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FAO ANIMAL PRODUCTION AND HEALTH

guidelines

CRYOCONSERVATION OF ANIMAL GENETIC RESOURCES

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CRYOCONSERVATION OF ANIMAL GENETIC RESOURCES

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS
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Foreword

Livestock agriculture is in a period of tumultuous change and upheaval. General economic development, and population growth and mobility, have increased demand for livestock products, but have also placed pressures on the sustainability of rural environments and animal production systems. Livestock keepers will need to increase their efficiency to meet the rising demand while continually adapting their animal genetic resources to changing economic and environmental conditions. The genetic diversity necessary to allow this adaptation is in a state of decline, and the genetic resources that remain are not utilized in the most efficient way. *The State of the World's Animal Genetic Resources for Food and Agriculture* (FAO, 2007a) confirmed that a significant proportion of the world's 7 000+ livestock breeds are at risk of extinction and that many countries lack the technical capacity to ensure the proper management and sustainability of their animal genetic resources.

To address these problems, the Member Nations of FAO developed the *Global Plan of Action for Animal Genetic Resources* (FAO, 2007b) (*Global Plan of Action*), which was adopted at the first International Technical Conference on Animal Genetic Resources for Food and Agriculture in Interlaken, Switzerland, in September 2007. The *Global Plan of Action* contains four strategic priorities areas that provide a basis for enhancing the sustainable use, development and conservation of animal genetic resources throughout the world. It calls on FAO to continue to provide technical guidelines and assistance and to coordinate training programmes in order to support countries in their efforts to implement the *Global Plan of Action*.

Conservation of animal genetic resources is the third Strategic Priority Area of the *Global Plan of Action*. Conservation involves both *in vivo* maintenance and management of genetic diversity within livestock populations that are actively contributing to the livelihoods of their keepers or that are maintained in small numbers on research or demonstration farms and *in vitro* storage of genetic material that can be used at a later time to increase diversity in live populations or re-establish a population. A previous FAO publication on conservation – *Secondary guidelines: management of small populations at risk* (FAO, 1998) – covered both types of conservation. However, given the advances in technology and in the availability of information that have occurred during the past decade, the present guidelines will be complemented by a separate publication on *in vivo* conservation.

The development and operation of a gene bank for cryoconservation of animal genetic resources requires technical capacity in genetics, reproductive physiology, cryobiology and data management. Coordination among a wide group of stakeholders is also essential. These guidelines were developed to provide an overview of the fundamental issues involved in developing and operating gene banks as elements in comprehensive national strategies for the management of animal genetic resources.

Acknowledgements

Seven international scientists were responsible for the majority of the technical content of these guidelines: Harvey Blackburn, Robert Godke and Phil Purdy (United States of America); Sipke-Joost Hiemstra and Henri Woelders (Netherlands); Arthur Mariante (Brazil); and Flavia Pizzi (Italy). All of these persons make substantial contributions to the management of animal genetic resources in their respective countries, including the operation of national or regional gene banks.

The guidelines were reviewed, tested and validated at training workshops held at the National Gene Bank of Tunisia, the Escuela Superior Politécnica del Litoral in Ecuador and the Centre for Genetic Resources of the Netherlands, which were organized, respectively, with the local assistance of M'Naouer Djemali and the International Center for Agricultural Research in the Dry Areas (ICARDA), Paul Herrera Samaniego and Paul David Silva, and Sipke-Joost Hiemstra. In addition to FAO, significant financial support was provided by the United States Department of Agriculture, the European Regional Focal Point for Animal Genetic Resources and the various host organizations. More than 120 scientists, technicians and decision-makers attended these workshops.

The guidelines were prepared under the supervision of Paul Boettcher, with the full support of the Chief of FAO's Animal Genetic Resources Branch, Irene Hoffmann, and of officers of the Animal Genetic Resources Branch: Badi Besbes, Beate Scherf, Roswitha Baumung and Dafydd Pilling. Administrative and secretarial support was provided by Kafia Fassi-Fihri and Silvia Ripani.

FAO would like to express its thanks to all these individuals, and to those not mentioned here, who generously contributed their time, energy and expertise.

The goal and structure of the guidelines

These guidelines are intended to serve as a decision aid with respect to the various cryoconservation options that are available, and to provide technical guidance on the design and establishment of animal gene banks. The guidelines are written under the assumption that a decision has already been taken that cryoconservation will make a valuable contribution to a programme for conserving the animal genetic resources of interest. The advice provided is intended to be relevant to all species of domestic livestock, but species-specific guidance is given where appropriate. Much of the information may also be relevant to cryoconservation of wild relatives of livestock and to other wildlife species. Thus, countries may consider developing joint gene banks for both domestic and wild animals.

The guidelines focus on cryoconservation of animal genetic resources. Matters related specifically to *in vivo* conservation, as well as general issues of conservation, are presented in a separate publication – In vivo *conservation of animal genetic resources* – forthcoming in this series.

The guidelines are intended to provide the technical background information needed by countries wishing to set up, implement and monitor gene banks. Although reading all sections is recommended, certain sections are aimed at specific stakeholders with specific technical interests and responsibilities.

The terms "cryoconservation" and "cryopreservation" are both used frequently throughout the guidelines. Although these words are in some cases interchangeable, an effort has been made to restrict the use of "cryopreservation" to the actual process of freezing biological material for long-term storage. "Cryoconservation" is used to refer to the conservation of animal genetic resources through the use of cryopreserved germplasm.

Section 1 reviews reasons for conserving animal genetic resources and compares the various conservation options that are available. This is intended to help the reader confirm that cryoconservation will be a valuable component in a plan for conserving the animal genetic resource(s) under consideration (assuming a conservation programme of some kind is needed).

Section 2 discusses what must be done before the freezing and storing of germplasm can start, i.e. the preparation, implementation and organization of gene banks.

Section 3 discusses the objectives that can be addressed by gene banking programmes.

Section 4 describes the various types of germplasm and tissue that can be cryopreserved, as well as the uses to which they can be put. This is intended to provide a basis for informed choices regarding the type of material to store.

Section 5 discusses requirements for, and costs of, establishing gene banks of various sizes and degrees of technological sophistication.

Section 6 deals with the genetic issues that need to be considered when designing and implementing a cryoconservation programme, considering in particular the amounts

of various types of germplasm that need to be stored in order to capture desired amounts of genetic diversity. Biological material undergoes a number of (sometimes drastic) changes when subject to cryopreservation, and some of these will decrease the viability of the conserved germplasm.

Section 7 describes the process of cryopreservation at cellular level, and the possible effects that the process may have on the stored material. This overview is intended to provide the basic information needed to diagnose and avoid damage to genetic material during the cryopreservation process.

Section 8 describes methods for collecting and cryopreserving various types of genetic material from various species of livestock.

Section 9 addresses the health and sanitary issues that must be considered when establishing and operating gene banks for animal genetic resources in order to help prevent the conservation of potentially dangerous pathogens along with the valuable genetic material.

Section 10 describes documentation and database requirements for storing information on individual animals and on the samples of genetic material stored in the gene bank. To be of use, material stored in the gene bank must eventually be thawed and used to create new animals. Therefore, good organization and annotation of the stored material are essential.

Section 11 addresses the legal issues associated with cryoconservation. Although animal genetic resources can be considered a public good, the animals from which germplasm is taken for cryoconservation are usually privately owned. Ownership may or may not change during the gene banking process, but the terms of agreement between gene banks and the breeders providing germplasm must be explicitly defined.

Section 12 discusses priorities for capacity building and the need to train livestock keepers and extension workers. It also discusses the need for the inclusion of cryoconservation and related topics in higher education curricula.

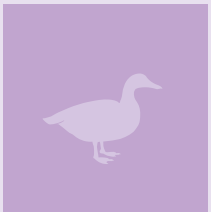
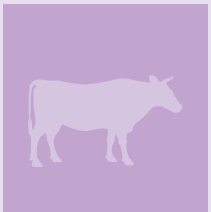
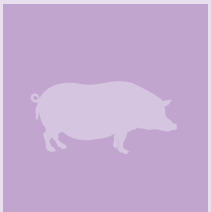
The main sections are followed by a series of appendices, which provide step-by-step instructions on procedures for collection and cryopreservation of germplasm.

Abbreviations and acronyms

AI	artificial insemination
AnGR	animal genetic resources (for food and agriculture)
AV	artificial vagina
BSA	bovine serum albumin
CASA	computer assisted sperm analysis
DNA	deoxyribonucleic acid
ET	embryo transfer
FAO	Food and Agriculture Organization of the United Nations
FSH	follicle stimulating hormone
ICSI	intracytoplasmic sperm injection
IVF	*in vitro* fertilization
MTA	material transfer agreement
N_e	effective population size
OCM	oocyte collection medium
OIE	World Organisation for Animal Health (Office International des Epizooties)
OMM	oocyte maturation medium
PBS	phosphate-buffered saline
PHE	penicillamine, hypotaurine and epinephrine
SCNT	somatic cell nuclear transfer
TUGA	transvaginal ultrasound-guided oocyte aspiration

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).

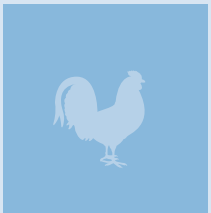
conserving genetic diversity by keeping live animals outside their original or usual production environments (*ex situ – in vivo*) will not always guarantee that the genetic diversity of the breed is maintained. Thus, *in vivo* conservation should be complemented by cryoconservation of germplasm. In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

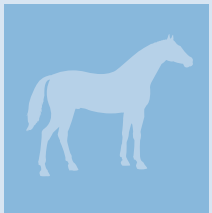
WHEN IS CRYOCONSERVATION THE BEST OPTION?

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.

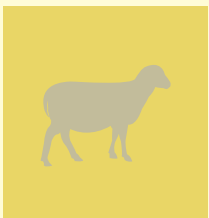
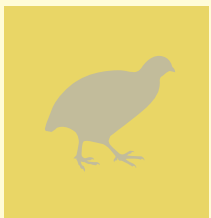
Two key features will help livestock conservation projects attract funding from international agencies:

- 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

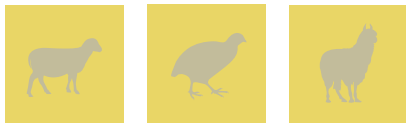
Gene banks and their collections of germplasm and tissue can have multiple functions and objectives. While the primary function of gene banks is conservation of AnGR for use in the medium or long term, the material stored may also be used for other purposes. For example, such material can be used to 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

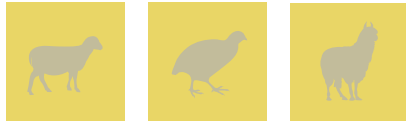
The primary motivation for developing cryopreserved germplasm collections is to have the capacity to maintain and enhance the genetic diversity of *in situ* populations. One common measure of genetic diversity is effective population size (N_e), which is usually smaller than the absolute population size.

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.

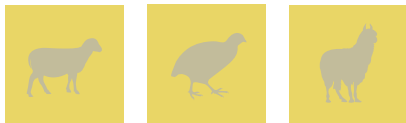
COLLECTION GOALS

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.

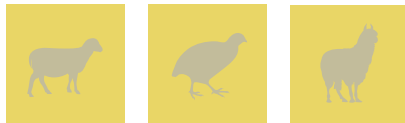
Breed reconstitution

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.

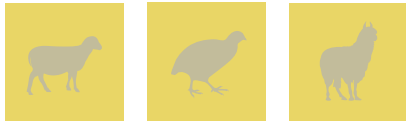
In summary, when building a collection of cryopreserved material, the following three principles should be borne in mind (FAO, 1984):

- 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

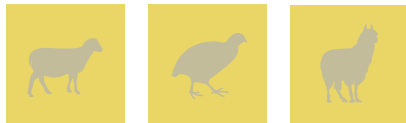
Gene banks can support the livestock industry by assisting in the development of new breeds and/or incorporating alleles of interest into *in vivo* populations. Having collected the breadth of genetic variation for specific breeds, a gene bank can work with state or private breeders in developing new 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

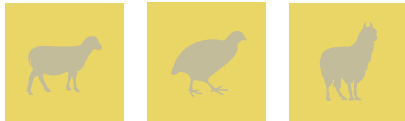
As genetic change occurs in the *in situ* population, the core collection will need to be revitalized. As a result, gene bank managers have to decide whether or not to form an "historic collection" of germplasm or to de-access the material (i.e. to remove the material from the gene bank, implying the 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).

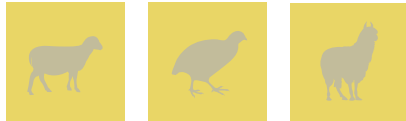
UTILIZING THE WORKING COLLECTION

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-

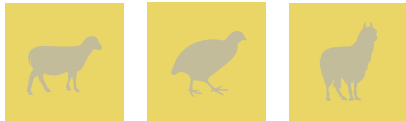


plasm needs to be continually added and de-accessed. The working collection is usually the most variable element of a breed's germplasm collection. Major considerations in determining the size of the working collection include the amount physical storage space available in the gene bank, the number of 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

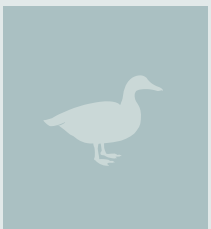
A common but largely misdirected criticism of gene banks is that the germplasm may lose relevance to the livestock industry over time as *in vivo* populations change. This criticism assumes that once a collection is developed, new acquisitions are not made. However, in the case of plant gene banks, collections 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

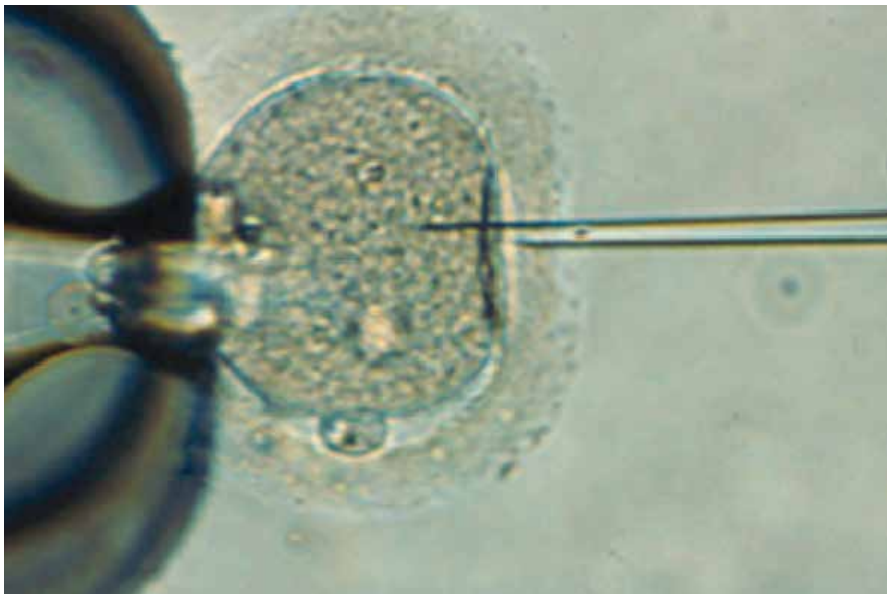


PHOTO CREDIT: R.A. GODKE

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

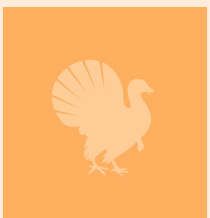
Mouse oocytes have been derived from embryonic stem cells (Hubner *et al.,* 2003). If this methodology can be further developed for livestock species, it could have important implications for oocyte and embryo cryopreservation.

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).

SECTION 5

Establishing a gene bank – physical structure and costs



Establishing a gene bank – physical structure and costs

Gene banking operations and facilities are likely to vary substantially from country to country, in terms of both the size and capacity of the bank and the types and amount of equipment needed. These factors are dependent upon the quantities of germplasm to be placed in the gene bank, which will in turn depend upon the objectives of the gene bank, the range of species and breeds to be conserved, and the financial resources available for the conservation programme. For the purposes of these guidelines, the types of facilities and equipment needed to operationalize a gene bank are subdivided into three size categories: small, medium and large.

In many circumstances, the elements needed to establish a gene bank will already be in place. For example, some countries have state-owned and operated AI centres, or they may have an existing gene bank for plants or wild animals. If such operations already exist, the AnGR gene bank can be incorporated into these programmes. Such an approach would certainly facilitate the development of a cryoconservation programme and the collection of material from some livestock species (i.e. those served by the AI centres). However, additions to existing facilities and equipment may be necessary. Health and sanitation issues must be considered, especially for any facilities that might be shared between wild and domestic animals.

Some features are necessary for the smooth functioning of a gene bank regardless of its size. These are the physical plant (i.e. the actual building and other structures), durable equipment, security arrangements, centralization and accessibility. Specialized human resources are also critically important.

THE PHYSICAL PLANT

The characteristics of the building housing the gene bank will depend greatly upon the total size of the facility and its operations. For example, if animal and plant gene banks are housed together, the type of buildings required will be different from those needed for a single-purpose facility for AnGR. For livestock, specific components of a single- or multiple-use facility, such as the laboratories for germplasm acquisition, processing and cryopreservation, do not need to be located in the same physical place.

Developing the gene bank will usually require the services of a professional architect. If no buildings are yet in place, professional assistance with the development of specific blueprints and construction plans will clearly be needed. If buildings exist but need to be redesigned or refurbished, this will also usually require the employment of an architect and/or engineer.

The gene banking process comprises three main activities: 1) collection of the germplasm; 2) processing and freezing of the germplasm; and 3) storage of the germplasm.

Although all of these activities can be undertaken at the same location, each requires its own separate facilities. In theory, a gene bank need only involve a place for storing germplasm (i.e. if the germplasm is provided from elsewhere). However, in most situations, a gene bank will have the infrastructure needed for at least two, if not all three activities.

Animal housing and collection facilities

Many gene banking operations will find it practical and convenient – if not absolutely necessary – to have a dedicated facility for holding animals while their germplasm is collected. Depending on the species and the type germplasm that is to be conserved, it may not be possible to collect a significant quantity of germplasm during a single intervention with an individual animal. For example, even with superovulation, only a few embryos can be obtained from a cow in a single intervention. Thus, the process will need to be repeated several times for each donor. Superovulation generally requires administering a regime of hormones over the course of several days prior to embryo collection. This is more practical if the donors are kept in a central facility. In the case of semen donors, training the animals for collection will usually increase yield and quality. For countries aiming to adhere to World Organisation for Animal Health (OIE) standards for export, holding facilities will be needed for quarantining animals prior to collection and monitoring their health after collection (for further information visit the OIE web site http://www.oie.int).

Building materials. The choice of materials for internal and external construction of the gene bank building is important. The building must be strong enough to withstand environmental challenges specific to the location as well as the normal wear and tear caused by the animals housed at the facility. The internal surfaces must be able to resist both the physical actions of the animals and the effects of regular sanitizing. Thus, the materials used must be impermeable to water and able to withstand repeated cleaning with sanitizing chemicals. The flooring must be coarse enough for the animals to maintain stable footing when germplasm is being collected (e.g. when bulls mount a teaser animal).

Multiple buildings. Preventing the spread of disease among the animals held at the facility and to the germplasm stored in the bank is critical. Therefore, the facility should have a system for quarantining all incoming animals. If possible, the collection facility should have multiple buildings. This allows the establishment of a quarantine system based on an "all in/all out" policy. Under such a system, once one group of animals leaves a given building, it can be cleaned and the next group of animals can then be brought into the empty, sanitized building and quarantined. Once the quarantine is lifted, the animals can proceed back and forth to a separate collection building, but never again enter a non-sanitized housing unit. Animals with questionable health must always be housed separately from healthy animals and never intermingle directly with them or utilize common spaces such as the collection facility. In addition, if the facility is intended to hold several species, the design must allow each species to be kept apart in different and separated buildings.

Environmental control. The facility should be designed so that the air flows in one direction through the building. This limits the potential for "dead spaces" created by air

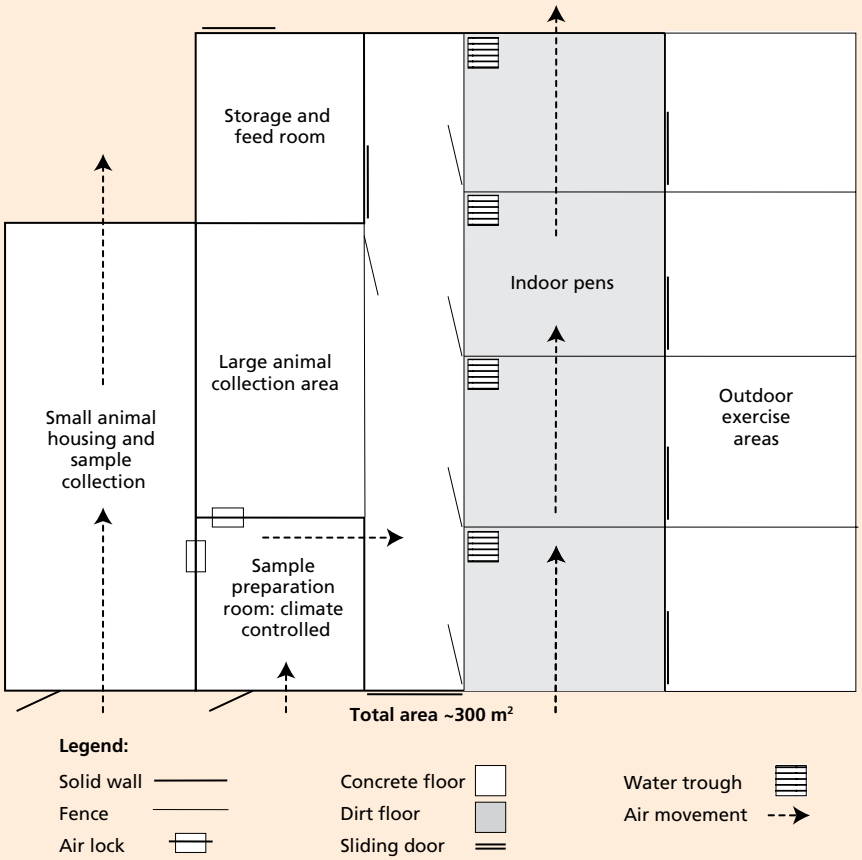
flowing in multiple directions. It also ensures that only fresh air will enter the building and that air laden with animal by-products (methane, urea, etc.) will be vented outside, thus minimizing the load of contaminants within the facility and improving the health of the housed animals. Proper ventilation will, in addition, help maintain the appropriate humidity within the facility. Temperature control is also important. Depending on the local climate, heating and/or cooling systems may be needed in order to maintain the indoor temperature within the range of thermal comfort of the species being kept. In particular, the animals must be protected against high temperatures, which can adversely affect sperm production, especially in pigs. In addition, the intensity of light and the periods of light and dark may have to be controlled in order to meet the physiological and behavioural needs of the animals.

Biosecurity. Ensuring the biosecurity of the site should undoubtedly be one of the most important concerns of a gene bank manager. The following principles should be considered:

- *Location.* The decision as to where to construct the gene bank should be based on a detailed study of potential locations. If possible, the facilities should be situated in a low animal-density area. The animal-holding buildings should be situated at least 3 km from other farms or similar sources of biological risk, and at least 1 km from the nearest main roads or railways, especially if the nearby roads and railways are frequently used for transporting livestock.
- *Perimeter fence.* The installation must have a perimeter fence to prevent the entry of non-authorized persons and domestic or wild animals.
- *Animal loading bay.* The loading bay for the arrival and departure of donor animals poses a great risk to the biosecurity of the site. It should be situated outside the perimeter fence and some distance from the animal housing units. In addition, trucks bringing feed to the facilities should unload in storage and transfer areas outside the perimeter fence.
- *Clean and dirty areas.* The facility must be clearly divided in two physically separated areas: the dirty area (buildings for animals, collection pens, and storage areas for waste materials, feed, etc.) and the clean area (laboratories, instrumentation rooms, germplasm storage facilities and offices). If these two facilities are in the same building, only one indirect connection between the clean and dirty areas should be allowed, ideally through a sanitary air lock in the wall between collection pens and laboratories. Locker rooms with changing facilities and showers should be located between the two areas.
- *Staff.* Members of staff should work exclusively in the gene bank and not have contact with other farms or facilities that house animal species from which material is stored in the gene bank. The clothes and footwear used in the laboratories should be stored in the changing facilities and not used or otherwise taken outside.
- *Waste management.* The management of waste from the facility should be organized so as to reduce the risk of disease transmission. Storage facilities from which waste is loaded and transported away must be outside the perimeter fence.

BOX 4

Example animal holding and collection facility



(cont.)

- *Pest control.* Common pests include rats, mice, birds and flies. The control of rats and mice is greatly facilitated by adequate construction and maintenance of the facilities, particularly by preventing access to feed and water. Traps and rodenticides can be used if problems are discovered. Bird-proofing nets in windows and other openings will limit the entry of birds, although controlling them in open-sided buildings or outdoor pens is very difficult. Regular cleaning of pens, strategic use of insecticides and proper ventilation are important elements of fly control.
- *Dead animals.* Even with the greatest care and precaution, it is likely that donor animals will occasionally die at the facility. Carcasses should be taken away as quickly as possible and stored outside the perimeter fence until they are collected. Wild or stray birds and other animals should be kept away from the carcasses.

The figure illustrates a basic facility designed for housing multiple types of animals, such as several rams or 20 to 30 roosters, and for collecting and processing semen samples and embryos or other genetic materials. The freezing and storage rooms will ideally be physically separated from the animal housing and handling facilities for sanitary reasons. The facility is equipped with indoor and outdoor areas for the larger animals, which enable the animals to be moved with minimal handling to a collection area. Also included are three rooms that are self-contained: the small animal room, the storage and feed room, and the sample preparation room. Having these self-contained rooms allows the creation of "micro-environments" to fit specific needs. For example, the small animal room will need to have a daylight regimen; the feed room will need to be contained so as to eliminate the risk of rodent infestation; and the sample preparation room will need to have an environmental and temperature control system that decreases the risk of cold-shocking semen samples.

The airflow of the facility is illustrated in the figure by the dotted arrows. As described above, the small animal room is separated from the rest of the facility (except for an airlock for the passage of samples into the sample preparation room) to minimize the spread of by-products (faeces, feathers, etc.). Therefore, this room has its own air-handling unit, which provides fresh air and an exhaust system. For the same reasons, the large area of the facility has its own air-handling and exhaust system. The air-handling system of the sample preparation room vents into the larger facility and the vented air is then removed by a shared exhaust system.

Note that the facility shown in the figure includes only a single large animal-housing building. Although this is not ideal, it still allows the implementation of several biosecurity and general safety measures. An all in/all out procedure still can be followed, i.e. all the animals in a group of donors arrive and leave together and the building is cleaned and left unused for a few days between each group. The system allows different species to be alternated so that multiple species are not present at the same time. Multiple pens allow for male animals to be housed in separate pens. Finally, the animal handling and sample collection areas and sample preparation room have separate entrances, so the activities in each can be the respective responsibilities of different persons. This ensures that the processing technician does not come into direct contact with the animals.

Pens. Working, handling and containment pens (indoor and outdoor) should be constructed with the following factors in mind:

- *Size.* The pens and gates should be appropriate to the size of the animals and allow them to be moved in and out with minimal handling. For example, if the pens are too large, a lot of time may be needed to herd or coax the animals to the collection site, which will cause stress in the animals and decrease the quality of the germplasm samples collected.

- *Individual pens.* Male donors should be kept in individuals pens, but they should have visual contact with other animals. The objective of this arrangement is to avoid aggression between the males and subsequent injury, but to allow the development of social relationships that help reduce stress.
- *Materials.* Pens should be of high quality and durable enough to withstand the repeated stresses caused by the animals.
- *Automatic systems.* When financially feasible, the holding pens should ideally have automatic systems for supplying water and feed to the animals and for removing waste. Reducing human contact helps to prevent the animals from becoming stressed.
- *Safety.* Pens should be free from sharp edges and jutting angles that may injure animals or their handlers. Pens should be constructed with escape routes that handlers can use if the animals become violent.

Field collection

In many countries some collection of germplasm in the field is likely to be necessary, even if the country has a complete gene banking facility including infrastructure for animal holding and germplasm collection. Field collection is likely to be more common in large countries that have a single gene bank (because transporting animals to the central facility will be more expensive) and in countries where pastoral or other pasture-based production systems are predominant. Field collection requires particular protocols and advanced planning, as well as some specialized equipment. It is advisable to prepare a specific protocol to be followed during field collection and to train technicians in best practices for off-site collection. The national gene bank at the Canadian Animal Genetic Resources Laboratory for Cryobiology has a specific set of standard operating procedures for field collection of germplasm. These procedures are summarized in Appendix A.

Equipment. The following equipment and reagents are required for efficient and sanitary collection and/or freezing of germplasm in field conditions:

- disposable coveralls
- disposable boots
- single-use examination gloves
- reusable and sealable plastic bags
- indelible permanent markers
- paper towels or absorbent paper
- frozen ice packs
- shipping container
- packaging tape and dispenser
- portable incubator
- microscope
- microscope slides
- haemocytometer
- semen straws and filling/sealing equipment
- Styrofoam boxes
- liquid nitrogen storage tank

- gloves and tools for handling liquid nitrogen
- liquid nitrogen dry-shipper
- pre-addressed shipping labels (to the gene bank)

Safety. Field collection of germplasm should whenever possible be done by a team of technicians rather than by a single technician. Interacting with large animals always involves some risk of injury, particularly when the animals are from rangeland production systems and may therefore not have regular human contact. Surgical tools and liquid nitrogen can also cause injury. Cellular phones should be carried for general communication and to contact emergency services if injuries occur. When collection is undertaken in a remote, unfamiliar area, local authorities should be notified in advance and maps and geographic information system equipment should be carried (the latter also serves for recording the location of the sampling site).

As with collection at a dedicated facility, the animals should be restrained in a manner that is safe for both animals and technicians, and care should be taken to minimize the stress caused to the animals. Biosecurity protocols should be followed in order to prevent the transmission of disease from location to location (see also Section 9).

Germplasm processing and freezing laboratories

Although the processing and freezing laboratories may be part of the same physical structure as the animal collection and holding facilities, there must be a distinct physical barrier between the two areas, as the processing and freezing area must have a higher level of sanitation. The laboratory should be designed for maximum efficiency. In particular, it should be kept as small as possible so as to reduce the amount of maintenance and cleaning required. The various workstations should be arranged in such a way that the germplasm samples move from workstation to workstation in a logical order.

Essential features of the laboratory include the following:

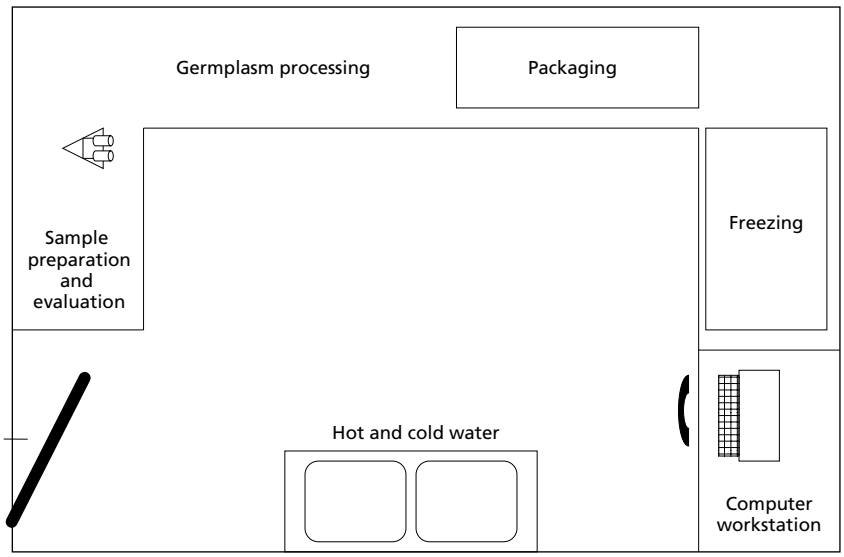
- washable work surfaces, floors (non-slip) and walls;
- sufficient lighting and ventilation;
- hot and cold running water as well as purified water;
- sufficient numbers of water-protected electrical sockets for all fixed and portable equipment; and
- adequate storage for consumable materials.

Figure 2 shows an example of a general layout for a simple germplasm processing laboratory. The various workstations are compact in order to increase the efficiency of the processing activities, which begin at the left of the diagram and then move step-by-step in a clockwise direction until the samples are finally packaged and frozen. Computing facilities are readily available (lower right of diagram), both for the operation of any software that may be necessary (e.g. for calculating dilution rates and inputting information about each sample into the central database – see Section 10).

Long-term storage room

In general, the long-term storage room for cryopreserved germplasm can and should be physically separated from any laboratory. Such an arrangement will increase the physical

FIGURE 2
Example design of a germplasm processing and freezing laboratory



Total area ~20 m²

security of the collection. By having physical barriers the potential for unauthorized access to the collection can be better controlled. Many existing gene banks have found this to be a desirable arrangement because it means that the long-term liquid nitrogen tanks do not have to be individually locked. Nonetheless, gene bank managers may, after weighing the advantages and disadvantages, decide that locking individual tanks should be a part of their standard operating procedures.

Material stored in a gene bank is a highly valuable resource and must therefore be safeguarded against loss. For this reason, it is strongly recommended to maintain two separate storage facilities in different geographic locations.

Liquid nitrogen is a dangerous material (see Box 5) and specific precautions must be taken, both in handling liquid nitrogen and in the design of storage facilities. If there is a leak of liquid nitrogen, there will be a risk that people involved in filling tanks and placing or retrieving samples, or simply working in the storage areas, will be asphyxiated. For this reason, designing the ventilation system should be a critical priority in the development of the long-term storage space. In general, nitrogen in its gaseous state is heavier than normal atmospheric air, so if liquid nitrogen spills the concentration of nitrogen will be greatest close to the floor. Therefore, ventilation ducts need to be placed at or near floor level. Conversely, air inflow ducts need to be placed high in the room (potentially at ceiling level) to help insure that nitrogen gas is forced out of the room through the lower exhaust vents.

BOX 5

Liquid nitrogen safety

Liquid nitrogen is an extremely dangerous substance to handle. Two major safety risks are:

- freezing or "burning" of skin upon contact; and
- hypoxia (lack of oxygen) and respiratory distress.

Liquid nitrogen has a temperature of -196 °C. It should never be allowed to come into contact with the skin. Protective gloves should be worn as well as standard lab coats. Shorts and open shoes or sandals should be avoided. Eye protection should be worn. Specially designed "tongs" should be used for handling the straws and containers used for storing germplasm. The low temperatures alter the physical properties of substances, so only tools specifically designed for work with liquid nitrogen should be used. If liquid nitrogen does come into contact with a person's skin, the affected area should be flooded with cold water and cold compresses applied. Medical attention should be sought immediately if eyes are affected or if the skin becomes blistered.

To prevent the possibility of hypoxia and respiratory problems, handling and storage areas should be well ventilated. Nitrogen vapour is heavier than air, so exhaust fans should be positioned so as to remove gas from near the floor. The handling and storage areas should also be fitted with oxygen meters that warn of the presence of excess nitrogen. An oxygen concentration of less than 19.5 percent is considered unsafe. If people become dizzy, everyone present must evacuate to a well-ventilated area. Victims of hypoxia must be moved to safety immediately and given artificial respiration and medical assistance.

Transport of liquid nitrogen presents special dangers. Great care must therefore be taken. Liquid nitrogen is an additional source of risk in road traffic accidents. Always ensure that tanks are safely secured. Transport of liquid nitrogen in the passenger compartment of a vehicle should be avoided. If this is not possible, one or more windows should be kept open.

It is also important to have adequate ventilation in the rooms where samples are frozen with liquid nitrogen. In both spaces, it is strongly recommended that oxygen sensors be installed and regularly tested.

SIZE AND CAPACITY REQUIREMENTS FOR GENE BANKS

The space required for gene banks can be extremely variable and may change as the mission of the gene bank changes or as the collection grows. It is important to note that the space requirements are relatively minor. Furthermore, the costs of developing the space should be amortized over appropriate depreciation timelines (e.g. more than 20 years).

Small repositories

Small repositories have limited space, but in some countries they will easily be large enough to meet the needs of national cryoconservation programmes. Facilities of this size can be expected to process and cryopreserve up to 500 straws of semen in a day. Typical dimensions of a small repository are as follows:

- wet lab (20 to 30 m^2);
- capacity to cool and refrigerate samples (to 5 to 15 °C, depending on the species) – cold room (10 m^2) or cooler cabinet (2 m^2) or Styrofoam box (0.6 m^2);
- cryotank storage room (20 m^2) (sufficient for approximately 300 000 straws);
- office for database management (12 m^2).

Medium-sized repositories

Medium-sized repositories should have a few additional features that not only increase the amount of germplasm they can store, but also the quantity of germplasm (principally semen) they can cryopreserve in a day (i.e. more than 500 straws per day). In fact, such facilities are likely to have automated equipment for straw labelling and filling and therefore be able to process and cryopreserve thousands of straws of semen per day. Typical dimensions of a medium-sized repository are as follows:

- wet lab (30 to 60 m^2);
- ability to cool and refrigerate samples – cold room (10 m^2) or cooler cabinet (2 m^2) or Styrofoam box (0.6 m^2);
- cryotank storage room (25 to 50 m^2) (approximately 400 000 to 600 000 straws);
- office for database management (12 m^2);
- other office space (12 m^2).

Large repositories

Large repositories have substantial capacity to cryopreserve semen, embryos and DNA and thereby meet their national mandates. Such facilities have the equipment needed to increase the quantity of germplasm frozen to several thousand straws per day. In addition, to the equipment necessary for cryopreserving samples, large repositories will also have equipment for analysing the quality of the germplasm cryopreserved: for example a computer-assisted sperm analysis (CASA) unit and flow cytometers. Typical dimensions of a large repository are as follows:

- wet lab (greater than 60 m^2);
- ability to cool and refrigerate samples – cold room (10 m^2) or cooler cabinet (2 m^2) or Styrofoam box (0.6 m^2);
- cryotank storage room (greater than 50 m^2) (>600 000 straws);
- office for database management (20 m^2);
- other office space (50 m^2);
- capacity to store excess liquid nitrogen or even to make liquid nitrogen.

RECOMMENDED EQUIPMENT AND ESTIMATED COSTS

The following basic equipment is needed for cryopreserving and storing samples:

- glassware for holding semen and measuring volume
- water bath
- microscope (phase-contrast for semen; stereoscope for embryos)
- centrifuge
- equipment for cooling samples (Styrofoam or plastic cooler, cooler cabinet)
- supply of pure or ultra pure (for embryos) water
- osmometer
- pH meter
- equipment for determining sperm concentration (one or more of the following):
  - spectrophotometer (fixed or portable)
  - Makler counter chamber (or disposable counting chamber)
  - haemocytometer
- temperature measuring devices
- straw labelling machine
- straw filling and sealing equipment
- freezing equipment (manual or programmable)
- carbon dioxide incubator (for embryos)
- laminar flow benches (for embryos)
- dry liquid nitrogen shipping tanks
- long-term liquid nitrogen storage tanks

Box 6 describes the types of liquid nitrogen tanks that will be needed. It is important to remember that it is not necessary to purchase all the liquid nitrogen tanks that will be needed over the long term at the same time. The tanks can be bought on a gradual, as-needed, basis.

GENE BANK SECURITY

Germplasm collections can be viewed as a national asset and thereby warrant appropriate levels of security. Building security consists of several aspects, including:

- **Safety of the germplasm from unauthorized access.** This can be achieved by controlling access to the room and the tanks themselves. In addition, gene bank managers should maintain records of which persons have access to the room(s) where collections are stored and when those persons accessed the room(s).
- **Structural integrity.** The structure of the gene banking facility should be sufficiently robust to withstand any environmental challenges that are conceivable in the local area (e.g. high winds, earthquakes, extreme temperatures, fires, floods). Construction in vulnerable areas, such as floodplains, should obviously be avoided.
- **Plans and equipment with which to continue operations in the event of major systems failures.** For example, generators for electricity and alternative sources of liquid nitrogen should be considered.

BOX 6

Liquid nitrogen tanks

The number and size of liquid nitrogen tanks needed for a gene bank will depend upon how much germplasm is expected to be stored. The capacity of tanks available for purchase will be specified in terms of numbers of straws, but it is also important to consider the quality of the tanks. Choosing to buy a given tank because the purchase price is low may not be the optimal financial strategy in the long term. Higher-quality (and thus more expensive) tanks will usually last longer and usually use less liquid nitrogen. They will also be less subject to leaks and other accidents that result in the loss of stored material. The value of the stored germplasm will usually far exceed the cost of the storage tank.

Any gene bank will need three types of tanks:

- long-term storage tanks;
- vapour shippers (for transporting germplasm); and
- storage tanks (for storing liquid nitrogen itself).

Long-term storage tanks are the most important part of a gene bank's equipment. Remember that the stored germplasm may be unique and irreplaceable. The tanks' liquid nitrogen consumption should be as low as possible, because liquid nitrogen is one of the major costs of maintaining a gene bank. In addition, it is recommended that individual tanks have electronic monitors that measure temperature and liquid nitrogen levels and sound an alarm if either parameter is outside set limitations. The size of tank required can vary greatly depending upon the size of the repository and how much germplasm is to be stored.

Vapour shippers are designed for the safe transportation of germplasm. They contain a hydrophobic absorbent that holds the liquid nitrogen, repelling moisture and humidity and thus maximizing the holding time (usually two to three weeks).

The third type of liquid nitrogen tank required are those used for storing surplus liquid nitrogen for future use in the other types of tank. Such tanks also serve as a buffer if regular supplies of liquid nitrogen are interrupted. Thus, the decision as to what size of storage tank is needed should take into account the level of security required given the frequency and reliability of liquid nitrogen deliveries, or alternatively the production schedule of the gene bank's own liquid nitrogen generation plant.

CENTRALIZATION AND ACCESSIBILITY

The development of the gene bank will be easier if it is located in an area that has sufficient infrastructure to ensure its smooth and continuous operation. For example, collection efforts will be facilitated if the gene bank is located near to major highways or airport hubs. Depending on the country, this may or may not be in or near the capital city. If the country has several well-connected cities, those found in the parts of the country that have the largest livestock populations will be the preferable options. If the country is large enough

TABLE 6
Estimated equipment costs by gene bank size

Item	Small		Medium	Large
	Necessary	Optional		
Long term liquid nitrogen tanks	$5 000 to $40 000		$40 000 to $120 000	> $120 000
Shipping tanks	$3 000		$3 000	$3 000
Equipment for straw filling	$500		$40 000	$40 000
Equipment (ranging from Styrofoam box to programmable freezer) for freezing samples	$200		$200 to $20 000	$200 to $20 000
Microscope	Compound $500		Phase contrast $5 000	Phase contrast with fluorescence $15 000
Centrifuge		$10 000	$10 000	$10 000
Spectrophotometer (fixed or portable)		$2 100	$2 100	$2 100
Makler counter chamber	$850		$850	$850
Haemocytometer	$200		$200	$200
pH meter	$1 200		$1 200	$1 200
Osmometer		$8 000	$8 000	$8 000
Water bath			$800	$800
Total	$11 450 to $46 450	$31 500 to $66 550	$111 350 to $211 150	$201 350 to $221 350

Note: costs are shown in United States dollars at 2012 prices.

to have notable regional differences in climate, situating the gene bank in a relatively colder region can help to minimize liquid nitrogen evaporation and thereby reduce the cost of maintaining the gene bank.

Other factors to consider in choosing the site for a gene bank include the extent to which public – and private – sector arrangements can be made for germplasm collection and processing and the location of important stakeholders. For example, AI centres may already be operating in the country, and the national germplasm programme may be able to develop arrangements with the AI companies to acquire germplasm from their facilities. By formulating such arrangements, the national programme can focus its own attention and resources on breeds and species for which there is a lack of commercial infrastructure for collection. As noted above, existing gene banks for plants or wild animals are also potential collaborators. In addition to aiding the collection effort, the presence of collaborating institutions may increase the accessibility of inputs such as liquid nitrogen.

HUMAN RESOURCES

The number and type of personnel needed to operate the gene bank will vary with the size of the facility. Common to all sizes, however, is the need for three different disciplines: genetics; reproductive physiology and cryobiology; and information systems and database development. Minimally, the gene bank will require:

- a curator (who will likely have a genetics background);
- technical support to cryopreserve germplasm; and
- technical support to develop a database and enter information into the database.

The curator has to develop targeted collection goals for each species and breed of interest. Obviously, this task is a significant responsibility for one person. Thus, in formulating plans for the development of the collection, the curator may need to seek input from a wide array of livestock expertise. Such consultations should involve not only the research community, but also the livestock industry, including groups involved in raising various livestock breeds.

As gene bank activities increase in size and scope, more staff will be needed. Much of this increase will be because of the increased flow of germplasm into the repository. However, as the size of a gene bank increases, additional technical needs will also arise. There may be a need to use a broader range of reproductive and cryopreservation technologies on a wider range of species. There may also be a need to quantify and understand the genetic diversity in the gene bank, which may require the use of molecular genetic techniques. With additional requirements comes the need for additional scientific support. Of course, many of these additions can be addressed by integrating conservation activities with those of pre-existing laboratories that have the necessary expertise (e.g. laboratories that specialize in molecular genetics). Nevertheless, the gene bank absolutely needs to have a reasonable level of competence with respect to these additional technologies if it is to fulfil its conservation mission.

Proper training is a critical factor in ensuring the smooth operation of gene banks. Regardless of his or her particular role, every gene bank employee should have an initial course of orientation and training at the beginning of their period of employment. Thereafter, training must be continual, with particular emphasis given to biosecurity.

CONTINUITY OF OPERATIONS

As with any important national resource, gene banks need to have procedures in place for handling the collection in the event of an emergency. Such emergencies might include the loss of electricity, flooding, earthquake, civil unrest, or a disease outbreak that affects the animal or the human population. If such an event take places, it is important to have a predetermined plan providing a set of guiding actions that can minimize the impact of the emergency on the maintenance of the collection.

To ensure the sustainability of the gene bank in the event of a disaster, the following points should be taken into account (some of these are mentioned above in the subsection on gene bank security, but their importance cannot be overstated):

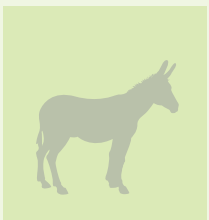
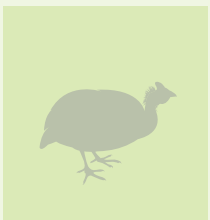
- Establish the gene bank in a location where hazards such as earthquakes, floods and tornados are minimized.

- Split the collection into two parts and store them in geographically separated facilities. This provides potential protection against earthquakes, floods, tornados, etc. The decision on whether or not to split the collection will depend on the finances available and the level of security afforded by the primary bank. In general, only the facilities for long-term storage (core and historical collections) will need to be replicated, so the costs of having two gene banks will be much less than double the cost of a single bank.
- Consider developing contingencies for moving the entire collection, or a predetermined subset of the collection, in the event of civil unrest.
- Establish minimum numbers of staff required to report to the gene bank in the event of an emergency.
- Develop plans for acquiring supplies (e.g. liquid nitrogen) from alternative sources.

Contingency plans should be subject to periodic review in order to ensure that they are still viable.

SECTION 6

Developing gene bank collections


Developing gene bank collections

Developing and updating gene bank collections is a long-term endeavour that involves several processes. Major steps include understanding the dynamics of the AnGR population of interest, determining the status of the population (e.g. whether numbers have dropped to a predetermined critically low level), establishing collection targets for germplasm (semen, embryos or oocytes) or tissue, and selecting the animals from which samples will be obtained for the collection.

As mentioned in Section 1, the choice of which breeds and animals to include in the gene bank will depend upon the country's objectives for AnGR management (preferably set out in a national strategy and action plan for AnGR) and the country's capacity to obtain and store the samples. In countries that do not have an existing gene bank, conservation needs for all the country's AnGR should be reviewed as a first step in determining whether establishing a gene bank would be an appropriate part of the country's conservation strategy. Factors to consider include both the accessibility of the animals for collection and the state of technical and financial capacities within the country for processing and storing the samples.

Primary considerations for all gene banks have to be the need to acquire sufficient quantities of germplasm to reconstitute the targeted populations and the need to ensure that the samples collected sufficiently represent the genetic diversity of these populations.

CHOICE OF POPULATIONS TO INCLUDE IN THE GENE BANK

Before initiating collection activities, gene bank managers will have to assess the various breeds and species in the country in order to determine where to start. More details on identifying breeds at risk and prioritizing AnGR for conservation will be presented in the forthcoming FAO guidelines on *in vivo* conservation of AnGR. Such decisions can be made on the basis of breeds' population size, potential genetic uniqueness, economic importance and/or cultural importance. In general, the choice of breeds for conservation should be a group decision undertaken by the National Advisory Committee on AnGR (FAO, 2011b) or similar committee, in consultation with the wider stakeholder community. Nevertheless, gene bank managers and curators should be allowed flexibility to be pre-emptive and initiate the collection of germplasm from breeds that are viewed to be in an extreme state of risk or critical to the country's livestock sector.

Various quantitative measures can be used for deciding which breeds should be prioritized for inclusion in collections. In general, two primary factors determine the conservation priority of a breed:

- level of risk of extinction; and
- conservation value.

Risk status

From a quantitative perspective, risk status can be thought of as expected future population size. The best measurable indicator of future population size is the current population size. Data on past population size can improve the prediction of future population size, as trends can be extrapolated into the future. Unfortunately, breed-level population data are often not available, especially where breed associations have not been established. Although many countries undertake periodic censuses of livestock, very few of these censuses record which breeds the animals belong to. As a result, it may be relatively easy to obtain a general overview of the national livestock industry, but gaining accurate information about the status of breeds within species is likely to be more difficult. The best way to overcome these difficulties is to initiate a national programme of surveying and monitoring for AnGR as an element of the country's national strategy and action plan for AnGR. As well as supporting decision-making at national level, this will enable regular reporting of population data to FAO via the Domestic Animal Diversity Information System (DAD-IS: http://www.fao.org/dad-is) and thereby contribute to reporting on global status and trends of AnGR. FAO has produced guidelines on *Surveying and monitoring of animal genetic resources* (FAO, 2011c) to assist countries in the task of obtaining data on their AnGR.

If the availability of data on national breed populations is insufficient, gene bank managers (in consultation with relevant national working groups on AnGR) may wish to initiate their own protocols for surveying and monitoring AnGR, provided the necessary financial and technical support is available. If new surveying initiatives are planned, the sampling procedure must be well designed. A statistician should be enlisted to design an appropriate sampling strategy. Further advice can be found in the above-mentioned guidelines (FAO, 2011c). If breed associations are present in the country, collaboration with them is recommended, as these organizations typically keep track of annual registrations and may be willing to share this information.

Factors other than population size can also influence risk status. Breeds that are distributed across more farms and/or across a wider geographic area tend to be at less risk than breeds with more concentrated distributions. When a breed is maintained by large numbers of livestock keepers, the action of a single individual will have less impact on the population size. Wider geographical distribution decreases the risk that the entire population may be wiped out by a single geographically concentrated catastrophic event, such as a disease outbreak. Increased cross-breeding and increased inbreeding also put AnGR at risk – even if the number of animals stays the same, the diversity may decrease.

Conservation value

As discussed in Section 1, conservation of AnGR may be undertaken to meet various objectives. These objectives and their relative importance are likely to vary from country to country. In turn, breeds will vary in terms of their relative contributions to meeting these conservation objectives. The following factors will influence the conservation value of a given breed (Ruane, 2000):

Genetic uniqueness. Genetic distinctiveness is an important criterion for establishing conservation priorities. Understanding the genetic history of particular breeds, or formally estimating genetic distances among breeds, will assist in determining breed uniqueness.

Genetic variation within a breed. Genetic variation gives an AnGR the capacity to adapt and allows for genetic response to selection. Conserving the most genetically diverse breeds is the most efficient way to conserve the diversity of a species.

Traits of economic importance. Breeds that are genetically superior for traits that are economically important (at present or in the foreseen future) should receive priority in conservation. Prioritization decisions require evaluation of both current and potential future importance of particular breed characteristics and performance.

Unique traits. Breeds with special behavioural, physiological or morphological traits should be given high priority for conservation.

Adaptation to a specific environment. The adaptation of breeds to specific environments is likely to be under some genetic control. Thus, the conservation of AnGR that thrive in specific environments (e.g. those that are in some way harsh) may be important. Breeds that perform valuable environmental services will also often be priorities for conservation.

Cultural or historical value. Breeds with special cultural or historic values merit consideration for conservation.

Species a breed belongs to. The above criteria are important in selecting breeds within species; some consideration should also be given to the species to which the breeds belong when considering which genetic resources to conserve and which approach should be applied. For example, in some countries, particular species may be especially important (e.g. alpacas in Peru). With regard to cryoconservation, the technology and procedures for cryopreservation are generally more advanced for cattle than for other species, meaning that costs are lower and success rates are higher. Considering species in conservation decisions therefore helps ensure that financial resources are fairly allocated to all important livestock species and accounts for the costs and likelihood of success of different conservation options.

Accounting for all of these factors can be quite difficult. Clearly, prioritization of breeds for collection and entry into the gene bank can be done in a number of different ways, and the approach chosen will depend on national circumstances. Advisory committees of experts, including stakeholders from industry and breeders' associations, can advise the gene bank about the risk status of various populations and their genetic, economic and cultural importance.

Molecular markers can be used for evaluating genetic distances and breed diversity (see FAO, 2011a). Various objective methods have been proposed for incorporating molecular measures of diversity into conservation decisions (see review by Boettcher *et al.,* 2010). These methods typically allow for simultaneous consideration of risk status and the various genetic and non-genetic factors listed above. Their use will be described in more detail in the forthcoming FAO guidelines on *in vivo* conservation of AnGR, because prioritization should be done as part of an overall conservation plan rather than just for cryoconservation.

Although formal prioritization methods may increase the efficiency of conservation decision-making, a willingness to be flexible in establishing collections is also needed so as not to miss unexpected needs and opportunities when they arise. When particular AnGR are at high risk of extinction, or if they can be collected a very low cost, collecting them for cryoconservation can be justified even if no formal prioritization analysis has been undertaken.

COLLECTION TARGETS FOR RECONSTITUTING POPULATIONS

Once the decision has been taken as to which breeds and populations should be collected for the gene bank and the type of germplasm to collect, the next step is to determine the amount of germplasm needed. The quantity will vary depending on the conservation goal, the type of germplasm and the species. In general, the goal of reconstituting extinct populations will require the greatest amount of germplasm.

Targets for cryoconserved semen

When semen is cryoconserved, the principal method for reconstituting a breed or population is through backcrossing (Figure 3). Starting with females of a common breed, four backcross generations allow the reconstitution of more than 90 percent (four generations ~94 percent; five generations ~97 percent)[4] of the nuclear genome of the conserved breed or population. The following subsections describe how semen can be used to reconstitute a population and the amount of germplasm needed to do this.

The length of reproductive cycles and conception rates influence the speed and efficiency with which populations can be reconstituted. To better establish collection goals, the manner in which the cryoconserved material will be used to reconstitute a breed needs to be quantified. Previous publications (FAO, 1998; Boettcher *et al.,* 2005) described a single backcrossing approach, such as that shown in Figure 4. In this approach, females are used as mothers only once in their lives. This allows the breed to be reconstructed with maximum speed and simplicity. All animals of the same age will be of the same generation and will have the same proportion of genetic material from the reconstituted breed. Because females are used only once, the amount of semen needed for reconstituting a population is quite large. This is primarily because of the 50:50 sex ratio that must be assumed, which in the early generations results in a large number of excess males.

Some livestock species, including cattle, buffalo, small ruminants and horses, have longer reproductive cycles and/or smaller family sizes than others such as pigs and poultry. Advanced strategic planning with regard to mating has the potential to drastically improve the efficiency of gene banking and breed reconstitution. The mating plan shown in Figure 5 uses a slightly different backcrossing scheme. The approach allows females in the first, second and third backcross generations to be mated up to three times. This gives these animals a much greater chance of producing a female offspring. Expanding the mating opportunities of the later generations means that less semen is used, and decreases the cost of maintaining the animals used in reconstituting the breed. The principal drawback of this approach is that it will take slightly (e.g. one to three years) longer to reconstitute the population.

[4] Proportion of target breed recovered = $1 - 0.5^b$, where b is the number of backcross generations.

FIGURE 3
Population reconstitution with cryoconserved semen

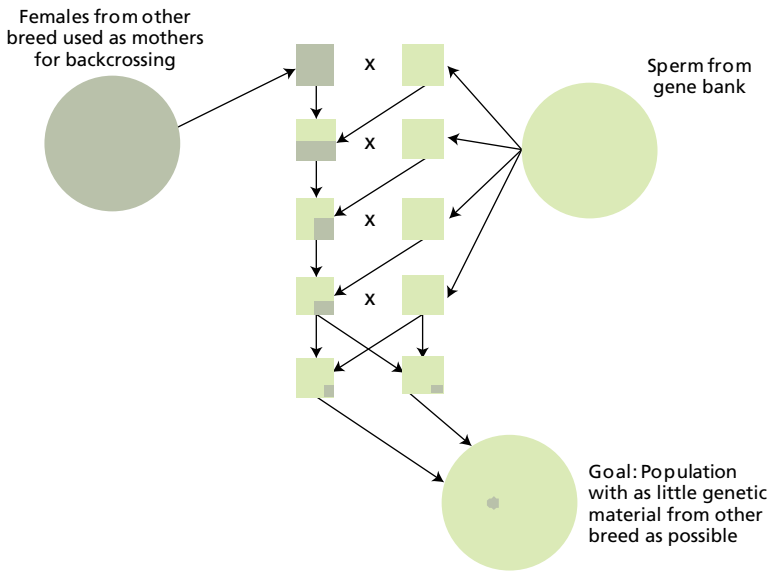
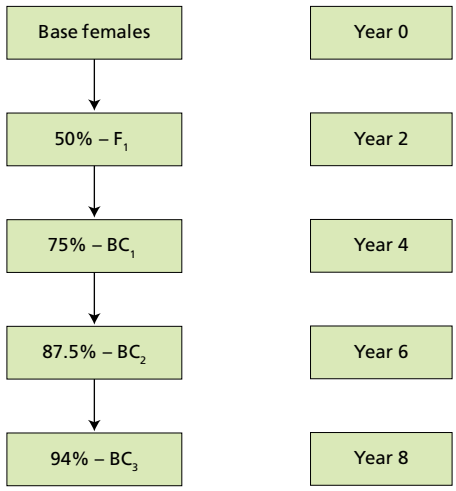
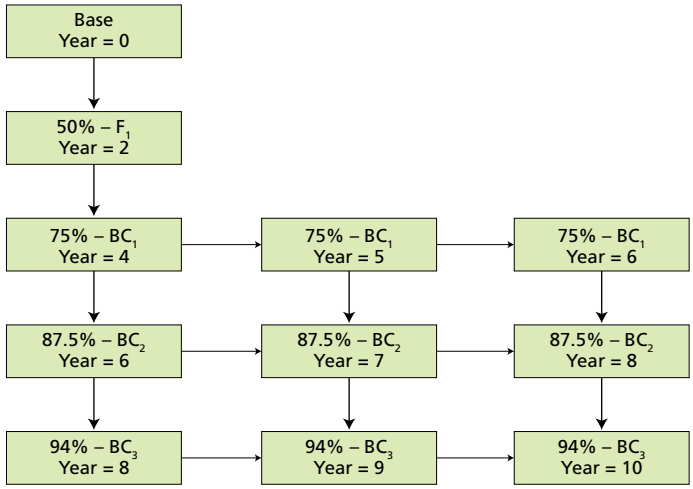


FIGURE 4
Standard backcrossing plan for breed reconstitution (using cattle as an example)



Note: BC = backcross (numbers indicate the first, second and third backcross generations).

FIGURE 5
Alternative efficient mating plan for breed reconstitution



Note: BC = backcross (numbers indicate the first, second and third backcross generations).

Ruminants and horses. Table 7 shows the quantities of semen needed for a "150 percent breed replacement plan" in which a breed is reconstituted using the breeding plan shown in Figure 5. A "150 percent breed replacement plan" means that the gene bank will save 1.5 times the amount of germplasm that is expected to be necessary to reconstitute a breed (i.e. to obtain 25 males and 25 females with 94 percent of the genetics of the conserved breed). Planning at the 150 percent level provides flexibility and additional germplasm that may be needed to compensate for lower than expected pregnancy or survival, excess animals of one sex, or other failures in the reconstitution process. The table shows targeted quantities of semen for various rates of pregnancy (which will vary according to circumstances). Another variable shown in Table 7 is the number of founder-breed females used to start the reconstitution process. The final number of animals with 93.75 percent of the targeted genome that will be generated (a critical factor in the success of the reconstitution process) is also shown (in parenthesis) in Table 7. Note that the reconstituted population sizes are also estimates of N_e. For several of the conception rates and initial numbers of animals bred shown in the table, the N_e is below 50, i.e. below the recommended threshold (see Section 3). However, this deficiency can be addressed by having semen form extra males in the repository, which can be used to broaden the genetic base of the newly regenerated breed.

Estimates of the quantities of semen required per male are presented in Table 8. For many mammalian species, the quantities shown in Table 8 are achievable in a single collection. Nevertheless, gene bank managers should follow a conservative approach and store samples from at least two collections (separated by at least two weeks) from each male.

With cattle, and potentially other species in the future, the utilization of sexed semen would dramatically change the projected amount of semen needed to reconstitute popula-

TABLE 7
Number of semen doses required to reconstitute a breed of cattle, small ruminant or horse

Founder females (N)	Pregnancy rate			
	0.4	0.5	0.6	0.7
75	...*	...	449 (26)	460 (37)
100	...	564 (22)	599 (35)	615 (49)
150	771 (17)	846 (33)	897 (53)	...
200	1 029 (23)	1 128 (44)	...	...
250	1 287 (29)	1 410 (55)	...	...
300	1 544 (34)	...	...	...
350	1 800 (40)	...	...	...
400	2 058 (46)	...	...	...

Note: The figures in parenthesis show the effective population size of the reconstituted population.
The quantity of semen is calculated at the 150 percent level (see main text for explanation).
* Missing values indicate that results are not practical, resulting in either too few (upper-left corner) or too many (lower-right corner) animals or requiring large quantities of semen.

TABLE 8
Number of doses per male required to reconstitute a breed of cattle, small ruminant or horse

Founder females (N)	Pregnancy rate											
	0.4 Males (N)			0.5 Males (N)			0.6 Males (N)			0.7 Males (N)		
	25	50	100	25	50	100	25	50	100	25	50	100
75	...	...	...	...	...	...	18	9	5	19	10	5
100	...	...	...	23	12	6	24	12	6	25	13	7
150	31	16	8	34	17	9	36	18	9	...	...	...
200	42	21	11	46	23	12	...	...	...	...	...	...
250	52	26	13	57	29	15	...	...	...	...	...	...
300	62	31	16	...	...	...	...	...	...	...	...	...
350	72	36	18	...	...	...	...	...	...	...	...	...
400	83	42	21	...	...	...	...	...	...	...	...	...

Note: Quantity of semen is calculated at the 150 percent level (see main text for explanation).

tions. The figures in Tables 7 and 8 assume a sex ratio of 50:50. If sexed semen were used, the number of doses could be reduced by 30 to 45 percent.

Another option that countries may consider as a means of decreasing the number of doses of semen (and time for reconstitution) is to accept a larger proportion of the founder breed in the final "reconstituted" population. For example, if a ratio of

TABLE 9
Number of straws of semen required to reconstitute a breed of pig

Founder females (N)	Pregnancy rate		
	0.4	0.5	0.6
15	2 880 (56)	2 760 (66)	2 520 (72)
25	4 800 (96)	4 560 (108)	4 200 (126)
50	9 600 (192)	9 000 (225)	8 400 (252)

Note: The figures in parenthesis show the effective population size of the reconstituted population. The quantity of semen is calculated at the 150 percent level (see main text for explanation). Number of straws is based on an assumption of 20 0.5 ml straws per insemination, with a total of 1 billion cells per insemination.

seven to one (87.5 percent) of the reconstituted and founder breeds is acceptable, the number of backcross generations can be decreased to three and the amount of semen required can be decreased by 30 percent or more. This option may be particularly attractive in situations where reaching the goals shown in Table 7 would be difficult for practical reasons.

Pigs. The relatively high reproductive capacity and shorter gestation period of pigs enables quicker breed reconstitution than can be achieved in cattle and horses. Because of the prolificacy of the species, large numbers of animals are not needed in order to start the reconstitution process. However, because of dilution protocols, more straws of semen are required per insemination than are needed in other species. To a certain degree, the innate reproductive ability of the sow compensates for the occasionally high mortality of pig sperm cells during the cooling and cryopreservation process. In Table 9, the numbers of straws of semen needed for reconstitution are shown, as well as the numbers of offspring reconstituted at the 93.75 percent level. The data are based on the assumption that six piglets from each litter reach breeding age. This is a very conservative estimate given the results obtained by Spencer *et al.* (2010), who reported a 74 percent pregnancy rate and an average litter size of 11 when females were bred through AI. Quantities of semen needed per male are presented in Table 10. As with the other species, these quantities can potentially be obtained from a single collection, but a more conservative practice is to obtain two collections from each boar.

Chickens. In contrast to programmes for mammalian species, chicken conservation schemes may give less emphasis to breeds as categories. Although breeds are important, industrial populations consisting of distinct lines may (depending on the country) be of greater importance. In addition, poultry breeders and scientists have created numerous research populations, many of which can be categorized by Mendelian traits controlled by single genes or by quantitative traits.

Plans to reconstitute chicken populations need to account for the fact that cryopreservation has been problematic in recent years because of the contraceptive effects of glycerol on hen fertility. Multiple inseminations have been employed to overcome this problem and were a recommended in the previous guidelines on conservation (FAO, 1998). Other means

TABLE 10
Number of straws per boar required to reconstitute a breed of pig

Founder females (N)	Pregnancy rate								
	0.4 Donor males (N)			0.5 Donor males (N)			0.6 Donor males (N)		
	25	50	100	25	50	100	25	50	100
15	116	58	29	111	56	28	101	51	26
25	192	96	48	183	92	46	168	84	42
30	384	192	96	360	180	90	336	168	84

Note: The quantity of semen is calculated at the 150 percent level.

of solving this problem include using alternative media and performing intramagnal instead of intravaginal insemination (Purdy *et al.,* 2009). By using the intramagnal approach, multiple inseminations are not needed and the number of units of semen needed to reconstitute a population can be reduced significantly (Blackburn *et al.,* 2009).

Table 11 outlines the semen resources needed to create secure reserves of chicken genetic resources in gene banks. Various objectives for the eventual use of the stored materials are considered and the data assume the use of intramagnal insemination. Semen resources required for the reconstitution of a breed are presented in the first column of data. These figures were calculated based on a number of assumptions regarding the efficiency of reproduction and the survival of the resulting offspring. Specifically, the assumptions used in the calculations include:

- two fertile eggs produced per hen per insemination;
- 1.4 fertile eggs hatched per hen per insemination;
- 85 percent of hatched chicks become adults;
- two inseminations per 0.5 ml straw; and
- 50:50 sex ratio among surviving chicks.

Furthermore, the data assume an efficient mating system similar to that shown in Figure 5 for ruminants and equines. In the initial generations, females are allowed to produce only a single offspring, after which (the third generation for reconstitution of a breed) they are used to produce multiple offspring. This system decreases the number of excess males in the early generations.

Many research institutes around the world have developed specific within-breed lines that differ either for a single gene or for a single polygenic quantitative trait. Cryoconservation of germplasm from such lines may also be of interest, either to protect against loss or for economic efficiency (i.e. maintenance *in vitro* rather than *in vivo*). Reconstitution of such lines will require fewer resources, because a smaller amount of genetic variation needs to be captured in the donor population. The amounts of material that need to be banked in order to be able to reconstitute such lines are shown in the second and third columns of data in Table 11. Note that populations with single genes of interest can be reconstituted in one or two generations.

TABLE 11
Resources required for chicken gene banks to address various conservation objectives

	Objective for use of stored material		
	Reconstitution of a breed (five generation backcross)	**Reconstitution of a research line with a given quantitative trait (five generation backcross)**	**Single gene introgression**
Total straws used[a]	257	127	7
Initial number of hens	140	100	14
Inseminations for entire reconstitution process	513	254	14
Generation number to start multiple intramagnal inseminations per hen (inseminations/hen)[b]	3 (3)	4 (3)	0 (0)
Final number of birds in the reconstituted population (generation number)	62 (5)	44 (5)	16 (1)
Minimum number of straws for 150 percent reconstitution	386[c]	191[c]	11[c]

[a] Based upon a motile sperm concentration of 200 × 10^6 (Purdy *et al.,* 2009).
[b] Generation 3 and 4 hens will have 87.5 percent and 93.7 percent of the genome of interest.
[c] Assumes a 0.5 ml straw and two inseminations per straw.

Embryo use in breed reconstitution

As noted in Section 4, embryos have some advantages and disadvantages relative to semen with regard to the reconstitution of a population. Their principal advantage is the speed with which breeds can be reconstructed (less than five years). In addition, the use of embryos ensures the conservation of a breed's entire genome, whereas when only semen is used the mitochondrial genome is lost and a certain proportion of the founder breed used for backcrossing will be present in the reconstituted population. Embryos could also be particularly important for breeds with extremely unique characteristics that would be very difficult to re-establish by using semen in a backcrossing scheme. The Angora goat is such an example. Re-establishing the Angora's fibre quality through crossing with a founder population of goats that produce low-quality fibre would be very problematic from an economic standpoint, as the fibre from initial backcross generations would be of very little economic value.

However, embryos are significantly more expensive to collect and require greater technical capacity than semen (Gandini *et al.,* 2007). Moreover, embryo transfer (ET) is not possible in all species of livestock. Biologically, the embryo offers the complete genetic complement of the breed. However, the genetic combinations formed when making the embryos can become dated. Gene bank managers should be aware that this may affect the utility of embryos after long-term cryostorage.

Table 12 shows the number of embryos that need to be cryopreserved in order to obtain a reconstructed population of 25 breeding males and 25 females, depending on the survival of the embryo from thawing to birth and the subsequent survival of the animal

TABLE 12
Number of cryopreserved embryos required to reconstitute a breed population

Embryo survival (thawing to birth)	Offspring survival (birth to breeding age)			
	0.6	0.7	0.8	0.9
0.2	625	536	469	417
0.3	417	358	313	278
0.4	313	268	235	209
0.5	250	215	188	167
0.6	209	179	157	139
0.7	179	154	134	120

Note: The quantity of semen is calculated at the 150 percent level. Reconstituted population is assumed to have at least 25 males and 25 females. Numbers of embryos (n_{emb}) were obtained according to the formula $n_{emb} = 1.50 \times 25 (0.5 \times s_e \times s_o)$, where 1.50 is a multiplier used to obtain the 150 percent level, 0.5 is the sex ratio, and s_e and s_o are survival rates to birth and breeding age, respectively (Gandini and Oldenbroek, 2007).

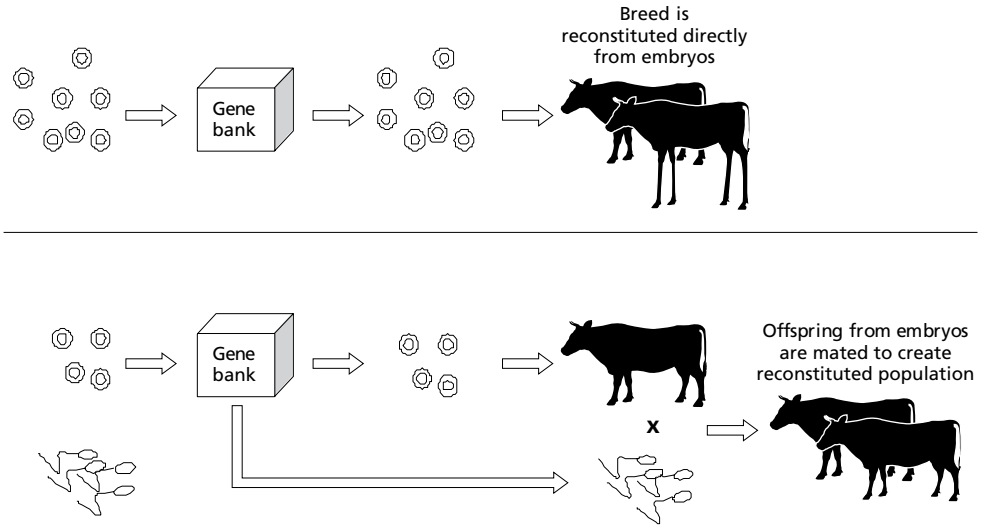
from birth to breeding age. Ideally, the numbers of embryos per donor will be nearly evenly distributed across at least 25 donor females, each mated to a different male (or multiple males), to capture the maximum amount of diversity possible from the existing population.

In cases where sampling 25 donors is not possible (or simply to economize), another option that may be considered is a hybrid scheme in which a combination of semen and embryos is stored (Boettcher *et al.,* 2005). In this strategy, fewer embryos are required than are listed in Table 12. Embryos are used to produce a population initially consisting of fewer than 25 breeding males and 25 breeding females, but these animals are subsequently mated with each other and with the stored semen in order eventually to obtain a population of the desired size after one or more years (Figure 6). This approach can reduce costs relative to storing only embryos while still allowing the maintenance of the mitochondrial genome and usually decreasing the time required for breed reconstitution relative to the backcrossing scheme utilized when only semen has been stored (Figures 3 to 5).

Nonetheless, a hybrid scheme requires some compromises. Although the reconstitution will usually be faster than that achievable with stored semen, multiple breeding cycles are nevertheless required to reach the final goal of 25 animals of each sex. Using embryos, this goal can be achieved in a single breeding cycle. Furthermore, genetic relationships in the reconstructed population increase (and N_e decreases) as the number of embryos decreases, as does the probability of failing to reconstitute the new population. Therefore, a hybrid scheme with fewer than 30 percent of the number of embryos required for an embryo-only reconstitution plan (Table 12) is not recommended. To increase N_e, the sires whose semen is stored in the bank should be different from the sires of the stored embryos.

A conservative approach to developing a germplasm collection would be to acquire semen samples as described in the above subsection on "targets for cryoconserved semen" and, where possible, to acquire embryos opportunistically and thereby enhance the quality

FIGURE 6
Reconstitution using only embryos versus a reduced number of embryos and semen



of the banked material. Collecting and maintaining sufficient embryos in the gene bank to generate 10 to 20 females will speed reconstitution and ensure that mitochondrial DNA is captured in the collection (Boettcher *et al.,* 2005).

Cryopreservation of embryos is most effective in ruminants. Pig embryos can be cryopreserved, but the success rates are lower. In poultry, embryo preservation is not yet possible. Therefore, to capture the genetic complement of a poultry population primordial germ cells have to be harvested and cryopreserved (see Section 4). Moore *et al.* (2006) demonstrated that primordial germ cells can be cryopreserved effectively. However, successfully inserting these cells into recipient eggs is problematic; the success rate is about 4 to 8 percent (Petitte *et al.,* 1990).

Before establishing a genetic reserve in an embryo bank, the number of available donor animals should be determined and a decision taken regarding whether these donors can produce a sufficient number of embryos to allow eventual reconstitution of the targeted breed(s). Ideally, embryos from at least 25 donor females mated to 25 different sires should be included in order to increase the genetic variability of the reconstituted population to the recommended level. The final decision will depend on the availability of donor females and the costs of the system used to access the germplasm. For example, if the gene bank has to buy the donor females from the breeders, the acquisition of exactly 25 donors may be financially optimal. However, if the breeders are paid for each collection (or if no payment for access is necessary), then the cost of sampling more than 25 donors may be similar to

the cost of sampling exactly 25 donors. In the case of at-risk breeds, gaining access to 25 donor females may be problematic.

Multiple embryo recoveries may be needed from each superovulated donor female. Embryo recovery following superovulation is notoriously variable, especially in cattle. The numbers of embryos per collection from a well-managed donor may range from zero to 40, with an average of five or six transferable embryos. In addition, females that do not respond well to superovulation during the first collection have a tendency not to respond well in subsequent attempts. Therefore, it is important that males used for matings are not associated with the same female throughout the collection period; otherwise, one or a few sires may dominate in the re-established breed. Repeated use of the same sire on a given donor female will also result in the banking of multiple full-sib embryos. There is an implicit penalty associated with storing full sibs: deleterious genes passed on by an individual of one sex penalize not only its own future contribution to the population but also that of its healthy mate. Avoiding full-sib embryos helps spread the risk (Woolliams, 1989).

Breed reconstitution with oocytes and semen

When oocytes and semen are stored in the gene bank, the number of oocytes required can be determined by using a slight modification of the formula used to obtain the figures presented in Table 11:

$$n_{ooc} = 1.5 \times 25\ (0.5 \times s_e \times s_o \times s_{ivf})$$

where n_{ooc} is the number of oocytes to be banked, 1.5 is a multiplier used to obtain the 150 percent level, 0.5 is the sex ratio, s_e and s_o are survival rates to birth and breeding age, and s_{ivf} is the success rate of the IVF procedure used to obtain an embryo from an oocyte.

The number of doses of semen stored should be sufficient to fertilize all the stored oocytes. A conservatively high estimate is one dose of semen per oocyte stored. However, a single dose can fertilize multiple oocytes. Ideally, the number of males from which semen is stored should be at least as large as the number of female donors of oocytes.

UTILIZATION OF GENE BANK MATERIAL IN LIVE CONSERVATION AND BREEDING

As noted in Section 3, in addition to reconstituting a breed after its extinction, gene bank material can be used for several other purposes, both in the short and the long term. First, the use of gene bank material for breeding animals in live populations can be helpful in controlling inbreeding rates or in revitalizing populations. Controlling the rate of inbreeding in a population is important for maintaining genetic variation. The N_e of a reconstituted population should be at least 50, which corresponds to a rate of inbreeding of 1 percent per generation. Gene bank collections can play an important role in maintaining the genetic variation of a breed and can actually increase the N_e of a breed if used properly. Box 7 presents an example of how this approach is being applied in the Netherlands. Obviously, semen taken from the bank for this purpose must not come from males that are part of the live breeding population. When managing a small population, three to ten males should be identified each year and their semen should be stored for future use. At least 20 doses of semen from each male should be conserved, with quantities increasing

BOX 7

Use of gene bank semen for revival and support of the breeding programme of the endangered Dutch Friesian Red and White cattle breed

In the 1800s, the cattle population in the province of Friesland in the Netherlands consisted mainly of Red Pied cattle. During the past century, however, preference for the Black and White phenotype, followed by sustained import and crossing with Holstein-Friesians, resulted in a sharp decline in the Red Pied population, so that only 21 Red and White individuals (4 males and 17 females) remained in 1993. At that point in time, a group of owners started the Foundation for Native Red and White Friesian Cattle. A breeding programme was developed in collaboration with the newly created national gene bank for farm animals. Semen from sires preserved in the gene bank in the 1970s and 1980s was used for breeding. Male progeny were raised by breeders, who were granted a subsidy from the gene bank. Semen from these males was collected, frozen and later used under new contracts. The breed increased in number, reaching 256 registered living females and 12 living males in 2004. In addition to the living populations, more than 10 000 doses of semen from 45 bulls are stored in the gene bank and kept available for AI. The combination of the living population and the gene bank stock results in a much larger N_e than is represented by the living population only.

depending on the reproductive capacity of the species (low capacity → more doses) and population size (larger population → more doses). At least 100 doses per sire should be conserved for species with low reproductive capacity such as cattle and horses. Because this practice will involve the use of "old" germplasm, it limits the amount of genetic progress that can be made for a given trait. However, the main objective is to maintain a high level of genetic diversity in the population.

Second, the material can be used in a cross-breeding system for introgressing specific characteristics into live populations. Breeders may want to introgress desirable characteristics of a cryoconserved breed into an existing breed. Introgression can be based on phenotypic information, and the desirable characteristics can be maintained in the subsequent generations by continuous selection. Alternatively, the genes underlying the desirable characteristics can be identified and molecular markers used to maintain the desirable genomic regions or traits. Introgression or crossing cryoconserved populations with live populations can be used to produce completely new breeds. Introgression generally involves only a single cycle of breeding to the conserved breed that provides the desired gene. In subsequent generations, the live animals are bred *inter se* or backcrossed to purebred animals of the live population. Therefore, the number of doses that need to be stored for this purpose will depend on the number of females in the live population that will be subject to crossing to initiate the introgression process. Box 8 describes the use of banked germplasm to introgress genes into a herd of pigs used for research.

BOX 8

Reconstituting a research pig line

Gene banks have an important role in backing up research populations. Purdue University in the United States of America had developed a line of pigs that were either homozygous or heterozygous for both the Napole and Halothane genes, which negatively affect pork quality in animals with the homozygous recessive genotype. In 2003, Purdue decided to discontinue this population and chose to have samples of semen from three carrier boars frozen and banked by the National Animal Germplasm Program. In August 2007, the University decided to re-establish a population in which the recessive homozygous condition was present so that it could be used to research meat quality. Samples of the semen stored with the National Animal Germplasm Program were therefore transferred back to Purdue and sows were inseminated. The results were a 100 percent pregnancy rate and an average litter size of 7.7 pigs. The resulting boars were genotyped, and 14 of 25 were found to be heterozygous for both genes. With the F_2 population, several boars were homozygous for both mutant genes. This case was the first in which a livestock research line was cryopreserved, discontinued, and re-established using germplasm frozen and stored by a gene bank.

For any breeding programme, regardless of the population size, periodic cryogenic storage of genetic material is recommended as a backup in case (genetic) problems occur. A cryo-aided live scheme can be very beneficial, especially for populations with a low N_e, mainly because it will result in prolonged generation intervals and therefore a larger N_e. Intensely selected breeds can have a small N_e even if the actual number of animals is very large (Bovine HapMap Consortium, 2009). It is important to collect new genetic material regularly (at least once each generation interval) in order to maximize genetic diversity.

The plan for collection and utilization of material from a gene bank must consider the characteristics of the breed in question, including the characteristics of its production system in addition to its genetic and phenotypic characteristics. This is especially important for breeds that differ greatly from the norm. Most scientific research on reproductive physiology, cryobiology, breeding and animal husbandry has been undertaken on international transboundary breeds kept under intensive management. To ensure optimal results when utilizing stored germplasm, complementary research to establish breed-specific protocols may be prudent. Box 9 presents an example from France in which the use of gene bank semen from two at-risk breeds did not yield the results expected because of differences between the characteristics of the breeds and those of the international transboundary breeds upon which protocols (and expectations) were based.

SELECTION OF INDIVIDUALS FOR CRYOPRESERVATION

As described in Section 3, the first target for germplasm collections is to enable the reconstitution of a population with an N_e of 50 animals. However, for the gene bank manager

BOX 9

Using frozen biological material from at-risk breeds: discrepancy between theory and practice

France has efficient conservation programmes for at-risk pig breeds, which include both *in situ* and *ex situ* conservation. Semen was collected from males from the unique bloodlines of all the at-risk breeds, mostly in the 1980s (semen pellets) and the 1990s (straws). The living populations of two local pig breeds (Bayeux and Blanc de l'Ouest) have suffered tremendous losses of genetic variability since the 1980s, prompting the respective breeding associations to contact the French National Cryobank and request permission to use pelleted semen from the collection (four males per breed). The quality of the semen in the pellets was evaluated as poor in Blanc de l'Ouest and good in Bayeux. This semen was used for mating four sows from each breed, with the primary goal of obtaining new breeding males. In the case of the Porc Blanc de l'Ouest breed, the four sows produced litters ranging in size from 6 to 13 live piglets, and semen from two of the males was collected for AI in 2007. However, in the case of the Bayeux breed, only two females were born from the four inseminated sows. The failure of the programme in the Bayeux breed was a result of several factors. The Bayeux is usually raised in an extensive production system and the sows behaved very aggressively when herded. Sanitary issues also played a role, as all the animals came from smallholdings where hygienic standards were lower than those in standard commercial operations. Last but not least, the physiology of the Bayeux sows was very different from that of sows from typical commercial lines. The protocols applied in this case, such as heat grouping, choice of insemination dates and so forth, were based on procedures that yielded optimal results for mainstream commercial breeds. This experiment yielded an important lesson. The French National Cryobank and breed associations have now agreed to develop special protocols tailored to each breed and to consider breed-specific measures in any future programmes for the use of stored genetic material.

the issue quickly turns to which animals within a breed should be sampled for the repository, and for which of the collection categories. Genetics are often among the primary considerations, but reproductive and sanitary aspects must also be accounted for.

Genetic aspects

Here the major consideration is to select animals that are as unrelated as possible to each other in genetic terms. This can be done in several ways. The appropriate option will depend upon the availability of information and resources.

- When pedigree information is available, simple procedures can be used to ensure that animals are not closely related, such as not selecting animals with common grandparents.
- More formal analyses can also be undertaken, such as the application of genetic contribution theory to select the least-related group of germplasm donors (Meuwissen, 2002).

Clustering approaches can be used to group animals that are closely related and identify clusters that are genetically distant from one another (see Box 10).

- Donors should be chosen from within lines if line breeding is practised.
- With or without pedigree information, various molecular DNA approaches can be used to determine the genetic uniqueness of animals or subpopulations within a breed. A principal obstacle to such approaches is that they require wide sampling of the animals within the breed, and many more animals will need to be genotyped than are actually needed for the gene bank. Alternatively, gene bank managers may consider collecting germplasm samples concurrently with taking blood or tissue samples and then utilizing the resulting genotypic data to enable more effective use of the stored material to decrease genetic relationships in the reconstituted population.
- Genetic markers can also be used to identify introgression from other breeds, the level of which may differ among subpopulations or geographical areas. This type of information is useful in determining how genetically pure the targeted animals are.
- If no reliable animal registration is available and resources are insufficient for the use of molecular genetics, donors should be carefully chosen based on their geographical location, phenotype and herd history.
- Particularly when pedigree information is not available, donor animals should be chosen from different areas and herds; genetic flows (i.e. exchange of animals) among herds and areas should be taken into account. One option is to collect along line transects drawn across maps of the regions of the country where the breed is located. Adequate geographic spacing of the collection sites should help ensure that the level of genetic relationships among collected animals is low.
- Even when animals are taken from geographically distant locations, owners should be interviewed to determine how unrelated their animals are from the immediate surrounding population and from other more distant flocks or herds.

Reproductive aspects

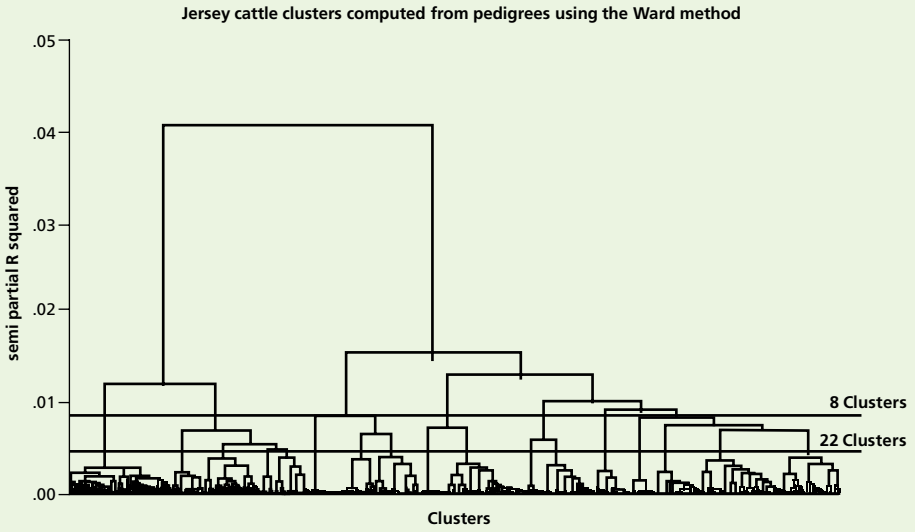
Only a small sample of animals can be represented in the gene bank, and with limited amounts of germplasm. Therefore, the gene bank manager must sample the animals that have the potential to yield the greatest number of offspring from the germplasm stored.

- Before the final decision about which animals to chose as donors, all candidates should be subject to a clinical and andrological or gynaecological evaluation. During this evaluation special attention should be given to animals that are selected as donors because of their genetic traits but which may be subfertile.
- When choosing female donors, animals with a good reproductive history should have a higher priority than animals that have a history of poor reproduction or have never given birth.
- When choosing male donors, priority should be given to animals that are known to produce semen that is of good quality after it is frozen and thawed.
- Both male and female donors must have morphological and behavioural characteristics that facilitate the collection of genetic material.

BOX 10

Selection of bulls for the National Animal Germplasm Program in the United States of America

Cluster analysis was used to evaluate the bulls of the Jersey breed stored in the United States of America's gene bank (Blackburn, 2009). Pedigrees of the 537 bulls in the gene bank were evaluated, along with pedigrees of the most popular bulls in 2004 and 2005. Genetic relationships among the bulls were used to assign them to clusters, which were visualized in a dendrogram or "tree" diagram (see the figure below).



(cont.)

Sanitary aspects

It is critically important that the conserved germplasm does not transmit pathogens into the future along with its genetic information. Thus, strict sanitary standards should be followed.

Donor animals should be clinically inspected to confirm that they are healthy and free from contagious and infectious diseases. They should fulfil all requirements established by OIE in terms of infectious and contagious diseases that may be transmitted through semen and/or embryos (see Section 9). To meet all the OIE standards, it may be necessary to quarantine animals to confirm their health status prior to collecting germplasm.

COLLECTION OF COMPLEMENTARY BIOLOGICAL MATERIAL

In addition to its role in promoting national food security, the gene bank has an opportunity and responsibility to collect germplasm, or tissue samples, for DNA analysis or other research purposes. It is advisable to undertake this type of activity at the same time as other germplasm collection activities. For example, it takes little additional effort to collect

The figure demonstrates how it is possible to define any given number of clusters (e.g. 8 or 22 clusters) by drawing a horizontal line across the dendrogram. The number of bulls in each cluster, the mean genetic relationship per cluster, and the number of bulls in the gene bank from each cluster were calculated for 22 clusters (see the table below). Several clusters were poorly represented in the repository (e.g. clusters 4, 15, 16 and 21) and efforts were therefore made to acquire samples to fill those gaps in the collection. A similar procedure could be followed in the initial selection of animals for a gene bank, by choosing similar numbers of animals from each cluster.

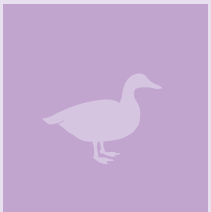
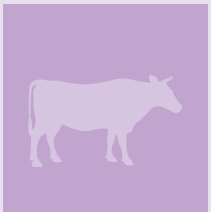
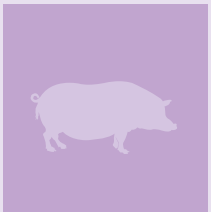
Cluster	n	Mean Rel.	No. in Rep.
1	350	.18	85
2	98	.20	30
3	451	.05	105
4	50	.23	2
5	115	.35	8
6	214	.21	38
7	193	.25	16
8	198	.11	13
9	342	.21	42
10	161	.20	37
11	116	.36	17
12	126	.22	36
13	142	.19	7
14	116	.33	15
15	62	.26	
16	104	.20	2
17	156	.26	35
18	105	.33	22
19	70	.30	18
20	86	.28	14
21	49	.22	2
22	46	.22	10

Note: n = number of bulls per cluster.
Mean Rel. = mean additive genetic relationship within the cluster.
No. in Rep. = number of bulls selected for the gene bank from each cluster.

blood samples for health tests and future DNA analysis at the time of germplasm collection. Such efforts will, in the long term, increase the utility of the collection maintained by the gene bank.

SECTION 7

Basic principles of cryopreservation

Basic principles of cryopreservation

Spermatozoa were the first mammalian cells to be cryopreserved successfully (Polge *et al.,* 1949). This success was due to the serendipitous discovery by Polge and co-workers of the cryoprotective effect of glycerol. Since then, many methods have been developed for various types of cells, tissues and organs. Much progress in the field has come from empirical work as well as from fundamental cryobiology. Increased understanding of the causes of cryo-injury has continually helped to improve cryopreservation methods. Research into fundamental cryobiology has provided the basis for new cryopreservation methods such as vitrification.

The two most commonly used cryopreservation methods for animal germplasm are slow-freezing and vitrification. These are quite different methods, but relate to the same physico-chemical relationships. The differences between the two can be explained by first describing what happens during slow freezing.

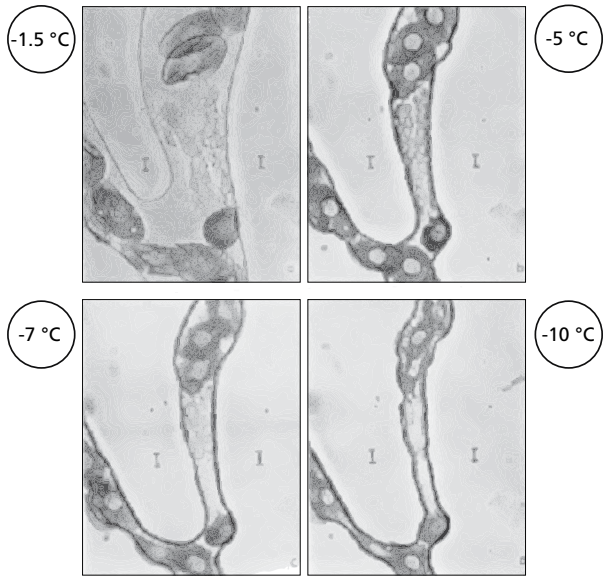
SLOW FREEZING

In slow-freezing, cells in a medium are cooled to below freezing point. At some stage, ice masses containing pure crystalline water will form. What remains between the growing ice masses is the so-called unfrozen fraction, in which all cells and all solutes are confined (see Figure 7). The concentrations of sugars, salts and cryoprotectant (e.g. glycerol) increase, while the volume of the unfrozen fraction decreases. The increase in osmotic strength causes an efflux of water from the cells. Slow cooling is needed in order to allow sufficient efflux of water to minimize the chance of intracellular ice formation. As cooling continues, the viscosity of the unfrozen fraction ultimately becomes too high for any further crystallization. The remaining unfrozen fraction turns into an amorphous solid that contains no ice crystals.

Chilling injury and cold shock

The first challenge in cryopreserving cells from homeotherm (warm-blooded) animals is in cooling the cells below body temperature. Cells may be damaged by very rapid cooling (cold shock) or be damaged by low temperature *per se* (chilling injury). Behaviour and function of membrane lipids and proteins may be affected by temperature. For example, membrane lipids that are normally in a liquid crystalline state may solidify at non-physiological temperatures, which can change their function and begin processes such as cryocapacitation of the production of reactive oxygen species that increase damage to membranes. Decreasing the temperature may cause an imbalance in cellular processes because the rate of one process may be affected more strongly than that of another. One example is the disintegration of the metaphase spindle of oocytes caused by a change in the dynamic equilibrium of the association/dissociation of the tubulin filaments.

FIGURE 7
Frog erythrocytes in the "unfrozen fraction", which is enclosed by growing masses of ice



Source: Rapatz and Luyet (1960).

Supercooling

In slow-freezing methods cells are brought into a suitable freezing medium and cooling is continued below the freezing point of the medium. Ice formation does not necessarily start at the freezing point. Small ice crystals have a lower melting/freezing point than "bulk" ice, due to their large surface tension. Spontaneous ice nucleation will in most cases occur after the solution is supercooled to a temperature between -5 and -15 °C. Thereafter, ice will grow rapidly in all directions, and the release of the latent heat of fusion will cause the sample to warm up abruptly until the freezing/melting temperature of the solution (i.e. of the remaining unfrozen fraction) is reached. At this point, the ice formation will stop, or will proceed at a rate governed by the rate at which the heat of fusion is transported from the sample. Finally, the sample can "catch up" again with the lower temperature in the freezing apparatus. From a practical perspective, this means that the cells undergoing cryopreservation in a typical semen straw have to withstand a series of large and abrupt temperature changes.

Conditions in the unfrozen fraction

Cells are faced with very high concentrations of solutes in the unfrozen fraction. Dehydration and high salt concentration may result in loss of stability in the membranes or denaturation of proteins (Tanford, 1980; Crowe and Crowe, 1984; Hvidt and Westh, 1992; Lovelock, 1953). Moreover, high salt concentrations may cause extracellular salts to enter the cells, a process known as "solute loading" (Daw *et al.,* 1973; Griffiths *et al.,* 1979).

The fast efflux of water causes a rapid decrease in the volume of the cells to approximately 50 percent of their original volume. This leads to structural deformation of the cells. Further mechanical stress may be caused by cells being confined in very narrow channels of unfrozen solution and squeezed between growing masses of ice (Rapatz and Luyet, 1960).

The influence of cryoprotectants

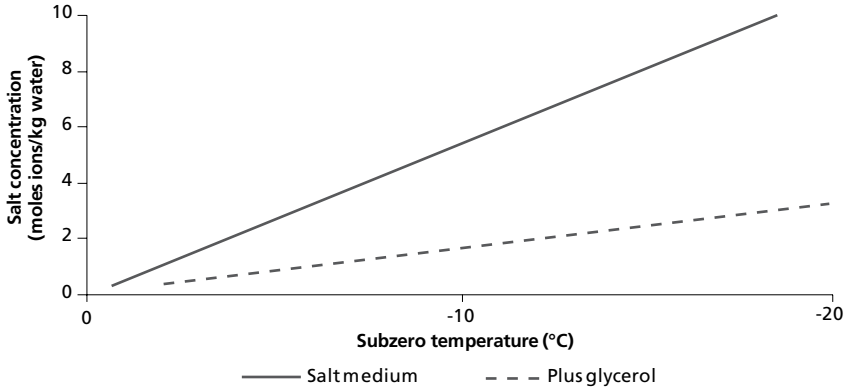
At all practical cooling rates, the total solute concentration (which is measured in moles per kg water) is determined only by the subzero temperature (Figure 8). When the initial freezing medium contains only salts (electrolytes), salt concentrations in the unfrozen fraction will reach extremely high levels as the temperature decreases. In contrast, in a medium that contains a large proportion of non-electrolytes, the total solute concentration at each subzero temperature will be the same as that found at the equivalent temperature in a medium containing only salts; however, the salt concentration will be much lower.

Sugars can be used as non-electrolyte solutes, but they will only affect the extracellular salt concentration. Moreover, high concentrations of impermeable solutes impose osmotic stress on the cells already before freezing. This is much less the case when a membrane permeable solute, such as glycerol, is used rather than a non-permeable solute. When cells are brought into a hypertonic glycerol medium, water will leave the cells because of the osmotic pressure difference. However, at the same time, glycerol will enter the cells. After a short period of equilibration, the cells will have regained their original volume. The osmotic stress imposed by a hypertonic glycerol solution is therefore much smaller than that imposed by a hypertonic sugar solution. Hence, glycerol can be used at greater concentrations than sugars without damaging the cells. A substantial initial glycerol concentration in the medium means that part of the extracellular and intracellular water is replaced by the glycerol. Hence, the amount of ice formed is lower, the unfrozen fraction remains larger, the degree of shrinkage of the cells is limited, and the electrolyte concentration in the unfrozen solution and in the cells will be relatively small (see Figure 8). The mechanisms through which other membrane permeable substances, such as ethylene glycol and dimethyl-sulfoxide (DMSO), provide cryoprotection are similar to those involving glycerol.

There are additional mechanisms through which polyols, such as like glycerol and several sugars, provide cryoprotection. These substances can stabilize lipid membranes by hydrogen bonding with the polar head groups of membrane lipids (Crowe and Crowe 1984; Crowe *et al.,* 1985), which is especially important under severely dehydrated conditions. In addition, these substances may affect the mechanical properties of the unfrozen fraction, especially its viscosity and glass-forming tendency.

The degree to which cells shrink and re-swell after addition of a membrane-permeable cryoprotectant depends on the concentration of the cryoprotectant and the relative permeability of the membrane to water and to the cryoprotectant (Kleinhans, 1998). For instance, bull sperm shrink very little when brought into a freezing medium with glycerol (Chaveiro *et al.,* 2006), whereas bovine embryos react much more strongly. Upon thawing, removal of the cryoprotectant has the opposite effect on cells: they first swell and then they shrink again. This may lead to damage if the cells expand too much. Damage due to over-swelling of cells can be prevented by stepwise removal of the cryoprotectant.

FIGURE 8
Effect of subzero temperature on salt concentration in the presence and absence of glycerol



Note: The total solute concentration (salts plus non-electrolytes) is a function of subzero temperature. Thus, the presence of non-electrolytes, such as glycerol, results in a lower salt concentration in the unfrozen fraction and inside the cells (Mazur and Rigopoulos 1983).

The influence of cooling rate

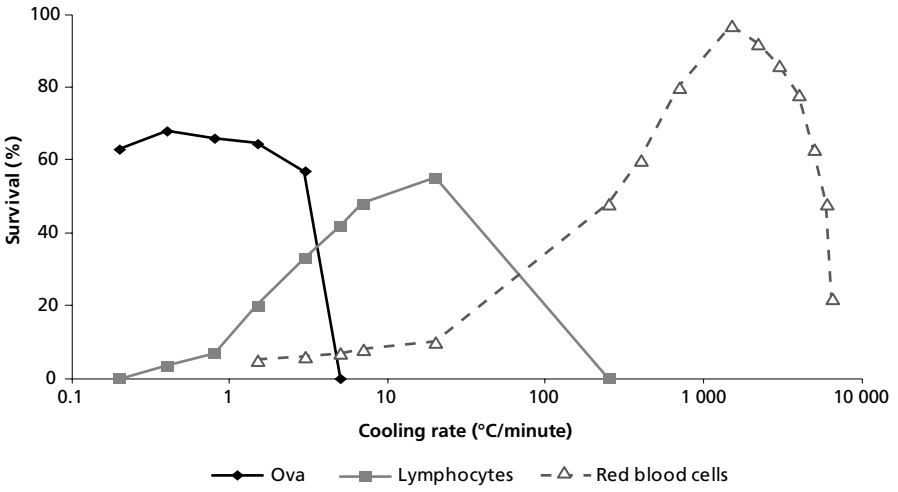
A general observation in the cryopreservation of cells and other biological systems is that each system has a specific optimal cooling rate, with decreased survival at cooling rates that are too low (slow-cooling damage) or too high (fast-cooling damage) (Mazur *et al.,* 1972).

Ice growth is a rapid process, but transport of water through the cell membrane is relatively slow, because the membrane acts as a resistance barrier. Therefore, as cooling and extracellular ice growth continue, the liquid water of the unfrozen fraction remains very close to equilibrium with the ice, but the intracellular water lags behind. This means that the water concentration (i.e. the chemical potential of water) is too high for thermodynamic equilibrium, and there may be a risk of intracellular ice formation.

The optimal cooling rate falls in a range that is neither too fast nor too slow. When cells are cooled very slowly, the intracellular water lags behind only a little, and the risk of intracellular ice formation is minimal. However, it also means that the dehydration of the cells is maximal, which is not desired. At higher cooling rates, intracellular dehydration, intracellular solute concentration and shrinkage of the cells is less excessive. Moreover, the cells are exposed to the unfavourable conditions for a shorter period of time. However, when cooling rates are increased too much, the dehydration may not be fast enough to prevent intracellular ice nucleation (Mazur, 1963, 1985; Mazur *et al.,* 1972). Fast-cooling damage can also be caused by other factors. For instance, it has been proposed that rapid water flow through membrane pores could lead to an uneven distribution of pressure on the membrane (Muldrew and McGann, 1993, 1994). Fast-cooling damage could also result from the very sudden changes in size, shape and ultrastructure, caused by the rapid efflux of water (Woelders *et al.,* 1997).

Different cells or other biological materials (embryos, tissue pieces) may have different optimal cooling rates. The optimal cooling rate of cells is largely determined by their volume

FIGURE 9
Effect of cooling rate on survival of different kinds of cells



Note: Cells may have a specific optimal cooling rate, i.e. they have a lower survival rate at cooling rates that are too low (slow-cooling damage) and at a cooling rates that are too high (fast-cooling damage) (Mazur, 1985).

and their membrane surface area (volume to surface area ratio), and by the permeability of the membrane to water and to cryoprotectant (see Figure 9).

Interactions of cooling rate with thawing rate and cryoprotectant concentration

The optimal cooling rate may depend on various other factors, such as the cryoprotectant concentration and the thawing rate. It has been observed in semen from a number of species that the combination of fast cooling and slow thawing is particularly damaging to the cells. (Rodriguez *et al.,* 1975; Fiser, 1991; Henry *et al.,* 1993; Woelders and Malva 1998). If intracellular ice nucleation occurs at a low temperature and cooling proceeds rapidly, it may be that the cytoplasm turns into glass before the intracellular ice crystals grow to a significant size, thus causing only sublethal, or no, damage. During slow thawing, the small crystals can grow and subsequently damage the cells (Rall *et al.,*1984). In addition, cells may be damaged by extracellular restructuring of ice masses, a process known as "recrystallization" (Bank, 1973).

Programmable and non-programmable freezers

Biological material can either be frozen using quite simple, non-programmable, freezers or using more sophisticated, programmable, freezers (see Figure 10). Although programmable freezers are more expensive, they do not necessarily yield more satisfactory results, especially for experienced technicians and cryobiologists. Therefore, the choice between

FIGURE 10
Example of a programmable freezer

PHOTO CREDIT: IMV TECHNOLOGIES, L'AIGLE, FRANCE

Note: the freezer is about 1 metre in height.

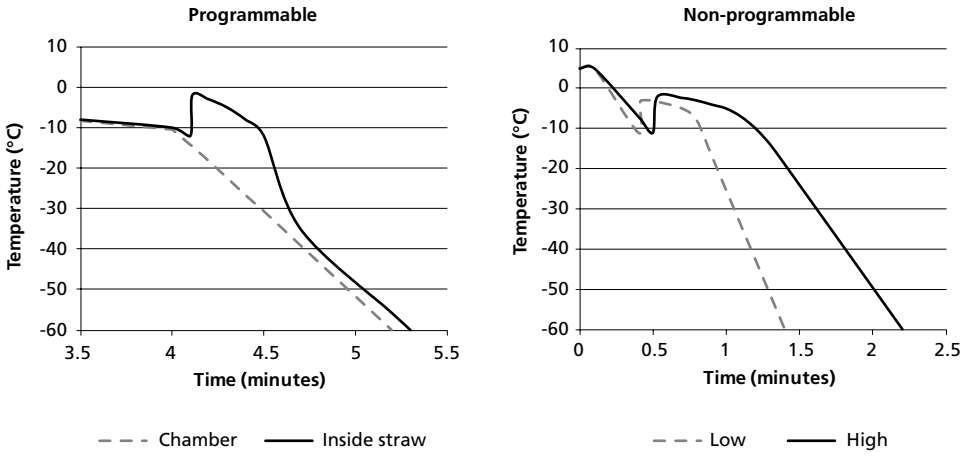
programmable and non-programmable systems will depend on the financial resources available and the experience of the technicians. In some cases, even the most experienced technicians prefer the operating simplicity of programmable models.

In most programmable freezers, the straws or vials are cooled by cold nitrogen vapour. The temperature inside the cooling chamber can be accurately controlled and the time course of the temperature can be programmed. However, the time course of temperature inside the straws may be different due to the generation of heat of fusion (Figure 11).

In non-programmable freezers, the straws may be cooled by being exposed to vapour (or a cold surface) at a constant low temperature. An example of a simple system is the freezing of straws placed on a rack in a Styrofoam box partially filled with liquid nitrogen without ventilation. The height of the straws above the liquid nitrogen determines the rate of heat exchange. Alternatively, straws can be placed on a piece of Styrofoam that floats on the liquid nitrogen (e.g. Dong *et al.,* 2009). The thickness of the Styrofoam piece determines the rate of heat exchange.

Generally in such systems, the rate of heat exchange is governed by the temperature difference between the inside and the outside of the straw, and by the extent of heat conduction. The latter depends strongly on the volume to surface ratio of the straw or vial and the rate of (forced) ventilation. Therefore, it is difficult to compare one type of non-programmable freezer with another, or to know the actual freezing rate obtained with any given non-programmable apparatus. Optimal conditions have to be determined by experimentation.

FIGURE 11
Examples of freezing curves in programmable and non-programmable freezers



Left panel: A typical freezing programme in a programmable freezer (dotted line) and the corresponding freezing curve measured inside the straw. Note that the temperature inside the straw doesn't necessarily follow the programmed chamber temperature and that in parts of the curve the cooling rate may be higher than anticipated.
Right panel: In a non-programmable freezer (Styrofoam box), the constant temperature outside the straw results in sigmoidal freezing curves. The overall steepness of the freezing curve can be changed by choosing a different vapour temperature, for example, by changing the height of the straws above the liquid nitrogen (Woelders and Zuidberg, personal communication).

Non-programmable systems do, however, have an advantage. The cooling curve (the time course of cooling and freezing) is, by default, of the form theoretically predicted to be optimal for slow freezing (Woelders and Chaveiro, 2004), with relatively low cooling rates directly after ice formation begins and higher cooling rates later. The bulk of the ice formation happens in the temperature range between the freezing point and -10 °C, and consequently most of the water efflux from the cells must also take place in this temperature range. Thus, the heat of fusion liberated during ice formation slows the cooling exactly at the point when cells need extra time to export intracellular water. The overall steepness of the freezing curve can be adjusted in non-programmable systems by choosing the height of the straws above the liquid nitrogen, which is proportional to the temperature of the vapour around the straws (Figure 11). In more sophisticated systems with forced ventilation and adjustable preset vapour temperatures, the rate of heat exchange can be adjusted by choosing the preset vapour temperature.

VITRIFICATION

The term "vitrification" refers to any process resulting in "glass formation", the transformation from a liquid to a solid in the absence of crystallization. According to this definition, cells that are properly slow frozen become "vitrified".

If, in slow-cooling methods, cells ultimately become vitrified, how do so-called vitrification methods differ? Vitrification methods involve the use of a medium that has a very high solute concentration to begin with. Thus, ice cannot form in any part of the sample. As no ice forms, cooling does not have to be slow. In fact, it may be beneficial to cool very rapidly. The vitrified state and the associated physico-chemical conditions obtained using vitrification methods, are to some extent similar to those obtained by slow cooling, but the way of reaching this point is quite different.

Chilling injury and cold shock

As in the case of slow-freezing methods, vitrification methods can damage cells or tissues through cold shock and chilling injury. Depending on the material and the protocol used, however, cells or tissues may be rapidly cooled from a temperature at which chilling injury and cold shock play no role (e.g. room temperature). Extremely high rates of cooling from such a temperature to the vitrified state seem to be able to "outrun" cold shock and chilling injury. For example, rapid cooling seems to prevent disintegration of the metaphase spindle of oocytes.

Cryoprotective agents

In vitrification methods, cells or tissues are brought into a medium that has a very high concentration of cryoprotective agents, also known as cryoprotectants. If the concentration of solutes is high enough, vitrification solutions will solidify to a glass without any risk of intracellular or extracellular ice formation during cooling or warming, independently of the cooling and warming rates used. However, the very high concentrations of cryoprotective agent needed for vitrification may cause damage due to abrupt osmotic changes, extremely low water potential or chemical toxicity. According to the description provided by Rall (1987), the embryos are first equilibrated with 25 percent vitrification solution at room temperature. Then the embryos are cooled to 4 °C and transferred to 50 percent vitrification solution and then to 100 percent vitrification solution. They are then rapidly packed and transferred into liquid nitrogen. The stepwise increase of cryoprotective agent concentration reduces osmotic effects, while the low temperature and rapid transfer help prevent damage by chemical toxicity. In addition, chemical toxicity may be reduced by using mixtures of various permeant CPAs, or addition of non-permeant CPAs (60 g/litre polyethylene glycol) (Rall, 1987) or 60 g/litre bovine serum albumin (BSA) (van Wagtendonk-de Leeuw *et al.,* 1997).

Reduction of cryoprotective agent concentration at high cooling rates

Solutions that have a solute concentration lower than that of classical vitrification solutions have freezing points below which there is a significant tendency to form ice crystals. But when the solution is cooled very rapidly, there is simply no time for ice formation. Below a certain temperature, the solution becomes so viscous and stiff that ice formation becomes impossible, and the solution turns into "metastable" glass. The solute concentration needed for metastable vitrification decreases as a function of increasing cooling rate. The most recent vitrification procedures, therefore, make use of high cooling rates in order to reduce the concentration of CPAs and thereby decrease the damage caused by osmotic stress and chemical toxicity.

The cooling rate can be increased in several ways. One is to reduce the volume of the sample to be vitrified. An early example of this approach is the open pulled straw method (often abbreviated OPS) (Vajta *et al.,* 1998, 2000a,b). Even smaller sample volumes have been used on electron microscope grids, so-called hemi-straws, nylon loops (cryoloops) or polypropylene strips (Cryotop® – Kitazato Supply Co., Fujinomiya, Japan) (Kuwayama, 2007). The Cryotop system allows a volume of 0.1 µl to be vitrified.

In addition to reducing the sample volume, a faster cooling rate can be achieved by heat transfer to a liquid that does not boil. Liquid nitrogen at its boiling point (-196 °C) will generate nitrogen gas when it absorbs heat. This will create a film of gas that insulates the sample from the liquid nitrogen. Liquid nitrogen at its freezing point (also known as "nitrogen slush") doesn't have this disadvantage. It can be produced with an apparatus called Vit Master® (IMT Ltd, Ness Ziona, Israel) (Arav *et al.,* 2002).

In metastable vitrification procedures, it is also essential that the warming (i.e. thawing) of the sample is very rapid. If warming is slow, ice crystals can form while the temperature is between the vitrification temperature and the freezing point of the vitrification solution.

Most recent vitrification protocols make use of these ultra-rapid approaches in order to reduce cryoprotective agent concentrations and prevent cold shock and chilling injury. Current vitrification solutions (Liu *et al.,* 2008; Morató *et al.,* 2008) have much lower solute concentrations than those used in classical vitrification solutions (e.g. VS3, Rall 1987). As described in Section 4, very good results are currently obtained when using these approaches for vitrification of oocytes and embryos. Recent studies with pig and cattle oocytes have indicated that the Cryotop system gives better results than the open pulled straw system (Liu *et al.,* 2008; Morató *et al.,* 2008).

FREEZE DRYING

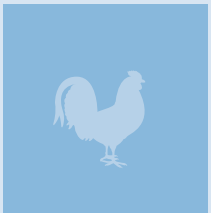
Storage of freeze-dried biological material is extremely cost efficient, as no expensive and bulky liquid nitrogen containers are necessary. Furthermore, it is safe. The material may be stored at ambient temperature and, unlike cryogenic storage, there is no risk of equipment malfunction or of personal injury from liquid nitrogen. On the negative side, however, freeze drying generally reduces cell viability. Therefore, standard insemination procedures generally cannot be used for freeze-dried sperm. However, freeze-dried sperm have been successfully used to produce live offspring using ICSI in mice and rabbits (Wakayama and Yanagimachi 1998; Liu *et al.,* 2004). In addition, freeze-dried somatic cells have been successfully used to produce apparently healthy embryos using SCNT (Loi *et al.,* 2008a, 2008b). However, there have so far been no reports of cloned offspring produced by SCNT using freeze-dried somatic cells. Thus, while freeze drying is potentially useful for gene banking of genetic resources with the objective of regenerating live animals and recovering lost breeds, this would require further development and optimization of procedures. Conversely, freeze-dried gametes and somatic cells can already be used for conservation of germplasm intended for use in (genetic) research.

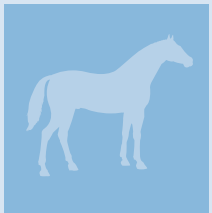
The key to freeze-drying is to bring the material to a vitrified glass state in which the glass transition temperature is higher than ambient temperature. The first step is to bring the biological material to a vitrified state. The next step is to apply a vacuum to the material,

which results in sublimation of any ice that may be present and further decreases the water content of the vitrified material. This increases the glass transition temperature, which ultimately reaches a level higher than the ambient temperature. Thus, at the end of the process the material can be stored at ambient temperature while remaining in the stable glass state. Obviously, the initial freezing/vitrification procedure, and the medium used, should be optimized so as to ensure the survival of the germplasm throughout this phase. In addition, the medium composition must be optimized so as to prevent the cells from being damaged by the effects of the further dehydration of the material.

SECTION 8

Collection of germplasm and tissues





Collection of germplasm and tissues

Whether germplasm is collected on farm or at collection facilities will depend on the conditions within country, the availability of resources and the accessibility of the targeted animal populations. Collection and processing procedures will differ widely depending upon the type of germplasm being collected and the donor species. This section presents an overview of collection procedures for the various types of germplasm, including information particular to each of the major livestock species. Further details regarding germplasm processing and cryopreservation are presented in the appendices.

SEMEN

Bull, ram, buck goat, buck rabbit and stallion semen is typically collected using an artificial vagina (AV), whereas boar semen is usually collected by using the hand-glove method. These methods are usually performed on animals that have been trained for semen collection and are thus habituated to contact and interaction with humans and the collection equipment. Electro-ejaculation may be considered for animals that have not been properly trained, although it should be avoided in the case of boars and stallions. If a male has died before sufficient semen has been collected or if a male can be sacrificed because its genetic material is considered more valuable than the live animal, epididymal semen can be collected directly from the testes. In poultry, the abdominal stroking technique can be used for semen collection. For a scientific review of semen collection methods for various mammals, see Watson (1978). For the basic supplies and equipment needed for semen collection and freezing, see Appendix B.

Collection with an artificial vagina

Different types and sizes of AV are available for use in different species and breeds within species (bull, ram, buck goat, stallion and buck rabbit). Before collection, the AV should be prepared with a large-enough volume of warm water to ensure sufficient physical pressure to stimulate the glans penis of the male. The inner wall of the AV should be between 42 and 48 °C, depending on the body temperature of the animal, and should remain within the same temperature range throughout the semen collection process. The collection liner of the AV should then be lubricated with a non-spermicidal sterile gynaecological lubricant.

The use of a "teaser" animal of the same species is recommended. The teaser animal facilitates collection by allowing the donor male to mount and ejaculate in a way similar to natural mating. A live teaser may also be used to increase arousal, by allowing the donor to follow the teaser while it is led around the collection area immediately prior to collection.

For stallions, collection via AV is often performed by using a "phantom mare" rather than a live teaser animal. A phantom mare is an object constructed to mimic the size and shape of a real horse. For rabbits, the teaser is typically a female and the AV is affixed at the vulva.

Once the teaser is readied, the donor male is allowed to mount one to two times for false collections. The false mounting procedure will allow the male to become aroused and increase the final volume of the ejaculate. During these false collections the penis of the male is diverted to protect the teaser animal and does not enter the AV.

Once aroused, the male is allowed to mount and the penis is guided so that it enters the AV. The AV should be held at a height and angle that allow for easy and comfortable entry by the penis of the donor male. The male is allowed to thrust and remain on the teaser until ejaculation is complete. The AV is then taken into the laboratory and the semen is processed for insemination or cryopreservation.

Safety of the personnel, donor and teaser animals should be a primary concern during semen collection with an AV, especially in the case of large species. The teaser animal should be securely restrained. The collection area must provide secure footing for both the semen donor and the teaser. For stallions in particular, protective head gear, such as a helmet, should be worn by all persons handling the animals prior to, during and following collection.

Electro-ejaculation

In general, the AV method is preferred for semen collection, as it tends to yield the highest-quality semen and cause the least stress in the animal. However, in some situations where the donor male cannot be trained for conventional collection, such as at remote sites in the field, collection via electro-ejaculation is the most practical option (bull, ram, buck goat, not stallions).

Prior to the electro-ejaculation procedure, the collection tubes (or cones) will need to be prepared. In the case of bulls, the conical glass tubes need to be insulated using a 37 °C water jacket. Ram- and buck-semen collection tubes can be handled similarly or simply kept insulated by the hand of the collecting technician.

The probes of electro-ejaculators differ in size according to species, as do the specific collection methods. Operating instructions differ according to the manufacturer, and must therefore be carefully followed. Training and experience are needed in order to be proficient in semen collection using this methodology.

For cattle, electro-ejaculation is performed with the bull restrained in a standing position. The probe is lubricated and inserted into the rectum with the metal electrodes facing ventrally (downward). The electro-ejaculator is turned on and the voltage increased (manually or automatically) in small increments until the bull maintains an erection. The oscillating voltage peaks are then continued until semen is ejaculated and collected in a clean vessel.

In the case of bucks and rams, the animal is placed on his side and the penis is extended from the sheath by stretching the sigmoid flexure. The penis is grasped with sterile gauze and the glans penis (with its urethral process) is diverted into a 50 ml disposable tube. The lubricated electro-ejaculator is then inserted into the rectum of the animal and used to gently massage the accessory glands by exerting a downward pressure on the bottom of the rectum. This pressure should be applied for 10 to 15 seconds prior to turning on the

electro-ejaculator. After massaging, the electro-ejaculator is turned on for three to eight seconds and then the animal is allowed to rest for 15 to 20 seconds. Massaging the accessory glands in between stimulation will cause the male to ejaculate. Stimulation is usually not performed more than three times during the collection process and at least one hour is allowed between collections. After ejaculation, the semen is covered to maintain its temperature and taken to the laboratory for processing.

Gloved-hand collection technique

For collection of pig semen, the boar is first allowed to mount a teaser animal or mounting dummy. The penis needs to be fully extended prior to semen collection. The protruding penis is then grasped so that the ridges of the penis are between the collector's fingers and pressure can be applied to the glans penis with the smallest finger of the collector's hand. After the initial fractions of the semen are ejaculated, the sperm-rich portion (which has a milky appearance) should be collected into a 37 °C insulated container covered with two layers of sterile gauze to remove the gel fraction. The remaining fraction is then ready for further processing. For an overview of methods for evaluating the quality of boar semen, see Woelders (1991) or Colembrander *et al.* (2000).

Abdominal stroking

In poultry, semen collection is performed by the abdominal massage method described by Burrows and Quinn (1935). This procedure works best when done by two persons working together. One person carefully restrains the bird between his or her arms and body, while the second person collects the semen. This person massages the abdomen of the bird with firm rapid strokes from behind the wings towards the tail. The animal's readiness to ejaculate is indicated by the tumescence (erection) of the phallus. At this point, the handler gently squeezes the cloaca with two or three fingers, expressing semen through the external papillae of the ducti deferentis and into a pre-warmed tube held underneath the cloaca. The person collecting the semen must be careful, because the close proximity of the penis and cloaca increases the likelihood of the semen being contaminated with faeces, urates and bacteria, which have a detrimental effect on semen quality.

Semen can be collected from mature males twice or three times per week. Either a graduated glass funnel-shaped tube or a standard graduated glass tube can be used. Preferably, the semen extender (e.g. Lake extender – Lake, 1960) and the collection tubes should be placed in a 25 °C incubator for processing.

Epididymal sperm collection

Over the years, attempts have been made to harvest epididymal sperm from live intact males (mammalian species) either by catheterization or by flushing the lumen of the cauda (tail) of the epididymis with a hypodermic needle and a plastic syringe. Of these two approaches, catheterization of the cauda epididymis in the mature male is reported to be the most successful. In most males, the catheterization procedure is successful, but frequent post-surgical problems with the indwelling catheter have meant that this approach has remained relatively unpopular.

The most often-used approach to harvesting epididymal sperm is to surgically remove the testes from the male before, or shortly after, death. With this approach, the excised testes are placed in a sterile resealable (zip-lock) plastic bag, then into an empty Styrofoam cooler to maintain them at body temperature, and then transported to the laboratory for processing. For longer-distance transport, the testes can be chilled. The temperature during transport is usually adjusted by placing ice or ice packs in the bottom of the cooler. However, the temperature of livestock testes upon arrival at the laboratory (i.e. before processing) should not be below 5 °C.

The most common method of harvesting sperm is by slicing (with a scalpel) across the cauda portion of excised epididymis that has been placed into a sterile Petri dish containing a sperm medium (Guerrero *et al.,* 2008, 2009). A second approach is to make two incisions in the cauda and use a 30 ml syringe attached to a plastic tube to flush the sperm from the lumen into a dish with a retrograde flow of sperm medium (Saenz *et al.,* 2008). The cauda epididymis of goats can be sliced and suspended in Salomon's one-step freezing medium at a one to four dilution rate. After two filtrations (75 and 50 µm) through a nylon grid at room temperature, sperm suspension is free from any somatic cells and ready for further processing (Ehling *et al*., 2006). In some species (e.g. dogs) it is possible to collect viable sperm from intact testes that have been frozen (-20 °C) in a plastic zip-lock bag shortly after collection and then thawed (Graff *et al.,* 2000).

Shipping and processing of collected semen

Following collection, the samples can be immediately cryopreserved or transported to a laboratory (up to 24 hours holding time) for cryopreservation. The protocols for processing are species-specific, and, thus, specific media are required. Detailed information on processing and freezing samples is presented (by species) in Appendix C. Evaluation of semen is an essential aspect of processing. Proper evaluation prevents the freezing of semen that is not viable, and allows possible problems with semen donors to be identified quickly. General guidelines for semen evaluation (valid for all livestock species) are presented in Appendix D.

When semen is collected on farm or in the field, the samples must be maintained in a temperature-controlled environment during transport to the processing centre. A shipping container should, therefore, be prepared immediately before the collection of the semen. A standard shipping container has both an inner and an outer Styrofoam box. The outer box will hold numerous sealed reusable frozen ice packs as well as the inner Styrofoam box. The ice packs are placed in the outer box prior to the collection of the semen samples. After the samples are collected, the ice packs are placed into the inner box. For bulls, rams and buck goats, the number of ice packs must be sufficient to cool the samples to 5 °C. For stallions and boars, the samples need to be cooled to 15 °C. The number of ice packs needed in order to reach these temperatures has to be determined on a case-by-case basis, as different types of commercial ice pack have different cooling capacities because of their differing sizes and volumes.

EMBRYOS

Production, collection, processing and freezing of embryos are more demanding than the equivalent procedures for semen, and a greater level of training and experience is required.

The following subsections address major issues in the cryoconservation of embryos. FAO has previously produced manuals on ET in several species including cattle (FAO, 1991a), buffalo (FAO, 1991b) and sheep and goats (FAO, 1993). In addition, commercial manuals are available for purchase, for example from the International Embryo Transfer Society[5].

Superovulation of donor females

Ideally, to maximize N_e in a reconstituted breed, each female embryo donor will produce at least one male and one female offspring. To reach this goal, each female will obviously need to produce multiple embryos. To increase the number of embryos per collection, donor females are administered various hormone agents (gonadotropin-like) to stimulate the ovaries to produce multiple ova for fertilization and embryos for collection. Early reports in cattle, sheep, goats and pigs described the use of pregnant mare serum gonadotropin (often abbreviated PMSG) at various dose levels to superovulate donor females. Pregnant mare serum gonadotrophin is extracted from the serum of pregnant mares after 40 days of gestation. A single intramuscular injection on pregnant mare serum gonadotrophin has potent follicle stimulatory activity. For information on the use of this agent in donor cattle, see reviews by Elsden *et al.* (1978) and Saumande *et al.* (1978). This agent (now termed equine chorionic gonadotropin or eCG) is still the agent of choice in pigs and often in sheep and goats. However, equine chorionic gonadotropin has a long half life, and often over-stimulates the ovaries of donor cattle. Therefore, equine chorionic gonadotropin is no longer the agent of choice for cattle in North America, although it is still used in countries where other gonadotropic agents are not commercially available.

Today, follicle stimulating hormone (FSH) has become the agent of choice for superovulating donor cattle and is also used in sheep and goats. FSH has a much shorter half life in the circulation and is, therefore, usually administered by twice-daily injections for three to five days (see Monniaux *et al.,* 1983; Armstrong, 1993; Mapletoft *et al.,* 2002). However, success using once-daily injections in cattle has also been reported (e.g. Looney *et al.,* 1981; Bo *et al.,* 1994). *Bos indicus* cattle appear to be more sensitive than *Bos taurus* cattle to FSH. Various modifications to techniques for superovulating *Bos indicus* cattle have been developed and are now in use (see Baruselli *et al.,* 2006, 2008; Bo *et al.,* 2008).

Information on various superovulation procedures for cattle and buffalo is presented in FAO training manuals (FAO, 1991a, b). Some of the more commonly used superovulation schemes for cattle donors today are presented in Appendix E of these guidelines. Current recommendations for optimum fertilization and successful embryo transfer are that one or two inseminations per donor cow with one or two units of good-quality semen per insemination are needed (see Schiewe *et al.,* 1987).

It is very important to select the appropriate number of embryo-donor females to match each sire in the breeding schedule and thereby to improve rates of genetic variability in the cryoconservation programme (e.g. Woolliams, 1989). The data presented in Table 3 (Section 4) give an indication of the number of transferable embryos that can be obtained after a single superovulation treatment and embryo recovery, as well as estimates of the number

[5] http://www.iets.org

of embryos obtainable from one donor female during one year. However, the figures vary quite considerably from animal to animal. Some females simply do not respond to the stimulatory agents, or stop responding. In addition, they may develop physiological conditions that make it difficult to retrieve the embryos. Thus, although 25 donor females and 25 donor males is the recommended minimum, a larger number of candidate females may be needed, because of the likely failure to obtain embryos from some donors.

The expected rates of success in both collection and transfer must be considered when determining the number of embryos to collect and store. Experienced ET professionals can be expected to achieve cattle embryo recovery rates greater than 75 percent, with four to eight good-quality bovine embryos per donor collection. Using good-quality embryos for transfer, 65 to 80 percent pregnancy rates can now be expected in well-managed cattle operations. Expected pregnancy rates from ET in a variety of livestock species are presented in Table 3.

Stages of embryo development

Embryos develop through various morphological stages after *in vivo* fertilization. As the embryos divide, the number of embryonic cells (blastomeres) per embryo increases as they migrate through the reproductive tract of the female (Table 13). It is important to know when the embryos can be expected to be in the uterus of the superovulated female, so that the embryos can be obtained from the uterine horns through non-surgical recovery.

An embryo technician must be able to recognize not only the stage of embryo morphological development, but also to assess embryo quality before selecting and transplanting embryos. The ability to make this judgment can be developed only with experience gained in assessing and grading embryos in the laboratory. For reviews on assessing embryo quality and classifying embryos, see the classic training publications by Lindner and Wright (1983) and Robertson and Nelson (1998).

TABLE 13
Location of the embryo following oestrus

Number of days from standing oestrus	Morphological stage	Location
1	Fertilized ovum	Oviduct
2–5	2- to 16-cell stage	Oviduct
3–4	Early morula	Oviduct
4–5	Compact morula	Distal uterine horn
5–6	Early blastocyst	Distal uterine horn
6–7	Blastocyst	Distal uterine horn
7–8	Expanded blastocyst	Distal uterine horn
8–9	Expanding hatched blastocyst	Distal uterine horn
9–10	Hatched blastocyst	Uterine horn*

*After hatching the embryo begins to migrate towards the middle portion of the uterine horn.

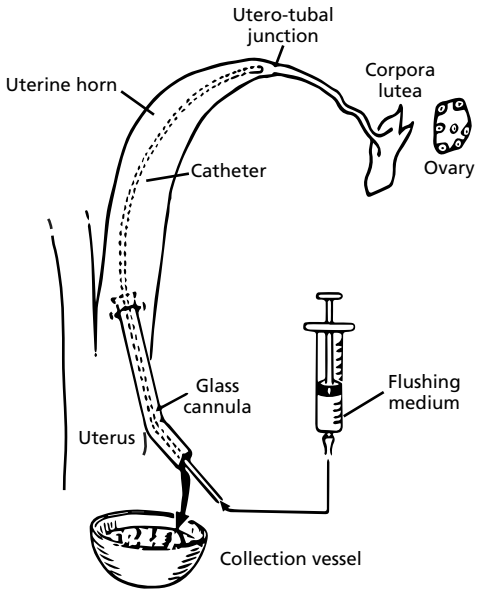
Embryo collection

Livestock embryos are collected from donor females by flushing the reproductive tract using a physiological flushing medium. The most often-used flushing medium for cattle is phosphate-buffered saline (PBS), which can be obtained by mixing commercially available dry packets with water or purchased as a ready-prepared solution. Various other media are also commercially available. In some species (e.g. cattle, horses and buffaloes) harvesting donor embryos is most often done using a non-surgical standing method, but in other species (e.g. pigs, sheep and goats) a surgical approach is usually required (see review by Betteridge, 1977).

Surgical embryo collection. Today, surgical embryo collections in pigs, sheep and goats are usually done at commercial ET units. Information on surgical procedures available for sheep and goats is provided in FAO (1993). In addition, see Kraemer (1989) or Baldassarre and Karatzas (2004). Over the years, research reports have described various non-surgical approaches to embryo collection and transfer in these species (see reviews by Foote and Onuma, 1970; Betteridge, 1977). However, in most cases, the number of embryos recovered per collection and the pregnancy rates per embryo transferred are lower than those achieved using the standard surgical approaches.

Figure 12 is a simple diagram of the surgical collection procedure for sheep and goats. The procedure is typically performed laparoscopically via the insertion of a glass cannula and catheter into the uterus. The uterus is flushed by injecting an appropriate medium using a syringe

FIGURE 12
Commonly used approach for collecting embryos (morulae and blastocysts) from sheep and goats



Drawing courtesy of R. A. Godke.

attached to the catheter. The injected liquid causes the embryos to flow out of the uterus into the collection vessel. The embryos can then be identified, processed and cryopreserved.

Embryo collection in pigs is also usually done surgically. Given that pigs naturally produce multiple offspring, superovulation is usually not practised, as embryo viability may be reduced. Collection is performed five to eight days after insemination, via laparotomy at a mid-ventral position. The animal is maintained under general anaesthesia while the procedure is performed. At this point, embryos will be in the blastocyst stage. Collection can be done earlier, at the four- to eight-cell stage, but in this case the embryos will require further culture before transfer and additional manipulation (e.g. delipidation).

Non-surgical embryo collection in cattle. Today, virtually all cattle embryos collected in the field and in-clinic by commercial ET companies are collected by a simple, non-invasive non-surgical procedure (see FAO, 1991a). Non-surgical embryo collection and transfer pose little risk to the cow, and greatly reduce the time needed for harvesting embryos. The drawback of non-surgical embryo collection is that embryo recovery rates may be a little lower if collection is done by a less-experienced technician.

There are two basic approaches to non-surgical recovery of embryos from cattle (see review by Chapman and Godke, 2004).The body of the uterus and uterine horns can be flushed simultaneously using a single flushing procedure, often referred to as "uterine body flushing" or "body flushing". Alternatively, each uterine horn can be flushed separately using two flushing procedures, a process known as "uterine horn flushing" or "horn flushing". Flushing with either of these approaches usually recovers 50 to 90 percent of available ova/embryos, depending on the experience of the technician. The potential number of embryos available for collection can be determined through palpation or ultrasonic examination based on the number of corpora lutea present on the ovaries of the donor animal. However, rectal palpation of donors with a large number of ovulations yields a rather imprecise estimate. It is therefore recommended that, if possible, ultrasonography be used to evaluate the ovaries of the donor prior to the embryo collection procedure. A list of equipment used in non-surgical embryo collection and transfer procedures in cattle is provided in Appendix F.

When using the body flush procedure, a Foley catheter is inserted through the cervix and into the uterine body. The cuff is then inflated and pulled back against the internal os of the cervix. The catheter is connected to a "Y" connector that allows medium to flow through the catheter, out of the cow and into an embryo filter apparatus where the embryos are captured. During flushing, the uterus and the horns are allowed to fill with medium until turgid. They are then manually massaged, and embryos are recovered as the uterus is drained. This filling and draining process is repeated until the volume of flushing medium fluid allotted to the female is depleted. The body flushing procedure uses about 1 000 ml of flushing medium for each donor animal

With independent horn flushing, the catheter is passed through the cervix and into the uterine horn. The tip of the catheter should be placed anterior to the external bifurcation of the uterus, half to three-quarters of the distance through the lumen towards the distal tip of the uterine horn. The cuff of the catheter is then inflated and 750 ml of flushing medium is allowed to flow into the horn. Manual manipulation is then used to recover the embryos

and the medium from the horn. When one horn has been flushed, the cuff is deflated and the catheter removed. It is then placed into the contralateral horn and the same flushing procedure is repeated. This approach uses about 1 500 ml of medium per donor animal.

Hay *et al.* (1990) conducted a comparative study of body and horn flushing for the recovery of embryos. On average, a greater number of embryos were obtained through horn flushing, but the difference was not significant. Given that the difference between the two approaches was not significant, the conclusion was that the technician should choose which approach to use based on his or her preference and proficiency. A modified approach to uterine flushing in cattle – termed the "shallow uterine horn flushing technique" – has recently been reported successful in dairy heifers (Sartori *et al.,* 2003).

The potential advantages of a single-embryo collection procedure (i.e. without superovulation) for on-farm use in cattle should not be overlooked. With such an approach, the donor female can remain on the farm, thus reducing the risk of disease transmission. Moreover, no ovarian stimulating agents are needed. The approach can be expected to cause less stress in the donor animals and, therefore, allow them to maintain a constant level of productivity. The collection method is the same as that for superovulated donor females. However, there is usually less uterine endometrial swelling (caused by the hormone stimulating agents), and this means that a less-experienced technician has a better chance of harvesting the seven or eight day-old embryo. A single-embryo approach may be particularly useful when reconstituting populations using a combination of stored semen and embryos or as part of *in vivo* conservation programmes. In such circumstances, large numbers of offspring per living female are not necessarily desired, as they would increase the genetic relationships among the animals in the live population. Sexed semen can be used to increase the probability of obtaining offspring of the desired sex.

Many factors can adversely affect recovery rates, including poor nutritional status of the donor, improper (over- or under-) hormonal stimulation of the donor, failure of the fimbria of the oviduct to pick up the ova, use of poor-quality semen to inseminate the donor cow, failure of embryos to enter the uterus after fertilization, and failure to collect the embryos during the flushing procedure. Many of these factors may be associated with inexperience on the part of the technicians.

Non-surgical embryo collection in horses. Successful production of live offspring following ET in horses was first reported in the early 1970s in the United Kingdom and Japan. The non-surgical embryo collection and transfer procedures used today in the mare are easier to perform than those used in the cow. The basic non-surgical collection and transfer procedures used in the mare were reported by Colorado State University (Imel *et al.,* 1981). Several modifications have subsequently been introduced to improve the procedure (see Wilcher and Allen, 2004). The latter-stage horse embryo is large enough to see even without a microscope.

In some countries, embryo collection and transfer technologies have been held back by the rules and regulations of various breed associations, whereas in other countries the use of these technologies is increasing rapidly. The use of ET in horses has become particularly common in Brazil.

Although the mare can be given hormones to superovulate her ovaries, donor mares tend to produce fewer oocytes for fertilization (range of two to four) post-treatment than donor cows. Usually, no more than one embryo is produced from a donor mare per cycle for potential embryo collection. To obtain early-stage embryos (single ovulated, < 300 µm in diameter) for cryobanking would require more than 130 mare cycles to harvest 100 embryos. By superstimulating the mares the number of cycles needed to produce the same number of embryos can probably be reduced to some extent.

General recommendations on embryo collection. To maximize efficiency, the collection, processing and storage of embryos must be carried out by trained professionals. In fact, many countries will have specific regulations on who can perform embryo collection. Technicians will need to undergo special training on sanitation and specific techniques.

Donor animals, if possible, should be subject to quarantine and/or health testing prior to collection. At the time of collection, the donor animals must also be kept as clean as possible. Body parts that will be accessed and manipulated during the procedure (e.g. tail and vulval area) should be washed and dried. Before surgical collection, hair should be clipped from incision sites and the area must be washed, rinsed and disinfected. Animals must be well restrained and treated in a manner that avoids stress and does not compromise their welfare.

The embryo-collection team (usually two or three technical people) needs to have access either to well-maintained, clean and sanitary permanent facilities, or to a mobile laboratory, where embryos can be collected, evaluated, processed and packed. The processing laboratory needs to be clean and equipped with an appropriate working space, electricity, temperature-controlled incubator(s), microscope(s) and other technical equipment and supplies (see Appendix F). Small equipment must be sterilized between collections and single-use disposable materials are recommended for sanitation purposes, when possible.

The direct disease risk associated with embryos, depends very much on the handling of the embryos by the collection team. This places great responsibility on the team, who must be competent in collecting, processing and storing embryos according to the relevant defined protocols. Given this major responsibility, and to ensure that the work is always done to high standards, it is recommended that a procedure for approving and officially recognizing members of embryo collection teams be introduced.

The potential health risk can be large if the recommended procedures regarding collection and handling are not followed precisely. It is very important to review the International Embryo Transfer Society recommendations for the sanitary handling of *in vivo*-produced embryos before beginning embryo collection (see Stringfellow, 1998) as well as the OIE standards (see Section 9 and http://www.oie.int). If gene banking involves transboundary movement of embryos, particular attention should be paid to the OIE standards.

Abundant results from worldwide research on the risks of disease transmission via embryos are available for cattle. Less information is available for sheep, goats and pigs, and information is almost non-existent for other species. Any embryo collection should be preceded by an extensive clinical examination of the donor animal for the presence of diseases. Its herd or flock mates should also be checked, as should the general environment in which the animals are kept. The clinical examination may eliminate a potential donor from

consideration or indicate that a treatment needs to be applied. The results of the examination may also influence the precise protocol applied for superovulation and recovery, as good results can only be expected from perfectly healthy animals.

The disease risk may be lower in some species than others, but this should not influence the level of attention to the animals' health status. For more information on disease control in embryos see FAO (1985).

Conventional embryo freezing

Embryos are usually frozen when they are at the morula or blastocyst stage, which, depending on the species, is reached by five to nine days after fertilization. After collection, embryos are placed into a hypertonic solution containing a cryoprotective agent, such as glycerol or ethylene glycol (see Leibo, 1992). These agents mildly dehydrate the embryo before and during the cooling process. Today, most livestock embryos are frozen in 0.25 ml or 0.50 ml plastic straws, similar to those used for freezing bull semen.

Because embryos are a collection of many interacting individual cells, whereas each sperm consists of a single cell, the freezing protocols for embryos are generally more sophisticated than those for semen. For more details on the procedures for cryopreserving livestock embryos, see Appendix G. Cellular properties often vary between species and between the stages of embryonic development. Thus, to minimize damage to the embryo and optimize survival rates, it is important to ensure that the cryopreservation procedure accounts for the particular characteristics of the targeted species (Rall *et al.,* 2000) (see Section 7 for basic principles of cryopreservation). The most often-used embryo freezing method is slow freezing, which is based upon a reversible dehydration of the cells that prevents the most damaging effects of intracellular ice crystallization. Most technicians who use the slow-freezing technique use an automated embryo-freezing machine. These machines can be adapted to work under field conditions.

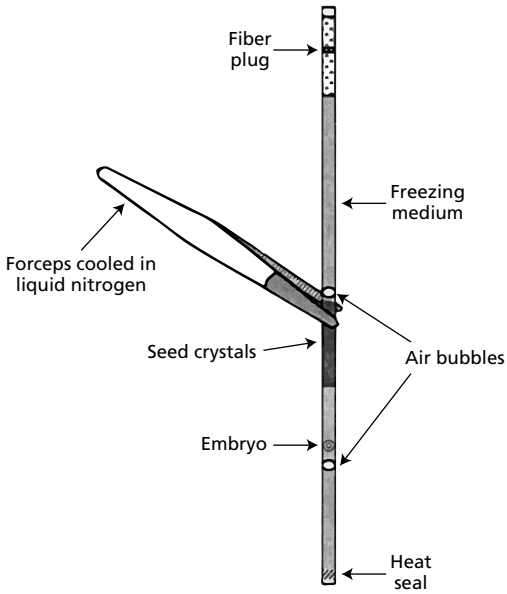
After the embryo and cryoprotectant are placed in the plastic straw, one critical step in the freezing process is "seeding", which is the act of purposefully inducing ice-crystal formation in the cryoprotectant solution surrounding the embryo (Figure 13). After embryos are cooled to approximately -35 °C, they are plunged into liquid nitrogen for storage at -196 °C. A summary of the methods and their applications is presented in Leibo (1992) and Rall (1992).

Several factors have been shown to be critical in determining the success or failure of cryopreservation:

1. the quality of the embryo – as estimated from its morphology examined under a stereomicroscope;
2. the time from embryo collection to the onset of freezing, which should be no longer than three to four hours; and
3. the appropriateness of the freezing and thawing procedure for the type of embryo being cryopreserved.

Field reports indicate that *Bos indicus* cattle embryos do not survive the freezing process as well as *Bos taurus* embryos. The greater lipid content found in most *Bos indicus* embryos at freezing may explain the lower post-thaw survival rates (see Ballard *et al.,* 2007; Looney *et al.,* 2008).

FIGURE 13
Using a forceps to induce ice-crystal formation (seeding) in a plastic straw



Drawing courtesy of R. A. Godke.

Cryopreservation of embryos using vitrification

As explained in Section 7, vitrification is a process that uses the rapid increase in the viscosity of solutions during freezing to obtain a glassy solid phase, both inside and outside the cells, without the formation of ice crystals (see Rall and Fahy, 1985; Rall, 1992). Vitrification involves the use of a high-concentration mixture of cryoprotective agent. Embryos placed into vitrification solutions are plunged directly into liquid nitrogen, saving valuable time and eliminating the need to purchase an embryo freezing machine (Vajta and Kuwayama, 2006; Vajta and Nagy, 2006). For further details on vitrification procedures, see Vajta *et al.* (2005) and Vajta and Kuwayama (2006). Although vitrification is a quick procedure and does not require special equipment, it can be technically more demanding and typically yields pregnancy rates that are 10 to 15 percent lower than those obtained using slow freezing until the technicians have gained enough experience to master the technique.

Success rates with vitrification in cattle are now approaching the rates achieved with conventional embryo freezing (Seidel and Walker, 2006). Commercial kits for vitrification of livestock embryos are now available. As vitrification methodologies improve, there are some indications that the approach may have some advantages over standard slow-freezing procedures in cattle (Vajta *et al.,* 1997; Visintin *et al.,* 2002). The future for vitrification technology appears promising, especially for embryos that have lower viability following conventional cryopreservation, such as pig embryos and embryos produced via IVF. At present, good success is being reported using vitrification to cryopreserve horse oocytes.

Embryo sexing and genetic diagnosis technology

Sexing and selecting embryos prior to cryoconservation may decrease the costs of storage, and particularly of subsequent thawing, transfer and production of offspring, especially if a greater proportion of animals of a particular sex is desired in the future.

One simple approach to gender determination is to bisect the embryo and identify the sex of one of the halves. Once the sex is established, the remaining half of the embryo can be transferred to a recipient female (e.g. Nakagawa *et al.,* 1985; Herr and Reed, 1991). Using another approach, White *et al.* (1987) bisected bovine embryos and then sexed one of the half-embryos of each pair by using an H-Y antibody procedure. Then both the half-embryos of the pairs were transferred to a different recipient animal. The success rate for embryo sexing was 90 percent, and there was no significant difference in pregnancy rates between the sexed half-embryos and control half-embryos (47 percent vs. 44 percent).

Studies using polymerase chain reaction (PCR) technology on fresh and frozen–thawed animal embryos clearly indicate that embryo biopsy techniques can be used for embryo sexing (Peura *et al.,* 2001; Kirkpatrick and Monson, 1993) without reducing post-biopsy transfer pregnancy rates. With today's embryo-sexing technology, only a few cells from the trophoblast of the embryo are needed for the *in vitro* procedures. In fact, the equipment and the supplies needed to sex bovine embryos are commercially available for use by veterinarians and livestock producers worldwide. If the instructions of these commercial embryo sexing kits are carefully followed, reported success rates approach 100 percent for cattle embryos.

At present, research efforts are directed towards minimally invasive embryo biopsy approaches for harvesting cells to be used in identifying potential genetic abnormalities and diseases prior to transferring the embryo. In the near future, cells from embryonic biopsy will be used by breeders and AI companies to identify genotypic and/or phenotypic traits of the embryo by using quantitative trait loci (commonly abbreviated QTL) and genomic selection technology. The potential for using genomic information to select the appropriate embryo to transfer would provide significant benefits to commercial breeders. Various companies have recently started making genome-based technology available on a commercial basis to livestock producers. With regard to cryoconservation, technologies for genomic selection of embryos will be particularly useful for cryobanking undertaken with the objective of gene introgression (see Section 6). For general cryoconservation programmes, these technologies may also be useful for selecting animals or embryos with the aim of maximizing the amount of genetic variability conserved in the gene bank (de Cara *et al*., 2011).

OOCYTES

Two approaches can be used for collection of oocytes. Conventional oocyte collection consists of harvesting oocytes from ovaries that have been removed from a donor female. Transvaginal ultrasound-guided oocyte collections (TUGA), on the other hand, consists of removing oocytes from the ovary of a living animal. The choice between the two approaches will depend on a number of factors, including technical capacity, financial resources and the value of the donor female.

Conventional oocyte collection

Oocytes are often harvested from slaughterhouse ovaries for research purposes. This can also be an option for cryoconservation, especially if the genetic characteristics of the donor animals (i.e. beyond their breed) are not important and the germplasm is expected to remain within the country (in which case strict adherence to OIE sanitary standards for export is not necessary). The ovaries of the donor females are collected immediately after slaughter, placed in reclosable plastic zip-lock bags and kept warm *en route* to the laboratory. In general, livestock ovaries should not be cooled, as this dramatically reduces the success rate of embryo production from IVF. Individual bovine oocytes are generally aspirated from small, medium and large follicles (see Appendix H), subsequently matured, fertilized and cultured, and then either frozen or transferred fresh to a recipient. Usually, four to twelve oocytes per ovary can be harvested from cows (using a sterile needle and plastic syringe). Once collected, the oocytes are evaluated for quality and placed in oocyte maturation medium overnight (e.g. 20 to 23 hours for cattle oocytes) in preparation for *in vitro* maturation and IVF procedures (see examples in Appendices I and J). This methodology can be used to collect oocytes from nearly all species of livestock and is relatively simple, inexpensive, and highly recommended for training students and laboratory personnel.

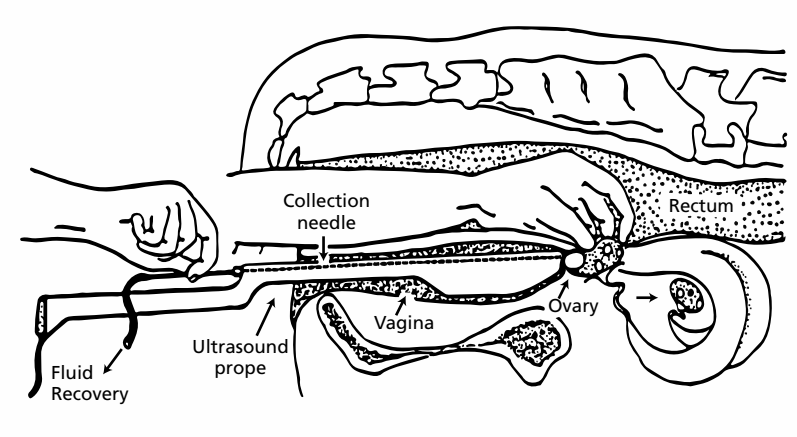
This oocyte harvesting procedure could be used to produce offspring from females that unexpectedly die or suffer incapacitating injuries or from old or clinically subfertile animals. A novel conservation strategy might be systematically to recover the ovaries from all the females of an at-risk breed when they die or go for slaughter. These oocytes could then be fertilized in the laboratory and the subsequent embryos frozen for transfer to recipient females at a later date. In the event of the compulsory slaughter of a herd because of a non-viral disease outbreak, it is still possible to produce clean embryos (using the International Embryo Transfer Society and OIE animal-health and embryo-handling procedures) that can later be used to re-establish the herd.

Transvaginal ultrasound-guided oocyte collection

Oocytes can be collected from live donors via follicle aspiration by using one of three basic surgical procedures. The first is the standard laparotomy technique to expose the ovaries, which can be performed using various anatomical approaches in all large livestock species. Although this method has been successful in cattle and horses, today it is most often used in sheep, goats and pigs. The second approach is the endoscopic technique, which is also used most commonly in sheep and goats, but has also been used for follicle aspiration in cattle and horses. The third approach uses transvaginal ultrasound-guided oocyte recovery (TUGA), which is now most commonly used in cattle, buffalo and horses. TUGA is often referred to by its common name, "ovum pick-up" (OVU). All three of these approaches to oocyte collection require the services of a skilled technician; a descriptive overview of the TUGA procedure is presented below.

TUGA was originally developed in humans to retrieve oocytes by using ultrasonography to evaluate the ovary and guide a hollow needle transvaginally into each visible ovarian follicle (e.g. Wikland and Hamburger, 1984; Dellenbach *et al.,* 1985). The needle is attached to a source of vacuum and is used to aspirate fresh *in vivo* oocytes from the

FIGURE 14
Transvaginal ultrasound-guided oocyte aspiration (TUGA) in the cow



Drawing courtesy of Dr. A. Meinjtes.

follicle, which are then subjected to *in vitro* maturation, IVF and then *in vitro* culture procedures. The procedures for humans were modified for harvesting oocytes from live cattle (e.g. Callesen *et al.,* 1987; Pieterse *et al.,* 1988, 1991) and other species. TUGA is now routinely used in cows, doe goats, mares and more recently in sows and large, hoofed, wild animal species.

TUGA can expand the time that animals can be reproductively active. For example, both pregnant cows and mares continue follicle wave development during early to mid-gestation. A novel approach is to take advantage of these developing ovarian follicles to produce IVF offspring from oocytes during the early stages of pregnancy. This procedure does not compromise the pregnancy, and oocyte yield actually tends to be greater than from non-pregnant animals (Meintjes *et al.,* 1995b; Cochran *et al.,* 1998a,b). This procedure can be especially useful for larger animals, because they tend to have only one offspring per pregnancy and their relatively long gestation periods mean that potential donors pass through long periods when they cannot be used for embryo production. In addition, TUGA can be used on animals before they reach sexual maturity. Oocytes from prepubertal sheep and cattle have produced IVF offspring (see Looney *et al.,* 1995; Bols *et al.,* 1999). Oocytes from near-term bovine foetuses and new-born calves are being harvested for IVF procedures, but at the time of writing these guidelines no offspring have been produced through this approach.

To retrieve the oocytes for IVF, a trained professional inserts an ultrasound-guided stainless steel needle through the wall of the vagina near the cervix to extract the oocytes from the follicles visible on the ovaries. The procedure is conducted on the small, medium and large follicles on both ovaries of the donor female.

TUGA in cattle. The donor cow is restrained in a suitable holding chute and administered an epidural block. A convex ultrasound 5 megahertz (MHz) sector transducer is fitted onto the distal end of a specially designed plastic handle and used to visualize the ovaries on the screen of the ultrasound monitor. The plastic handle (with a latex protective covering) is inserted into the vaginal canal, and then the ovary is grasped *per rectum* and placed against the transducer in the vagina (Figure 14).

Follicles are identified as black (hypoechoic) circular shapes on the monitor screen. An 18-gauge, 55 or 60 cm-long needle is inserted through the needle guide in the plastic handle. This needle is connected to a suction pump by means of polyethylene tubing that passes through an embryo filter or into a 50 ml conical-shaped test tube for collection of the follicular fluid containing the oocytes. The basic solution used for this procedure is PBS, with 10 percent bovine serum, antibiotics and heparin added. Using this aspiration method, oocytes are recovered from 60 to 70 percent of the medium to large-size follicles punctured, with an average of three to ten oocytes per non-stimulated donor female. A significant training period is required in order to become proficient in using this procedure in cattle.

Aspirations are usually performed once a week, but have been performed twice a week for up to three months in cows (Gibbons *et al.,* 1994; Broadbent *et al.,* 1997) with no overt effects reported in the donor females. With TUGA and IVF, more embryos can be produced in a shorter period of time than is possible using conventional ET. The procedure can realistically be repeated on the same cow three to four times a month. In addition, using TUGA for oocyte collection does not require any hormone treatment of the donor. The frequency of recovery can be much greater than that for embryo collection after superovulation (up to 80 recoveries during one year in cattle compared with no more than six collections when embryos are collected non-surgically). Oocytes can be harvested from donor cows at any time of the oestrous cycle, including at standing oestrus and the growth phase of the first follicular wave (Paul *et al.,* 1995). Although superovulation is not necessary, the number of oocytes collected per female can be increased by treating the cow with gonadotropic hormones prior to the aspiration procedure. *In vitro* production of embryos generally results in one to three embryos for transfer per oocyte collection procedure in non-stimulated donors.

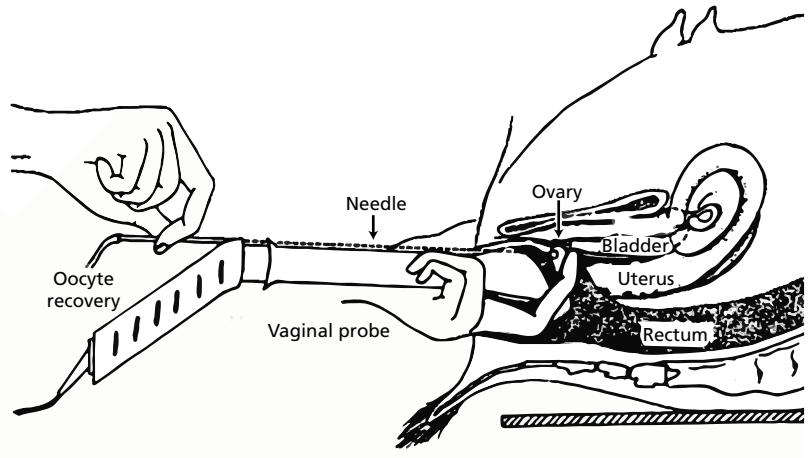
TUGA in buffalo. Similar oocyte collection procedures to those used in cattle are now being developed for buffaloes in various parts of the world. Again, the primary objective is to fertilize the collected oocytes *in vitro* for fresh transfers or for cryopreservation. The successful use of TUGA for harvesting oocytes has been reported in the swamp buffalo (Pavasuthipaisit *et al.,* 1995; Techakumphu *et al.,* 2004; Promdireg *et al.,* 2005) the Italian Mediterranean buffalo (Boni *et al.,* 1996) and the Murrah buffalo (Gupta *et al.,* 2006). The basic bovine IVF procedures are being fine-tuned for embryo production from buffalo oocytes.

TUGA in the horse. The horse has presented a unique problem for researchers working on assisted reproductive technology. As noted above, although embryo collection and transfer are relatively simple in the mare, superovulation is generally ineffective. Due to the unique anatomical structure of the horse ovary, usually only a single oocyte matures to an ovum and ovulates at the appropriate time during each oestrous cycle. Also, for some as yet unknown reason, typical IVF procedures have not worked consistently in the horse.

Even though only one follicle normally matures and ovulates during an oestrous cycle, mares are thought also to have one or two waves of multiple follicles during the cycle. This developing follicle population makes it possible to use TUGA to collect oocytes from live mares for the production of embryos for transfer (see Brück *et al.,* 1992; Cook *et al.,* 1992). The first foals obtained from oocytes aspirated from live mares were produced using ICSI (see Section 4). After ICSI, embryos are surgically transferred at the two- to four-cell stage into the oviducts of suitable recipients, because the culture of IVF-derived equine embryos has not yet been perfected.

The aspiration set-up for mares is similar to that used in cattle, but with some modifications. Briefly, mares require sedation instead of an epidural block, and a 12-gauge needle is used to puncture the follicles. Extra rinsing of the follicle is necessary in the horse, as the oocyte is usually well-embedded in the follicle wall. In such cases, the needle recommended is a double-lumen needle. This allows the follicular fluid to be aspirated and the medium used again to rinse the follicle (two to four times per follicle). The follicular fluid is collected into a 500 ml bottle, and later passed through the standard embryo filter. Using this modified method, oocytes have been successfully recovered from mixed-breed cyclic horses and ponies (Meintjes *et al.,* 1995a), pregnant mares (Meintjes *et al.,* 1994, 1996) and from free-ranging zebras in South Africa (Meintjes *et al.,* 1997). Oocyte recovery rate usually ranges between 50 and 75 percent of follicles punctured per mare. After IVF or sperm injection procedures, developing two- to four-cell stage embryos are transferred surgically into the oviducts of recipient mares. The ICSI procedure (Section 4) appears to be the method of choice, at present, for producing horse embryos in the laboratory.

FIGURE 15
Ultrasound-guided oocyte aspiration in goats



Source: drawing courtesy of R.A. Godke; reprinted from *Theriogenology*, 51, Graff, K.J., Meintjes, M., Dyer, V.W., Paul, J.B, Denniston, R.S., Ziomek, C. & Godke, R.A. Transvaginal ultrasound-guided oocyte retrieval following FSH stimulation of domestic goats, 1099–111, Copyright (1999) with permission from Elsevier.

TUGA in small ruminants. *In vitro* production has also proven successful in goats. Transvaginal aspirations have been performed on cyclic and non-cyclic adult does with good success (Graff *et al.,* 1999). Although the oocyte recovery rates usually range between 60 and 80 percent of the follicles punctured per donor female, there are some problems with the aspiration of ovarian follicles from goats using TUGA. First, the ovaries cannot be grasped *per rectum* for optimum visualization with ultrasonography. Second, as the ovaries cannot be easily grasped, it is more difficult to puncture follicles and aspirate the oocytes. Although the methodology for puncturing the follicle is similar to that in the cow and the mare, the doe must be sedated, put under anaesthesia and then placed in dorsal recumbency (Figure 15).

Manual pressure is placed on the abdomen in an effort to stabilize the ovaries for aspiration. The ultrasound probe, which is smaller than that used for cattle, buffaloes and horses, is inserted into the vagina with the convex transducer at the distal end of the handle. The aspiration proceeds with no need for extra rinsing of the follicles to recover the oocytes.

Oocyte recovery is usually a little slower than desired because not all follicles can be visualized, and not all follicles visualized can be adequately punctured due to the difficulty involved in securing the ovary. Offspring have been obtained from frozen–thawed goat embryos produced using the transvaginal aspiration procedure together with IVF methods (Han *et al.,* 2001). Although this non-invasive procedure requires expertise and patience, it is an important technology that involves less risk of ovarian adhesions or death than the standard surgical method for harvesting oocytes from goats. An efficient TUGA method for harvesting oocytes has not, as yet, been developed for sheep.

TUGA in other species. TUGA has also been used successfully in other animals, with modifications made primarily to account for anatomical differences among species. For example, TUGA been used successfully in adult pigs (Bellow *et al.,* 2001), llamas (Brogliatti *et al.,* 2000) and various other hoofstock, such as red deer (Berg *et al.,* 2000), sika deer (Locatelli *et al.,* 2006), the rare bongo antelope (Pope *et al.,* 1998; Wirtu *et al.,* 2009) and the African eland (Wirtu *et al.,* 2009). Therefore, this technique may be particularly useful for managers of gene banks that preserve both domestic and wild animal genetic resources.

SOMATIC CELLS

As discussed in Section 4, collection of tissues other than germ cells and embryos can be useful for gene banking, either for the production of new animals (through SCNT) or to obtain genetic and health-related information about the animals sampled (DNA isolated from cells).

Tissue

Somatic cells for subsequent use in DNA analyses or SCNT can be sampled from the tissue of live animals or from animals shortly after death (Silvestre *et al.,* 2004). Because the requirements for the two objectives (DNA or SCNT) differ, separate protocols have to be used for each. For DNA, one approach is to use a sterile scalpel blade to aseptically remove thin strips of skin from the body surface (e.g. shoulder area) of an animal and then place them in a pre-labelled sterile screw-top vial for transport to the processing laboratory. Tissue can also be easily obtained from the peripheral border of the ear of a live animal (or immediately after the animal's death) using a sterile hole punch. Prior to freezing (or

vitrification), the tissue samples should be wrapped in blotting paper moistened with PBS and maintained at 4° C to minimize degradation of the samples. As an alternative to cryo-preservation, tissue samples can be preserved through dehydration by storing each sample in a glass vial containing a grain of silica gel. In addition, for DNA analyses in the relatively short term (less than two years) hair samples may be an option. Hairs must be plucked from the roots and then desiccated, placed in separate labelled containers for each animal and stored in a dry environment. Various protocols for DNA extraction exist, but the simplest approach is to use a commercial kit designed for use with the specific tissue.

Collection of tissue for SCNT needs to follow a more stringent protocol than that used for samples intended for DNA analyses. Appendix K presents two protocols, the first for simple and low-cost field collection and freezing (Groeneveld *et al.,* 2008) and the second for situations in which samples will be processed in a laboratory. In both cases, the same procedures are generally applicable to all species, particularly to mammalian species.

Blood

Blood samples collected from live animals or from animals shortly after death can be used for DNA analyses or for cloning via SCNT. The DNA from the blood of mammals comes from the white cells only, because the red cells do not have nuclei. Birds have DNA in both red and white cells, and thus a smaller volume of blood is required from birds than from mammals in order to obtain ampoule quantities of DNA for analyses. Blood is relatively simple to obtain in quantities that provide sufficient DNA for genetic analyses. Collection of blood from the jugular or caudal vein with a needle and vacutainer tube is the common procedure in mammals. Blood from poultry is usually sampled from veins in the wing.

It is recommended that two vials of whole blood (total of 10 to 14 ml) be collected at the time of collecting any other germplasm from animals selected for the gene bank; this will reduce the risk of accidental loss of all samples from an individual animal. White blood cells can be harvested from fresh whole blood following centrifugation. The buffy coat (the thin middle layer that forms when blood is centrifuged) is carefully pipetted from the sample and divided into at least two small pre-labelled sterile vials for use in nuclear transfer. The vials are then frozen in nitrogen vapour and stored in liquid nitrogen.

If only DNA is needed, it can be extracted using commercial kits, following their specific procedures. For long-term storage, the extracted DNA should be put into labelled aliquots of 50 µl, with a concentration of 200 µg/ml. Division into aliquots will provide repeated access while avoiding freezing and thawing of the entire sample. The DNA may be safely stored at 4 °C for two months before being divided into aliquots, provided the preparation is pure enough. Otherwise rapid transfer to long-term storage is necessary. For long-term storage, DNA can be maintained at -20 °C or lower (-80 °C or in liquid nitrogen), but the latter is not necessary.

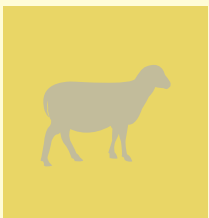
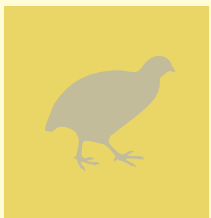
Gonadal tissue (poultry)

As discussed in Section 4, recent studies have reported that ovarian tissue can be harvested from immature female chicks, frozen, thawed and transferred back to other young females (see Song and Silversides, 2006). Subsequently, newly hatched chick testicular tissue has

been harvested and transplanted successfully to host chicks, resulting in live offspring born from sperm derived from the donor testicular tissue (see Song and Silversides, 2007b). The procedures used for cryopreservation of ovarian and gonadal tissue are presented in Appendix L.

SECTION 9

Sanitary recommendations


Sanitary recommendations

Collection and banking of AnGR can present various complications. Animals to be sampled will likely originate from different farms, and this presents the possibility of disease transmission when the animals or their germplasm come in contact with each other in the central gene bank. At-risk breeds will likely be found in only a few locations, leaving little opportunity for selection of donor farms on the basis of sanitary conditions. Disease outbreaks may present both an urgent need to collect germplasm from animals in the affected area and a health and sanitation risk for the gene bank. Each country will need to balance its breed conservation needs and its compliance with national and international health regulations. Decisions will be based, in part, on the types of diseases that are present and how contagious, virulent and damaging to animal production they are. Certainly, animals that have a highly contagious and possibly fatal disease, such as foot-and-mouth disease, should not be collected except in the most dire of circumstances (i.e. if no non-infected animals exist).

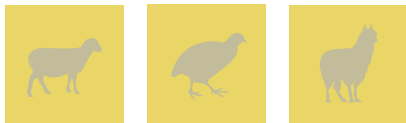
The diseases of concern to gene bank managers will vary from country to country, as will the health regulations that gene banks have to follow in developing their collections. However, if the gene bank is interested in distributing germplasm to other countries, especially those with widely divergent health concerns, attention must be paid to OIE health regulations (http://www.oie.int). International transfer of germplasm that does not conform to these regulations could put a country at risk of losing its OIE export status.

The primary issue for a gene bank collecting germplasm in the field is to minimize the risk of spreading diseases from farm to farm while collecting germplasm from animals belonging to different owners. Additionally, efforts must be made to reduce the risk of spreading diseases during the utilization of germplasm that the repository has collected and cryopreserved.

A high standard of health testing and of sample preparation is easier to achieve if germplasm is collected in an AI centre or other controlled environment. Maintaining the required protocols is more difficult when collecting is done in the field. In certain instances it may be necessary to obtain a waiver from national or international veterinary agencies so that germplasm from breeds at risk can be collected and cryoconserved. Obviously, conditions in the field may mean that maintaining animals in quarantine for any length of time is impractical. Even if there are no such problems, quarantining animals in the field increases collection costs. A potential means of addressing the risk of collecting germplasm from infected animals in the field is to draw blood samples from the donor animals and then have the blood samples tested for the relevant disease(s).

The following sources serve as excellent reference material on sanitary requirements for the collection, testing and processing of germplasm:

- OIE recommendations and regulations, set out in its Terrestrial Animal Health Code, covering the collection, testing and processing of germplasm samples for international exchange (http://www.oie.int/index.php?id=169&L=0&htmfile=titre_1.4.htm);



- AI centre and semen import and export protocols of the United States of America's National Association of Animal Breeders (http://www.naab-css.org/guidelines/ and http://www.aphis.usda.gov/regulations/vs/iregs/animals); and
- European Union regulations for AI centres and international exchange of germplasm (http://ec.europa.eu/food/animal/index_en.htm).

Although the above-listed regulations and protocols may not apply directly to all countries, applying the procedures described will improve sanitary safety and thus reduce the risk of disease transmission.

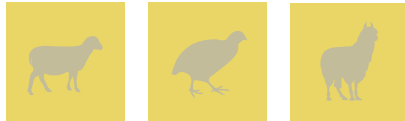
COLLECTION AND PROCESSING FACILITY

It is likely that the animals brought into a collection centre will remain there for only a relatively short period of time. Quarantine and health testing procedures should be as cost-effective as possible. In order to minimize the risk of disease transmission, three components of the collection facility have to be addressed:

1. As animals enter the collection facility they should be maintained in isolation buildings or pens for a specified period of time during which they can be tested for various diseases, placed on selected rations and trained for collection. Ideally, personnel that handle quarantined animals should not be involved in the care or collection of animals that have already been tested. As noted in Section 5, for ease of management, the quarantined area should be subject to an all-in all-out policy, i.e. if any animal fails to meet the predetermined health criteria, none of the animals in the group should be accepted.
2. Once animals have passed the health tests they should be moved into the main facility, where they will be maintained and their germplasm collected. At this stage in the collection process, the main health concerns relate to the potential for introducing a disease from outside the facility. Such risks can be minimized by requiring animal handlers to follow specific sanitation protocols and by keeping rodents and wild birds out of the facility. The risk of contamination from bedding can be addressed by cleaning animals prior to collection. Even though the animals in the collection facility will have passed through quarantine, equipment for germplasm collection such as AV and collection tubes should be kept clean and changed for each animal.
3. After collection, samples of semen and other genetic materials are transferred to a laboratory. Protocols for biosafety of at least Level 2 (see http://www.cdc.gov/biosafety/publications/bmbl5/BMBL.pdf) need to be in place in order to maintain minimal sanitation standards and prevent any cross-contamination between samples.

FIELD COLLECTION

Although germplasm collection in a closed facility is desirable for control of health and sanitation, in some instances it may be more practical and cost effective, or even necessary, for gene banks to perform collections in the field. During field collection, collectors must respect the sanitation of each collection site, and minimize the risk of spreading diseases. Shipping boxes and other supplies used at one collection site should never be used at other sites. The boxes and supplies may be reused after they have been sanitized, but only at the same collection site. If frozen materials arrive at the gene bank, the liquid nitrogen tank or



dry shipper should be sanitized with a 10 percent bleach solution after it has been warmed to room temperature.

If personnel travel from collection site to collection site, then very specific sanitation practices should be implemented. The undercarriage and tires of vehicles should be washed, preferably with a disinfectant, after visiting each site. Likewise, the boots of the personnel should be disinfected, or covered with disposable boot covers that are discarded after leaving each site. Clothing should also be laundered or changed between collection sites or disposable suits should be worn. Polyvinyl or nitrile gloves should be worn, and changed between handling different animals.

Equipment such as syringes and needles should never be used on more than one animal, and must be disposed of properly, according to local regulations, following use. Non-disposable equipment, such as electro-ejaculators, must be sanitized and rinsed between animals in order to avoid spreading pathogens.

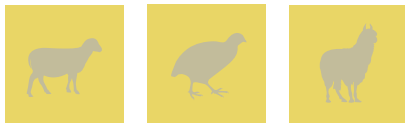
DISEASE TESTING

As mentioned above, germplasm should not, if at all possible, be collected from animals that are clearly infected with a highly contagious disease. In general, it is preferable to avoid sampling animals that are affected by any kind of disease. Hence, quarantine of all animals entering a collection centre is recommended. Infected animals may not show any outward symptoms of disease even if they spend time in quarantine. For this reason, it is advisable to collect samples of blood or other tissues (e.g. nasal smears) that can be used in more comprehensive tests for the presence of disease agents.

STORING SAMPLES

Cross-contamination of samples in liquid nitrogen is possible, but this has only been found to be of consequence in studies where the contaminant was placed in the tank (Grout and Morris, 2009). Otherwise, the probability of contamination seems to be very low. Nevertheless, because pathogen transmission is possible, care should be taken to minimize any risk of cross-contamination. It is critically important to consider how the material will be handled and stored. For example, semen straws can be sealed with a variety of substances, including polyvinyl alcohol (PVA) powder, clay and metal ball bearings, or by heat sealing. While PVA powder, clay and ball bearings are inexpensive, the quality of the seal they provide is inferior to that achieved by heat sealing, and consequently the potential for contamination through leakage or rupturing of straws is greater.

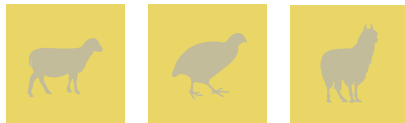
If the sanitary status of previously stored samples is questionable, then the semen straws or other storage devices can be cleaned with ethanol and allowed to dry following thawing. However, a more important question may be how to deal with newly collected and frozen samples from sources where biosecurity standards may be lower than desirable, such as those collected in the field rather than at an approved collection centre. In such circumstances, separate storage of samples that have uncertain sanitary status should be considered. For maximum security, distinct storage sites can be used. At minimum, samples of unknown status should be stored in separate tanks. In addition, specific liquid nitrogen tanks should be designated for quarantine purposes. Then, for example, samples



not received from facilities that are known to be sanitary, such as designated collection facilities or AI centres, can easily be segregated and held in a dedicated liquid nitrogen tank until their sanitation status is determined. If samples collected in the field are considered to be suspect in sanitation and health terms, they can be quarantined until the results of blood tests from the sampled animals demonstrate a clean bill of health. In some countries, local regulations may stipulate that germplasm from different species have to be stored separately, regardless of the samples' health status and the site of collection.

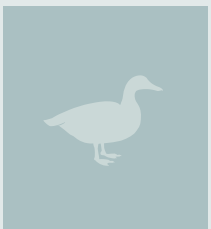
Segregating germplasm according to its health and sanitary status is of particular importance when there is a possibility that germplasm may be transferred internationally and adherence to OIE standards is critical. Even if possible international transfer is not a factor, given the potentially high value of stored germplasm and the fact that it may in the future be the only means by which to recover a lost breed or support the maintenance of a highly endangered breed, the risks involved in storing samples with known and unknown levels of sanitation together must be given serious consideration.

Regardless of the system used, information on the sanitary status of each individual sample must be included in the gene bank database and information system (see Section 10). In this way, all samples can be tracked and managed properly regardless of their storage site.



SECTION 10

Database and documentation


Database and documentation

Proper and accessible documentation is vital for the future use of any stored gene bank material. An essential aspect of this is to develop and implement a database that can be used to catalogue, summarize, query and retrieve the information needed to establish and operate the gene bank. Basic information about gene bank collections should be easily accessible without the need for any additional information from outside the database. The database will be essential for managing routine gene bank operations (e.g. quality-control testing, sample identification, sample location, current inventory, movement among collections and de-accession) and to support management decisions.

The database will serve as the primary tool for receiving, storing and exchanging information about samples in the collection. The outflow of information is just as essential as the input of information. Potential requestors of germplasm must be able to view the contents of the collection. To ensure broad access, the database needs to be linked to the internet. Internet access helps to promote awareness of the country's AnGR programme and makes it easier for users to access information and make use of the stored germplasm.

Databases for gene bank management can be very diverse. For example, a very basic information storage system can be set up using a spreadsheet program. More complicated databases can be developed by using computer software specifically designed for database construction. With such software, a broad array of databases, differing vastly in complexity, can be developed.

From the first phases of planning the gene bank, it is essential to recognize the important role that the database will play both in day-to-day management and in allowing potential users to access up-to-date information on the material in the collection.

COMPONENTS OF A GENE BANK INFORMATION SYSTEM

All databases have a tabular structure, with the tables linked to each other either through one-to-one relationships or through one-to-many relationships. Initial design of the database should involve close cooperation between gene bank managers, database developers/operators and potential user groups. This serves to ensure that users' information needs are identified and accounted for in the development of the database. Once needs have been identified, it is usual to develop a graphic scheme illustrating the various relationships within the database. As well as the database itself, a number of additional elements, necessary for inputting and extracting data, need to be put in place. These will include:

- data input screens that facilitate data input and mimic data-collection forms;
- data edit screens that allow changes to be made to data elements, and include automated features that allow changes to more than one record at a time;
- record review capability (facilitates the recall of individual or group records);

- capability to summarize elements in the database and calculate statistics that may be of interest to users or managers;
- capability to query the database, i.e. to extract specific pieces of information;
- output options that allow users to choose how the data they request are presented in tabular or graphical form, as well as the type of file to which the data are exported;
- data entry and edit control capability that allows database managers to control who can enter and edit data in the database; usually this is accomplished by making access to entry and edit functions password protected; and
- inter-operability – there may be a need or desire to link the gene bank database to databases operated by other national or international agencies and exchange information between them.

In addition to the items listed above, serious consideration must be given to the extent to which the information in the database should be made available on the internet. It may be decided that some information, such as where certain samples are stored in the repository, should not be made publicly available. Such information can be password-restricted or subject to some other form of access control. Generally, the access granted to outside users of a gene bank database will be "read-only".

When planning the construction of the database, gene bank managers should be aware of opportunities that exist to utilize database systems that have been, or are being, developed by other gene banking groups. Database development requires specific expertise that may not be available in all institutions or countries. Therefore, the use of existing databases and software packages or joint development of a database across countries may be a desirable option. In addition, using an existing database application will usually facilitate inter-operability. Examples of databases already developed by countries and regions to document cryoconserved material include the following:

1. Supported by the European Commission, the EFABISnet project developed the CryoWEB database tool (Duchev *et al.*, 2010). This tool has already been implemented in a number of European countries and is integrated with the EFABIS breed database. The CryoWEB database software is available under a free GPL license and can serve as a basis for further adaptation or development (see http://cryoweb.tzv.fal.de/).
2. The National Animal Germplasm Program in the United States of America, EMBRAPA of Brazil, and Agriculture and Agri-Foods Canada have joined together to develop an internet-based database for management of germplasm collections, which also offers the option of performing cross-country comparison of germplasm collections (see http://www.ars.usda.gov/Main/docs.htm?docid=16979).

DATABASE INFORMATION SET

In developing descriptors for the database, each country must determine what information it wants to maintain and what is needed to describe thoroughly the samples maintained in the repository. Tables 14 and 15 provide a list of minimum and recommended information fields that should be completed for every donor animal and sample in the gene bank. Breed-level information (final four rows of Table 14) needs to be collected once per breed, but should be directly available for each animal of each breed.

TABLE 14
Donor animal information: recommended minimum and additional database fields

Trait	Type	Minimum	Recommended	Comments
Animal identification				
Owner identification	Alpha-numeric	X		
Repository identification	Alpha-numeric	X		
Association identification	Alpha-numeric		X	
Markings			X	e.g. tattoo
Animal birth date	Alpha-numeric		X	
Sex	Alpha-numeric	X		
Source				
Breeder name	Alpha		X	Contract with original owner (if any) attached to the database
Owner name	Alpha	X		
Geographic location (geographical coordinates or mailing address)	Alpha-numeric	X		
Taxonomy				
Species	Alpha	X		
Breed	Alpha	X		
Population	Alpha	X		
Environment	Alpha		X	e.g. arid, semi-arid, humid, subhumid, temperate, subtropical highland
Management system	Alpha		X	e.g. pastoralist, ranching, mixed crop–livestock, small-scale landless, industrial landless
Phenotypic measures				
Body weights	Numeric		X	Birth weight, weaning weight, mature weight
Visual identifiers	Alpha-numeric		X	Colour, horns, photograph
Production measures	Alpha-numeric		X	Milk yield, fleece weight, litter size, etc.
Genetic measures				
Pedigree	Alpha-numeric		X	3 generations if possible
Genetic test results	Alpha-numeric		X	e.g. Halothane, Scrapie
Genetic markers	Alpha-numeric		X	Microsatellite, SNP
Breeding values	Numeric		X	e.g. production traits
Breed information				
Census data	Numeric		X	
Phenotypic descriptors	Alpha-numeric		X	Average weights
Genotypic descriptors	Alpha-numeric		X	Known genetic attributes
Production systems	Alpha-numeric		X	Production systems where the breed is prevalent

TABLE 15
Sample information: recommended minimum and additional database fields to be associated with animal identification

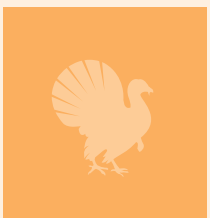
Trait	Type	Minimum	Recommended	Comments
Collection				
Date				
Location				
Sample quality				
Semen				
-Temperature at arrival in lab	Numeric	X		
-pH at arrival in lab	Numeric	X		
-Pre-freeze motility	Numeric		X	
-Post-thaw motility	Numeric	X		
Embryo				
Grade before freezing				
Stage of development				
Quality after freezing				
Straw information				
Identification	Alpha-numeric	X		
Freeze date	Numeric	X		
Species	Alpha-numeric	X		
Breed	Alpha-numeric	X		
Storage information				
Kind of straw or pellet or else	Alpha-numeric	X		
Tank	Numeric	X		
Placement in tank	Numeric	X		
Collection method				
Semen	Alpha-numeric	X		
Embryo	Alpha-numeric	X		
Oocyte	Alpha-numeric	X		
Somatic cells	Alpha-numeric	X		
Freezing protocol used*				
Semen	Alpha-numeric	X		Detailed protocol attached to the database
Embryo	Alpha-numeric	X		
Oocyte	Alpha-numeric	X		
Somatic cells	Alpha-numeric	X		
Sample ownership				
Semen	Alpha-numeric	X		
Embryo	Alpha-numeric	X		
Oocyte	Alpha-numeric	X		
Somatic cells	Alpha-numeric	X		
Sample sanitary status				
Semen	Alpha-numeric	X		Details of diagnostic tests attached to the database
Embryo	Alpha-numeric	X		
Oocyte	Alpha-numeric	X		
Somatic cells	Alpha-numeric	X		

* Thawing instructions may be included as additional information.

Obtaining these descriptors may be difficult, and in some situations some of them may not exist. A potential solution is to obtain information while performing field collections. One such approach is to develop survey sheets that germplasm collectors can use to obtain the required information by interviewing livestock keepers or can be given to the livestock keepers to complete and return.

SECTION 11

Legal issues – contracts and access



Legal issues – contracts and access

In the development of country-based gene banks there may be need for various types of agreements covering the acquisition of germplasm or tissue and the dispersal of the materials when they are requested by potential users. The agreements should delineate the rights and responsibilities of the gene bank, the users of the gene bank's germplasm/tissue and (where relevant) the donors of the samples. Because of the potential legal ramifications, the gene bank must have clear policies and procedures for drawing up such agreements. Such policies may be established by the gene bank management or may be established at a higher level, such as through national legislation. For example, in the case of United States of America's genetic resources system, the Congress enacted legislation[6] stating that material in the public collection will be distributed to requestors free of charge. With such a policy in place, the gene bank has clear guidance on one aspect of germplasm distribution. However, as described in this section, such a policy only covers one aspect of germplasm release. Each country needs to establish a clear set of criteria for all aspects of acquisition, storage and use of gene bank material.

In developing policies and general agreements for acquiring and dispersing germplasm, a suggested guiding principle is that these instruments should facilitate the sustainable use, development and conservation of AnGR and the enhancement of the country's livestock sector. In other words, conditions placed upon the acquisition or release of germplasm should not be so restrictive as to put valuable AnGR at risk by impeding the development of germplasm collections or the use of the material stored in the repository.

STRUCTURE FOR HANDLING AGREEMENTS

Gene banks have to contend with a range of different types of agreements concerning the acquisition and release of germplasm. Because of the long-term nature of the gene bank's mission and its close relationship with the livestock sector, it may be useful to establish an advisory committee of interested parties not employed by the gene bank. This committee may or may not be the same as the National Advisory Committee on AnGR (see guidelines on the *Preparation of national strategies and action plans for animal genetic resources,* FAO, 2009). The committee's mission would be to provide advice and recommendations on policies for acquiring and distributing germplasm. It would be able to provide the gene bank with advice on how contracts and, other agreements and policies, can be formulated in such a way as to garner support from industry and government.

[6] Public Law 101-624-Nov. 28, 1990 (http://awic.nal.usda.gov/public-law-101-624-food-agriculture-conservation-and-trade-act-1990-section-2503-protection-pets).

ACQUIRING GERMPLASM

Depending on the country, the exchange of AnGR may not be regulated by specific legislation but by more general property rights. This is often the case because, historically, in most countries individual livestock have been primarily considered private property (of an individual, group of individuals or company). As a result, owners have been able to breed and improve their livestock as they have deemed appropriate. Furthermore, livestock owners have, for centuries, generally been free to buy and sell animals for genetic improvement purposes (Wood and Orel, 2005). As biotechnologies such as AI have emerged, they have been used for marketing the genetic improvement breeders have made. To facilitate commercial exchange, buyers and sellers have used a variety of agreements and private contracts. Similarly, because the genetic material that gene banks target for inclusion in their collections will generally be owned by individual livestock breeders, agreements transferring ownership from the breeders to the gene bank will usually be required. Alternatively, the breeder may prefer an agreement that facilitates the holding of the germplasm by the gene bank without a transfer of ownership.

As a result of pre-existing practices for the exchange of AnGR within the country, gene bank managers may have to negotiate an arrangement with each owner from whom they wish to acquire germplasm samples. Such arrangements can take several forms:

1. The gene bank may buy the animal from the owner, thus obtaining unconditional rights to the AnGR.
2. The livestock owner may donate the sample of germplasm to the gene bank, and by doing so give up all claims to the germplasm donated.
3. The livestock owner may charge a fee for access to the animal and the germplasm collected. By doing this the owner may or may not forego further claim(s) on the germplasm collection.
4. The livestock owner may maintain ownership of the germplasm for a specified period of time while it is in the gene bank (also known as an embargo), after which the germplasm becomes the property of the gene bank. Such an approach can protect breeders, for a period of time, from competitors that may want to acquire the samples for the purpose of gaining an advantage. If the owners do not want to forego their rights to germplasm stored in the gene bank, managers have to determine whether material stored for a long time (and replaced with newer samples) should remain in the gene bank or be returned to the owner.

These approaches (particularly 3 and 4) may require the gene bank to formulate contracts – generally referred to as material transfer agreements (MTAs) or material acquisition agreements – for the transfer of the germplasm. The following elements are suggested for inclusion in such an MTA.

Property rights. The ownership of the cryopreserved material should be specified. The rights of the owner (donor) and the gene bank should be defined.

Costs of collection. The donor and gene bank will need to agree about the costs associated with collecting and freezing the germplasm.

Storage. There may be cases in which the gene bank regards particular germplasm as important to store, but has neither clear ownership of this material nor the potential to

acquire such ownership. In such circumstances, the gene bank may wish to arrange for the germplasm owner to pay a storage cost.

Access. Depending upon the particular interests of the donor, the MTA may need to stipulate specific conditions for accessing the germplasm (see arrangement 4 above). The simplest approach is to structure the agreement in such a way that any requestor must first obtain permission from the donor before the material is released. By doing this, any issues that may arise regarding the further use of the material will not involve the gene bank, thus maintaining its position as a neutral entity. Serious attention should be given to the objective of ensuring that the release of gene bank material does not harm the competitiveness of the breeder/provider of the germplasm.

Intellectual property rights. If the gene bank is established as a public good, research results obtained using the material in the gene bank should be publicly accessible without any claims on intellectual property. Such a position will also minimize or eliminate the gene bank's involvement with any type of benefit-sharing arrangements.

Veterinary/sanitary issues. The collection, transfer and use of germplasm may be affected by national health policies, and the gene bank will need to take these into account in the process of acquiring germplasm. The health status of the donor animal and the cryopreserved genetic material should be defined in the MTA. The MTA should contain a list of diseases for which the animal has been tested at the time of collection.

Storage sites and quality assurance. MTAs should include a short statement indicating that the gene bank will follow a set of best practices to ensure that the viability of the samples is maintained.

Data protection. The provider and gene bank may wish the MTA to include stipulations regarding what information about the cryopreserved material and the donor will be made publicly available.

ACCESS TO THE GENE BANK'S COLLECTION

In general, there are three primary reasons for accessing stored material:

- national public need;
- non-research related breeding of animals by non-government organizations or private entities; and
- research.

The type of use will determine from which collection category (see Section 3) the genetic material will be taken.

Requestor's actions

Potential users of gene bank material should initiate the process by submitting a written request outlining their need for germplasm. The written request should provide the following information about the germplasm needed and its intended use:

- legal entity and affiliation of the applicant;
- type and quantity of genetic material requested:
  - species;
  - breed;

  - number of animals;
  - name and registration numbers, if specific animals are requested;
  - type of germplasm;
  - quantity of germplasm;
  - justification for the type of germplasm requested;
- accurate information on the intended use:
  - for breeding purposes – a justification of the need to access stocks from the gene bank; this justification may need to include information on the structure, effective size and performance of existing populations;
  - for accessing DNA for research purposes – details about the project including objectives, collaborators and sponsors;
- types of benefits that could arise from obtaining access to the resources;
- the competence of the requestor to use the genetic material properly and maximize success; and
- agreement or waiver by the requestor to accept any risks associated with the health status of the genetic material and to take any subsequent measures necessary to avoid the spread of diseases.

For convenience, the gene bank may decide to prepare a standard form for requestors to compile, based on the above list.

The information provided will help the gene bank manager to decide whether or not release of material is justified and beneficial for the national programme of AnGR management. The manager may also need to determine whether the consent of the germplasm provider is needed, based on the terms of the MTA under which the germplasm was provided. When germplasm is to be used for generating live animals, the gene bank may want to consider requesting that the user redeposit germplasm from the resulting progeny. For germplasm used in DNA studies or for generating live animals, it is recommended that the recipient be required to submit any phenotypic or genotypic data arising from the project to the gene bank for entry into a publicly accessible database. The submission of such information to the gene bank can take place after it has been published.

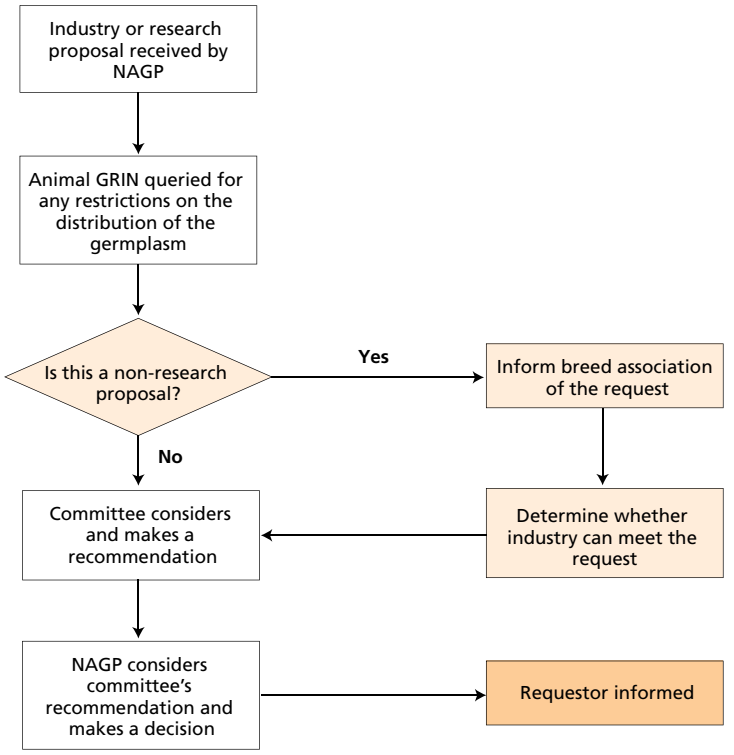
Meeting the request

Once a request for germplasm or tissue has been received and its merit evaluated, the gene bank must also determine whether it has sufficient quantities of the material available to fulfil the request without compromising the core collection (see Section 3). If the requested material is also available in the commercial sector, the cryobank should not allow use of its material.

National need. In the case of national need, the government may decide to withdraw germplasm from the relevant categories of the collection. In such a situation, the gene bank may want to convene a group of in-country experts and industry-related persons to provide recommendations and assist in facilitating the use of the germplasm.

Non-research and research requests. Non-research requests are usually those made by a segment of the livestock industry for the purposes of resolving a genetic resource issue. Requests for research purposes may come from either the public or the private sector.

FIGURE 16
Reviewing requests for animal germplasm and/or tissue – an example from the United States of America



Note: NAGP = the National Animal Germplasm Progam of the United States of America; GRIN = Germplasm Resources Information Network; "committee" refers to the advisory group that has the mandate to make decisions on release of material from the gene bank.

Source: adapted from Blackburn (2009).

The gene bank should establish a review process for dealing with both non-research and research requests. As an example, Figure 16 details the process used by the National Animal Germplasm Program in United States of America to review such requests (Blackburn, 2009).

INTERNATIONAL TRANSFERS OF GERMPLASM

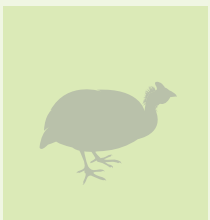
The primary mission of a national gene bank is to secure the integrity of national AnGR. Therefore, its operation and practices should be firmly established under the country's laws. Opportunities may exist to exchange germplasm across national boundaries. In such cases, the primary regulations involved are those related to animal health. The OIE has established protocols for transferring germplasm from country to country. These protocols have been used by member states of the World Trade Organization (WTO), in line with the Agreement on the Application of Sanitary and Phytosanitary Measures, to establish national measures

consistent with internationally "harmonized" standards, guidelines and recommendations. Aside from the need to conform to animal health regulations, the exchange of AnGR is mainly a matter of transferring private ownership (by private law contracts and customary law). In addition, signatory countries of the Convention on Biological Diversity need to ensure that any international transfer of AnGR is consistent with the terms of the Nagoya Protocol on Access and Benefit-Sharing[7].

[7] http://www.cbd.int/abs/text/

SECTION 12

Capacity building and training


Capacity building and training

The development of sustainable conservation programmes is only possible if it is combined with the development of human resources, institutions and long-term organizational support. Well-trained researchers and decision-makers are critical for creating awareness of AnGR-related problems and for implementing programmes to conserve and sustainably use AnGR.

Strategic Priority Area 4 of the *Global Plan of Action for Animal Genetic Resources* (FAO, 2007b) calls for development of a strong and diverse skills base to implement the *Global Plan* at national and international levels. The Convention on Biological Diversity calls for access to, and transfer of, technology (Article 16)[8]; exchange of information relevant to the conservation, management and use of biological diversity, including information on research, training, surveys and specialized knowledge (Article 17)[9]; and technical and scientific cooperation through, where necessary, appropriate international institutions, with special attention to capacity building (Article 18)[10].

The most important task in improving knowledge of AnGR and their management is to make sure that all major aspects of conservation and sustainable use are integrated into regular university curricula worldwide. Consideration should be given to both local and global aspects of animal production, given the importance of interaction among different genotypes and environments and among different species (including wild species and plants and micro-organisms) within the same environment. Students should be introduced to the agro-ecosystem approach to agriculture and livestock production. Closer collaboration between countries, both developed and less-developed, can be promoted via extended exchange programmes for students and teachers (Malmfors *et al.,* 1994). Vangen and Mukherjee (1994) suggested an integrated approach to university teaching of animal breeding and the genetics of conservation, particularly at postgraduate level. Training courses should also be organized for national administrators and heads of departments involved in policy decisions, industry personnel and facilitators of conservation programmes. The courses should aim to promote awareness of the importance of AnGR and explain the major steps in their characterization, documentation, conservation and improvement.

Including the following topics in higher education courses on AnGR will increase awareness and understanding of the importance of conserving and properly managing these resources:

Global threats and opportunities in the management of animal genetic diversity. This topic should cover the evolution and history of domestic species and breeds; the concepts of the breed and the population; the characteristics of animal populations in various

[8] http://www.cbd.int/convention/articles/?a=cbd-16

[9] http://www.cbd.int/convention/articles/?a=cbd-17

[10] http://www.cbd.int/convention/articles/?a=cbd-18

parts of the world; and present economic development trends. Livestock production systems in various regions of the world and the prospects and constraints facing different animal populations in relation to environmental and socio-economic conditions should also be addressed.

Understanding genetic diversity and factors affecting genetic variation. Education in factors affecting the dynamics of genetic variation in small populations is of great importance. The concept of the rate of inbreeding (and hence N_e) and its relation to the dynamics of genetic variance (and other measures) over time is important.

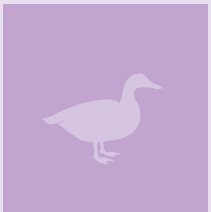
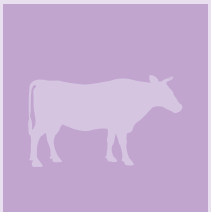
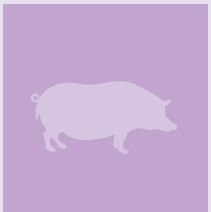
Characterization and documentation of animal populations. In any programme aiming to conserve AnGR for future utilization, as well as in other aspects of AnGR management, characterization and documentation of living populations and any cryoconserved material is extremely important. This should include the distribution of the populations, and their characteristics in relation to defined environments. It is also important to know how to monitor population changes and measure genetic relationships between breeds and to organize and utilize databanks.

***Ex situ* strategies and methods for conservation of animal genetic resources.** Courses on cryoconservation should cover methods for storing frozen semen, embryos, oocytes, cell cultures and DNA, as well as objectives for conservation programmes, methods of collection, sample sizes and record keeping.

Reproductive biotechnology. Having personnel who have received training in reproductive biotechnology will allow countries to undertake cryoconservation programmes independently. Furthermore, it will allow developing countries to take advantage, via technology transfer, of the extremely rapid development that is occurring in the field of advanced biotechnology in developed countries.

Sanitary and legal issues related to access and exchange of germplasm. Many aspects of AnGR management, including the operation of gene banks, require awareness and knowledge of national and international policies affecting the exchange of AnGR.

References and appendices

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Appendix A

Specialized procedures for field collection of germplasm[12]

Specific standard operating procedures have been developed for each of the methods and items of equipment used to collect semen and embryos. These standard operating procedures must be followed appropriately.

If samples are to be shipped to the gene bank via courier service, rather than transported by the collection team, contact the nearest courier service company prior to arriving at the collection site and check their latest drop-off times for overnight delivery to the gene bank. These time limits will dictate the working schedule for collection, initial evaluation, packaging and delivery of the collected germplasm.

When arriving at the site of collection, first introduce yourself to your contact person(s) (e.g. owner, breeder or staff member) and discuss clearly the detailed logistics of how the animals will be safely handled and the germplasm collected, handled, analysed and delivered to the gene bank.

Be prepared to answer questions and make sure (verbal agreement) that your contact person is comfortable with the procedures and protocols to be used on the selected animals. Make sure the contact person at the collection site duly completes and signs any written agreements involving ownership of the germplasm (e.g. material transfer agreement or donorship certificate). Sign the agreement (on the behalf of the gene bank) prior to starting collection. Make sure a clean and closed-in environment is available for handling and analysing the collected germplasm.

After the logistics are understood and confirmed, put on the necessary personal protective equipment. Respect the local biosecurity protocols (if applicable).

Unload and arrange the equipment for collection, handling and analysis of germplasm before proceeding to collect the germplasm. Follow the appropriate standard operating procedures for these steps. Make sure the incubator and the necessary media are at the proper temperatures before proceeding.

Use any waiting time (e.g. time required to warm media) to converse with your contact person about the procedures. Continue to encourage questions. Make sure your contact person understands that you value and care about the donor animals, and that all necessary precautions are being taken to minimize their stress and pain. Explain in detail any special procedures, such as electro-ejaculation or embryo flushing, and how the animal may react. Make sure your contact person is comfortable with the procedures.

[12] Adapted from a document provided courtesy of the Canadian Animal Genetic Resources Laboratory for Cryobiology.

When ready to proceed, arrange the collection area. Make sure all necessary precautions are taken to safely constrain the animal and that all persons involved are in a safe working environment.

Proceed with the collection of the germplasm following the appropriate standard operating procedure.

When the germplasm has been collected, immediately transfer and store it in the portable incubator and move it to the clean and closed-in area for subsequent procedures (e.g. evaluation, processing and packing or freezing).

Analyse the quality of the collected germplasm under the microscope. Take this time to show your contact person what the sample looks like under the microscope. The person may never have seen live embryos or motile sperm cells before. Semen concentration can be evaluated using a haemocytometer.

If a problem is encountered, re-collect germplasm from the animal.

If the germplasm cannot be transported or shipped immediately to the gene bank, proceed with the freezing procedures using the appropriate standard operating procedure for semen or embryos. If the collected germplasm can be shipped to the gene bank, proceed with preparing the collected germplasm for shipping to the gene bank.

Accordingly, prepare (freeze or pack) the collected germplasm following the appropriate standard operating procedure.

For frozen germplasm, use a dry-shipper, which should be prepared before visiting the collection site.

For fresh germplasm, transfer the collected material to 15 ml or 50 ml tubes (depending on the volume of the collected material).

Place the tube in a reusable and sealable plastic bag containing shredded absorbent paper and seal the plastic bag.

Place frozen ice packs on the bottom of the shipping container and cover with several layers of absorbent paper (1 cm thick) to avoid direct contact between the germplasm and the ice pack.

Place the sealed plastic bag containing the germplasm on top of the absorbent paper and cover with several more layers of absorbent paper. Secure the shipping container with packaging tape. The shipping container must be spill-proof to be accepted by the courier service company.

Place a shipping label (addressed to the gene bank) on the shipping container and contact the courier service company for pick-up and delivery. Otherwise, deliver the material yourself.

Upon arrival of the collected germplasm at the gene bank, a subsample should be analysed to determine the viability of the material after the cryopreservation process, following the appropriate standard operating procedure. A brief report, including viability, quality and the number of straws processed, should then be prepared and sent to the contact person at the collection site.

Appendix B

List of equipment needed for semen collection and freezing

MAJOR EQUIPMENT AND FACILITIES

- animal handling facilities
- adequate dedicated workspace for semen processing (clean, dry, climate controlled)
- warming cabinet (electric)
- microscope (10X eyepiece with 10X and 43X objectives)
- digital balance (6 kg ± 1 g)
- warm water bath (electric)
- sperm-counting equipment (haemocytometer or spectrophotometer)
- straw filler (preferably with attached label printer)
- semen freezing unit
- semen liquid nitrogen storage tanks (pre-tested)
- source of liquid nitrogen (very important to have a reliable source all year round)

SMALL EQUIPMENT AND CONSUMABLES

- coveralls and boots
- disposable gloves and boot covers
- helmets (head protection), especially for semen collection from stallions
- artificial vaginas, cones and collection tubes
- lubricant
- glass-lined thermos bottles
- thermometers (centigrade)
- semen diluents, extenders and cryoprotectants
- plastic semen straws
- assorted glassware and plasticware
- artificial light

Appendix C

Procedures for cryopreservation and thawing of semen from common livestock species

The following procedures require technical expertise. Before widespread implementation in a national conservation programme, the gene banking team should test the procedures on a small group of animals (preferably of the same breed). The test should include semen collection, freezing and thawing, and insemination to obtain a successful pregnancy.

CRYOPRESERVATION OF CATTLE SEMEN

For a review of bull semen collection, processing and handling, see the classic laboratory manual by Herman *et al.* (1994).

Freezing

1. The collected material should contain approximately 5 to 15 × 10^9 sperm per ejaculate. Avoid temperature changes in the semen during transfer to the processing laboratory. In the laboratory, use a water bath to maintain the temperature at approximately 35 °C.
2. Visually inspect the semen to ensure proper colour (usually milky white) and freedom from abnormalities (e.g. blood, pus). Filter the semen if contaminants such as hair or dung are visible.
3. Microscopically evaluate semen for motility (> 60 percent), and proportions of live sperm (> 70 percent) and abnormal (< 30 percent) sperm (see Appendix D).
4. Evaluate sperm concentration (haemocytometer or spectrophotometer) and determine the final volume needed for a diluted concentration of ≥ 100 × 10^6 sperm/ml.
5. Dilute the semen to the proper volume using the one-step or two-step procedure:
   One-step procedure
   - Add the entire sample to the volume of One-Step Diluent (milk + 20 percent egg yolk, 7 percent glycerol plus antibiotics required to yield a final concentration of 100 µg of tylosin, 500 µg of gentamicin, 300 µg of lincomycin, and 600 µg of spectinomycin in each ml of total volume) required to obtain the desired final sperm concentration.
   - Cool to +5 °C within one hour and maintain for at least two hours.

   Two-step procedure
   - Add Diluent A (milk + 10 percent egg yolk + antibiotics + 3 percent glycerol) to the semen sample in a progressive manner (over 15 minutes at 35 °C) until half the final volume is obtained.

- Cool to +5 °C within one hour.
- Add Diluent B until the final volume is reached (Diluent B consists of Diluent A +11 percent glycerol).
- Keep at +5 °C for two hours.
- For the above methods, protein-free products without milk or egg yolk are commercially available. Using these can reduce sanitation risks.

6. Fill pre-printed 0.25 ml straws with semen (25 to 30 million sperm/straw).
7. Transfer straws to liquid nitrogen vapour at -70 °C to -100 °C for nine minutes.
8. Transfer straws to liquid nitrogen tank and store.

Thawing

1. Before inseminating animals using a given batch of semen, thaw one straw to check for quality.
2. Thaw straws directly in a water bath at +37 °C for 30 seconds.
3. Inseminate cows and heifers transcervically ~12 hours after onset of standing oestrus.

CRYOPRESERVATION OF BUFFALO SEMEN

Freezing

1. Collect sperm (5×10^9 to 10×10^9 sperm per ejaculate) at 35 °C and transfer to the laboratory.
2. Avoid temperature changes in the semen after collection by placing the sample in a water bath (35 °C).
3. Evaluate sperm visually and microscopically.
4. Measure concentration and determine final volume for a concentration of 100×10^6 sperm/ml.
5. Add Diluent A (milk +10 percent egg yolk + antibiotics + 3 percent glycerol) to the semen sample in a progressive manner (over 15 minutes at 35 °C) until half the final volume is obtained.
6. Cool to +4 °C within 1.5 hours.
7. Add Diluent B up to the final volume (Diluent B consists of Diluent A + 11 percent glycerol).
8. Keep at +4 °C for four hours.
9. Meanwhile, fill pre-printed 0.5 ml straws with semen (about 50–60 million sperm/straw).
10. Cool from +4 °C to -140 °C in five minutes in liquid nitrogen vapour; then plunge into liquid nitrogen.
11. Transfer straws to liquid nitrogen storage.

Thawing

1. Thaw a sample from each batch to check for quality prior to use for AI.
2. Thaw straws directly in a water bath at +35 °C for 30 seconds.
3. Inseminate females transcervically 12 hours after onset of oestrus.

CRYOCONSERVATION OF SHEEP SEMEN

Freezing

1. The collected semen should contain about 4×10^9 sperm per ejaculate and should be maintained at 37 °C.
2. Evaluate semen visually and microscopically. Semen should be white and quite viscous.
3. Select only those ejaculates with mass motility above 10 percent and less than 30 percent abnormal sperm.
4. Evaluate sperm concentration and determine final volume for a concentration of 400×10^6 sperm/ml.
5. Dilute the semen to the proper volume using a one-step or two-step procedure. For a discussion of semen diluents and extenders for ram semen, see Paulenz *et al*. (2002).

   One-step procedure
   - Add the entire sample the volume of One-Step Diluent (300 mM Tris, 28 mM glucose, 95 mM citric acid, 2 percent [v:v] glycerol, 15 percent egg yolk, 1 mg/ml of streptomycin sulfate and 0.06 mg/ml of benzylpenicillin) required to obtain the desired final sperm concentration.
   - Cool to 4 °C within one hour and maintain for at least 1.5 hours.

   Two-step procedure
   - Add Diluent A to the semen at 30 °C to obtain 60 percent of the final volume (Diluent A consists of 25.75 g of lactose in 250 ml bi-distilled water + 20 percent egg yolk).
   - Cool progressively to +4 °C over two hours (0.2 °C/minute).
   - Prepare Diluent B: Reconstitute milk from a non-fat powder source (4 g into 100 ml bi-distilled water) and adjust pH to 6.6 with a Tris solution (20 g of tri-sodium-citrate-5.5 H_2O into 70 ml H_2O); then mix nine volumes of the resulting solution with one volume of glycerol.
   - Add Diluent B up to final volume (Diluent B consists of Diluent A +11 percent glycerol).
   - Add Diluent B in three equal parts, over 30 minutes, at 4 °C up to the final volume.
   - Keep the semen for 90 minutes at +4 °C.
6. Fill 0.25 ml plastic straws with semen.
7. Place straws horizontally in liquid nitrogen vapour at -75 °C for eight minutes.
8. Transfer directly into liquid nitrogen at -196 °C and store.

Thawing

1. Thaw straws in a water bath at 37 °C for 30 seconds.
2. Assess semen viability: mix one volume of sperm to four volumes of a sodium citrate solution (20 g of tri-sodiumcitrate-2 H_20 in 70 ml bi-distilled water) at 38 °C and estimate the proportion of motile sperm after five minutes and after two hours: only sperm with more than 30 percent of living spermatozoa at two hours should be used for insemination.
3. Proceed to surgical or non-surgical insemination of pre-synchronized recipients.

CRYOCONSERVATION OF GOAT SEMEN

Freezing

1. Collected semen should contain about 4×10^9 sperm per ejaculate when sampling occurs in season. Semen should be kept at 32 °C for transfer to the laboratory and processing.
2. Evaluate semen visually for any abnormalities.
3. Wash sperm with a Krebs Ringer Phosphate Glucose Solution (0.9 percent NaCl, 1.15 percent KCl, 1.22 percent $CaCl_2$, 2.11 percent KH_2PO_4, 3.82 percent $MgSO_4$-$7H_2O$, 5.24 percent glucose) by mixing one volume sperm with nine volumes of the washing solution at 28 °C to 32 °C, followed by centrifugation at 500 g for 15 minutes at 20 °C.
4. Discard the supernatant, and evaluate the semen (wave motion, concentration). Select only those ejaculates with a mass motility greater than 60 percent.
5. Calculate the final volume (V). Repeat centrifugation under the same conditions at 20 °C.
6. Dilute the semen to the proper volume using a one-step or a two-step procedure:
   One-step procedure
   - Add the entire sample to the volume of One-Step Diluent (300 mM Tris; 28 mM glucose; 95 mM citric acid; 2 percent [v:v] glycerol; 2.5 percent egg yolk; 1 mg/ml of streptomycin sulfate and 0.06 mg/ml of benzylpenicillin) required to obtain the desired final sperm concentration (≥ 200 million sperm per ml).
   - Cool to 4 °C within one hour and maintain for at least 1.5 hours.

   Two-step procedure
   - Prepare Diluent A: 80 ml of a sodium citrate solution (194 mg glucose +3.52 g sodium citrate +1.05 g streptomycin +50 000 IU penicillin in 100 ml distilled water) supplemented with 20 ml egg yolk.
   - Add V/2 of Diluent A to the pelleted sperm at 20 °C.
   - Cool to +4 °C within 30 minutes (at 0.5 °C/minute).
   - Add V/2 Diluent B (Diluent A + 14 percent v:v glycerol) in three successive steps with ten minute intervals. Diluent should also be at +4 °C.
7. Fill 0.25 ml plastic straws with semen.
8. Freeze straws in liquid nitrogen vapour for five minutes.
9. Plunge directly into liquid nitrogen and store.

Thawing

1. Thaw straws in a water bath at 37 °C for 30 seconds.
2. Assess post-thaw motility.
3. Proceed to insemination of does.

CRYOCONSERVATION OF HORSE SEMEN

Freezing

1. Collect sperm (~8×10^9 sperm per ejaculate) and filter on gauze.
2. Dilute semen to an approximate concentration of 50×10^6 sperm per ml in 37 °C holding medium. Various suitable media can be used. One example is SMED (100 ml

of nanopure water, NaCl at 37 mM, KCl at 10 mM, KH_2PO_4 at 0.07 mM, $NaHCO_3$ at 35.7 mM, $MgSO_4$ at 2.4 mM, HEPES at 10 mM, $CaCl_2$ at 1.7 mM, fructose at 84.3 mM and glucose at 5.5 mM supplemented with 0.3 g of bovine serum albumen at a pH of 7.2).

3. Centrifuge the extended semen (800 × g for 9 minutes or 400 × g for 13 minutes).
4. Remove the supernatant by aspiration.
5. Suspend the resulting pellets in a small amount of SMED and determine the sperm concentration.
6. Redilute the sample to 400 × 10^6 sperm per ml in skim milk–egg yolk extender (SMEY) (154.8 mM glucose, 4.2 mM lactose, 0.5 mM raffinose, 0.85 mM sodium citrate dihydrate, 1.25 mM potassium citrate, 29.8 mM HEPES, 51.5 mg/ml of skim milk powder, 1 mg/ml of ticarcillin with 2 percent egg yolk).
7. Cool the sample to 15 °C in a shipping container, which will allow it to be held for up to 24 hours.
8. After holding, further cool the sample to 5 °C over two hours.
9. Dilute with SMED-glycerol, so that the final dilution is 4 percent glycerol (up to a 1:1 dilution [v:v]).
10. Load the samples into printed straws.
11. Freeze in liquid nitrogen vapour (4.5 cm above the liquid for ten minutes)
12. Plunge into liquid nitrogen for storage.

Thawing

1. Thaw eight 0.5 ml straws (400 × 10^6 sperm) together in a water bath at +37 °C for 30 seconds.
2. Sperm should be deposited, usually daily, into the uterine body during the oestrous period.

CRYOCONSERVATION OF PIG SEMEN

Freezing

1. When collecting boar semen, it is critical to discard the first emission of sperm and to keep only the second (about 200 ml, rich in sperm, with 40 × 10^9 in total).
2. Filter the semen through gauze to eliminate the bulbo-urethral secretions.
3. Semen should be kept at 37 °C until processing, which should be done as quickly as possible. Ideally, semen will be extended within 15 minutes.
4. Two approaches can be applied, depending on whether collection is in the field or in the laboratory:
   In the field:
   - If semen is collected in the field, the ejaculate should be quickly diluted in 37 °C Beltsville Thawing Solution (BTS) (205 mM of glucose, 20.4 mM of sodium citrate, 14.9 mM of $NaHCO_3$, 3.4 mM of EDTA and 10 mM of potassium chloride), placed in the shipping container and cooled to 15 °C.
   - Upon arrival at the laboratory, centrifuge the samples at 800 × g for ten minutes.
   - Combine the pellets and determine the sperm concentration.

- Dilute samples using BF5 Cooling Extender (CE) (52 mM of TES, 16.5 mM of Tris [hydroxymethyl] aminomethane, 178 mM of glucose with 20 percent egg yolk at 325 mOsm) to 750 × 10^6 sperm per ml.
- Cool semen to 5 °C over 2.5 hours.
- Diluted semen with BF5 freezing extender (91.5 percent of CE, 6 percent glycerol, 2.5 percent Equex Paste [v:v]) to 500 × 10^6 sperm per ml.

At a semen collection and processing centre:

- Dilute one volume of sperm with one volume of Diluent A (anhydrous dextrose, 37 g; tri-sodium-citrate-2H_2O, 6 g; $NaHCO_3$, 1.25 g; EDTA diNa, 1.25 g; KCl, 0.75 g in 1 litre of bi-distilled water).
- Cool to 15 °C within two hours.
- Centrifuge at 800 × g for 20 minutes at 15 °C.
- Remove supernatant, which is diluted seminal plasma.
- Resuspend the pellet of sperm with about 10 volumes of Diluent B (8.5 g of fructose, 0.15 g of $NaHCO_3$, 0.015 g of cysteine, 116 ml of bi-distilled water, 34 ml of egg yolk and 1.69 g of equex STM [Nova Chemicals]) to obtain a concentration of 3 × 10^9 sperm per ml.
- Cool the suspension to 5 °C over two hours.
- Add one volume of the diluted sperm solution to one volume of Diluent C (Diluent C consists of Diluent B + 6 percent glycerol); Diluent C must be added in three steps to give a final concentration of 3 percent glycerol and 1.5 × 10^9 sperm per ml.
- Keep at 5 °C for about 90 minutes.

5. Load the semen into 0.5 ml straws.
6. Place straws horizontally at 5 cm above the level of boiling liquid nitrogen for 15 minutes (this will ensure a freezing rate of about 20 °C/minute down to -145 °C.
7. Plunge the straws into liquid nitrogen and store.

Thawing

1. Thaw straws in a 38 °C water bath for 20 seconds.
2. Mix the content of seven straws with 95 ml of Diluent A at 38 °C to obtain one dose for one AI.
3. Inseminate the sow within one hour after this dilution (5.3 × 10^9 sperm per AI).
4. See Pursel *et al.* (1975) and Almlid and Johnson (1987) for more information.

CRYOCONSERVATION OF RABBIT SEMEN

Freezing

1. Collect semen.
2. Prepare Diluent A. In 100 ml bi-distilled water, dissolve 3.028 g of Tris (trishydroxymethylaminomethane), 1.25 g of glucose, 1.67 g of citric acid-H_20, and 5 ml of DMSO (dimethyl-sulfoxide); add one volume of egg yolk to four volumes of solution (Vicente and Viudes-de-Castro, 1996).
3. Add four volumes Diluent A to one volume semen.
4. Progressively cool the diluted semen to +5 °C over one to three hours.

5. Prepare Diluent B. In 100 ml of bi-distilled water, dissolve 8.25 g of lactose and 1.3 ml of glycerol; add 20 percent egg yolk (one volume egg yolk to four volumes of solution).
6. Add one volume of Diluent B pre-cooled at +5 °C to one volume of diluted semen.
7. Fill 0.5 ml straws with semen.
8. Keep for ten minutes at +5 °C.
9. Freeze straws horizontally in liquid nitrogen vapour for three minutes at -120 °C.
10. Plunge the straws directly into liquid nitrogen and store.

Thawing

1. Thaw the straws in a water bath at +37 °C for one minute.
2. Inseminate does intravaginally, followed by an intra-muscular injection of 0.2 ml gonadotropin releasing hormone (GnRH).

CRYOPRESERVATION OF CHICKEN SEMEN

Two methods for cryopreservation of chicken semen are presented below. The primary difference is in the media used.

Method I

With this method the semen can be used directly for insemination after thawing. No need first to wash the semen free of the cryoprotectant.

Freezing

1. Prepare the media.

Table C1. Base medium – Lake's diluent*

	Molecular weight	g/litre	mmol/litre
Sodium-L-glutamate•H_2O	187.13	19.2	102.6
Magnesium acetate•4H_2O	214.46	0.7	3.3
Fructose	180.16	8	44.4
Potassium acetate	98.2	5	50.9
Polyvinylpyrrolidone	40 000	3	0.08

*Described in Lake (1968) as "Solution 1".
Note: The final pH of the medium should be 6.9.

The medium can be prepared with or without the cryoprotectant DMA (dimethylacetamide); the two variants are known as Lake-DMA and Lake's diluent, respectively. DMA concentration is 1.8 mol/litre = 157 g/litre = 16.7 volume percentage. To prepare Lake-DMA, take 15.7 g (or 16.7 ml) of DMA and add Lake's diluent to a total volume of 100 ml.

2. Store media in closed vessels to prevent evaporation and place in a temperature-controlled cool box at 5 °C.
3. Collect semen and transport it to the laboratory for further processing and freezing. All further handling is performed at 5 °C (cold room or open top cooler cabinet).

4. Determine the concentration of the non-diluted semen (spectrophotometer or haemocytometer).
5. Diluted semen with Lake's diluent to the desired concentration (e.g. 1.8×10^9 sperm/ml).
6. Add half a volume of Lake-DMA to one volume of semen and fill into 0.25 ml straws.
7. Freezing may be performed in a programmable freezer with a constant rate of 50 °C/minute) (maximum rate of most freezers) or in static liquid nitrogen vapour, 1–2 cm above the level of the liquid nitrogen.

Thawing

1. Remove straws from the liquid nitrogen and place in a 5 °C water bath.
2. Move the straws vigorously through the water for 30 seconds. Do not thaw bundles of straws as this will slow the thawing rate. Despite the low temperature of the water bath, the thawing rate will be high enough (average thawing rate from -190 to + 5 °C is 500–600 °C/minute).

Method II

With this method the cryoprotectant must be washed from the thawed semen prior to insemination.

Freezing

1. Collect semen (1.5×10^9 sperm per ejaculate).
2. Mix three volumes of semen (an ejaculate is about 300 µl) with four volumes of Diluent A (0.7 g of magnesium acetate [tetra-hydrated] + 19.2 g of sodium glutamate + 5.0 g of sodium acetate + 8.0 g of fructose + 3.0 g P.V.P [MW 10 000 to 15 000] in 1 litre of bi-distilled water).
3. Cool diluted semen immediately over 20–30 minutes to +5 °C (0.5 °C/minute).
4. At +5 °C, add one volume of diluted semen to one volume of Diluent B (Diluent A +11 percent glycerol). This gives a final concentration of 300×10^6 sperm/ml.
5. Equilibrate over 30 minutes at +5 °C.
6. Fill 0.25 ml straws with semen.
7. Freeze at a rate of 7 °C/minute from +5 °C to -35 °C, and at a rate of 8 °C/minute from -35 °C to -140 °C.
8. Plunge into liquid nitrogen and store.

Thawing

1. Prepare Diluent C: 0.8 g magnesium acetate (tetrahydrated) +1.28 g of potassium citrate +19.2 g of sodium glutamate +6.0 g of fructose +5.1 g of sodium acetate in 1 litre of bi-distilled water.
2. Prepare Diluent D: 0.8 g of magnesium acetate (tetrahydrated) +1.28 g of potassium citrate +15.2 g of sodium glutamate +6.0 g of glucose +30.5 g of B.E.S (N,N-bis-2 hydroxyethyl-2-amino-ethanesulfonic acid) +58 ml of NaOH (1M/litre) in 1 litre of bi-distilled water.

3. Thaw straws in a water bath at +5 °C for three minutes. Open and transfer semen to a glass beaker. Mix one volume of semen with 20 volumes of Diluent D, still at 5 °C.
4. Remove glycerol by centrifugation at 700 x g at +5 °C for 15 minutes.
5. Discard the supernatant and add one volume of sperm pellet to one volume Diluent D at +5 °C, and proceed to insemination of the hens.

CRYOCONSERVATION OF TURKEY AND DUCK SEMEN

For the present, it is recommended that turkey and duck semen samples be treated in the same way as rooster semen. However, ongoing research is expected soon yield species-specific protocols (Woelders, 2009). For insemination of turkeys, three straws per insemination are recommended.

Appendix D

Guidelines for basic semen analysis

Cryopreservation of semen can be a valuable step in ensuring the long-term survival of a given AnGR. However, these efforts will be futile and a waste of resources if the sperm is not fertile. Therefore, collected semen should be evaluated for quality and viability prior to processing and freezing (Jeyendran, 2000).

Semen should be evaluated as soon after collection as possible. Exposure to temperature changes, light and contaminants are generally detrimental to semen quality. Specific equipment, such as computer-aided semen analysis (CASA) systems can be used for automated evaluation of motility parameters; however, subjective evaluation under a microscope is also done, and technicians should be trained in these techniques.

Three basic characteristics should be addressed when evaluating semen and estimating sperm viability:

1. sperm concentration;
2. motility; and
3. morphology.

SPERM CONCENTRATION

Concentration is most accurately estimated with specialized equipment, such as a spectrophotometer. Counting can also be done manually, under the microscope, using a haemocytometer. A haemocytometer is a thick glass slide with two vessels serving as counting chambers. Each chamber is marked with a grid pattern etched into the glass, creating a background of squares. Diluted semen (usually 1:100) is pipetted into the chambers and the haemocytometer is viewed under a microscope. Because counting is easier and more accurate when the sperm are immobile, sperm are usually killed by including a small quantity of formaldehyde in the diluent. By counting the number of sperm within a sample of squares in the grid and considering the size of the squares of the grid and the dilution rate, the concentration and number of sperm in the original sample can be estimated. This information can then be used to determine the proper quantities of semen extender needed to obtain the desired concentration of sperm in the semen to be packed and cryopreserved. Sperm concentration can also be used as an indicator of the health of the semen donor (low concentration may indicate a health problem).

MOTILITY

The movement of the sperm should be checked: first, because movement indicates that the sperm are alive; and second, because motility is correlated with fertility. Two types of motility are usually evaluated – gross motility and individual motility.

Gross motility

1. Place a drop of diluted semen on a pre-warmed slide (37 °C) and examine sperm at 10X under a standard or phase-contrast microscope.
2. Look for general movement of the sperm with rapidly moving waves and individual swirls of sperm within the waves.

Individual motility

1. Place, on a pre-warmed slide, a drop of semen diluted (1:10) in saline solution, citrate or extender. When CASA equipment is used, chambers of a special design are needed (Makler chambers).
2. Position a cover slip over the mixture and examine under ≥40X magnification.
3. Estimate the proportion of individual sperm that are moving progressively forward (so-called "progressive forward motility"). This can be done by randomly picking ten or more sperm in different areas of the slide, counting those with forward motility and dividing by the total.
4. Although motility and its correlation with fertility may vary by species, the following figures can be used as a general guideline:
   - > 70 percent = very good
   - 50 to 60 percent = good
   - 40 to 50 percent = satisfactory
   - 30 to 40 percent = acceptable, but undesirable
   - < 30 percent = unsatisfactory

MORPHOLOGY

Abnormally shaped or damaged sperm are less likely to be capable of fertilization than normal sperm are (Berndtson *et al.,* 1981). Mixing the semen with a stain (e.g. eosin-nigrosin) highlights the sperm so that abnormalities can be readily identified under a microscope. Two kinds of abnormalities can be defined: primary abnormalities, which are assumed to have occurred in the testes; and secondary abnormalities, which arise in the epididymis or ejaculate. The proportion of normal sperm should be > 70 percent.

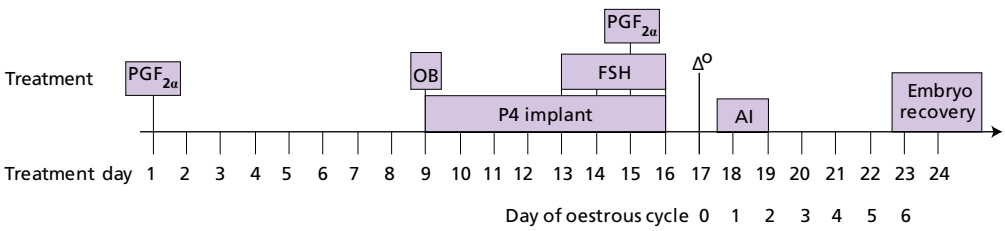
1. Place a drop or stripe of stain on a warmed microscope slide.
2. Add a small amount of semen.
3. Mix the semen and the stain with another slide and then use the narrow edge of the second slide to smear the mixture across the first slide.
4. Cover the mixture with a cover slip and examine under 1 000X magnification (oil immersion).
5. Examine the sperm for abnormalities, including the following:
   - abnormally shaped (tapered or pear-shaped) or sized (too large or small) heads;
   - missing or stump tails;
   - coiled or bent tails;
   - detached or creased (folded-over) acrosome;
   - clumping of multiple sperm; and
   - plasma droplets on tails.
6. Count at least 100 sperm and calculate the proportion (%) of abnormalities.
7. Discard semen if the proportion of abnormalities is too high (more than 30 percent).

Appendix E

Commonly used superovulation schemes for donor cattle embryo recovery

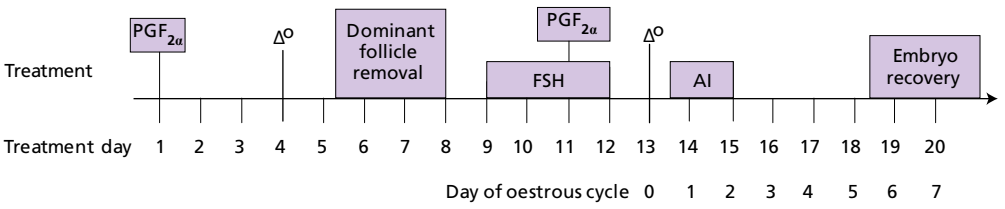
Figures E1 and E2 show two commonly used superovulation schemes.

FIGURE E1
Superovulation scheme 1



$PGF_{2\alpha}$ = prostaglandin F_2 alpha.
OB = 2 to 5 mg of oestradiol benzoate injected. Note that using this agent may be illegal in some countries.
Δ^o = standing oestrus.
P4 Implant = intravaginal progesterone implant (commercially available in most countries).
FSH = follicle stimulating hormone.

FIGURE E2
Superovulation scheme 2



$PGF_{2\alpha}$ = prostaglandin F_2 alpha.
Δ^o = standing oestrus.
P4 Implant = intravaginal progesterone implant (commercially available in most countries).
FSH = follicle stimulating hormone.

Appendix F

List of equipment and supplies needed for non-surgical embryo collection and transfer in cattle

EQUIPMENT

- animal holding chute
- temperature-controlled water bath
- liquid nitrogen tank
- stereo microscope with a heated stage
- Cassou gun and sheaths

RENEWABLE SUPPLIES

- boots (washable)
- coveralls (washable or disposable)
- plastic gloves (disposable)
- plastic foot covers
- paper towels
- disinfectant liquid
- liquid soap
- Petri dishes
- small sterile plastic embryo dishes
- scissors
- lidocane
- donor semen (2 to 4 units)
- plastic syringes 10 or 12 ml and needles for tailblock procedures
- flushing medium (Delbecco's phosphate-buffered saline)
- holding medium (e.g. TCM-199, a commercially available medium)
- foetal calf serum (commercially available)
- plastic straws for the embryos (e.g. 0.25 ml)
- straw labelling equipment (preferably with label printer)
- liquid nitrogen
- record book (very important!)

Appendix G

Technical procedures for cryopreservation and thawing of livestock embryos

It is anticipated that the team responsible for the cryopreservation and/or thawing of embryos will have demonstrated their technical expertise before implementing an embryo recovery and banking programme. Freezing cattle, goat and sheep embryos is now a common practice for in-field use. Freezing early-stage horse embryos is practised commercially, but freezing later-stage equine embryos has still has not been widely mastered. Freezing pig embryos is more difficult, although there have been some recent advances in this area. For these reasons, only procedures for ruminant and horse embryos are outlined here.

CRYOPRESERVATION OF BOVINE EMBRYOS

Freezing

1. Collect embryos non-surgically from a superovulated donor female at day seven of the oestrous cycle, evaluate them for morphological development, and assign them an embryo-quality grade. The embryos should be at the compact morula and blastocyst stages if the procedure is progressing correctly.
2. Maintain the embryos in a clean environment that is free from possible contaminants and maintained at around room temperature (20 to 30 °C). Freezing should be done as soon as possible after collection (i.e. within four to six hours).
3. While grading quality, check that the zona pellucida is intact on all embryos (under 50X light microscope) and that embryos are free from adherent material.
4. Wash the embryos from one donor (no more than ten embryos) in five consecutive baths of phosphate-buffered saline (PBS) solution containing broad-spectrum antibiotic and 0.4 percent bovine serum albumin. Use different glass and plastic ware for each donor and new micropipettes for each subsequent wash.
5. Treat the embryos to two washes with trypsin (60 to 90 seconds in total) to remove or deactivate any viruses. Trypsin wash is sterile porcine-origin trypsin (1:250) in Hank's balanced salt solution at a concentration of 0.25 percent.
6. Wash the embryos an additional five times in PBS – antibiotic solution with 2 percent bovine serum albumin.
7. Equilibrate the embryos at room temperature for ten minutes in PBS with 10 percent foetal calf serum and 10 percent glycerol.
8. Place the embryos between two or four air bubbles in 0.25 ml sterile, pre-labelled plastic straws. Most often, one embryo is cryopreserved per straw.

9. Place straws horizontally in a freezing unit and cool from room temperature to -7 °C at a rate of 5 °C/minute.
10. Induce seeding at -7 °C by contact at the extreme end of the straw with liquid nitrogen-cooled tweezers, and freeze the embryo to -35 °C at a rate of 0.5 °C/minute.
11. Plunge the straws directly into liquid nitrogen and then store them in liquid nitrogen at -196 °C.

Thawing

1. Select the appropriate straw from the liquid nitrogen storage tank. Important! Do not bring the straws up above the frost line of the liquid nitrogen tank (the neck of the tank) until the correct straw is identified for embryo transfer.
2. Thaw the straw rapidly in a water bath at 20 °C for 30 seconds or 39 °C for 8 to 25 seconds, depending on the initial embryo-freezing rate. Then cut the ends of the straw and remove the embryo. Rehydrate the embryo in 1M (molar) sucrose solution for ten minutes; then reduce the sucrose concentration in a stepwise procedure.
3. Prepare the Cassou gun and clean the perineal region of the recipient. Transfer the contents of one straw (one embryo) into the uterine horn corresponding to the corpus luteum of a day-seven recipient female.

CRYOPRESERVATION OF GOAT AND SHEEP EMBRYOS

Freezing

1. Follow steps 1 to 6 of the bovine protocol.
2. Equilibrate embryos collected from one donor female at room temperature for ten minutes in PBS with 10 percent foetal calf serum and 10 percent cryoprotective agent. Ethylene glycol can be used as a cryoprotectant for sheep, whereas glycerol is to be used for goats.
3. Place one or two embryos between two or four air bubbles in a 0.25 ml sterile pre-labelled plastic straw.
4. Place the plastic straws horizontally in the freezing unit and cool from room temperature to -7 °C at the rate of 5 °C/minute.
5. Induce seeding at -7 °C and freeze embryos to -30 °C at a rate of 0.3 °C/minute.
6. Plunge straws directly into liquid nitrogen. Store the straws in liquid nitrogen at -196 °C.

Thawing

1. Select the appropriate straw from the liquid nitrogen storage tank. Important! Do not bring the straws up above the frost line of the liquid nitrogen tank (neck of the tank) until the correct straw is identified for embryo transfer. Thaw one straw at a time and transfer the embryo(s) before thawing the next straw.
2. Thaw the straw rapidly in a water bath at 20 °C for 30 seconds. Then cut the ends of the straw and remove the embryo. Rehydrate the embryo in 0.5M sucrose solution for ten minutes then reduce the sucrose concentration in a stepwise procedure.
3. Proceed to transfer the embryo into a prepared female.

CRYOPRESERVATION OF HORSE EMBRYOS

Freezing

The following procedure is the slow-freezing approach of Czlonkowska *et al*. (1985). Commercial vitrification kits are also an option.

1. Collect embryos approximately six to seven days after insemination. Transport them to the processing laboratory and wash them in PBS solution.
2. Equilibrate embryos collected from one donor female at room temperature by washing four separate times (for ten minutes each wash) in solutions of 2.5, 5, 7.5 and 10 percent glycerol in PBS.
3. Load each equilibrated embryo into a 0.25 ml straw in a drop of 10 percent glycerol solution between two air bubbles.
4. Seal the straw and place vertically in a freezing rack.
5. Cool the straw from room temperature to -6 °C at a rate of 1 °C /minute.
6. Hold the straw at -6 °C for five minutes and then seed with forceps cooled in liquid nitrogen.
7. Freeze the straw by reducing temperature to -33 °C at a rate of 0.3 °C /minute.
8. Hold the straws at -33 °C before plunging into liquid nitrogen.
9. Store the straws in liquid nitrogen.

Thawing

1. Remove the straw(s) from the tank and expose them to air for 10 seconds.
2. Submerge the straws for one minute in a 35 °C water bath.
3. Expel the embryos into a PBS solution containing 10 percent glycerol and 0.25M sucrose.
4. Wash out the cryoprotectant by incubating the embryos for ten minutes each in solutions of 0.25M sucrose in PBS with progressively decreasing concentrations of glycerol (7.5, 5, 2.5 to 0 percent).
5. Proceed to embryo transfer.

Appendix H
Bovine oocyte collection procedures

TECHNIQUE I – COLLECTION FROM DISSECTED OVARIES VIA "SLASHING"

Materials

- oocyte collection medium (OCM) – see Table H1
- L-glutamine
- BSS + heparin
- Pen/Strep
- 1 × saline solution (0.9 percent) – see Tables H3 and H4
- Petri dishes
- bench top paper
- 400 ml beaker
- scalpel handle
- scalpel blades (sizes #11 and #20)
- haemostat
- mouth pipette (optional)

Preparation for oocyte collection

1. Prepare Oocyte Collection Medium + supplements (OCM+ – see Table H2).
   a. Add the following to 1 litre of Oocyte Collection Medium (OCM – see Table H1)
      - BSS + heparin (stock) 20 ml
      - Pen/Strep (e.g. Gibco 15140-122) 10 ml
      - L-glutamine (e.g. Gibco 25030-081) 10 ml
   b. Place OCM+ at room temperature at least two hours before the arrival of the ovaries.
2. Set up working area for collection (one station per person).
   a. Cover bench top with paper.
   b. Place the following on the bench top:
      - 400 ml beaker
      - scalpel handles
      - scalpel blades (sizes #11 and #20)
      - haemostats
      - 1 × saline solution brought to room temperature to wash ovaries

Procedures

1. Clean the ovaries with 1 × saline solution.
2. Slash the ovaries to release oocytes.

a. Add 150 ml OCM+ to each beaker.
b. Attach a haemostat to the base of the ovary. Cut the excess tissue away from the ovarian stalk by using a #20 scalpel blade and blot off blood with absorbent tissue.
c. Slash follicles in the size range 2–8 mm. Hold the ovary above the beaker and make several small incisions to each follicle using a #11 scalpel blade. Follicular fluid and blood in the collection medium may result in clotting of the medium, thereby rendering it impossible to retrieve oocytes. To prevent clotting, avoid slashing large follicles (> 10 mm) and corpora lutea. Once all the follicles on the ovary have been slashed in one direction, go back over it and slash each in the opposite direction, making an X through each follicle. This effectively opens the follicles and allows the oocytes to be washed out.
d. Submerge the ovary in OCM and swirl it several times. Repeat this process until all ovaries have been processed, with a maximum of ten ovaries per person.

3. Identify and isolate oocytes
a. Once a group of ovaries has been processed, fill the beaker with OCM+ and incubate it at room temperature for five minutes to allow oocytes to settle.
b. Bathe the outside of the beaker with ethanol and place the beaker on a stable surface and allow oocytes to settle again for a few minutes.
c. Using aseptic technique, slowly aspirate OCM from the top of the beaker down to 50 ml. Be careful to not disturb the oocytes on the bottom of the beaker. Stop immediately if this occurs and allow the oocytes to settle again. Fill the beaker again with OCM+ and let it settle for another five minutes. Then slowly aspirate down to 50 ml.
d. Transfer the remaining media, with oocytes, to two grid plates. Wash the beaker with about 20 ml of OCM and add to the grid plates.
e. Collect cumulus oocyte complexes as fast as possible using a mouth pipette. Place the retrieved cumulus oocyte complexes into the first Petri dish containing OCM+ for further washing.
f. Transfer the cumulus oocyte complexes from the first dish to the next, leaving all debris behind (repeat twice to assure that oocytes are clean of debris).

Video demonstrations of the above techniques can be found at http://www.animal.ufl.edu/hansen/ivf/Videos.htm courtesy of Dr P. Hansen, University of Florida.

TECHNIQUE II – COLLECTION VIA ASPIRATION

Materials

- holding medium (HM) – see Table H5
- 1 × saline solution (0.9 percent)
- Petri dishes
- bench top paper
- 50 ml conical tube
- 10 ml plastic syringe
- 20–22 gauge needle
- mouth pipette (optional)

Preparation

1. Set up working area for collection (one station per person) by covering bench to with paper.
2. Place the following materials on the bench top:
   - 50 ml conical tube
   - tube holder
   - 10 ml air syringe
   - 20–22 gauge needle
   - saline brought to room temperature to wash ovaries

Procedures

1. Clean the ovaries with 1 × saline solution.
2. Extract the oocytes from the ovaries via aspiration.
   a. Use the syringe to aspirate every follicle in the size range 2–8 mm.
   b. Deposit the follicular fluid slowly in the 50 ml conical tube.
   c. After aspirating all the ovaries, allow the oocytes to settle.
   d. Using a Pasteur pipette, slowly aspirate the oocytes from the bottom of the 50 ml tube. Be careful not disturb the oocytes.
   e. Place the oocytes in a grid dish containing enough HM to cover the dish.
   f. Wash the oocytes with HM three times.
   g. Collect cumulus oocyte complexes as fast as possible using a mouth pipette. Place the retrieved cumulus oocyte complexes into the first Petri dish containing OCM+ for further washing.
   h. Transfer the cumulus oocyte complexes from the first dish to the next, leaving all debris behind (repeat twice to assure that oocytes are clean of debris).

PREPARATION OF DIFFERENT MEDIA FOR OOCYTE COLLECTION

Table H1. Oocyte collection medium (OCM) (without supplements)

Ingredient	Quantity/litre	Location
Medium 199 with Hank's Salts (e.g. Sigma M-0393)	1 bottle = 10.6 g	Refrigerator
$NaHCO_3$ (e.g. Sigma S-5761)	0.35 g	TC cabinet*
HEPES (e.g. Sigma H-3375)	5.95 g	TC cabinet

*TC cabinet = temperature controlled cabinet.

1. Mix Medium 199, HEPES, and $NaHCO_3$ with 0.95 litres MilliQ H_20.
2. Using 10M NaOH, adjust pH to ~7.4 and bring the volume to 1 litre.
3. Sterile-filter the medium into bottles.
4. Store at 4 °C for up to three months.
5. Label as "OCM" or "OCM - supplements", including date of preparation.

Table H2. Oocyte collection medium (OCM) (with supplements)

Ingredient	Amount	Location
BSS + heparin (stock)	20 ml	Freezer
Pen/Strep (e.g. Gibco 15140-122)	10 ml	Freezer
L-glutamine (Stock)	10 ml	Freezer
OCM - supplements (Stock)	1 litre	Refrigerator

1. On the day of use, add BSS + heparin, Pen/Strep and L-glutamine to the OCM.
2. Date and label "OCM + Supplements".
3. Make 500ml if only one person is slashing – half of Pen/Strep and L-glutamine aliquots can be refrozen.

Table H3. 10X saline stock solution

Ingredient	Amount	Location
NaCl	90 g	TC cabinet*
MilliQ H_2O	1 000 ml	

*TC cabinet = temperature-controlled cabinet.

1. Combine ingredients and mix thoroughly.
2. Sterile-filter the solution.
3. Date, label "10 × saline", and store at 4 °C.

Table H4. 1X transport saline 0.9 percent (prepare from 10 × solution)

Ingredient	Amount	Location
10 × saline	100 ml	Refrigerator
Pen/Strep (Gibco 15140-122)	10 ml	Freezer

1. Mix ingredients and fill with MilliQ H_2O to 1 litre.
2. Date, label and store at 4 °C.

Table H5. Holding medium (HM) – HEPES Talp

Ingredient	Amount	Location
BSA, fraction V (Sigma A-3311)	120 mg	Refrigerator
HEPES-TL	39.2 ml	Refrigerator
Na pyruvate (Sigma P-5280)	0.4 ml	Refrigerator
Pen/Strep (Gibco 15140-122)	0.4 ml	Freezer

1. Combine and mix ingredients, ensure that the final pH is approximately 7.4.
2. Sterile-filter the medium.
3. Date, label "HEPES-Talp" and store at 4 °C for one week.

Appendix I
In vitro maturation of bovine oocytes

MATERIALS

- Petri dishes
- mouth pipette (optional)
- heat-pulled Pasteur pipette
- oocyte maturation medium (OMM – See steps 1 and 2 under "Preparation" below)
- medical grade mineral oil
- warming plate
- incubator
- laminar flow hood

PREPARATION

1. Add 8.835 ml of Medium 199, 20 µl of FSH stock, 125 µl of LH stock, 1 ml of foetal bovine serum (FBS) and 100 µl of Pen/Strep to a 15 ml tube. Filter the medium through a 0.4 µm membrane (see Zhang *et al.,* 1992).
2. Add 10 µl oestradiol stock.
3. Prepare 25-µl maturation droplets of filtered OMM on a Petri dish (five to nine droplets per dish), cover with mineral oil and equilibrate in an incubator (5 percent CO_2) for at least three hours.
4. Prepare two to four 75 µl wash droplets of OMM on a separate Petri dish.

PROCEDURE

1. Rinse the cumulus oocyte complexes at least twice in the 75 µl droplets containing OMM.
2. Transfer ten cumulus oocyte complexes to each 25 µl droplet of OMM under oil.
3. Incubate for 22 hours at 39 °C and 5 percent CO_2.

It is essential that the oocytes be collected, washed and incubated in OMM as quickly as possible to ensure maximum developmental rates. This entire process should never exceed two hours.

Blood is toxic to oocytes and embryos, so it is imperative that they be washed thoroughly to remove blood prior to transfer to OMM.

Appendix J
Bovine *in vitro* fertilization protocol

MATERIALS

- centrifuge carriers
- Percoll (ENHANCE-S Plus)
- sperm-TL (Table J1)
- IVF Talp (Table J4)
- HEPES Talp (Table J5)
- SP Talp (Table J6)
- penicillamine, hypotaurine and epinephrine (PHE) medium
- heparin
- CR1aa medium
- 15 ml conical tubes
- Petri dishes
- mouth pipette (optional)
- heat-pulled Pasteur pipettes
- sterile pipette tips and pipettor
- microcentrifuge tubes (1.5 ml)
- medical grade mineral oil
- laboratory tissues (e.g. Kimwipes®)
- standard haemocytometer
- water bath
- incubator
- centrifuge
- stereomicroscope
- laminar flow hood

PREPARATION

1. Prepare PHE medium.
   a. Prepare primary stocks of 2 mM penicillamine (3 mg/10 ml saline), 1 mM hypotaurine (1.09 mg/10 ml saline) and 250 mM epinephrine (1.83 mg dissolved in 40 ml of the following solution: 165 mg 60 percent Na lactate syrup, 50 mg Na metabisulfate and 50 ml H_2O).
   b. Combine 5 ml of 1 mM hypotaurine, 5 ml of 2 mM penicillamine, 2 ml 250 mM epinephrine and 8 ml saline and sterile filter.
   c. Aliquot PHE medium into sterile microfuge tubes and store in a light-resistant container at -20 °C indefinitely.

2. Move PHE medium (400 µl) and heparin (200 µl) from freezer to oven (39 °C). PHE medium should be covered with aluminium foil (light sensitive).
3. Make fertilization microdrops.
   a. Make five 44 µl drops of fertilization media (IVF-Talp) in each 35 mm dish. Cover with pre-warmed and pre-gassed mineral oil (ten oocytes per drop).
   b. Make four 70 µl drops of fertilization media in a 35 mm dish (washing medium). Cover with mineral oil.
   c. Equilibrate in CO_2 incubator (39 °C) for at least two hours.
4. Fill one conical tube with ~10 ml HEPES-Talp. Label the tube.
5. Fill one conical tube with ~5 ml of IVF-Talp. Label the tube.
6. Fill one conical tube with 5 ml SP-Talp. Label the tube.
7. Also prepare one conical tube with 8 ml HEPES-Talp.
8. Transfer tubes of HEPES-Talp (cap tightly) and SP-Talp (cap tightly) to the water bath (39 °C).
9. Transfer IVF-Talp (cap loosely) to CO_2 incubator.
10. Prepare Percoll Gradient: Label one conical tube "Percoll Gradient" and fill the tube with 100 µl of sperm TL and 900 µl of ENHANCE-S Plus.
11. Carefully transfer Percoll Gradient to the water bath.

PROCEDURES

1. At 22 to 24 hours post-maturation, thaw one straw of semen in water at 39 °C for 30 seconds. When getting semen straws out of the liquid nitrogen tank, be sure not to raise any of the other straws above the frost line. Use special semen forceps.
2. Dry a straw, hold it in a laboratory tissue to keep it warm and prevent exposure to light! Cut off the sealed ends and slowly layer thawed semen on top of the Percoll Gradient. Centrifuge at 1 200 rpm for 20 minutes.
3. Check the viability of the thawed semen. Dilute one drop of semen with SP-Talp and place 5 µl of the suspension on a slide. View at 40X magnification to assure that motile sperm are present.
4. While the centrifuge is running, pour 1 ml of HEPES-Talp (from conical tubes in CO_2 incubator) into a Petri dish (35 mm). Remove oocytes from each well of OMM plate and transfer to the dish containing HEPES-Talp.
5. Transfer the oocytes to the washing medium (from step 3b in Preparation).
6. Transfer up to ten oocytes into each 44 µl fertilization microdrop. Return the IVF plate with the microdrops to the incubator when finished. You only have 15 minutes to wash and transfer all oocytes to the microdrops. Set a timer and ask for help if necessary.
7. After the centrifuge stops, carefully remove the carrier with the Percoll Gradient from the centrifuge. There should now be a sperm pellet, if not you must completely start again with new gradient and semen.
8. Within the laminar flow hood, aspirate the Percoll down to the sperm pellet. Slowly add the 5 ml of pre-warmed SP-Talp to the conical tube containing the sperm pellet. Transfer the tube to the second pre-warmed centrifuge carrier and centrifuge at 1200 rpm for an additional ten minutes.

9. After the centrifuge stops, aspirate the SP-Talp down to the sperm pellet. Return the conical tube with the sperm pellet to the water bath.
10. Determine sperm pellet concentration (see standard haemocytometer procedure).
    - Gently swirl the sperm pellet to mix the sperm with any remaining medium. Use a clean pipette tip to transfer 5 µl of sperm into 95 µl of water; pipette gently to mix.
    - Clean the haemocytometer and coverslip by washing with water followed by 70 percent ethanol; dry with a tissue.
    - Using a new pipette tip, transfer 10 µl of diluted sperm into each chamber (each side) of the haemocytometer.
    - Use 40X magnification to count sperm cells in the five squares arranged diagonally across the central square on one side of the haemocytometer. Use an event counter to keep track of how many cells are counted. Record the count.

    a. Continue counting on the second side of the haemocytometer, counting the five diagonally arranged squares to obtain the total haemocytometer count. If the count of one side varies more than 10 percent from that on the other side, then the diluted sample was not properly mixed. Repeat the procedure starting at step a. When the count is consistent, record the total count and continue the procedure.

    b. Clean the haemocytometer and coverslip with water followed by ethanol.
11. Prepare sperm suspension for insemination.

    Note that the final sperm suspension used for IVF is composed of fertilization medium and sperm pellet produced by Percoll separation. The following instructions summarize and simplify the determination of quantities of pelleted sperm and medium required:

    a. Use the following formula to calculate the volume of sperm pellet needed per 300 µl of final sperm suspension, assuming that 1×10^6 sperm/ml is the desired concentration in the final fertilization medium, using the formula:
       - 7 500/x = µl of sperm pellet to make 300 µl of final sperm suspension when inseminating with 1×10^6 sperm/ml; where x is the average haemocytometer count (total haemocytometer count divided by two).

    b. If a concentration other than 1×10^5 sperm/ml is desired, then the volume of the sperm pellet must be adjusted to accommodate the difference. To adjust this volume perform the following calculation:
       - Sperm concentration desired/1×10^6 (sperm/ml) = sperm concentration adjustment factor.
       - Multiply the volume of the sperm pellet by this adjustment factor to yield the volume of sperm pellet needed to prepare 300 µl of final sperm suspension at the desired concentration.
       - Example: if a bull requires 2×10^6 sperm/ml rather than 1×10^6 sperm/ml, 2×10^6 sperm/ml/1×10^6 sperm/ml = adjustment factor of 2.
       - (adjustment factor) × (µl of pellet needed for 1×10^6 sperm/ml) = µl of sperm pellet needed to yield 2×10^6 sperm/ml in 300 µl of final sperm suspension providing 2×10^6 sperm/ml in the fertilization microdrop.

    c. Calculate the volume of fertilization medium needed in the final sperm suspension (i.e. by subtracting the volume of the sperm pellet from 300 µl).

d. Place the calculated amount of fertilization medium into an Eppendorf microcentrifuge tube. Then add the calculated amount of sperm pellet to the tube. Sperm stick to plastic, so add the fertilization medium to the tube first. Mix gently by pipetting up and down several times within the tube. Immediately begin fertilizing the oocytes in the microdrops, as the pH of this solution will change rapidly.

12. Fertilization
    a. Add 2 µl heparin (for a final concentration of 2 µg/ml of heparin in the fertilization medium), 2 µl of PHE medium and 2 µl of final sperm suspension to each fertilization microdrop.
    b. Record the time and the date on each IVF microdrop plate.
    c. Incubate for 18 hours at 39 °C in a humidified atmosphere of 5 percent CO_2 in air.
13. Embryo culture
    a. Make five 20 µl microdrops of CR1aa in a 35 mm dish. Cover the drops with oil. Incubate for at least 20 minutes to the allow medium to equilibrate.
    b. Thaw one vial of hyaluronidase (1 mg/ml). Place the solution in a 1.5 ml conical tube. Incubate the tube in the water bath for about two minutes.
    c. Place the oocytes (which are hopefully zygotes/embryos) in the tube containing the hyaluronidase solution. Each tube can accommodate up to 300 embryos.
    d. Vortex the oocytes at maximum speed for three minutes.
    e. Transfer the oocytes to one 35 mm dish containing HEPES-Talp. Rinse the tube with HEPES-Talp to capture all embryos.
    f. Wash the oocytes four times in CR1aa.
    g. Place ten oocytes in each microdrop of CR1aa.
    h. At day three, transfer the oocytes into new microdrops of CR1aa.
    i. Check development at day seven.

MEDIA PREPARATION

Table J1. Sperm-TL

Ingredient	Final (mM)	mg/100ml	Location
NaCl (Sigma S-5886)	100	582	TC cabinet*
KCl (Sigma P-5405)	3.1	23	TC cabinet
$NaHCO_3$ (Sigma S-5761)	25	209	TC cabinet
NaH_2PO_4 (Sigma S-5011)	0.29	3.48	TC cabinet
HEPES (Sigma H-3375)	10	238	TC cabinet
Lactic acid (Sigma L-7900)	21.6	183.4 µl	Refrigerator
Phenol red (Sigma P-0290)	1 µl/ml	100 µl	TC cabinet
$CaCl_2$•$2H_20$ (Sigma C-7902)	2.1	29	Desiccator
$MgCl_2$•$6H_20$ (Sigma M-2393)	1.5	31	Desiccator

*TC cabinet = temperature-controlled cabinet.

1. Add NaCl, KCl, NaHCO3, NaH2PO4, HEPES, lactic acid, and phenol red to a beaker. Bring volume to 80 ml with double distilled water (ddH2O) and dissolve completely.
2. CaCl2•2H2O and MgCl2•6H2O should be dissolved in a small amount of ddH2O before being added to other ingredients.

3. Check to ensure that pH = ~7.4 and then adjust volume to 100 ml with ddH2O.
4. Sterile-filter the resulting solution into a bottle.
5. Date, label "SP-TL", and store at 4 oC for up to two weeks.

Table J2. IVF-TL

Ingredient	Final (mM)	mg/100ml	Location
NaCl (Sigma S-5886)	114	666	TC cabinet*
KCl (Sigma P-5405)	3.2	23.5	TC cabinet
$NaHCO_3$ (Sigma S-5761)	25	210.4	TC cabinet
NaH_2PO_4 (Sigma S-5011)	0.34	4.08	TC cabinet
Lactic acid (Sigma L-7900)	10	84.92 µl	Refrigerator
Phenol red (Sigma P-0290)	1 µl/ml	100 µl	TC cabinet
$CaCl_2•2H_20$ (Sigma C-7902)	2	30	Desiccator
$MgCl_2•6H_20$ (Sigma M-2393)	0.5	10	Desiccator

*TC cabinet = temperature-controlled cabinet.

1. Add NaCl, KCl, NaHCO3, NaH2PO4, lactic acid, and phenol red to a beaker.
2. CaCl2•2H2O and MgCl2•6H2O should be dissolved in a small amount of ddH2O before being added to the other ingredients.
3. Bring the volume to 80 ml with ddH2O, and dissolve completely.
4. Check for pH ~7.4 and then adjust the volume to 100 ml with ddH2O.
5. Sterile-filter into a bottle.
6. Date, label "IVF-TL", and store at 4 oC for two weeks.

Table J3. HEPES-TL

Ingredient	Final (mM)	mg/500 ml	Location
NaCl (Sigma S-5886)	114	3330	TC cabinet*
KCl (Sigma P-5405)	3.2	120	TC cabinet
$NaHCO_3$ (Sigma S-5761)	2	84	TC cabinet
NaH_2PO_4 (Sigma S-5011)	0.34	20.4	TC cabinet
HEPES (Sigma H-4034)	10	1,200	TC cabinet
Lactic acid (Sigma L-7900)	10	424.6 µl	Refrigerator
Phenol red (Sigma P-0290)	1 µl/ml	500 µl	TC cabinet
$CaCl_2•2H_20$ (Sigma C-7902)	2	150	Desiccator
$MgCl_2•6H_20$ (Sigma M-2393)	0.5	50	Desiccator

*TC cabinet = temperature-controlled cabinet.

1. Add NaCl, KCl, NaHCO3, NaH2PO4, HEPES, lactic acid, and phenol red to a beaker.
2. CaCl2•2H2O and MgCl2•6H2O should be dissolved in a small amount of ddH2O before being added to the other ingredients.
3. Bring the volume to 450 ml with ddH2O and dissolve completely.
4. Check for pH ~7.4 and then adjust volume to 500 ml with ddH2O.
5. Sterile-filter into a bottle.
6. Date, label "HEPES-TL", and store at 4 oC for two weeks.

Table J4. IVF-Talp

Ingredient	Amount	Location
BSA, EFAF (Sigma A-6003)	60 mg	Refrigerator
IVF-TL	9.8 ml	Refrigerator
Na pyruvate (Sigma P-5280)	100 µl	Refrigerator
Pen/Strep (Gibco 15140-122)	100 µl	Freezer

1. Combine and mix all ingredients; pH should be ~7.4.
2. Sterile-filter the resulting solution.
3. Date, label "IVF-Talp", and store at 4 oC for up to one week.

Table J5. HEPES-Talp

Ingredient	Amount	Location
BSA, Fraction V (Sigma A-4503)	60 mg	Refrigerator
HEPES-TL	20 ml	Refrigerator
Na pyruvate (Sigma P-4562)	0.2 ml	Refrigerator
Pen/Strep (Gibco 15140-122)	0.2 ml	Freezer

1. Combine and mix all ingredients; pH should be ~7.4.
2. Sterile-filter the resulting solution.
3. Date, label "HEPES-Talp", and store at 4 oC for up to one week.

Table J6. Sperm–Talp (SP-Talp)

Ingredient	Amount	Location
BSA, Fraction V (Sigma A-4503)	60 mg	Refrigerator
SP-TL(Specialty Medium BSS-009-D)	9.5 ml	Refrigerator
Na pyruvate (20mM Stock)	0.5 ml	Refrigerator
Pen/Strep (Gibco 15140-122)	100 µl	Freezer

1. Combine and mix all ingredients; adjust pH to ~7.4.
2. Sterile-filter the resulting solution.
3. Date, label "Sperm-Talp", and store at 4 oC for up to one week.

Table J7. 10X SP-TL (for Percoll Gradient)

Ingredient	g/100 ml	Location
NaCl (Sigma P-5886)	4.675	TC cabinet*
KCl (Sigma P-5405)	0.23	TC cabinet
NaH_2PO_4 (Sigma S-5011)	0.348	TC cabinet
HEPES (Sigma H-4034	2.38	TC cabinet

*TC cabinet = temperature-controlled cabinet.

1. Combine and mix ingredients. Do not adjust pH!
2. Bring volume to 100 ml with double distilled H2O.
3. Sterile filter.
4. Date, label "10X SP-TL", and store at 4 oC for up to one month.

Appendix K

Harvesting tissue samples for cryoconservation of somatic cells

Collection of somatic cells for gene banking can be done via two approaches: 1) collection and cryopreservation in the field; and 2) field collection with processing and freezing in the laboratory. The choice will depend on the situation in the given country. Protocols for both approaches are described below.

IN THE FIELD (SAME FOR BOTH PROTOCOLS)

1. To prevent the infection of tissue samples, ensure that the conditions for sampling are as clean as possible, especially the working area for placing the sampling equipment.
2. Identify the animals to be sampled.
3. Restrain each animal and record as much of the following information as possible (see Section 9 for more details about data collection):
   - site-specific data, including GPS coordinates;
   - animal-specific information (Section 10), including a digital photo; and
   - sample-specific information (e.g. vial and animal numbers).
4. Clean and disinfect the area of skin from where the sample is to be taken.

PROTOCOL 1 – ONE-STEP COLLECTION AND FREEZING IN THE FIELD[12]

In the laboratory prior to field collection

1. Prepare a cryoprotectant medium by mixing 70 percent PBS and 30 percent of 87 percent glycerol, yielding a final concentration of 26.1 percent glycerol.
2. Store aliquots of the mixture in small, tightly capped vials (2–3 ml: e.g. centrifuge tubes) sufficient for five or six tissue samples (0.3 ml per sample).

Required equipment

- specialized ear tagger with tissue biopsy punch (see example in Photo K1)
- combination ear tag/sample cap (see example in Photo K2)
- portable cryotank with liquid nitrogen
- micropipette and tips
- plastic storage vials with cryoprotectant (prepared as described above)
- latex gloves and disinfectant spray (e.g. ethanol 70 percent)
- animal restraining equipment

[12] Groeneveld *et al.* (2008).

PHOTO K1
Ear tagger

PHOTO CREDIT: CAISLEY INTERNATIONAL GMBH

PHOTO K2
Ear tag / sample vials

PHOTO CREDIT: CAISLEY INTERNATIONAL GMBH

Collection procedure

1. Fill the sample cap of the ear tag with 0.3ml of the cryoprotective medium and secure it in position in the ear tagger.
2. Place the ear tagger[13] in the proper position on the ear of the animal and collect the sample by squeezing firmly the handles of the ear tagger. This will push the ear biopsy into the sample cap.
3. Remove the sample cap containing the ear biopsy from the tagger and shake it gently to ensure the cryoprotectant medium fully covers the ear biopsy stored inside.
4. Place the sample cap directly into the portable cryotank.
5. The samples may be moved to a larger tank for long-term storage upon return to the gene bank. This must be done as quickly as possible to avoid the risk of thawing.

PROTOCOL TWO – FIELD COLLECTION AND LABORATORY PROCESSING

Required equipment

- sampling tool (e.g. surgery kit, scalpel, hole punch or ear notcher/tagger)
- tweezers
- sample vials
- laboratory gloves and disinfectant spray (e.g. ethanol 70 percent)

[13] Ear taggers and similar sampling tools can be purchased commercially from various sources, including the following: www.prionics.com, www.caisley.de, www.biopsytec.com and www.prosampler.com

- animal restraining equipment
- transport container with ice (e.g. Styrofoam box)

Collection procedure

1. Use the chosen tool to obtain a skin sample (e.g. ear notch, skin biopsy).
2. Insert the sample into a sterilized, labelled storage vial.
3. Place the vial into the transport container.
4. Return to the laboratory for further processing and long-term storage.

Processing procedure

1. Slice the tissue sample into small pieces preferably 0.25 to 1.0 cubic mm.
2. Submerge in phosphate-buffered saline (PBS) supplemented with 80 mg/ml of streptomycin sulfate, 60 000 units/ml of benzylpenicillin and 20 000 IU/ml of potassium penicillin G.
3. Wash the samples (four to five pieces) in 20 percent foetal calf serum-PBS (FPBS).
4. Place pieces in vitrification solution (20 percent [v:v] ethylene glycol, 20 percent [v:v] dimethylsufoxide in FPBS).
5. Load the mixture into 0.25 ml French straws (1.5 cm of FPBS, air bubble, samples in 4 cm of vitrification solution, air bubble and 1.5 cm of FPBS).
6. Seal the straws.
7. Plunge the straws vertically into liquid nitrogen.

THAWING SAMPLES (BOTH PROTOCOLS)

Samples are thawed by holding the straw or sample cap in liquid nitrogen vapour for ten seconds and then submerging them in a 23 °C water bath for five seconds. The sample is then expelled into a dish, diluted with 0.25 M sucrose in FPBS for five minutes and then placed in FPBS (without sucrose) for five minutes. Samples can then be cultured for SCNT or used for DNA extraction.

Appendix L

Collection and freezing of poultry gonadal tissue

Storage of gonadal tissue is a procedure recently adopted for cryoconservation of special research lines of poultry at the public research institutes in Canada. The cryoconservation procedure is outlined below (adapted from Silversides *et al.,* 2008).

COLLECTION OF OVARIAN TISSUE

1. Euthanize female chicks and open the abdominal cavity.
2. Locate the ovary, which can be recognized as an irregularly shaped structure attached to the dorsal wall on the left side of the abdominal cavity.
3. Detach the ovary by using a fine forceps or tweezers.
4. Remove the connective tissue from around the ovary and dissect the cortical part.
5. Cut the ovary into two or three pieces.

COLLECTION OF TESTICULAR TISSUE

1. Euthanize male chicks and open the abdominal cavity.
2. Locate the testes, which are worm-like organs on both sides of the dorsal part of the abdomen.
3. Detach the testes by using a fine forceps or tweezers.
4. Remove any connective tissue.
5. Cut each testes into four or five pieces (each about 1.0 to 1.5 mm^3).

TISSUE CRYOPRESERVATION (SAME FOR BOTH TESTES AND OVARIES)

1. Following removal, place the gonads in a solution of 1.0 ml of phosphate buffered saline (8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4 in 1 litre of H_2O at pH 7.4) and keep on ice until freezing (within four hours).
2. For freezing, prepare a cryoprotectant solution of Dulbecco's Modified Eagle's Medium (Sigma Chemical Co., St. Louis, MO, United States of America) containing 10 percent (v:v) dimethylsulfoxide and 10 percent (v:v) foetal bovine serum.
3. Use the fine forceps to transfer the tissue into a 0.5 ml cryo straw.
4. Inject the cryoprotectant into the straw by using a long needle.
5. Eliminate bubbles within the straw and then seal.
6. Allow the temperature of the straw contents to equilibrate by maintaining them at room temperature for 20 minutes, periodically inverting the straws three to five times.

7. Freeze the straws according to the following programme:
   - Reduce temperature to 10 °C (at -1 °C/minute).
   - Reduce temperature from 10 °C to -7 °C (at -0.5 °C/minute).
   - Hold at -7 °C for ten minutes.
   - Initiate ice nucleation with a forceps cooled in liquid nitrogen.
   - Reduce temperature from -7 °C to -55 °C (at -0.5 °C/minute).
   - Plunge the straws into liquid nitrogen.

Appendix M
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The *Global Plan of Action for Animal Genetic Resources*, adopted in 2007, is the first internationally agreed framework for the management of biodiversity in the livestock sector. It calls for the development of technical guidelines to support countries in their implementation efforts. Guidelines on the *Preparation of national strategies and action plans for animal genetic resources* were published by FAO in 2008 and are being complemented by a series of guideline publications addressing specific technical subjects.

These guidelines on *Cryoconservation of animal genetic resources* address Strategic Priority Area 3 of the *Global Plan of Action* – "Conservation" and particularly complement the guidelines on In vivo *conservation of animal genetic resources* which will be published in the same series. They have been endorsed by the Commission on Genetic Resources for Food and Agriculture.

The guidelines describe, in logical chronological order, the process of establishing a programme for cryoconservation of animal genetic resources, starting with the process of confirming the decision to implement a cryoconservation programme. The task of organizing the institutions needed to meet the chosen conservation objectives is then described. This is followed by an overview of the types of germplasm that can be cryoconserved, and technical details regarding the physical plant required to set up a gene bank and the quantities of germplasm required to ensure the capture of sufficient genetic variability. Principles of cryopreservation are then explained from a biological point of view and cryopreservation procedures discussed for different species and tissue types. The main body of the guidelines concludes with sections on sanitary measures, data management, legal issues and capacity building. Appendices provide detailed protocols and lists of equipment and reagents for collection and cryopreservation of various tissues for a range of common livestock species.

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