RFP EUROPE
European Regional Focal Point
For Animal Genetic Resources

Guidelines for the Constitution of National Cryopreservation Programmes for Farm Animals

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The **European Regional Focal Point** (ERFP) is part of the UN Food and Agricultural Organisation's (FAO) officially recognised global network on Animal Genetic Resources (AnGR) for Food and Agriculture. It was set up with the financial backing of the French Government, as part of the FAO's Global Strategy for the Management of AnGR for Food and Agriculture to encourage greater co-operation between the official National Co-ordinators for AnGR in the 37 European States. The ERFP is funded by donations from at least 10 donor Governments within Europe and has the full support of the European Association of Animal Production (EAAP). It is recognised by the European Commission as being the expert body, with the backing of the EAAP, on AnGR issues affected by EU legislation.

The ERFP plays a vital role in bringing together European National Co-ordinators on AnGR and encouraging co-operation in AnGR conservation activity across Europe. In November 2002, a first call for actions was launched by the ERFP, which resulted in the funding of two projects. One of these was entitled 'Development of Guidelines for cryopreservation of AnGR in Europe', which has resulted in this publication.

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Table of Contents

Foreword

On behalf of the Steering Committee of the ERFP, it gives me great pleasure to commend this publication to you. It is the result of 12 months dedicated work and represents the collaborative effort of National Co-ordinators and researchers expert in cryopreservation from 11 European countries.

The working group, who has developed these Guidelines, took up the difficult task of assembling all the relevant issues regarding cryopreservation of AnGR into practical Guidelines. Some issues are not new, but the way they are presented is intended to make them easier to pick up and use. Other issues are relatively new to the European (and global) AnGR community and will be of real interest to those engaged in ex situ conservation.

The authors wish to point out that these Guidelines should be considered as a growing document to be added to as new information comes to light. They have been developed by a limited number of experts in a short period of time, based on the state of knowledge and technology available to them in 2003. We hope you agree that the working group has produced a valuable document that you will find both informative and useful. However, it needs to be borne in mind that such Guidelines can never be complete and that they will need to be updated regularly.

As the FAO's First Report on the State of the World's AnGR for Food and Agriculture process gains momentum and countries throughout Europe begin to develop new or existing national action plans for the conservation and sustainable use of their indigenous breeds of livestock, the question of ex situ conservation of those breeds will arise. We trust that these Guidelines will become an essential reference document for all those considering setting up or renewing gene banks whether policy makers, NGOs, research institutes or private organisations. We also hope that National Co-ordinators throughout the world will also find them helpful.

Particular thanks are due to Sipke Joost Hiemstra who has been the driving force behind this project but he would not have been able to succeed in producing the Guidelines without the enthusiastic support and contributions of the other members of the working groups to whom we are all indebted.

Mike Roper

Chairman, ERFP Steering Committee

Authors and participants

Two major efforts have resulted in this document named 'Guidelines for the Constitution of National Cryopreservation programmes. First of all, on February 23rd 2003, the Workshop on Cryopreservation of Animal Genetic Resources in Europe was organised in Paris, during the 'Salon International de l'Agriculture'. This workshop brought together specialists in ex situ conservation from all over Europe to present papers with their experiences and to pool their knowledge. The Paris workshop was actually the starting point for the ERFP Working Group, which had the task 'to develop practical Guidelines for cryopreservation programmes'. The contribution of the Paris workshop to the final result of the Working Group has been substantial. Therefore, acknowledgements are due to the French organisers of this workshop and in particular to Dominique Planchenault.

From March 2003, the ERFP Working group has been developing these practical Guidelines for the constitution of national cryopreservation programmes as was agreed upon by the ERFP-Steering Committee late 2002. A group of 13 experts and national co-ordinators for AnGR met in Lelystad on 12/13 June 2003 to discuss the outline, initially proposed by Thomas Schmidt, Coralie Danchin-Burge, Eildert Groeneveld and Sipke Joost Hiemstra, and started to add content. All participants contributed substantially to the design of the Guidelines which have been developed since then. Participants Lelystad-meeting (for personal details see Annex I).:

- Coralie Danchin-Burge, France
- Barbara Gajda, Poland
- Ilma Grigaliunaite, Lituania
- Sipke Joost Hiemstra, the Netherlands
- Asko Maki-Tanila, Finland
- Alfredo Martin, Spain
- Mihály Pásztor, Hungary
- Flavia Pizzi, Italy
- Thomas A. Schmidt, Germany
- Saffron Townsend, United Kingdom
- Morten Walløe Tvedt, Norway
- Jack Windig, the Netherlands
- Henri Woelders, the Netherlands

At the end of the meeting in Lelystad, the Netherlands, the working group decided that each chapter should be further developed by a limited number of authors. During summer 2003, first drafts of chapters have been exchanged between members of the working group and it resulted in the first full draft of the Guidelines by August 2003. At the annual workshop of National Co-ordinators for AnGR in Rome, August 2003, the progress of the project was presented by the project co-ordinator (S.J. Hiemstra). During this workshop in Rome it was decided to finalize and further improve the Guidelines before the end of the year 2003. For each chapter, one responsible person was appointed, who took the lead for the particular chapter. Contributions of the main authors to different chapters are listed below.

List of authors

Chapter 1, 2	Introduction and fundamental considerations	S.J. Hiemstra and G. Gandini
Chapter 3	Institutional setting and organisation	C. Danchin-Burge and S. Townsend
Chapter 4	Intake of genetic material	G. Gandini and T. Schmidt
Chapter 5	Freezing and storage	F. Pizzi, H. Woelders and B. Gajda
Chapter 6	Use of genetic material	S.J. Hiemstra and A. Maki-Tanila
Chapter 7	Sanitary/veterinary requirements	A. Martin and C. Danchin Burge
Chapter 8	Documentation	T. Schmidt and E. Groeneveld
Chapter 9	Legal issues	M. Walløe Tvedt and A. Maki-Tanila

Acknowledgements

The whole document should be considered as a concerted action with significant contributions from many persons, which is illustrated in the paragraph above authors and participants. Furthermore, many other persons contributed with comments and suggestions to draft versions of the Guidelines. Among others we would like to acknowledge Ricardo Cardellino (FAO), Louis Ollivier and the EAAP Working Group on Animal Genetic Resources in particular for their comments and suggestions.

Secondly, it would not have been possible to develop these Guidelines without financial support of many organisations and governments. In particular we want to acknowledge those countries who support the ERFP financially and the French government and the organizing committee of the workshop in Paris and last but not least those organisations, national or regional governments who made it possible for the persons mentioned in the paragraph above to contribute to the Paris workshop and to the development of these Guidelines.

1 Introduction

Late 2002, the Steering Committee of the European Regional Focal Point (ERFP) for Animal Genetic Resources (AnGR) in food and agriculture, decided to stimulate a ERFP-project called 'Development of Guidelines for cryopreservation of AnGR in Europe'. The main objective of this project was to develop a set of practical Guidelines and to exchange state of the art knowledge and experiences. These Guidelines are intended to help individuals or organisations, which would like to start or further develop a cryopreservation programme on a national level. It is also intended to be a useful tool to create awareness among policy makers, politicians and other stakeholders with regard to the relevance and best practise of cryopreservation initiatives.

The document can be used as a handbook or checklist and gives recommendations, references and specific examples. The Guidelines are meant to support decision-making regarding the design of national cryopreservation programmes (NCP). Organisational, operational, technical (genetics, cryobiology), legal and veterinary aspects are covered, being the major issues with respect to cryopreservation programmes. Governmental, industry, breeders groups and non-governmental organisations' initiatives can all be part of a NCP. An important criterion for inclusion of different cryopreservation initiatives should be that they contribute to long-term conservation of national genetic diversity through cryopreservation.

Cryopreservation is considered here as a tool to conserve genetic variation on a long-term basis: the use of cryopreservation for everyday use (such as AI for dairy cattle) is not discussed here. However, the long term conservation objectives can also mean that short term use of gene bank material is needed or allowed, for example to support in situ conservation programmes.

Occasionally terms such as cryobank and cryoreserve are used, which refer to the physical collections under a national cryopreservation programme. The Guidelines cover all types of breeds, strains, lines or varieties: local/regional, national and international; commercial/main stream and rare/endangered. Another important basic assumption is that we consider animal genetic resources (breeds) as dynamic entities, since animal breeding is a continuous process. Chapter 2 starts with 'Fundamental considerations'. Chapter 3 follows with considerations regarding the 'Institutional setting and organisation'. Chapters 4, 5, 6, 7, 8 and 9 deal with 'Intake of genetic material', 'Freezing and storage', 'Use of genetic material', 'Sanitary aspects', 'Documentation' and 'Legal aspects', respectively. Each chapter closes with a conclusive summary and a list of references.

The Guidelines are based on literature and experiences and are not meant to replace (for example) the FAO secondary guidelines for development of national farm animal genetic resources management plans. These Guidelines are developed from a European perspective, but may also be useful in other regions of the world. Because of on-going developments in research and in development of cryopreservation programmes, this document should be seen as a permanent draft, which needs regularly updating.

2 Fundamental considerations

It is the main responsibility of a nation to preserve its genetic diversity on a long-term basis (CBD, 1992). Animal production is vital to mankind and conservation of animal genetic diversity is a way to secure our future. Consequently, before embarking upon a NCP programme, nations need to develop the best suitable individual strategy or policy for conservation of farm animal genetic resources. In order to develop the best country-specific strategy, many choices have to be made.

During previous centuries, local, national and international breeds have been developed. Many breeds have evolved into a strong position (local, regional, national and/or international) because of specific characteristics, production efficiency or adaptations. At the moment, a decreasing number of international breeds are responsible for a growing percentage of the world food production. This has resulted in a growing number of rare breeds or a decline in the number of breeds overall. In Europe and on a global level, genetic erosion is taking place and breeds are being lost or put at risk. Both between and within breed variation is under pressure as a result of selection, breed replacement or genetic drift.

Therefore, conservation efforts are strongly needed. Conservation efforts need to cover both within and between breed variations. We also distinguish between in situ and ex situ conservation programmes. In general, in situ conservation is preferred as a mechanism to conserve genetic resources. In order to be successfully conserved a breed has to evolve and adapt within its changing environment and the best way to conserve a breed is to create a (current) need for its product or function and to develop the breeds in the desired direction.
However, ex situ conservation is considered to be a very important tool to avoid irrecoverable loss of breeds or genes, to re-establish a breed, to secure our resources from sanitary accidents, to support breeding in small populations and to conserve genetic variation (genes, traits or breeds) in selection programmes. Ex situ conservation can be carried out through live preservation (for example in zoos) and cryopreservation (in liquid nitrogen). These Guidelines will cover only cryopreservation.

2.1 Objectives of cryopreservation

In general, cryopreservation of genetic material from domestic farm animals can have different objectives. Although the main objective of a NCP is to address long-term conservation needs, there are also short or medium term objectives. Objectives for cryopreservation need to be defined in terms of future use of the cryobank material:

1. To support populations conserved in vivo:
   - as a back-up in case genetic problems occur in the living population (e.g. loss of allelic diversity, inbreeding, occurrence of deleterious genetic combinations)
   - to increase effective population size of small populations and reduce genetic drift
2. To reconstruct breeds, in case of extinction or loss of a substantial number of animals
3. To create new lines/breeds, in case of breed extinction
4. As a back-up, to quickly modify and/or reorient, the evolution/selection of populations
5. For research

Items 1 to 3 generally apply to endangered populations or protection of small populations. Genetic material is stored to guarantee the survival of a breed or breed genetic variation. Gene introgression (e.g. upgrading programmes) and creation of synthetic lines are particular examples of usage as outlined in item 3.
Item 4 refers to non-endangered, selected populations, to strengthen the selection of a new target in a breed. Item 5 includes several research objectives, for example retrospective population genetic analyses.
The specific aims of a national gene bank may vary according to national strategies, availability of material and budgets.

Conclusive summary

Starting the development of a NCP one very important question need to be answered first:

- What is or are the main objective(s) for the cryopreservation programme?

Literature

CBD, 1992: Convention on Biological Diversity.
FAO, 1998: FAO Secondary Guidelines for development of national farm animal genetic resources management plans 1998 – management of small populations at risk pp. 59-71

3 Institutional setting and organisation

The design of the National Cryopreservation Programme (NCP) in terms of responsibilities, organisation, funding and management is one of the crucial factors for the (future) success of any NCP.

3.1 Fundamental considerations for NCP organisation

Before setting up a NCP it is necessary to fully understand how the genetic resources are managed in the country in question.

3.1.1 Territorial organisation

First of all, it should be remembered that on the European or international level, no rules exist (so far) in relation to how a NCP should be organised, but recommendations exist (these Guidelines; FAO, 1998; OECD, 2003). The NCP should link up with initiatives like the OECD Biological Resource Centres (or similar), and ensure that country specific requirements are brought to the attention of 'universal' initiatives, so that regional or global perspectives are attained and accommodated.

The territorial and regional organisation (counties, departments, regions, provinces, autonomic communities, etc.) must be taken into account in the organisation of a NCP. This territorial component has to be taken into account in the inventory of all the potential genetic resources stakeholders. As an example, the original stud/herd/flock book owner country or place of breed origin cannot always be defined at a country level and an international organisations/federation may exist instead: for example, Huzul horses are the product of a region which is part of different countries so an international organisation had to be set up for this breed. Obviously the Hungarian NCP will have to include this international partner in their programme. On the other hand, associations may exist only at a regional/local level.

An efficient NCP requires regional and national cooperation between authorities and organisations. In particular, some 'universal' standards are required to manage the NCP collections. It is important to check that on both a regional and local level new legislation is not going to impede the NCP in some way.

3.1.2 Listing the NCP partners

When setting up the NCP, it is necessary to list all the potential stakeholders. Usually one or several stakeholders will take the lead to develop a programme and take major responsibility for the objectives of the NCP, but it is essential that the ideas/objectives/proposals be presented to all potential stakeholders.

The possible stakeholders can either own genetic material or not, or be NGOs, private or public organisations. All types of collections of different sizes should be able to become involved in the NCP. Public-private partnerships are possible and even advisable. The partnership should focus on long-term responsibility, and have a long-term management structure put in place from the beginning so that changes in public or private policy do not cause the NCP to fail. Overall, efforts should be to combine resources to build an effective NCP from existing structures.

Setting up a NCP – The Dutch, French and UK experiences

When comparing the Dutch and French cryobank, there is in both cases a strong involvement of both government and the animal production sectors.

In the Dutch example, private organizations have founded the Gene Bank Foundation for Farm Animals (SGL) in 1993, but the involvement of the government has strongly increased since, mostly financially. This has lead to the transfer of the collections in another structure, with joint responsibility of the Centre for Genetic Resources, the Netherlands (CGN) and SGL. CGN is an independent unit of Wageningen University and Research Centre, which carries out so called 'statutory tasks' regarding animal genetic resources and associated activities for the Ministry of Agriculture, Nature Management and Food Quality.

In the French example, the Ministry of Agriculture was leading the project of creating the French cryobank, but private organizations such as the AI centres federation and the breeders associations federation were involved right from the beginning.

In the UK, a NCP for rare breeds (The National Archive) was formally established in 2002/3 by the national NGO, the Rare Breeds Survival Trust (RBST) and is stocked through an ongoing programme of collection, carried out with the help of rare breed associations. The RBST has sole responsibility for funding, maintaining and organising collections for the NCP, and controls access to the resource. A NCP for mainstream sheep breeds (the NSP semen archive) is also currently under separate development by UK Government in association with national sheep industry stakeholders.

The main stakeholders that should be part of the NCP are:

- *Governments*
  Governments should have first and general responsibility for maintaining genetic resources of the country (cbd, 1992). In practice, they are usually most involved in the protection of endangered breeds. This should mean that governments or governmental institutions play a major role in the NCP.
- *NGO or NGO network*
  In some countries, the Government may not support rare breed conservation. When there is no or little governmental involvement, a NGO or NGO network may exist and can take national or regional responsibility for setting up a NCP.

Government or NGO involvement in the NCP is mostly political and financial. Often they are not the owners of genetic resource but they can participate in the NCP collection by financing in part or totally the collection and cryo-preservation of genetic material owned by the following structures:

- *Breeding associations*
  Breeding associations or their members are usually the owners of the genetic resource. Breeding associations should be involved in the NCP. Official herd books are well recognized by EU regulation and they are often supported by national governments, so co-responsibility for long-term conservation should be sought as a return for their established role. In the case of poultry and rabbits, fancy breeder associations need to be considered.
- *Private companies*
  For several farm animal species (poultry, pig, but also rabbit, fish and dairy cattle), international breeds or important national breeds, private companies are responsible for breeding programmes. Even if most of the stock is held by breeders (who are part of a breeder association), the sire scheme (dairy cattle) or the dam scheme (pig, poultry and rabbit production) is led by corporate companies, which means that these companies keep genetic material that might be of interest for the NCP. For these private companies, participating to the NCP is a secure way to preserve genetic variability and protect their genetic material from infectious disease outbreaks.
- *Research institutes and/or agricultural schools/universities*
  Research institutions and/or agricultural schools/universities can be involved in rare breed programmes or they can be owners of experimental populations resulting from research projects. This type of genetic material can be of great value in term of genetic diversity. Also, researchers are often closely involved in establishing NCP Guidelines and research on genetics, collecting, storing and freezing techniques. They usually have a good network of international experts in their area and they can be useful to monitor any major advancements in science and technology development. Their networks can help to build collaboration between NCP's from different countries. Finally, their facilities may be used to realise some cryopreservation operation (including storage) for the NCP, because the breeding industry is not always able or willing to achieve this specific task. For example, in the UK, most boar studs are run by commercial companies who will not collect boars from stocks other than their own because it contravenes their high bio security arrangements. Thus, it was very difficult to collect from local pig breeds because access to mainstream facilities was not possible, and other options for boar collection were eventually used.

However, if the NCP consists of various collections that are funded and maintained by different organisations (as opposed to central government), each are likely to have their own set of guidelines/budget that may not require/allow a NCP that meets all the recommendations found in these Guidelines. In this case, priority setting may not be universal among organisations with similar collections.
Furthermore, if there is no strong governmental involvement in the NCP there is a possibility that the scope of the NCP cannot be wholly determined by national objectives, since resource and jurisdiction may be limited.

3.2 Management of a NCP

The NCP needs to be managed at different levels. Besides operational management of day-to-day activities, steering and advisory committees are required to supervise the programme. A wide and active participation of as many stakeholders as possible is necessary and should result in agreement of a permanent management structure/committee to oversee management of the NCP in the long-term. Major funding organisations also need to be involved in the setting up of the NCP conservation strategies and priorities.

3.2.1 Promoting agencies

All major stakeholders should act as promoters to develop the NCP. In general, a joint responsibility between government or NGOs and the private sector should be the goal. It is recommended that the government take the long-term responsibility for conservation of farm animal diversity, because the NCP will represent an important national resource. The government should give high priority to cryopreservation of rare breeds, but also to those that are otherwise genetically distinct or locally adapted. On the other hand, breeding organisations should be at least partially responsible for cryopreservation of the existing genetic variability of selected, mainstream populations.

3.2.2 Advisory Committee(s)

Two types of advisory committees should be considered to lead the NCP:

- *A Platform for genetic resources*
  On a national level a platform can advise the responsible Ministry on policies regarding genetic resources:
  - Covering plants, animals (incl. fisheries), forestry, micro-organisms
  - Setting priorities between plants, animals (pets breeds and farmed wild animals included in some countries, neglected in others), micro-organisms and forestry
  - Discussing in situ and ex situ policy relationships
  - Representing the country at the European level (by joining the ERFP) and international level (by sending experts at the FAO when needed)

  As an example France set up a Genetic Resources Board (BRG) to provide a national frame on genetic resources (www.brg.prd.fr)
- *An Advisory Committee on Animal Genetic Resources (AnGR)*
  This Committee is responsible for the actual design of the NCP. In-situ programme and in situ /ex situ relationships can also be examined in more detail by this Committee.
  The Advisory Committee on AnGR should comprise:
  - The responsible Ministry or Ministries (e.g. Agriculture, Environment)
  - Private sector (e.g. breeding companies)
  - NGOs (e.g. breeding organisations, societies for rare breeds, research groups, farmers unions)
  - Relevant research sectors (e.g. university departments involved in reproductive biology, populations genetic conservation)

  Tasks should include:
  - Establishment of institutional organisation of the NCP
  - Appointment of a Steering Committee/Boar
  - Inventory of farm animal genetic resources
  - Prioritisation between species and between breeds
  - Development of broad terms of reference for the NCP (objectives, use of NCP, access/benefit sharing, funding, etc.)
  - Cost-benefit analysis
  - Representation of the NCP at the national and international level

3.2.3 Steering Committee / Board

The Steering Committee is responsible for the development, management and operation of the NCP and should report at intervals to the Advisory Committee.
Tasks should include:

- Building a communication network with respect to the cryopreservation programme
- Sound project management
- Germplasm/donor database development and management
- Develop and execute a working strategy (e.g. timescale, budgets, mechanisms for access to material, etc.)
- Quality assurance and control
- Sourcing and using best relevant expertise to address technical issues
- Identifying funding sources for the operations
- Finding contractors to carry out the germplasm collection
- Deciding on storage sites and obtaining site licenses where necessary

If needed, a *Scientific Committee* can also be created to help the Steering Committee. It should be designed by the Advisory Committee and will have representatives from different research institutes in areas such as cryopreservation techniques, population genetics, and sanitary recommendations. etc. In order to be representative, experts need to cover all species.
Tasks of the *Scientific Committee* should include:

- Giving scientific advice on the NCP targets and priorities
- Supporting the development of the NCP guidelines
- Evaluation of NCP in scientific terms and making regular feedback recommendations
- Carrying out a permanent scientific watch on all topics related to the NCP

3.3 Funding

The agreement between stakeholders on how to finance the cryopreservation activities is an essential element of the management structure of the NCP. To ensure support for or consensus on the NCP on a long-term basis, it is recommended that combined financial involvement of government, NGOs and commercial sector be obtained.
It should be remembered that from a European perspective, EU-regulations might give opportunities for funding of the creation of *ex-situ* collections in European countries. At the international level, linkage to the creation of Biological Resources Centres (oecd, 2003) may also result in government subsidies for a NCP.

3.3.1 Financial involvement of stakeholders

Careful consideration should be given to how funding organisations are involved with the steering committee and what their rights are regarding the NCP. Financial involvement of stakeholders can be in terms of money, material, labour and/or logistics.

The amount of budget necessary will depend on the way the NCP manages to finance:

- The collection of biological material and cryopreservation (species/breeds covered, facilities, techniques, logistics, etc.)
- The maintenance of the collections (buildings, tanks, liquid nitrogen, insurance, etc.)
- The appointed management staff of the NCP

For the first two points, the NCP costs will depend strongly on the practical situation in a country for a given species. If AI centres are collecting males and freezing semen on a daily basis, it may be possible to have some biological material collected and stored by the AI centres free of charge or at a low cost. On the other hand, if no AI centres exist for collecting and freezing semen for a specie, the NCP will have to run a programme from scratch to get biological material, which will be costly and time consuming. Overall, logistics is also a key point. Sometimes transfer of material to storage sites looks cheap, but it is rather time and cost consuming. Strong involvement of the stakeholders in this process is the key to success.

3.3.2 Cost-benefit analyses

Analysis of costs and (future) benefits is not an easy task, but should be done in order to underpin the long-term cryopreservation objectives of a NCP. The NCP needs to find a balance between minimization of operational costs and quality assurance/guarantee of the future potential benefits. Since a NCP has a patrimonial goal, it is necessary to have a high quality level of the stored biological material, but too high a quality (mostly if the sanitary restrictions are very strict) may forbid the cryopreservation of many important potential donors. The balance between the quality/availability of biological material and the donors that should be stored has to be finely tuned

A 'classic' cost-benefit analysis should include:

- Assessment of individual NCP objectives
- Assessment of potential future benefits within objective
- Government versus breeding company versus NGO funding
- Analysis of operational costs
- The desired quality level

Some examples of practical considerations are as follows:

- Type of material to be collected
  Theoretically it makes better sense in the long term to freeze embryos rather than semen for species with a long generation interval. Yet, in cattle, semen is usually collected on a daily basis in AI centres and large quantities can be stored in cryobanks at low costs.
- Amount of material to be collected
  The optimal size of collections is very much dependent of practical considerations (logistics, room available in the storage sites, maintenance costs, etc.) A combination of careful donor selection procedure (according to the conservation objectives of the NCP) and minimisation of volumes collected and stored (no more than the NCP conservation objectives require as a minimum) should be kept in mind at all times to ensure cost-effectiveness. The volume of the stored genetic material should be as small as possible whilst containing the largest possible genetic diversity.
- Insurance
  Insurance of a NCP is required to cover two resources – the genetic resource and the capital resource. The genetic resource can be insured by storage at multiple sites, but the capital resource (i.e. cost of site, fixtures and the amount required to collect material from as many animals as are already held) can usually only be insured by conventional means. Costs of premiums may be very high for this type of cover, and may need to be considered in light of the capital at stake.
- International context
  International cooperation between NCP's could be established for international breeds such as, in cattle, Holstein, Brown Swiss etc., to make sure that the same type of genetic material is not stored in different countries. Periodic assessment of the whole collection (every ten years seems to be a good interval) should be established in order to detect oversized collection.

Example

In the French collection, dairy bulls are entered in the collection every year if they have outstanding EBV values or outstanding pedigrees. It is quite likely that after 10 years of sampling some bulls sampled on EBV traits will be redundant and could be removed from the cryobank.

- Priorities between and within breeds
  These can be achieved only following practical considerations that take into account all available information (degree of endangerment, support from breeders, co-ordination among breeders, available (cryo)technology, logistics, etc.), and may change over time as more information or resource becomes available. It is important that priority setting is re-evaluated periodically (every five years seems to be a good interval, but could be longer depending on the degree of fluctuation in trends in individual regions/countries).
- Choice between the best value contractors/operators
  As stated previously, the NCP needs to use existing structures to be as effective as possible and the least costly. As mentioned above, a duplicate collection is necessary to insure the genetic resource being collected. Sometimes, for practical reasons, it is impossible to have only two locations for the cryobank storage and the numbers of storage sites are multiple. Yet, in order to minimize management costs and operational costs, the number of storage sites needs to be small. It is often possible to request that contractors store a proportion of material as part of the contract agreement for collection.
- Time parameter
  Costs involved in the creation of a NCP are, of course, relevant on a short-term basis, whereas the use of the NCP has to be foreseen on a medium or long-term basis.

3.4 Some examples of existing NCPs

In several European countries, NCP's have been developed or are under development (personal communications, 2003).

Spain

The 'Comité de Reproducción y Banco de Germoplasma Animal', attached to the 'Dirección General de Ganadería' of the Ministry of Agriculture, Fisheries and Food and created in December 1998, carries the main responsibility for co-ordination of the National Cryobank. The Committee meetings are once a year with representatives of Central and Regional Government, INIA (National Institute of Agriculture and Food Technology and Investigation), AI Centres and Breeders Organizations.
The proposal of technical requirements to co-ordinate the constitution of the different Germplasm Banks is one of the roles of the Committee. Among its functions is also to propose specific regulation for Animal reproduction and Germplasm Banks.

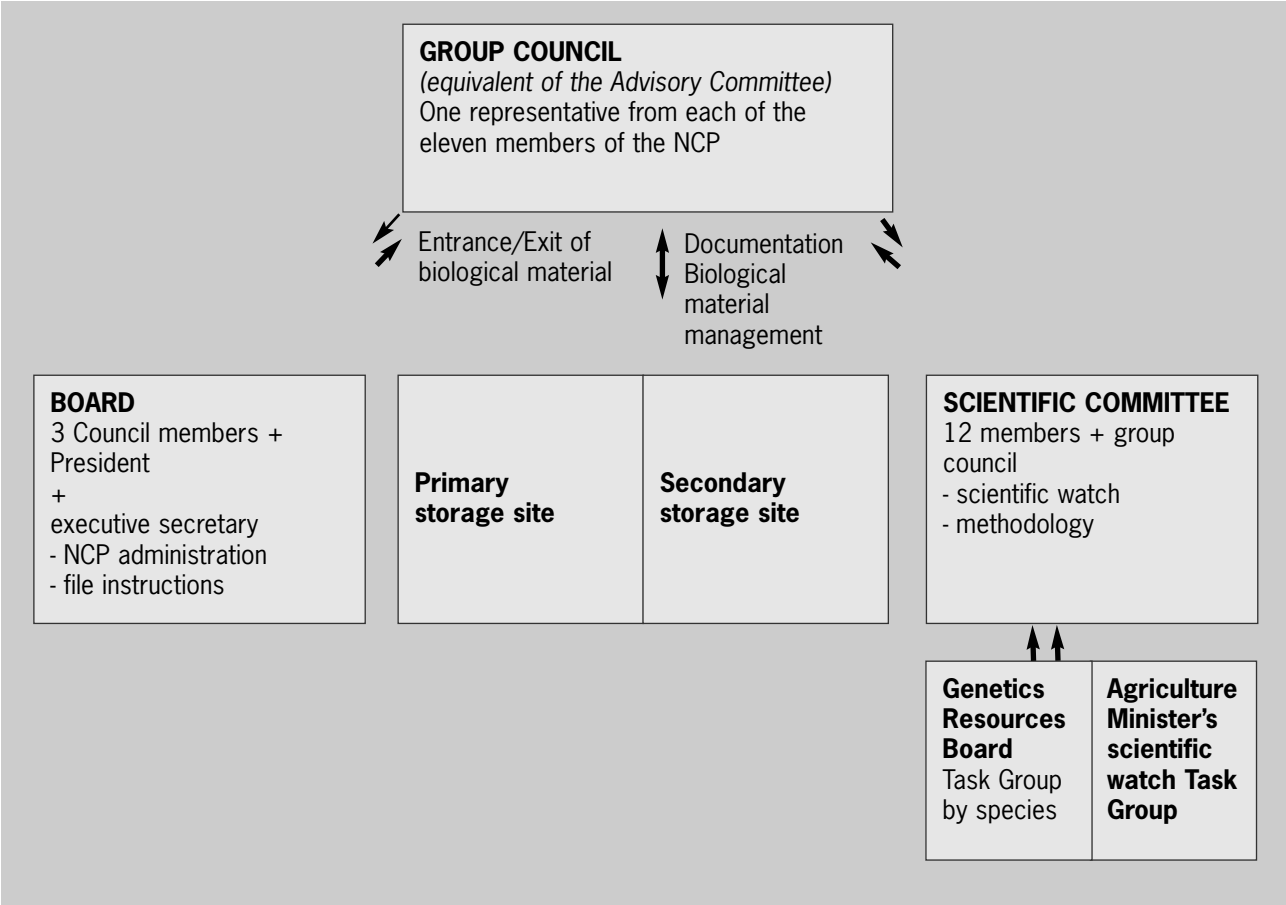
United Kingdom

In the UK, responsibility for an ex-situ conservation programme for all rare breeds currently rests with the national rare breed NGO, the Rare Breeds Survival Trust (RBST). In 2001 a National Appeal was launched by the Trust to raise UK £2.5 million, to include provision for a germplasm store (National Archive) for all 63 rare breeds of UK livestock and equines. Currently, collection of semen is well underway, and within five years it is anticipated that all breeds will be represented within the Archive such that they will be insured against future breed catastrophe. The Archive also makes sufficient material available for ongoing in-situ rare breed conservation programmes. All archive design, ownership and terms of release rest with the RBST. Responsibility for collection of material for commercial or mainstream breeds currently rests with individual breeding companies or organisations, although UK Government has recently agreed to collect semen from scrapie susceptible components of all UK sheep breeds as part of the UK National Scrapie Plan.

The Netherlands

In the Netherlands, ex situ conservation is a shared responsibility between the Ministry of Agriculture, Nature and Food quality and the private sector/breeding industry. The private sector has organized ex situ conservation activities through the Dutch Gene Bank Foundation for farm animals (SGL). Breeding industry and the livestock production sectors are represented in the board of SGL. The Ministry gave the mandate to the Centre for Genetic Resources of Wageningen University and Research Centre (CGN) to conserve and promote sustainable use of animal genetic resources. CGN is responsible for further development of ex situ conservation programmes and works closely together will all relevant stakeholders and with the board of SGL and seeks scientific and practical advice from a diverse group of experts and organisations.

France



Conclusive summary

The design of a NCP should take into account:

- The territorial organisation of the NCP
- All relevant stakeholders/partners and their respective roles
  - Government
  - NGOs
  - Breeding associations
  - Breeding industry
  - Research institutes and/or agricultural schools/universities
- The management structure of the NCP
  - Promoting agencies
  - Advisory Committee(s)
  - Steering Committee/Board
- Funding of the NCP
  - Stakeholder involvement
  - Cost-benefit analysis

Literature

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4 Intake of genetic material

This chapter discusses some genetic aspects of cryopreservation. It recalls the aims for storing genetic material, it reviews the criteria to choose species and breeds to be included in the cryobank and it discusses sampling strategies. The material to be frozen is function of the aims of the specific cryopreservation programme, of the available funds and field constraints. Due to the numerous sources of variation, the Guidelines do not provide exact numbers and recipes for the material to be stored but they rather intend to furnish instruments to define type and amount of material as a function of the specific situation.

In chapter 2, the possible aims for storing genetic material are listed as fundamental considerations to develop a cryopreservation programme. As sampling strategies vary for the different uses or aims, this chapter starts with a recall of these possible aims. Genetic material of a given breed could be stored:

- To support populations conserved in vivo:
  - as a back-up in case genetic problems occur in the living population (e.g. loss of allelic diversity, inbreeding, occurrence of deleterious genetic combinations)
  - to increase effective population size of small populations and reduce genetic drift
- To reconstruct breeds, in case of extinction or loss of a substantial number of animals;
- To create new lines/breeds, in case of breed extinction
- As a back-up, to quickly modify and/or reorient, the evolution/selection of populations
- For research

4.1 Selection of species and breeds

4.1.1 Species

The question which species the cryobank should be focused on, is somehow different from the question which breed has to be conserved because among species distinctiveness is not a matter of discussion. The decision is related to aims and strategies of the specific cryopreservation programme. These Guidelines are focused on farm animals. FAO includes in its World Watch List for Domestic Animal Diversity (Scherf, 2000) the following species: buffalo, cattle, yak, goat, sheep, pig, horse, ass, dromedary, camel, alpaca, llama, guanaco, vicuna, domesticated deer species, rabbit, chicken, duck, turkey, goose, Muscovy duck, guinea fowl partridge, pheasant, quail, pigeon, ostrich, nandu, emu and cassowary. Attention is also given to the close wild relatives and ancestors of the domestic species. This list is considered an open-ended list.

Among the current cryopreservation programmes we observe a variety of interests. The American Livestock Breeds Conservancy (Sponenberg and Christman, 1995) considers cattle, ass, goat, horse, pig, sheep, chicken, duck, goose and turkey. The Dutch cryobank has its main focus on cattle, horse, pig, sheep and chicken, so far. The French bank also includes rabbit and fish (Danchin-Burge and Hiemstra, 2003). The Austrian cryobank covers, among others, some autochthonous wild fish species, which were regularly used for human nutrition in the past (Fischerleitner, 2002). The US National Animal Germplasm Program plans to include salmon and catfish (Blackburn, 2003). The German national programme considers, as a first step, (Nationales Fachprogramm, 2003) cattle, horse, sheep, goat, pig, rabbit, chicken, duck, goose, turkey, and pigeon, followed by bee, fish and some farmed wild species such as red and fallow deer. The Lithuanian national programme (personal communication) considers cattle, horse, pig, sheep, goat and goose and plans to also include bee, salmon, Lithuanian hound and wild boar.

None of the species above mentioned should be on principle excluded from a national cryobank. The choice of the species can be guided by the following:

- State of the art of freezing techniques in the species;
- Present and future values for the specific country (i.e. contribution to national agricultural production and added agricultural values);
- Available expertise and infrastructures;
- Costs and available funds;
- Conservation value of the populations available/planned for storage;
- Degree of endangerment of the population's available/planned for storage.

Co-ordination among countries in choosing the species is strongly recommended to avoid meaningless repetitions.

4.1.2 Breeds

The term 'breed' is used here as any unit of potential conservation interest, including commercial and local, registered and not registered, endangered and not at risk breeds and lines. The increased rate of loss of breeds and the limited resources available require prioritisation of actions and optimal allocation of available funds. The primary focus in farm animal diversity is on conservation of breeds, and between-breed genetic diversity is generally proposed as a major criterion to orient conservation priorities.

To optimise the genetic diversity stored in the cryobank, a decision-taking tool we can use is the so-called 'marginal diversity', originally proposed by Weitzman (1992, 1993), later applied to farm animal genetic resources (Thaon d'Arnoldi et al, 1998; Ollivier and Foulley, 2002; Simianer, 2002; Reist-Marti et al, 2003). This approach uses the pair wise genetic distances between the elements of a set of breeds (for a review on genetic distances see eding and laval, 1999; to estimate genetic distances several programme packages are available like Phylip (Felsenstein, 2000), DISPAN (FAO 1998b), Genetix (Belkhir et al., 2000), Fstat (Goudet, 2001), Arlequin ver. 2.0 (Schneider et al. 2000)). It then evaluates the relative contribution of each breed to the total genetic diversity of the set and the expected loss of diversity by loosing any specific subset. The Weitzman approach also allows estimating the diversity expected after a given period of time, taking into account the extinction probabilities of each breed, and computing the relative gain in diversity expected after a given period from improving the survival probability of any breed or specific subset of breeds. If we assume that cryo-preservation reduces to zero extinction probabilities, the marginal diversity approach can be used to support decisions on breeds to be cryopreserved. The Weitzman approach has been criticised as it ignores within breed genetic diversity and alternative systems have been proposed (e.g. Eding and Meuwissen, 2001; Caballero and Toro, 2002); A method for using the Weitzman approach in order to combine both between and within breed diversity has been also indicated (Ollivier and Foulley, 2002).

FAO proposes to select those breeds which are 'thought to be the most distinct genetically from throughout the complete geographic range' (FAO, 1998b) and to establish a centralized selection of the most distinct breeds. Decisions taken locally risk losing the advantages of the global approach. The national programme should always look at the widest possible picture, including at least the neighbouring countries.

If cryopreservation also aims to support in situ conservation, other criteria, in addition to genetic diversity between and within breed and degree of endangerment, should be taken into account for prioritizing conservation actions. Ruane (1999) listed six criteria to select a breed for conservation:

- degree of endangerment
- adaptation to a specific environment
- traits of economic importance
- unique traits
- cultural and historical value
- genetic uniqueness of a breed

The socio-economic role of the breed, its contribution to management of natural agro-ecosystems and the likelihood of success of the conservation programme should also be considered. Such a wider approach opens two questions: how can we measure all these elements and how can we weight the different criteria for practical decisions? The ongoing development of FAO and EAAP breed databases will provide a tool to store and monitor breed information useful to prioritise conservation actions. Countries should make all efforts to collect detailed and accurate information. A methodology to measure breed cultural value has been recently proposed (Gandini and Villa, 2003) and more research is needed to measure breed aspects such as adaptation and role in environment management. Regarding the second question it has been proposed (Ruane, 1999) to use the different breed aspects and values as independent selection levels. This means that a breed, which scores for a very high value for a specific criterion should be, prioritised regardless its score in other criteria.

4.2 Selection within breeds

Type and amount of genetic material to be stored are functions of aims of storage, available funds, local constraints and availability of biological material, and all these elements can vary across time and countries. Efficiency and costs of freezing and reproduction techniques are progressively improving in some species. Among the aims of cryopreservation programmes, breed reconstruction is generally considered as the maximum guarantee against risk of breed loss and it has been more investigated. However, other aims, such as creation of new lines and breeds, research or supporting populations conserved in vivo, may be equally likely. For all these reasons, these Guidelines do not give exact figures on the material to be stored. They intend to provide tools for developing cryopreservation plans and instruments to guide the choice on what and how much material to store and from which donor animals. These Guidelines should be considered complementary to FAO Secondary Guidelines for the Development of National Farm Animal Genetic Resources Management Plans – Management of Small Populations at Risk (FAO, 1998c).

4.2.1 Selection of donor individuals

If in the breed there is no reliable animal registration, donor animals should be carefully identified on phenotype and herd history, taking into account possible more or less recent crossing activities with other breeds. In these cases, genetic markers can also be used to identify gene introgression from other breeds, the level of which may differ among sub-populations or areas.

Donors should fulfil all sanitary requirements (see chapter 7) and have morphological and behavioural characteristics to facilitate the collection of genetic material (e.g. past experiences can negatively affect efficiency in semen collection).

Different cryopreservation aims may require storage of different types of genetic variation, which can be accomplished by selecting donors randomly or by specific criteria:

- *Representative sample* – To store a representative sample of the breed genetic variation, sampling should be random, also with respect to genetic relationships among individuals. In practice, this can be achieved by selecting:
  - on pedigree information, animals distantly related, for example without common grandparents;
  - animals from different areas and herds, considering genetic flows (i.e. exchange of animals) among herds and areas;
  - animals from within lines if line breeding is practised

  Storing a representative sample of the breed is the most common case.
- *Specific genotypes /* alleles – If storage of specific genotypes or alleles is required (e.g. to modify and reorient the evolution / selection of the population, for gene introgression or to create new lines / breeds), individuals with specific genotypes can be selected by genetic markers, estimates of breeding values, phenotype and pedigree information.
- *Maximal genetic variation* – Specific cases might require to store a sample of the maximal genetic variation of the breed. If pedigree information on the candidate donor animals is available, individuals that minimise average group coancestry should be chosen. A description of the founder population can be obtained by founder analysis and gene dropping techniques, and then we can choose those individuals that maximise founder variation. Molecular markers can be used, when pedigree information is not available or scarce, to estimate genetic relationship and to identify directly genotype variation (for a review see Toro and Maki-Tanila, 1999)

If all necessary animals cannot be found in the population, in order to store the desired genetic variation most effectively, it might be necessary, through appropriate mating, to produce specific donor animals.

4.2.2 Number of donor individuals

The number of individuals used as donors of the material affects the amount of genetic variation stored in the cryobank:

- using heterozygosity as parameter of genetic variation, the percentage of breed heterozygosity retained in the bank is $1 – (1/(2N))$, where N is the number of donor individuals. The use of 25 donors, corresponding to 98 % of heterozygosity retained, has been often suggested (e.g. Smith, 1984). Higher and lower numbers of donors can be considered, in relation to budget and donors availability (e.g., to find 25 male donors it can be a difficult task in many end angered breeds). The number of donors can be also influenced by the amount of material (number of semen doses or embryos) that can be obtained from each donor, that might depends by the species characteristics or by specific conditions (e.g. semen collected from animals at slaughter or from untrained animals). These Guidelines assume, as a general rule, a minimum of 25 donor animals;
- when we are interested in capturing allelic diversity, the probability to have in storage a specific allele is a function of its frequency (p) in the sampled population and of the number of donor individuals (N), as $1 – (1\text{-}p)^{2N}$;
- to include in storage particular genotypes and pedigree lines, specific numbers of donors could be required.

In populations of small size it might be necessary, through appropriate planning of mating, to produce specific female and/or male donor animals.

4.2.3 Type of material

Biological materials partly differ in the genetic information they carry and the effectiveness to achieve conservation aims (e.g. Gandini and Oldenbroek, 1999):

- through backcrossing, semen can be used to reconstruct the breed, but some percentage of the genes from the founder female population will remain in the reconstructed breed. For example, to achieve over 95 % of genes of the breed to be reconstructed, 5 generations of grading-up are needed;
- with semen and somatic cells, cytoplasmic effects of the breed will be lost or altered;
- with oocytes, compared to embryos, it is still possible to choose the desired mating;
- DNA storage has been proposed for gene transfer, but these techniques still pose many difficulties. DNA is not useful for re-establishing animals or breeds. DNA storage can be useful for research to support conservation decisions, to identify the genetic structure within and between populations, for characterisation and into identifying single genes effects. Other type of material can be stored in the cryobank for related conservation purposes, such as blood and serum, e.g. for future veterinary screening on the material stored. Storage of DNA, blood or serum is not further discussed here.

Table 4.1 provides an update of FAO Guidelines (1998c) of the state of the art of cryopreservation techniques, which includes the efficiency at freezing and after freezing, for the major species within the European context.

Table 4.1: State of development of cryopreservation techniques, by species: + routine technique available; 0 positive research results, – not feasible in the present state of art; ? unknown; * some research hypotheses

Species	Semen	Oocytes	Embryos	Somatic cells
Cattle	+	+	+	0
Sheep	+	0	+	0
Goat	+	0	+	0
Horse	+	0	0	0
Pig	+	0	0	0
Rabbit	+	?	+	0
Chicken	+	-	-	0
Fish - some species	+	*	*	*
Dog	+	?	?	0
Cat	0	0	0	0

Since the sheep Dolly was recreated from udder somatic cells it is well known that by cloning methods we can re-establish animals from their somatic cells. Some other species were cloned by this method as e.g. horse, cattle, pigs, and by cross-species nuclear transfer (Loi et al., 2002). Storage of somatic cells is a cheap and very effective method to keep a wide range of genetic variation with a limited budget. But up to now we cannot use it as a regular method for re-establishing animals or breeds. Considering future developments of scientific knowledge and the relatively low costs, somatic cell freezing could be considered. Freezing of oocytes is possible in cattle, but efficiency of in vitro development after fertilisation and survival rate after freezing are still low. Then, oocytes' freezing is not advisable as an alternative technique to embryo storage (see chapter 5).
In conclusion the available tools are semen and embryos storage, although somatic cells and oocytes freezing could be used if no alternatives are possible and as additional tools.

4.2.4 Amount of material

The amount of material to be stored is primarily a function of the aim or multiple aims of the cryopreservation programme.
It is recommended to duplicate the amount of material and to store each duplicate at different storing sites in order to reduce risks of loss associated to fire, problems to the storing plant or natural catastrophes. Then, each figure on number of cells, semen doses or embryos to be frozen given below must be doubled.

4.2.4.1 Breed reconstruction

Random demographic factors, such as herd culling for sanitary reasons or farm disappearance, and negative demographic trends can severely reduce the size of small populations and lead them to extinction. Storage of material allowing breed reconstruction is an insurance against risk of breed loss. However it should be noted that breed reconstruction might have some limits:

- because of the costs associated to large storage, the reconstructed breed will necessarily go through a bottleneck that implies loss of genetic variation and poor selection opportunities for some generations;
- material is often stored when the breed is already severely endangered, which means that its genetic variation has been probably already eroded.

To estimate the amount of material to be stored for breed reconstruction, the following aspects should be first considered:

- Number of donors – Following the indications given in 4.2.2, we assume at least 25 donor animals, equally contributing to the genetic material to be stored. These animals will be the founders of the reconstructed population.
- Size of the population at reconstruction – The size of the population at reconstruction relates i) to its potential for growth and development and ii) to the possibility of minimising loss of genetic variation during the reconstruction process. We assume to reconstruct, from the 25 founders, a population of 25 females and 25 males. The reconstructed population in first generation will have an effective population number (Ne) of 50, corresponding to an inbreeding rate per generation of 1%. FAO (1998c) suggests as minimum objective the re-establishment of the breed with 12 males and 12 females, from 25 donor animals.

Semen

Semen is used to reconstruct the breed through series of backcrosses, starting from a group of females of another breed. The number of doses to be stored is a function of (Ollivier and Renard, 1995):

- number of doses needed per parturition (d);
- expected lifetime production of fertile females by each female (f). This is function of expected number of parturitions (np), number of females born alive, survival rate and selection from birth to breeding age. The values

of these parameters vary among species, breeding conditions (field or controlled) and breeding schemes (age at culling can be set to modify the time needed for breed reconstruction, e.g. culling after first parturition);
- percentage of the genome of the stored breed that we want to have in the reconstructed breed, that is function of the number of generations of grading up. The % of genome retained is given by $1 – 0.5^n$, where n is the number of generations of grading up (table 4.2);
- number of females (NF) and males we want to reconstruct, above assumed as 25 per sex;

Table 4.2: Mean, standard deviation (SD) and upper bound (UB) of the genome from the group of females used for reconstruction that remain in the reconstructed breed, as a function of number of generations of grading up (from Hill, 1993).

Generation of grading up	1	2	3	4	5	6	7
Mean genome	0.50	0.25	0.125	0.063	0.031	0.016	0.008
SD	0	0.035	0.029	0.02	0.014	0.009	0.006
UB	0.5	0.32	0.18	0.10	0.06	0.03	0.02

The number of females to be inseminated during the reconstruction process can be computed as $F = NF(r + r^2 + ... + r^n)$, where n and NF are defined above, and r is the inverse of the expected lifetime production of fertile females by each female (1/f) during the grading up process. The number of semen doses to be stored can be computed as:
$D = dFnp = dNF(r + r^2 + ... + r^n)np$
where d and np are defined above. Number of parturitions (np) is function of both the species demography and the breeding scheme used for reconstruction (i.e. maximum number of parturitions before culling the females). At the last generation of grading up, besides the 25 females, 25 males will also be generated.

Table 4.3 shows that the number of females to be inseminated during the reconstruction process can vary as a function of *f* (from 0.4 to 2) and the percentage of the genome of the stored breed that we want to retain in the reconstructed breed (from 93.8 to 98.4). It is worth pointing out that for $f < 1$, by increasing the number of backcrosses, the number of semen doses growths exponentially. Then, as a function of both the feasibility of obtaining large numbers of semen doses and available funds, the percentage of the genome to recover from the stored breed might vary among species and country conditions.

Table 4.3: Number of females to be inseminated during the reconstruction process according to percentage of genome recovered and expected lifetime production of fertile females by each female (*f*).

No. grading up generation	Mean recovered genome (%)	*f*					
		2*	1.5*	1	0.8	0.6	0.4
4	93.8	70	79	100	180	420	1,586
5	96.9	74	87	125	257	742	4,027
6	98.4	76	93	150	352	1,278	10,131

* = these reconstruction schemes assume the use of semen from all 25 founder males in last two generations, to equalise their genetic contribution in the reconstructed breed.

To minimise loss of genetic variation during the process of breed reconstruction, some additional considerations are necessary. When female reproduction potential is low, 25 or more successful matings are expected each generation, therefore a balanced use of the 25 founder males can be easily accomplished. When female reproduction potential is high, in order to minimize costs, reconstruction schemes with a number of successful matings per generation lower then 25 could be used and a subset of males will be used at some generations. In this case, males should be used with attention in order to equalize their genetic contributions during the grading up process to avoid losses of genetic variation (Gandini et al., 2001). The number of doses needed (d) per parturition is a function of conception rate and insemination strategy (e.g. two doses in the pig species). The expected number of parturitions per females (np), which is function of the species demography and of the breeding scheme, influences *f* and the number of years necessary for reconstruction. Assuming, as an example, d equal to 3 and np equal to 4, and considering the need to duplicate the amount of material to be stored, the total number of doses based on the number of females reported in Table 4.3 may vary from 1,680 to 243,144. Finally, the number of doses to be collected per donor is given by the total number of

doses divided by the number of donors. In fact each founder should contribute equally to the new population. The pig situation has been detailed by Labroue et al (2001).
Considering also that some within family selection is advisable during the reconstruction process, when the number of breeding females that can be obtained per dam is low (e.g. cattle or horse species), the number of semen doses to reconstruct the breed can be very high. If the number of doses that can be obtained per donor is low (e.g. horse species) it may be necessary to have a large number of donors, often not available in small-endangered populations. Then, in some species the re-establishment of the breed via embryos may be necessary or more desirable.

Embryos
As for semen, embryo storage could aim to produce a total of 25 breeding females and 25 breeding males, from at least 25 donors. As minimum objective, the re-establishment of the breed with 12 males and 12 females, from 25 donor animals, has been also suggested (FAO, 1988c).

The number of embryos to be stored (NE) is a function of:
i) the number of breeding animals we want to produce (N),
ii. survival rate of embryos after freezing/thawing (SR_1),
iii) survival rate after transfer, measured at 45-60 days (SR_2),
iv) survival rate from 45-60 days to birth (SR_3), v) survival rate from birth to breeding age (SR4). Then:
$NE = (N)\ 1/(SR_1\ SR_2\ SR_3\ SR_4)$
With unsexed embryos, considering the recovery of 50 embryos, the probability of being either female or male is 0.5 with a standard error of 0.07. A minimum number of 300 unsexed embryos stored per breed has been recommended (Ollivier and Renard, 1995; FAO, 1998c).

Semen plus embryos
Storage that combine semen and embryos can be necessary considering that:
i) with storage of only semen in some species large numbers of doses can be required, so that it might difficult or impossible to obtain in small breeds,
ii) in small breeds the number of females available as donors can not be sufficient to provide the number of embryos required with the strategy that uses embryos only.
Moreover storage of combinations of semen and embryos can be envisaged in some species to reduce cryo-conservation costs (see lömker and simon, 1994 for cattle).
The optimal combination of number of embryos and semen doses should be derived considering availability and costs of semen and availability and costs of embryos in the specific context. The above considerations on minimum number of donors and size of the breed at reconstruction apply also here.

4.2.4.2 Development of new lines / breeds
An important use of the cryopreserved material is to allow new lines or breed's development. This can be realised by using the stored semen to create a certain number of males and females, with a given percentage of genes from the stored breed, to be used as breeding animals. After this selection it will take place. The computation of the number of the semen doses required to create the initial population can be done as indicated for breed reconstruction (see paragraph 4.2.4.1.)
If, for example, a new breed is to be developed from a gene pool that initially has 75% of the genes from the cryopreserved breed, as in a FAO (1998c) hypothesis, n will be equal 2, corresponding to two grading up generations. The requirement for selection might be that a number of females and males higher than the hypothesised 25 in case of breed reconstruction will be available as candidates. Moreover, we might freeze material to be used to develop more than one breed.
Then, as function of the desired percentage of genes from the cryopreserved breed (number of generations of grading up), of the desired number of females and males initially create and of the number of new breeds we want to allow for, the number of semen doses required might be lower or higher then those expected for breed reconstruction. In the particular case of gene introgression, in addition, the number of doses will be also function of the genotype of donor animals, if they are heterozygous or homozygous for the gene of interest.

4.2.4.3 Supporting populations conserved in vivo
Cryopreservation programmes may have the objective to support populations in vivo:
- *As a back up in case genetic problems occur* - When a population goes through a bottleneck it is likely that some deleterious genes increase their frequency. If frequency is high and population size is small, deleterious genes might have a large impact on population survival. Rate of removal of deleterious genes is function of the possibility of identifying carriers (by means of phenotype, DNA and/or pedigree analysis) and of the numbers of new genotypes (e.g. semen doses) we can introduce. Semen is the material of choice. It is difficult to provide figures of the number of doses needed, due to the numerous assumptions to be made. FAO (1998) provides some indications at this regard, suggesting a number of doses necessary to mate 100 females for two generations. Attention should be paid to avoid creating bottlenecks by using a small number of parents;
- *To increase the effective population size* – Frozen semen and embryos can be used to minimise inbreeding and

genetic drift in small-managed populations and the combination of live and cryopreservation can be a powerful tool in conservation of small populations (Meuwissen, 1999). Sonesson et al. (2002) proposed a scheme where semen is collected from the first two generations and used alternatively on dams, allowing a reduction of the rate of inbreeding. More research is needed to optimise the use of frozen semen and embryos for combined in situ and ex situ conservation management schemes. Type and amount of frozen material will be function of the specific population and adopted management scheme.

4.2.4.4 Back up to quickly modify and/or reorient the evolution /selection of the population.

The storage of original or extreme genotypes can be of use to quickly modify or reorient the evolution /selection of a selected population. It has been suggested to store original and extreme genotypes (Verrier et al., 2003) that can be identified as:

- extreme breeding values for production or functional recorded traits
- rare genotypes (alleles, haplotypes or genotypes)
- animals representing specific founders or pedigree lines.

Semen is the material of choice.

The diffusion of these genotypes can be realised by using directly the stored semen. A specific example can be the storage of material from dual-purpose cattle, reoriented to beef or milk production.

4.2.4.5 Research

The storage of gametes and embryos can be of interest for various research areas, including:

- to document the genetic structure of a population at a given time (Verrier et al., 2003)
- to identify single genes of large effects (FAO, 1998c).

In both cases semen can be the material of choice.

4.2.5. Frequency of collection

Breeds evolve through time. In particular when the aims of cryopreservation are 'to support populations conserved in vivo', 'back up, to quickly modify and/or reorient the evolution/selection of the population' or 'to document the genetic structure of a population at a given time' an obvious question is how often the breed should be sampled. Frequency of collection is particularly important in these cases but the question is legitimate also in case of storage for breed reconstruction. Storage can be planned at specific generation intervals (Verrier et al., 2003) considering:

- the selection intensity in the population
- the rate of evolution of the population genetic structure
- risk of occurrence of genetic problems
- costs and available funds
- occurrence of opportunities for collection

Conclusive summary

Sampling strategies in cryopreservation programmes vary for the different uses or aims of these programmes. Selection of species and breeds should be done in the context of the national conservation programme (NCP) for farm animal genetic resources. When the set of species and breeds eligible to be included in the cryobank have been selected, the following questions arise:

Is there any existing cryopreserved collection likely to be included into the national cryobank?

- Before deciding on which biological material should be stored, a careful investigation should be made to register all the cryopreserved collections that could be included into the cryobank. The existing biological material might not be the NCP first choice, but time and money can be saved if this material is taken into account. The existing collection will then influence type and amount of the 'new' biological material to be collected and stored. This analysis, whenever possible, should be extended to countries that have the same or similar breeds.

Which donor individuals?

- Donor animals can be chosen at random or following specific criteria. In the first - most common - case, random also refers to genetic relationship among individuals, i.e. mean relationship among chosen donors should be equal to mean relationship in the breed. It might be necessary to produce specific male and female donors.
- If there is no animal registration, donor animals should be carefully identified on phenotype and herd history.
- All sanitary requirements should be fulfilled, wherever possible (see chapter 7).

How many donor individuals?

- A minimum number of 25 donors are suggested. Logistics financial and reasons might lower this number, but this will affect genetic variation stored. It might be necessary to produce specific male and female donors. Higher numbers of donors will be necessary when the number of doses produced per donor is not sufficient to obtain the required total number of doses.

Which type of biological material?

- The available materials are semen and embryos. Considering the species and the aims of storage, the number of doses needed by storing semen or embryos or a combination of semen and embryos should be computed. Considering the specific context and costs and available funds, the most opportune material can be chosen.
- A cost-benefit analysis should be also included before the final decision of material is chosen. Woelders et al. (2003) provides such an analysis.
- Freezing oocytes and somatic cells is advisable only if no alternatives are possible, or as additional tools.

How many doses?

- The amount of material to be stored is a function of the aim or multiple aims of the cryo-conservation programme. At first the aims of the storage should be discussed within the NCP. Guidelines how to compute the number of doses have been given above.
- Considering the species and breeds, which are candidates for cryopreservation in a specific country, a simulation study is recommended that include for specific sets of aims the number of doses required and the relative cost-benefit analysis. This analysis will affect the decision how many species and breeds can be included in storage.
- It is recommended to duplicate the amount of material and to store each duplicate at different storing sites in order to reduce the risk of total loss by e.g. fire, technical problems in the storing plant or any natural catastrophe.
- Future samplings are recommended as well to update the stored material, as required by the aims of storage and the specific breed situation.

Time schedule for sampling?

- For genetic and practical reasons, or financial requirements, it may be necessary to follow different time schedules in sampling.
- Sampling schedule can be adapted to specific opportunities that might arise to reduce collecting costs.

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5 Freezing and storage

In this chapter, the choice and availability of infrastructure for a NCP and freezing protocols for different species and different types of genetic material will be covered.

5.1 Choice and availability of infrastructure

If available, the national cryobank could make use of the existing infrastructures for collection, freezing, storage of semen or embryos. In most cases these will be facilities of commercial organisations (AI centre or industry), but in specific cases they could consist of private facilities such as of individual hobby breeders or non-profit organisations. Such infrastructures are available in most European countries for cattle, sometimes also for pigs, sheep, goats and horses. For collecting semen, the infrastructure may be available for poultry and rabbits. For some species, or in some regions, such infrastructures are not available, for instance as a result of the type of husbandry, e.g. extensive farming of pigs in southern Italy.

It is highly recommended that a nation wishing to have a NCP, should have a centre, or several regional centres, disposing of all the necessary facilities for collection, freezing and storage of semen and embryos. This should include facilities to keep animals, e.g. for training males and collection of semen. Also, this centre should have the expertise and network to support regional efforts, e.g. to acquire the animals needed for obtaining the germplasm. Local (regional) subsidiaries could possibly be used to collect and freeze the germplasm locally. In many cases, facilities at research institutes are being used for cryopreservation. Presently, most cryopreservation in Poland is done by a research institute. Concerning embryos research institutes may have the needed expertise, like for OPU, IVF, ET, and cryopreservation. However sometimes the capability to handle large numbers of animals and samples is a problem for these institutes. Also, an important concern is that gene banking maintained at a research institute could be perceived as introducing veterinary/sanitary risks. In the Netherlands, for example, a clear separation between research and gene banking activities had to be implemented to avoid such problems.

Therefore, it is definitely preferable to have available, or to establish a national centre for gene banking, ideally with the availability of an animal facility at close hand. It may not be always necessary to maintain all the expertise of assisted reproductive technology and cryopreservation available all times in this centre. Instead expertise and facilities may be acquired for a given period when needed (e.g. a four-year project to establish a collection of embryos).

General statements about funding a NCP are discussed in chapter 3.3. Concerning funding for freezing and storage of genetic materials, a distinction must be made between the use of existing facilities and the establishment of new facilities. Existing facilities would largely consist of commercial facilities, such as AI or ET centres. There may be relevant incentives for such breeding organisations to participate in an NCP effort.
Funding of a national facility for gene banking is most likely to come from the national and/or regional government, as the government has a general responsibility to conserve farm animal diversity (CBD, 1992). As discussed in chapter 4 gene banking could also fulfil the purpose of commercial enterprises, e.g. as a backup stock in case of calamities, or to reorient the selection of populations. Consequently, part of the facilities could be funded by these companies, e.g. as an initial investment, or in the form of a levy to pay for freezing or storing genetic material. In order to minimize costs and increase efficiency, collaboration between Biological Resource Centres (OECD, 2003) must be taken into account: a BRC can be multi species and multi gender.

5.2 Freezing protocols by species and by type of material

The choice of which genetic material should be stored is in large part determined by the feasibility and efficiency of cryopreservation procedures for each material. The genetic information carried by different materials and their effectiveness in achieving conservation aims are discussed in chapter 4. In this paragraph, general statements on cryopreservation and state of the art knowledge on freezing protocols for different species and genetic material are illustrated.

5.2.1 Equipment and personnel

Cryopreservation procedures are not high technology demanding or do not need very expensive equipments. However, given that biological material is put in a “suspended animation that eventually leads to successful fertilization” (Watson, 1995) a high level of expertise is needed. To guarantee the success of the process each step must be carefully performed by skilled personnel.

5.2.2 Security

Spermatozoa and embryos stored in liquid nitrogen retain their fertilising potential virtually indefinitely, provided that the storage temperature is maintained. Because the stored germplasm may be unique and cannot be replaced when lost, the storage containers of a NCP should be equipped with an alarm system. However, the transfer of biological material between storage sites is a weak link if the operators responsible for this transfer are not experienced. Transfer logistics of biological material need careful planning. A regular supply of liquid nitrogen must be guaranteed to maintain the storage condition.

5.2.3 Storage of samples /packaging

Semen and embryos can be frozen in straws. In some species semen can be successfully frozen also in pellets. However in order to ensure a reliable labelling of the samples, and to prevent contamination, we recommend packing semen in straws rather than using the 'pellet' freezing technique. Small (0.25 ml) or medium (0.5 ml) straws can be used according to the species: e.g. medium straws (0.5 ml) for freezing horse semen while small straws (0.25 ml) can be used for buck semen. Both, small or medium straws, are suitable for freezing cattle semen. For some species more than one straw per insemination dose may be needed.

In animals with a bad veterinary status the use of CBS™ straws, with both ends sealed, can provide a dual sanitary warranty: the straw content cannot be contaminated by the outside environment, and the environment cannot be contaminated by the straw content (Guérin,1998). However semen freezing protocols in CBS™ straws are not yet tested, moreover packaging (filling, printing, etc.) in these straws is expensive, difficult and time consuming. Moreover the use of these straws does not obviate the need to keep such collections separate from collections with a certified veterinary status.

Packaging of semen in straws should preferably be done by an automatic filling machine and the straw be sealed by ultrasound. In specific conditions (e.g. cryopreservation in a research lab or local subsidiaries) commercially available semiautomatic filling and sealing system can be used.

For freezing embryos traditional (0.25 ml) straws are used in slow freezing procedures whereas for vitrification (fast freezing) of cattle and pig embryos open pulled straws (OPS) technology can be used (Vajta, 2000).

5.2.4 Identification

Given that a reliable identification of the sample is a major objective of the bank, a precise labelling procedure must be adopted. Straws must be labelled with a straw printer. Identification elements for semen straws should include: species, breed, identification of animal, country, collection centre, and freezing date. The labelling of embryos straws should contain the embryo transfer organization code, species, breed, donor identification, identification of the male, freezing date, number of embryos contained. Additional information such as the stage of development of the embryo and the quality of embryos can be added. The labelling should be in accordance with the recommendations on straw identification stated in *Secondary Guidelines Conservation* (FAO,1998a).

5.2.5 Freezing procedure for semen

Semen can be frozen easily and with adequate sperm survival in cattle, horse, pig, sheep, goat, rabbit, chicken and fish (Woelders et al., 2003). Semen collection can be a problem in animals kept free ranging, e.g. in boar, sheep and poultry. Using epididymal spermatozoa could be an alternative. Epididymal spermatozoa can be frozen equally well as ejaculated semen and has been successfully used for IVF in human, mouse, rat and cat, and in a number of livestock species i.e. pigs and goats (Rath and Niemann, 1997; Ikeda et al., 2002; Romar et al., 2003), cattle (Henault et al., 1995), and goats (Blash et al., 2000). In the goat epididymal sperm was also used successfully for AI, albeit with poor results compared to frozen-thawed ejaculated semen (Blash et al., 2000). The collection of fish semen may be difficult in some species, however if sacrificing the male of such species is not a problem, then the technique is very well possible.

Generally, media for semen cryopreservation have the following features:

- Provide a suitable osmotic balance (usually close to physiological osmolarity). The bulk osmotic support may be ionic (salts) or non-ionic (sugars).
- Provide membrane permeable cryoprotective agents, like glycerol, dimethyl sulphoxide DMSO, dimethylacetamide DMA, dimethylformamide DMF.
- For mammalian species they contain lipid and protein components like milk or egg yolk that protect cells against cold shock, chilling injury and cryoinjury.
- Inhibit bacterial growth (e.g. antibiotics) in accordance with veterinary regulations or other health considerations.
- In some (but not all) media, specific energy substrates are present (glucose, fructose).
- Some (but not all) freezing media contain a pH buffer. In some media the bulk osmotic support happens to be provided by a pH-buffering salt (Tris-citrate).

Although new techniques for freezing semen of domestic species are continuously being developed, the adoption of the freezing procedures listed below is highly advisable because these are well tested under field conditions.

Cattle

Tris-based medium is commonly used for freezing bull semen. Tris-based medium can be purchased sterile in a concentrated form, which can be mixed with pure water and pasteurised egg yolk (van Wagtendonk-de Leeuw et al., 2000). This medium is a modification by De Leeuw et al. (1993) of the Tris-based egg yolk medium containing glycerol (Davis et al., 1963). Bull semen can be also successfully frozen using a skim milk diluent in combination with 7% glycerol and antibiotics (FAO, 1998a). There have been many attempts to reduce the risks of transmission of harmful pathogens associated with the use of egg yolk included in the diluents. However, suitable alternatives have not been as successful as either milk or egg yolk (De Leeuw et al., 1993; van Wagtendonk-de Leeuw et al., 2000). Some diluents containing soy bean extract replacing the egg yolk fraction are commercially available (Wagtendonk-de Leeuw et al., 2000; Vishwanath et al., 2000).

Freezing cattle semen can be performed using controlled programmable or static vapor freezers. In this case freezing Packaging should preferably be done by a commercially available filling machine and be heat-sealed (or sealed by sonication). We recommend to use the specific straws for a given species, when available (e.g. cattle, pig). A reliable identification for each semen doses must be adopted. Straws must be labelled with a straw printer. The identification should contain the species, breed, ID of animal, country, collection centre, date of collection. Generally all labelling in accordance to ICAR recommendations on straw identification of stored samples and also stated in the Secondary Guidelines Conservation (FAO,1998).
can be done by cooling extended semen to 5° C, followed by placing the racks with the straws on nitrogen vapour at –80° C, and plunging the straws into liquid nitrogen two minutes later.

Buffalo
Buffalo spermatozoa are more susceptible than cattle to freezing damage. This is probably due to low membrane phospholipid content of buffalo spermatozoa and the loss of lipid during freezing thawing. This problem may be partially solved by using Tris-fructose-egg yolk diluents containing 6% of glycerol to cryopreserve buffalo spermatozoa (for review Sansone et al., 2000).

Sheep
Tris (300mM) – citric acid (94.7mM) - glucose (27.75mM) – egg yolk (15%) – glycerol (5%) diluent added with antibiotics elaborated by Salamon and Visser (1972) is recommended for the frozen storage of ram semen (Salamon and Maxwell, 2000). Season exerts a significant influence on semen freezability in ram (D'Alessandro and Martemucci, 2003).

Goat
Buck sperm can be frozen with good survival when semen is collected during the breeding season or during the non-breeding season if the bucks are under are under light treatment. The semen is pre-extended in Krebs Ringer Phosphate Glucose (KRPG), centrifuged 2 times and finally re-suspended in milk-based extender with glycerol (7%) (Corteel, 1977). Centrifugation is needed to remove seminal plasma, which contains an enzyme that causes alteration of the plasma membranes of the spermatozoa after interaction with lipids and phospholipids of milk. Semen packaged in small (0.25ml) straws is frozen on liquid nitrogen vapours or in a programmable freezer.

Pig
The Westendorf method (Westendorf et al., 1975) or minor modification thereof is still widely used for boar semen cryopreservation. At collection, ejaculates are diluted 1:1 in Beltsville Thawing Solution (BTS). After 3h at 15° C the diluted semen is centrifuged, the supernatant discarded and the pellet re-suspended in lactose–egg yolk extender (LEY) (80 ml of lactose 11% solution and 20 ml egg yolk). The resuspended semen is then cooled to 5° C over a period of 90 min. Then, LEY containing 9% glycerol (v/v) and a synthetic detergent ('Orvus es Paste' or 'Equex') is added to give a final concentration of 3% glycerol. Recently developed freezing procedures provide interesting results for their use in the creation of semen banks (Thilmant, 1997; Bussière et al., 2000 refs. in Pizzi et al., 2001).

Horse
Methods and extenders are amply described for this species (Cochran et al., 1984; Martin et al., 1979; Samper and Morris, 1998; Vidament et al., 2000). In spite of some differences, the freezing extenders are mainly composed of milk, egg yolk and glycerol. Individual variability of stallion semen freezability is quite high. Therefore, semen of specific males may not be freezable. However, actually in the laboratories of the National Studs in France, 85% of the stallions have sperm that can be frozen with success. Anyway, the control of sperm quality after thawing is recommended. A threshold of 35% rapid sperm (motile sperm, velocity >30µm/sec) after freezing/thawing, evaluated by computer assisted sperm analysis (CASA), is applied to reject ejaculates (Palmer and Magistrini, 1992).
The 0.5 ml straws are frozen in liquid nitrogen vapours or in a programmable freezer.

Ass
A basal medium containing glutamine at 80 mM, 10% (v/v) quail egg yolk and 4% (v/v) glycerol was successfully used to improve post-thaw total and progressive motility and sperm velocity in a endangered donkey breed (Trimeche et al., 1998). Pregnancies were obtained only when the glycerol was diluted (1/2) before artificial insemination. The results were obtained on a few numbers of females and actually new experiments are conducted in the French National Studs.

Rabbit
Rabbit semen can be successfully frozen. Rabbit sperm are sensitive to cryoprotectants containing hydroxyl groups such as glycerol and DMSO (Weitze, 1977). For this reason, cryoprotectants containing amides or methyl groups have been tested. Egg yolk-acetamide extender (20% egg yolk (v/v) and containing 1M acetamide) together with mechanical seeding is recommended (Chen and Foote, 1994).

Poultry
The highest fertility rates after artificial insemination with frozen-thawed chicken semen were obtained with rapid cooling of semen with DMA as cryoprotectant and pellet packaging. (Tselutin et al.,1999). Nevertheless, as stated previously, semen packaged in pellets is not recommended in the creation of a semen bank, given the low level of security in sample identification and sanitary requirements. Therefore a standardization of this method, coupled with straw packaging, was chosen as a reference for gene bank applications in local breeds of chicken in France (Blesbois and Labbé, 2003).

Current procedures of turkey semen cryopreservation do not allow achieving satisfactory levels of fertility; however, with minor adaptations, such procedures can be used to create sperm banks in this species (Blesbois and Labbé, 2003).

Gander semen can be successfully frozen using DMA as cryoprotectant (Lukaszewicz, 2001), whereas semen cryopreservation of other domestic birds like ducks is still not feasible.

Fish
Methods for successful sperm cryopreservation are available for most if not all domestic fish, although with a high variability among males and ejaculates. Each species needs a specific extender and cryoprotectant (refs. in Blesbois and Labbé, 2003)

5.2.6 Freezing of oocytes
The freezing of oocytes is not recommended for storing genetic material in an NCP because the state of the art is simply not sufficient for any of the mentioned mammalian species (Shaw et al., 1993; Critser et al., 1997). Concerning other species, researches are needed on cryopreservation of avian oocytes and embryos to allow a long-term preservation of sex-linked genes bearing the w chromosome (only carried by females in avian species) (Blesbois and Labbé, 2003).

5.2.7 Freezing of embryos
Flushing and embryo transfer are routine in cattle (Hahn, 1990; Hasler, 1992). To induce super ovulation, different products such as pituitary extracts (porcine and ovine) and human menopausal gonadotropins (hMG) can be used. In some countries (e.g. Germany) the use of pituitary extracts is outlawed, in this case economically viable alternatives are eCG (equine chorionic gonadotropin) and hMG. Some authors state that super ovulation may lead to lower embryo quality, even though it may be more efficient (Mapletoft et al., 2002; Kanitz et al., 2002). Embryos can be successfully produced in vitro (IVP) from oocytes collected by Ovum Pick Up (Galli et al., 2001) in different categories of animals (heifers and cows) or from ovaries collected at slaughter (Galli et al., 2003). Recent developments have made embryo collection (from slaughtered animals, or by surgery) and ET possible in pigs (Hazeleger and Kemp, 2001). Embryo freezing methods (conventional 'slow freezing' or 'rapid freezing' such as vitrification method) have been routinely applied for some time now in cattle. Similar methods are also adequate for sheep and horse. Vitrification methods for pigs embryos, which seem to be adequate, recently become available (Vajta et al., 2000; Dobrinsky et al.,2000; Berthelot et al., 2000). For rabbit embryos conventional freezing procedure (Joly et al, 1994) or cryopreservation by vitrification (Gajda, 1996) are available.

5.2.8 Freezing of somatic cells
At present, due to the low level of efficiency of nuclear transfer technology the storage of somatic cells is considered only if no alternatives are available, such as cryopreservation of semen and embryos in developing countries (FAO, 1998b). Hair follicles, obtained by skin biopsy, have been proposed as a source of somatic cells. Somatic cells could be frozen and stored in addition to semen and embryos. The rationale for this is that collecting and freezing somatic cells require less expertise and facilities then other biological materials like semen, embryos. Moreover somatic cells are more tolerant to accident occurring during collection and storage. The use of these cells in future should be considered, only after sufficient progress is made in cloning methods, to ensure that no permanent DNA aberrations are introduced in the population. Mitochondrial genes are not conserved (as with semen). Cells can also be used as a source of DNA. If no DNA is stored in the cryobank, any biological material can be a source of DNA for research experiments. It is quite simple to thaw somatic cells, culture them, refreeze some cells and use the rest to extract DNA.

Conclusive summary
Before starting collection and storage of genetic material the following elements should be considered carefully:

Facilities for collection, freezing and storage
- Whenever available a national cryobank could make use of the existing infrastructures such as breeding organization (AI and ET centre) or research institute.
- For all the other situations (species, regions), a nation wishing to have an NCP, should have a centre, or several regional centres, disposing of all the necessary facilities for collection, freezing and storage of semen and embryos.

Funding

- Existing facilities: breeding organisations should have relevant incentives to participate in an NCP effort.
- Establishment of new facilities: funding for a national facility for gene banking is most likely to come from the national and/or regional government, as the government has a general responsibility to conserve farm animal diversity. Gene banking also serves the purpose of commercial enterprises, consequently, could be partially funded by private companies

Freezing protocols by species and by type of material

- Equipment and personnel: cryopreservation procedures do not need high technology or very expensive equipments but a high level of expertise is needed. To guarantee the success of the process each step must be carefully performed by skilled personnel
- Security: spermatozoa and embryos stored in liquid nitrogen retain their fertilising potential virtually indefinitely provided that the storage temperature is maintained. Transfer logistics of biological material need careful planning. A regular supply of liquid nitrogen must be guarantee. An NCP should have an alarm system connected to the tanks of storage
- Storage of the sample: semen and embryos can be frozen in straws. In some species semen can be successfully frozen also in pellets. However for a reliable identification and sanitary warranty packing in straws is recommended. Packaging should be done by an automatic filling-sealing machine.
  For freezing embryos traditional (0.25 ml) straws are used in slow freezing procedures whereas for vitrification (fast freezing) of cattle and pig embryos open pulled straws method can be used
- Identification: a reliable identification of the sample is a major objective of the bank. Straws must be labelled with a straw printer. Identification elements are: species, breed, ID of animal, country, collection centre, and freezing date. Labelling of embryos straws should also contain donor and male identification and the number of embryos contained
- Freezing procedure

Semen

- New techniques for freezing semen of domestic species are continuously being developed. However it is highly advisable to use freezing procedures that are well tested under field conditions. Semen can be frozen with adequate sperm survival in almost all the domestic species (cattle, horse, pig, sheep, goat, rabbit, chickens and fish). Semen collection could be a problem in animals kept free ranging. Using epididymal spermatozoa could be an alternative. Epididymal spermatozoa can be frozen and have been also used for IVF in cattle, pigs and goats, and for AI in goats.

Oocytes

- Freezing of oocytes is not recommended for storing genetic material in a NCP because the state of the art is simply not sufficient for any of the mentioned mammalian species. In avian species researches are needed on cryopreservation of oocytes and embryos.

Embryos

- Flushing and embryo transfer are routine in cattle. Induction of super ovulation by pituitary extracts and human menopausal gonadotropins can be used. Cattle embryos can be successfully produced in vitro from oocytes collected by Ovum Pick Up (from heifers and cows) or from ovaries collected at slaughter. In pigs, embryo can be collected after slaughter. In addition, surgical and non-surgical methods for collection of embryos and for ET have become available. Embryo freezing methods (conventional 'slow freezing' or 'rapid freezing' such as vitrification method) are routinely used in cattle. Adequate methods are also available in sheep and horse and since recently also in pigs and rabbit.

Somatic cells

- Somatic cells can be stored if no alternatives are available or in addition to semen and embryos. Their use should be considered in the future, provided that sufficient progress is made in cloning methods. Mitochondrial genes are not conserved (as with semen).

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6 Use of genetic material

Different objectives of cryopreservation programmes have been described in chapter 2 and 4. These objectives relate to (future) use of the genetic material. Also, intake decisions and agreements with providers of genetic material relate to the objectives and (future) use. In order to be able to give guarantees to providers of genetic material and to meet the objectives of the cryopreservation programme, clear, consistent and appropriate rules or procedures need to be defined for (future) use.

Access to the cryobank depends first of all on property rights and rights of disposal, which has been agreed upon with the provider of the material. On the other hand, the cryobank has to define operational procedures and criteria for use of genetic material from the cryobank. The first paragraph of this chapter deals with property rights and rights of disposal. In the second paragraph possible operational procedures and criteria are listed.

6.1 Property rights and rights of disposal

Access to cryobank collections depends on the ownership and ownership related limitations and criteria for use. Although chapter 9 will cover legal aspects more in detail, the following elements should be considered with respect to (future) use:

- Entry or access to collections can be different for public, private and/or public/private collections. For collections in the public domain, public interest is an important criteria for use of cryobank material. Entry to private collections shall always comply with individual private rulings or contracts between cryobank and private partners. Some collections can be considered as partly private and partly public.
- Providers of genetic material to the gene bank may ask for an embargo period with respect to use of this material. This means that use of genetic material from the cryobank is not allowed within this (embargo) period. The main reason for embargo periods is that access to NCP collections may be in conflict with current market interests. An embargo period can restrict use until possible restrictions for competitive reasons are clarified. Conversely, market interests should not dictate use of the collection.
- Because of limited public or national funding, public institutions or a NCP may seek private funding. It may help to finance public collections if marketing of material or storage facilities is carried out in a controlled manner. However, if parts of the collection is made available for sale, this should be done with due consideration given to original rights and contracts and with consent and involvement of the donors. Private organisations may seek material from public collections for marketing purposes, which should be carefully monitored.

6.2 Operational criteria and procedures

Applicants of cryobank material need to fulfil criteria and have to apply for genetic material from the cryobank according to strict procedures.

- A cryobank may want to distinguish between base collections or permanent collections and active collections. Active collections are accessible if applicants fulfil the application criteria. It is advised to avoid access to the base collections, unless there is a breed catastrophe. For minimum numbers of samples/individuals reference is made to chapter 4. Especially if collections or breeds are poorly characterised, it is useful to allow provision for extra material to be used in research.
- Cryobanks have to optimize sample quantity in relation to expected and allowed use of genetic material (base collections versus active collections). Definition of thresholds is necessary to make clear at which quantity of genetic material access is prohibited. It is also advised to set replacement rules for users of the collection. The cryobank can ask for replenishment in case of interesting offspring and/or decreasing number of samples in the cryobank. In this case, users have to agree to cooperate in order to collect material of offspring of cryobank material in the future, at least when there is limited number of samples/individuals left in the cryobank.
- With respect to access to cryobank material, the NCP should distinguish between different types of material (e.g. semen versus embryos). For example semen should primarily be used for supportive breeding and embryos for re-establishment of the breed.
- In general, applicants have to be acceptable for the NCP and their request needs to fulfil the criteria of the cryobank and should comply with the objectives of the particular collection. Specified requests need to be judged by the cryobank organisation, making use of criteria set by the major stakeholders involved.
- Individuals as well as different types of organisations (organised groups, communities, companies, universities, etc.) can be user of cryobank material. The different users have a different legal status and may have different purposes for use of cryobank material.
- It is important for a NCP to monitor the use of cryobank material and to be able to report on the use of cryobank material. Mainly because of sanitary and legal risks associated with cryopreservation and use, it is recommended to implement a quality assurance and control system.
- A NCP should avoid responsibility in the event of an accident occurring during or as a result of entry to the collection. An application form should contain a declaration of the applicant, taking over the responsibilities and knowing the eventual (sanitary) risks associated with use of cryobank material.
- The NCP has to decide on costs of cryobank material and handling and distribution costs (for different types of users).

- It is recommended that cryobank and user agree on a 'Material Transfer Agreement' (MTA), which regulates the use of material taken from the cryobank; it regulates the legal relationship between the cryobank and the receiver of the material. Chapter 9 describes the recommended content of MTA's more in detail.

Conclusive summary

A NCP shall define procedures and criteria for use of genetic material from the cryobank, taking into account:

- The objectives of the NCP
- Differences between base collections and active collections
- Different types of users
- Legal aspects associated with the cryobank material
- Sanitary risks associated with the use of cryobank material
- Operational limitations

7 Sanitary/veterinary requirements

Cryopreservation programmes should comply with national and international sanitary requirements in order to be allowed to use the genetic material, which has been conserved, in a cryobank in the (near) future.
For each species, breed, biological material and sanitary status, it has to be decided:
- How collection takes place and subsequent treatment.
- How storage is organised and based on what considerations (veterinary rules, use of specific storage material).
- The limitations for use.

In general, the collected material, collection and treatment must comply with veterinary rules. It is important to categorise the genetic material to be cryopreserved in terms of compliance with regulations. Several categories can be established.
- Products that comply with OIE recommendations. For this category, there will be no future limits for their use within EU and other regions, provided that the requirements are very similar to those included in EU regulation.
- Products that comply with the EU regulation. There will be no future limits for their use within EU territory, unless EU regulations change.
- Products that cannot comply with EU legislation. The future use of these products is subject to an agreement between the national Veterinarian Authorities and the owners. A sample of serum of each animal from which material has been collected should be kept.
- Products not regulated by any legislation. It is advisable to make an agreement between owners and Veterinarian Authorities, based on scientific research.

In general, we must take into account several premises:
- The existence, at international level (OIE, International Embryo Transfer Society), of recommendations, which, each country, is free to follow or not.
- The existence, at EU level, of legislation regulating the intake and storage of semen, ova and embryos of some domestic species (bovine, porcine, equine, ovine, and caprine). These rules are compulsory for EU-members. There are no existences, however, of any regulation for somatic cells and DNA.

The rules can change in some emergency situations (disasters, outbreaks of infectious diseases, and so on). Also, some breeds with very low population size may not be able to comply with the rules. However, animals belonging to these breeds must comply with those rules setting measures to prevent diseases recognised as zoonoses (i.e. officially free of tuberculoses and free or officially free of brucellosis) and notifiable diseases.
In order to avoid sanitary risks and to guarantee future use of the collected material, it is advisable that (as much as possible) the highest sanitary standards should be used. However, in many cases, because of practical or budgetary reasons, it is not realistic to choose for these high standards and not necessary from a conservation perspective only.

In the following two paragraphs, recommendations are described for collection, treatment and storage of respectively embryos/oocytes and semen. These recommendations are based on EU regulations, OIE recommendations and other sources. The recommendations for semen and embryos/oocytes will be followed by a paragraph, which deals with implications of the sanitary status for future use.

7.1 Collection and treatment of embryos and oocytes

There is a number of important diseases in livestock production systems where sanitary approaches have been shown to be effective in providing a high safety standard in collecting embryos/oocytes for a cryobank. The following elements, which can be applied to mammalian species, are covered in the next paragraphs:
- General rules for embryo collection and embryo production teams
- Requirements for donor and recipient animals
- Risk management
- Conditions applicable to the storage, quarantine and transport of embryos
- Conditions to be applied to micro manipulated bovine embryos

7.1.1 General rules for embryo collection and embryo production teams

The purpose of veterinary control of in vivo and in vitro derived embryos is to ensure that specific pathogenic organisms associated with embryos are controlled, and transmission of infection to recipient animals and progeny is avoided.
- The embryos can be produced in vivo and collected by an embryo collection team or produced in vitro fertilisation by an embryo production team, both approved by an official veterinarian.
- The collection, processing and storage of embryos must be carried out either by a veterinarian team or under its responsibility by one or more technicians who are competent and trained by the veterinarian team in techniques of hygiene and disease control.
- The collection team must have facilities and equipment for collecting, processing and treating embryos at a permanent or in a mobile laboratory. Recording of its activities must be carried out and kept for inspection.

- In case of in vitro production, it can be advisable to have a permanently sited processing laboratory. It is also advisable to have handy suitable equipment for collection and transport of biological material if ovaries and other tissues are collected in an abattoir.
- The production/collection team must not operate in an infected zone for FMD, Contagious bovine pleuropneumonia, African horse sickness, African and Classical swine fever, as well as the permanently sited laboratory.
- Equipments in contact with embryos must be disposable and cleaned, disinfected and sterilised before their use.
- Identification of each straw/vial containing embryos (date of collection, breed, identifications of donors), in line with international standards of the International Committee for Animal Recording (ICAR) and International Embryo Transfer Society.
- Microscopical examination of embryos.
- Laminar flow facilities available when required.
- Use of antibiotics in media for recovery and storage of embryos. The media used should be sterilised by approved methods

7.1.2 Requirements for animals

- Both donor and recipient females must come from herds/flocks free or officially free of Tuberculosis, Brucellosis, Bovine leucosis and show no signs of main infectious diseases, as IBR/IPV, FMD, Bluetongue, Sheep and goat pox, Classical swine fever, African horse sickness, Swine vesicular disease, Pathogenic enterovirus encephalomyelitis.
- The veterinary administration should have knowledge of the herd/flock of origin of the donor animals, which must not be situated in an infected zone for the 30 days before and after embryo collection, with regard to FMD, Rinderpest, Small ruminant pest, Contagious bovine pleuropneumonia, African horse sickness, African swine fever and Classical swine fever.
- Semen used for insemination should have been produced and processed in accordance with the previsions of paragraph 7.2.2.
- If the semen donor for embryo production is dead or the health status of the semen donor concerning a particular infectious disease or diseases was not known at the time of semen collection, additional tests must be required on the inseminated female after embryo collection to verify that these infectious diseases were not transmitted. Otherwise, an aliquot of semen from the same collection date must be tested.
- Preferably, the donors should not be carriers of lethal recessive genes.

7.1.3 Risk management

Irrespective of animal species, there are three phases in the embryo transfer process that determine the final level of risk (in general, very low):

- The first phase comprises the potential for embryo contamination and depends basically on the health status of the herd/flocks and the donors from which embryos are collected.
- The second phase covers risk mitigation using internationally accepted procedures for processing of embryos, including washing of embryos (only embryos from the same donor must be washed together), additional washes with trypsin (if necessary) and assuring the integrity of the zona pellucida.
- The third phase encompasses the risk reductions resulting from post collection surveillance of the donors, testing of embryo-collection fluids and non-viable embryos, or other samples such blood or serum

7.1.4 Conditions applicable to the storage, quarantine and transport of embryos

- Embryos should be frozen and stored in fresh liquid nitrogen in cleaned and disinfected tanks or containers.
- The embryos should be stored in sealed sterile ampoules, vials or straws under strict hygienic conditions at a storage place officially approved.
- Only embryos from the same donor should be stored together in the same ampoule, vial or straw.
- Ampoules, vials or straws must be sealed at the time of freezing.

7.1.5 Conditions applicable to micro manipulated bovine embryos

These rules cover embryos which have been subjected to biopsy, splitting, transgene injection, intracytoplasmic sperm injection, nuclear transplantation or other micromanipulation which breach the integrity of the zona pellucida.
For these kinds of embryos, apart from complying with the rules described above, a variety of specialised microsurgical instruments may be used. Though, any cutting, penetrating or breaching of the integrity of the zona pellucida is an action that can alter the health status of an embryo. To maintain health status during and after micromanipulation, some conditions should apply:

- Any product of animal origin and other media constituents used in the micromanipulations of embryos, their culture, washing and storage should be free of pathogenic micro-organisms.
- All the equipment should either be of a single use type or should be sterilised between embryos.

7.1.6 Legal issues and recommended literature

- Council Directive 89/556/EEC, on animal health conditions governing, intra-Community trade in and importation from third countries of embryos of domestic animals of the bovine species, amended by Council Directive 93/52

and Commission Decision 94/113. This Directive shall not apply to embryos derived by transfer of nuclei.
- Council Directive 92/65/EEC, laying down animal health requirements governing trade and imports into the Community of animals, semen, embryos and ova not subjected to animal health requirements laid down in specific Community rules referred to in Annex A (I) to Directive 90/425/EEC.
- 3rd Manual of the International Embryo Transfer Society.
- Existing sanitary rules and limitations for cryopreservation programmes (Guérin and Pozzi, 1998).
- OIE Terrestrial Animal Health Code, 11th edition-2003.

7.2 Collection and treatment of semen

To provide a high safety standard for semen collection and treatment, rules need to be applied in several areas, which are explained in the next paragraphs:
- General considerations
- Conditions applicable to artificial insemination centres
- Conditions applicable to semen storage centres
- Conditions applicable to semen collection facilities
- Conditions applicable to semen laboratories
- Conditions applicable to testing of bulls
- General considerations for hygienic collection, handling, packing and storage

7.2.1 General considerations

The purposes of official sanitary control of semen production are to:
- Maintain the health of animals on an artificial insemination centre at a level which permits the international distribution of semen having negligible risk of infecting other animals or humans with specific pathogenic organisms that can be transmitted by semen
- Ensure that semen is hygienically collected, processed and stored

7.2.2 Conditions applicable to artificial insemination centres

- The *artificial insemination* centre should be comprised of animal accommodations areas (incl. isolation facilities for sick animals), semen collection room, semen laboratory, semen storage areas and administration offices. A quarantine station needs to be attached to the centre but in different location from other facilities.
- The centre should be officially approved by the Veterinarian Administration, under supervision of the veterinarian authority and subject to regular inspections.
- A register shall be kept of all the animals present, giving for each animal its identification, date of birth, date of entry.

7.2.3 Conditions applicable to semen storage centres

- Additional to the previous paragraph, storage centres must be supervised in order to be able to keep a record of all movements of semen and of the status of the donor animals.
- Only semen collected at approved collection centres is stored in approved storage centres, without any contact with other semen.

7.2.4 Conditions applicable to semen collection facilities

- Separate and distinct areas for accommodating fully health tested resistant animals, semen collection, feed and manure storage and isolation of suspect animals.
- Apart from animals associated with semen production, only animals necessary for handling are allowed, provided that they are kept separated when semen is collected.
- Measures to prevent the entry of wild animals.
- Personnel at the centre needs to be technically competent.
- Visitors, tracks and vehicles used for transporting animals and from the semen collection facilities should not be allowed to enter the facilities or it must be strictly controlled.
- Facilities need to be cleaned and disinfected regularly.

7.2.5 Conditions applicable to semen laboratories

- Physically separated from semen collection facilities, with separate areas for artificial vagina cleaning, semen evaluation, processing, pre-storage and storage.
- Personnel should be technically competent and must observe high standards of personal hygiene.
- Visitors to the laboratory should be prohibited or restricted to a minimum and subject to formal authorisation.
- Laboratories should be constructed with materials that permit cleaning and disinfections. These operations must be done at the end of each workday. Regularly, treatments against rodents and insects are needed.
- Only semen collected from donors having a health status equivalent to or better than the donors at the semen collection facilities should be processed in the laboratory.

7.2.6 Conditions applicable to testing of males and teaser animals

Animals (both donors and teasers) can enter an AI centre only if they fulfil the requirements laid down by the

veterinarian administration:

- Animals should originate from herds/flocks, which are not subjected to any movement restrictions on health grounds.
- Pre-quarantine testing for Brucellosis (B. melitensis, B. ovis and B. bovis), Tuberculosis, Bovine viral diarrhoea-mucosal disease (BVD-MD) and Infectious bovine rhinotracheitis-infectious pustular vulvovaginitis (IBR/IPV), and free from clinical signs of the following diseases: BSE, FMD, Contagious agalactia, Contagious caprine pleuropneumonia, Caseous lymphedenitis, Paratuberculosis, Scrapie, Pulmonary adenomatosis, Maedi-Visna or Caprine arthritis/encephalitis (CAE), Classical swine fever or Aujeszky's disease as specified in the Terrestrial Animal Health Code of OIE.
- Testing in the quarantine station prior to entering the semen collection facilities for Tuberculosis, Brucellosis, BVD-MD, Campylobacter fetus, Trichomonas foetus IBR/IPV, Ovine epididymitis, Maedi-Visna, CAE, Border Disease, Contagious caprine pleuropneumonia and Bluetongue.
- Testing for BVD-MD prior to the initial dispatch of semen from each serologically positive bull.
- Testing of frozen semen for IBR/IPV in AI centres not considered as IBR/IPV free.
- Testing programme for animals resident in the semen collection facilities. All animals resident in the semen collection facilities should be tested at least annually for the following diseases: brucellosis, bovine tuberculosis, BVD-MD, Campylobacter fetus, Trichomonas foetus and IBR/IPV, Maedi Visna, CAE, Bluetongue, Swine vesicular disease, African swine fever, Teschen disease and Vesicular stomatitis, as described in the Terrestrial Animal Health Code of OIE.
- It may be required to include in their veterinary prophylactic programmes a number of other diseases.
- Preferably, the donors must not be carriers of lethal recessive genes.

7.2.7 General considerations for hygienic collection, handling, packing and storage of semen

Although there is no evidence that a population of common bacteria normally present in the semen can result in a decrease of fertility, the introduction of large numbers of particular combinations of exogenous micro flora may weaken immune defences, leading to an infectious process. So, excessive contamination of semen must be avoided by the application of some recommendations.

- Conditions applicable to the management of animals: kept under hygienic conditions, coat cleaned and preferably short (except hairs of the preputial orifice, which aid the drainage of urine).
- Conditions applicable to the collection of semen: floor of the mounting area, hindquarters of the teaser, artificial vagina easy to clean and to disinfect, collecting tubes sterilised and disposal gloves for the person collecting the semen.
- Conditions applicable to the handling of semen and preparation of semen samples in the laboratory: any products of animal origin used in the treatment of semen, including diluents and additives, shall originate from a source free from animal health risk, or be treated prior to use to render the product safe. A mixture of antibiotics should be included. Buffer solutions employed in diluents should be sterilised. Receptacles used for storage and transport (straws, vials) must be suitable disinfected and sterilised before the start of any filling operation. The cryogenic agent should not previously have been used for other products of animal origin.
- Semen straws/vials should be sealed and code marked according with the international standards of ICAR.

7.2.8 Legal issues and recommended literature

- Council Directive 88/407/EEC, laying down the animal health requirements applicable to intra-Community trade in and imports of semen of domestic animals of the bovine species.
- Council Directive 2003/43/EC, amending Directive 88/407/EC, including semen storage centres in the scope of the regulation.
- Council Directive 90/429/EEC, laying down the animal health requirements applicable to intra-Community trade in and imports of semen of domestic animals of the porcine species.
- Council Directive 92/65/EEC, laying down animal health requirements governing trade and imports into the Community of animals, semen, embryos and ova not subjected to animal health requirements laid down in specific Community rules referred to in Annex A (I) to Directive 90/425/EEC.
- Existing sanitary rules and limitations for cryopreservation programmes (Guérin and Pozzi, 1998).
- OIE Terrestrial Animal Health Code, 11th edition-2003.

7.3 Implications of sanitary status for future use

In paragraphs 7.1 and 7.2 recommendations are given, based on EU-regulations and other international recommendations, which intend to minimize sanitary and veterinary risks associated with handling of semen and embryos/oocytes. At least EU-regulations have been set up from the perspective of international exchange of genetic material and to minimize the risk of spreading of diseases. The regulations do not necessarily meet conservation or biodiversity objectives in the first place.

Although minimisation of sanitary risks is always important, it may not always be necessary on a national, regional or farm level, to comply with strict EU regulations. Since cryopreservation will have a medium or long-term objective, and sanitary/veterinary requirements will change, this is a serious issue to consider for cryopreservation programmes.

In this paragraph, implications of use of genetic material from a cryobank with different sanitary status, including

tracking of the donor sanitary status, is described. We need to distinguish between:
- Use of material with an approved sanitary status
- Use of material with no sanitary status

7.3.1 Use of material with an approved sanitary status

Semen, embryos and oocytes should be obtained, if possible, from EU certified AI centres. The material must be treated according to regulations and can thus maintain the same EU status as the material used or stored by the AI station. However the regulations can be interpreted in different ways, which can be more convenient to set up a cryobank. As an example, in some countries, an agreement have been made such as that their freezing facility is to be considered a subsidiary of an AI station. On the other hand they are allowed to freeze biological material from a different species the next day, provided the facility and devices are cleaned and all materials are sterilised.
One possible problem is that EU regulation can and probably will change in the (far) future. The consequence is that material of the "old" highest status, which is of course still really perfect material, simply loses its status and cannot be put in one tank together with newer, complying material. However, and in some EU Directives (see intracommunity trade of bovine embryos), a 'whereas' exists excepting to comply with the rules such material collected before entering in force the Directive. A similar criteria should be considered in future regulations and so, no legal restriction should exists for his future use in the EU countries, providing that new material comply with the higher sanitary status and no physical contact exist between them.

7.3.2 Use of material with no sanitary status

It is always allowed to collect and store material (unless under some special conditions, e.g. epidemic types of diseases). In case there is no national regulation that means that the germplasm that is collected will simply have no status. Unless the material will be used, there is no problem but this means at least that you cannot export the material to countries with national regulation. Additionally in some cases it can mean you are not allowed to use the material in your own country except with special permission (biological material collected from an animal with a disease listed by the OIE).
However, it is strongly advised to avoid mixing material without status with other material that has a certified status, for else all material will lose the status.
However, we must take advice that if certain veterinary rules are not respected, intra-community trade or export will be forbidden. In that case it means that each country needs to launch its own cryobank for similar breeds that exist in different countries, which can result in an increase of costs.
If specific permission is required to use the material, the procedures are not very transparent or well described. Actions on EU or national level are recommended to get this clear. Official recognition of national gene banks for farm animals by the veterinary inspection would be helpful. This would not automatically give specific prerogatives privileges to the cryobank, but would help in clearing procedures to get permission for use of the material.
CBS straws could be used in order to exclude contamination from straw to straw. However, material with different status or material of different species should not be mixed and therefore be stored in at least separate tanks.
Consequently, it is recommended to keep separate tanks for:
- Quarantine per species per status
- Longer term storage per species per status.
  All the lower status than present EU-certified status can be put together (but separate per species).

7.3.3 Tracking the donor sanitary status

After collection of genetic material for the cryobank, the frozen biological material should be kept in a separate quarantine tank for four weeks until the veterinary status of the donor animals is ascertained. It is also advised to collect serum from the donor animals in order to track the sanitary status whenever necessary (for example when the sample is used). Storage of serum may require a separate tank unless this is not required by regulations (may be different when CBS straws are going to be used for the sera). Instead of or in addition to serum collection, it can be advised to have a declaration of the veterinary of the collection centre that the donor animals did not develop any signs of disease in the 4 weeks quarantine period.

Conclusive summary

- Cryopreservation programmes have to decide on sanitary aspects related to collection, treatment, storage and use for different species, breeds, biological material and sanitary status.
- Collections can have different sanitary status, which varies from 'compliance with EU/OIE regulations' to 'not regulated by any legislation'.
- In general it is advisable to follow EU/OIE regulation where possible, in order to avoid sanitary risks and to guarantee future use.
- The sanitary status of cryobank material has implications for current and future use
- It is necessary to be able to track the donor sanitary status from collection to future use and to establish a quarantine period at the moment of collection.
- General sanitary/veterinary agreements must be made between responsible organisations, those centres involved in collecting/storing material and the owners of the material.

8 Documentation

8.1 General rules

Complete documentation is vital for the future use of any stored material. Because we do not know how far in the future the samples will be reactivated we need to keep all those data about the donors and probes, which are essential for their use. We have to keep in mind that breeding associations, institutions, and as well governmental conditions can change at all including the countries themselves. World keeps changing but the cryobank should be able to fulfil all requirements far in the future and as much as possible without links to other organisations or databases which may not exist anymore. Therefore vital documents should be kept as well in digitised form.
The aim of a clearly structured and well documented database is that at any time in the future at least all basic information is available and understandable without any additional use of other information from outside the database or links to other databases. Data security should be as well worked out on the highest common level, including mirrored data stored at least at two different places to avoid their loss.
If there is any special need for special genes or traits far in the future firstly those animals or those breeds have to be re-established which give us the highest expectations to fulfil our interest. Less data means less chance to select the right animals which means spending more time and more money to get any feedback of the cryobank. Therefore, good documentation increases the value of the cryobank and the relation between costs and income from any recreation of animals. A gene bank without any documentation is like a high-tech machinery without any instruction manual. And this lack would be increased if we have a national gene bank distributed to different places without the adequate data answering the question: What is stored at which place?
All these requirements mentioned above were taken into account when the international project CryoIS was established on the basis of the European project **APIIS**, an **A**daptable **P**latform **I**ndependent **I**nformation **S**ystem (Groeneveld 2003). CryoIS is as an open source software available for any country starting a new national cryobank and would help to establish international co-operation.

8.2 Data and documents to be recorded

Essential information on a national cryobank can be grouped into the following categories:
- Donor animal
- Physical type of the genetic material
- Location of the material in storage
- Veterinarian status of the material
- Legal status of the material

Donor animal
Across all species and breeds we need to know some information about any donor animal despite the question which material we will store, e.g. semen, embryos, or somatic cells. Always we need:
- Species
- Breed
- Sex
- Birth date or collection date (at least one of them)
- Identification number of donor animal

In all cases of doubt concerning the taxonomy of species and in particular breeds, FAO regulations used e.g. in the FAO database DAD-IS and the listing of Mason (1996) should be followed (see as well FAO 1998a, 1998b).
Additionally it is mostly useful to have information about:
- Breeder
- Owner
- Pedigree, two generations, at least sire and dam

Type of the genetic material
Genetic material can be stored in many different confections, mainly: semen, embryos, oocytes, somatic cells, and other genetic material like sera and processed DNA.
Semen
The physical storage of semen has to be described. Two points are mainly to store:
- Kind of straw or pellet (e.g. 0.25 ml or 0.50 ml)
- Confection of the semen (e.g. dilution rate) or number of sperm per dose

Embryos
At least information on the physical kind of the pellets should be stored in the database. Way of collection and the development status can be added. Due to sanitary Mason and reliability of identification, embryos are usually frozen in straws. Identification elements of embryos should be in accordance with FAO and IETS standards.
Oocytes
From genetic standpoint we need the same information as for semen. Because of the up to now not regular use of oocytes at all some more technical points concerning collection and processing should be kept.

Somatic cells
We need to know the same information about the donor of somatic cells as for any embryo or semen donor.
Other genetic material
DNA, processed in different ways, blood sera, and hairs are in many cases kept for different purposes mainly for the proof of the parentage and for different molecular analyses. In the same way as described for the semen we need to keep all important information about the donor animals, collection, storage and processing.

Location of the material
Facility
Postal address, and so on.
Refrigerator and cryo container
Type and location, like room number, individual container no. and so on.
Canister
Number and position of the canister in the cryo container where the sample is located. As well the stage in the canister, if more than one stage is used, or e.g. the colour of the goblet if necessary for individual identification.

Veterinarian status of the material
The veterinarian status can be recorded as meeting the EU regulations like 88/407/EU for bovines.

Legal status of the material
The gene bank contains samples with different legal or contractual status, e.g. as regards dissemination, this should be recorded.

8.3 Technical organisation of the database

Developing and implementing a database on a national gene bank normally goes well beyond the capabilities and resources available to those establishing the gene bank. Thus, joint development of a gene bank database aimed at. One such afford is CryoIS developed and installed at the Dutch National Gene Bank and at the Institute for Animal Science in Mariensee. The software is available under a free GPL license and can serve as a basis for further development (Groeneveld 2003).

Conclusive summary

- A gene bank without consistent documentation is worthless.
- Data should be stored on:
    - donor animal
    - type of material
    - veterinarian status
    - legal status of material
    - location of samples in storage
- Instead of writing a cryo database from the scratch available software should be used. Also, co-operation in further development of a database should be done.

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9 Legal issues

Cryobank related legal aspects are often under-estimated. Intake, use, storage and access to the material all raise legal issues that should be thought thru from the perspective of a cryopreservation programmes. Various aspects regarding property rights in general need to be considered, in order to guarantee long-term viability of the cryopreservation programme.

Different levels of legal instruments that are relevant to ownership and use of genetic resources must be in mutual agreement on a national level (Cardellino, 2003):
- International treaties and conventions, which the country has signed.
- National legislation covering genetic resources, if existing
- Material transfer agreements or commercial transactions between providers and users of genetic material, regulated by common law

During the last decades, there has been an increasing focus on property rights to genetic resources both in domestic legislation and in international law. This is less so for animal genetic resources than for plants. There is no parallel intellectual property protection for animals as for plant varieties. Patent law is applicable for transgenic animals and for utilisation of animal genes and cells. It is not unlikely that the focus at intellectual property rights to animal genetic resources and animals is going to increase in the future. Also there are international legal instruments dealing with genetic resources in general. The Convention on Biological Diversity and the Bonn Guidelines of the CBD cover genetic resources in all kinds of biological material not excluding farm animals.

Legal issues are relevant at the national and international level and are relevant for the public and private sector. From the perspective of the cryobank a so called 'Material Acquisition Agreement' (MAA) can be drafted for the purpose of setting the conditions for the intake of material and a 'Material Transfer Agreement' (MTA) can be used to regulate the access to, use of and disposal of the material in the bank. For both these legal instruments, private law agreements need to be developed within the framework of the NCP to establish a certain and predictable legal situation. The MAA or MTA agreements should specify which uses of the genetic material provided are permitted and which are prohibited (Cardellino, 2003). There may be provisions as to whether the resources or its derivatives are allowed to be commercialized or not. There may also be specifications as to generation of rights over the genetic resources or any other resources derived from them. The sharing of benefits must also be specified. The MAA and MTA agreements should reflect the interests of the major stakeholders, e.g. financing organisations and owners of genetic resources. The MAA and MTA should be formulated as standard-contracts to avoid individual negotiations related to each sample. When implementing agreements, one should consider the consequences triggered by breach of its terms.
In the following paragraphs different types of property rights and also the desired content of MAA and MTA will be discussed.

9.1 Property rights and rights of disposal

Three types of property rights can be of relevance for the three phases of the life of material in a collection - entry, storage and end use - and should therefore be considered:
- Contract based restrictions
- Physical property rights
- Intellectual property rights

The following paragraphs describe these property rights and also discuss public versus private collections, other (new) rights and considerations regarding storage of the material.

9.1.1 Contract based restrictions

When animal genetic resources are transferred, the right of the receiver of the material depends upon the right of the provider. Typically such transfer is based upon mutual understanding of the transferred rights rather than a written contract. Occasionally the parties to a transfer have concluded mutual agreement. Such contractual terms follow the general principle in contract law and are only valid or enforceable between these two parties. This has the implication that a private law agreement cannot bind all future successors of the material. Agreements in animal breeding can for example be specifying that a farmer cannot use the offspring from one particular bull for breeding. A contract has limited legal effect and forcibility upon 3rd parties. For instance, material constituting a part of a collection may have limitations regarding access and use, depending upon the terms specified when the material was collected. Therefore, it is important for a cryobank to ensure that the terms imposed when conducting sampling are in accordance with the intended use of the material. One remedy regarding 3rd parties is to set as a condition in the agreement that the party to the agreement is obliged to impose the same terms in his agreements with 3rd parties. Such obligation might be combined with sanctions upon the party is not complied with.

9.1.2 Physical property rights

The provider of the animal genetic resources can have the title to the material according to domestic property legislation. Property legislation will determine the right he has towards the genetic material and will be setting the

frames for the contractual rights to transfer the right to others. This can be illustrated with an example: the farmer holds a right to use his animals for breeding purposes, unless he has undertaken any contractual obligations not to use the offspring in further breeding. Existing ownership rights to the material is also enforceable to 3rd parties. It is not dependant upon mutual agreement. Property rights to animal genetic material depend upon either EU legislation, domestic statutory legislation or domestic customary law. In many cases property rights to animal genetic resources is not dealt with particularly in the legislation. This is for example the case in the Nordic countries, see the report A Nordic Approach to Access and Rights to Genetic Resources (Tvedt, 2003).

9.1.3 Intellectual property rights

Whereas property rights to animal genetic material is closely related to the right to the individuals, intellectual property rights (IPR) as patent rights can entail a commercial exclusive right that is not bound to the individual. It can target a specific gene or a particular characteristic.
The object of an IPR is abstract and will depend upon how the right is defined under the intellectual property right regime, e.g. a patent claim. Presently, there are only few patents in use in animal breeding, e.g. test-day model. There are several applications pending on QTL findings. This situation can however change, as it has done for plant breeding. Therefore, a cryobank should have a strategy how to tackle intellectual property right-related issues when arising.

9.1.4 Public or private collections

The three types of property rights mentioned in the previous paragraphs can be hold by different types of stakeholders. Relevant property rights should be considered for different types of NCP collection material. Rights will depend upon pre-determined conservation programmes, which could be either public or private. Usually, the interests of a breeders group or society will have to be taken into account in either case.
We can distinguish between public, private and combinations of these two types of collections. Collections of a national cryobank funded by the government can in general be considered as public collections. On the other hand, private or co-operative collections exist, where breeding companies, NGOs, breeding associations, etc or individual farmers have ownership of the material in the cryobank. Combinations of public and private may also be the case. Government funded collections may for example be subject to restrictions of stakeholders/providers with regards to rights to use the material. Furthermore, regional or international collection can take place in the public domain but can also be subject to private partnerships across countries.

9.1.5 Other rights

New rights should be considered at appropriate intervals. For example 'farmers rights', 'animal breeders rights', 'animal keepers rights' or 'trademarks' may come into the picture when we want to recognize the rights of (former) owners. These concepts are not extensively discussed and developed with regards to animal genetic resources. If they are developed, they should take due consideration to encourage conservation efforts and exchange of animal genetic resources.
There is a need for regulating or specifying property rights which are specifically related to the conservation value of a national resource. Specific valuable information could be available regarding the unique genetic characteristics of indigenous resource, based upon research carried out by one party who is willing to share the information, but would like to retain rights to it. Examples on this are animals which have exceptional breeding values for fertility or disease resistance and which are owned by breeding organisations and made available for research.

9.1.6 Material storage

If the cryobank/owner(s) of the genetic material and the storage facility are two different organisations, a contract can be necessary that will specify each organisation's commitment and responsibility for the transfer of the material from one facility to another and the storage of the material.

Example
In the UK, within the National Archive run by the RBST (see section 3.4.4), the following procedure is applied to all incoming samples:
All rare breed societies are advised of the purpose of the National Archive and procedure for entry (including provision of pedigree data and health status requirements), and are invited to ask members to offer animals for collection based on the following agreed allocation of material collected:

55% National Archive (permanent storage) – ownership RBST
30% National Archive (in situ conservation breeding) – ownership usually RBST, but can include other breed organisation
15% To owner of animal being offered for collection

Owners subsequently make independent applications to the RBST to have collections made from their animal(s). In addition to the conditions above, as part of the overall agreement between RBST and the owner, the RBST covers transport costs to and from the collection centre up to a certain predetermined amount. All insurance and contractual arrangements regarding the animals in relation to the collection procedure are made separately between the owner and the organisations contracted by the RBST to carry out the collections. The current protocol is designed to cater for semen collection only, and may be modified as additional germplasm is added to the Archive.

9.2 Material Acquisition Agreement & Material Transfer Agreement

The Bonn Guidelines of the CBD (CBD, 2002) suggest elements for Material Transfer Agreements (MTA's) directed at contracts between Parties to the CBD (countries that have signed the CBD). These suggested elements can also serve as background for any MTA, MAA or similar contract regulating access to genetic resources in a repository, such as a cryobank (Cardellino, 2003). The Bonn Guidelines also contain possible descriptions of benefits arising from the use of the material.

For cryopreservation programmes on a national level, model (standard) material acquisition agreements (MAA) or material transfer agreements (MTA) should be developed for intake, use and access to genetic material. The main reason to develop such an MAA is to ensure that the objectives of the cryobank is left with stabile working conditions and a legal situation that ensures its long-term perspectives. A main objective of an MAA is to establish clear expectations with regards to the potential outcome for the provider of the material and with regards to the uses to which the donor can expect the material to be used.

Material Transfer Agreements (MTA) regulates the use of material taken from the cryobank. Thus, it regulates the legal relationship between the cryobank and the receiver of the material. It follows the material when there is a request for use of cryobank material. A model (standard) MTA should be developed for use of genetic material under responsibility of a national cryopreservation programme and a list of criteria need to be developed to be able to deal with requests for genetic material from the cryobank. The MTA should consider mostly the same issue as the MAA but be adjusted to the particular situation where material is given from the cryobank rather than collected for the cryobank.

An overall topic that should be regulated in the MTA is the rights conferred to the receiver when the genetic material is transferred. The MTA will be a contractual right and as we discussed under 9.1, it is mainly enforceable between its parties, e.g. the cryobank and the receiver. Thus, the cryobank should take into account that there might arise particular issues if the receiver wants to transfer the material to third parties. Besides regulating this legal relationship, the MTA can regulate the right of the receiver to use the transferred genetic material in inventions that later are covered by intellectual property rights.

9.2.1 Material acquisition agreement (MAA)

The following elements should be part of a MAA:

- Property rights
  The MAA should specify the ownership of the germplasm sample. The NCP should clarify existing property rights of incoming material. All three property rights categories should be reviewed (see section 9.1). There may be differences in property rights between donor/animal and the collected genetic material. The cryobank should specify the ownership to the material. Clear instruction to both owner and NCP should be made with regard to individual rights. Property rights of cryobank material need to be a balance between the rights of the (original) owner and the rights of the person/organisation who pays for the collection. In addition, it may be the wish of certain donors to take additional samples from an animal after NCP requirements have been met. Consideration to how this need is to be met should be given.
- Restrictions in use of collected genetic material (entry to collection)
  This should be described carefully in setting the agreement. If potential competitive problems between provider and cryobank related to the use of the collected material are discussed, an embargo period for use of specific material can be a solution. NCP should avoid exclusive user rights for participants belonging to (partially) public funded cryopreservation programmes. From the perspective of the cryobank the MAA should provide for maximum freedom with regards to the right to use of the material. It should at least ensure the cryobank to have the legal right to use the material in accordance with its objectives. In this respect, the cryobank should have in mind the possible change in use of its collections in the future.
- Rights of subsequent donors
  Where cryobank material is used there are naturally offspring born from the cryobank material. The owner of cryobank offspring has no exclusive rights or property rights, unless specified. This aspect should be regulated in Material Transfer Agreements. A standard article should be that cryobank, provider and future user should not claim any intellectual property rights on the derived material, if that is in the interest of the cryobank. This point may become even more valid when somatic cells are stored and possibly used for cloning.
- Veterinary/sanitary aspects
  In the signed MAA both the provider and the cryobank should define the veterinary status of the material and confirm the status and its implications. MAA should contain a list of diseases, which have been checked before entry to the cryobank. In the case of transfer of existing cryoreserves into the National Cryobank, current EU or national regulations may prevent material from being used if it was collected under legislation that has been superseded. It is therefore important that all details are checked on incoming samples against the current ability to use the sample for NCP purposes.
- Benefit sharing arrangement in case of (future) benefits.
  All possible future benefits, arising from the use of the cryobank material must be covered in the MAA (including cloning from somatic cells). One particular issue could be to specify the consequences of the cryobank turning

towards subsequent commercialisation of the material.
- Storage sites and quality assurance
  For safety reasons, collection material should be located in a minimum of 2 storage sites. The NCP should ensure that it is satisfied that all sites operate according to its existing guidelines.
- Data protection
  Restriction rights on the donor's information should be considered (e.g. Data Protection Acts)
- Other
  Consideration should be given to what would happen if an organisation (provider or cryobank) stops the NCP activities. These considerations should cover what would happen to ownership and who would have the right of first refusal. Some thoughts should be given on international or at least European cooperation in case a cryobank stops its activities.

9.2.2 Material transfer agreements (MTA)

Objectives of the CBD are to promote conservation of biological diversity, the sustainable use of its components and the fair and equitable sharing of benefits arising out of utilization of genetic resources, including by appropriate access to genetic resources and by appropriate technologies (Article 1, CBD, 1992). Access must be on 'mutually agreed terms' (both supplier and recipient must agree on terms and conditions of transfer) and subject to 'prior informed consent' (provider country can decide to grant or refuse access).

Applications for access to genetic material from a cryobank should contain a number of basic elements (according to CBD, Cardellino, 2003):
- Legal entity and affiliation of the applicant
- Type and quantity of genetic resources to which access is sought
- Accurate information regarding intended use
- Kinds and types of benefits that could come from obtaining access to the resources

The NCP has to set criteria for judging applications for use of genetic material from the cryobank:
- The cryobank must adhere to the conditions set out in the MAA. For example what type of uses and under which restriction do the collection and owner(s) of the collection allow use? There can be a significant difference (for example) between privately organised collections of 'main stream' breeds and national collections of endangered breeds in terms of accessibility to the material (long term commercial value versus emergency value). Therefore, these issues must be considered in each case or from species to species.
- The volume of genetic material that is requested should be in comparison to the total stored volume and the remaining storage should not go below a certain threshold.
- To make sure that the collection is not depleted by the successive use, the user needs to give back an equivalent amount of genetic material. The NCP has to check if the user has the financial and/or logistic ability to replace the used cryobank material
- If the requested cryobank material is also (still) available in the commercial sector, the cryobank should not allow use of cryobank material.
- If necessary there need to be consent of the former owner of the cryobank material
- NCP is advised to give a serious advisory role to breeder's societies.

A model MTA could exist of the following elements:
- Property right and use right issues, as discussed above
- The cryobank may want to have access to the offspring from the cryobank material that has been given upon request. If this is the case, it should be stated in the MTA. In this respect the cryobank might want to address to what extent the receiver shall have property right to offspring and to what extent the user shall have the right to claim any intellectual property rights and to what extent the derived genetic material shall be made available back for the cryobank. Moreover it should be clear if the recipient is allowed to exclude others from using the same material. Another way to define the rights of the cryobank could be that users should always (be willing to) give an equivalent amount back to the cryobank.
- Confirmation of veterinary status of the requested material and the implications of this status for (future) use. User has to sign that he/she is aware of the veterinary restrictions and risks with respect to use of specific cryobank material.
- Consent of the former owner of cryobank material (if necessary)
- Benefit sharing arrangement in case of (future) benefits (Reference: CBD, 2002)

Conclusive summary

- NCP's need to develop legal instruments in order to guarantee long time viability of the cryobank
- Property rights and rights of disposal are critical elements
- Standard or model Material Acquisition Agreements and Material Transfer Agreements have to be developed

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Abbreviations

ABS	Access and Benefit Sharing
AI	Artificial Insemination
AnGR	Animal Genetic Resources
BRC	Biological Resources Centre
CBD	Convention on Biological Diversity, Rio de Janeiro, 1992
EAAP	European Association for Animal Production
EBV	Estimated Breeding Value
ERFP	European Regional Focal Point
ET	Embryo Transfer
EU	European Union
FAO	Food and Agricultural Organisation of the United Nations
FMD	Foot and Mouth Disease
IPR	Intellectual Property Rights
IVF	In Vitro Fertilization
MAA	Material Acquisition Agreement
MTA	Material Transfer Agreement
NCP	National Cryopreservation Programme
NGO	Non Governmental Organisation
OECD	Organisation for Economic Cooperation and Development
OIE	Office International des Epizootie
OPU	Ovum Pick Up