slow and balanced adaptation to the conditions in which it is maintained. However, commercially important breeds are often subject to high selection pressures and larger than desired levels of inbreeding (a few top sires fathering many offspring), while commercially less-important breeds often have a small population size and are threatened by genetic drift and extinction. Moreover,

[1] http://www.cbd.int/convention/articles/?a=cbd-08

[2] http://www.cbd.int/convention/articles/?a=cbd-09

TABLE 1
Conservation techniques and objectives

Objective	Technique		
	Cryoconservation	***Ex situ – in vivo***	***In situ***
Flexibility of a country's animal genetic resources to meet future changes:			
• insurance against changes in production conditions	++	+	++
• safeguard against diseases, disasters, etc.	++	-	+
• opportunities for genomic research	++	++	++
Genetic factors:			
• allowing continued breed evolution/genetic adaptation	-	+	++
• increasing knowledge of phenotypic characteristics of breeds	+	++	+++
• minimizing exposure to genetic drift	++	-	+
Sustainable utilization of rural areas:			
• opportunities for development in rural areas	-	+	+++
• maintenance of agro-ecosystemdiversity	-	-	++
• conservation of rural cultural diversity	-	+	++

+++ = highly relevant to the objective; ++ = moderate relevance; + = low relevance; - = not relevant.
Source: adapted from Gandini and Oldenbroek (2007).

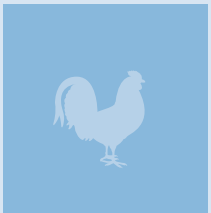
conserving genetic diversity by keeping live animals outside their original or usual production environments (*ex situ – in vivo*) will not always guarantee that the genetic diversity of the breed is maintained. Thus, *in vivo* conservation should be complemented by cryoconservation of germplasm. In other words, long-term *in situ* conservation programmes may benefit from the existence of a germplasm repository.

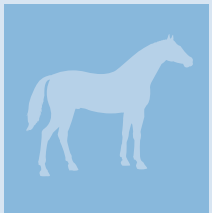
WHEN IS CRYOCONSERVATION THE BEST OPTION?

As a result of preparing their national strategies and action plans (NSAPs) for AnGR, countries should have identified which of their AnGR require conservation and what the objectives of conservation programmes for these resources should be. Based on these objectives – and taking into account the state of national technical capacity and infrastructure for cryoconservation, and the amount of capital available to invest in an AnGR gene bank – countries should determine which AnGR (if any) should be targeted for cryoconservation. Table 1 presents a comparison of the three main conservation strategies, indicating which are preferable with respect to a number of conservation objectives. The number of plus signs (+) indicate the relevance of the technique to the objective, whereas minus (-) indicates that the technique is of little relevance. The information refers to well-designed conservation programmes. For example, *in situ* conservation programmes will not be relevant for safeguarding against disease and disasters if all herds are concentrated in a small geographic area. Cryoconservation will actually introduce genetic drift if germplasm is not collected from a sufficiently large number of animals.

SECTION 2

Implementation and organization





Implementation and organization

Once the decision to establish a cryoconservation programme has been taken, preparation and planning can begin. A well-planned and maintained cryoconservation programme can play a key part in maintaining genetic variability within a given livestock population and essentially prevent its complete extinction. Nevertheless, a gene bank should in most cases be regarded as a form of insurance against the loss of genetic variability or extinction, and thus as a complement to (rather than a substitute for) programmes for the management of AnGR *in vivo*.

Although a facility for cryoconservation of germplasm can be relatively quickly and inexpensively established to "save" breeds that are at great risk of extinction, long-term maintenance of a gene bank requires continuous resources. Thus, it is essential that plans are put in place to ensure the ongoing provision of these resources.

SHAPING NATIONAL STRATEGIES AND ACTION PLANS

Conservation is only one of the four strategic priority areas of the *Global Plan of Action for Animal Genetic Resources*, and cryoconservation is only one among several options available for conserving AnGR. Ideally, gene banks should be established within the framework of a national strategy and action plan (NSAP) for AnGR (or similar national programme for AnGR management). FAO has developed guidelines on the preparation of NSAPs (FAO, 2009), which recommend that countries establish National Advisory Committees on AnGR. Where they exist, such committees should either take responsibility for planning the gene bank or create a dedicated task force of experts to deal with gene banking. Many countries have existing gene banks, but as yet no formal national strategy and action plan. The existing gene banks should, of course, be accounted for in the development of the national strategy and action plan. Moreover, while a coherent national strategy is important, countries should not allow at-risk AnGR to be lost while waiting for the establishment of their national strategy and action plan if the losses can be prevented by creating a gene bank.

ORGANIZATION AND INSTITUTIONS

No single organizational and institutional system will be ideal for all situations and countries. The optimal system will depend on a wide variety of factors, including the infrastructure and related institutions that already exist, the technical capacity of available personnel, the species to be targeted by the cryoconservation programme, the stakeholders that will be involved, and level of government versus private support. For example, a breed association will have a vested interest in establishing a gene bank for conservation of its

particular breed, whereas governments may assign priority to conserving the populations that are most critical for national food security. Therefore, evaluating major institutions and stakeholders in terms of their goals and their capacity to contribute to conservation programmes will be an essential step in the initial phases of developing a national strategy and action plan for AnGR.

Establishing linkages among institutions will be critical to maximizing efficiency in a cryoconservation programme. Collaboration with artificial insemination (AI) centres will be highly beneficial in many developing countries, as these centres will have the technical capacity and infrastructure needed for collecting, freezing and storing germplasm, as well as a reliable source of liquid nitrogen. In other situations, collaboration across species or among different ministries may be particularly important. For example, it may be efficient to have a national gene bank for all the different kinds of organisms that need to be cryoconserved, including not only livestock, but also wild animals and/or plants (assuming health and sanitary regulations can be addressed adequately).

When the characteristics of a country require the establishment of multiple gene banks for livestock, good communication links among the gene banks and between them and the National Focal Point for the Management of AnGR are important. Effective communication will help the National Focal Point ensure that the country's conservation goals are being met without duplication of activities. Although the advice offered in these guidelines is generally presented in terms of how to establish and operate a single national gene bank, one practical option that countries may wish to consider is a "virtual" gene bank. This would involve keeping different types of germplasm (e.g. varying according to species or breed) in different locations, but having a central database to monitor inventories.

PARTICIPATION OF STAKEHOLDERS

A number of different groups of stakeholders have particular roles to play in the establishment and operation of a cryoconservation programme. Each of these stakeholder groups must be consulted in the planning phases of the programme so that their responsibilities can be outlined and their consent to collaborate obtained.

The state

Within the government, overall responsibility for the conservation of AnGR will usually lie with the ministry of agriculture. In countries where responsibility for biological diversity as a whole is vested in other ministries, such as those of the environment or natural resources, close liaison and coordination among the various ministries is important. Such relationships must be clarified in the development of the national strategy and action plan for AnGR. The government as a whole ultimately influences cryoconservation programmes through budget allocation, whether by directly supporting state-owned and operated gene banks or by providing grants to private institutions that operate gene banks for the public interest. In many developing countries, AI centres are financially supported by the ministry of agriculture. Universities and research institutes, as public institutions, may be entrusted with the cryoconservation of breeds at risk (possibly to be used, in part, for research purposes).

As described in the preceding subsections, the state is responsible for overseeing the development of the national strategy and action plan, establishing a National Advisory Committee on AnGR or a cryoconservation task force and coordinating national activities involving all stakeholders. It is has a role to play in providing funding and training, promoting communication, and facilitating regional and international collaboration.

Individual livestock keepers and breed associations

Private livestock keepers will typically be the initial owners of the individual animals whose germplasm is to be cryoconserved. Thus, engaging with them will be critical to the success of the gene bank. Individual livestock keepers may provide information about the origins of breeds and animals, and thus assist in the process of selecting stocks that are as genetically unrelated as possible. Formal agreements must be drawn up outlining the terms of any compensation for the provision of germplasm to the gene bank. The agreements should also cover rights to future access to the stored material and set out any conditions surrounding this access (see Section 11).

Breed associations, such as cooperative-breeding and herd-book associations, are clearly interested in the long-term well-being of their respective breeds and may organize and financially support cryoconservation activities. Whether or not this is the case, support from breeders' organizations is needed in order to obtain good survey information for use in selecting the animals whose germplasm is to be deposited in the gene bank and for the general success of the conservation scheme. Other non-governmental organizations (NGOs) may also be able to contribute to cryoconservation programmes, in particular through grassroots interaction with farmers and breeders. Breed conservation is the specific objective of some NGOs.

Private companies

Commercial breeding companies, processing companies and agricultural support services may become increasingly interested and involved in cryoconservation activities (particularly pig and poultry businesses) in order to maintain the variation of breeds and the possibility of accessing these breeds easily when producing new founder lines. Private companies continue to seek additional genetic resources from outside the company, and are likely to conserve genetic material that may hold future promise. They also undertake research that directly benefits themselves. They may have infrastructure available to host a public gene bank. Clearly, if private facilities are to be used for a public gene bank, it will be critically important to establish precise legal agreements regarding access and benefit sharing in order to ensure total transparency (see Section 11).

The National Coordinator for the Management of AnGR

The National Coordinator for the Management of AnGR (National Coordinator) will be an important partner. The National Coordinator is likely to be a member of the National Advisory Committee on AnGR. In all countries, the National Coordinator should be kept informed about all cryoconservation activities, as he or she will be responsible for reporting this information to FAO.

FUNDING AND ATTRACTING SUPPORT FOR PROJECTS

As mentioned above, direct stakeholders – such as the state, breeders' associations and private companies – will generally be expected to provide most of the financial support for the gene bank. However, other sources of funding may be necessary. In order to develop plans that may attract funding and wider support, the relevance of the gene banking activities to the implementation of the Convention on Biological Diversity must be clear, which implies relationships between the targeted livestock and conservation of biological diversity in general, sustainable use and equitable sharing of benefits arising from use. Funding from national governments and cooperating international bodies will be more likely if the gene banking project is clearly of relevance to multiple aspects of government policy (e.g. agricultural, environmental, cultural or social policy, or – in the case of draught animals – energy and transport).

Documenting the wider importance of a local breed may mean that rather than being regarded merely as a commodity subject to market-driven economic forces, it can be valued according to the principles of the Convention on Biological Diversity. If such broader values are recognized, participation in conservation projects (financing or contribution of in-kind services) may extend beyond agencies concerned with agriculture and livestock to those concerned with environmental issues and indigenous cultures. Increased awareness among the general public – who are increasingly urban in their lifestyles – of problems affecting rural communities can also play an important role in influencing funding decisions.

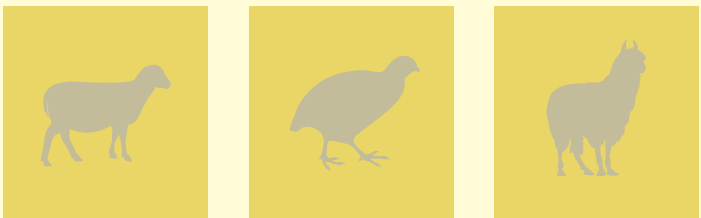
Two key features will help livestock conservation projects attract funding from international agencies:

- being part of a national strategy for the conservation of biodiversity and the environment as a whole, including wild animals, plants and forests, water, soil and microbes; i.e. projects are likely to be regarded more favourably if they do not view livestock in isolation from their environments; and
- supporting indigenous communities who wish to continue their traditional lifestyles. The needs of indigenous people have growing international recognition because it is now acknowledged that many indigenous people have been practising sustainable lifestyles for millennia. Hence, projects that aim to encourage the use and conservation of traditional breeds with intimate ties to such communities are more likely to be viewed favourably.

It is difficult to get long-term funding from international aid agencies. Therefore, it is important that governments commit themselves to providing the financial support needed to keep conservation projects in operation over the longer term. Finally, conservation projects should not only provide the support needed to prevent the targeted breeds from becoming extinct, they should also seek to identify and promote means by which the future use of the breeds can become more self-sustainable.

SECTION 3

Objectives of cryoconservation programmes



Objectives of cryoconservation programmes

Cryopreservation allows the storage of biological material without deterioration for at least several thousand years (Mazur, 1985) and probably much longer. This means that the wealth of genetic diversity present in the world today can be put into long-term storage in a biological "safe deposit vault".

GENE BANKING

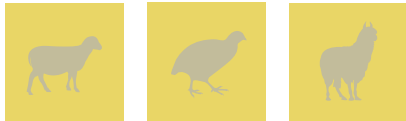
Gene banks and their collections of germplasm and tissue can have multiple functions and objectives. While the primary function of gene banks is conservation of AnGR for use in the medium or long term, the material stored may also be used for other purposes. For example, such material can be used to introduce genetic diversity into *in vivo* populations and thereby reduce inbreeding levels and broaden breed diversity in the event of a genetic bottleneck. It can also be used to provide flexibility to the livestock industry when particular selection strategies are found, after the fact, to be less appropriate than initially envisioned.

Developing gene banks with multiple functions is beneficial because it increases the potential returns. Clearly, gene bank managers should take the potential for multifunctionality into account as they plan and execute collection strategies (ERFP, 2003).

One common reason for establishing a gene bank is to provide the possibility of recreating breeds or breeding lines if they are lost as the result of a calamity. Storage of germplasm for this purpose is typically long term, and does not involve frequent use of the stored material or necessitate regular updating of the collection.

A second potential purpose is to support *in vivo* conservation. Frozen semen and embryos can be used to minimize inbreeding and genetic drift in small managed populations; the combination of live animals and cryopreserved germplasm can be a powerful tool in conservation (Meuwissen, 1999). Sonesson *et al.* (2002) proposed a scheme in which semen is collected from the first two generations of a breeding programme and then used in an alternating manner to breed females of subsequent generations. By using semen of bulls from the founder population (generation = 0) exclusively for breeding females born in odd-numbered generations and semen of bulls from the subsequent generation (generation = 1) on females born in even-numbered generations, Sonesson *et al.* (2002) reported a significant decrease in inbreeding relative to conventional approaches that did not use stored semen.

Material stored in a gene bank may also serve as a backup that can be used if genetic problems occur. A decrease in effective population size (N_e – see Box 1) and the resulting high level of inbreeding can increase the frequency of specific deleterious alleles that were not apparent in the larger population. Although most individuals are likely to carry some



BOX 1

Maintaining genetic diversity

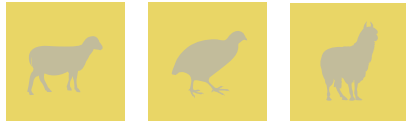
The primary motivation for developing cryopreserved germplasm collections is to have the capacity to maintain and enhance the genetic diversity of *in situ* populations. One common measure of genetic diversity is effective population size (N_e), which is usually smaller than the absolute population size.

N_e is "the number of breeding individuals in an idealized population that would show the same amount of dispersion of allele frequencies under random genetic drift or the same amount of inbreeding as the population under consideration" (Wright, 1931). An idealized population is one in which all members have an equal opportunity to produce offspring. Sex ratio has such a major influence on N_e that a population consisting of four males and four females has an N_e of eight, which is the same as the N_e of a population composed of two males and 100 females.

An N_e of 50 or larger has been recommended for breed conservation (FAO, 1998). At this level, the rate of inbreeding is 1 percent per generation. However, for gene banks to reach their full potential, additional genetic considerations need to be accounted for. For example, gene banks should sample enough animals to capture rare alleles within the respective population, and thereby ensure that their collections cover the range of phenotypes needed in order for them to be used for corrective mating or as a basis for introducing the genotypes needed for adapting breeds to future market demands.

When reconstituting a breed from germplasm collections, significant attention must be given to the mating plan, so that after backcrossing has been completed the genetic relationships are minimized and the constant N_e maintained. A simple approach is to use approximately equal numbers of doses of semen from each bull. The N_e can be enhanced by not using semen from a portion of males in the gene bank during the reconstitution process. These males will then be unrelated to the reconstituted animals and it will be possible to use their samples to increase the N_e further once the reconstitution process is completed.

deleterious alleles, the effects of such genes are usually recessive and only observed in the homozygous state. Low N_e increases the probability that mates will share a common ancestor, and thus the risk that they will carry the same deleterious allele(s) and that their offspring will receive the allele(s) from each parent. This does not only happen in at-risk breeds. It can also happen in commercial breeds with large populations (e.g. when a very small number of sires are responsible for a very large number of offspring). In such cases, the N_e of the gene pool is very small even if the real population is large. Gene bank resources may be needed to dilute the deleterious alleles in the population by introducing genotypes (e.g. in the form of semen doses) from the original population (i.e. the larger population that existed before selection).



A fourth important use of cryoconserved material is in the development of new lines or breeds, or for quickly modifying or reorienting the evolution or selection of a population. For instance, storage of original or extreme genotypes can provide the means to redirect the genetic trend of a selected population. Verrier *et al.* (2003) suggested storing genotypes that have extreme breeding values for specific traits, carry rare alleles or represent particular founders or pedigree lines. An example would be storing material from a traditionally dual-purpose cattle breed that was later reoriented to beef or milk production.

Finally, gene banks can serve as the primary source of material for scientists performing DNA research. Storing isolated DNA along with germplasm in a gene bank can allow researchers to access genomic information more quickly and without potentially damaging the valuable germplasm. It can also provide access to genetic material from common sets of animals for genotyping research. Gene banks can also supply multigenerational samples, which can be very useful in such studies.

Because of the broad range of uses to which a gene bank's collection can be put, it may be useful to subdivide the genetic material from each breed into various categories.

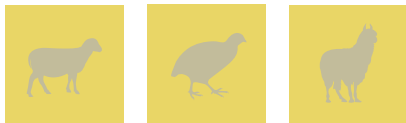
COLLECTION GOALS

The collection goals for each of the above-described gene banking functions differ substantially and are also dependent upon on the difficulty involved in acquiring the particular germplasm. Collection goals exist at the animal and breed level, and it is important to be flexible in meeting both criteria. The standard recommendation is that a conserved population (including a newly reconstituted breed) should have an N_e of 50, so that the rate of inbreeding can be held at 1 percent per generation (FAO, 1998). However, Meuwissen and Woolliams (1994) showed that the N_e needed in order to conserve genetic diversity could vary from 31 to 250 animals, depending on the mating system and other factors such as the level of inbreeding depression rates or response to selection to improve fitness.

When aiming to conserve specific alleles, FAO (1984) points out that by preserving semen from 50 unrelated males, a repository has a 63 percent chance of capturing alleles with a frequency of 0.01.[3] This estimate may give some gene bank managers cause for concern if they are attempting to capture unique and potentially unidentified alleles. By increasing the number of unrelated males to 100, the probability of capturing a rare allele at the 0.01 level increases to 87 percent. Deciding whether to increase the number of males in the collection requires the gene bank manager to weigh the trade-offs between the costs of the additional collections (both acquisition and storage), the additional protection afforded and the broader goals of the repository. In some breeds, the whole live population may be smaller than the targets mentioned above, in which case samples may be taken from a large proportion of the male population, if not from all the males.

Experience at some gene banks has shown that for some breeds acquiring the targeted number of males is relatively easy. This is mainly because certain breeds are available for collection at AI centres or are readily accessible on local farms. However, for some at-risk

[3] Probability of capture = $(1-p)^{2(N)}$, where p is the frequency of the desired allele and N is the number of males with germplasm in the repository.



breeds that are widely dispersed geographically, the potential for collecting from multiple animals is limited. In such cases, acquiring samples from the recommended number of animals will be difficult in a short period and will have to be considered a long-term goal.

Minimum collection goals should be established. As described later in this section, the material in a gene bank may be categorized into separate "collections", the most critical of which is the core collection, which contains the germplasm necessary for reconstituting breeds. To establish minimum targets, gene bank managers need to determine how the germplasm will be used. Enabling potential breed reconstitution should be the most important consideration. Mating strategies may differ across species and as a function of whether semen or embryos are used in the reconstitution process. Furthermore, collection goals are heavily influenced by the reproductive efficiencies that can be achieved in the process of reconstitution. This aspect of the process is critical because it directly affects collection targets. As reproductive efficiency increases, collection targets can be decreased.

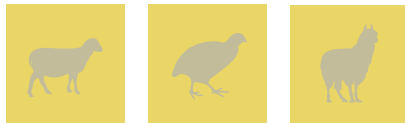
Breed reconstitution

According to FAO (1984), the number of parents sampled and the amount of material (semen doses, embryos, etc.) stored will depend on the intended eventual use of the stock. If the stock is to be used for pure breeding or as a maternal breed in a cross-breeding programme, inbreeding (leading to inbreeding depression) and loss of genetic variation (leading to lower responses to subsequent selection) should be avoided. In most cases, collection of germplasm from all individuals will not be possible, and a sample of animals will have to be selected. The sampling process will clearly result in a loss of genetic variation relative to the whole population (i.e. loss of the unique alleles from non-sampled animals). FAO (1984) recommended limiting this loss to a maximum of 2 percent of the total genetic variation. A loss of 2 percent is equivalent to an N_e of 50 in the reconstituted population, and would be met by collecting semen from 25 unrelated sires, or by 25 parental pairs with frozen embryos. For comparison, a loss of 2 percent of the total genetic variation is similar to the loss due to inbreeding over about four generations of selection for many breeds of livestock in practice.

Thus, moderate numbers of donors and quantities of germplasm are likely to be adequate for most gene banks, though these numbers might be increased in practice to provide a margin of safety. The number of frozen embryos or semen doses that need to be stored from each mating or each sire depends on the level of reproductive success achieved with the respective type of frozen material and on the amount of testing, multiplication and additional use to which the conserved stocks will be subject.

FAO (1984) was the first to suggest a minimum number of 25 donors, and many later manuals, including FAO's *Secondary guidelines for development of national farm animal genetic resources management plans: management of small populations at risk* (FAO, 1998) and more recently the European *Guidelines for the constitution of national cryopreservation programme for farm animals* (ERFP, 2003), have agreed with this number.

In determining the number of donor animals to include in a cryoconservation programme, FAO (1998) assumes that every animal is valuable and has a utility in terms of the amount of genetic diversity it provides to the conserved gene pool. Each additional animal adds a



marginally smaller amount of genetic variability to the collection. Therefore, one can expect eventually to reach a threshold above which the benefit of the additional variability saved is less than the costs of sampling and conserving the additional genetic material. FAO (1998) established this threshold at 25 unrelated males for semen collection, and 25 unrelated males and 25 unrelated females for somatic cells or parents of embryos. If the number of animals available is below the threshold, then they should all be selected for inclusion in the programme irrespective of the relationships among them. Sampling from more than 25 animals is, of course, beneficial if resources permit, although in some cases more animals do not necessarily yield more genetic variation (i.e. if many of them are closely related). To obtain DNA, 40 individuals should be sampled (as recommended in the FAO guidelines *Molecular genetic characterization of animal genetic resources* – FAO, 2011a). The same males can be used for both semen collection and embryo collection (i.e. as sires of embryos). The same individuals can be used for embryo collection, somatic cells and DNA. For DNA, it is recommended that if there are fewer than 25 individuals of one of the sexes available, then extra individuals from the other sex should be sampled to bring the total number of DNA samples stored up to 50.

ERFP (2003) also suggests collecting semen from a minimum of 25 donors. From a practical standpoint, achieving these recommended numbers of germplasm donors may be difficult, if not impossible. Logistical issues, small population size and financial constraints may necessitate that fewer than 25 animals are sampled. This will decrease the amount of genetic variation stored. In such cases, the recommendation is simply to collect as much germplasm as possible. When many breeds must be conserved on a limited budget, collecting germplasm from fewer than 25 animals per breed from all breeds may be preferable to collecting germplasm from 25 animals from a proportion of the breeds. Another practical approach that can be considered if resources are limited (in terms of available animals or finances) is to set 25 donors as a longer-term goal (i.e. to be achieved over several years). Section 6 addresses the numbers of doses (straws of semen or embryos) that will be needed for meeting various conservation goals. More than 25 donors may be necessary when the number of doses produced per donor is not sufficient to allow the required total number of doses to be obtained.

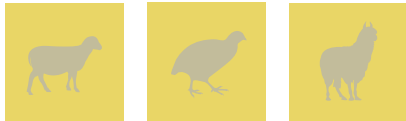
In summary, when building a collection of cryopreserved material, the following three principles should be borne in mind (FAO, 1984):

- conserve small amounts of germplasm from many donor animals rather than large amounts from few donors;
- choose donors that are as genetically and phenotypically diverse as possible; and
- store the breeds as pure lines rather than gene pools, so as to allow the use of the unique combinations of traits and flexibility in the combination of stocks.

It is also important to duplicate the material and store the sets of samples at separate locations in order to reduce risks of loss (see Section 5).

Supporting *in vivo* conservation, within-breed selection, and introducing variation into existing populations

Gene banks have the potential to bolster *in vivo* conservation efforts. Their primary role is to serve as the ultimate backup for *in situ* populations in the event of worst-case scenarios in which an entire breed is lost (e.g. in the event of civil conflict or widespread drought).



There are less extreme circumstances in which it may be desirable to utilize gene banks, such as those in which a breed or population may benefit from the introduction of genetic variation. As noted above, the stored material may be useful for eliminating deleterious genes or accessing genes and gene combinations that have become valuable due to a change in selection goals. This particular function has several aspects.

- At-risk breeds face the threat of reduced genetic variation and high inbreeding levels, which may result in a loss of fertility and general vigour. Introducing variation from gene banks has the potential to alleviate these problems. However, it is only possible to use a gene bank for this purpose if it contains samples from animals that have a lower than average genetic relationship to the population. This can be achieved by selecting donors that are as unrelated as possible.
- Some livestock breeding populations have been selected for one specific trait to the exclusion of others, and as a result lack the genetic variation required to effectively alter phenotypes in response to new market conditions (e.g. changes in the value of fat in milk). Re-introduction of genotypes present before the selection started can help overcome this problem. Therefore, insuring that collections contain as much genetic variability as possible should be an important objective.
- With the advent and use of various DNA technologies, it has become possible for gene banks to store samples from animals of known genotypes for traits of interest. Having genotypic information will facilitate the use of the material contained in the collection.
- Storage and use of samples containing rare alleles can also support *in vivo* populations directly or indirectly through research activities.

Capturing specific alleles

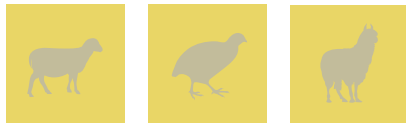
Gene banks can support the livestock industry by assisting in the development of new breeds and/or incorporating alleles of interest into *in vivo* populations. Having collected the breadth of genetic variation for specific breeds, a gene bank can work with state or private breeders in developing new breeds that better fit current or near-term market trends. The oldest material in the bank may be particularly helpful in such a project, as these samples will have become dated with respect to contemporary selection goals and may represent a unique set of animals and genes.

As more information is obtained through genomics research, linking DNA variation to phenotypes will increase opportunities to scan gene bank material for alleles of interest, which in turn can be used to form new breeds or be incorporated into existing breeds (Womack, 2005).

A relatively small amount of germplasm may be sufficient for utilizing a specific allele. Depending on the species, and on fertility and survival rates, as few as 20 to 30 doses of semen can have a rapid impact on the target population.

COLLECTION CATEGORIES

As gene banks are established and the acquisition of germplasm begins, the need for different collection categories becomes apparent. For example, the gene banks of France, the Netherlands and the United States of America established different collection categories to



meet their projected needs (Danchin-Burge and Hiemstra, 2003; Blackburn, 2004, 2009). The purpose and size of the categories may vary depending upon the needs of the country and its livestock industries. By developing such categories, the gene bank manager can better establish collection goals and know how well they are being met. The examples presented below are based upon experiences in developing germplasm collections. Gene bank managers may wish to consider using these or similar categories to assist in the management of the banked material.

Core collections

The term "core collection" has multiple definitions in the conservation community. In these guidelines, the term is used to describe germplasm collected and stored for potential use in critical situations (e.g. reconstitution of an extinct breed, introduction of genetic variability into a living population to resolve a genetic crisis such as an extreme population bottleneck, or elimination of mutation that poses a threat to the population). The core collection is not necessarily static; rather it may be updated as needed to ensure that the genetics contained in it meet the requirements of the livestock keepers raising the respective breed. Because viability, pregnancy and survival rates are variable, it is suggested that the size of a core collection should be equivalent to at least 150 percent of the amount of germplasm that is expected to be necessary for reconstituting the breed.

Historic collection

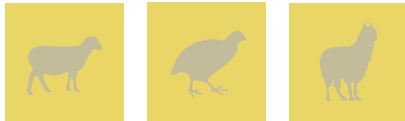
As genetic change occurs in the *in situ* population, the core collection will need to be revitalized. As a result, gene bank managers have to decide whether or not to form an "historic collection" of germplasm or to de-access the material (i.e. to remove the material from the gene bank, implying the destruction of the germplasm). Such material still has potential value, as it may prove useful if selection goals change drastically. It can also be used for DNA research, as well as in research projects looking for genes or gene functions, and studies on genetic diversity.

Working collection

The "working collection" is the most dynamic subset of the gene bank and provides ready access to germplasm for creating research populations of animals, introducing unique germplasm if selection goals need to be modified mildly or developing a new breed.

Evaluation collection

The gene bank manager must quantify the success of the cryopreservation process for each animal sampled for the gene bank, particularly in the case of semen. This can be done using an "evaluation collection". The evaluation should be done soon after freezing and repeated if there is any concern that the samples may have been compromised in some fashion. A relatively small portion of the cryopreserved germplasm from each animal is used for this purpose (e.g. two to ten straws of semen). Samples from the evaluation collection may also be used to test for evidence of pathogens in the cryopreserved material. Deep-frozen germplasm is assumed to be practically 100 percent resistant to deterioration, at



BOX 2

Viability of germplasm after long-term storage

It is assumed that once good-quality germplasm is frozen in liquid nitrogen it should remain viable indefinitely. However, this assumed viability has not been systematically evaluated over long periods of time.

In order to study the viability of old frozen semen, Carwell *et al.* (2009) inseminated 40 pure-bred lactating Angus cows and heifers and 88 lactating cross-bred beef cows with frozen–thawed semen from 25 pure-bred Angus bulls processed during three time periods (1960–1975, 1976–1991 and 1992–2002). The study showed that overall pregnancy rates did not differ across the time periods in the Angus or the cross-bred cows. The authors concluded that the semen collected from the 1960s through to 2002 was still viable and produced similar pregnancy rates in artificially inseminated beef cows.

After transferring 414 sheep embryos stored for 13 years, Fogarty *et al.* (2000) concluded that embryos cryopreserved for a considerable number of years can be successfully thawed and transferred to recipient ewes to reconstitute a sheep population.

least within the reasonable time-horizons required for AnGR banking. Various studies have demonstrated that storage time has no detectable effect on the viability of cryopreserved germplasm (see Box 2). Nevertheless, for prudence, it is recommended periodically to thaw and check a small portion of the collection for viability (at least once every ten years).

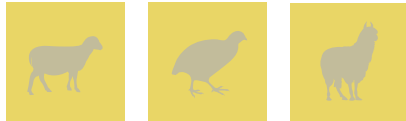
UTILIZING THE WORKING COLLECTION

Establishing a working collection is a prerequisite for all the above-described uses to which AnGR stored in a gene bank may be put. Germplasm stored in the working collection can be much more freely accessed for use. Important aspects of constructing and maintaining a working collection include:

1. establishing rules for transferring germplasm that is no longer actively needed from the working collection to the core collection;
2. determining when quantities of germplasm from a given donor exceed requirements for the core collection and can be moved to the working collection; and
3. locating and obtaining samples of interest to deposit in the working collection, such as those from animals that may have unique gene combinations relative to the live breeding population.

The working collection will usually comprise primarily semen. In some countries it may be advantageous to establish linkages with AI centres and obtain germplasm samples from animals currently being collected for AI and/or to agree to accept samples that no longer have commercial value.

Because of its broad array of uses, the working collection may be larger than the core collection for a specific breed. Furthermore, gene bank collections are not static, as germ-

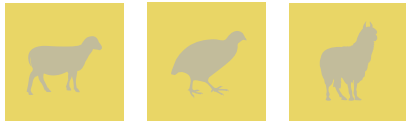


plasm needs to be continually added and de-accessed. The working collection is usually the most variable element of a breed's germplasm collection. Major considerations in determining the size of the working collection include the amount physical storage space available in the gene bank, the number of cryotanks the facility can accommodate and the recurrent financial requirements. A reasonable estimate is that working collections for single breeds will range in size from 50 to 200 animals and from 500 to 1 000 insemination doses.

REFRESHING THE COLLECTIONS

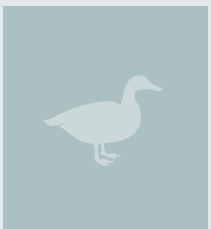
A common but largely misdirected criticism of gene banks is that the germplasm may lose relevance to the livestock industry over time as *in vivo* populations change. This criticism assumes that once a collection is developed, new acquisitions are not made. However, in the case of plant gene banks, collections are continually expanded with new varieties. A similar approach is envisioned for livestock; samples would also be acquired from single animals where seemingly new or otherwise interesting mutations become evident. As well as adding new germplasm samples to the repository, there may also be a need for gene banks to de-access material over time and as more information is garnered about the uniqueness and utility of the samples in the collection. De-accession may also be necessary because of financial or physical constraints. De-accession is a difficult undertaking and a gene bank needs a well-established protocol before initiating the process. Potential reasons for de-accessing samples include:

1. genetics of the sample are too closely related to other samples in the collection (e.g. half-sibs or closer);
2. the post-thaw quality of the sample is low and similar genetics are in the repository; and
3. samples are dated, and are judged to be of less value than more current samples that are being collected.



SECTION 4

Potential use of different types of germplasm and tissue


Potential use of different types of germplasm and tissue

Potential means of conserving genetic diversity include storing semen, embryos, oocytes and somatic cells. There are clear differences in the present-day feasibility and practicality of these options.

Semen has been successfully frozen and is widely used in AI. Sperm-sexing technology has been developed for livestock and is being introduced to the commercial market in some countries. Banking sexed semen could potentially decrease the number of doses required for storage, depending on the goals of the gene bank, but would increase the cost per dose substantially.

Embryos are also widely used for gene banking. However, up to now they have been used in fewer livestock species than semen has. For breed reconstitution, embryos have an advantage over semen in that they allow the recovery of the entire genome (i.e. no backcrossing is required) and thus the reconstitution can be accomplished in a single generation.

Oocytes are the female germ cells or gametocytes. They may be considered for storage in gene banks. Ideally, oocytes will be frozen along with semen from the breed to be conserved, as otherwise backcrossing with semen from another breed will be needed to reconstitute an extinct population. Techniques for freezing and thawing oocytes are less well developed and refined than those for semen and embryos, and require further field evaluation. An advantage of oocytes over embryos is that by using *in vitro* fertilization (IVF), desired matings can be selected at the time of thawing, rather than at the time of freezing. Cryopreservation of ovaries could be another way of conserving AnGR, either as a source of oocytes or as a source of ovarian tissue for transplantation (see below).

Somatic cells may be a prudent backup in circumstances where cryoconservation of gametes and embryos is not financially or technically feasible or has very low success rates. In its most simple application, banking somatic cells requires only the collection and direct freezing of a piece of tissue, such as a section taken from the ear. Since the creation of "Dolly" the sheep, the first mammal produced by cloning somatic cells (Wilmut *et al.,* 1997), this technology has been used to produce live offspring in many domestic species, including cattle, goats, pigs and horses. If production of live animals from somatic cells is developed to a point at which it becomes both reliable and economical, then preservation of somatic cells would become an attractive option for cryoconservation of AnGR.

Nuclear DNA storage for gene transfer has been proposed, but the techniques involved still pose some difficulties. At present, DNA is not used for re-establishing live animals but can be useful in characterization studies to support conservation decisions, including evaluation of the genetic structure within and between populations and identifying genes that affect productivity and adaptation. See the FAO guidelines *Molecular genetic characteriza-*

tion of animal genetic resources (FAO, 2011a) for more information. DNA analysis can also improve management of gene bank inventories.

Other types of material such as blood and serum can be stored for animal evaluation purposes (e.g. for future veterinary diagnostic screening and evaluation).

SEMEN

A major advantage of using semen for cryoconservation is that existing technologies allow it to be collected and used in many animal species. Moreover, for a number of species – notably cattle, small ruminants, horses and pigs – AI centres with dedicated animal housing and semen collection and processing facilities exist in many countries. The existence of such centres may facilitate the acquisition, storage and future use of semen. If dedicated facilities are not available, field collection may be an option; it may be the only means available for collecting material in areas where extensive livestock keeping is practised.

Relative to other types of germplasm, semen has the disadvantage that only a single complement of chromosomes is preserved. If a breed is cryoconserved only in the form of semen, and all living animals belonging to the breed are lost, then the only means of reconstituting the breed is to inseminate founder females from another breed of the same species (or the female gamete in the case of other reproductive techniques). A series of backcrossings is then required to restore the breed to its (nearly) original genetic status. By using only the conserved semen (i.e. from the breed that is being recovered) in each generation, the percentage of genes from the founder breed decreases logarithmically, while the percentage of genes from the breed being recovered increases. This means that enough semen must be available to inseminate the required number of animals in the series of consecutive crosses. To restore the "original" genotype of the lost breed, at least four to five generations of backcrossing will be required, depending on the level of purity desired in the restored population (Ollivier and Renard, 1995) (see Section 3). Marker-assisted breeding can help speed this process to a certain extent. DNA markers can be used to select progeny that contain the greatest percentage of the recipient-strain genome in each generation, a process known as "speed congenics" (Hospital *et al.,* 1992; Wakeland *et al.,* 1997).

In addition to the need for backcrossing, the use of semen has the disadvantage that mitochondrial genes are not preserved. Variation in mitochondrial genes between breeds and within breeds has been established (Loftus *et al.,* 1994; Troy *et al.,* 2001). In avian species, the use of semen to restore a breed would result in the complete loss of the W chromosome from the breed, as the male is the homogametic sex and has no W chromosome.

In some cases, semen collection through standard procedures can be problematic, for instance when the animals cannot be trained for the procedure. In such cases, the collection of epididymal sperm post-mortem may be a good alternative. Epididymal sperm can be collected from a number of species, but species differ in terms of the numbers of insemination doses that can be produced per male. For example, rams have quite a high semen yield in terms of the number of doses per animal (e.g. Ehling *et al.,* 2006). In addition, epididymal ram sperm has been shown to have good freezability and good fertilizing ability in cervical and in laparoscopic inseminations. Details of various semen-collection procedures are presented in Section 8.

TABLE 2
Comparison of non-surgical and surgical artificial insemination in livestock when using frozen–thawed semen

Parameter[a]	Cattle	Sheep	Goats	Pigs	Horses
Non-surgical artificial insemination					
Pregnancy rate (%)	45–75	15–50	20–55	60–90	35–70
Difficulty[b]	1	5	4	2	1
Recommended for use?	Yes	Yes	Yes	Yes	Yes
Surgical artificial insemination					
Pregnancy rate (%)	65–85	55–85	55–85	85–90	60–80
Difficulty	4	1	1	2	3
Recommended for use?	No	Yes[c]	No	No	No

[a] Ranges are estimated from multiple scientific and in-field sources. The values vary depending on the expertise of the technicians, the level of animal nutrition and the management practices.
[b] Difficulty of the insemination procedure, with 1 being the easiest and 5 being the most difficult to perform.
[c] Assuming that an experienced surgical team is available to conduct the procedure.

Techniques for the use of stored semen in AI vary from species to species. Both surgical and non-surgical procedures have been developed for most major livestock species. The amount of training needed in order to become proficient in each technique varies from species to species, as does the rates of success. Table 2 gives an overview of parameters for surgical and non-surgical AI in various species, including expected pregnancy rates. The AI approach that will be used and the corresponding pregnancy rates should be considered when determining the number of doses of semen to conserve (See Section 6).

EMBRYOS

Since the birth of normal offspring from cryoconserved mouse embryos were first reported in 1972 (Whittingham *et al.,* 1972), similar successes have been achieved in more than 16 mammalian species, including all the major livestock species. In the species for which collection and transfer techniques are available and operational, embryo banking is a very good option for conserving genetic diversity, and the offers fastest way to restore an original breeding population, including both nuclear and mitochondrial genetic information. Embryo technology, however, is usually more costly and requires greater technical capacity than gene banking with semen.

While the birth of live offspring from frozen–thawed embryos has been reported in most common livestock species, the difficulty involved and the expected success rates vary from species to species. The greatest success has been achieved in cattle, a species in which cryopreservation of embryos has become a routine procedure. Both slow-freezing and vitrification protocols (see Section 7) are effective (van Wagtendonk-de Leeuw *et al.,* 1997). The success of cryopreservation is dependent on the stage of the embryo. Especially good results are obtained with blastocysts.

TABLE 3
Non-surgical versus surgical embryo collection

Embryo collection type	Cattle	Sheep	Goats	Pigs	Horses[c]
Non-surgical					
Difficulty[a]	1	5	4	3	1
Percent of treated females with ≥1 embryo per collection	85	<20	<35	<35	80
Transferable embryos per collection (n)	4–8	0–3	0–3	0–5	≤1
Collections per year (n)[b]	3–6	1–2	1–3	2–4	4–6
Recommended for use?	Yes	No	No	No	Yes
Surgical					
Difficulty[a]	5	2	2	1	4
Percent of treated females with ≥1 embryo/collection	85	75	80	95	<80
Transferable embryos per collection (n)	4–8	3–8	4–9	10–25	≤1
Collections per year (n)[b]	3	1–2	1–2	2	3
Post-surgical adhesions	+++	+++	++++	++++	+
Recommended for use?	No	Yes	Yes	Yes	No

Ranges presented are estimated from multiple scientific and in-field sources. The values are based on the use of superovulated donors, technicians with the appropriate expertise, and optimal donor nutrition and animal management practices.

[a] Difficulty of the procedure, with 1 being the easiest and 5 being the most difficult to perform.
[b] Post-surgical adhesions generally dictate the number of surgical collections per female during her life time. The number of surgeries per female may be designated by governmental regulations and/or an institutional review board.
[c] Frozen–thawed equine embryos >300 µm in diameter rarely produce a pregnancy following transfer.

Procedures for cryoconservation of buffalo embryos have largely been developed by adapting techniques used in cattle, but fewer resources have been spent on development and refinement and as a result success rates are generally much lower. The potential for cryopreserving sheep and goat embryos is similar to that in cattle (e.g. Fogarty *et al.,* 2000; Rodriguez Dorta *et al.,* 2007). Cryopreservation of horse embryos is somewhat less efficient (Ulrich and Nowshari, 2002). Of all the major livestock species, cryopreservation of pig embryos has long been the most problematic, because pig embryos are extremely sensitive to chilling and have high lipid content. It continues to be the case that pig embryos are the most difficult to freeze and thaw to produce offspring. However, recent studies have focused on overcoming these problems and have produced successful methods for cryopreserving pig embryos (e.g. Nagashima *et al.,* 1995; Vajta, 2000a); live piglets have been obtained (e.g. Dobrinsky *et al.,* 2000; Nagashima *et al.,* 2007).

In addition, species differ with respect to the difficulty of collecting and transferring embryos and whether surgical or non-surgical collection procedures are preferred. Table 3 compares surgical and non-surgical collection for major livestock species. Table 4 compares

TABLE 4
Non-surgical versus surgical embryo transplantation by species

Transfer type	Cattle	Sheep	Goats	Pigs	Horses
Non-surgical					
Success rate[a] (%)	50–80	10–15	10–15	5–10	55–80
Success rate frozen (%)	50–65	<10	<10	<10	10–20
Difficulty[b]	1	5	4	3	1
Recommended for use?	Yes	No	No	No	Yes
Surgical					
Success rate[a] (%)	55–80	50–65	50–65	60–85	60–80
Success rate frozen (%)	50– 65	35–65	35–65	25–60	10–20
Difficulty[b]	3	3	3	2	2
Recommended for use?	No	Yes	Yes	Yes	No

[a] Ranges presented are estimated from multiple scientific and in-field sources. They values are based on the use of superovulated donors, technicians with the relevant expertise, and optimal donor nutrition and animal management practices.
[b] Difficulty of the procedure, with 1 being the easiest and 5 being the most difficult to perform.

transfer procedures. The information presented in the two tables should be considered when planning a gene banking programme, both in choosing the type of germplasm to store and in deciding upon the quantity needed. In addition to the five major mammalian species included in the tables, other livestock species for which live offspring have been obtained from cryopreserved embryos include the dromedary (Nowshari *et al.,* 2005) and rabbit (Naik *et al.,* 2005). Pregnancies have been reported in the llama (Aller *et al.,* 2002) and red deer (Soler *et al.,* 2007).

OOCYTES

As in the case of using embryos, restoring a lost breed or genotype by using cryopreserved oocytes plus semen would not require backcrossing. In the last ten years, considerable progress has been made in the cryopreservation of oocytes. For a long time, IVF rates with cryopreserved oocytes in humans and in other species had been poor due to:

1. release of cortical granules, which causes the zona pellucida to become impenetrable to spermatozoa; and
2. disintegration of the metaphase II spindle.

IVF rates have improved since the introduction of intracytoplasmic sperm injection (ICSI) (Palermo *et al.,* 1992).

In the most recent years, both slow-freeze and vitrification protocols (see Section 7 for more details) seem to be giving excellent results in humans, and the two are considered to work equally well (Porcu and Venturoli 2006), although there may be more enthusiasm lately for vitrified oocytes (Jain and Paulson, 2006). Fewer data are available from livestock

than from humans. This may in part be due to species-specific problems, but it may also indicate that there may have been less incentive to develop and use cryopreservation methods for oocytes in livestock species than in humans. Viable oocytes have been recovered after freezing and thawing in a great number of animal species, i.e. cattle, pigs, sheep, rabbits, mice, monkeys, humans (as reviewed by Critser *et al.,* 1997), goats (Le Gal, 1996), horses (Hochi *et al.,* 1996) and buffaloes (Dhali *et al.,* 2000). Successes have been reported with post-thaw oocyte maturation, fertilization and embryo development in a number of species. Live-born young from embryos produced from cryopreserved oocytes have been reported in cattle (e.g. Abe *et al.,* 2005 and Otoi *et al.,* 1995) and horses (Maclellan *et al.,* 2002) as well as in several model species. Freezing the oocytes of avian and fish species has not been successful, largely because of the large size, high lipid content and polar organization (vegetal and animal pole) of bird and fish ova.

In vitro production of embryos using fresh oocytes and fresh or frozen semen, and subsequent cryopreservation of embryos, is an alternative option that may be considered. In fact, it may be the most feasible option for most species at present (see below).

SOMATIC CELLS

Somatic cells (e.g. skin fibroblasts) can be readily cryopreserved. Collection of suitable somatic cells is straightforward. Cryopreservation protocols for somatic cells are relatively simple and do not require controlled-rate freezing equipment. This means that establishing the collection is easy and cheap, and can make somatic cells a very attractive option for gene banking, especially for countries with many breeds and/or limited resources. Viet Nam, for example, has adopted cryoconservation of somatic cells as a primary component of its AnGR conservation programme (see Box 3). However, the complexity and costs involved in using somatic cells are much greater than those for other types of cryopreserve material. Utilization involves cloning and culturing the cells after thawing (or prior to freezing), reprogramming the nuclei, collecting oocytes by ovum pick-up or from slaughtered animals, culture and *in vitro* maturation of the oocytes, enucleation of the oocytes, transfer of the somatic nucleus to (or fusion of the somatic cell with) an enucleated oocyte, culture of the resulting embryos, and transfer into recipients of the same species. The use of nuclear transfer means that the original mitochondrial genotype is lost. In addition, cloning involves some ethical issues, as there are lingering concerns about the welfare of cloned animals and the safety of their food products. Many countries have not yet approved the consumption of products from cloned livestock.

Live offspring have been obtained from cloned embryos in a number of livestock species including sheep, goats, cattle, water buffaloes, pigs, horses, mules, camels, deer, rabbits, cats and dogs. However, in cattle and sheep only a small proportion of embryos produced using somatic cells develop into live young – typically less than 5 percent – although the efficiency is gradually increasing. A significant proportion of pregnancies are aborted, and full-term pregnancies often result in malformed young. For pigs and horses, greater success rates are reported, with near-normal rates of malformation in the young. Viable litters of cloned pigs are now obtained routinely by transferring large numbers of somatic cell nuclear transfer (SCNT) embryos into each recipient. In fact, a number of companies offer

BOX 3

Cryobanking of somatic cells in Viet Nam

Somatic cells offer a convenient solution for collection and long-term storage of AnGR under adverse conditions with limited infrastructure. As such, cryobanks of somatic cells can serve as a back-up source of genetic material for regenerating at-risk livestock breeds. With the assistance of researchers from Germany (Groeneveld, *et al.,* 2008) a protocol for collection and banking of somatic cells was developed and tested in Viet Nam. The procedure was developed so as to be applicable across all mammalian livestock species. It involves collecting samples using specially designed ear taggers (often used for disease diagnosis or DNA testing) with an integrated tag and vial system that cuts a skin sample from the ear and deposits it in the vial while simultaneously attaching the tag to the ear. The researchers also used a hand-held global positioning system to capture geographical coordinates, and a digital camera to take individual photos of each animal. Breed information was also recorded. The procedure was tested in a pilot study that collected and cryoconserved samples from six local Vietnamese populations of pigs (three breeds), goats (two breeds) and sheep (one breed). The materials for the gene banking (e.g. ear taggers and tags, liquid-nitrogen tanks and computer) required an initial investment of around 3 000 euros, whereas the variable costs of conserving 50 samples from one breed (ideally, 25 females and 25 males) were less than 1 000 euros. Sampling was undertaken by the Institute of Agricultural Sciences of South Viet Nam with the assistance of local government agencies and 300 samples (6 breeds × 50 animals) were collected in two months. After three months, the viability of the cells was tested by culturing cells from a subsample of 23 vials; 100 percent viability was observed. Although local scientists do not have extensive experience in cloning, the banked cells ensure that the six breeds can be reconstituted in the future if the *in vivo* populations are lost. Somatic cell cryoconservation has since been extended to other species and breeds.

cloning as a commercial service and supply biopsy kits that livestock keepers can use to collect a skin sample and send it to the cloning company for immediate generation of cloned animals or long-term storage for future use. In general though, present cloning techniques frequently introduce errors that affect embryonic and foetal development. Costs tend to be high for most species, with the possible exception of pigs. However, on a long time horizon, such as might be expected for reconstituting an extinct breed, increased understanding of nuclear reprogramming is likely to make cloning more reliable and efficient, and hence less costly. Such developments would allow somatic tissue cryopreserved today to be used successfully and efficiently in the future. Therefore, cryobanking of ear or other skin tissue can be considered a cheap method of ensuring the conservation of valuable genotypes for the more distant future.

CRYOPRESERVATION OF DNA FOR GENETIC ANALYSES

DNA carries the genetic information from the male and the female, which is transmitted to the next generation by syngamy of two gametes. This information is coded by units of DNA termed genes, which can be identified, mapped onto segments of the chromosomes and isolated through basic molecular procedures. Researchers are now using stored somatic-cell nuclear DNA to conduct various genetic analyses of animal populations.

In the future, the characterization of genes on various chromosomes will likely be an integral part of conservation (Allendorf *et al.,* 2010). One of the more immediate applications of DNA lies in determining the underlying genetic structure of populations. Various methodologies (e.g. restriction fragment-length polymorphisms, microsatellites, single nucleotide polymorphisms and direct sequencing) are routinely available for rapidly screening populations for genetic variation. They provide a previously unimaginable level of detailed information. The additional knowledge obtained on the partitioning of genetic variability can inform conservation decisions, and has already been used to set conservation priorities in wild species. Furthermore, such techniques can provide information on the levels of genetic admixture within a breed, or on the levels of introgression from other populations or breeds, thereby providing an indication of the level of genetic erosion through cross-breeding (Bradley *et al*., 1994). These uses are taken up in more detail in the FAO guidelines on *Molecular genetic characterization of animal genetic resources* (FAO, 2011a).

In addition, the transfer of genes from one individual to another has attracted a great deal of interest among researchers and pharmaceutical companies. Although progress in this area has been considerable, much of the initial promise, especially in livestock species, has not been realized. Difficulties include regulating gene expression at the correct stage in development and incorporating genes into the correct tissues. Many production traits of interest are modulated by multiple gene expression rather than by a single gene. The cohesive regulation of all these genes is complex and not yet fully understood. How functionally related, yet disparate, genes might be transferred into an individual and regulated in a manner that does not compromise normal function and animal welfare is still unclear.

In the very long term, regeneration of an organism from nothing more than its DNA may be possible. In fact, with refinement in DNA synthesis, regeneration of an individual based only on the DNA sequence may theoretically become feasible. However, such *in silico* conservation would require a great deal of technological advancement and can currently be recommended only as a complement to established *in vitro* and *in vivo* approaches.

CHOOSING THE GENETIC MATERIAL TO STORE

The type of material chosen for preservation in the gene bank may depend on the purpose of the gene bank, for example, whether the gene bank is intended to serve as support for *in vivo* breeding and conservation schemes (Sonesson *et al.,* 2002) or is simply intended to preserve present-day biodiversity for "eternity" (or at least for improbable emergency situations in the finite future). In the former case, semen (and embryos), which can be updated periodically and can also be regularly taken from the gene bank and readily used in the field, are the most practical options. In the case of gene banking for "eternity", methods allowing cheap and fast collection of as many species and breeds as possible are desirable. Where financial resources

TABLE 5
An overview of some characteristics of several ways to cryoconserve genetic material

Characteristic	Semen	Semen and oocytes	Embryos	Somatic cells
Number of samples needed to restore a breed[a]	2 000[b]	100 of each	200	Tissue[c] from ≥60[d] animals (30♂ and 30♀)
Backcrossing needed	Yes	No[e]	No	No
Mitochondrial genes included?	No	Yes	Yes	No
Collection possible in livestock species?	Mostly, not always	Yes, in some species. Operational for bovines	Yes, in some species. Operational for bovines	Always
Cost of collection[f]	$$	$$	$$$$	$
Cryopreservation possible?	Yes	Still in experimental stage	Operational in bovines, horses and sheep. Promising in pigs. Impossible in poultry	Yes
Utilization	Surgical or non-surgical insemination backcrossing for ≥4 generations	*In vitro* maturation/ IVF[g] followed by surgical or non-surgical ET	Surgical or non-surgical ET	Transfer into enucleated oocytes, followed by surgical or non-surgical ET
Current feasibility	High	Medium	High, depending on available resources	High for conservation, Low for regeneration of offspring. Future development seems likely

Adapted from Woelders *et al.* (2003).
[a] See Section 6 for more specific details.
[b] Dependent on species, reproductive efficiency, and other factors.
[c] Sections of at least 2.5 × 2.5 mm.
[d] To obtain a final population of ≥50 animals (25♂ and 25♀), while accounting for failures in cryopreservation and cloning.
[e] Yes, if only oocytes are stored.
[f] Cost is proportional to the number of $; $ = low cost and $$$$ = extremely high cost.
[g] IVF = *in vitro* fertilization, ET = embryo transfer.

and the relevant expertise and facilities are available, embryos are usually the best choice. When such resources are not available, collection and cryopreservation of somatic cells should be considered a reasonable option (Groeneveld *et al.,* 2008). Table 5 summarizes the characteristics, advantages and disadvantages of cryoconservation via different types of germplasm.

ADVANCED PROCEDURES AND THEIR CURENT POTENTIAL IN CRYOCONSERVATION

Ongoing research into the biology of gametes and embryos will likely give rise to new methods of recreating individuals from frozen material (see Holt, 1997; Gilmore *et al.,* 1998; Holt and Pickard, 1999; Woelders *et al.,* 2003; Gosden, 2005; Johnson, 2005). The following subsections describe options that can already be considered today for use in cryobanking. These approaches generally require a great deal of expertise and/or are only applicable in

a limited number of species. Nevertheless, as in the case of SCNT, future developments are likely to increase their efficiency and decrease costs, and thus it may be appropriate to take the potential use of these methods into account when taking decisions on the type of germplasm to store and the method of preservation to use.

In vitro fertilization with frozen–thawed semen

Initial *in vitro* production utilized oocytes collected from slaughterhouse ovaries. This worked well during early experimentation, when large numbers of immature oocytes were needed in order to develop *in vitro* laboratory procedures and to train technicians. In the 1980s it was proposed that the application of *in vitro* production in animals would likely involve genetically valuable breeding stock and possibly be used for preserving diversity in endangered wild animals (Loskutoff *et al.,* 1995).

The first frozen–thawed IVF embryo-derived calves were produced in the United States of America (Zhang *et al.,* 1993). Oocyte collection from live donors and IVF procedures became commercially available to dairy and beef cattle producers in the early 1990s (Bousquet *et al.,* 1999). With thousands of bovine offspring produced worldwide, IVF with frozen sperm is now used routinely in commercial cattle embryo transplant units. However, the success rate for frozen IVF embryos is lower than for fresh IVF embryos. Although years of research have been devoted to this technique, IVF methodology is still being tested and fine-tuned for both dairy and beef cattle.

IVF is a multistep process that requires a well-equipped laboratory and a skilled technician. As the name implies, the IVF procedure involves harvesting the oocytes from the donor's ovaries and fertilizing them *in vitro*. The resulting embryos are held in an incubator for seven or eight days and then frozen or transferred non-surgically to recipient females at the same stage of their oestrous cycles. With improvements in oocyte maturation and sperm maturation methods, IVF rates of bovine oocytes are expected to be higher than 85 percent (e.g. Zhang *et al.,* 1992). The pregnancy success rate for good-quality IVF-derived frozen bovine embryos usually ranges from 35 to 50 percent.

Identifying appropriate and efficient *in vitro* culture systems for IVF-derived embryos seems to be one of the major bottlenecks to IVF application in other livestock species at present. Although the first IVF offspring in sheep, pigs and goats were reported in the mid-1980s (Cheng *et al.,* 1986; Hanada, 1985), the IVF procedures developed have not been accepted by the commercial livestock industry, primarily because of the high cost of the process.

IVF in horses has not, as had been expected, developed to a level at which it can be used in the field. Although the births of several foals produced by IVF were reported in France in the early 1990s, repeatable IVF protocols for horses are not available at present. Attempts by many others to produce IVF foals have not been successful, making it clear that more research is needed. The reasons for the low success rate of equine IVF remain unclear. Equine oocytes have a thick zona pellucida compared with other species, and *in vitro* maturation of these oocytes takes longer than in other livestock species (Hinrichs *et al.,* 1993). The thick zona pellucida of the oocytes may act as a barrier to sperm prepared *in vitro*. The zona pellucida found in *in vitro* maturation oocytes may also be altered by the culturing process.

This factor, in addition to inadequate sperm cell preparation, probably contributes to poorer than expected IVF embryo production rates in the horse.

Because the use of IVF in livestock (particularly horses) appears to be hindered by problems with *in vitro* maturation and *in vitro* sperm zona penetration, other assisted reproductive technologies, such as zona drilling, zona renting, subzonal sperm injection and intracytoplasmic sperm injection are now under investigation for use in livestock (Gao *et al.,* 2004; Guerrero *et al*., 2008; Chiasson *et al.,* 2010). There is still much to be studied and learned about the use of assisted reproductive technologies to maximize reproductive potential in genetically valuable animals (Hansel and Godke, 1992).

Attempts have been made to use IVF procedures to cross-fertilize different bovine species. In a recent study, an attempt was made to use African buffalo sperm for IVF of domestic cattle ooctyes (Owiny *et al.,* 2009). Although fertilization did occur with some cattle oocytes, very little development occurred thereafter.

With further development and fine-tuning to improve the repeatability of oocyte retrieval and the culture of oocytes before or after cryopreservation, IVF procedures will likely become very competitive with, or superior to, conventional embryo collection and cryopreservation for use in AnGR gene banking, because of the cost and labour advantages.

Intracytoplasmic sperm injection (ICSI)

Research groups in many countries have attempted to develop techniques for producing offspring from microinjection of sperm cells into unfertilized ova (e.g. Uehara and Yanagimachi, 1976; Markert, 1983). The first ICSI experiments in mammals were conducted in rodent species. The premise of the technique was that the ovum of the female would be activated by a microinjected sperm. However, results have been variable. The first live offspring was produced by ICSI into the ooplasm of rabbit ova (Hosoi *et al.,* 1988). IVF and normal cleavage of *in vitro* maturation oocytes were first reported in cattle following ICSI with *in vitro* capacitated sperm (Younis *et al.,* 1989). The first transplant offspring in livestock (live calves) from ICSI of bovine oocytes were reported in Japan (Goto *et al.,* 1990). Varying levels of success have been reported since.

ICSI is more advanced in horses than in other livestock species. Among the first successes were pregnancies produced using oocytes taken from horse ovaries obtained from abattoirs (Squires *et al.,* 1996), non-pregnant mares (e.g. Meintjes *et al.,* 1995a; McKinnon *et al.,* 1998) and pregnant mares (Cochran *et al.,* 1998a, 2000). Today, the ICSI procedure is used routinely in mares that have low fertility via conventional means.

Although ICSI is quite successful in horses, the technology is not yet ready for routine use in most livestock species. However, as in the case of conserving somatic cells for future cloning, if germplasm is to be conserved for long-term storage, it is probably feasible to store semen and wait for the development of the technology needed to use it. Once the technology has been developed to the point at which its use becomes routine, ISCI has the potential dramatically to increase flexibility. For example, mistakenly thawed bull semen could be refrozen, thawed again, and then used for ICSI. Recently, a calf has been produced using ICSI with frozen–thawed bovine epididymal sperm (Guerrero *et al.,* 2008). ICSI also

FIGURE 1
Intracytoplasmic sperm injection procedure

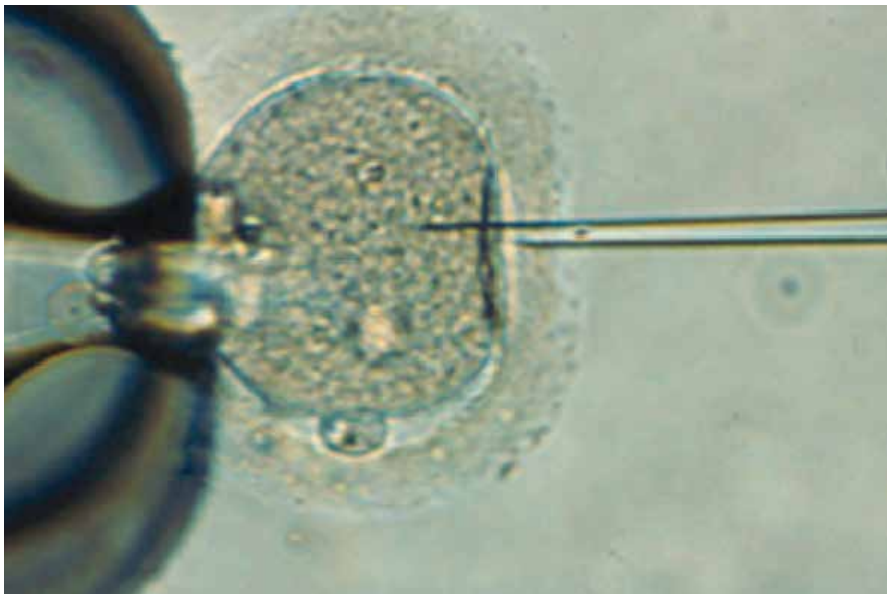


PHOTO CREDIT: R.A. GODKE

has the potential to be used with freeze-dried semen, which would eliminate the need for cryoconservation in liquid nitrogen and greatly facilitate *ex situ* conservation of AnGR in countries where sourcing liquid nitrogen is a problem.

FUTURE PROSPECTS FOR CRYOCONSERVATION

Depending on the species and the conservation goals, current technologies offer a wide range of options in terms of the tissue types that can be cryoconserved. The future is likely to see an expansion of these options as research improves protocols and results in greater efficiency.

Cryopreservation of ovaries and other gonadal tissue

Cryopreservation of ovaries could be another means of conserving AnGR. In humans, ovary banking is routinely used to prevent loss of fertility during cancer treatment. In addition, it is considered an effective method for cryobanking strains of mice used for research.

Cryopreserved ovaries, or parts of ovaries, may be used as a source of oocytes. Oocytes can be harvested from heterotopically grafted (i.e. grafted to tissues other than the ovary) ovaries for subsequent IVF to produce embryos. The embryos must then be transferred to a recipient animal. Alternatively, cryopreserved ovary tissue or whole ovaries can be grafted orthotopically (i.e. grafted to ovarian tissue) into a recipient animal in order to restore the animal's fertility. The animal can then be mated and will produce offspring carrying the genotype of the ovary donor.

The production of live offspring after orthotopic transplantation of sliced frozen mouse ovarian tissue was first reported more than 50 years ago. Since then, there have been reports of successful orthotopic allografting of mature and juvenile mouse ovaries to recipient mice (e.g. Candy *et al.,* 2000). Recently, Japanese quail ovarian tissue was successfully frozen, thawed and transplanted into young chicks, which then subsequently produced live offspring after mating (Liu *et al.,* 2009). Restoration of fertility after grafting cryopreserved ovarian tissue has also been achieved in larger animals, for example, using vitrification of (hemi) ovaries in sheep (e.g. Bordes *et al.,* 2005). Song and Silversides (2006, 2007a) successfully obtained offspring from procedures involving cryopreservation and subsequent transplantation of both male and female gonadal tissue in poultry.

Cryopreservation of ovaries may offer new possibilities for livestock production systems, but requires additional research and development, especially for use in mammalian species. Further improvements are needed in the cryopreservation procedure and the grafting technique. However, as with cloning and somatic cells, advances in these techniques can be expected to occur in the interim if ovaries are cryopreserved for long-term storage.

Even with technological advances, cryopreservation of ovaries or ovarian tissue may remain less efficient than cryopreservation of embryos as a method for *ex situ* conservation of livestock genetic diversity. First, obtaining the ovaries requires either laparoscopy or sacrificing the donor animal. Second, making use of the cryopreserved material to produce offspring requires surgical expertise and facilities for grafting the thawed cryopreserved ovaries into recipient animals.

As in the case of oocytes, the storage of ovaries would require either complementary cryopreservation of semen from males of the same breed or the application of a backcrossing strategy similar to that required when only semen is used (but using male founders or semen from another breed rather than females). In mammalian species, such a strategy would result in the loss of genetic material on the Y-chromosome. Given the generally lower cost and greater ease of preserving semen, preserving only oocytes or ovaries would be logical only in specific instances, such as when no males are easily available or if the reconstituted breed will be used only as a maternal line in a cross-breeding programme.

Embryonic stem cells

Embryonic stem cells are undifferentiated embryonic progenitor somatic cells that have been cultured *in vitro* and frozen for later use. Such cell lines have been established in laboratory species and are being used to generate transgenic animals carrying cross-species or tailored genes. The advantage of these cells is that they can be frozen, thawed and then multiplied through numerous cell cycles. In the animal species (i.e. mice and primates) where true embryonic stem cells have been identified, they are obtained relatively easily from cultured young embryos (inner cell mass of the blastocyst stage) or early-stage germ cells (e.g. primordial germ cells) and can be kept frozen for future use.

If embryonic stem cells are introduced into an activated enucleated oocyte, or possibly an embryo at the beginning of its development, they can influence subsequent cell development in various body tissues throughout life. Embryonic stem cells could thus be potential vectors for the transmission of genetic characteristics. However, even though there has

been an intensive research effort in recent years, there is at present no convincing evidence of the existence of true embryonic stem cells in livestock species. If these cells and adult somatic stem cells could be used in livestock species with a reasonable rate of success, then this new stem cell technology would be a useful tool in cloning, clinical veterinary medicine and even in the conservation of genetic diversity.

Spermatogonia

These cells reside within the basal layer of the seminiferous tubules of the testis and have the capacity to give rise to spermatozoa. Beginning before puberty and continuing in the adult animal, spermatogonia undergo continuous replication, thereby, maintaining their number in a process known as stem cell renewal. It has been shown in mice (Brinster and Zimmermann, 1994) that these stem cells, when isolated from the testes of donor animals, can be processed and used to repopulate another testis without evidence of immuno-rejection.

Kimura and Yanagimachi (1995) reported the development of normal mice from oocytes injected with secondary spermatocyte nuclei. Frozen–thawed testicular tissue from day-old chicks has been transplanted into host chicks, resulting in the production of live offspring from the donor tissue sperm (Song and Silversides, 2007b). Spermatogonia transfer could potentially be used to pass genetic material from one generation to the next, and when combined with cryopreservation, offers a means of saving genes from male animals for future generations.

Primordial germ cells

Although efforts have been made over the years to produce gametes and offspring from primordial germ cells (e.g. Tsunoda *et al.,* 1989; Chuma *et al.,* 2005), it is only recently that increased success has been reported (in fish and in birds). In chickens, primordial germ cells usually migrate to the gonadal ridge via the blood stream between days four and six of incubation. During this migration stage, the primordial germ cells can be harvested from the blood of a chick embryo, and then cultured and transferred to other developing chick embryos, resulting in germline transfers (Etches, 2010).

In quail, male or female primordial germ cells have been successfully transferred into chick embryonic gonads, subsequently replacing the host germ cells (e.g. Ono *et al.,* 1996). Germline chimeras have also been reported, with host quail that have subsequently produced live offspring from the donor-quail germ cells (Kim *et al.,* 2005). Using germline transplantation, live offspring have been produced by surrogate birds from other avian species (e.g. pheasant) (Kang *et al.,* 2008). This research area holds promise for future cryoconservation systems.

Parthenogenetic and IVF embryo reconstruction

Various attempts over decades of research on parthenogenetic embryo production have only produced limited embryo development. One attempt to recover female germplasm used embryo micromanipulation techniques to make chimeric embryos, each with a combination of material from a parthenogenetic bovine embryo (from one breed) and an IVF bovine embryo (from a second breed) (Boediono *et al.,* 1999). The reconstructed chimeric embryos

were then transferred to recipient cattle and resulted in live offspring, each of which had a chimeric genotype (parthenogenetic and IVF) and exhibited the distinct coat colour patterns of both breeds (phenotype). In a breed or species with no males remaining, it would be possible to try to save the germplasm by producing a female offspring from reconstructed embryos in an effort to produce oocytes from the at risk-breed portion of their chimeric ovaries.

Gametes derived from embryonic stem cells

Mouse oocytes have been derived from embryonic stem cells (Hubner *et al.,* 2003). If this methodology can be further developed for livestock species, it could have important implications for oocyte and embryo cryopreservation.

Cloned embryo reconstruction

Poor placental development has, in part, been blamed for the loss of cloned pregnancies during early and late gestation in recipient cattle. It has been proposed that if it were possible to exchange the placental tissue (embryonic trophoblast) of the cloned embryos with material from a non-cloned embryo via embryo reconstruction, this might enhance normal foetal development *in utero* and thus produce more viable cloned bovine offspring. Therefore, efforts are underway to use embryo reconstruction of IVF-derived and SCNT-derived cattle embryos to improve nuclear transfer production rate (e.g. Murakami *et al.,* 2006).